Malignant hypertension in the transgenic Ren-2 rat

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Doctor of Medicine

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May 1995

Declaration

I declare that all the work presented in this thesis is my own except where stated otherwise, and it has been entirely composed by myself

May 1995

Acknowledgments

Specific thanks must go:

The Scottish Hospitals Endowment Research Trust (Mrs Robina Menzies-Smith Medical Research Scholarship) and the A.F.R.C for financial support.

Gillian Brooker for teaching me how to use the telemetry system

Dr JJ Morton at the Department of Medicine, Western Infirmary, Glasgow for plasma renin and Angiotensin II assays

Nicola Burns and Dr Brent Williams at the Department of Medicine, Western General Hospital, Edinburgh for the aldosterone assays

Dr Lynn Manson and the staff at the Department of Haematology for the preparation of blood films

Dr Stewart Fleming and staff at the Department of Pathology for sectioning and staining of tissues in preparation for histological examination

Dr Yuri Kotelevtsev at the Centre of Genome Research for help in the design of the analytical cross described in chapter 4 and to Barbara Young for performing the initial mapping experiments.

Dr John Firth and Mrs Kay Yeates, John Radcliffe Hospital, Oxford for performing the RNase protection assays for endothelin mRNAs

Dr Jean-Paul Clozel at Hoffman-la Roche, Basle, Switzerland for kindly giving us the endothelin antagonist, Bosentan to use in this study.

I would also like to thank a number of people who were sources of inspiration and help, in particular Murielle Veniant, Matt Sharp, Louise Anderson, Hazel Davidson-Smith, Donald Ogg and of course Allan Cumming.

Finally without John Mullins' help and support none of this would have been possible He has my admiration and gratitude. Thank-you John.

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 3.5.10)

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Degree Doctor of Medicine (MD) *Date* 20th February 1995

Title of Thesis Malignant hypertension in the transgenic *Ren*-2 rat

No. of words in the main text of Thesis 45,000

The transgenic rat line TGR(mREN2)27 has been previously shown to develop severe hypertension as a consequence of over-expression of the mouse Ren-2 renin gene (Mullins et al 1990). It was observed that an alteration in phenotype occurred in hybrids (HanRen2/Edin--) derived from crossing homozygous *Ren*-2 transgenic rats with the Edinburgh Sprague-Dawley strain of rats. Investigations into this phenotypic change revealed it to be due to spontaneous development of malignant phase hypertension. Furthermore the incidence of the malignant hypertensive phenotype was altered by the genetic background into which the transgene was introduced.

The introduction of this thesis has reviewed the literature on the genetics of human essential hypertension and rat models of genetic hypertension, the role of the kidney in essential hypertension and the pathogenesis of malignant hypertension. Techniques in molecular biology which include transgenesis have been used to investigate the role of individual genes in blood pressure regulation. In this context the literature concerning the transgenic rat line TGR(mREN2)27 was extensively reviewed.

The heterozygote cross HanRen2/Edin-- was found to develop malignant phase hypertension within a relatively narrow age range. Seventy-three percent of male and 52% of female HanRen2/Edin-- developed malignant hypertension. In contrast, other heterozygote crosses HanRen2/Han-- and HanRen2/Lew-- had an incidence in males of 18% and 0% and in females of 4% and 0% respectively. Telemetry was used to record blood pressure continuously in unrestrained conscious rats and demonstrated an accelerated rise in blood pressure in rats with clinical features of malignant phase hypertension. Histopathology showed fibrinoid necrosis and myo-intimal proliferation of afferent arterioles and small renal arteries. An associated deterioration in renal function occurred with a rise in plasma urea and creatinine. TGR(mREN2)27 normally have a suppressed renal renin-angiotensin system but in malignant phase affected animals had a significant elevation of plasma renin, angiotensin II and aldosterone. Immunohistochemistry demonstrated increased renin at the site of the afferent arterioles near the vascular poles of glomeruli. Blood films demonstrated a microangiopathic haemolytic anaemia. A genetic basis for the differing incidence of malignant phase between the three heterozygote crosses was further supported by the results of an analytical cross set up to segregate Edinburgh Sprague-Dawley alleles. Results suggested that malignant phase hypertension complicated benign hypertension due to the effects of one or possibly two genetic loci.

A further study looked at the role of endothelin in malignant hypertension. Previous investigators had suggested that endothelin may be involved in the pathogenesis of malignant phase hypertension. RNase protection assays demonstrated increased expression of endothelin-1 mRNA in kidneys from malignant hypertensive rats. Chronic inhibition of endothelin receptors using an oral non-specific endothelin receptor antagonist (Bosentan) did not prevent or reduce the transition from benign to malignant phase hypertension. It would therefore appear that endothelin synthesis occurs in response to the transition to malignant hypertension but it is not a central initiating factor.

In conclusion, this is a representative model showing many of the characteristics of malignant phase hypertension in humans. The differing incidence between transgenic *Ren*-2 crosses appeared to be a consequence of genetic factor(s). This may therefore be another example of a genetic pre-disposition to develop target organ damage from hypertension.

Contents

CHAPTER 1

Introduction

1.1 Genetics of Essential Hypertension

Hypertension has been defined by the World Health Organisation criteria to be present if systolic blood pressure (SBP) is 140 mmHg or more and/or diastolic blood pressure (DBP) is 95mmHg or more. As such, it has been shown to affect up to 24% of all individuals from populations within developed societies, and up to 50% of people over fifty years of age[1]. Resulting complications, namely cardiovascular, cerebrovascular and renal, exert a significant impact on health care resources. A recent survey has shown that only 53% of all hypertensives are on treatment for hypertension in order to prevent secondary complications[1], but despite that it still remains the commonest reason for initiating life-time medication[2]. In 95% of cases, hypertension is a primary condition labeled as essential hypertension, on the basis that no underlying or secondary causes for the elevation in blood pressure (BP) such as renal artery stenosis, renal parenchymal disease, hyperaldosteronism or phaeochromocytoma have been diagnosed[3]. However the causes of elevated BP in essential hypertension remain unclear.

Hypertension does not have a defined phenotype, but is simply the tail of a skewed frequency-distribution curve for blood pressure (BP)[4]. The distribution of measured blood pressure amongst individuals from a population is a consequence of their biological diversity, their adopted lifestyle and errors or variation in the measurement. Various arbitrary values have been used to define hypertension of differing severities in the clinical setting, but measured BP has been shown both to increase with age and be influenced by situational effects at the time of measurement. Assessments of the distribution of BP within populations have often been based on single measurements thereby potentially over-estimating the true prevalence of hypertension[5]. In early studies complications such as heart failure, impaired renal function or retinopathy were often labeled as a symptom complex equating with 'hypertension' and subsequently included in the analysis of some populations leading to falsely high estimates[5,6].

Ward (1990) has reviewed many of the studies performed, since the observation by Morgagni in 1761 of a father and son dying of 'apoplexy' and cerebral haemorrhage respectively, which suggested that both familial and environmental factors might be important[7]. Apparent familial aggregation of high blood pressure or

its complications could be a consequence of common environmental, genetic or combined factors but also it could be due to the influences of age, sex and other confounding genetic/environmental factors such as obesity. These might not be randomly distributed through affected families potentially introducing bias. Many early studies of 'essential' hypertension should also be interpreted with caution in view of a number of factors. The medical technology of today was not available to early investigators, and reports of quite severe hypertension in children[8] does suggest that secondary causes, e.g. vesicoureteric reflux and nephropathy may not have been adequately excluded from the groups studied.

Between populations, blood pressure differences appear to be influenced more by environmental and socio-cultural factors in particular dietary sodium intake, physical activity, obesity and psychosocial stress[7]. Though genetic factors may play a role, for example by influencing sodium handling[9] or the degree of obesity, 'westernization' of lifestyle is clearly a major factor. However within groups from diverse ethnic and genetic backgrounds, the presence of familial aggregation has prompted investigators to look for a genetic aetiology. Platt (1947) found an excess of 'hypertension' in parents of hypertensive probands, compared to the parents of normotensives, but relied on histories suggestive of hypertension related complications[10]. Reported transmission of high blood pressure through three generations[8], together with an apparent partition in the frequency distribution curve of blood pressure measurements from siblings of hypertensive patients giving a bimodal curve with approximately equivalent portions, prompted him to suggest that blood pressure was inherited by a single autosomal dominant gene[10,11]. However it was shown by Murphy (1964) that apparent bimodality or polymodality could arise from a number of errors including observer differences, digit preference in BP recordings selecting against intermediate values, the class intervals used in presenting the data, and small sample sizes, even when taken from a population with a unimodal distribution[12]. Therefore conclusions about inheritance could not be made from such analyses.

From a larger number of subjects in the 'Precursors of Hypertension and Coronary Disease Study', John Hopkins University, both SBP and DBP were shown to have distributions with a positive skew which could be restored to a binomial distribution following logarithmic transformation[6]. Hamilton et al (1954) recognised the need for adjustment of BP for age and sex, and found no natural dividing line between hypertensives and normotensives[13,14]. They did however find a consistent familial aggregation with a regression coefficient for SBP of 0.22 and of DBP 0.18 irrespective of whether the probands were normotensive or hypertensive[15]. In a larger

population-based study taking a random sample from the general population in Rhondda Fach, UK, it was estimated that approximately 33% of SBP and 20% of DBP was genetically determined. No suggestion of bimodality was seen for BP, but it was accepted by the authors that single gene inheritance could account for a very small percentage of cases[16]. Other population-based studies from Tecumseh, USA, and Framingham, USA have found variable but significant regression coefficients for first degree relatives of hypertensive probands[7].

If genetic factors were important in the aetiology of essential hypertension, then they potentially could be operating prior to the clinical diagnosis, hence it was of interest to ascertain whether familial aggregation of BP existed from childhood. A study performed on children aged 2-14 years from 192 families did demonstrate familial aggregation. This was made by observing that the distribution of mean family scores (in standard deviation units (SDU) where one SDU equals the individual's BP minus the mean for their specific age and sex group divided by the SD of BP in that group) differed significantly from the expected normal distribution (which would have a standard deviation of one and mean of zero). Stronger regression coefficients existed between child propositi and their siblings compared with their mothers[17]. A weak correlation between infant blood pressure and maternal blood pressure has been found at birth, but a significant relationship with paternal blood pressure was not found until one month old[18]. At birth, infant blood pressure could be affected by the intra-uterine environment which in turn may be influenced by both maternal genes and environment. A significant contribution to any expected correlation in BP between parents and offspring and between offspring could be a consequence of a shared environment. In the case of DBP, 67% of the expected correlation was calculated to be due to environment and 33% to be due to shared genes[19].

Pickering and Platt became engaged in a highly publicised debate over the inheritance of essential hypertension, but most have come to support Pickering who proposed a polygenic inheritance together with an environmental influence[10,11,5,4,16,20]. It has now been estimated that between 20-60% of the population variability in BP is genetically determined[7,21].

Recently clear examples of autosomal dominant inheritance of hypertension mediated by single gene defects have been described. Glucocorticoid remediable hyperaldosteronism (GRA) does not reliably exhibit clinical features or blood chemistry that would have allowed clear distinction from essential hypertension after standard investigations. Diagnosis of GRA rests on finding elevated urinary excretion of steroid metabolites 18-hydroxycortisol and 18-oxocortisol. Lifton et al identified linkage of GRA with an RFLP arising from a fusion event occurring between the

ACTH responsive regulatory sequences of the 11β hydroxylase gene and the coding sequences of the aldosterone synthase gene. This has resulted in ectopic expression of aldosterone synthase in the adrenal zona fasciculata which responds to stimulation by ACTH rather than angiotensin II (Ang II) or potassium[22].

Liddles Syndrome (pseudoaldosteronism) has long been recognised as having an autosomal dominant mode of inheritance with constitutive activation of the Na^+H^+ exchange channel in the distal tubule[23]. Recently polymorphisms for the gene coding for the β subunit of the epithelial sodium channel have been found to be linked with hypertension in five kindred with the syndrome[24]. It is therefore quite possible that other single gene defects may be identified causing hypertension in certain kindred[25].

1.2 Rat models of Genetic Hypertension

High blood pressure in humans may result from the interaction of a number of genes with each other (epistatic) and with the environment (ecogenetic)[26], but analysis of the trait is complicated by its quantitative nature, incomplete and age related penetrance and sexual dimorphism. The genetic heterogeneity of the human race and the difficulty in controlling environmental factors makes the investigation of the genetics of hypertension in humans extremely problematic. Animal models of genetic hypertension have therefore been developed, derived from inbred strains and when maintained in controlled environments, they can provide a potential tool for study (Table 1.2.1).

A number of genetically hypertensive rat models have been developed by selective breeding from relatively hypertensive pairs taken from originally normotensive rat strains to create inbred hypertensive strains. It was assumed that by selecting hypertensive animals and with further breeding and selection for high BP, inbred strains with a hypertensive phenotype would result from segregation of hypertensinogenic genes. Subsequent investigation into the genotypic differences between the new hypertensive strain and normotensive control strains might then identify candidate loci. Studies looking at the physiology of these hypertensive rat strains might also point to intermediate phenotypes and mechanisms by which blood pressure homeostasis might be disturbed. In reality this has not been so simple. A number of candidate genes have been suggested on the basis of both studies in human essential hypertension and in rat models of genetic hypertension (reviewed in references[34,35]).

Table 1.2.1.

Hypertensive rat	Control	Original strain	Reference
GH (Dunedin)	N	Otago Wistar	Smirk & Hall 1958 [27]
SHR	WKY	Wistar	Okamoto & Aoki 1963 [28]
Dahl SS	Dahl SR	Sprague-Dawley	Dahl, Heine & Tassinari 1962 [29]
Dahl SS/Jr	Dahl SR/Jr	Sprague-Dawley	Rapp 1984 [30]
SBH	SBN	Sabra rat	Ben-Ishay, Saliternick & Welner 1972 [31]
LH	LN, LL	Sprague-Dawley	Dupont, Dupont, Froment et al 1973 [32]
MHS	MNS	Wistar	Bianchi, Fox & Imbasciati 1974 [33]

Table 1.2.1 - A summary of the origins of genetic hypertensive rat strains developed by selective breeding. Abbreviations: GH = Genetically hypertensive strain (Dunedin), N = normotensive colony; SHR = spontaneously hypertensive rat, WKY = Wistar-Kyoto; Dahl SS = salt sensitive, Dahl SR = salt resistant; Dahl SS/JR = inbred salt sensitive strain, Dahl SR/JR = inbred salt resistant strain; SBH = Sabra hypertension prone, SBN = Sabra hypertension resistant; LH = Lyon hypertensive, LN = Lyon normotensive, LL = Lyon low blood pressure; MHS = Milan hypertensive strain, MNS = Milan normotensive strain.

The spontaneously hypertensive rat (SHR) was a classic example. It was developed by Okamoto et al (1963) by selecting a breeding pair with a relatively higher blood pressure from a colony of Wistar rats. Continued breeding and selection for more than twenty generations resulted in the inbred hypertensive SHR strain[28]. A second inbred strain was bred without selection from the same original outbred Wistar colony and designated Wistar-Kyoto (WKY) and this strain has been used extensively as the 'normotensive' control for both physiological and genetic studies. It was subsequently noted that a differing incidence of cerebral haemorrhage existed between sub-strains of SHR. Selection by maintaining lines derived from parents that subsequently developed stroke has resulted in the stroke prone SHR strain (SHRSP)[83].

Table 1.2.2

Candidate gene	References
Renin	36, 37, 38, 39
Angiotensinogen	40, 41, 42, 43, 44
Angiotensin II receptors	45, 46, 47
Angiotensin Converting Enzyme	48, 49, 50, 51, 52, 53
Nitric oxide synthase	54, 55, 56
Endothelin	57, 58, 59
Endothelin-A receptor	60
Kallikrein gene family	61, 62
Sodium-lithium counter transport	63
Sodium-hydrogen exchangers	64, 65
Sodium-potassium ATPase	66, 67, 68
Atrial natriuretic peptide (ANP)	69, 70
ANP/guanylyl cyclase A receptor	71
Adrenergic receptors	72, 73
Insulin receptor	74, 75,
α - adducin	76
SA gene	77, 78, 79, 80, 81, 82

Table 1.2.2 Candidate genes implicated in BP regulation and human and/or rat genetic hypertension.

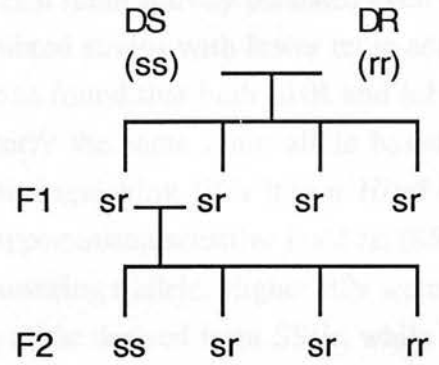
In SHR, the kidney has been implicated as the source of hypertension. Donor kidneys from five week old SHRSP (stroke-prone SHR strain) or WKY rats were transplanted into bilaterally nephrectomised F1 (SHRSP X WKY) recipients. By eight to ten weeks of age, recipients of SHRSP kidneys showed both elevated BP and plasma creatinine compared to WKY kidney recipients[84]. However further studies have suggested that neither changes in circulating or renal renin-angiotensin system (RAS) activity contribute to the post-transplant hypertension in F1 hybrids receiving an SHR-SP kidney[85]. SHR showed a relatively thickened arterial media and a greater increment in renovascular resistance in response to noradrenalin (though reduced sensitivity) compared to WKY even at a pre-hypertensive stage at four to five weeks of age[86]. Using laser-Doppler flowmetry it has been shown that renal papillary blood flow is reduced in the pre-hypertensive SHR, but that direct intra-medullary administration of Captopril at a dose (5 mg/kg/day) that does not lower systemic BP resulted in an increase in papillary flow, natriuresis and a lowering of blood pressure[87]. The ability of vasodilator prostaglandins, prostacyclin and PGE_2, to

counteract both angiotensin II (Ang II) and Thromboxane A_2 induced renal vasoconstriction was reduced in young SHR compared to WKY[88,89]. Relatively higher levels of renin mRNA have been found in kidney, liver, brain, adrenal and heart of five week old SHR compared to WKY by ribonuclease (RNase) protection assay. This increment persisted in liver, brain and adrenal tissue taken from twelve week old SHR compared to WKY, but similar levels were found in kidney and reduced levels in SHR heart and aorta by this stage[90].

Evidence in SHR of increased levels of brain Ang II, renin and hypothalamic angiotensinogen mRNA have also implicated the brain RAS in hypertension. Antisense inhibition, using oligonucleotides complementary to mRNA coding for the target protein, are thought to inhibit effective translation by hybridization to the mRNA. This approach was used to study the effects of specific inhibition of both angiotensinogen and Ang II receptor (AT_1) mRNA by intracerebroventricular (icv) administration of the respective oligonucleotides. These measures resulted in normalisation of BP[91,92]. It has also been suggested that in SHR compared with both WKY and Sprague-Dawley (SD), that an overactive kallikrein-kinin system particularly in the rostral ventrolateral medulla, is involved in BP regulation[93,94].

The inbred Dahl salt-hypertension sensitive (DS) rat and salt-hypertension resistant (DR) strains were developed from selective breeding from outbred SD[29]. DS rats show a suppressed RAS with low plasma renin activity (PRA) and renal and adrenal tissue renin contents existing from the pre-hypertensive stage. DS rats have 15% fewer glomeruli than the DR strain and reportedly show an inappropriate renal vascular response to a high salt diet[30]. The two inbred strains were crossed and a subsequent F1 intercross produced an F2 population (Fig 1.2.1) which was raised on a high salt (8% NaCl) diet from weaning and the blood pressure response determined. A 1.2 kb insertion/deletion (I/D) polymorphism was found in the first intron of the renin gene which was able to differentiate the cloned s (DS) and r (DR) alleles. Using a specific probe for this I/D area, a significant difference in SBP was observed between the three possible genotypes in males, with an increasing blood pressure correlating with increasing dose of the s renin allele, though accompanying differences in plasma or tissue renin were not looked for in the F2 generation[36]

Fig 1.2.1



F2 progeny are derived from an F1 intercross (all heterozygous at any one locus). Resulting offspring in F2 will have one of three possible genotypes - in this case homozygous ss or rr, or heterozygous sr.

The Lyon hypertensive strain (LH) was developed by selective breeding from Sprague-Dawley stock. Normotensive and hypotensive inbred strains were also generated and subsequently combined to produce an inbred normotensive control strain (LN)[32,95]. In addition to hypertension, LH rats show altered lipid profiles with an elevated plasma cholesterol, and raised plasma insulin and insulin:glucose ratio. In an F2 cross derived from Lyon LH and LN progenitors, blood pressure was significantly below mid-parental values suggesting a recessive phenotype, with the degree of genetic determination for SBP estimated at 28% and DBP 13%. Positive but weak relationships were found in the F2 population which suggested cosegregation of MBP and total cholesterol (r = 0.393), phospholipids (r = 0.245) and triglycerides (r = 0.245)[96]. In F2 rats, two microsatellite markers were found to show significant linkage with both diastolic and systolic blood pressure. One of these was contained within the renin gene locus on chromosome 13, but a marker contained within the carboxypeptidase gene locus showed the highest LOD score statistic for linkage with pulse pressure[97].

A *Bgl* II RFLP occurs in the rat renin gene as a consequence of a variation of the number of tandem repeats (VNTR) of a 38bp sequence in the first intron. A number of renin alleles have now been identified in different hypertensive and normotensive rat strains[39]. Using a labeled probe complementary to the conserved region of the tandemly repeated sequence on Southern blots of *Bgl* II digested DNA, it was identified that inheritance of one or two SHR renin alleles in an SHR/Lewis F2 cross resulted in higher MBP compared to F2 rats inheriting only Lewis alleles[37]. In sixteen week old normotensive Brown Norway (BN) rats, renal renin activity is increased compared to SHR, and the inheritance of the BN renin allele in recombinant

inbred strains was associated with increased renal renin activity and a tendency to a lower BP compared to those inheriting the SHR allele. Interestingly, the difference in renal renin activity persisted even when blood pressure was matched in recombinant inbred strains with lower renin activity linked to inheritance of the SHR allele[98]. It was found that both SHR and inbred Dahl salt- hypertension resistant rats (SR/Jr) carry the same renin allele based on assessment of the first intron VNTR and a distinguishing fifth intron *Hind* III site. Hence in an F2 cross of the inbred salt-hypertension sensitive Dahl rat (SS/Jr) and SHR, SHR contributed the blood pressure lowering r allele. Higher BPs were therefore seen in F2 progeny homozygous for the s allele derived from SS/Jr, while lower BPs were observed in rats homozygous for the r allele derived in this cross from SHR[39].

Therefore significant linkage has been demonstrated between the renin gene and high BP in three strains of inbred genetic hypertensive rat, the SHR, the LH strain and the Dahl SS strain. No linkage has been found between the angiotensinogen locus and either hypertension or cardiac hypertrophy in an F2 cross of SHR-SP and WKY rats using a polymorphism that distinguished the two strains[99].

Two groups have identified loci that show linkage with basal BP variation and stronger linkage with BP variance in response to sodium loading in F2 SHRSP/WKY crosses. Hilbert et al (1991) used a combination of minisatellite probes, microsatellite markers and an RFLP previously identified in the SHRSP renin gene[49]. Jacob et al (1991) developed a series of microsatellite markers for the rat based on simple sequence repeats (SSR)[48]. In common to both studies, a locus on chromosome 10 named BP/SP-1 or Bp1 respectively showed significant linkage disequilibrium with sodium loaded BP. Both groups identified the locus to be in the region of the angiotensin converting enzyme gene which by its very nature has always been a prime candidate. Screening cDNA libraries from SHRSP and WKY has shown that the sequences for the respective ACE cDNAs differed by 5 nucleotides in the coding regions, only one of which resulted in an amino acid substitution ($Lys_{207} \rightarrow Arg_{207}$) in SHR-SP. No significant effect on function was apparent as enzymatic analysis revealed similar Km and V_{max} for ACE extracted from lung tissue from the two strains[100]. Another study failed to identify linkage between ACE alleles and hypertension in an F2 (SHR x WKY) population using CA repeat elements to differentiate ACE alleles, but linkage was found in an F2 (Dahl SS/Jr x MNS) population[71].

Phenylethanolamine N-methyl transferase (PNMT) is involved in catecholamine synthesis and has been found to be elevated in the brains of SHR-SP. The gene for the enzyme has been mapped to the region 17q22-17q24 in humans

which is within the conserved linkage group syntetic to the region in the rat where the locus BP/SP-1 (or Bp1) was found[101]. The PNMT gene has now been located to Chromosome 10 in the rat, but no sequence differences were identified between the cloned genes obtained from SHRSP and WKY genomic DNA libraries, including 1.0 kb of 5' sequence containing known regulatory regions and all exons[102].

Weaker linkage was found with two chromosome X markers (BP/SP-2) with F2 progeny homozygous for the WKY allele, as opposed to the SHR allele, having the higher blood pressure[49]. Weak linkage with baseline DBP and sodium loaded BP was found for a locus on Chromosome 18, but the authors have suggested that more evidence is required to confirm genetic linkage with this region[48].

In addition to the RAS, other systems potentially involved in BP homeostasis have been implicated. An RFLP marking the kallikrein gene family or closely linked genes (using a pancreatic kallikrein cDNA probe which cross hybridizes with a number of closely related kallikrein genes) has demonstrated that inheritance of the SHR allele in recombinant inbred strains derived from SHR and BN progenitors, is associated with significantly greater median SBP, DBP and MBP[62].

In SHR, the S_A gene was identified as a candidate gene on the basis of isolation of a cDNA by differential hybridization from an SHR kidney cDNA library using cDNA probes prepared from SHR and WKY rat kidneys[77]. It was subsequently localized to rat chromosome 1 showing tight linkage to both rat myosin light chain 2 and leukosialin genes[81]. Increased expression of an mRNA species was seen in SHR kidney compared to that from WKY at 4 weeks, but not at 16 weeks. At 16 weeks of age, liver expression is only seen in SHR[77]. In contrast, a 10-fold reduction in expression was seen in SHR brain compared to WKY brain[78]. To test whether the S_A gene was related to hypertension, an F2 cross from SHR-SP and WKY found the SHRSP allele to be linked with increased systolic blood pressure on sodium loading, but no linkage with basal blood pressure was identified[81]. In an F2 cross between SHR and WKY, the SHR allele did cosegregate with hypertension[79]. However no effect of inheritance of the SHR S_A allele on basal blood pressure was seen in F2 males from an SHR/Lewis cross, but a small increase in MBP did occur with inheritance of the SHR derived allele compared to the Lewis allele in females[78].

Unfortunately the finding of considerable genetic heterogeneity between WKY strains available from different commercial sources, and even from within a single source, has suggested that this strain was distributed before full inbreeding had been achieved[103]. Divergence between SHR and WKY has also been found to be high using a method of RFLP analysis following digestion of genomic DNA with restriction enzymes and hybridization with a number of probes[104]. Comparison of

DNA 'fingerprints' by Southern Blot analysis of six different VNTR sequences showed that the median percentage of bands shared by SHR and WKY was only 54%. Relatively little genetic heterogeneity in the Dahl strains has been found, with Dahl SS rats sharing 80% of bands with the Dahl SR strain[105]. This would be greater than that expected between a parent and F1 offspring (50%) or a parent and first backcross generation (75%). More recently evidence of genetic contamination of the inbred Dahl salt sensitive (Dahl SS/Jr) strain with apparent phenotypic variability has been reported and could potentially call into question many studies that have used these strains[106,107]. However there still appears to be a close genetic relationship between hypertensive and control Lyon strains on the basis of the low percentage of informative microsatellite markers (11% of 241 markers) that detected allelic differences between the Lyon strains[97].

Selective breeding for a phenotype will result in the segregation of hypertensive genes and in the SHR it has been estimated that perhaps between three and six loci are responsible[108]. In an F2 (Dahl SS/Jr x MNS) population, it has been estimated that approximately half of the genetic variance is explained by a combination of the effects of ACE, GCA (guanylyl cyclase A) receptor and renin gene loci[71]. The segregation of 'hypertension' genes is likely to be accompanied by cosegregation of other loci which may become fixed in the 'hypertensive' strain, and therefore the presence of a genetic polymorphism at a specific locus does not necessarily imply causality[109].

Interestingly the 'genetic' hypertension of SHR could be prevented by pre-treating females with the ACE inhibitor, captopril until they were mated at two months of age. They remained off treatment thereafter and their offspring were found to show a significantly reduced basal BP at both 4 and 9 months of age though their responses to exogenous Ang II (icv) and intravenous (iv) Ang II or Ang I were not significantly different to controls whose parents had not received any pre-treatment with ACE inhibitors prior to mating[110]. This serves as an example of the persisting difficulty in separating genetic and environmental contributions even in animal models.

1.3 Genetics of Human hypertension

The findings of linkage between candidate genes and hypertension in animal models of genetic hypertension can not be directly translated into the human situation. The techniques described above have been applied to the humans with necessary modification in view of the inability to direct mating to generate optimal genetic crosses for study! Association studies seek a difference in the observed frequency of

an allele at a marker locus comparing affected cases to unaffected, unrelated controls looking for evidence of the loss of expected random segregation of alleles in a population. An allele is said to exhibit linkage disequilibrium with a trait if it occurs at a significantly higher frequency in the affected group compared with the unaffected control group. However linkage of an allele does not imply causality. The allele may simply lie close to and be in linkage disequilibrium with the allele which is responsible. If the groups chosen for study are not relatively homogenous, with differences for example in ethnicity or gender between case and control groups, this may result in apparent but artefactual linkage[111]. In a condition like essential hypertension with an expected genetic basis, comparison of affected cases with a positive family history and unaffected cases with a negative family history may improve the likelihood of identifying a relevant genetic marker, and secondly reduce the risk of error arising from delayed penetrance of the phenotype.

Linkage studies test for a departure in the random segregation of both traits and genetic markers in families and look for evidence of excess sharing of a particular allele in affected relatives that is not consistent with random Mendelian segregation[111]. Sibling or relative pairs may be examined, but analysis can be complicated by incomplete or delayed penetrance of the phenotype. Both these methods are very dependent on a low likelihood of recombination events (<1%) occurring between the marker and the trait. Association studies with non-specific markers are unlikely to be helpful due the low sensitivity of the method, but identification and use of functionally significant gene polymorphisms, which lie close to or within candidate gene loci, as specific markers may improve the likelihood of a positive result (Table 1.2.2). The development of increasingly detailed genetic maps will result in the availability of more informative markers in the future[35,25].

Multifactorial determination of a trait will also weaken the power of these methods, and in hypertension, either by restricting the analysis to severe hypertensives or by using intermediate phenotypes such as plasma renin concentration, total body sodium or ACE activity, the likelihood of detecting significant linkage may be improved. In the case of polygenic disease, the relatively small contribution to the overall phenotype made by a single locus may make its effects too small to detect by allele sharing methods. A different approach is to search directly for mutations in candidate genes. Sequence variations do not always alter function as they may not alter the amino acid sequence or they may occur in non-coding regions, but a mutation can be followed through a pedigree and its relationship with a phenotype be ascertained[22,35,21].

The finding of renin gene polymorphisms in rat models of genetic hypertension, as reviewed above, prompted Soubrier et al (1990) to look for similar findings in essential hypertensives with a positive family history (at least one affected parent and one sibling) compared with normotensives with no family history. RFLPs were detected after digestion of genomic DNA with three restriction endonucleases followed by probing with a renin cDNA fragment and a 5' genomic DNA fragment, but no significant difference in allele frequencies were seen between hypertensive and normotensive subjects in the French Caucasian population studied[112]. A similar result was found in a Japanese family with a high incidence of essential hypertension[38].

A linkage study using a dinucleotide repeat sequence 3' of the angiotensinogen gene, looked at hypertensive sibling pairs from Salt Lake City and Paris and found a significant excess of shared alleles in male Parisian hypertensive siblings, but greater statistical significance was achieved when severe hypertensives from both populations were analysed together[40]. DNA sequence analysis of fifteen angiotensinogen variants identified five with 5' nucleotide substitutions and ten with silent or missense variants. Only variants with the resulting amino acid substitutions M235T and T174M (occurs with M235T) showed linkage disequilibrium, and both elevated BP and increased plasma angiotensinogen levels were found in heterozygote and homozygote M235T carriers[40]. In two association studies performed on Caucasian and Japanese populations, the M235T variant again occurred at a higher frequency in hypertensives and was associated with elevated plasma angiotensinogen[41,44]. Substrate level may well be important as the K_m for the enzymatic cleavage of angiotensinogen by renin is close to the plasma level. Two groups have reported linkage of the angiotensinogen locus with pre-eclampsia, using the 3' dinucleotide repeat sequence in a linkage study and the M235T variant in an association study [42,43].

However both the M235T and T174M polymorphisms are at some distance from the cleavage sites of the human angiotensinogen molecule. Significant linkage has been demonstrated between hypertension and the angiotensinogen locus using a 3' dinucleotide repeat sequence in sixty-three UK families with two or more members with essential hypertension, but in this study no specific linkage was demonstrated with either the M235T or T174M polymorphisms[113].

An insertion/deletion (I/D) polymorphism within intron 16 of the ACE gene has been found to account for approximately half of the total phenotypic variance in serum ACE in a population of healthy, normotensive Caucasians, with higher serum ACE in those homozygous for the deletion (DD) allele[114]. Despite the findings of linkage between the ACE gene, or a locus in close proximity to it and hypertension in rats, linkage has not been found in the majority of studies in human

populations[115,52,51,53]. However in one report, linkage of the I allele was described in essential hypertensives with a positive family history when compared with normotensives with a negative family history[50].

Interestingly this insertion/deletion (I/D) polymorphism in the ACE gene has been found to be linked with myocardial infarction (MI)[116], parental history of MI[117], hypertrophic cardiomyopathy[118], ischaemic and idiopathic dilated cardiomyopathy[119]. The DD genotype appeared to act as an independent risk factor for MI and was associated with a significant increase in plasma ACE[116]. In groups matched for age, sex and BP, a statistically significant association between LVH by ECG criteria and the ACE DD genotype was apparent in men, with a stronger association observed in normotensives[120]. This has raised the interesting possibility of screening populations for underlying genetic risk factors for development of hypertension related target organ damage, and might also have implications for the strategies directed at primary prevention of MI[121-123], particularly where it may be possible to modify the gene-environment interaction[20]. However some investigators have found that the DD genotype may be overestimated due to the occasional failure of the insertion allele to amplify using the polymerase chain reaction in DNA samples from heterozygotes, so mistyping ID as DD[124].

The S_A gene, a candidate gene for hypertension on the SHR chromosome 1, was found to have a human counterpart on chromosome 16 with a deduced amino acid sequence showing 84% identity with the deduced rat sequence which suggests that the gene may well be functional and under selection pressure. No function has yet been ascertained despite searching for sequence homology with known proteins, but 30.6% homology was seen with acetyl coenzyme A synthase. A *Pst* 1 polymorphism was found to be linked with hypertension in a middle-aged Japanese population but not linked with potential confounding traits such as body mass index, plasma cholesterol or fasting glucose[80]. However other investigators have found no such linkage between hypertension and this RFLP or with other markers for the S_A gene[82]. No intermediate phenotype or gene product has been identified as yet and the role of S_A remains obscure, but it may simply be a marker for another gene in close proximity.

Two studies have shown linkage of hypertension but not obesity with the insulin receptor (R1-) allele based on an *Rsa* 1 RFLP arising from the insertion of a CA repeat sequence in intron 9[74]. This is of interest in view of the association of insulin resistance and essential hypertension. Conflicting results were obtained using a microsatellite polymorphism located in intron 2, where derived allele frequencies showed similar distributions between hypertensive and normotensive subjects suggesting that the causative polymorphism may lie closer to intron 9 than to intron

2[75]. Recently linkage has been described between a marker in close proximity to the α-adducin locus and hypertension in an association study[76]. This membrane skeleton protein has previously been implicated as a potential factor in the genetic hypertension of the MHS rat by altering ion transport[125].

These results support the concept that variants of 'candidate' genes may contribute to the heritable aspects of an individual's variation in BP and affect their response to the environment. The important contribution to BP homeostasis of the RAS and the finding of genetic polymorphisms occurring in components of this system in both rat models and in humans, has led to the desire to study the contribution of individual genes, in particular by subtle alterations in the regulation of their expression and function. New methods, in particular transgenesis (section 1.6), have been developed which could help identify how hypertension might result from altered gene regulation and function[126]. The physiological and pathological roles of the RAS and the kidney in BP homeostasis and in hypertension will be considered next.

1.4 The Renin-Angiotensin System and its regulation

Renin is a single chain glycosylated carboxypeptidase of MW 41 kD. Unlike most members of the aspartyl protease family it is quite substrate specific, cleaving angiotensinogen with optimal function at pH 5.5-6.0. The active site of the renin molecule is located in a cleft between two almost symmetrical lobes (reviewed in [127]).

Human and rat genomes contain one renin gene located on Chromosome 1. These are expressed highly in the kidney, but in mice high expression is also found in the submaxillary glands (SMG). Some strains of mice e.g. C57BL/6 and Balb/c have one renin gene (*Ren*-1c), but certain strains such as DBA/2J and Swiss have two, namely *Ren*-1d and *Ren*-2d[128-131]. *Ren*-2d is thought to have arisen as a consequence of a gene duplication event occurring between 2.75 - 7.2 million years ago and it encodes a thermolabile, non-glycosylated isozyme which is expressed at high levels in the granular convoluted tubules of the SMG[132]. *Ren*-1d is expressed in two gene strains principally in the JGA cells of kidney at an approximately equivalent level to *Ren*-2d expression in the SMG on a per cell basis. Both *Ren*-1d and *Ren*-2d mRNA levels were found to be approximately equivalent in kidneys of two-gene strains (DBA/2J) by Field and Gross (1985) using primer extension analysis[133]. This conflicted with earlier findings suggesting that *Ren*-1d was mainly responsible for renin synthesis in the kidney of two-gene strains on the basis of thermostability analysis of kidney renin activity and the observation that antibody raised against SMG

renin failed to reduce renin activity in kidney[132]. *Ren*-1d expression was found at significantly lower levels in SMG compared to *Ren*-2d on a per cell basis[134] (Table 1.4.1).

Table 1.4.1

Tissue	Cell type	*Ren*-1c	*Ren*-1d	*Ren*-2d
kidney	JGA cells	+++	+++	+++
SMG gland	granular convoluted tubule cells	+	+/-	+++
Adult adrenal	Zona fasciculata, X zone	+/-	++	++
Testes	Leydig cells	+	++	+
Ovary	Theca, corpus luteum	?	++	++

Differential expression of mouse renin genes on a per cell nucleus basis - adapted from Sigmund & Gross (1991)[135].

All mouse renin genes consist of 9 exons and 8 introns with relatively high homology existing between cDNA sequences, 97% between *Ren*-1c and *Ren*-2d and 99% between *Ren*-1c and *Ren*-1d[135]. Sequence divergence is found between *Ren*-1d genes and *Ren*-2d in the 5' region, but it is not clear that this region is responsible for the differing tissue-specific expression of the mouse renin genes[134-137]. Renin gene expression does appear to be influenced by hormone levels showing higher levels of expression particularly in male mouse SMG, but also in female rat and mouse liver[138].

Approximately 88% homology exists between mouse *Ren*-1 (m*Ren*-1) and rat renin (r*Ren*) genes, and 78% homology between m*Ren*-1 and human renin (h*Ren*) genes at the cDNA level. At the amino acid level, all three mouse gene products exhibit ~97% homology with the most significant differences between the *Ren*-1 and *Ren*-2 gene products occurring at three asparagine-linked glycosylation sites, which may account for the differences in glycosylation and thermostability[139], (reviewed in [135]). The physiological role of *Ren*-2 in two gene strains of mice is not clear and awaits the results of experiments designed to knock out individual renin genes[140], but *Ren*-1c, *Ren*-1d and *Ren*-2d derived mRNA have all been seen to respond appropriately to dietary NaCl by an RNase protection assay measuring steady state levels of mRNA[141]. Tissue distribution of rat renin transcripts is similar to mouse *Ren*-1, but no expression is found in the rat SMG[136,138].

In cells of the juxta-glomerular apparatus (JGA), translation of renin mRNA produces pre-prorenin which then undergoes cleavage of its amino terminal 20 amino acid signal sequence and glycosylation during passage through the endoplasmic reticulum to give prorenin. Prorenin is then packaged in the Golgi apparatus, and a

proportion is directly secreted as prorenin in vesicles via the constitutive pathway while other protogranules coalesce to form larger secretory granules[142]. The constitutive secretion of prorenin appears to be dependent on the rate of transcription of the renin gene. In the secretory granules the prosegment is cleaved by the action of lysosomal enzymes, most probably cathepsin B in the kidney[143], and removal of the prosegment results in exposure of the active site or cleft.

The main site of synthesis of renin in the kidney is within the specialised cells of the juxtaglomerular apparatus (JGA). Juxta-glomerular (JG) cells are derived from modified smooth muscle cells which adopt the morphology of secretory cells with between five and twenty cells located in the afferent arteriolar wall of the vascular pole of the glomerulus. Electron microscopy studies have suggested that renin secretion from granules involves exocytosis in response to stimulatory events. JG cells are innervated by renal sympathetic nerves and via β_1 adrenoceptors, rapid release of the renin contained in granules occurs resulting in a rise in PRA. Stimulation of the renal nerves reduces both renal blood flow (RBF) and glomerular filtration rate (GFR) and increases renin mRNA particularly at higher levels of stimulation. Such effects can be reduced or blocked by pre-administration of the β_1 antagonist, atenolol[144]. Likewise renal denervation or β-antagonists result in a reduction in both kidney preprorenin mRNA content and renin immunoreactivity[145].

Tubular epithelial cells of the thick ascending Loop of Henle and distal tubule form the macula densa in association with modified extra-glomerular mesangial cells. The macula densa is thought to sense distal tubular sodium delivery and an increase in distal tubular luminal NaCl concentration results in a reduction in renin synthesis and release. Postulated mediators include nitric oxide (NO), as suggested by both *in vivo* inhibition of renin release in response to L-NAME, an NO synthase inhibitor[146] and stimulation in response to NO liberators such as sodium nitroprusside in cultured mouse renal JG cells[147]. A constitutive form of NO synthase (NOS I) has been identified in the macula densa[148] and there is evidence to support a mediator function for NO in afferent arteriolar constriction, JG cell renin release, mesangial cell and podocyte contractility and hence glomerular haemodynamics and tubulo-glomerular feedback[149]. An adenosine (A1) receptor analogue had been shown to mimic the effect of increased NaCl delivery inhibiting renin release, but though adenosine itself did induce inhibition of renin secretion in a dose responsive way, the maximum degree of inhibition attained was considerably less than that resulting from increased luminal NaCl delivery itself in isolated perfused rabbit kidney JGA. This suggested that adenosine alone could not be the sole mediator of the macula densa response[150].

An increase in the number of secretory JG cells, their renin mRNA content and post-translational processing and release of renin occurs in response to stimuli such as low plasma sodium, diuretic use, ACE inhibition and in pathological conditions such as renal artery stenosis, malignant hypertension or in the clipped kidney of experimental two kidney - one clip (2K-1C) rats[151-156,127]. A decrease in whole kidney renin mRNA content occurs in the contralateral kidney of the 2K1C hypertensive rat, and this effect is mimicked by exogenous infusion of Ang II by osmotic minipump into uninephrectomized rats. This supports a role for Ang II in down-regulating renin gene transcription[157,158]. However the clipped kidney is also exposed to high Ang II but an elevated renin mRNA content is found suggesting that additional factors, perhaps raised intra-renal pressure might be necessary.

In addition to JG cells, renin has also been found in proximal tubular cells. This could be due to glomerular filtration and endocytosis of filtered renin, peritubular delivery or synthesis de novo. Though previous investigators had failed to identify renin transcripts by in situ hybridization, Moe et al (1993) showed the presence of renin mRNA in cultured rabbit proximal tubular cells (PTC) and microdissected rat proximal tubules. Contamination of the PTC culture with JG cells could not be excluded, but renin synthesis by JG cells in PTC culture media could not be demonstrated. Stimulation by five to six days pretreatment with enalapril was however necessary to detect renin mRNA in the microdissected tubules[159].

In plasma from humans and other species, circulating active renin is derived from the kidney, but cryoactivation (0-4°C), trypsin activation or acidification of plasma increases this five to ten fold due to conversion of 'inactive renin' to renin. The nature of 'inactive renin' has been a subject of much debate (reviewed in [160]). Measurement of prorenin has been problematical due to the variability in values observed as a consequence of the methods used for blood retrieval, the type of anaesthesia administered or the method used for prorenin activation[161]. Bilateral nephrectomy in rats removed all circulating active renin, and significantly reduced but did not abolish 'inactive' renin. This therefore suggested that 'inactive' renin may be derived from or sequestered in other tissues with similar findings being reported in humans[162] . It has been reported however that the remaining Ang I generating activity measured in plasma of nephrectomized animals is not due to either prorenin or renin, but to an alternative molecule which elutes from a G3000SW column as a single peak with a MW of 65,000, close to the MW of angiotensinogen (compared with MW 48,000 of prorenin and MW 40,000 for active renin in normal rat plasma)[163]. These findings were supported by immunological data using polyclonal antibodies directed against the pro-fragment of prorenin, which confirmed the absence of prorenin from

nephrectomized rat plasma[163]. This Ang I immunoreactive material (AI-IM) which interferes with radioimmunoassay of renin, is thought to be generated by trypsin digestion of angiotensinogen, which is inevitably higher in nephrectomized plasma[162]. Radioimmunoassays which do not include a step for AI-IM removal by a cation exchange resin should therefore be interpreted with caution[162].

In another study of trypsin activated plasma from nephrectomized rats, a renin inhibitor CH-732 inhibited Ang I generating activity by approximately 20%, compared with 75 to 90% inhibition using N-ethylmaleimide which inhibits sulphydryl enzymes. It was concluded that the prorenin concentration in 24 hour nephrectomized rat plasma was only 10% of that seen in intact rat plasma with the majority of prorenin-like activity present being due to an unknown sulphydryl enzyme[164].

The role of prorenin remains to be clarified and has been the source of some controversy, partly due to the methodological problems briefly commented on above which may have overestimated prorenin levels following nephrectomy, thereby leading to a search for physiological functions and extra-renal sites of synthesis. The finding of prorenin in high levels at extra-renal sites e.g. placenta and ovary have led some investigators to postulate either direct involvement in tissue RAS, as no *in vivo* extra-renal 'convertase' has yet been identified[165,166], though a number of candidates exist that could function as local prorenin activating enzymes (reviewed in [167]). Other putative roles for prorenin have been suggested, including a vasodilator function opposing renin mediated vasoconstriction[168] and as a growth factor contributing to cardiac hypertrophy following tissue uptake of circulating prorenin by the heart[160].

The substrate of renin is angiotensinogen which is primarily synthesized in the liver and cleaved by renin to release the decapeptide Ang I. Species specificity of the cleavage reaction appears to be a consequence of the amino terminal amino acid sequence around the cleavage site[169]. Rat and mouse angiotensinogen cDNAs have been cloned and the deduced amino acid sequence is 453 residues with a signal sequence of 24 residues[170,171]. Sequencing of the human angiotensinogen gene from a genomic DNA library has shown the gene to be 12 kb in length with five exons and four introns and like rat and mouse genes, considerable similarity with other serine protease inhibitors e.g. α_1-antitrypsin, and anti-thrombin exists. Putative regulatory sequences in the 5' flanking region respond to glucocorticoids, oestrogens, cAMP and mediators of the acute phase response resulting in increased plasma angiotensinogen levels[172,173]. Rat hepatocyte cultures have been used as an *in vitro* system and results have suggested that interleukin-6 (IL-6) may be a mediator of the increase in angiotensinogen synthesis seen in acute inflammation, but that this effect appears to depend on the presence of glucocorticoid, and may be partly mediated via an increase

in intracellular cAMP[173]. Regulatory mechanisms of angiotensinogen gene activation have also been studied in adipose tissue using a pre-adipocyte culture transfected with a mouse angiotensinogen promoter-CAT (chloramphenicol acetyl transferase) reporter chimaeric construct[174]. Induction of adipocyte differentiation resulted in accumulation of angiotensinogen mRNA. Constructs with deletion mutants of a specific 5' sequence (-96 to -52) within the promoter region exhibited reduced adipocyte differentiation coupled activation[175].

Angiotensinogen mRNA has been identified in a number of extra-hepatic tissues including kidney, brain, spinal cord, adipose tissue and aorta. Two studies have shown that sodium depletion induced by low dietary sodium and frusemide resulted in an increase in renal angiotensinogen mRNA. One group found no significant effect of low sodium on hepatic mRNA while another found a significant increase[176,177]. *In situ* hybridization of kidney has demonstrated angiotensinogen mRNA to be localized mainly to the proximal tubules with increased expression in response to salt depletion[178]. In the 2K1C rat, whole kidney angiotensinogen mRNA levels were not significantly different in either the clipped or the contralateral kidney when compared to respective control kidneys from sham operated animals, in contrast to the observed changes in renin mRNA[157]. However angiotensinogen mRNA, measured by densitometry of Northern blots (relative to βactin mRNA) was found to increase at relatively low levels of renal nerve stimulation possibly concurrent with the increase in JGA cell renin release[144].

ACE is a zinc protease which catalyses the hydrolysis of dipeptides from the carboxyl terminus of polypeptides. It converts the inactive decapeptide Ang I to the active octapeptide Ang II. It also inactivates bradykinin by two sequential dipeptide hydrolysis reactions. Two ACE isoforms are derived from a single gene by transcription from two alternative promoters resulting in the somatic form, a glycoprotein of 170 kD, and the germinal form with a lower MW of 110 kD consisting of the active carboxyl terminal domain. Somatic ACE gene expression and enzymatic activity are found in highest concentrations in vascular endothelium and in absorptive epithelial cells of the small intestine, renal proximal tubular epithelium and epididymus. The germinal form is uniquely expressed in a stage-specific pattern in developing spermatozoa[179]. In all species, both forms consist of an intracellular hydrophilic carboxyl-terminal sequence and a trans-membrane hydrophobic domain indicating that ACE exists as an integral membrane protein. *In vivo* soluble circulating ACE is found though the source is not clear, but it is thought to be primarily derived from vascular endothelium lining pulmonary capillaries. An active soluble from of ACE is produced by cultured Chinese Hamster Ovary (CHO) cells by proteolytic

cleavage at the carboxyl terminal region (Arg-1137 and Leu-1138)[180]. Along with the other components of the RAS, ACE mRNA transcripts have been found in whole kidney. Specific patterns of mRNA expression relating to development have been reported in kidney, heart, aorta, lungs and liver along with a changing profile of serum ACE activity with age[181].

Exogenous infusion of Ang II has been found to exert a negative feedback effect reducing both ACE activity and pulmonary mRNA levels. However the resulting change in BP and possible effects of Ang II on other neuroendocrine systems does not exclude the possibility that this may be an indirect effect. ACE inhibition with Quinapril caused a statistically insignificant fall in plasma Ang II, but both pulmonary and serum ACE activities were significantly reduced while an increase in pulmonary ACE mRNA resulted. In contrast testicular ACE activity and mRNA content were unaffected reflecting the fact that the ACE inhibitor did not cross the blood-testis barrier[182].

The product of the proteolytic cleavage by ACE on Ang I is the active octapeptide Ang II. The physiological and potentially hypertensive effects of Ang II can be summarised in Fig 1.4.1. Ang II is a potent vasoconstrictor with a preferential effect on the efferent arteriole producing a modest decrease in GFR, profound decrease in RPF, while filtration fraction, MBP and renal vascular resistance increase in response to infusion[183]. The peptide exerts a net anti-natriuretic effect by inducing retention of sodium by direct action on Ang II receptors in the proximal tubule[184]. Via AT_1 receptors, Ang II appears to be involved in both basal and stimulated aldosterone synthesis and release[185-187]. A tubulo-glomerular feedback system exists whereby afferent arteriolar tone and glomerular filtration rate responds to distal tubular perfusion pressure and sodium delivery and this is modified by altered peritubular Ang II concentration[188]. Ang II has been reported to act centrally causing increased thirst and appetite for salt, and release of ACTH, β-endorphin, prolactin, luteinizing hormone and vasopressin[189] and act peripherally on presynaptic receptors stimulating catecholamine release[190,191].

Fig 1.4.1.

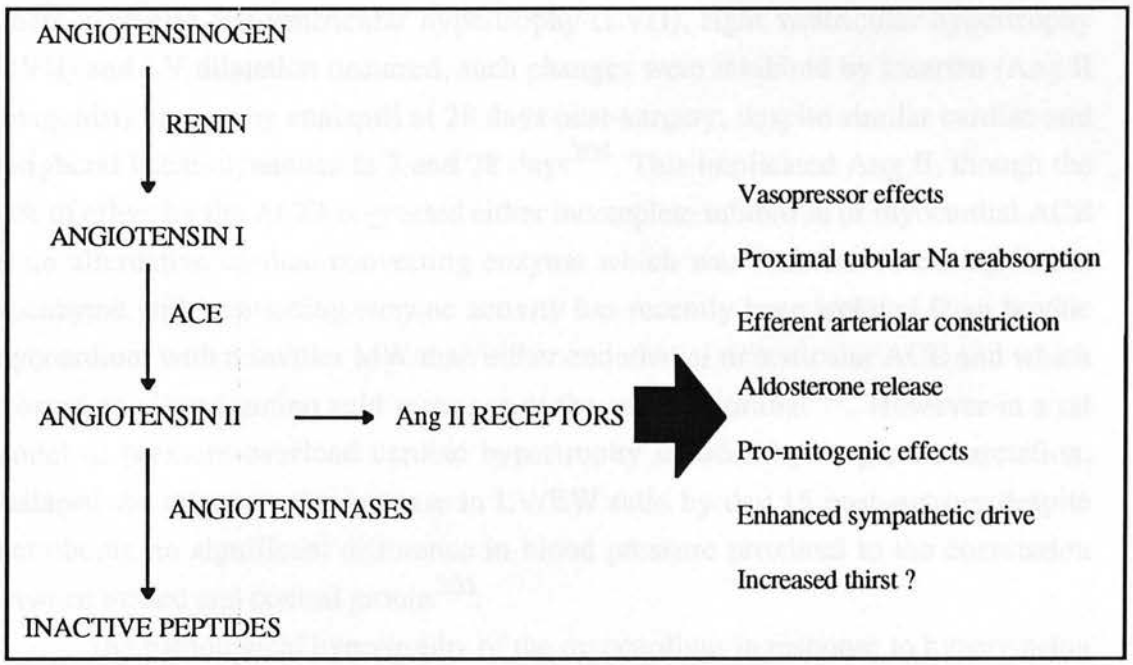


Fig 1.4.1 Mechanisms by which RAS activity and Ang II generation may elevate BP.

The pro-mitogenic actions of Ang II, like other growth factors PDGF, FGF-1, and TGFβ_1, may via induction of proto-oncogenes including c-*myb*, c-*myc*, c-*fos* and c-*jun*, be partly responsible for the smooth muscle cell hyperplasia occurring in myo-intimal proliferation, vascular wall remodeling and left ventricular hypertrophy in response to hypertension or endothelial damage[192-198]. Following endothelial cell injury and denudation by balloon catheterisation in rats, the normal process of smooth muscle cell proliferation and migration into the intima was decreased by continuous treatment with an ACE inhibitor (ACEI), cilazepril[199]. A greater inhibition of neointimal thickening seen with an ACEI compared to a calcium antagonist at equi-hypotensive doses[200]. Balloon catheter induced endothelial damage leads to both increased medial expression of angiotensinogen mRNA, maximal at one week post injury, and the appearance of angiotensinogen mRNA in the neointima of the damaged vessel[201]. Rat cardiac angiotensinogen gene expression is also seen to increase in non-infarcted myocardium following coronary artery ligation[202].

In response to hypertension, smooth muscle cell hypertrophy is seen to predominate over hyperplasia in walls of larger conducting vessels. *In vitro* Ang II has been shown to induce hypertrophy but not hyperplasia of cultured, quiescent and relatively differentiated, rat thoracic aortic smooth muscle cells (in serum-free medium) and this effect was blocked by a specific Ang II antagonist. Ang II did not however

potentiate the growth response to 10% fetal bovine serum or partially purified platelet derived growth factor[203]. In a rat model of volume overload cardiac hypertrophy where eccentric left ventricular hypertrophy (LVH), right ventricular hypertrophy (RVH) and LV dilatation occurred, such changes were inhibited by losartan (Ang II antagonist) but not by enalapril at 28 days post-surgery, despite similar cardiac and peripheral haemodynamics at 7 and 28 days[204]. This implicated Ang II, though the lack of effect by the ACEI suggested either incomplete inhibition of myocardial ACE or an alternative cardiac converting enzyme which was resistant to enalapril. An isoenzyme with converting enzyme activity has recently been isolated from bovine myocardium with a smaller MW than either endothelial or testicular ACE and which showed an altered amino acid sequence at the amino terminal[205]. However in a rat model of pressure-overload cardiac hypertrophy induced by surgical coarctation, enalapril did attenuate the increase in LV/BW ratio by day 15 post-surgery despite there being no significant difference in blood pressure proximal to the coarctation between treated and control groups[206].

The pathological hypertrophy of the myocardium in response to hypertension involves both cardiomyocytes and non-myocyte cells, in particular fibroblasts. These may contribute to the impaired diastolic relaxation and contractile function with reduced ventricular compliance seen with increasing fibrillar collagen content of the ventricular wall. Some studies have suggested that circulating Ang II and aldosterone may be implicated in this cardiac fibroblast response[207,208].

Most of the known systemic effects of Ang II appear to be mediated by AT_1 receptors which are G protein coupled with a seven transmembrane domain structure and which either activate phospholipase C or inhibit adenylate cyclase. Two isoforms have been identified, AT_{1A} and AT_{1B}, with differing tissue distributions[167]. A second receptor (AT_2) has been found which is expressed highly in embryonal tissues, possibly having a role in vascular growth during development, but in addition it is expressed at low levels in adults[167]. Interestingly low protein feeding in rats has been shown to increase the binding of Ang II to isolated glomeruli as a consequence of increased receptor number, associated with an increase in AT_1 receptor mRNA expression as demonstrated by Northern blotting and *in situ* hybridization in cortex and medulla. No change in receptor affinity was reported[209]. A reduced RPF and GFR but an increased intra-renal vascular resistance and a fall in PRA occurred in response to the low protein diet. Such changes were reversed by ACE inhibitors or losartan (a specific AT_1 receptor antagonist).

The classic view of the RAS has been of a circulating endocrine system whereby circulating angiotensinogen produced by the liver was cleaved by plasma

renin to produce Ang I which was then converted to Ang II on passage through the lungs and other vascular beds. Active circulating Ang II was then available to act on target tissues and exert its physiological effects. This view has been challenged by the increasing amount of evidence suggesting that local RASs are present and operating within tissue types in a paracrine fashion and are not dependent on either circulating enzyme or substrate levels (reviewed in [210-213,189]). The efficacy of ACE inhibitors in 'normal' and 'low' renin essential hypertensives, referring to patients with normal or low plasma renin activity has suggested that either ACEI lowered blood pressure by methods independent of the RAS or they inhibited ACE and reduced Ang II generation within tissues where PRA did not reflect the level of RAS activity[214]. In the heart, adrenal, testes, and brain various investigators have identified the necessary components, in particular renin, angiotensinogen and ACE mRNAs[215,216,211]. Even within the kidney, there is evidence for a local RAS[217], possibly acting within the proximal tubule with both angiotensinogen and renin mRNA reported together with ACE activity in the apical brush border[159].

Evidence for a cardiac RAS is more controversial[213,218]. Tissue culture experiments e.g. stretch induced cardiac hypertrophy in neonatal cardiac myocytes was associated with production of Ang II[213], though this may not reflect the *in vivo* situation. In rat models of myocardial infarction, left ventricular hypertrophy[219] and in the LV of aging rats[220], up-regulation of cardiac ACE and angiotensinogen mRNA levels have been reported, though there may be species variation. However the lack of convincing and reproducible demonstrations of renin mRNA in the normal, healthy heart has been the missing link for a cardiac RAS[138]. PCR artifact or non-specific hybridization conditions during Northern blot analyses can erroneously identify mRNA species (reviewed by [218]). The presence of renin in cardiac tissue could reflect either *in situ* synthesis or uptake of circulating renin. Binephrectomy led to the disappearance of tissue renin activity suggesting that uptake was the main source, but persistence of Ang II in anephric rats particularly in aorta, heart and adrenal did suggest that Ang II could be formed by alternative pathways in rats e.g. cathepsin G or chymase could cleave Ang I to give Ang II[167,205]. However in pigs, 30 hours after nephrectomy there was no detectable renin, Ang I or Ang II in either plasma or in LV tissue suggesting that in the healthy heart, uptake of circulating renin accounted for any angiotensin peptides detected in atrial and ventricular tissue[221].

1.5 The kidney in essential hypertension

In many primary renal diseases such as glomerulonephritis, pyelonephritis or polycystic kidney disease, hypertension is a common finding. What is the role however of the kidney in essential hypertension? This question was addressed by Luke (1993) in his review[222]. The three main areas of experimental evidence implicating the kidney lie with the ability to transplant hypertension or normotension with the kidney[84,223], the presence of abnormal renal vascular responses during the pre-hypertensive state[224], and the association of high sodium chloride intake with hypertension in epidemiological studies[225,222]. The first two points are dependent on the assumption that there is no secondary damage to the kidney occurring during the pre-hypertensive period when there is no measurable systemic hypertension, but if any such damage was present, it might alter the kidney's response to vasoactive agents or its ability to excrete a sodium load. The findings of normal exchangeable sodium, plasma volume and extracellular fluid (ECF) volume in established essential hypertensives are found with an observed right shift and resetting of the pressure-natriuresis curve[226]. Increased sodium intake and/or a degree of sodium retention may initially lead to an increase in ECF volume, an increase in cardiac output and GFR but no change in peripheral resistance. An initial consequence of this may be increased ouabain-like sodium/potassium ATPase inhibitory activity[225] and synthesis and release of atrial natriuretic peptide (ANP)[227,177]. A raised intracellular sodium could, as a consequence of the reduced sodium gradient result in an increase in cytosolic calcium and enhanced smooth muscle contractile activity possibly accompanied by a compensatory increased natriuresis induced by ANP. Hence the transition to a state of normal ECF volume, normal cardiac output but raised peripheral resistance could result, as seen in established essential hypertension. Thereafter adaptive structural changes in vessel walls may maintain the elevation in blood pressure[228, 229].

It has also been postulated that altered renal haemodynamics and the renal RAS may be central to inducing hypertension even in apparent 'normal' or 'low' renin hypertension (reviewed in [224]). Laragh (1992) discussed the concept of a sub-population of relatively ischaemic nephrons which might show increased renin synthesis relative to the basal blood pressure and fail to suppress plasma renin activity in response to a stimulus such as a sodium load. There is some pathological evidence in favour of this[166]. Patients with 'normal' renin may in effect show an inappropriately high plasma renin activity for their blood pressure. The efficacy of ACE inhibitors in apparent low or normal renin essential hypertension supports this hypothesis. Lower GFR and RBF in young, pre-hypertensive four week old SHR

correlated with higher BP during the development phase of hypertension in eleven week old SHR, but not with BP in established hypertension (sixteen weeks old). These haemodynamic alterations were shown to be genetically linked to hypertension in an SHR/WKY F2 population, and appeared to relate to the higher PRA and greater sensitivity to ACE inhibition found in four week old SHR compared to WKY[230]. Again in an SHR/WKY F2 population, it was observed on regression analysis that afferent arteriolar diameter at seven weeks of age correlated with SBP at twenty-three weeks of age ($r = -0.36$) suggesting that a specific renal pathology was in some way responsible for development of genetic hypertension[231].

Stop-flow pressure (SFP) tubuloglomerular feedback responses have indicated that physiological increases in renal interstitial Ang II concentration, independent of circulating Ang II, enhance tubuloglomerular feedback response sensitivity. A lower glomerular capillary pressure is seen at equivalent end proximal tubular fluid flow rates in the presence of increased interstitial Ang II[232,188]. Therefore any increase in single nephron GFR (SNGFR) is attenuated in the presence of increased interstitial Ang II. Together with the Ang II mediated decrease in distal tubular sodium delivery (due to proximal tubular sodium reabsorption), this results in a sustained reduction in distal tubular sodium delivery. The vasoconstrictor effects on afferent and efferent arterioles and on glomerular capillaries (via mesangial cell contraction) act together to reduce SNGFR and glomerular plasma flow, and hence further reduce distal tubular flow. The actions of aldosterone on the distal tubule form another mechanism by which Ang II leads to a sustained reduction in sodium excretion.

Other authors have argued that the primary renal abnormality initiating essential hypertension is a congenital reduction in filtration surface area leading to a limited capacity to excrete a sodium load[233]. Reduced nephron number arising from disease or surgery will often give rise to systemic hypertension. In the clinical situation, progression of hypertension may well result from a further reduction in nephron number as a consequence of glomerular hypertension and progressive hypertensive glomerulosclerosis. MHS rats show smaller kidney to body weight ratios compared with the MNS from 25 days to 100 days of age[234]. SHR do not however show a statistically significant reduction in nephron number and though slightly lower values for the glomerular capillary ultrafiltration coefficient were seen compared with WKY, there was no significant difference in filtration surface area or permeability[235]. SHR exhibit a similar ability to exhibit a sodium load[236].

In summary there are a number of reasons to implicate the RAS in essential hypertension. The finding of linked polymorphisms of candidate genes for components of the RAS to hypertension in both humans and experimental animal

models, the known physiological effects of RAS activation leading to elevation in blood pressure, the likely involvement of the kidney in hypertension, and the subsequent adaptive structural changes that may in part be Ang II dependent has directed attention towards this system. It is important to bear in mind that hypertension is a polygenic disease and other factors are undoubtedly involved. This interest in the RAS in essential and genetic hypertension has therefore led to the application of transgenic technology in order to study gene function and regulation *in vitro* and *in vivo* (reviewed in [237,238]).

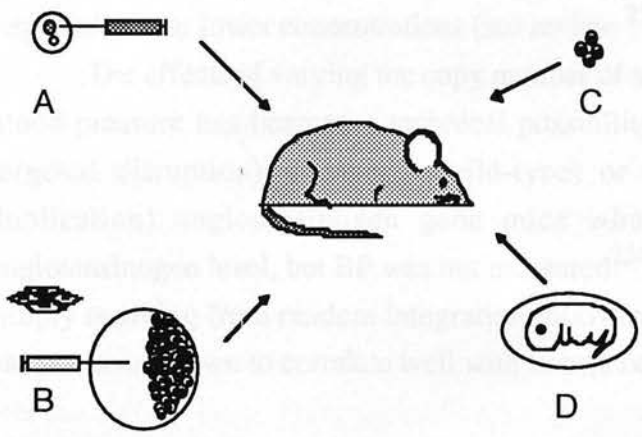
1.6 Transgenesis: Materials and Methods

A transgene may be defined as a gene or gene construct introduced into a species by one of a number of methods and following stable chromosomal integration, the transgene is transmitted through the germ line by Mendelian inheritance to subsequent generations[239, 240]. For experimental purposes most transgenic animal work has been done in mice and more recently in rats. Developments of the technology has led to extension of the work into larger animals which has offered increased opportunity for both experimental and commercial use[241]. The effects of manipulating an individual gene within a complex system and the subsequent genetic and environmental interactions giving rise to the resulting phenotype can then be studied *in vivo*.

The genetic material forming the transgene construct may consist of DNA from either the same species or from a different species. A gene's own promoter sequence can be used to direct appropriate tissue specific and temporal expression. Alternatively an heterologous promoter can be used to direct expression of a transgene to specific tissues, or allow expression to be switched on in response to a defined and controlled stimulus. For example the α1-antitrypsin promoter can be used to direct expression principally to the liver, thereby resulting in synthesis and secretion of the gene product from hepatocytes[242]. Metallothionein promoters can respond to dietary heavy metals mediated by heavy metal sensitive response elements within the promoter sequence. These can circumvent problems of transgene expression during foetal development or allow the study of the effects of transgene function starting at a defined time point. Regulatory elements within promoter sequences of genes can be studied using 'reporter' gene constructs where a promoter sequence is linked to a gene encoding an easily detectable enzyme[174,243]. These may be designed to express either a conveniently assayable product e.g. the Escherichia coli Lac Z gene codes for β-galactosidase which produces a blue colour on cleavage of the substrate X-gal (5-

bromo-4-chloro-3-indolyl-β-D-galactosidase), and the bacterial enzyme chloramphenicol-acetyl transferase (CAT) catalyses transfer of an acetyl group from acetyl CoA to chloramphenicol and hence promoter driven expression of a CAT reporter can be quantified by generation of acetylchloramphenicol[244]. Other markers of gene expression used have included SV40 which by production of T antigen leads to tumourigenesis[245]. Promoter-reporter constructs can then be designed with systematic deletions of putative regulatory regions within promoter sequences so enabling identification of the specific sequences which are important for determining regulation of gene expression (see review [240]).

Fig 1.6.1.



A Microinjection of a construct into the pronucleus of a fertilised oocyte.
B Genetically manipulated embryonic stem cells are created by homologous recombination or random integration and injected into a blastocyst.
C Retroviral infection of a four cell embryo.
D Retroviral infection of an embryo.
Embryos are then returned to pseudo-pregnant females.

Fig 1.6.1 Methods used for introduction of genetic material into the genome of the mouse.

Genetic material can be introduced by a number of methods which are summarised in Fig 1.6.1. Direct microinjection of DNA into the pronucleus of fertilised one cell embryos will be discussed in more detail below. Retroviral infection of 4-cell and later stage embryos is followed by re-implantation into a pseudo-pregnant foster mother. More recently techniques for inhibiting or abolishing endogenous gene function have been developed. Selective inactivation or mutation of a targeted gene can be performed *in vitro* by homologous recombination in embryonic stem (ES) cells[246,247] (reviewed in [248]). The gene of interest is interrupted by the introduction of a homologous sequence carrying a selectable marker, usually sequences which confer antibiotic resistance. Genetically altered ES cells carrying the mutation are then selected for by resistance to the relevant antibiotic e.g. neomycin, in culture and are then introduced into a blastocyst. Resulting progeny will be chimaeric with coat colour

being used to identify the relevant ES cell origin and confirm germ-line transmission of the mutated gene in subsequent generations. Gene knock-out technology and phenotype analysis is limited at present by the ability to culture ES cells only in certain strains of mice. Targeted disruption can be lethal during embryogenesis, but results of such studies are identifying new roles for some genes in development and apparent functional redundancy of others[249-252].

Partial loss of gene function can be achieved by introducing genes which produce high levels of antisense RNA which may interfere with pre-mRNA transport or hybridize to the target mRNA and interfere with splicing and translation. Inducible promoters have been used with some success in plants allowing some control over antisense mRNA production. Improved efficiency with this technique is being attempted by using antisense RNA with catalytic properties or ribozymes to cleave the target mRNA at lower concentrations (see review [253])

The effects of varying the copy number of a gene on a quantitative trait such as blood pressure has become a technical possibility with the report of one-copy (by targeted disruption), two-copy (wild-type) or three copy (by targeted tandem duplication) angiotensinogen gene mice who showed alterations in plasma angiotensinogen level, but BP was not measured[254]. However transgene copy number simply resulting from random integration following microinjection as detailed below has not been shown to correlate well with transgene expression[255].

Production of transgenic animals by microinjection

To obtain a maximal number of donor oocytes for microinjection, superovulation is necessary, aiming for a two to three fold increase in the number retrieved. This is achieved in mice by an intraperitoneal dose of pregnant mare serum gonadotrophin (PMSG), containing both follicle stimulating hormone (FSH) and luteinising hormone (LH) activity on Day -2 and human chorionic gonadotrophin (hCG) on Day 0, following which the females are placed with fertile males for mating[239,256]. Modification of this was found to be necessary in rats, who required continuous infusion of purified pig pituitary FSH administered by osmotic minipump subcutaneously for 50 hours prior to an intraperitoneal dose of hCG to induce superovulation[257].

The fertilized eggs are then removed, separated from surrounding cumulus cells by enzymatic digestion with hyaluronidase and maintained in culture medium in a CO_2 incubator. When the pronucleii are visible, microinjection of the DNA construct (1 μg/ml) is performed using a glass holding pipette and microinjection needle (<1 μm diameter) and approximately 2 pl of DNA solution is introduced, usually into the

larger male pronucleus. Fertilized eggs surviving this process are reimplanted into the oviducts of pseudopregnant females (mated with vasectomized males) and a variable proportion of the progeny born will then carry the transgene[258].

The transgene exists in the form of concatamers of DNA and randomly integrates into the genome of the recipient. Integration is tested by Southern blot hybridization and analysis of tail DNA from the resulting progeny using a transgene specific sequence as the probe or alternatively by polymerase chain reaction (PCR) using transgene specific sequences for primers. Multiple integration events can occur and this can be assessed by diagnostic Southern blot hybridization. Integration may disrupt endogenous gene sequences or function, and hence any phenotype observed in transgene positive (TG+) animals must arise in more than one TG+ littermate to be certain that an insertional mutagenic effect is not responsible[259]. In situations where insertional mutagenesis may have led to a phenotype, sequencing of the flanking regions around the integration site using the transgene DNA as a template for a probe, may lead to identification of endogenous genes that have undergone sequence rearrangement and are responsible for the new phenotype[260].

Expression of the transgene is then assessed by detecting mRNA by Northern blot analysis and *in situ* hybridization determining both tissue distribution and temporal patterns of expression. The protein product or other intermediate phenotype can be quantified and the resulting phenotype characterized. Breeding strategies can be developed to obtain animals homozygous for the transgene, segregate multiple insertion events or by crossing with a different transgenic line a new transgene can be introduced to create a double transgenic[261]. The species into which the transgene is introduced may directly influence transgene expression. For example strain specific modifiers may affect transgene methylation and hence expression and phenotype[262].

1.7 Studying the Renin-Angiotensin system using transgenesis.

As described above, two distinct renin genes were identified and subsequently cloned from the DBA/2 mouse[129], with *Ren*-2 lying upstream of *Ren*-1 producing a non-glycosylated thermolabile renin isozyme that appeared to have no effect on blood pressure[263]. In order to assess the physiological role and identify regulatory sequences of renin genes in mice, various transgenic mice have been made. *Ren*-1c mice transgenic for the *Ren*-1d gene showed appropriate tissue-specific expression[264], and suppression of transgene expression in the kidney in response to dietary sodium loading suggesting that the *Ren*-1d transgene construct contained the appropriate regulatory sequences[264].

A 24 kb *Xho* I genomic fragment containing the *Ren*-2 gene with 5.3 kb and 9.5 kb of 5' and 3' flanking sequence respectively was microinjected into fertilized pronucleii of one renin gene mice, homozygous for *Ren*-1c[265]. This almost recapitulated the situation found in two renin gene mice, but for the presence of the *Ren*-1c allele in place of *Ren*-1d. Expression of the *Ren*-2 transgene was qualitatively similar to endogenous *Ren*-2 expression in DBA/2 mice, with appropriate tissue specific and cell specific expression. Expression of the transgene was relatively higher in female adrenal glands, but lower in the SMG and kidney with levels approaching only 5-10% of those seen in the wild-type. Expression in female SMG was barely detectable but was induced appropriately to levels equivalent to those found in males by testosterone. The main difference between *Ren*-2 transgene expression in transgenics and *Ren*-2 in DBA/2 was the high constitutive expression of the transgene in the X-zone of the adrenal without an oestrus cycle dependent variation, compared with the normal cycling of expression seen between the zona fasciculata and X-zone[266]. This suggested that a further DBA/2 dependent *trans* acting factor was necessary for such hormonal regulation in expression or certain *cis* acting elements were not included in the microinjected construct. In a similar experiment a genomic fragment containing the same *Ren*-2d gene with only 2.5 and 3 Kb of 5' and 3' flanking sequence respectively was introduced into one renin gene mice of a different genetic background. In male transgenics, wild-type levels of *Ren*-2d transgene expression were achieved. In females relatively lower expression was seen in SMG and kidney but this responded to testosterone with increased expression[255]. Adrenal gland expression levels were not reported in this study. The genetic background of the recipient strain or the reduced length of flanking sequence in the construct may explain the disparity of observed transgene expression between the two studies.

TGR(mREN2)27

The 24 kb *Xho*-1 fragment of mouse genomic DNA containing the *Ren*-2d gene as described above[265] was microinjected into fertilised oocytes (F1 embryos of a Sprague-Dawley/WKY cross). Thirty-seven eggs were re-implanted and eight progeny were delivered. Five were transgene positive (TG+) by Southern blot, of which three (founders #25, #26, and #27) transmitted the transgene to the next generation. Four of the five TG+ founders were found to be hypertensive with SBP>200 mmHg by ten weeks of age confirming a transgene position-independent phenotype[267]. The normotensive TG+ founder #26 was found to be mosaic for the transgene however, inheritance of the transgene in her offspring was associated with blood pressure elevation comparable to the other positive founders. Significant

hypertension persisted in subsequent generations in two lines, #26 and #27[268], though male infertility in line #26, a probable consequence of transgene insertional mutagenesis, has prevented the subsequent generation of homozygotes in this line. The line TGR(mREN2)27 as derived from founder #27 has now been the subject of extensive investigation.

Serial measurements of SBP by tail cuff plethysmography of TGR(mREN2)27 rats (hemizygous for the transgene) showed an increasing blood pressure with age, attaining a plateau at nine to ten weeks. Initial characterization of the phenotype showed high plasma prorenin, and elevated adrenal tissue renin content[267]. Adrenalectomy with maintenance therapy of oral NaCl resulted in a fall in plasma prorenin to transgene negative (TG-) levels together with a fall in SBP suggesting that the adrenal was the principal source of the high circulating prorenin[269]. *In situ* hybridization and immunohistochemistry of adrenal gland showed both over-expression of the mouse *Ren*-2 transgene and renin protein to be principally in the zona glomerulosa and outer zona fasciculata. Northern blot and ribonuclease (RNase) protection assays also identified high transgene mRNA transcripts in the adrenal, ~2500 fold higher than endogenous rat renin gene expression, and between 10 and 500 fold higher expression in this tissue compared with any other tissues assayed[270]. Expression of the transgene in adrenal tissue, as shown by *Ren*-2 mRNA levels was high from birth and progressively increased with age[271].

Transgene expression in the kidney however, was highest at birth and decreased markedly at two to four weeks of age with further reductions occurring with developing hypertension. A similar profile was seen for endogenous rat renin expression with evident suppression in adult TGR compared to SD controls[271]. Suppressed kidney tissue renin and PRA was seen in TG+ compared to TG- littermates[267].

Detectable transcripts were also found in brain (cortex/septum), pituitary, aorta, mesenteric vessels, lung, testes, ovary, coagulation gland, thymus, small intestine, stomach and uterus, all being sites of normal *Ren*-2 expression in the mouse. However, *Ren*-2 expression was not found in the rat SMG gland suggesting an absence of necessary *trans* acting factors. Other notable sites of absent expression as determined by Northern hybridization included liver, heart, parotid gland, spleen and cerebellum[270]. Others have reported *Ren*-2 mRNA in the heart by RNase protection assay at the limits of detection[271]. In TG- controls, using a *Ren*-2 derived probe that hybridized to rat renin mRNA, endogenous rat renin gene expression has been only detected in kidney[270], but others have reported low levels of expression in adrenal, brain, pituitary and thyroid by RNase protection assay[271].

The underlying cause of hypertension in the TGR(mREN2)27 rat line is yet to be clarified. Interest has focused on the adrenal gland in view of the high transgene expression and concomitant elevation of adrenal tissue renin and prorenin which were predominantly transgene derived, while renal tissue renin and plasma renin reportedly remained suppressed[272,273]. In association with the elevated adrenal transgene expression, mineralocorticoid levels were altered with increased 24 hour urinary excretion rates for 18-OH cortisol, deoxycorticosterone (DOC) and aldosterone in TG+ rats from six to eighteen weeks of age, but not at thirty weeks, compared with age matched SD rats. Conflicting results were found for plasma measurements with decreased DOC, in both transgenic homozygotes and hemizygotes compared to SD controls while plasma corticosterone levels were similar in SD and transgenic homozygotes but were significantly elevated in hemizygotes. No significant differences were observed for either plasma sodium or potassium levels in the three groups[274]. Electron microscopy of adrenal tissue has revealed evidence of cholesterol accumulation in zona glomerulosa (ZG) cells. Studies on dispersed ZG cells from female transgenics showed a higher basal aldosterone secretion compared to cells from SD rats, but no difference between transgenics and controls in the net increase in aldosterone release in response to either Ang II or ACTH (adrenocorticotrophic hormone) stimulation was found[275].

An increase in active renin release, from cultured adrenal cells from transgenic rats was seen in response to both Ang II and raised intracellular Ca^{2+} [273]. Such mediators would normally be expected to elicit a negative feedback response on the kidney, suggesting that the normal negative feedback control does not operate on cultured adrenal cells derived from TG+ animals. Prorenin release *in vivo* was clearly stimulated by ACTH with a 10-fold rise in plasma levels compared with only a 4-fold rise in non-transgenics. However plasma renin concentration remained unaffected or fell respectively[274]. If transgene expression in the adrenal does lead to deranged mineralocorticoid synthesis and metabolism, it seems unlikely that it is the sole factor inducing hypertension as spironolactone therapy did not lower blood pressure or prevent the development of high blood pressure in TG+ rats despite treatment leading to an elevation of plasma renin concentration in both SD and TG+ animals[276,274].

Measurement of free intracellular sodium in intact lymphocytes from TGR(mREN2)27 using a sodium sensitive fluorescent dye method showed an increase relative to SD. This appeared to be secondary to reduced Na^+-K^+ATPase activity measured in erythrocytes, which also argued against a mineralocorticoid mediated effect[277]. It was not however established in this paper as to whether the animals studied had established hypertension or were pre-hypertensive.

Dexamethasone suppression of pre-hypertensive TGR(mREN2)27 did attenuate the BP rise, with a concomitant rise in mineralocorticoid levels, but a fall in plasma glucocorticoids (D. Ganten, unpublished observations)[278].

No evidence of increased insulin resistance in TGR(mREN2)27 hemizygotes has been demonstrated as shown by similar fasting plasma glucose and insulin levels together with equivalent fasting rates of hepatic glucose production and glucose utilization during a hyperinsulinaemic euglycaemic clamp[279].

The blood pressure lowering effects of both ACE inhibitors and Ang II antagonists in TGR(mREN2)27 have argued in favour of an Ang II dependent hypertension with salt/volume sensitivity[280-283,276]. Even within the kidney, studies have suggested that an inappropriately high Ang II level may modify tubulo-glomerular feedback responsiveness in *Ren*-2 transgenic rats[284]. While some groups, as outlined above, have reported suppression of plasma renin levels, an elevation in PRC and Ang II have also been reported in TGR(mREN2)27 rats compared to SD controls on a normal (0.1% Na) diet[285]. Possible explanations for discrepancies include the method of blood collection, use of anaesthesia, storage of blood or method for assay[161]. Nephrectomy in TGR resulted in an increase in PRC, in contrast to the fall to near-undetectable levels seen in SD suggesting mainly extra-renal sources of renin in TGR. Additional adrenalectomy blunted the rise by ~50% implicating the gland as the major but not sole source of plasma renin[285]. A fall in PRA and Ang II followed ovariectomy but no change in prorenin levels were observed. A concomitant fall in transcription of both the *Ren*-2 gene and endogenous r*Ren* in the kidney argued in favour of an ovarian hormone dependent regulation of gene transcription or a role for the ovary in conversion of prorenin to renin in TGR[286].

The finding of *Ren*-2 transcripts in vascular tissue together with the observation that a perfused isolated rat hind-limb preparation demonstrated increased release of both Ang I and Ang II in transgenics compared to SD has suggested that a major source may be the vascular wall. Bilateral nephrectomy prior to perfusion reduced angiotensin peptide release from SD, but not from TGR supporting a non-renal origin for the derived vascular wall renin in TGR[287].

The kinetics of the reaction of mouse renin acting on rat angiotensinogen have been studied and show a different pH optimum for the reaction when compared with endogenous rat renin acting on homologous substrate. The speed of reaction for mouse renin cleaving rat angiotensinogen was ten-fold higher than for the cleavage of mouse angiotensinogen, which appears to be due to the relative lack of substrate in the mouse [288]. In view of evidence suggesting that most circulating renin in TGR(mREN2)27 is transgene derived[288], this could explain an Ang II dependent

hypertension without a significant elevation of PRA, but the physiological significance of these *in vitro* findings need to be investigated further in an *in vivo* situation.

Initial comment on this transgenic rat suggested that it was a model of low plasma renin hypertension, with the implication that either disturbed adrenal mineralocorticoid or glucocorticoid regulation resulting from an overactive adrenal RAS or elevated prorenin might be responsible for the hypertension[289]. Further work has not clarified the issue, but the studies described above would support the suggestion that tissue (extra-renal) RAS activity is leading to an Ang II dependent hypertension. Much of the controversy may be a consequence of the methodological problems that exist in measurement of PRA and angiotensin peptides, but the relative lack of suppression of PRA, Ang I and Ang II as first described by Mullins et al (1990)[267] despite the very elevated BP is a hint of the likely involvement of the RAS.

Pathological changes of hypertension related target organ damage in transgenic heterozygotes were more apparent in males than females and developed from 4-6 months of age. These included progressive medial thickening in resistance arteries and arterioles. Thickness of the aortic wall was also increased, in particular the tunica media with disruption and formation of additional layers of elastic laminae. Hearts showed development of left ventricular hypertrophy (LVH) with thickening of the media of myocardial arterioles and perivascular fibrosis[290]. In the kidneys, vascular changes were prominent from four to six months of age, but fibrinoid necrosis was not described. Glomerulosclerosis, in some cases progressing to end-stage sclerosis, with areas of focal tubular atrophy were seen from six to eight months of age indicative of chronic hypertensive and ischaemic damage[269]. Increased proteinuria was found in 400g TGR (approximately four month old) compared to age-matched SD, which is a likely consequence of early glomerular damage[291,278].

In summary the TGR(mREN2)27 rat line has provided an interesting model of monogenic hypertension which has led to further work being directed towards developing a better understanding of renin gene regulation and function, and the development of strategies for ascertaining the relative contributions of prorenin, the adrenal gland, tissue RAS activity and species specific enzyme kinetics towards the hypertensive phenotype. Other transgenic models utilising other components of the RAS have been developed and have helped contribute to the overall picture. These will be described in brief below and are summarised in Table 1.7.1

Transgenic mice have been generated that carry the rat angiotensinogen gene as a genomic fragment with 1.6 kb of 5' flanking sequence. Four founders were generated, of which three transmitted the transgene to their progeny. Expression of the transgene was high in the liver in one founder together with elevated plasma

angiotensinogen and Ang II. Significant elevation of SBP was found in the high expressor line, but not in a low expressor line[292]. A similar experiment involved microinjection of constructs comprised of a mouse metallothionein I (MT-1) promoter fused to the rat angiotensinogen gene or rat renin gene, with the aim of a controlled induction of transgene expression by administration of zinc in the drinking water.

Table 1.7.1- Transgenic studies of the Renin-Angiotensin system

Transgene	Species	BP	Reference
Mouse *Ren*-1d	Mouse	→	141
Mouse *Ren*-2	Mouse	→	265
Mouse *Ren*-2	Rat	↑	267
Rat angiotensinogen	Mouse	↑	292
MT-1 Rat angiotensinogen	Mouse	→	293
MT-1 Rat renin	Mouse	→	293
Human renin	Mouse	→	294, 295
Human angiotensinogen	Mouse	→	294
Human renin	Rat	→	296
Human angiotensinogen	Rat	→	296

Summarises published transgenic experiments in rodents using renin and angiotensinogen constructs and their effects on measured blood pressure. (MT-1 =metallothionein promoter).

In contrast to the previous study, hypertension was not induced on expression of either transgene alone, but by crossing transgene positive mice to give double transgenics, hypertension was induced[293]. The species specificity of the cleavage reaction alluded to earlier, may well be responsible for the absent phenotype in the MT-1 rat renin transgenics, as it had been previously demonstrated that mouse angiotensinogen was resistant to cleavage by renin from other species including rat, hamster, rabbit, pig and human[297,169]. Therefore as expected mice transgenic for the human renin gene were neither hypertensive nor had an elevated PRA[295]. When they were crossed with mice transgenic for human angiotensinogen, resulting in double transgenic mice with coexistent human renin and substrate for cleavage, an elevated BP was observed. The resultant hypertension could be lowered by a specific human renin inhibitor (ES 8891), which had no effect on BP in non-transgenic mice, transgenic mice when carrying the human renin gene (TGM(hREN)) alone or human angiotensinogen genes (TGM(hAOGEN)) alone. In contrast, both ACE inhibition and

Ang II antagonists lowered BP in both the single and double transgenics and in non-transgenics[294].

The relative suitability of rats for physiological and pharmacological studies compared to mice has been a major incentive for developing transgenic lines despite the relative technical difficulties mentioned earlier. Rats transgenic for a genomic fragment containing human renin (TGR(hREN)) show elevated plasma human renin to levels ~12 fold higher than found in normal human plasma but no change in endogenous rat renin levels were found. Other parameters of the rat RAS e.g. prorenin, Ang II and angiotensinogen remained unchanged and systolic blood pressure was normal suggesting that there was no evidence of human renin cleaving rat angiotensinogen to produce Ang II or a hypertensive phenotype and there was no evidence of feedback inhibition on the endogenous rat RAS. Appropriate increases of both endogenous rat renin and human renin occurred on salt depletion suggesting that the required regulatory regions of the transgene were present. Transgenic rats carrying human angiotensinogen TGR(hAOGEN) exhibited levels of human angiotensinogen ranging from 2 to 8 fold higher than found in normal human plasma, but again the rat RAS parameters were unaffected. No evidence of cross-species enzyme-substrate interactions were found using specific human renin inhibitors, which could only inhibit the hypertensive response elicited by infusion of human renin but not rat renin into TGR(hAOGEN). Surprisingly double transgenics TGR(hREN X hAOGEN) did not develop spontaneous hypertension[296]. In an experiment using a different approach whereby the human renin gene was introduced and expressed in rat liver, following an in *vivo* gene transfer method utilising HVJ-liposomes (Haemagglutinating Virus of Japan or Sendai virus), elevation of blood pressure was found. This correlated with plasma human active renin levels and did respond to a specific human renin antagonist[298].

Recently the first study of targeted inactivation of a component of the RAS was published. Mice were made hypotensive and deficient in angiotensinogen by homologous recombination. It was found that BP was significantly lower in homozygous mutants (-/-) who had no detectable plasma angiotensinogen, while heterozygotes (+/-) had in fact a slightly higher level of plasma angiotensinogen relative to wild type (+/+) mice and no significant change in BP[299]. This may be due to an altered feedback effect.

An alternative transgenic experiment targeted production of the oncogenic T antigen protein to renin producing cells by using a construct comprised of the SV40 T antigen reporter with a promoter consisting of *Ren*-2 5' regulatory sequences. Expression of the construct in TG+ mice resulted in neoplastic transformation of

renin-expressing cells in SMG, adrenal gland, testes, and sub-cutaneous tissue. In kidney tissue either tumours developed or ischaemic features appeared with evidence of proliferation of smooth muscle cells in both medial and intimal layers of arterial walls, affecting in particular the interlobar, arcuate, and interlobular arteries and afferent arterioles resulting in luminal narrowing. In addition changes were seen in glomeruli with some evidence of cellular hyperplasia within the mesangial stalk. Such changes were found in four independent transgenic lines. Despite the afferent vascular pathology, both PRA and renal renin content were significantly reduced in TG+ mice, together with reduced expression of the endogenous renin gene *Ren*-1c. This inhibition of endogenous renin synthesis did not seem to be a function of Ang II mediated feedback inhibition[245,300]. An important finding from this study was the identification of the extent to which smooth muscle cells within the renal vascular tree have the capacity to synthesize renin, with the caveat that transformation of them had occurred. It has also allowed study of the physiological effects of inducing a primary renal vascular hyperplasia in the absence of hypertension.

1.8 Malignant phase hypertension

Malignant or accelerated phase hypertension classically presents with a rapidly rising blood pressure and deteriorating renal function. The term 'accelerated phase' was traditionally applied to patients with grade III hypertensive retinopathy (retinal haemorrhages and exudates) while 'malignant phase' hypertension was reserved for those who had grade IV retinopathy with papilloedema[301,302]. In the light of present knowledge regarding the underlying renal pathology in both situations, and with no clear distinction between them in terms of survival, such a demarcation is probably not warranted[303-305] and therefore in this thesis, the term malignant phase hypertension (MH) will be applied to the syndrome of clinical and pathological features observed with either Grade III or IV retinopathy.

Typical clinical features that have been described include headache, dizziness, visual blurring and central scotomata, fatigue, weight loss and nocturia of varying duration prior to diagnosis. Many patients however remained asymptomatic[306,302]. No specific symptoms indicative of impending MH have been described. Additional signs and symptoms resulting from renal impairment and hypertensive encephalopathy included nausea, vomiting, confusion, somnolence, focal neurological signs, ataxia and occipital blindness. Chest pain, orthopnea and exertional dyspnoea were frequently described in the presenting histories from an early series[301].

The incidence of MH as a complication of hypertension was difficult to ascertain in early studies as there was no clear data on the prevalence of benign hypertension in the population, and assumptions had to be made from population based studies [14]. Referrals of patients with MH to hospitals whose catchment areas could be estimated, then gave an incidence of about 1% across the complete age range[306]. Few further estimates have been made, but it was recognised that the incidence declined considerably in the early 1970s, particularly when occurring as a complication of essential hypertension[307]. Yaqoob et al (1991) reported eleven cases referred to a regional renal unit catering for a population of two million which suggested an incidence of MH with significant renal impairment amongst their hypertensive population of about 0.004%[308] though this is probably an underestimate as patients managed outwith the specified unit or those whom perhaps did not develop significant renal impairment would have been excluded.

The underlying cause of hypertension was relevant with a relatively smaller decline in incidence of MH as a complication of secondary forms of hypertension being reported[307]. Classification of Kincaid-Smith's series of 124 MH patients presenting from 1935 - 1955 showed that essential hypertension was the underlying cause in 42%, while renal artery stenosis only accounted for 3%[306]. Other studies have shown a similar contribution from essential hypertensives (38%), but up to 26% of cases were secondary to renal artery stenosis, and 25% due to renal parenchymal disease[307]. Overall the incidence of MH as a complication of essential hypertension has declined and now accounts for at most 10% of all cases[309]. This may be a consequence of increased treatment options for managing essential hypertension resulting in fewer refractory cases.

It was noted that relatively younger patients tended to be affected with the highest incidence of MH reported in the forty to fifty year age group with a falling incidence in older age groups representing a leftward shift of the age-distribution curve for MH as compared to that for hypertension in the population[301,306]. Though not significant, there was a suggestion that MH occurring in much younger patients tended to be due to renal parenchymal disease or chronic pyelonephritis, while renovascular causes were found in older men. The age range for essential hypertension complicated by MH was midway between. Increased susceptibility has been observed in blacks, though this may be a function of social factors leading to delayed diagnosis and management of underlying hypertension[310-313] or it may be due to underlying genetic differences.

Prognosis was extremely poor in early series with two year survivals of untreated MH ranging from 5-10%[301,306]. This compared with a five year survival of

untreated mild essential hypertension of >97%. Even early methods of treatment, consisting of sympathectomy, adrenalectomy or nephrectomy[314], reportedly improved this two year survival, and a series of studies of treated MH from 1957-1973 showed five year survivals ranging from 25 to 50% [311,302] and in 1986, five year survivals of 75% were reported in series of white patients[315,304].

The early reports of MH by Keith et al (1928) and Klemperer and Otani (1930) described the natural history of the condition without treatment. No marked macroscopic changes of the kidneys were seen apart from petechial haemorrhages over the cortical surface in occasional cases. The main renal findings were at the microscopic level, with diffuse changes affecting smaller arteries and arterioles, but sparing larger arteries. In particular, intimal hyperplasia with deposits of 'lipoids, fatty acids and calcium' were seen resulting in significant luminal narrowing[301]. In all cases renal involvement was apparent and profound, but lesions of varying severity were found in all other organs examined. Pathological examination of a further series of cases described, in addition to the dominant arteriolar changes, some degree of glomerular capillary tuft collapse, capillary thrombosis, glomerulosclerosis, evidence of some crescent formation, and tubular dilatation with occasional casts[316].

More recent studies have documented the light microscopic changes in considerable detail. Necrosis of medial smooth muscle cells, with loss of nuclei and deposition of fibrin was seen together with swelling of overlying endothelial cells and nuclear pyknosis. Luminal narrowing and thrombosis was seen, but the generally patchy nature of these changes was apparent. Intimal cellular hyperplasia more often affected afferent arterioles and was a lesser feature in interlobular arteries[306,317]. Light microscopic studies of affected kidneys showed that smooth muscle cells were responsible for the characteristic 'onion skinning' intimal proliferation, with concentric lamellae of basement membrane and granulofilamentous material and a thin layer of hypertrophic medial cells stretched around the circumference. Insudative lesions consisted of deposited granular protein within a pre-existent connective tissue matrix, though fibrin filaments were not typically found in the intramural lesions.

Electron microscopy showed that fibrinoid necrosis consisted of three components, fibrin, fibrinoid and cellular necrosis. Fibrinoid tended to be relatively electron dense and either granular or foamy, lacking the fibrillar structure of true fibrin. Cellular necrosis was not consistently present in all areas, and often fibrin and fibrinoid would be found lying between an intact endothelial layer and viable intimal and medial cells. Necrosis of individual muscle fibres were seen with occasional neutrophils. Arterial luminal thromboses consisted of both fibrin and platelets[317-320].

Affected glomeruli characteristically showed thickened basement membranes, with reduplication of an inner basement membrane in severe cases and relative shrinkage of the glomerular tuft. Occasionally in association with a necrotizing lesion of the afferent arteriole fibronecrotic, haemorrhagic or glomerulitic lesions were seen. The latter was typified by the presence of neutrophils within the glomerular capillaries, fibrin in Bowman's space and a glomerular epithelial proliferative response leading to crescent formation. Swelling and detachment of endothelial cells was seen. Hyperplasia and hypergranularity of the juxta-glomerular cells is reported[318,319,321,155].

It was observed that lesions occurring in blacks with MH tended towards a myointimal proliferative response in arterioles with marked hyalinization rather than fibrinoid necrosis. Likewise glomerular fibrinoid necrosis and proliferative changes were not common, though thickening of basement membranes, tuft collapse and deposition of eosinophilic material inside Bowman's capsular basement membrane were seen[322].

Other organs are affected to varying extents by MH. Post-mortem studies, radiological and echocardiographic studies have shown that the degree of cardiac hypertrophy is more dependent on the duration of antecedent hypertension rather than the severity of hypertension in MH[301,323]. Even with no evidence of LVH, patients may present with pulmonary oedema showing left ventricular functional abnormalities with impaired diastolic relaxation, abnormal filling and incoordinate systolic contraction despite normal cavity dimensions and ejection fractions. Left atrial enlargement was relatively more common than that found in benign hypertension, particularly in MH patients without a previous history of hypertension, suggesting that an inadequate time for adaptive processes to occur was responsible. Such functional changes were not completely reversible following treatment to lower BP[323]. Fibrinoid necrosis and vascular alterations have been observed in varying tissues including mesenteric arterioles, liver, spleen, pancreas, adrenals, cardiac vessels, brain and of course the retina though they remain most florid in the kidney [301,316,324,314,325].

Why does a situation of previously stable but elevated blood pressure transform to a situation where there is an accelerating rise in BP with a rapid deterioration in renal function, characterized by the described pathological changes. Volhard and Fahr described in 1914, a form of arteriosclerosis with inflammatory renal changes, thought to be responsible for a 'Kombinationsform' or malignant form of hypertension, and they postulated that a toxic factor superimposed on the atherosclerosis of small vessels was responsible for the clinical and pathological presentation. It was later suggested by the same authors that an ischaemic aetiology,

possibly prolonged vascular spasm, was occurring though it was apparent that inflammatory aetiologies could give rise to similar clinical presentations (reviewed in [316]). Sanerkin (1971) reviewed the evidence in favour of ischaemic or obliterative arteriolar spasm accounting for arteriolar and endothelial necrosis. Combined medial and intimal necrosis with intraluminal thrombosis was thought to result from an obliterative spasm lasting over three hours with failed reflow. Fibrinoid change occurred from continued seepage of plasma proteins through the necrotic vascular wall into viable surrounding tissue leading to the progressive eosinophilic change on light microscopy. Medial necrosis with preservation of endothelium could follow an obliterative spasm of a lesser duration. It was therefore suggested that the overall picture was of recurrent obliterative spasm affecting different groups of vessels[317].

It has been argued that exudative lesions may simply be a function of high blood pressure. The commonest precipitating factor in chronic essential hypertension has been said to be a sudden rise in BP, often due to sudden withdrawal from anti-hypertensive medication[302,313]. Other drug induced causes have included oestrogen containing oral contraceptives[326,327], corticosteroids, non-steroidal anti-inflammatory drugs, sympathomimetics, tricyclic antidepressants[328], ingestion of catecholamine precursors while on monoamine oxidase inhibitors and even the use of a non-selective β-antagonist in the context of a high circulating adrenalin during hypoglycaemia[329], all of which could acutely elevate BP. Secondary causes of hypertension, particularly those associated with labile BP are associated such as phaeochromocytoma, renin secreting tumours, and eclampsia[302].

It was observed that rapid resolution of the retinopathy occurred if blood pressure was reduced, though not necessarily to normotensive levels[314]. Protection of the kidney from the effects of high pressure from whatever cause, can occur naturally by virtue of a proximal renal artery stenosis. The expected pathological changes were not observed in the kidney distal to a stenosis while the contralateral kidney exposed to systemic hypertension showed fibrinoid damage[330]. Commencing anti-hypertensive medication prior to renal biopsy in MH resulted in altered histology with disappearance of fibrin deposits within three to four days, less evidence of necrosis, and thus more emphasis on intimal smooth muscle hyperplasia, variable tubular atrophy, and interstitial fibrosis[318,309]. These studies suggest that a loss of reno-vascular auto-regulation might well be important in the pathogenesis of transition to MH.

A possible role for inflammatory mechanisms in the initiation or triggering of MH is supported by the similarities observed between observed pathology in MH and certain clinical conditions with an obvious immune aetiology. Examples include

scleroderma, the vasculitides, haemolytic-uraemic syndrome and renal transplant rejection with renal lesions often developing in normotensive individuals[331,332]. Immunological parameters have been studied and an excess of HLA Bw35 and linked Cw4 was found amongst a mixed group of patients with MH compared to healthy blood donors, but this excess was not statistically greater than the small excess also found in patients with glomerulonephritis but without MH[333]. A small excess in HLA B-15 was observed in patients with a previous history of grade IV retinopathy, but this did not reach statistical significance ($p=0.088$)[334]. A higher percentage of MH patients had auto-antibodies compared to controls. Lymphocyte stimulation tests using arterial antigen indicated an increased T cell response and significantly elevated IgG and IgM, but not IgA levels in MH patients[335]. Whether these changes are primary or secondary to the vascular damage was not clear from these retrospective studies.

Smoking has been linked with an increased risk of developing MH, more advanced retinopathy, a greater risk of developing renal failure and a poorer prognosis[336-338]. It has been suggested that smoking may promote platelet aggregation, luminal thrombosis and hence endothelial damage.

Following initiation of MH, there is good evidence supporting a role for the RAS in maintaining the vicious circle of accelerating blood pressure, renal impairment and pathological damage (reviewed in [339,340]). A pressure induced diuresis and natriuresis could create a state of relative sodium depletion, thus activating the RAS. The afferent vascular pathology may result in a further stimulation of renin synthesis and secretion suggested by observed hyperplasia of the renin containing cells of the JGA[155]. Significantly elevated levels of aldosterone were reported in a series of fourteen patients with MH of varying duration despite being on treatment[341]. PRA, measured on admission in eight MH patients was found to be elevated in five and aldosterone was elevated in nineteen out of twenty-two[342]. Striking anti-hypertensive responses were reported in seven patients with MH and high PRA in response to propranolol, with the falls in BP correlating with both the reduction in PRA and aldosterone level[343]. The ACE inhibitor captopril given to MH patients lowered BP by reducing total peripheral resistance, and the response again correlated closely with pre-treatment PRA[344].

Various experimental animal models of MH have been described of which the majority rely on either surgical, pharmacological or dietary manipulation to induce transformation to MH. A proportion of both 1K1C (one kidney, one clip)[345] and 2K1C rats[324] develop pathological features of MH. In all 2K1C rats, the initial rise in BP occurred during a period of sodium retention, but in a sub-group of rats who developed features of MH, higher BP was associated with onset of sodium loss due to

both increased urine output and fractional sodium excretion. 2K1C MH rats showed both a higher PRA and renal renin content compared to 2K1C benign hypertensive rats, though there was a wide scatter in values[346]. Sprague-Dawley rats (SD) treated with aldosterone developed hypertension associated with suppression of plasma renin, but when given additional partially purified renin, they developed a syndrome typical of MH with focal necrosis of small arteries and arterioles[347]. Similarly exogenous rat renin given to uninephrectomized SD rats resulted in hypertension, a reduction in renal renin content, and by 10 days of treatment, hyalin deposition in glomerular tufts was evident with tubular dilatation, cast formation and arteriolar necrosis[348]. The absence of a reported elevation of renal tissue renin content in this study deserves comment with perhaps two possible explanations. The duration of MH was not clear from the study with no rise in BP apparent by Day 4 of treatment and tissues were obtained on Day 10 leading to the possibility that an inadequate time period had elapsed for malignant transformation of the hypertension to occur. Alternatively endogenous rat renin synthesis may have been suppressed by an overriding negative feedback, despite stimulation of the JGA following initial afferent vascular damage. In contrast rabbits given large doses of exogenous Ang II (0.9-1.8 μg/kg/min) for 3 days developed an initial rise in BP which was not sustained and subsequently developed renal impairment with rising urea and histological evidence of widespread ischaemic tubular necrosis, but both glomeruli and arterioles remained normal[349]. However SD rats infused with Ang II for over two weeks demonstrated a rising SBP to ~170 mmHg, and evidence of focal fibrinoid necrosis and proliferation of vascular smooth muscle cells particularly in afferent arterioles with tubular injury[350].

A proportion of stroke-prone SHR (SHR-SP) rats when given 1% NaCl to drink develop MH with fibrinoid necrosis. Involvement of the RAS was shown by the hypotensive response elicited by treatment with the Ang II antagonist (1-sarcosine, 8-alanine Ang II) on salt loaded SHR-SP, though such a response was not found in ordinary hypertensive SHR-SP given water. This implied that the 'benign' hypertension of SHR-SP was not Ang II dependent in contrast to salt loaded SHR-SP hypertension[351]. Chronic treatment with Enalapril dramatically improved survival in salt-loaded SHR-SP to >90% compared with 0% survival by 25 weeks of age in untreated controls. This was despite an ultimately higher BP being reached by the surviving treated group after 15 weeks of age in comparison with the BP in those untreated animals at the time of development of terminal cerebrovascular events. Renal pathology consisted of arterial and arteriolar necrosis and was evident in all untreated animals at the time of death, but was not found in any SHR-SP on the ACE inhibitor[352]. At a dose of 30 mg/kg/day, the Ang II antagonist Losartan both delayed

the rise in BP on salt-loading in SHR-SP, and prevented or delayed the appearance of arterial necrosis[353], but losartan also reduced the development of fibrinoid necrosis and arterial thickening in SHR-SP, even at a dose of 1 mg/kg/day which had no significant hypotensive effect[354]. Despite elevated adrenal renin activity in 5 week old SHR-SP compared with WKY, there was no significant difference in tissue Ang I or Ang II levels at this age, but by 25 weeks, when severe hypertension and occasional pathological evidence of focal fibrinoid necrosis was found[325], significantly higher adrenal tissue Ang I and Ang II levels are found in the SHR-SP[355].

Infusion of noradrenalin into dogs produced initial hypertension with RAS suppression, following which a transformation to MH occurred characterized by an abrupt rise in BP, sudden deterioration in renal function and fibrin deposition in the intima and media of renal vessels. Initial salt and water depletion led to hyponatraemia, and activation of the RAS coincided with the period of accelerating hypertension[356].

Uninephrectomized rats given deoxycorticosterone and 0.1% NaCl to drink, developed an initial benign hypertension associated with a positive sodium balance, rising BP and normal renal histology, but on transition to MH, weight loss, a negative sodium balance, a slight fall in BP and fibrinoid necrosis occurred. During both phases however, there was no evidence of an increase in plasma renin activity, nor in the malignant phase was there any BP response to Ang II antagonism[357]. However oral treatment with TCV-116, an Ang II (AT_1) receptor antagonist or enalapril for three weeks both decreased urinary protein excretion, and reduced the development of glomerular and arteriolar fibrinoid change in this model despite having no measured effect on BP. Though PRC was elevated in the treated groups, there was a small but not statistically significant reduction in kidney renin/GAPDH mRNA levels in the treated groups compared to the untreated control group given vehicle[358].

A small percentage of patients with MH also present with apparently normal PRA, and either elevated or normal aldosterone levels[342]. It has therefore been suggested that the trigger initiating MH might in fact be related to the natriuresis, and not necessarily be dependent on RAS activation[357]. In both experimental and clinical situations it has been observed that the correction of sodium depletion in MH may result in a fall in BP and recovery[359-361]. However in SHR-SP drinking 1% NaCl, chronic diuretic use reduced the incidence of pathological changes of MH but this was in the context of a coexistent anti-hypertensive effect[362].

Microangiopathic haemolytic anaemia is a well described feature of malignant hypertension in both the clinical situation[363-365] and in experimental models[357,366]. Fragmentation of erythrocytes result in the appearance of irregular shaped 'burr cells', schistocytes, spherocytes, microspherocytes and red cell fragments, without either an

intrinsic membrane abnormality or increased osmotic fragility being demonstrated[366]. Reticulocyte counts are consequently increased as a result of red cell damage. Similar pathology is seen in a number of other clinical states including haemolytic-uraemic syndrome/thrombotic thrombocytopenic purpura (HUS/TTP)[367], systemic lupus erythematosus, and in acute glomerulonephritis.

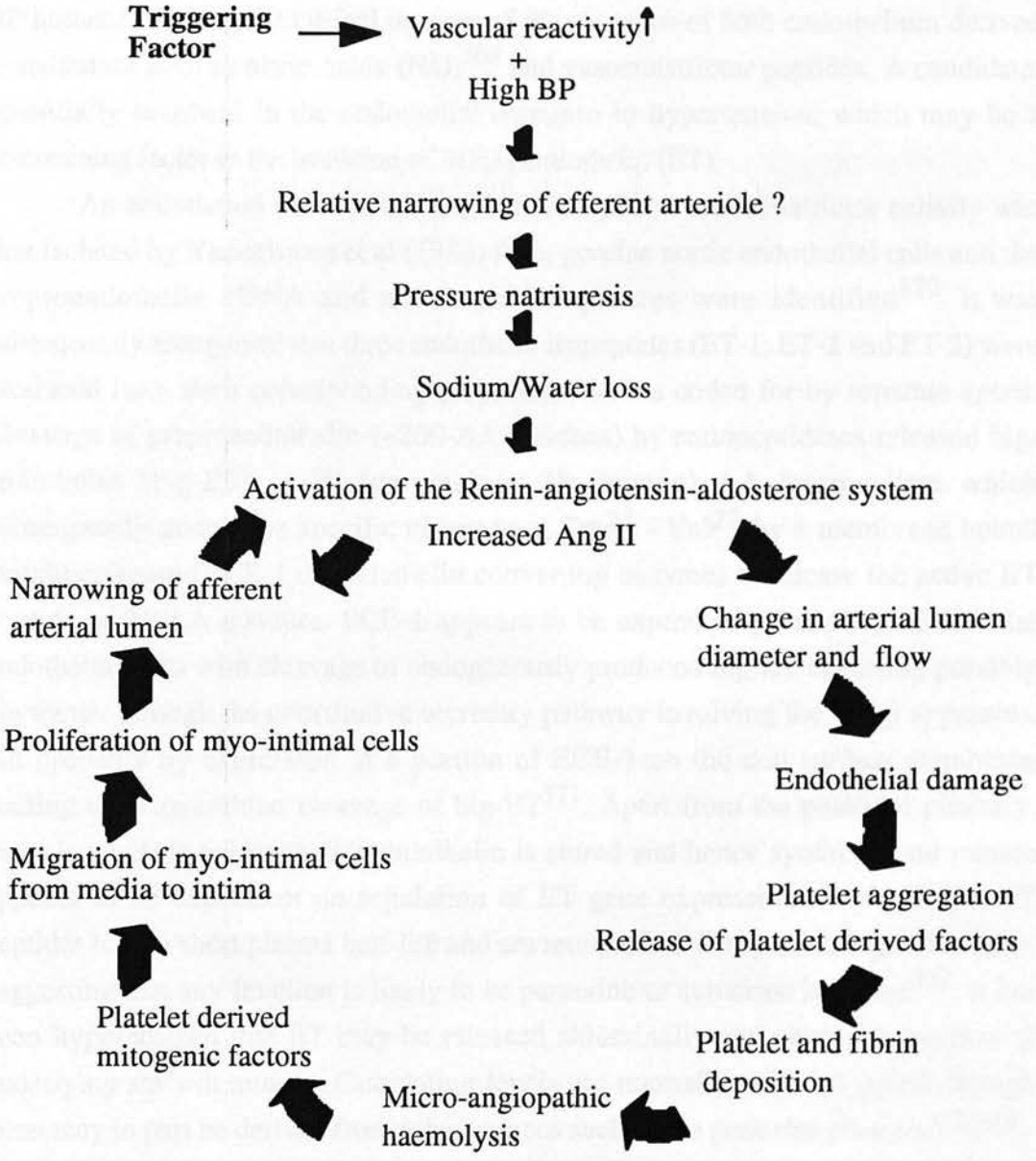
It has been postulated that either the passage of red cells through damaged arterioles due to fibrinoid necrosis leads to MAHA[363] or the onset of MAHA is important in initiating endothelial cell damage leading ultimately to fibrinoid necrosis[357]. The question has therefore been which comes first? The lack of correlation between haematological changes and either blood pressure or degree of retinal damage argued against the first of these hypotheses. It was found that red cell fragmentation correlated weakly with the degree of impairment of renal function[364,365]. These findings were supported by Isles et al (1984) who showed that similar haematological abnormalities were found in both MH and non-MH patients, as defined by the presence or absence of grade III/IV retinopathy, but with equivalent BP and renal impairment which suggested that uraemia might be responsible for the red cell fragmentation[365]. Some caution is required in the interpretation of this study as the MH group incorporated a spectrum of patients of which the majority had had between 1-10 weeks of treatment prior to blood sampling, and it has previously been shown that fibrinoid clears quickly following institution of treatment[309].

In contrast it was observed in the experimental DOCA/salt rat model of MH that changes of MAHA coexisted with a normal BUN (blood urea nitrogen) in a third of animals[366] suggesting that MH can develop without pre-existing uraemia. It would therefore appear that the role of MAHA in initiating MH is not clear, but that it is an important associated feature of MH which might contribute to the presentation[359].

In summary, the critical factors that lead to the transition from a benign or stable hypertension to malignant phase are not clear. To quote Kincaid-Smith (1958), "malignant hypertension may be determined by some new and unknown factor in addition to a high level of blood pressure"[306]. Considerable overlap in actual blood pressures are found between MH patients and benign hypertensives[368], but rate of rise may be important. The presence of a triggering factor acting in conjunction with high BP in benign hypertension may then create a situation of positive feedback with a vicious circle involving sodium depletion, afferent vascular pathology and RAS activation leading to an accelerating rise in BP (Fig 1.8.1). Logically, putative triggers might act to alter vascular reactivity to vasoactive hormones or affect endothelial cell permeability leading to plasma extravasation and myointimal proliferation which characterizes MH[305]. Which patients with existing essential hypertension or secondary

forms of hypertension are most at risk of developing MH would be the clinically important question to answer?

Fig 1.8.1



Schematic diagram illustrating possible processes involved in the pathophysiology of malignant phase hypertension. Adapted from Kincaid-Smith (1982)[309] and Houston (1989)[302].

1.9 Endothelin as a trigger factor in malignant hypertension

The factors which initiate the transition from benign to malignant phase hypertension, with the development of the characteristic pathological damage and activation of the renal RAS, have yet to be identified. The role of the endothelium in BP homeostasis may be critical in view of the presence of both endothelium derived vasodilators such as nitric oxide (NO)[369] and vasoconstrictor peptides. A candidate, potentially involved in the endothelial response to hypertension, which may be a determining factor in the initiation of MH is endothelin (ET).

An endothelial cell derived peptide with potent vasoconstrictor activity was first isolated by Yanagisawa et al (1988) from porcine aortic endothelial cells and the preproendothelin cDNA and amino acid sequences were identified[370]. It was subsequently recognised that three endothelin isopeptides (ET-1, ET-2 and ET-3) were produced from their corresponding preproendothelins coded for by separate genes. Cleavage of preproendothelin (~200 AA residues) by endopeptidases released big-endothelin (big-ET), a 39 (porcine) or 38 (human) AA intermediate which subsequently undergoes specific cleavage at Trp^{21} - Val^{22} by a membrane bound metalloprotease (ECE-1 or endothelin converting enzyme) to release the active ET peptide of 21 AA residues. ECE-1 appears to be expressed principally in vascular endothelial cells with cleavage of endogenously produced big-ET occurring possibly via transit through the constitutive secretory pathway involving the Golgi apparatus, but probably by expression of a portion of ECE-1 on the cell surface membrane leading to extra-cellular cleavage of big-ET[371]. Apart from the posterior pituitary, there is no clear evidence that endothelin is stored and hence synthesis and release appears to be dependent on regulation of ET gene expression. Furthermore, ET peptides have a short plasma half-life and are removed on first pass through the lungs, suggesting that any function is likely to be paracrine or autocrine in nature[372]. It has been hypothesized that ET may be released abluminally and cause contraction of underlying smooth muscle. Circulating levels are normally only 1-5 pg/ml, though these may in part be derived from other sources such as the posterior pituitary[373,374].

Studies have suggested that increased ET-1 gene expression and protein synthesis occurs in response to Ang II, ischaemia and thrombin *in vitro* [370,375,376], with mobilization of intracellular Ca^{2+} stores and activation of protein kinase C being implicated[375]. A transient increase in endothelial cell ET-1 gene expression has also been reported in response to low levels of shear stress[377,378] possibly mediated by actin filament disruption[379,380]. Aging endothelial cells *in vitro* have been shown to express increased ET-1 mRNA[381], whilst down-regulation of ET gene expression

was seen in cells of an endothelial cell line (transfected with preproendothelin cDNA) in response to NO donors[376].

Two functionally distinct receptors, ET-A and ET-B, have been cloned and are related to G protein coupled receptors[374]. A third ET-C receptor has been proposed. ET-A receptors preferentially bind ET-1 compared to the other two peptides, while ET-B receptors show equally high affinity for all three peptides but a lower efficacy. Both smooth muscle cells (SMC) and endothelial cells express ET-B receptors and these induce both direct vasoconstriction and a transient endothelium dependent vasodilatation, which probably results from endothelin induced release of NO and prostacyclin (reviewed in [374])[382,383], though conflicting data has been reported[384]. NO mediated effects appear to less important relative to those of prostacyclin in transient ET induced venodilatation as opposed to arterial vasoconstriction[382,383].

ET-A receptors are expressed on vascular smooth muscle cells and may mediate vasoconstriction through either activation of voltage dependent calcium channels, protein kinase C and/or intracellular Ca^{2+} release[58,385-387]. They may also weakly induce intimal and medial SMC proliferation via induction of protooncogenes including c-*fos* and c-*myc* (in common with Ang II but showing a supplemental effect *in vitro*)[388,389,390].

Heterogeneous ET-A receptor gene expression was found on intimal SMC just deep to the endothelium in 50% of internal mammary artery specimens taken from a group of hypertensive patients, with more severe intimal SMC proliferation and intimal thickening apparent in those that did express ET-A receptor mRNA. No hybridization signals were detected from the intima of normotensive controls[60]. However a study which looked at plasma immunoreactive ET-1 levels in groups of human essential hypertensives, ranging from mild untreated essential hypertension through to poorly controlled hypertension, no significant difference was found either between them or with normotensives[57]. No evidence of co-segregation of the ET-1 locus with either SBP or relative heart weight was found in two F2 populations, (SS/Jr X Lewis and SS/Jr X SHR) both raised on 8% sodium chloride diets, but linkage between ET-3 alleles and SBP and heart weight was demonstrated for the inbred Dahl SS/Jr strain[59]. However in the DOCA-salt hypertensive rat, it has been demonstrated that immunoreactive ET-1 content and ET-1 gene expression is increased in aorta and mesenteric vessels, despite normal circulating levels[391,392] but three weeks of treatment with the non-specific ET receptor antagonist, Bosentan, only slightly diminished the increment in BP with time compared with untreated DOCA-salt controls suggesting that BP in this hypertensive rat model was partially ET dependent. Bosentan also appeared to attenuate the reduction in lumen diameter and the increment

in media cross-section and media-lumen ratio that was normally seen with time in hypertensive DOCA-salt rats[393].

Exogenous administration of endothelin to anaesthetized rats resulted in an initial transient hypotensive response associated with a reduction in systemic vascular resistance, principally in musculo-cutaneous vascular beds, and a transient increase in cardiac index[394]. The initial vasodilatation did not occur in either renal or mesenteric vascular beds. The subsequent effects of exogenous ET-1 in rats and dogs consisted of systemic hypertension[394,395], renal bed vasoconstriction[396,397], decreased renal plasma flow and glomerular filtration rate[398,399], with glomerular hypertension and an overall increased filtration fraction as a consequence of preferential efferent arteriolar effects[400]. Such effects may however be both species and dose dependent[401]. Decreased glomerular ultrafiltration coefficient and single nephron filtration rates have also been reported in anaesthetized rats[402,403]. Sustained increases in both plasma renin activity and aldosterone concentrations were observed in dogs following intravenous ET, though these effects could have been mediated directly or via reduced sodium delivery to the macula densa as a consequence of a reduced urinary sodium excretion[404,398]. Both natriuresis[400] and salt/water retention have been reported in response to intravenous endothelin, though such effects again may be dose and species dependent[404,398,394]. Within the kidney both mesangial cell contraction and increased mitogenic activity have been reported in response to endothelin[402]. In isolated cultured mouse JGA cells, all three ET peptides inhibited cAMP stimulated renin release but had no effect on basal renin secretion. This appeared to be mediated by the ET-B receptor[405].

In humans arterial vasoconstriction, microvascular vasoconstriction and venoconstriction have been reported[406,383] and it is postulated that ET-1 mediated vasoconstriction may be normally attenuated by endothelium derived relaxant factors forming a local endothelial autoregulatory system[382], (reviewed in [374]). Continuous infusions of both ET-1 and ET-3 into SD rats could induce sustained increases in MBP possibly due to small increases in total peripheral resistance, though with the numbers of rats used, these did not reach levels of statistical significance with either peptide. Tachyphylaxis was not demonstrated[395]. In essential hypertension, both unchanged and increased basal plasma endothelin levels have been reported[57,407].

A pathological role for endothelin has been suggested in a number of clinical conditions. Reperfusion injury in ischaemic myocardium[408] and liver[409] have been shown to be aggravated by the production of ET. Gastric mucosal ulceration induced by intravascular ethanol has been shown to result from mucosal vasoconstriction mediated by ET-1[410]. In patients following sub-arachnoid haemorrhage, both

increased and unchanged levels of immunoreactive ET-1 (ir-ET-1) have been reported in the cerebro-spinal fluid, and it has been postulated that this could be involved in subsequent cerebral arterial vasoconstriction[411]. An increase in circulating plasma ir-ET occurs following haemorrhage in rats and is associated with an increase in total peripheral resistance that serves to maintain BP in haemorrhagic shock[412]. In a study looking at pre-eclamptic women, increased ET-1 mRNA levels were found in placental villous tissue compared to normal gestational placental tissue suggesting that there may be a role for ET in the placental vasoconstriction that results in foetal growth retardation[413].

In the kidney, experimental renal ischaemia led to increased ET-1 mRNA levels in the rat[414]. In another study performed in rats, supra-renal aortic cross clamping induced an increase in circulating ir-ET which could be abolished by prior nephrectomy suggesting that the kidney was contributing to the systemic rise in plasma ir-ET in this model[415]. ET has also been implicated in cyclosporin-induced renal vasoconstriction and nephrotoxicity. Elevated levels of urinary ET have been reported in a model of chronic renal failure in rats induced by renal mass reduction while chronic administration of an ET-A receptor antagonist (FR 139317) significantly reduced the progressive rise in urinary protein excretion observed in this model (reviewed in [416]). Similarly in a rat model of focal glomerulosclerosis, induced by puromycin aminonucleoside in conjunction with unilateral nephrectomy, progressive development of glomerular sclerosis was accompanied by increasing levels of mRNA for ET-1 and ET-A and ET-B receptors in the glomeruli. A low protein diet both reduced the prevalence of sclerotic glomeruli and attenuated the rise in ET-1, ET-A and ET-B receptor mRNA levels[417].

With the recognition that hypoxia, mechanical shear stress, thrombin and Ang II are all capable of inducing ET gene expression, it was of interest to ascertain the role of ET peptides in the transition to MH. In SHR-SP, a significant anti-hypertensive effect was seen in response to the selective ET-A antagonist BQ-123, but this was not seen in either SHR or normotensive WKY suggesting that ET did not have a significant role in maintaining hypertension in SHR, but that BP in MH may well be more sensitive to ET antagonists[418]. It was not however ascertained in this study whether pathological evidence of MH was present in SHR-SP. An anti-hypertensive response to the same antagonist was reported by a second group looking at SHR[419]. In two other rat models of MH, namely SHR treated with DOCA-salt for four or eight weeks and 2K1C rats treated with caffeine for six weeks, increased plasma ir-ET-1 levels were found relative to their hypertensive controls (WKY treated with DOCA-salt and sham-operated rats given caffeine respectively)[420]. However both MH models

had significantly higher BP and impaired renal function relative to their respective controls and histological confirmation of MH was not obtained. To date, a clear pathological role for ET has not been described, both in MH and in other clinical states where elevated plasma ET levels have been found[421].

The development of an animal model that would both spontaneously and predictably develop MH from a background of benign hypertension without the requirement for surgical, dietary or pharmacological manipulation would prove to be a valuable tool to address some of these issues. The following chapters describe the characterization and investigation of a spontaneous phenotype exhibited by TGR(mREN2)27 rats hemizygous for the *Ren*-2 transgene.

CHAPTER 2

Materials and Methods

2.1 Animals

Animal husbandry and all procedures were performed in accordance with the regulations set down by the Animal (Scientific Procedures Act) 1986. Rats were all housed in the Centre for Genome Research, and were maintained in controlled conditions with regulated temperature (18-20°C) and humidity (45-65%). A 12:12 hour light/dark cycle was used.

For studies requiring rats hemizygous for the *Ren*-2 transgene, male homozygote TGR(mREN2)27 rats were crossed with non-transgenic females. Male homozygotes used for breeding purposes were maintained on Lisinopril (dose 25 mg/l in drinking water). They had been previously confirmed to be homozygous for the *Ren*-2 transgene by Southern Blot analysis of tail DNA taken from at least three litters using a *Ren*-2 derived probe according to methods previously described[267]. After application of local anaesthesia (ethyl chloride B.P. jet), the distal 1 cm of tail was cut off pups at weaning and placed immediately on dry ice. Haemostasis was achieved with tissue adhesive (VetBond™). Tail DNA was prepared by digestion of cut tail in fresh tail buffer (50 mmol/l Tris HCl, pH 8.0; 100 mmol/l EDTA, 100 mmol/l NaCl, 1% v/v SDS) with Proteinase K 10 mg/ml (Boehringer) at 55°C. Phenol extraction was followed by precipitation in ammonium acetate (6 mol/l) and 70% ethanol washes. DNA was resuspended in TE (pH 8.0) before Southern analysis.

Strains used for crossing with homozygous *Ren*-2 males to obtain desired heterozygote crosses were Sprague-Dawley (SD) obtained from the Centre for Genome Research, Edinburgh UK; SD from the Central Institute for Laboratory Animal Breeding, Hannover, Germany; and Lewis rats from Harlan-Olac, Oxford, UK.

Pups were weaned at 21 - 25 days of age onto normal pelleted diet (CRM diet, SDS, Witham, Essex, UK) unless stated otherwise. Sodium content of this diet was 0.32% (64 mg/day assuming an average daily intake of 25 g of feed) and chloride content was 0.43%. For the study designed to exclude any effects of a small change in dietary sodium intake an alternative feed was used which had a sodium content of 0.2% (40 mg/day) and chloride content of 0.35% (CRM-X diet, SDS). There was no change in potassium content between the two diets. Tap water was given *ad libitum* to drink.

Animals were housed in plastic cages and bedded on shavings. They were kept in groups of two to three, unless on telemetry when they were housed singly.

2.2 Measurement of Blood pressure

Three methods have been employed in the studies described. Direct measurements were obtained by telemetry (Data Sciences International, St Paul, Minnesota, USA) or by carotid arterial catheterisation while indirect blood pressure measurements were obtained by tail cuff plethysmography. These methods will be described below, together with the study performed in order to validate indirect BP measurement by tail cuff plethysmography in this model.

(I) Telemetry

Continuous recording of blood pressure was desirable, but most conventional methods using direct BP recording have required anaesthesia, restraint or tethering. These methods have been shown to induce stress responses in rodents manifesting as an increase in body temperature and elevation of circulating catecholamine levels with a rise in BP, heart rate and oxygen consumption which may in part be strain dependent[422]. Indirect methods of BP measurement using tail cuff plethysmography therefore require animals to be frequently handled and accustomed to restraint or anaesthesia for the measurement to be taken. Only intermittent measurements can be made. However even with training, tail cuff plethysmography was still found to be associated with a significant rise in systolic blood pressure (SBP) which persisted through out the period of restraint and for at least 30 minutes after release, in a study which looked at the effects of this procedure on rats undergoing simultaneous direct BP recording by radio-telemetry[423].

These methods were therefore not considered suitable for measuring BP on a continuous basis in conscious, unrestrained and relatively unstressed animals. It was therefore decided to use a radio-telemetry system (Data Sciences International, St Paul, Minnesota, USA) which allowed continuous direct intra-aortic recording of SBP, mean blood pressure (MBP), diastolic blood pressure (DBP), heart rate (HR) and motor activity (Fig 2.2.1) with minimal disturbance to the animals following initial implantation of the recording devices. In addition it eliminated movement artefacts which are usually found with methods that employ either restraint or tethering[424,425].

Fig 2.2.1 Telemetry apparatus

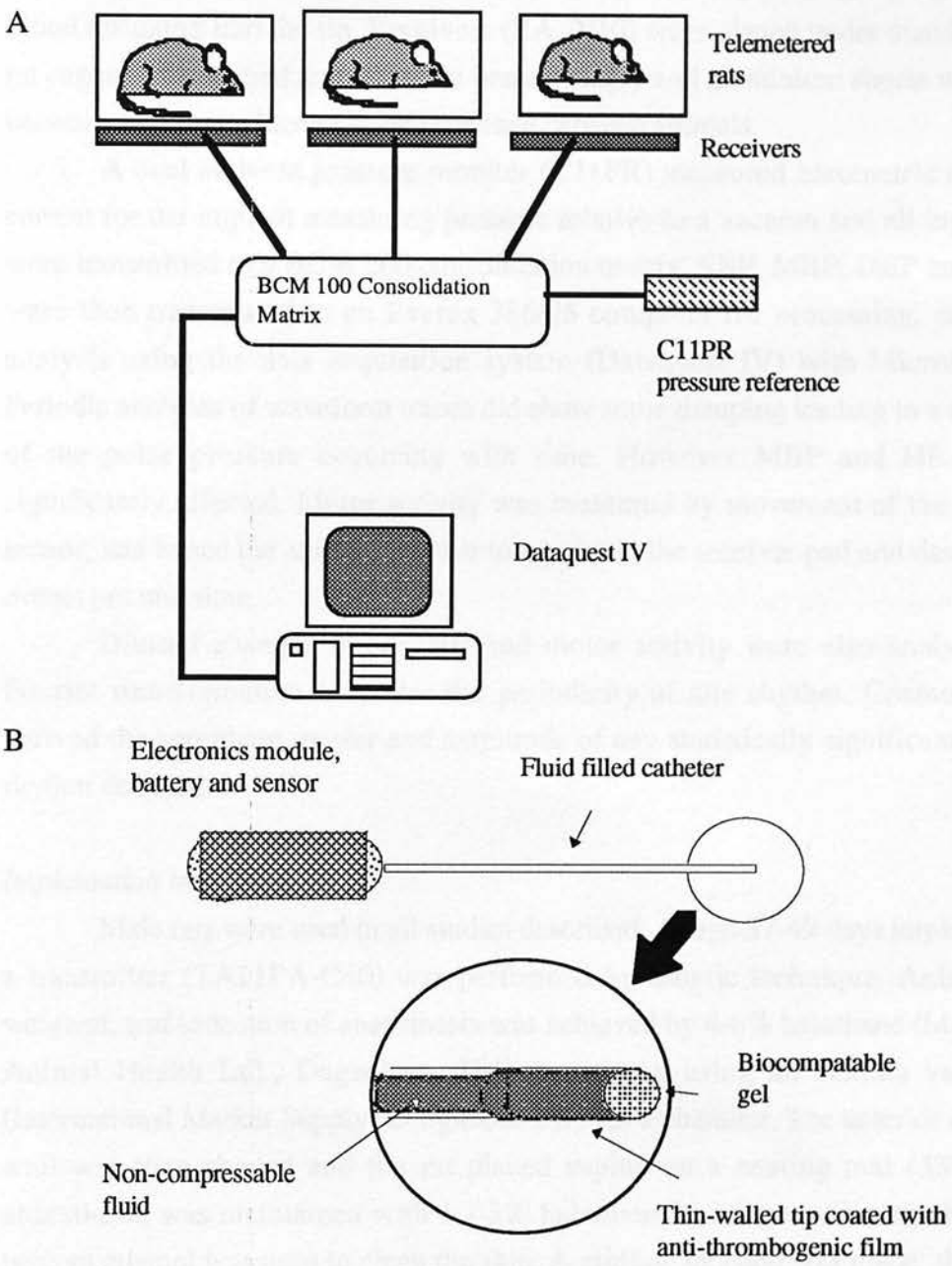


Fig 2.2.1 A represents the set-up for telemetric recording of SBP, MBP, DBP and motor activity. B illustrates the implant used (TA11PA-C40) and the details of the catheter tip (Adapted from [425]).

Equipment

The implanted transmitter (TA11PA-C40) weighing 9 g consisted of a module containing a battery powered sensor (a stable ion implant semiconductor strain gauge sensor). Arterial pressure was conducted to the sensor via a 0.7 mm diameter catheter.

To maintain patency, the distal 1 cm of the catheter was coated with an anti-thrombogenic film and the tip was filled with a biocompatible gel plug to prevent blood refluxing into the tip. Receivers (RA 1010) were placed under standard plastic rat cages. Telemetered animals were housed singly and aluminium sheets were placed between cages to reduce radio-interference between animals.

A dual ambient pressure monitor (C11PR) measured barometric pressure to correct for the implant measuring pressure relative to a vacuum and all input signals were transmitted to a BCM 100 consolidation matrix. SBP, MBP, DBP and HR data were then transmitted to an Everex 386/25 computer for processing, storage and analysis using the data acquisition system (Dataquest IV) with Microsoft OS/2. Periodic analyses of waveform traces did show some damping leading to a diminution of the pulse pressure occurring with time. However MBP and HR were not significantly affected. Motor activity was measured by movement of the implanted sensor, and hence the animal, relative to a point in the receiver pad and data stored as events per unit time.

Diurnal changes in BP, HR and motor activity were also analysed using Fourier transformation to assess the periodicity of any rhythm. Cosinor analysis derived the acrophase, mesor and amplitude of any statistically significant circadian rhythm detected.

Implantation technique

Male rats were used in all studies described. At age 37-49 days implantation of a transmitter (TA11PA-C40) was performed by aseptic technique. Animals were weighed, and induction of anaesthesia was achieved by 4-6% halothane (M&B, RMB Animal Health Ltd., Dagenham, UK) in oxygen using an Acoma vaporizer F (International Market Supply, Congleton, UK) via a chamber. The anterior abdominal wall was then shaved and the rat placed supine on a heating mat (33°C) while anaesthesia was maintained with 1 - 3% halothane in oxygen via a mask. Seventy percent ethanol was used to clean the skin. A midline incision was made through the skin and muscle layer and the peritoneal cavity was opened. Saline (0.9%) swabs were used to cover small and large bowel and the retroperitoneum was exposed. Using an operating microscope (Wild 650) the descending aorta distal to the origin of the renal arteries was dissected free from surrounding retroperitoneal fat by blunt dissection using a cotton bud. A tie (2/0 ethilon) was placed around the aorta distal to the origin of the renal arteries. About 2 - 5 mm above the iliac bifurcation the aorta was again dissected free of surrounding tissue and while aortic flow was transiently restricted by lifting the upper tie, the aorta was punctured with a 19 gauge needle tip

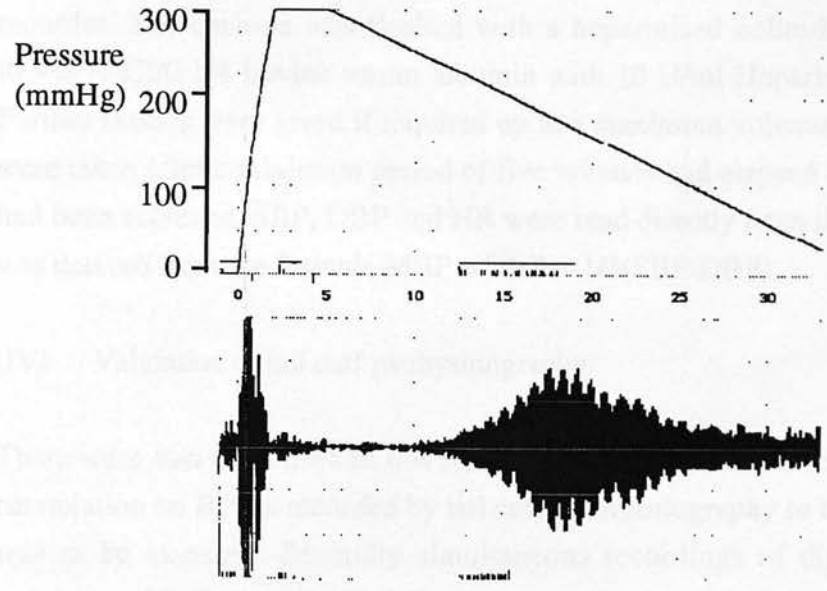
and the distal portion of the sensing catheter was inserted into the aorta and advanced up to the level of the upper tie. The sensing catheter was fixed *in situ* using tissue adhesive (VetBond™, Braun-Melsungen, Germany) with a 2 mm square piece of gauze placed beneath the catheter at the point of entry. The upper tie was released and removed. Patency of the sensing catheter was verified at this stage by eliciting an audible fluctuating note from a radio tuned to a low frequency AM band. Saline swabs were then removed and the transmitter module was placed in the abdominal cavity and sutured to the anterior abdominal wall. The muscle layer was sutured and skin was closed with surgical clips.

During the post-operative period rats were kept on heating pads until recovery and were usually eating and drinking within four hours. Antibiotics and anti-coagulants were not given prophylactically. During the course of the study no cases of overt infection of implanted transmitters occurred within six months of implantation. Where early complications of implantation did occur, such as haemorrhage or signs of distal aortic occlusion, animals were humanely put down. After animals had recovered from surgical implantation, data collection for analysis was commenced. An initial post-operative hypertensive period lasting up to three days was seen after which blood pressure was stable and a normal circadian rhythm of motor activity, HR and BP was observed.

(II) Tail cuff plethysmography

BP recording by tail cuff plethysmography was used for certain studies. Rats were anaesthetized with 2% halothane in oxygen with induction in a chamber and subsequent maintenance using a mask. Animals were placed in a prone position on a heating pad (33°C) for the actual measurement, but were not actively pre-heated. This avoided inducing heat stress, which has been shown to significantly elevate BP in rodents pre-heated to temperatures of 38°C before measurement[422]. The duration of anaesthesia prior to taking BP recordings was kept to a minimum and was similar for all animals. BP was measured using a cuff (size 3/8" or 7/16" depending on the size of the rat) placed around the tail with a photoelectric sensor (IITC Life Sciences, Woodland Hills, California, USA). SBP was defined as the pressure at which initial flow in the tail artery was detected. MBP corresponded to the pressure at which no further increase in the amplitude of the pulse pressure was detected. DBP was derived from the recorded SBP and MBP from the equation DBP = ((3XMBP) - SBP)/2, however both MBP and DBP measurements were dependent on not using an artefact filter (Fig 2.2.2)

Fig 2.2.2



Example of an analog recording obtained using an IITC system for measuring BP by tail cuff plethysmography. SBP corresponds to the pressure at which a pulse pressure is detected indicated by a tick mark. The upper panel shows the cuff pressure during controlled deflation starting at 300 mmHg. SBP in this case was 240 mmHg.

A minimum of five readings were taken from each animal to derive a mean value for recording at each time point. The cuff was inflated to 250 mmHg in all animals except where the SBP was greater than 200 mmHg, when the cuff was inflated to 300 mmHg. Deflation rate was controlled to 2 mmHg/second. Tail cuff inflation pressures were calibrated and checked using an aneroid sphygmomanometer before each session. Validation of tail cuff plethysmography was performed by simultaneous comparison with BP recorded by direct carotid arterial pressure measurement and the results are reported below.

(III) Direct carotid arterial catheterisation

Rats were anaesthetized with ketamine HCl 12 mg/100 g BW (Vetalar™, Parke-Davis) and xylazine 0.3 mg/100 g BW (Rompun™, Bayer). Rats were placed on a thermostatically controlled heating mat at 33°C and the anterior neck area was shaved and swabbed. The left carotid artery was exposed and a tie placed distally. A small clip placed proximally transiently occluded blood flow while a polyethylene cannula (PE20) was introduced through a small incision in the carotid arterial wall.

The cannula was tied *in situ*, the clip was removed and the cannula was attached to a pressure transducer (model CK-590, Gould, Cleveland, Ohio) connected to a chart recorder. The cannula was flushed with a heparinised colloid/crystalloid solution (0.9% NaCl/0.1% bovine serum albumin with 10 U/ml Heparin) prior to insertion. Further flushes were given if required up to a maximum volume of 1.0 ml. Readings were taken after a minimum period of five minutes had elapsed and a stable baseline had been achieved. SBP, DBP and HR were read directly from the chart, while MBP was derived from the formula MBP = DBP + 1/3(SBP-DBP).

(IV) Validation of tail cuff plethysmography

There were two objectives in this study. Firstly the specific effect of carotid arterial cannulation on BP, as recorded by tail cuff plethysmography in the anaesthetized rat, was to be assessed. Secondly simultaneous recordings of direct carotid arterial pressure and indirect tail cuff plethysmography at normal body temperature were made in order to validate the latter method as a means of measuring BP and HR.

Eleven male SD rats were used with body weights ranging from 130-250 g. They were anaesthetized with Vetalar™ (12 mg/100 g BW) and Rompun™ (0.3 mg/100 g BW) and cannulation of the right carotid artery was performed as described above. Two rats did not give a stable direct arterial BP measurements due to recurrent occlusion and damping. Their BP traces were deemed inadequate and were therefore excluded from further analysis.

Effects of carotid arterial cannulation on BP measured by tail cuff plethysmography

Between three and seven indirect measurements of BP were taken by tail cuff plethysmography from each of nine anaesthetized rats prior to commencing carotid arterial cannulation. Following surgery, from three to twelve further measurements were taken. Unpaired t - tests were used to compare SBP immediately pre- and post-arterial cannulation for each individual animal, with a p value <0.05 considered to be significant (Table 2.2.1)

In conclusion, there was no predictable BP response to the effects of surgery with four rats showing a significant rise in SBP, three showing no significant change and two showing a significant fall. On comparing pre-operative mean SBP ± standard deviation (SD) of 133.14 ± 30.3 mmHg with the post-operative mean of 141.67 ± 27.7) mmHg by paired t-test, the small increment was not significant ($p=0.423$).

Table 2.2.1

Rat	Pre-op SBP ± SD	Post-op SBP ± SD	Change in SBP	Unpaired t-test
1	163.3 ± 11.2	191.0 ± 2.8	↑	<0.001
2	95.7 ± 9.0	157.8 ± 21.7	↑	0.004
3	128 ± 12.2	123 ± 5.3	→	ns
4	130.8 ± 26.1	107 ± 10.9	↓	0.039
5	118.3 ± 5.5	134.6 ± 14.1	↑	0.048
6	145.5 ± 7.9	123.2 ± 9.1	→	ns
7	125.7 ± 8.9	157.7 ± 8.6	↑	<0.001
8	191.7 ± 8.1	165.1± 5.0	↓	<0.001
9	99.3± 6.8	115.5 ± 17.7	→	ns

BP responses of individual rats to carotid arterial cannulation as measured by tail cuff plethysmography.

Comparison of SBP, MBP, DBP and HR obtained by direct carotid arterial cannulation

From eight of the nine rats, an acceptable direct BP trace was recorded and simultaneous recordings of BP by tail cuff plethysmography and by direct carotid arterial cannulation were obtained. For each animal between five and twelve combined readings (mean 8.1) were obtained giving a total of 65 simultaneous recordings. A paired t-test directly compared the means and SD of the readings for SBP, MBP, DBP and HR obtained by the two methods (Table 2.2.2).

Table 2.2.2

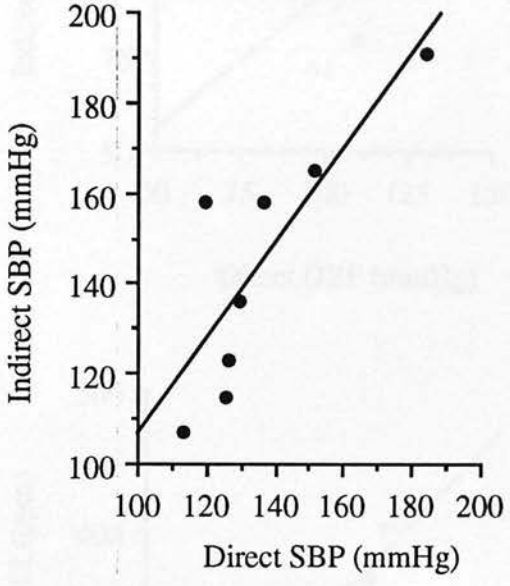
	r	p value	Direct BP ± SD	Indirect BP ± SD	p value
SBP	0.833	0.01	135.6 ± 22.5	144.1± 28.6	0.173 (ns)
MBP	0.643	ns	119.2 ± 18.2	112.6 ± 25.8	0.376 (ns)
DBP	0.529	ns	111.0 ± 16.4	99.0 ± 22.6	0.128 (ns)
HR	0.975	<0.001	344.4 ± 62.6	332.5 ± 56.1	0.053 (ns)

Correlation coefficient (r) with a p-value for significance is given for the linear relationships between simultaneous direct and indirect measurements. Means ± standard deviation (SD) of averaged direct and indirect measurements (n=8) are shown together with p values for comparison by a paired t-test. ns = not statistically significant.

Correlation coefficients between simultaneous measurements were calculated by least squares regression analysis (Fig 2.2.3).

Fig 2.2.3

A



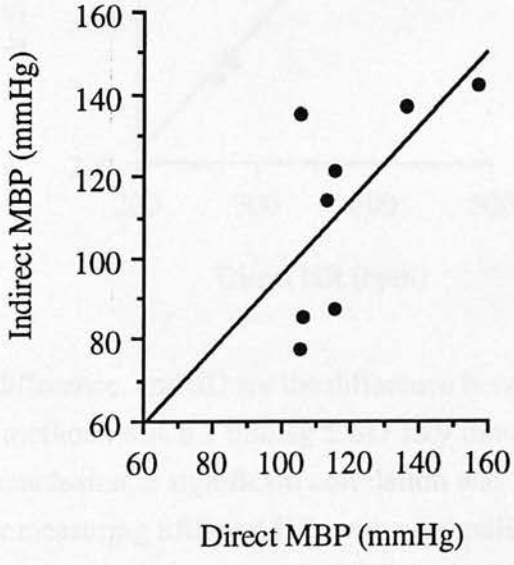
Least squares regression analysis of relationship between direct (carotid cannulation) and indirect (tail cuff plethysmography) measurements of A. SBP; B. MBP; C. DBP and D. HR. r^2 indicates the proportion of the total variation that is explained by the fitted model.

For SBP the fitted line y=0.23+1.1x

p=0.01

$r^2 = 0.69$

B.

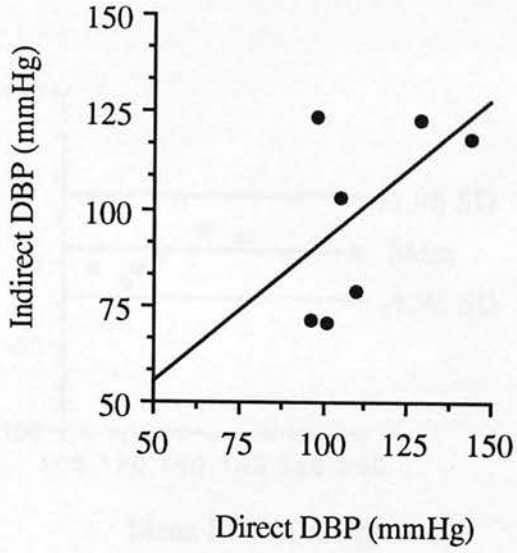


For MBP, y=4+0.9x

p = ns

$r^2 = 0.41$

C.

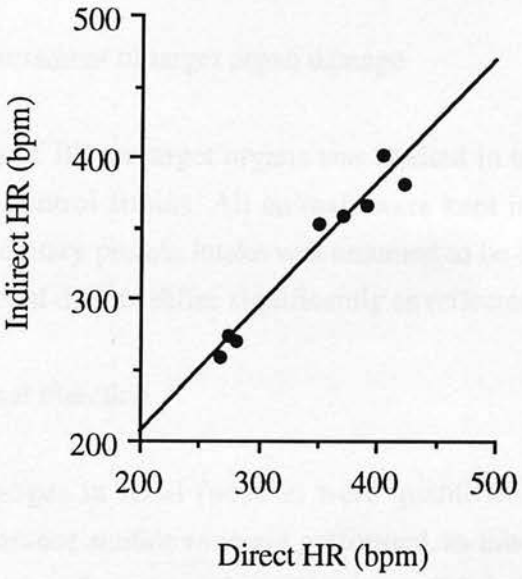


For DBP, y=18.2+0.73x

p = ns

$r^2 = 0.28$

D.



For heart rat, y= 30.1+0.9x

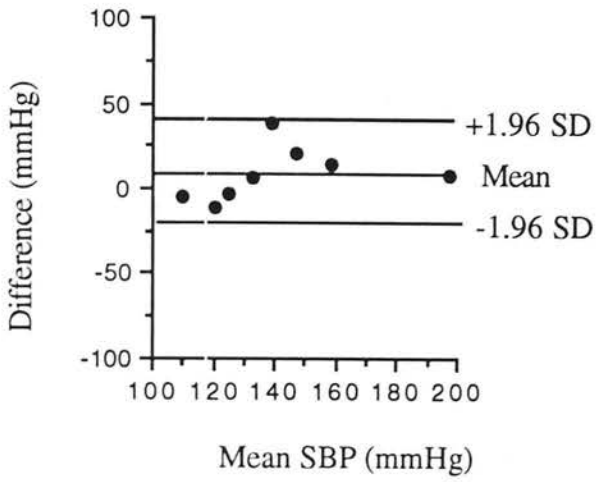
p<0.001

$r^2 = 0.95$

The mean difference, and SD for the difference between the average SBP as measured by the two methods was 8.5 mmHg ± SD 15.9 mmHg) (Fig 2.2 4).

In conclusion, a significant correlation was found between direct and indirect methods for measuring SBP and HR, with no significant difference in the mean values obtained. However the correlation coefficients for MBP and DBP were not statistically significant, despite the mean values obtained from the two methods being not significantly different. It was therefore decided that where BP was to be measured by tail cuff plethysmography, only SBP and HR data would be valid. It was recognised that direct comparisons of BP measured by different methods could not be justified.

Fig 2.2.4



Mean and SD of the differences for averaged SBP obtained by tail cuff plethysmography and by direct carotid arterial cannulation (Bland-Altman plot)[426]

2.3. Assessment of target organ damage

The effects of BP on target organs was studied in transgenic heterozygotes and non-transgenic control strains. All animals were kept in the same conditions as detailed above and dietary protein intake was assumed to be constant as intake of food between groups studied did not differ significantly as reflected by similar gains in body weight.

(I) Renal Function

Changes in renal function were quantified by plasma creatinine and urea. Formal clearance studies were not performed, as this was not practical in sick animals. Blood was taken from anaesthetized animals (2% halothane in oxygen) by either retro-orbital bleed using 50 µl microcapillary pipettes or by cardiac puncture using a 19 gauge needle and syringe. Blood was immediately put into lithium heparin (1 ml tube, Teklab M. L. Ltd., Durham, UK) on ice, spun at 4000 g for 10 minutes at 4°C and plasma was stored at -20°C before analysis. GEMENI Reagent kits (Electro-nucleonics, Fairfield New Jersey, USA) were used for assay of both plasma urea and creatinine using a Gemstar analyzer.

The urea assay utilized the enzyme urease (urea aminohydrolase) to hydrolyse urea to give ammonia and carbon dioxide. Quantification of generated ammonia was done by reaction with 2-oxoglutarate and NADH (reduced nicotine adenine dinucleotide) in the presence of glutamic dehydrogenase at 37°C to give glutamic acid and NAD. The rate of conversion of NADH (which absorbs light at 340 nm) to NAD

(no absorbance at 340 nm) is directly proportional to the initial concentration of urea. A standard with a known concentration of urea and distilled water allowed calculation of the urea concentration in the sample (10 µl).

Creatinine reacts with picric acid in alkaline buffer to form a yellow product. The absorbance change at 500 nm is proportional to the initial concentration of creatinine. 50 µl of plasma was mixed with 700 µl of sodium hydroxide and picric acid (equal volumes). Controls were provided by distilled water plus NaOH/picric acid and a known standard (of concentration 350 µmol/l) with reagents. In both assays distilled water blanks were used and quality control was verified with all assays by checking the QC factor (Standard concentration/ΔAbs/min of standard). For urea standards assayed (standard concentration = 13.3 mmol/l, n=15), the mean was 13.49 mmol/l with a variance of 0.04 and for plasma creatinine (standard concentration 177 µmol/l, n=20) the mean was 176.8 µmol/l with a variance of 63.4.

(II) Development of left ventricular hypertrophy

Two methods were used to assess development of left ventricular hypertrophy (LVH) in hypertensive *Ren*-2 transgenic heterozygotes and non-transgenic controls. Animals were sacrificed by rapid induction of anaesthesia (6% halothane in oxygen) in a chamber followed by cervical dislocation. The chest was then opened and heart and lungs were removed. The ventricles were dissected free from lungs, great vessels, mediastinal tissue and atria, blood was removed by gently rolling the heart on blotting tissue and the free right ventricular wall was removed by dissection. Direct weights of whole heart (WH) and left ventricle including septum (LV) were expressed as a ratio to body weight. Tissues were then placed in 4% formal saline for fixing prior to processing for histopathological examination.

A second method of measuring left ventricular mass was required that was not invasive and would therefore allow serial measurements in longitudinal studies. Assessment of left ventricular mass index (LVMI) using echocardiography has been well described in humans and the method compared with both radiological and angiographic determination of left ventricular wall thickness in patients and with LV mass measurements at autopsy[427-429]. Recently the technique as applicable to rats, has been described and validated by Jones et al (1992)[430].

It was therefore decided to compare this technique with LV weight as measured directly at sacrifice and if valid, use it in studies requiring serial measurement of LV mass. Echocardiography was performed using a Toshiba Capasee (Toshiba Medical Systems, Tokyo, Japan) with a 7.5 MHz wide band probe held

perpendicular to the anterior chest wall of anaesthetized (2% halothane in oxygen) animals lying on their back on a heating pad (33°C). The anterior chest wall was shaved, ultrasound couplant gel applied (Diagnostic Sonar, Livingston, UK) and a long axis view of the left atrium and ventricle was obtained on a 2D image. To standardize the long axis view for measurement, the apex of the LV, the aortic root, mitral valve and left atrium were all required to be visible (Fig 2.3.1). This reduced the potential for error arising from oblique transections of the ventricular wall at the time of measurement. From an M-mode scan, LV posterior wall (PW) and interventricular septal (IVS) thickness was measured from a video during maximal diastolic relaxation (end-diastole) at a level just below the tips of the mitral valve leaflets[428]. Three separate scans were performed obtaining one reading from each, and mean determinations for PW and IVS thickness and end-diastolic diameter (EDD) were obtained. Care was taken not to include papillary muscle in the estimated thickness of the posterior wall.

The LV mass was calculated from the formula below (1) based on the cube function formula which has approximated the shape of the LV to a prolate ellipsoid with a ratio of long axis to short axis length of 2:1.

(1) $LVM = 1.04\ (PW + IVS + EDD)^3 - EDD^3$ (Devereux 1986)[429]

This has been derived from the following formulae (Troy 1972)[427]:

(2) $LVCV = 4/3\pi(Dd/2)(Dd/2)(2Dd/2)$

where LVCV = end-diastolic chamber volume and Dd/2 = one half of the LV end-diastolic chamber dimension.

(3) $LV(C+M)V = 4/3\pi(Dd/2 + WTd)(Dd/2 + WTd)(2Dd/2 + WTd))$

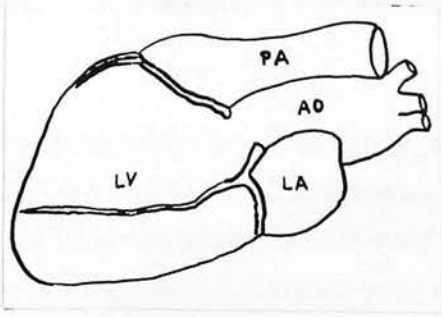
where LV(C+M)V = end-diastolic chamber volume plus muscle volume in ml, WTd = LV end-diastolic wall thickness. So LV muscle volume (LVMV) equals

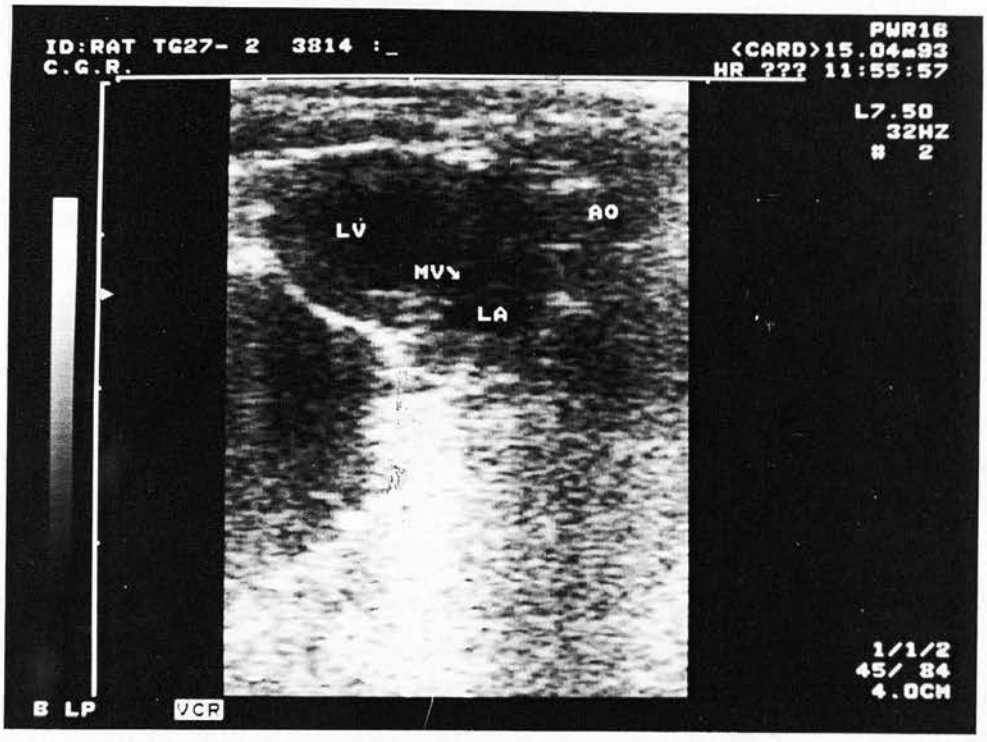
(4) $LVMV = LV(C+M)V - LVCV$

To derive LV mass (LVM) (g), the LVMV is multiplied by the specific gravity of heart muscle (1.05).

(5) $LVM = LVMV \times 1.05$

Fig 2.3.1





A two dimensional (2D) image in the longitudinal axis has been obtained by echocardiography. The top panel is a diagrammatic representation of the anatomy of the rat heart for reference. The lower panel shows the obtained image. LV = left ventricle; MV = mitral valve; LA = left atrium; AO = aortic root; PA = pulmonary artery. M-mode scans were taken from the level of the tips of the mitral valve leaflets.

Accordingly this method was used in the following study which directly compared LV mass as measured by echocardiography with direct LV weight.

(III) Validation of Echocardiography as a means of measuring LV mass

Methods

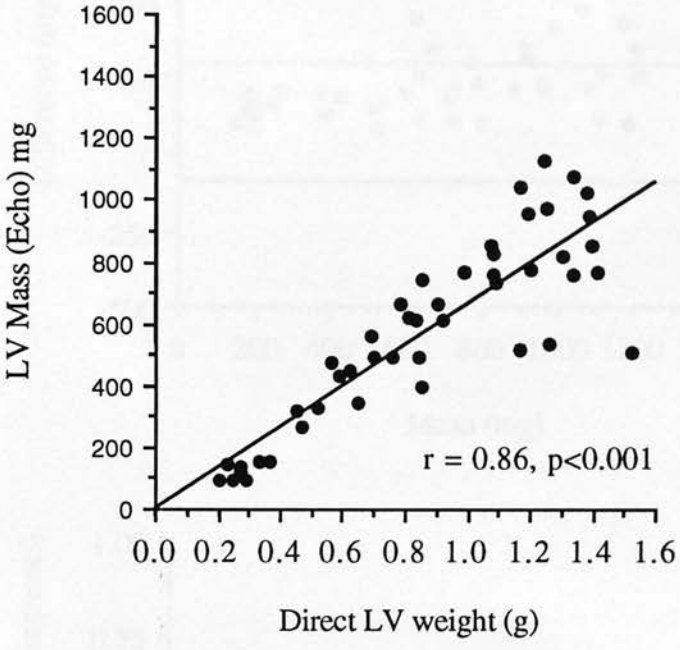
Hypertensive transgenic *Ren*-2 heterozygotes (HanRen2/Han--) (n=23) derived from crossing homozygous *Ren*-2 males with Hannover SD females (HanSD) and normotensive HanSD controls (n=21) were used for age-matched comparison at 25, 35, 45, 55, 65 and 75 days of age. Animals were kept according to the conditions as detailed above and fed on a normal (0.32%) sodium diet. On the day of sacrifice SBP and HR was measured in anaesthetized rats by tail cuff plethysmography as described above. A mean of five readings was taken. Rats were then placed on their backs and three echocardiographic measurements of LV mass were taken. Blood was obtained by cardiac puncture for later assay for the purposes of another study (Veniant, Whitworth, Menard et al, submitted for publication). Animals were then killed by cervical dislocation, the chest was opened and the heart removed. LV and IVS together were weighed directly after being blotted dry and dissected free of atria, right ventricle and great vessels.

Points were plotted for data pertaining to each animal, and a linear relationship with a correlation coefficient r of 0.86 ($P<0.001$) was observed between the two methods (Fig 2.3.2). Some increase in scatter was seen in animals with larger LVM or LV weight. However it has been well recognised that a correlation coefficient is not an adequate means of comparing two methods of measurement[426], and on plotting the relationship of the difference between the two methods with the mean value obtained, it was found that relative to direct measurements of LV weight, echocardiography systematically underestimated the LV mass relative to the LV weight, again with an increased discrepancy apparent between the two methods for larger LV weight/mass (Fig 2.3.3 A). Bias was calculated from the mean difference (d=287.1 mg) and the standard deviation of the difference (SD = 194.1 mg), but in addition the error was not normally distributed and so is illustrated after log transformation in Fig 2.3.3 B.

Intra-observer variation in the determination of LV mass using this method was assessed by repeated measurement of LV mass on one rat (HanSD male, 530g body weight). Ten independent measurements of LV mass were made, where each was based on the average of three determinations of IVS, PW and EDD as described

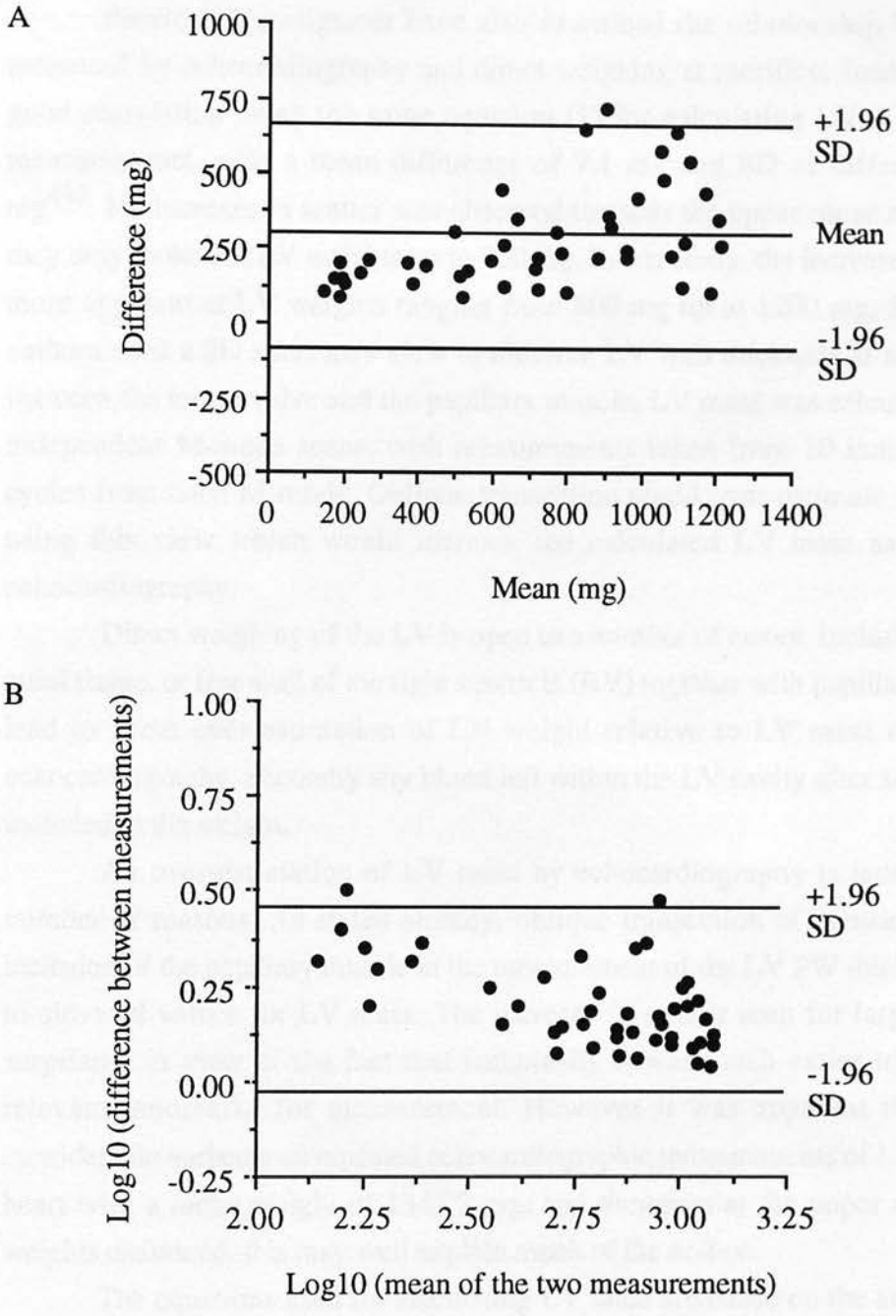
above. The mean LV mass was 1345.2 mg with a SD of 145.4 mg giving 95% confidence limits for the mean of 1060.2 - 1630.2 mg.

Fig 2.3.2



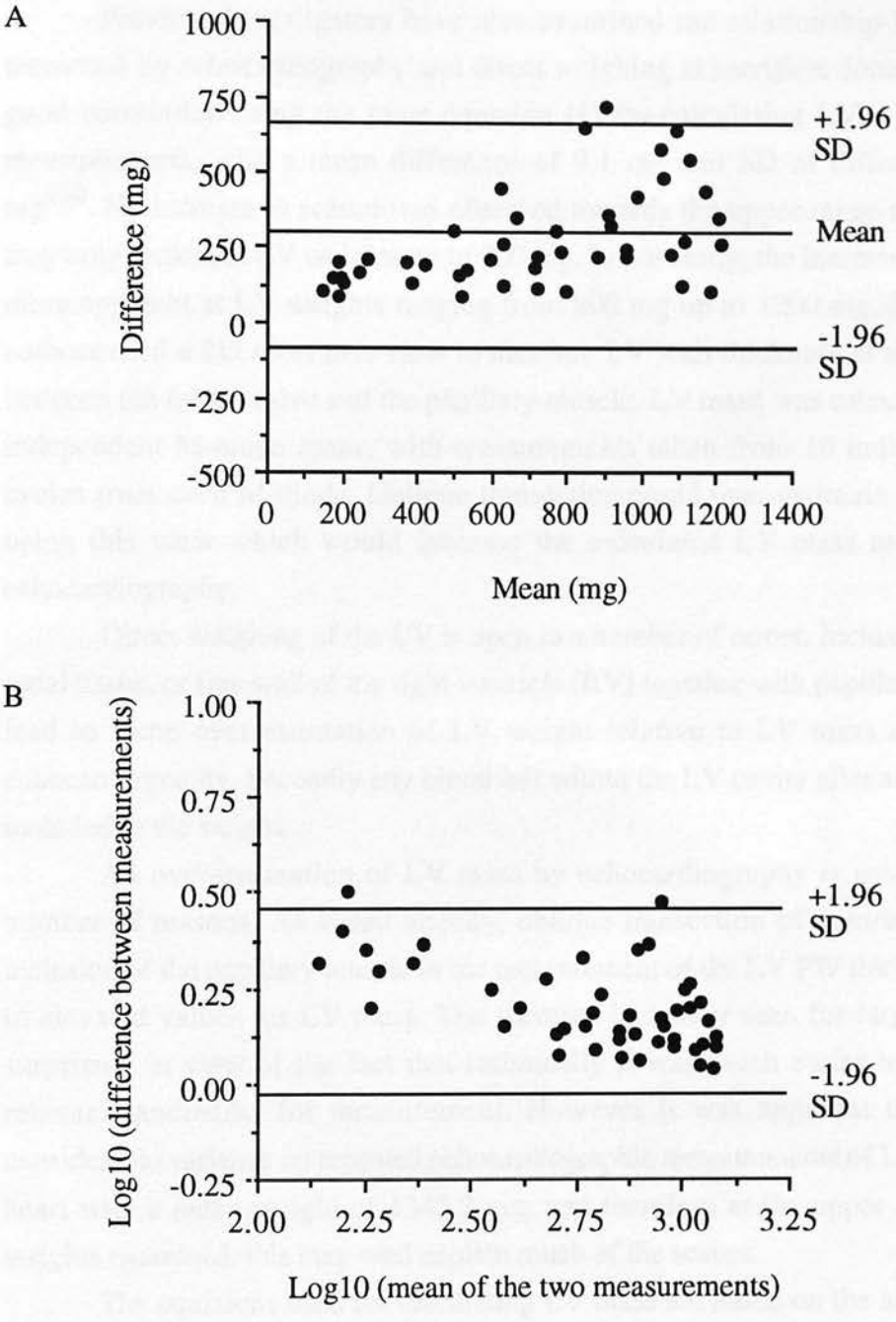
Least squares regression analysis of relationship between LV mass (mg) measured by echocardiography and direct LV weight (g). $y = 1.5 + 667x$, $r^2 = 0.76$.

Fig 2.3.3



A. Plot of the difference between the two methods of measurement against the mean of the two measurements (Bland-Altman plot). B. Log10 plot of the difference against the mean.

Fig 2.3.3



A. Plot of the difference between the two methods of measurement against the mean of the two measurements (Bland-Altman plot). B. Log10 plot of the difference against the mean.

Previous investigators have also examined the relationship between LVM measured by echocardiography and direct weighing at sacrifice. Jones et al found a good correlation using the same equation (1) for calculating LVM from M-mode measurements, with a mean difference of 9.1 mg and SD of differences of 56.6 mg[430]. No increase in scatter was observed towards the upper range of LV size, but they only looked at LV weights up to 900 mg. In our study, the increase in scatter was more apparent at LV weights ranging from 800 mg up to 1200 mg. Secondly these authors used a 2D short axis view to measure LV wall thickness at a level midway between the mitral valve and the papillary muscle. LV mass was calculated from two independent M-mode scans, with measurements taken from 10 individual cardiac cycles from each M-mode. Oblique transection could over-estimate wall thickness using this view which would increase the calculated LV mass as measured by echocardiography.

Direct weighing of the LV is open to a number of errors. Inclusion of remnant atrial tissue, or free wall of the right ventricle (RV) together with papillary muscle may lead to some over-estimation of LV weight relative to LV mass determined by echocardiography. Secondly any blood left within the LV cavity after sacrifice will be included in the weight.

An over-estimation of LV mass by echocardiography is more likely for a number of reasons. As stated already, oblique transection of ventricular wall and inclusion of the papillary muscle in the measurement of the LV PW thickness will lead to elevated values for LV mass. The increase in scatter seen for larger hearts was surprising in view of the fact that technically it was much easier to visualize the relevant landmarks for measurement. However it was apparent that there was considerable variance on repeated echocardiographic measurements of LV mass from a heart with a mean weight of 1345.2 mg, and therefore at the upper range of heart weights examined, this may well explain much of the scatter.

The equations used for calculating LV mass are based on the assumption that the LV is an ellipsoid with a regular conformation. Hence asymmetric LV hypertrophy would increase the error of the calculated LV mass. Various formulae have been developed and applied to correct for systematic errors arising from calculating LV volumes derived from the cube function, and some have been shown to systematically underestimate LV mass relative to anatomical mass[431], while others over-estimate [429]. The use of echocardiography in assessing LVMI in rats was also recently reported by Bruckschlegel et al. (1995)[432]. They obtained only one long axis view to measure LV

PW thickness and EDD and used a modified formula: LVMI = 1.04 ((EDD+2 X PW)^3 - EDD^3 - 14) /BW. However though they reported a reasonable correlation between LVMI obtained in this fashion and post-mortem weight ($r = 0.70$, $p<0.0001$), they have not applied the Bland-Altman analysis[426] to their measurements.

In conclusion, echocardiography has been shown in this study to have a linear relationship with LV weight measured directly post-mortem with a correlation coefficient of 0.86 ($p<0.001$), but as a method it under-estimated LV mass relative to direct weighing. Such an error may arise either from over-estimating LV weight on direct measurement due principally to inclusion of tissue other than the LV or from applying formulae derived for use in human echocardiography to the rat which may not be justified because of differences in LV shape. As a method for serial measurements of LV mass, it is satisfactory but interchangeable use of echocardiographic and direct assessments of LV mass may give rise to unacceptable error particularly with larger hearts. Echocardiography is also subject to inter-observer variation[428,429], and in longitudinal studies this would be an important and avoidable source of error. Intra-observer variation appears also to be a significant problem. The variable use of equations reported in the literature for determination of LVMI in rats by echocardiography together with the results above suggests that an improved formula specifically designed for use in rats is needed.

(IV) Measurement of Lung water content

Lung water content was measured as a crude indicator for the presence of pulmonary oedema arising from left ventricular failure. At sacrifice and immediately on removal of the heart and lungs, peripheral lung tissue was dissected free from major vessels and bronchi, lightly blotted and weighed. Lung tissue was then heated to dryness for at least 24 hours at 80°C and re-weighed. Percentage water content was calculated from (wet weight-dry weight)/wet weight.

(V) Histopathological examination of tissues

At sacrifice left ventricle, right ventricle, descending aorta, kidneys, brain and superior mesenteric artery were taken and fixed 4% formal saline. Tissues were processed using a Citadel tissue processor (Shandon Southern Products Ltd., Cheshire, UK) on an 18 hour programme passing through serial dehydration steps in graded ethanol concentrations (70%, 80%, 90%, 100% X3), followed by de-

alcoholisation with a clearing agent Histoclear™ (National Diagnostics, Atlanta, USA), X3, and paraffin wax immersion X2 at 60°C. One half kidney (longitudinal axis), LV, RV, brain (coronal sections at the level of the mid-brain), descending aorta and superior mesenteric artery were embedded in wax blocks (Raymond Lamb Blockmaster III embedding centre, London, UK) prior to sectioning and staining.

Sectioning, staining and mounting of tissues was kindly performed by personnel from the Department of Pathology, University of Edinburgh. Three µm sections were sectioned and stained according to methods as described elsewhere[433]. Haematoxylin & eosin stain was used for all tissues. Briefly sections were de-waxed in xylol, rehydrated through graded ethanols to water, stained with an alum haematoxylin, washed in running water, immersed in 1% HCl in 70% ethanol for 10 seconds, washed again for 5 minutes, stained with 1% eosin for 10 minutes and washed again. Slides were then serially dehydrated through graded ethanols, cleared with xylol and mounted with DPX (BDH Laboratory Supplies, Poole, UK).

In addition heart and kidney tissue were stained using a periodic acid-Schiff technique (PAS)[434] to specifically stain for tissue carbohydrates of extra-cellular matrix. To stain for fibrin, kidney tissue was stained with a standard MSB (Martius Scarlet Blue) stain[435]. With this method fibrin stains red, though early fibrin may appear yellow and old fibrin blue.

Sections of aorta and superior mesenteric artery were additionally stained with Elastic van Gieson (EVG) and brain with Luxol Fast Blue. All sections were then examined by light microscopy (Leitz Laborlux S) and photographed with a Leica Wild MPS46/52 camera.

(VI) Blood films

Selected animals were bled either by retro-orbital bleed or by cardiac puncture under halothane anaesthesia, and the blood collected into tubes containing EDTA (ethylene diamine tetra-acetic acid, Sarstedt Monovette 2.7 ml tubes). Full blood counts were performed automatically using a Coulter counter. Films were then spread and stained (Wrights stain) by personnel at the Department of Haematology, Royal Infirmary of Edinburgh. Films were examined under light microscopy, by an observer (L Manson) blinded to the clinical status of the animals, for the presence of spherocytes, microspherocytes, red cell fragments and a reticulocyte count was determined.

2.4 Renin-angiotensin system parameters

(I) Plasma RAS

Plasma renin concentration (PRC), angiotensin II (Ang II) and aldosterone assays were performed. Blood was obtained by either retro-orbital bleed or cardiac puncture from anaesthetized animals and put into lithium heparin (1 ml tubes Teklab Ml Ltd.) on ice, spun immediately at 4000g for 10 min at 4°C and plasma was then stored at -70°C before assay of PRC.

Radioimmunoassays (RIA) for PRC and Ang II were kindly performed by Dr J.J. Morton, MRC High blood Pressure Unit, Western Infirmary, Glasgow according to methods described previously[436-439]. Briefly for PRC assay, duplicate samples of rat plasma (3 µl) were incubated at 0°C and 37°C with a mixture (14 µl) of Ang I antiserum (1:80 dilution in 3 mol/l Tris, pH 7) and rat renin substrate in a ratio of 1:6. Rat renin substrate, shown to be free of renin, was obtained from bi-nephrectomized rats[438]. The enzyme incubation was terminated by rapid cooling in ice/water after 5, 30 or 60 minutes depending on renin content. Generated Ang I was then measured by RIA. 300 µl of cold Tris 0.5 mol/l, pH 7.5 was added to each sample followed by 10 pg of ^{125}I-labeled Ang I (in Tris 0.5 mol/l, pH 7.5). Samples were incubated at 5°C for 18 hours, and bound and free labeled Ang I was separated by the addition of plasma-coated charcoal with the free labeled fraction quantified by an automatic γ–counter[440]. Standard curves were prepared from dilutions of Asp^1-Ileu^5-Ang I from 323 to 2.5 pg in 0.2 ml 0.5 mol/l Tris (pH 7.5), 0.2 ml of anti-serum (1:80,000) and 10 pg of labeled peptide.

For Ang II assay, blood was collected into fresh Ωphenanthroline (0.05 mol/l)/EDTA (0.1 mol/l) inhibitor on ice in a ratio of 10 µl per 0.1 ml of whole blood to inhibit angiotensin converting enzyme activity and angiotensinases. The inhibitor consisted of 0.05 g 1-10 phenanthroline monohydrate (0.025 mol/l) (Sigma, UK) dissolved in 400 µl absolute ethanol and added to 0.464 g EDTA disodium salt (0.125 mol/l) (BDH Analar Laboratory Supplies, Poole, UK) and made up to 10 ml with water. Blood was then spun immediately at 4000 g for 10 minutes at 4°C and plasma stored at -70°C before assay. Plasma was passed through an extraction step using Sep-pak C_{18} cartridges previously washed with 5 ml methanol and 5 ml distilled water[439]. Plasma samples were then loaded and passed through the columns at a pressure of 10 psi. Ang II was eluted from the columns with 80% methanol in distilled water and the

extracts were dried under air and redissolved in Tris buffer (50 mmol/l, pH 7.5) before RIA.

Duplicate standard curves for Ang II were prepared from a stock solution of Val^5 angiotensin II amide ("Hypertensin", Ciba, Sussex, UK) at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0 pg in 200 µl Tris buffer (50 mmol/l pH 7.4). 400 µl of Tris/HCl buffer (50 mmol/l pH 7.4) was added to each of the plasma extracts which were thoroughly mixed, centrifuged (3000 rpm, 5°C, 10 minutes) and then 100 µl of extract was pipetted into duplicate tubes. Ang II antiserum (BPU) (100 µl of 1:30,000) was added together with 50 µl ^{125}I Ile^5 Ang II (Dupont) to both extracts and standards. Tubes for total counts were prepared from Tris buffer (100 µl) and Ang II label (50 µl). After thorough mixing, all tubes were placed in a fridge for 18 hours at 4°C. Bound and free label were separated using dextran-coated charcoal. Dextran T70 (0.282 g, Pharmacia) was dissolved in 50 ml of Tris/HCl buffer pH 7.4. Norit SX-1 charcoal (15 g) was added to 1.35 l of Tris/HCl buffer pH 7.4, dissolved on stirring, mixed with the Tris/dextran and stored at 5°C before use. Cold dextran/charcoal (1 ml) was added to all tubes which were then centrifuged (3000 rpm, 5°C, 10 minutes) and the supernatant discarded. The radioactivity present in the charcoal pellet (free fraction) was counted in an automatic γ-counter (LKB model 1260 gamma counter), the standard curve computed and hence values extrapolated for the plasma extracts. After adjusting for sample dilution, recovery and plasma volume final plasma concentrations were obtained in pg/ml.

Aldosterone was assayed from serum (obtained from whole blood spun at 4000 g for 10 minutes at 4°C) by N Burns and Dr B Williams (Department of Medicine, Western General Hospital, Edinburgh) by the method described previously[441]. 50 µl of plasma or 50 µl of standard (concentration range 0.06 to 32 nmol/l) were incubated in triplicate for 18 hours at 4°C with 200 µl of phosphate citrate buffer (pH 4, 0.05% bovine serum albumin) containing the radio-label ^{125}I-iodohistamine-aldosterone-3-mono-oxime and antisera (402L) in a final dilution 1:60,000. Standards were made up in charcoal stripped rat plasma. Free and bound radiolabel were also separated using dextran-coated charcoal, and the free fraction was quantitated using an LKB model 1260 gamma counter. Intra- and inter-assay variations were 7% and 14% respectively. The mean of the three determinations was used.

(II) Immunohistochemistry

Immunohistochemistry was performed on kidney using a polyclonal rabbit anti-mouse renin antibody (given by Dr D Campbell, Melbourne, Australia). Formalin fixed, paraffin embedded tissues were used, prepared as detailed earlier. Three μm sections were cut using a microtome (Anglia Scientific 0325), floated on to Superfrost microscope slides (Menzel-glaser, Germany) and baked for 5 minutes. Paraffin wax was then removed by 2 minute immersion of slides in serial baths containing Histoclear (3 baths), 100%, 95%, 70%, and 50% ethanol and finally distilled water. The slides were dried and wax was used to encircle the tissue section. Blocking solution (phosphate buffered saline (PBS)/5% goat serum) was layered on and left for one hour in a moist box containing PBS buffer. The blocking solution was aspirated off, and rabbit anti-mouse renin antibody was applied at a dilution of 1:2000 in PBS/2% goat serum for overnight incubation at 4°C in a moist box. The primary antibody was omitted for control sections which were processed in parallel. Three 2-minute washes with PBS/2% goat serum were followed by a 10 minute incubation with PBS/2% goat serum with 2% hydrogen peroxide to quench endogenous peroxidase activity. Three 2-minute washes with PBS/2% goat serum were then followed by incubation for 30 minutes with biotinylated goat anti-rabbit antibody (1:200) (Vectastain™, Vector Laboratories Inc., Burlinghame, CA, USA) in PBS/2% goat serum. After four washes with PBS, an immunoperoxidase detection system (Vectastain™ Elite ABC kit) was used in which a complex was formed between avidin DH (1:100) and biotinylated horse-radish peroxidase H (1:100) in PBS binding to the biotin of the secondary antibody. After incubation for 20 minutes at room temperature, further washes with PBS were followed by application of the chromogen diaminobenzidine tetrahydrochloride (DAB) dissolved in PBS and filtered through a 0.2 μm syringe filter, and hydrogen peroxide (3 μl/ml of 30%). A red-brown precipitate was formed by reaction with peroxidase on standing for 1-5 minutes. This was terminated by washing with UHP water and subsequently the slides underwent serial dehydration through graded alcohols and clearing with Histoclear before mounting. Counterstains were not applied.

Minimal background staining which was limited to red cells was encountered on using this antibody and secondary detection system. On omitting the primary antibody, no staining was seen.

2.5 Endothelin in malignant hypertension

(I) RNase protection assays for endothelin mRNA on whole rat kidney .

Animals

Male heterozygotes, hemizygous for the mouse *Ren*-2 transgene were bred and maintained as previously described. Hypertensive HanRen2/Edin--, HanRen2/Lew--, and non-transgenic, normotensive EdinSD were used (see Fig 3.1.1 for explanation of nomenclature).

Malignant hypertension was identified on the basis of clinical features as described in chapter 3 which include weight loss, apathy, fitting and which were confirmed by histopathological examination as previously described. Four groups of 8-10 week old rats were studied: (1) HanRen2/Edin-- with malignant phase hypertension (n=11), (2) age-matched HanRen2/Edin-- with benign hypertension but no signs of malignant phase (n=6), (3) HanRen2/Lew-- with benign hypertension (n=4), and (4) normotensive EdinSD (n=4). Animals were briefly anaesthetized by 2% halothane anesthesia and killed by cervical dislocation. Kidneys were quickly dissected out, snap frozen in liquid nitrogen, and stored at -70°C before RNA extraction.

RNA extraction

Total RNA was extracted from whole kidney for use in an RNase protection assay for endothelin mRNA quantification. At sacrifice, one kidney was immediately dissected out and the capsule removed, blotted dry, snap frozen in liquid nitrogen and stored at -70°C until RNA extraction was performed. A phenol/guanidium thiocyanate method was used as previously described[442]. Where appropriate, solutions used in RNA work were DEPC (diethylpyrocarbonate) treated as per standard protocols[443], but at a concentration of 0.01%.

Approximately one sixth kidney from each animal was homogenized in 4 ml of RNAzol™ B (Biotecx Laboratories, Inc. Texas, USA) in a Falcon 2059 tube on ice using a Polytron homogenizer. Four hundred µl of chloroform were added, mixed and left to stand on ice for 5 minutes. After centrifugation (3200 rpm at 4°C for 5 min) the upper phase was aspirated. An equal volume of isopropanol was added, tubes were mixed by inversion and left to stand at 4°C for 60 minutes to allow RNA to precipitate. After centrifugation at 10,000 rpm for 15 minutes at 4°C (Beckman Centrifuge, SS34

rota), the RNA pellet was washed with 70% ethanol (in DEPC treated water), mixed and spun for a further 15 minutes. After removal of ethanol, the dry pellet was resuspended in 100 µl of DEPC water. RNA concentration was quantified by spectrophotometry at wavelength 260 nm (Pharmacia LKB Ultrospec Plus). Integrity of extracted RNA was checked on ethidium bromide stained 1% agarose gels visualizing 5s, 18s and 28s ribosomal bands. 150 µg of total RNA was re-precipitated in 1/10th volume 3 mol/l sodium hydroxide and 2.5 volumes of 100% ethanol and stored at -70°C for RNase protection assay for endothelin mRNAs.

Endothelin RNase protection assay

The RNase protection assays for rat ETs -1, -2 and -3 were kindly performed by Dr J D Firth, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford according to the method previously reported[414]. In brief, uniformly labeled antisense RNA transcripts were generated by *in vitro* transcription using SP6 polymerase (Amersham) and α^{32}P-GTP (410 Ci/mmol/l, Amersham). In each case the riboprobe template contained genomic sequence which included a part of exon 2 of the gene of the relevant ET, which is the region coding for the mature peptide. For analysis of mRNA, precipitated total RNA from coded samples, were dissolved in hybridization buffer (80% formamide, 40 mmol/l PIPES, 400 mmol/l sodium chloride, 1 mmol/l EDTA pH 8) and RNA concentration determined by absorbance measurement at 260 nm using a DU-62 spectrophotometer (Beckman Instruments Inc.). One µg of RNA extracted from the human cell line K562, containing abundant α–globin mRNA, was added to 30 µg of rat kidney RNA in a final volume of 50 µl. RNA was denatured at 90°C for 10 minutes. Hybridization was performed overnight at 60°C with $2.5x10^5$ cpm of the appropriate ET probe and $2.5x10^5$ cpm of a probe specific for human α–globin. A comparison of the recovery of α–globin mRNA from individual samples allowed a correction to be made for any variation in efficiency of processing and gel loading. After hybridization, RNase digestion was carried out at 37°C for 30 minutes by the addition of 350 µl of solution containing 40 µg/ml RNase A (Boehringer), 10 mmol/l Tris (pH 7.5), 5 mmol/l EDTA and 300 mmol/l NaCl. This reaction was terminated by the addition of 60 µl of proteinase K (1 mg/ml) with 3% SDS and further incubation for 30 minutes at 30°C. Phenol-chloroform and then chloroform extractions were performed and the RNA fragments precipitated with 2.5 volumes of absolute ethanol. Precipitated RNA was dissolved in 5 µl of 80% formamide running buffer, and the reaction mix electrophoresed on a denaturing 8% polyacrylamide gel. After electrophoresis the gels were dried and subjected to autoradiography at -70°C,

following which the autoradiographs were aligned with their corresponding gels and the protected ET and α–globin mRNA bands excised. These were then counted using a flat-bed liquid scintillation counter (1205 Beta Plate TM, Pharmacia-Wallac OY, Turkey, Finland). Results were expressed as cpm (mean ± SD) after (a) subtraction of background (cpm derived from counting a sample containing no RNA) (b) correction for recovery of α–globin mRNA in individual samples (which did not vary over a range of more than 15% on any gel), and (c) correction for the cpm obtained from three external standards run on each gel. These external standards - containing 15 μg, 30 μg and 60 μg from a pool of RNA derived from the kidneys of normal SD rats - were required because the cpm obtained from any particular gel depended not only on the samples themselves, but also on the activity of the particular batch of probe used, which was made freshly each week.

Statistical analysis was initially performed using the Kruskal-Wallis test one way ANOVA, corrected for ties, to test the null hypothesis that there was no difference between the four groups. Where statistically significant differences were found, a Mann-Whitney U test, also corrected for ties, was applied between pairs of groups to identify where such differences lay. The p-value indicating statistical significance was taken to be < 0.05.

(II) Effects of an Endothelin antagonist, Bosentan, on transition from benign to malignant phase hypertension.

Animals

Male HanRen2/Edin-- were housed as described above in groups of three or four and fed a diet (0.32% sodium, Harlan-Olac, Bicester, Oxford) from weaning at 25 days. They were randomly assigned to a treatment group (n = 17), given Bosentan (Hoffman la Roche, Basle, Switzerland) at a dose of 100 mg/kg/day and a control group (n = 17) given the same diet without added Bosentan. The drug was thoroughly mixed with powdered foodstuff in aliquots of 1.75 g/kg. Correct dosage was verified by periodic weighing of both rats and food intake. Both groups had tap water *ad libitum* to drink. MH was defined as present on development of clear signs of the syndrome as previously described, together with light microscopic examination of renal tissue confirming the presence of fibrinoid necrosis and myointimal proliferation.

Blood pressure measurement

In order to determine the effect of Bosentan on BP in conscious transgenic *Ren*-2 rats, an additional group of 11 male rats (body weight 340 to 435g) had transmitters (TA11PA-C40) implanted under anaesthesia for telemetric recording of continuous arterial pressure as previously described in section 2.2 (I). At least two weeks after surgery, they were administered single doses of Bosentan 100 mg/kg/day, dissolved in 5% gum arabic (Fisons, Loughborough, UK) by gavage. Three rats were given further single doses of up to 175 mg/kg a week later to ensure that any increase in dose would not affect BP. MBP was recorded for 24 hours prior to and 48 hours after dosing in conscious unrestrained animals, and comparisons made between MBP at equivalent times of the day to correct for circadian rhythm[444,445].

During the study of chronic Bosentan treated *versus* untreated groups, systolic blood pressure (SBP) and heart rate (HR) were measured weekly in anaesthetized rats using the indirect tail cuff plethysmographic method (see section 2.2(II)). Five readings of SBP were obtained at each time point and a mean recorded for the individual animal. Body weight was measured weekly. Statistical comparison between groups was performed by Student's t test, with $p<0.05$ taken to be significant.

Effects of exogenous Endothelin

The effect of exogenous endothelin in treated and control animals was examined in order to show that treatment with Bosentan given mixed in food had effectively blocked endothelin receptors. On completion of the study at 105 days of age, random surviving Bosentan treated (n=3) and untreated animals (n=4) underwent BP recording by direct carotid pressure monitoring (see section 2.2 (III)). The internal jugular vein was cannulated for intravenous administration of porcine big-Endothelin (Sigma, UK) at a dose of 0.3 nM/kg in 0.9%NaCl/0.1% BSA at a final volume of 0.5ml/kg. After a stable baseline BP had been achieved, big-ET was given and the BP response was recorded over the following 60 minutes.

(III) Effects of chronic Bosentan treatment on development of LVH

In conjunction with the above experiment, HanRen2/Edin-- rats underwent measurement of LVMI using echocardiography using the method described in section 2.3 (II) at 25 days of age and from surviving rats at 60 days of age. Results obtained

from treated and untreated control groups, were compared by Students t-test at 25 and 60 days of age with $p<0.05$ taken to be significant.

CHAPTER 3

Characterization of a malignant hypertensive phenotype

3.1 Introduction

The transgenic rat line TGR(mREN2)27 was initially bred at the University of Heidelberg where the transgene was introduced into F1 embryos from a SD/WKY cross[267]. A colony of TGR(mREN2)27 was subsequently bred and maintained by back-crossing of transgene positive rats onto SD, referred to here as Hannover SD (HanSD), at the Central Institute for Laboratory Animal Breeding, Hannover, Germany. This strain has been well characterized producing a phenotype of severe, but benign hypertension as described in section 1.7. HanSD male rats homozygous for the *Ren*-2 transgene (HanRen2/HanRen2) were maintained on ACE inhibitors to control BP for breeding purposes. In order to develop a colony of rats hemizygous for the transgene at the Centre for Genome Research in Edinburgh, SD rats were obtained from N.I.M.R. Mill Hill, England, and established in Edinburgh (referred to as EdinSD) and these were then used to cross with homozygous HanRen2/HanRen2 to obtain F1 hybrids HanRen2/Edin--, hemizygous for the *Ren*-2 transgene (see Fig 3.1.1 for nomenclature).

Fig 3.1.1

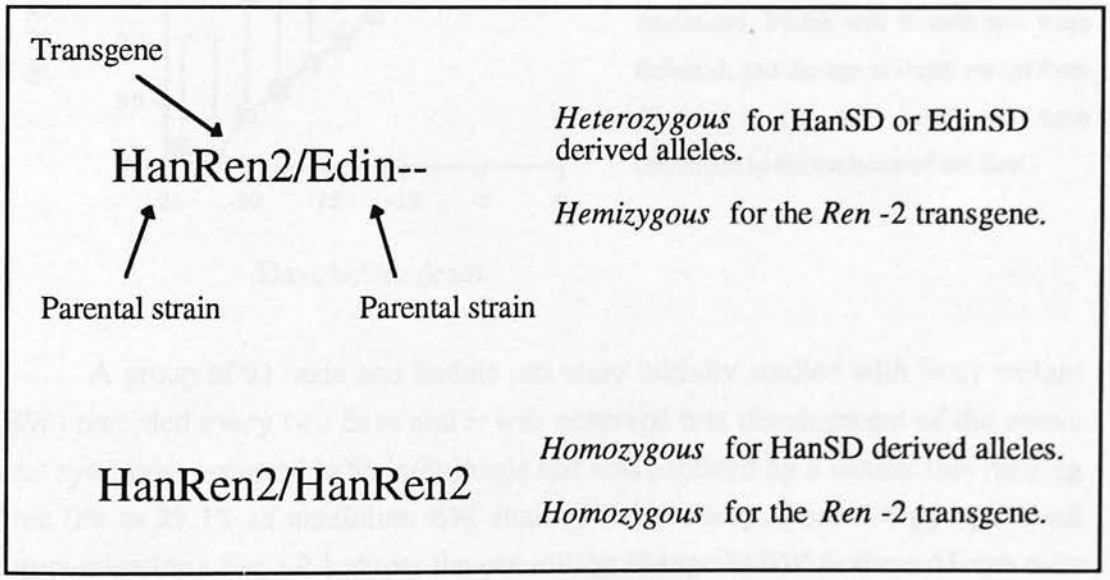


Fig 3.1.1 illustrates examples of the nomenclature that have been used to describe the genotype of rats used in the following studies. Parental strains are depicted as Edin for EdinSD, Han for HanSD and Lew for Lewis. Ren2 refers to the presence of the *Ren*-2 transgene, -- indicates absence of the

transgene. The term *hemizygous* refers only to the transgene, while *heterozygous* refers to all other alleles.

3.2 Description of the change in phenotype

HanRen2/Edin-- rats developed hypertension as previously described, but they exhibited an unexpected increase in mortality, with death occurring in a significant proportion of the animals by 100 days of age. It was observed that they became lethargic, adopting a characteristic hunched and apathetic posture with piloerection, though a smaller proportion exhibited signs of irritability and aggression. On occasion there were signs of neurological involvement manifesting as unilateral myoclonic seizures, hemiparesis or hemiplegia or with signs of retinal haemorrhage appearing as a darkened eye. Invariably weight loss occurred over a period of one to five days, together with evidence of a diuresis indicated by increased soiling of bedding.

Fig 3.2.1

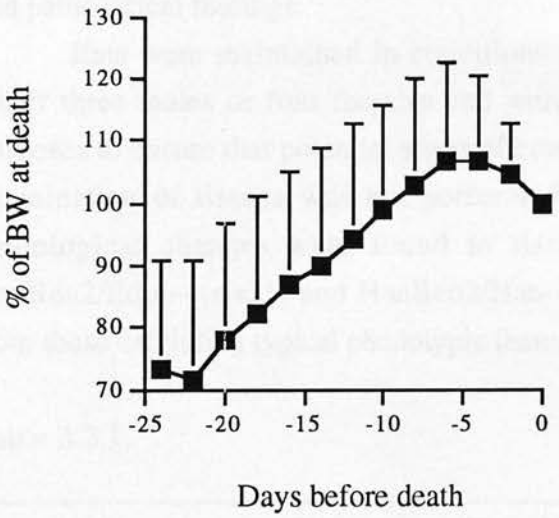


Fig 3.2.1 shows the change in body weight (BW) taken as a change in the %BW relative to the BW at the time of death (arbitrarily set at 100%). Data is expressed as group mean (± standard deviation). Males and female rats were included, and the age at death varied from 49 days to 105 days which will both contribute to the variance of the data.

A group of 91 male and female rats were initially studied with body weight (BW) recorded every two days and it was observed that development of the above fatal syndrome occurred in 51 individuals and was heralded by a weight loss ranging from 0% to 29.7% of maximum BW attained (Matt Sharp, Donald Ogg - personal communication). Fig 3.2.1 shows the percentage change in BW in these 51 rats over the 24 days prior to developing terminal features of the syndrome.

These observations prompted the following questions; what was the nature of the phenotypic change in this transgenic hypertensive rat line giving rise to an

unexpected increase in mortality? Why was it occurring and in particular was it related to a change in environment or was it an effect resulting from a change in the background strain in which the *Ren*-2 transgene was operating?

To address the potential environmental factors, a review of the conditions under which rats were kept in Heidelberg and in Edinburgh was made and apart from a small difference in the dietary sodium content with German rat chow containing 0.2% sodium as opposed to 0.32% in Edinburgh, conditions were otherwise the same. Animals were housed in groups in both centres and kept to a 12:12 hour light/dark cycle, with controlled temperature (18 to 20°C) and humidity (45 to 65%). The possible effects of a small change in dietary sodium intake on the development of hypertension and mortality rate were tested, and to examine whether genetic factors might be operating, three separate crosses were established, in which HanSD male rats, homozygous for the *Ren*-2 transgene (HanRen2/HanRen2), were crossed with non-transgenic EdinSD, HanSD and Lewis females. The genotypes of resulting progeny, hemizygous for the *Ren*-2 transgene, were therefore described as HanRen2/Edin--, HanRen2/Han-- or HanRen2/Lew-- respectively. The three heterozygote crosses were then compared with reference to mortality, physiological and pathological findings.

Rats were maintained in conditions described above, housed in groups of either three males or four females and were not used for any other experimental purposes to ensure that potential stress effects were minimised. Though pathological examination of tissues was not performed on all affected cases, characteristic pathological changes were found in tissues of all cases examined, namely HanRen2/Edin-- (n=31) and HanRen2/Han-- (n=7) which were randomly selected from those exhibiting typical phenotypic features (see section 3.3).

Table 3.2.1.

Heterozygote cross	Genotype of F1hybrid	Incidence of Malignant hypertension	
		Male	Female
HanRen2 X Edin SD	HanRen2/Edin--	86/117 (73.5%)	83/158 (52.5%)
HanRen2 X Han SD	HanRen2/Han--	7/39 (18%)	2/44 (4%)
HanRen2 X Lewis	HanRen2/Lew--	0/35 (0%)	0/31 (0%)

Table 3.2.1 shows the incidence of the phenotype (percentage) occurring by 100 days of age in the three heterozygote crosses, both for males and females, while maintained in identical conditions. 95% confidence limits were calculated for the incidence in each cross - for HanRen2/Edin-- males 65.7-81.3%, and for females 44.7-60.3%; and for HanRen2/Han-- males 6-30% and for females 0-10%.

The age range at which male and female HanRen2/Edin-- rats developed irreversible features of the phenotype is shown in Fig 3.2.2. The age ranges at which HanRen2/Han-- males (n=7) developed the phenotype was 56 to 87 days (median 70 days and mean 69.6 ± SD 12.0 days) while HanRen2/Han-- females (n=2) developed characteristic features at the ages of 58 and 80 days (data not shown).

Fig 3.2.2

A

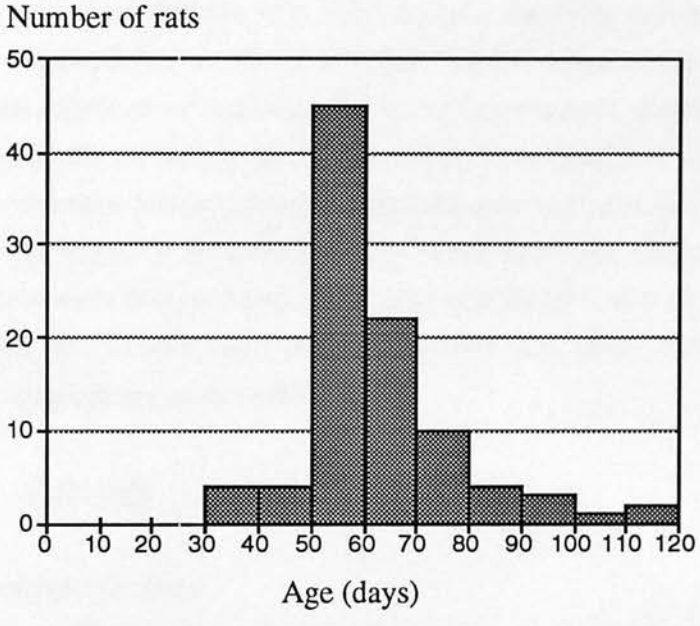
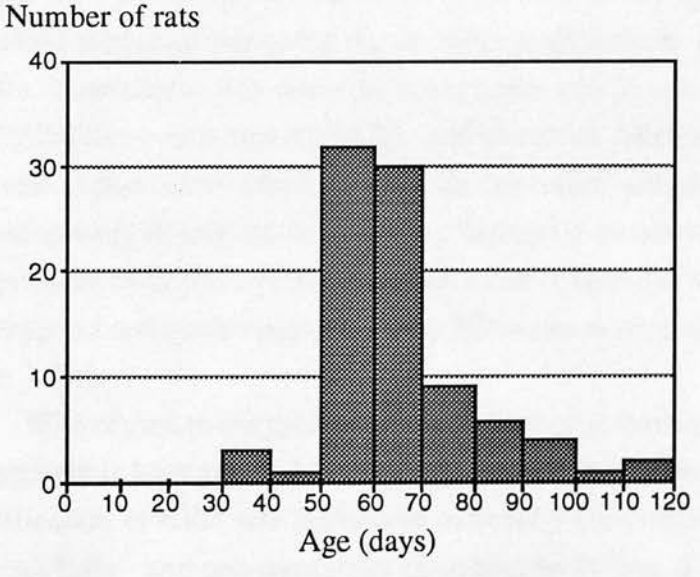


Fig 3.2.2 shows the age at death for male (A) and female (B) rats, against the number of animals.

Out of 117 male HanRen2/Edin-- rats, 86 had died by 100 days, and 92 died in total over a range of 33 to 113 days. The median age at time of death was 62 days, with a mean of 62.2 days ± SD 12.7 days.

B



Out of 158 female HanRen2/Edin--, 83 had died by 100 days and 91 died in total over a range 31 to 115 days. The median age at time of death was 62 days, mean of 65.1 days ± SD 15 days. No significant gender differences were observed for the age at onset of the syndrome or death.

3.3 Characterization of the change in phenotype.

A post-mortem study was performed on an initial series (n=197) of transgenic heterozygote animals HanRen2/Edin--, HanRen2/Han--, HanRen2/Lew-- and non-transgenic strains (EdinSD and HanSD) who had been maintained with free access to a normal diet (sodium content 0.32%) and tap water and were housed in groups of three (males) or four (females). At four, six and eight weeks of age (n= 3-8 per group) tissues were taken from age matched, healthy males from each heterozygote cross. They were anaesthetized with 6% halothane, killed by cervical dislocation and tissues were removed for weighing and histological examination as described earlier. A random selection of animals who had spontaneously developed clinical features as described above, including sudden weight loss, apathy or signs of cerebral irritation also underwent post-mortem examination after sacrifice (n=38). Kidney, heart, brain, aorta and superior mesenteric artery were removed; kidneys, whole heart and left ventricle were weighed and all tissues were fixed in 4% formal saline, embedded in paraffin wax blocks, sectioned and stained with H&E, MSB, PAS, EVG and LFB where appropriate as described earlier.

3.3.1 Pathology

Macroscopic findings

In general affected animals showed evidence of marked tissue dehydration. The main findings on macroscopic examination were seen in the kidneys with evidence of superficial petechial haemorrhage to varying degrees in the majority of affected animals. Haematuria was noted in some cases and in two animals examined (one HanRen2/Edin-- and one HanSD), unilateral or bilateral hydronephrosis was observed. Apart from these individuals, no other abnormalities were observed macroscopically in kidneys from healthy transgenic or non-transgenic SD. There was no significant difference in size between kidneys from the three different transgenic heterozygotes and pooled non-transgenic SD males at four, six and eight weeks of age (Table 3.3.1).

With regard to the cardiovascular system, left ventricular hypertrophy (LVH) was apparent in both affected animals and in healthy hypertensive transgenic animals. Quantification of LVH was performed in healthy HanRen2/Han--, HanRen2/Lew--, HanRen2/Edin-- and non-transgenic SD male rats at four, six and eight weeks of age (i.e. up to the age of expected onset of weight loss and phenotypic change). The indices of change in left ventricle (LV) weight measured were: (1) whole heart weight

to 100g body weight ratio (HW/BW), (2) LV weight to 100g BW ratio (LV/BW) and (3) LV to heart weight ratios (LV/HW) (Table 3.3.2).

Table 3.3.1

Strain	4 week n	**KW/BW ratio**	6 week n	**KW/BW ratio**	8 week n	**KW/BW ratio**
HanRen2/Han--	6	0.55 ±0.03	3	0.46 ±0.03	8	0.45 ±0.02
HanRen2/Lew--	3	0.56 ±0.04	5	0.50 ±0.03	5	0.45 ±0.03
HanRen2/Edin--	4	0.55 ±0.01	5	0.47 ±0.03	6	0.43 ±0.05
SD	8	0.53 ±0.03	6	0.44 ±0.03	8	0.40 ±0.04

Changes in kidney weight (KW) where KW equals the mean of the weights of the two kidneys and is depicted as a ratio to body weight (BW) at 4, 6 and 8 weeks of age. No statistically significant difference was found between the four groups.

Table 3.3.2

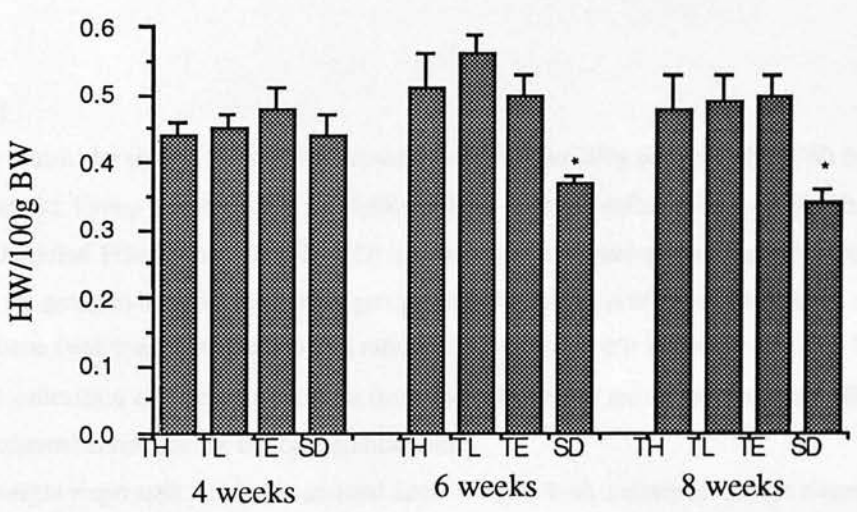
Strain **Age 4 weeks**	**n**	**HW/BW** **mean ± SD**	**LV/BW** **mean ± SD**	**LV/HW** **mean ± SD**
HanRen2/Han-- (TH)	6	0.44 ± 0.02	0.33 ± 0.01	0.75 ± 0.02
HanRen2/Lew-- (TL)	3	0.45 ± 0.03	0.32 ± 0.01	0.72 ± 0.05
HanRen2/Edin-- (TE)	5	0.48 ± 0.03	0.37 ± 0.01	0.80 ± 0.03
SD	8	0.44 ± 0.03	0.33 ± 0.02	0.79 ± 0.05
6 weeks				
HanRen2/Han-- (TH)	3	0.51 ± 0.05	0.39 ± 0.03	0.76 ± 0.4
HanRen2/Lew-- (TL)	5	0.56 ± 0.03	0.43 ± 0.04	0.75 ± 0.04
HanRen2/Edin-- (TE)	5	0.50 ± 0.03	0.41 ± 0.03	0.84 ± 0.02
SD	6	0.37 ± 0.01	0.30 ± 0.02	0.80 ± 0.04
8 weeks				
HanRen2/Han-- (TH)	8	0.48 ± 0.05	0.38 ± 0.02	0.78 ± 0.06
HanRen2/Lew-- (TL)	5	0.49 ± 0.05	0.40 ± 0.02	0.84 ± 0.02
HanRen2/Edin-- (TE)	6	0.50 ± 0.03	0.41 ± 0.03	0.84 ± 0.02
SD	8	0.34 ± 0.02	0.26 ± 0.01	0.78 ± 0.04

Changes in heart weight (HW) and LV weight as ratios to Body weight (100g) (BW) and changes in LV/HW ratio with age in three transgenic heterozygotes crosses and in normal Sprague-Dawley controls. Data shown as mean ± standard deviation (SD).

LVH was apparent in all three transgenic heterozygotes with statistically significant increases in both LV/BW and HW/BW ratios relative to normotensive pooled SD controls at six and eight weeks of age. However, no significant difference was seen between the three transgenic groups. Looking at LV/HW ratios, significant differences were elicited between HanRen2/Edin-- and both HanRen2/Han-- and HanRen2/Lew-- at four and six weeks, though the significance of this is not clear, since the ratios observed in normotensive SD were comparable to those seen in HanRen2/Edin--. However by eight weeks of age LV/HW ratios were significantly increased in both HanRen2/Edin-- and HanRen2/Lew-- compared to SD (Fig 3.3.1).

Fig 3.3.1

A



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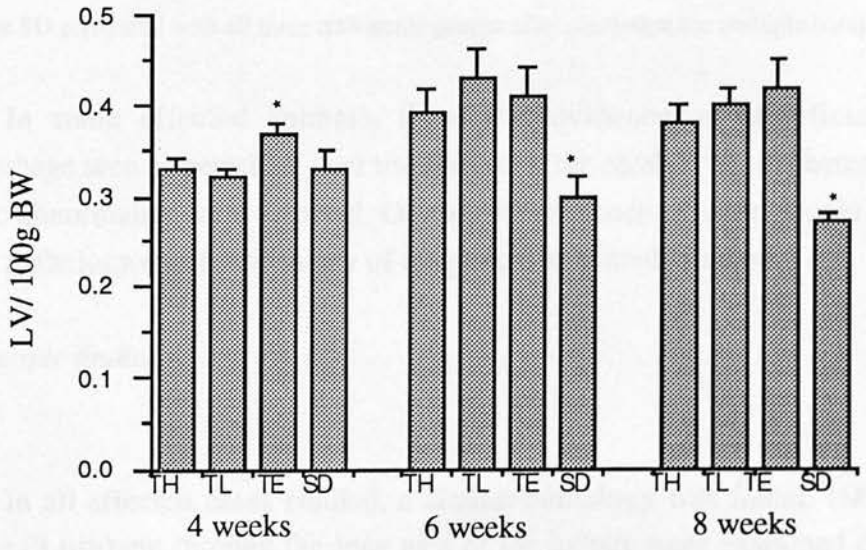

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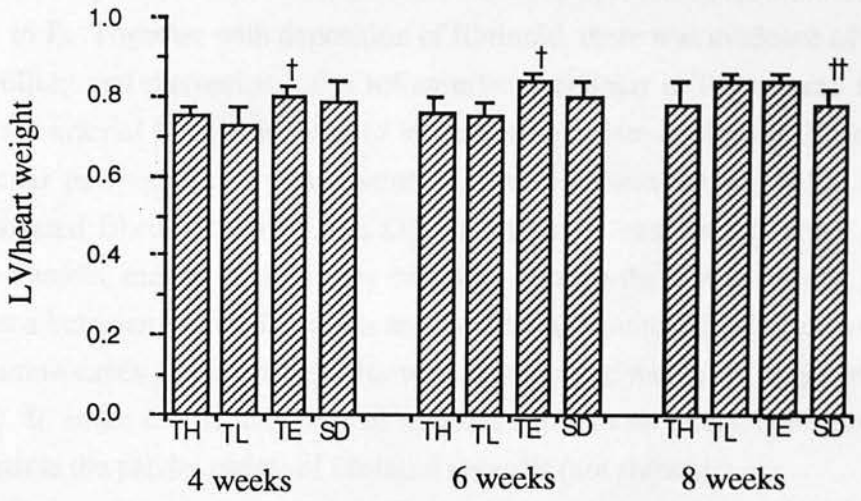


Fig 3.3.1

A. Total ventricular tissue (wet weight) expressed as a ratio to 100g body weight (BW) for rats aged 4, 6 and 8 weeks. Group means (± SD) for HanRen2/Han-- (TH), HanRen2/Edin-- (TE), HanRen2/Lew-- (TL) and pooled EdinSD and HanSD (SD) are shown. * indicates a significant difference ($p<0.05$) between the group indicated and all other groups after Bonferroni correction for multiple comparison.

B. LV tissue (wet weight) expressed as a ratio to 100g body weight in groups TH, TL, TE and SD as above. * indicates a significant difference ($p<0.05$) between the group indicated and all other groups after Bonferroni correction for multiple comparison.

C. LV weight expressed as a ratio to total heart weight, both measured as wet tissue weights for groups as above. † indicates a significant difference between TE and both TH and TL groups but not SD, after correction for multiple comparison. †† shows a significantly decreased LV/HW ratio at 8 weeks for SD compared with all three transgenic groups after correction for multiple comparison.

In some affected animals, there was evidence of superficial cortical haemorrhage seen as petechiae over the surface of the cerebral hemispheres. No other specific abnormality were detected. Of note, no evidence of macroscopic hepatic or splenic pathology was found in any of the groups of animals studied.

Microscopic findings

Renal:

In all affected cases studied, a similar pathology was found. H&E stained sections (3 μm) cut through the long axis of the kidney were examined under light microscopy. The main finding was of interlobular and afferent arteriolar damage with

evidence of myo-intimal proliferation and fibrinoid necrosis in all affected cases (Fig 3.3.2 A to J). Together with deposition of fibrinoid, there was evidence of endothelial cell swelling and disruption, mild inflammatory cellular infiltrates and thromboses within the arterial lumens leading to virtual or complete occlusion. No evidence of significant peri-vascular inflammatory infiltration was found. MSB stains also demonstrated fibrinoid (Fig 3.3.2, G&H). In larger vessels e.g. renal artery and arcuate vessels, medial hypertrophy was seen though there was usually no marked difference between affected animals and healthy age-matched hypertensive animals, but in some cases fibrinoid deposits were seen in the media of larger vessels (Fig 3.3.2 J). In some cases, longitudinal sections through an interlobular artery would demonstrate the patchy nature of fibrinoid necrosis (not shown).

Glomeruli in the majority of affected animals appeared to be mainly unaffected (Fig 3.3.2 G). In a few cases afferent arteriolar fibrinoid necrosis was associated with either fibrinoid necrosis or collapse of a glomerular tuft (Fig 3.3.2 H). . There was no evidence of mesangial proliferation and only occasional small and early crescent formation. Fibrin deposition was occasionally seen in Bowman's space. There was minimal tubular damage in the majority of cases with symptoms, but in both older affected animals and healthy transgenic hypertensive rats (>12 weeks old), features of tubular atrophy and glomerulosclerosis were seen and on rare occasions these were coincident with evidence of fibrinoid necrosis (Fig 3.3.2 B).

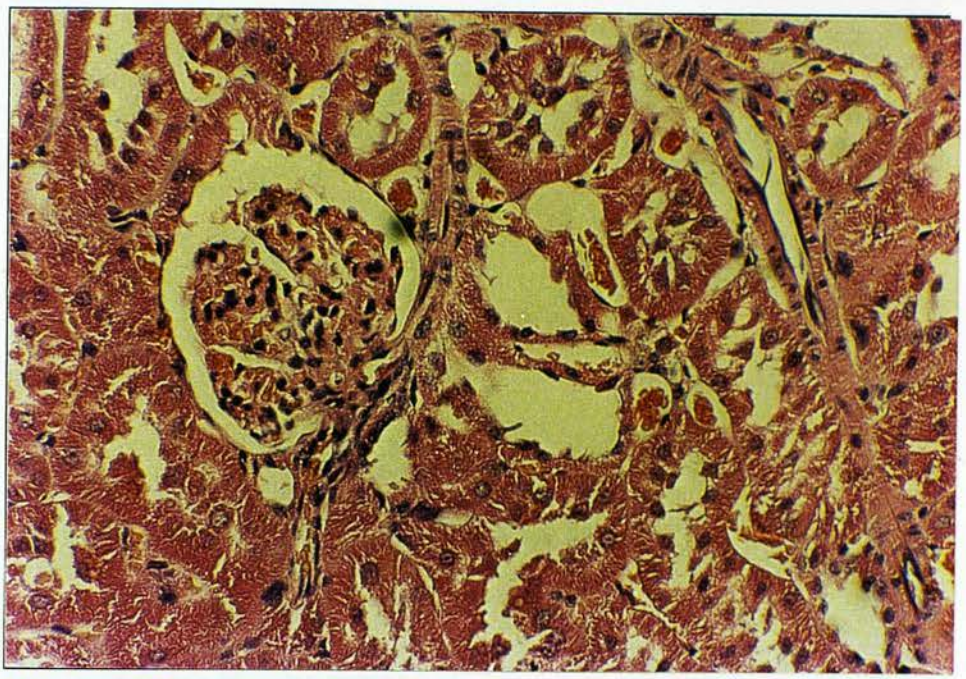
The overall impression arising from the histological examination of renal tissue from affected animals was that the changes were compatible with malignant phase hypertension (MH) and secondly, that the described lesions were of relatively acute onset and of a similar age suggesting that onset of, or transition to, MH was recent, with insufficient time to elapse for secondary ischaemic changes such as glomerular sclerosis and tubular atrophy to develop in the majority of cases.

Myocardium:

Three μm sections through left and right ventricular myocardium were examined after staining with H&E and PAS stains, and findings are illustrated in Fig 3.3.3 (A to H). LVH was apparent on light microscopy with cellular hypertrophy rather than hyperplasia observed when compared with non-transgenic controls, though this was not quantified.

Fig 3.3.2

A



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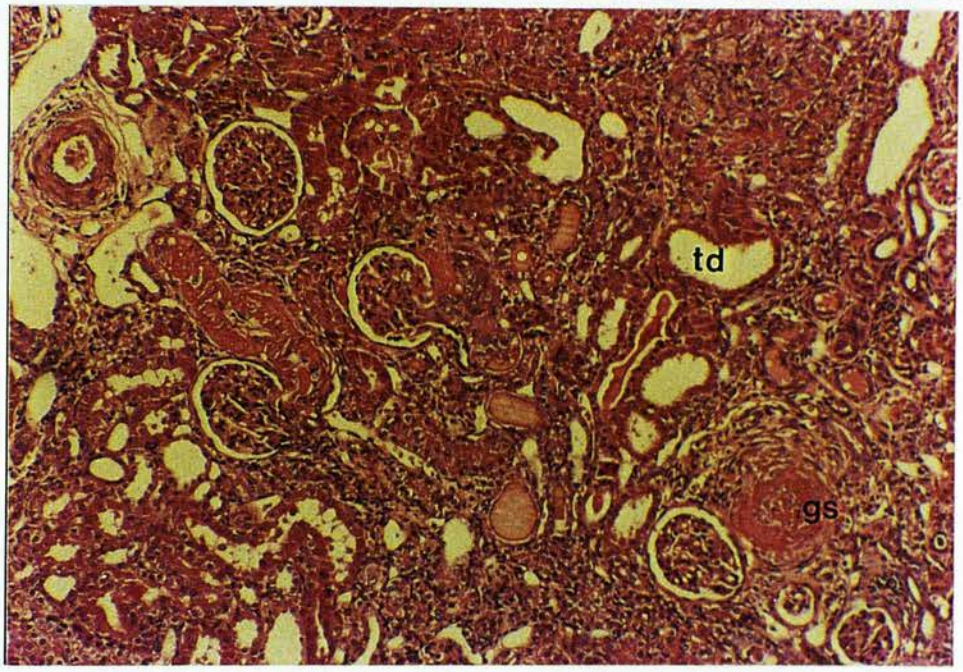
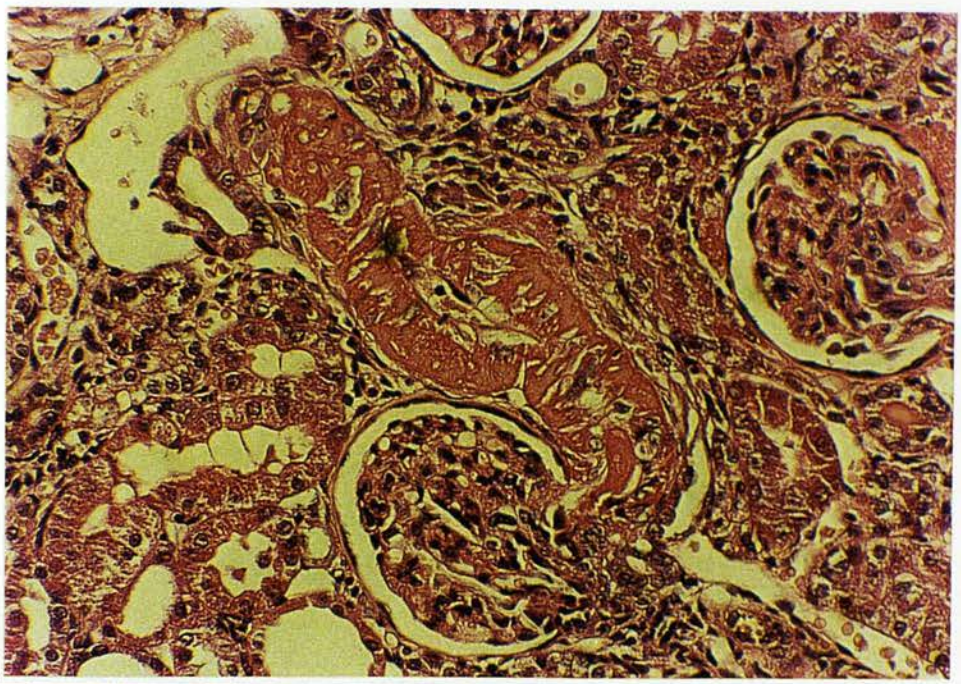


Fig 3.3.2 A - J are all photomicrographs from 3 µm sections of renal tissue fixed in 4% formalin before sectioning, staining and mounting.

A Section taken from a non-transgenic HanSD rat showing a normal afferent arteriole and glomerulus (H&E X250).

B Hypertensive damage in a HanRen2/Edin-- rat showing evidence of fibrinoid necrosis, with chronic hypertensive renal damage consisting of glomerulosclerosis (gs), arterial medial wall thickening, and tubular dilatation (td) (H&E X40).

C



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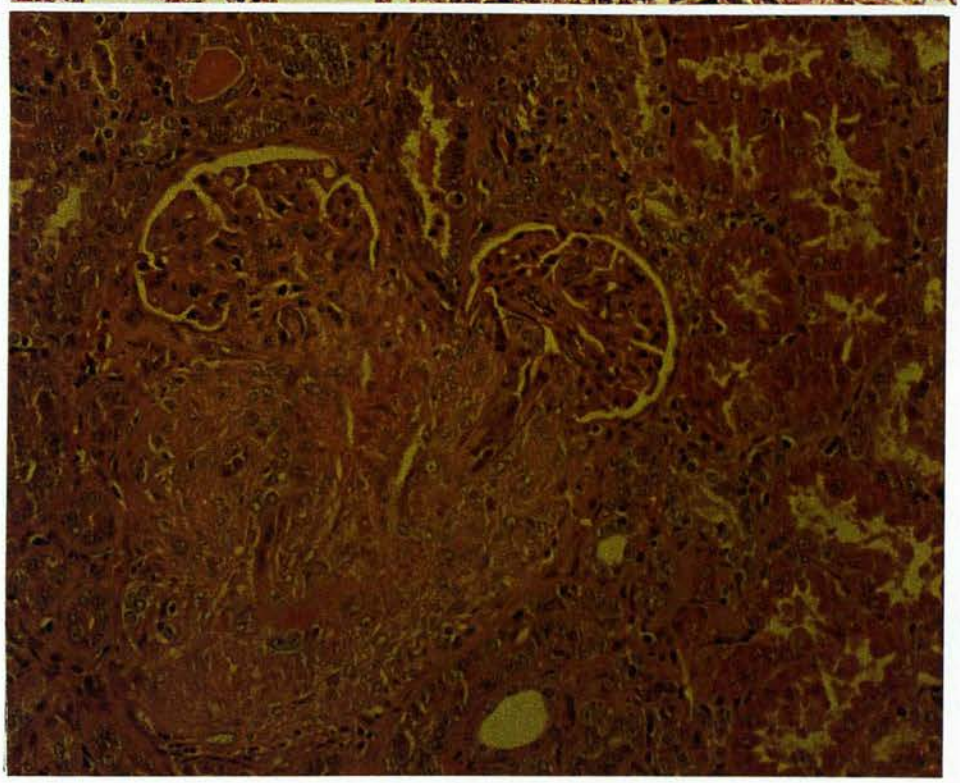
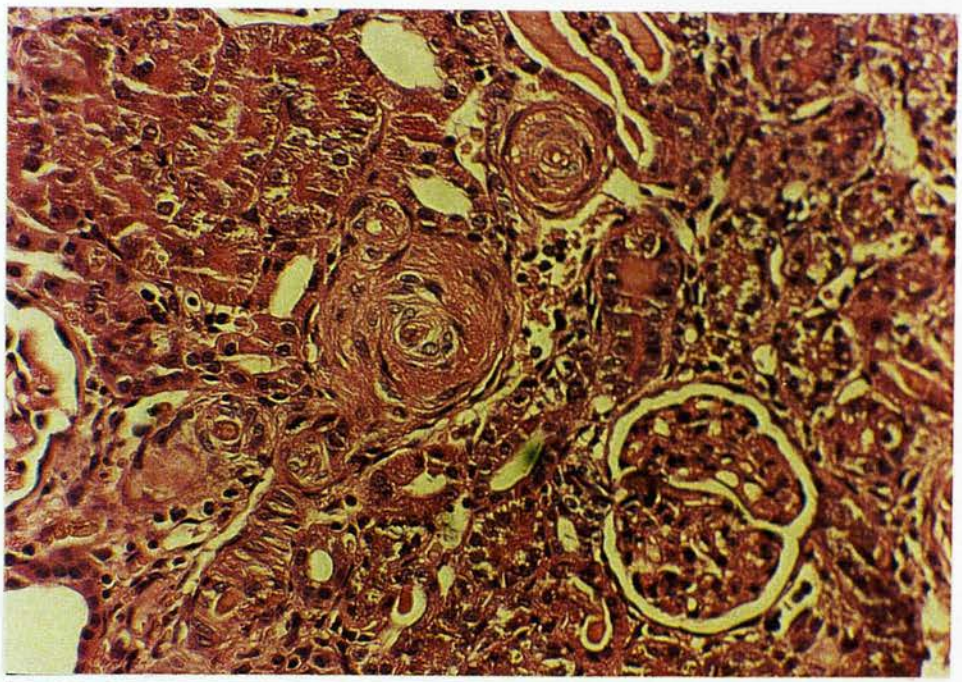


C Fibrinoid necrosis of an afferent arteriole from a HanRen2/Edin-- (H&E X250)

D Myo-intimal proliferation and fibrinoid necrosis affecting afferent arterioles supplying two glomeruli (H&E X250).

E



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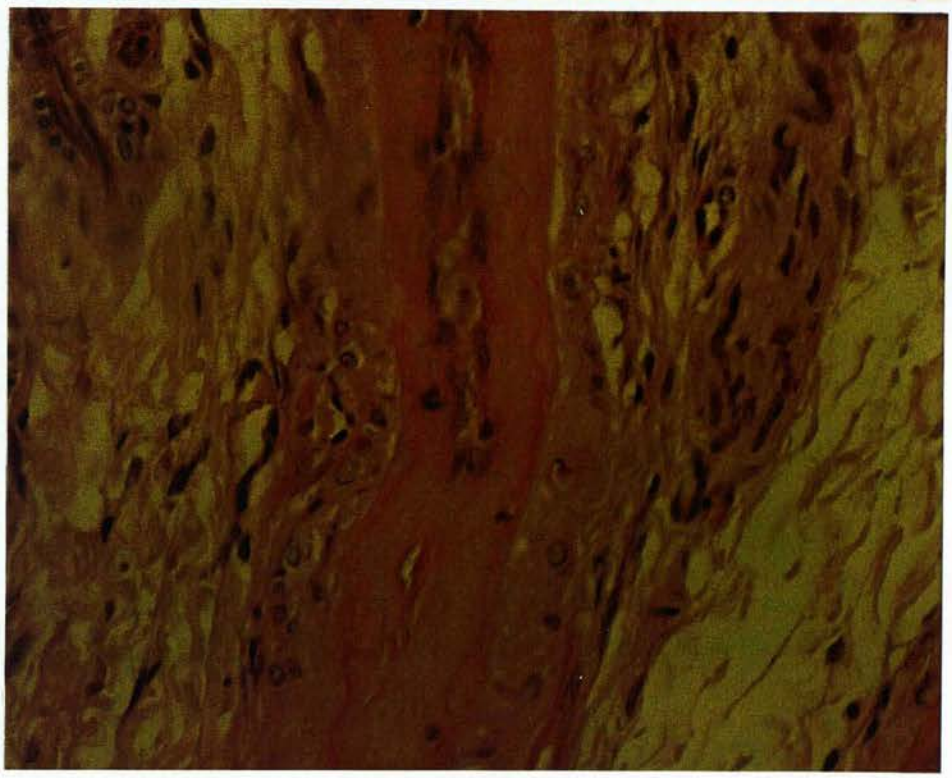
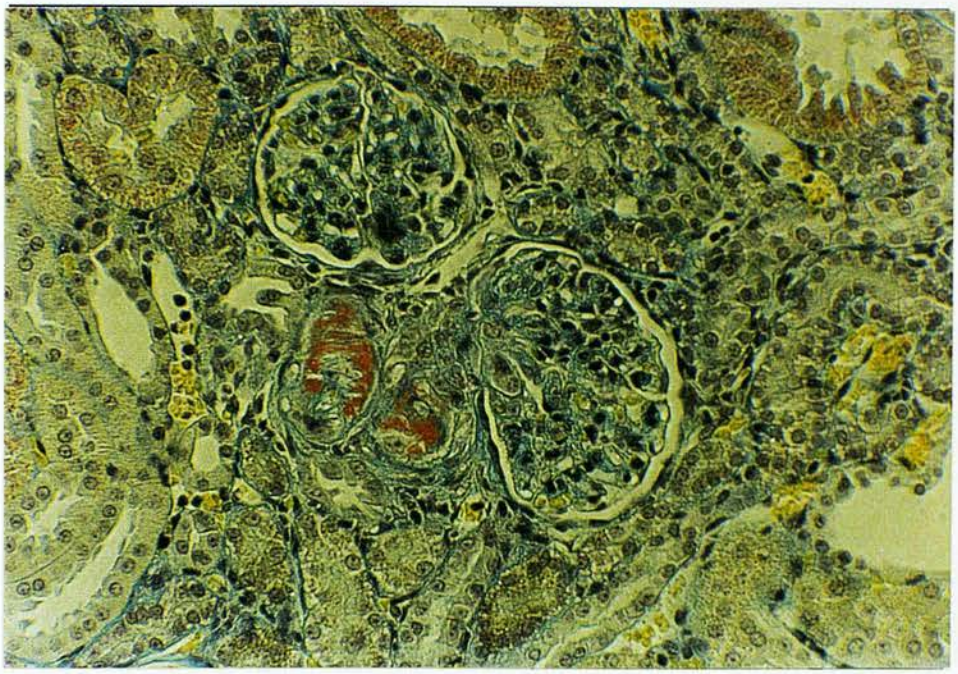


E Myo-intimal proliferation (H&E X250).

F Longitudinal section through an inter-lobular artery showing fibrinoid necrosis, with endothelial swelling and disruption (H&E X250).

G



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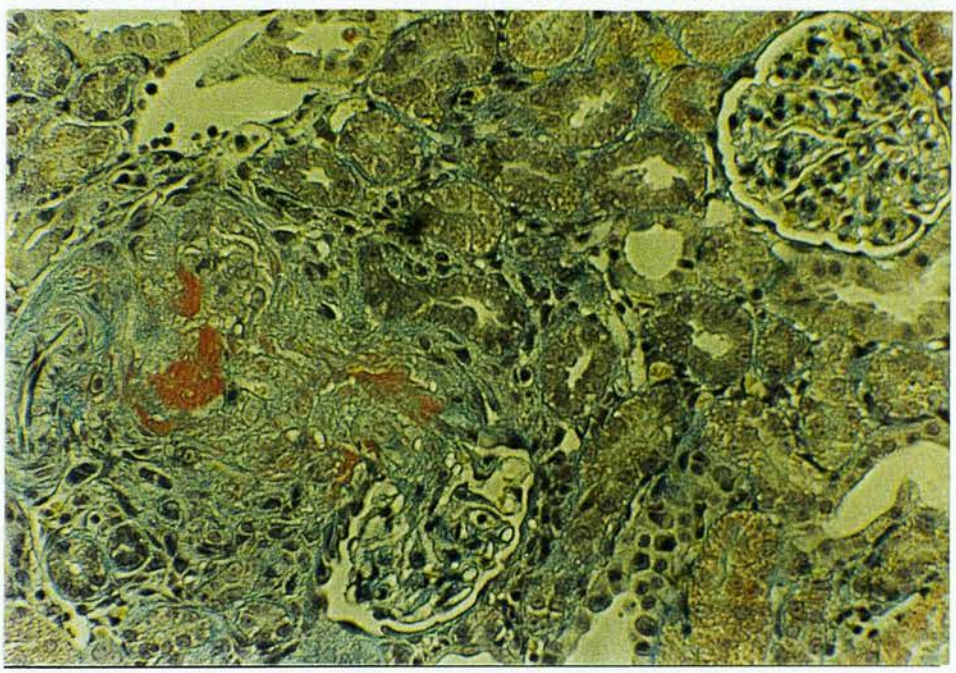
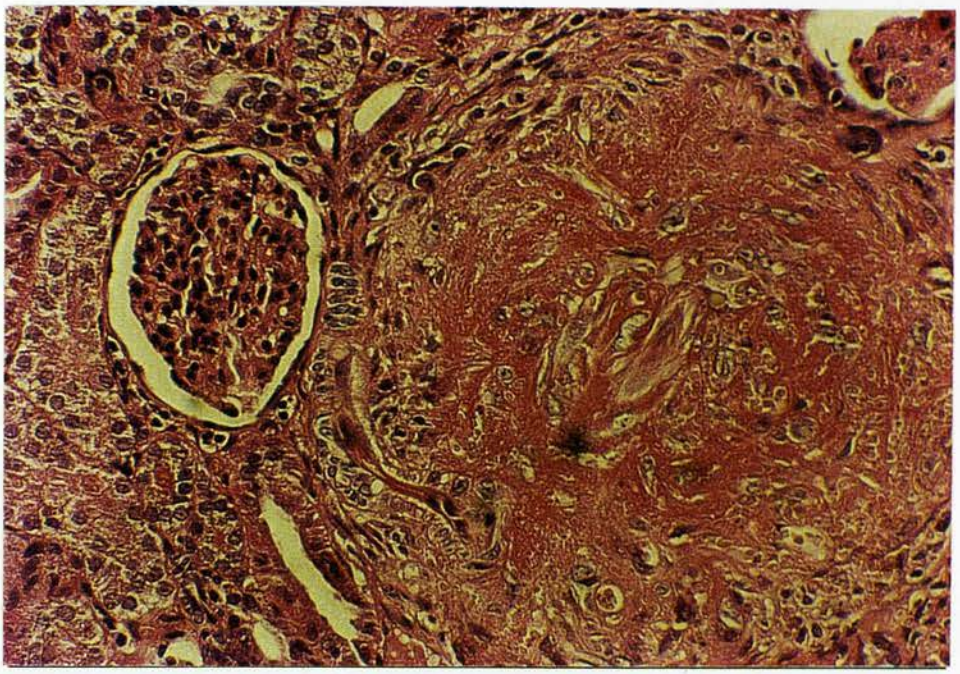


G Fibrinoid necrosis of afferent arterioles, while glomeruli appear undamaged (MSB X250).

H Firinoid necrosis of an afferent arteriole associated with tuft collapse of the glomerulus (MSB X250).

I



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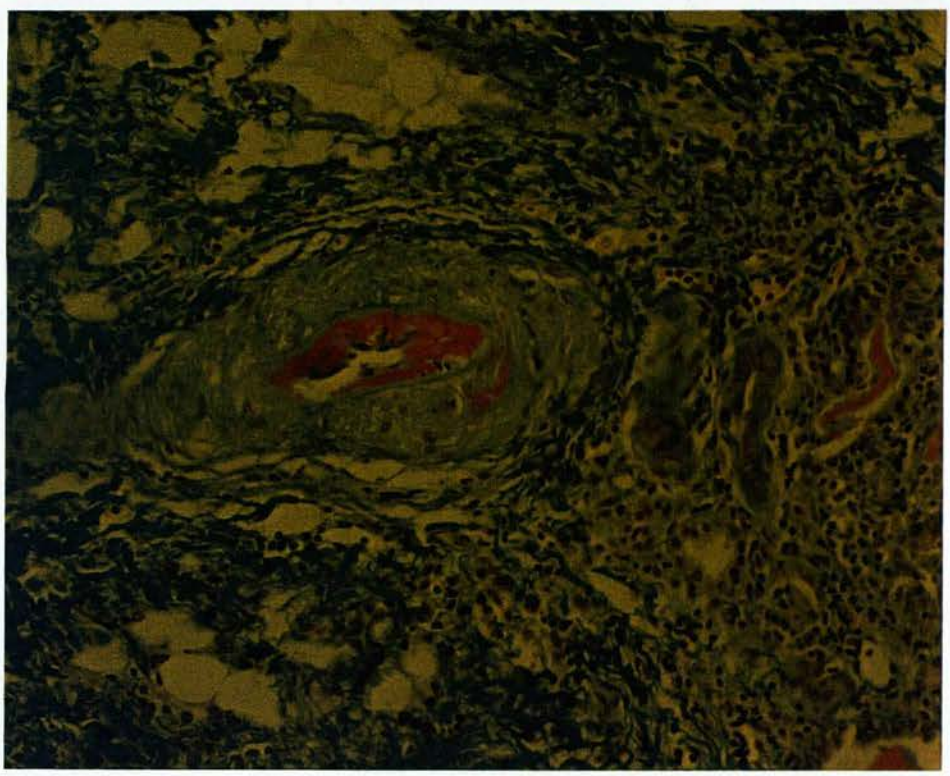
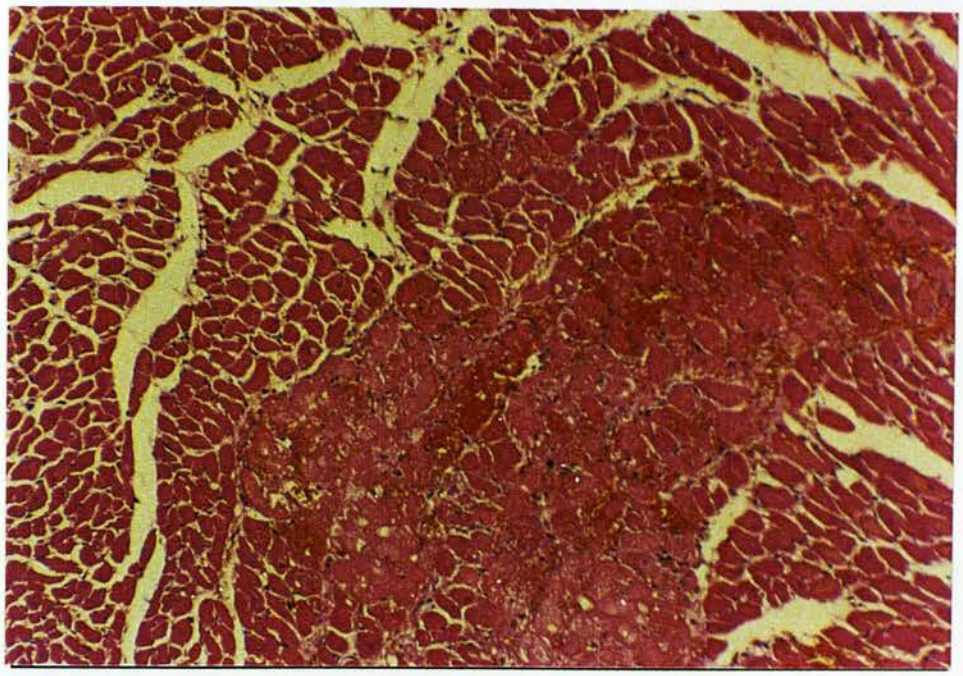


I Glomerulosclerosis with complete hyalinisation of a single glomerulus. Adjacent normal glomerulus for comparison (H&E X250).

J Fibrinoid necrosis affecting a larger artery in cross-section, principally with fibrinoid deposition in the sub-endothelial region, and also within the media. Significant luminal narrowing and occasional inflammatory cells adherent to the endothelium are seen (MSB X250).

Fig 3.3.3

A



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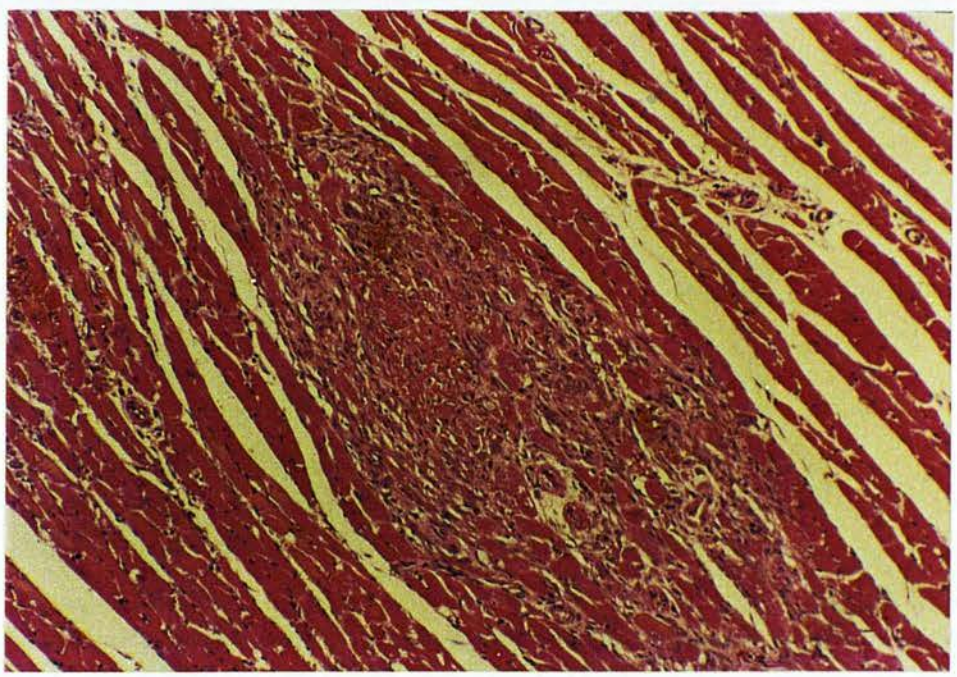
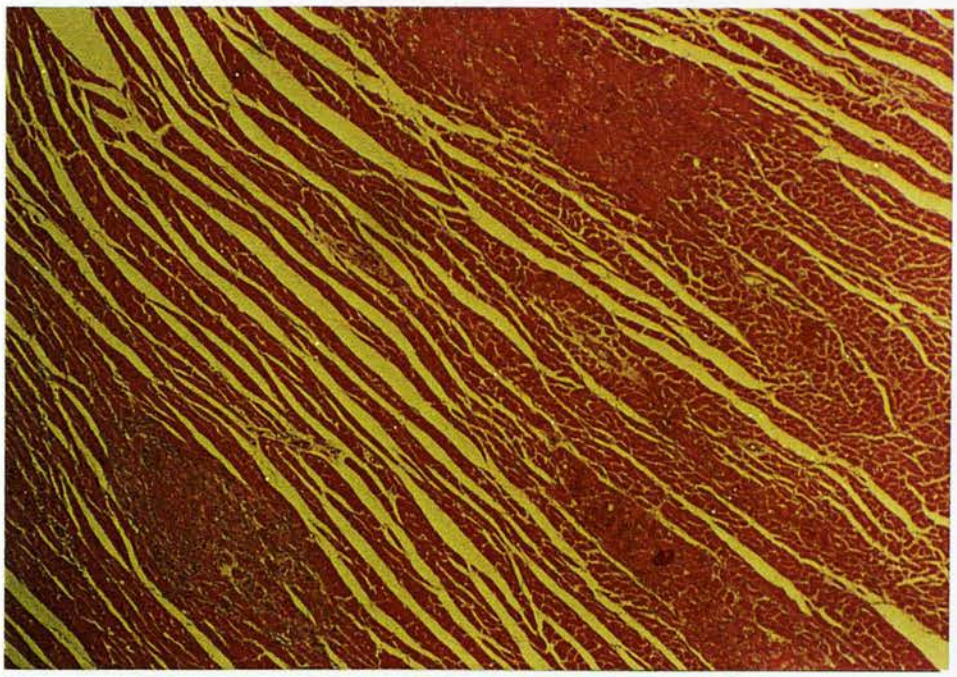


Fig 3.3.3 A-H are all taken from sections of myocardium from transgenic rats. Tissues were fixed with 4% formalin before sectioning, staining and mounting.

A An acute myocardial infarct (microscopic) is shown by the relative pallor of the cardiomyocytes, vacuolation and loss of cell borders together with evidence of haemorrhage within the infarct. Normal cardiomyocytes surround the infarct (H&E X100).

B A microscopic infarct showing evidence of an early healing response with inflammatory cell infiltration and formation of granulation tissue (H&E X100).

C



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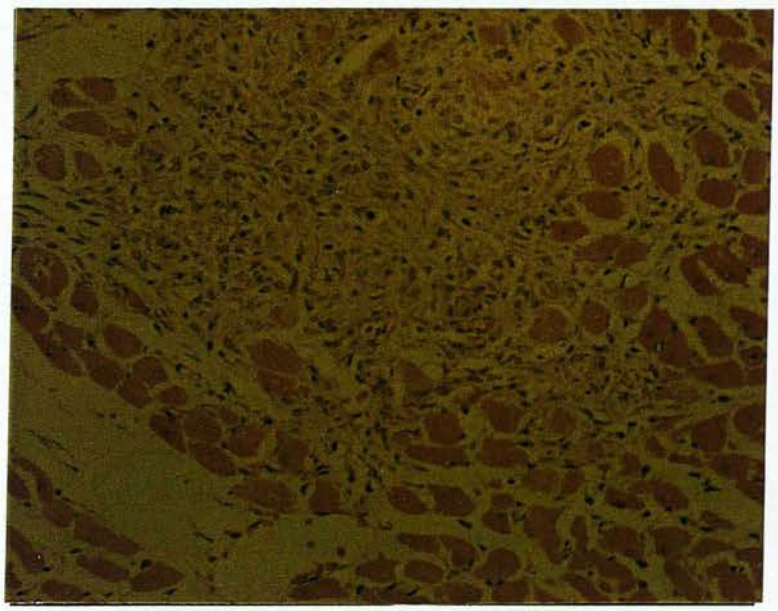
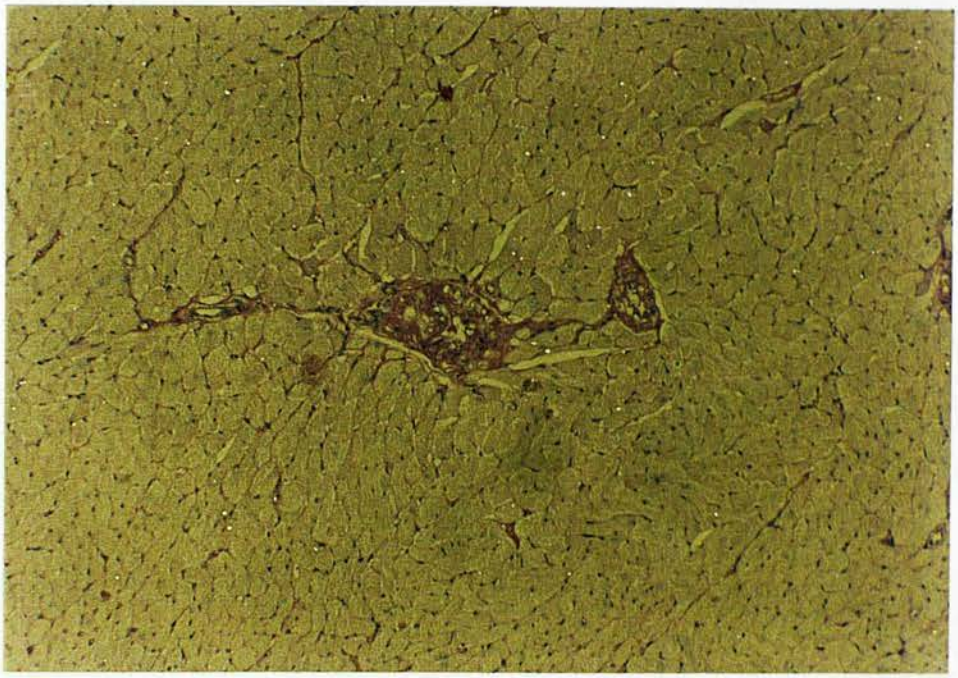


C Photomicrograph shows the concomitant presence of three microscopic myocardial infarcts of differing age within the same section. The upper (top right) infarct is acute with no evidence of a healing response, while the lower (bottom left) shows granulation tissue. (H&E X40).

D A more advanced stage in the healing process of an individual infarct is shown by the presence of a focal area of scar tissue (H&E X100).

E



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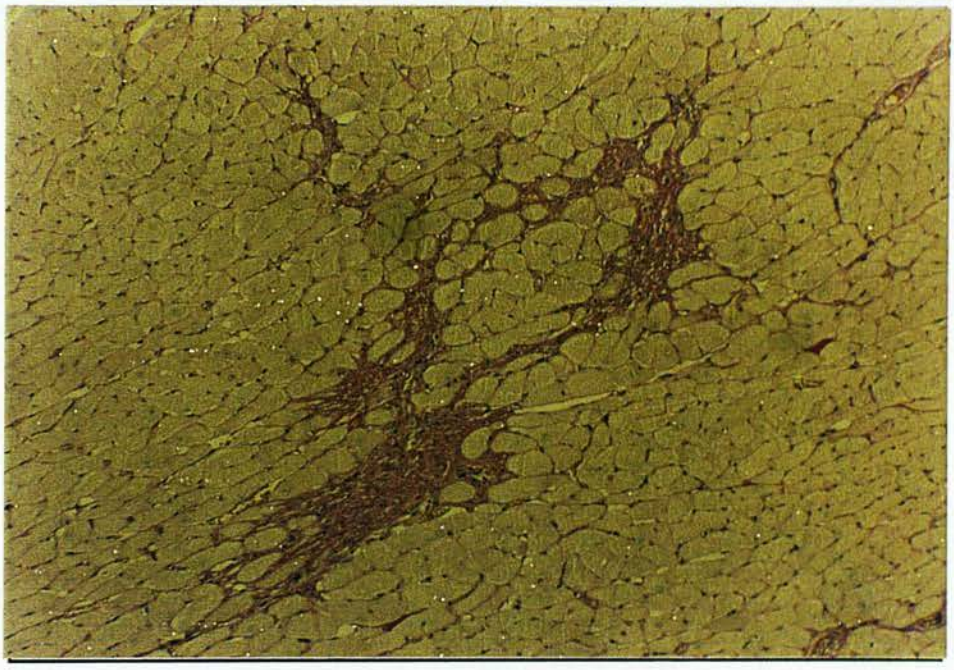
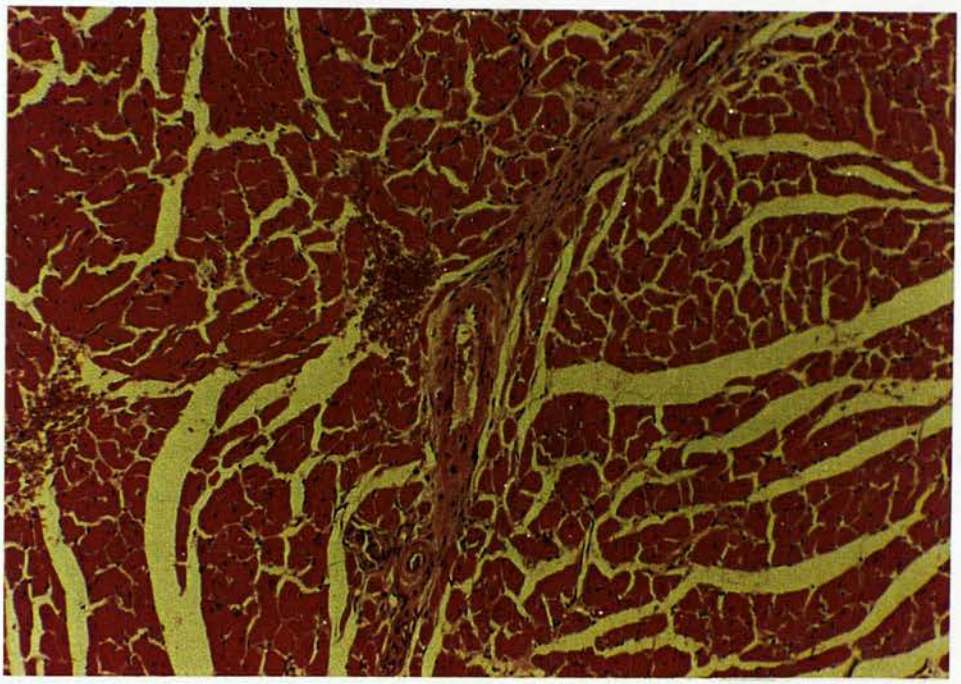


E Typical perivascular fibrosis (PAS X100).

F Interstitial fibrosis (PAS X100).

G



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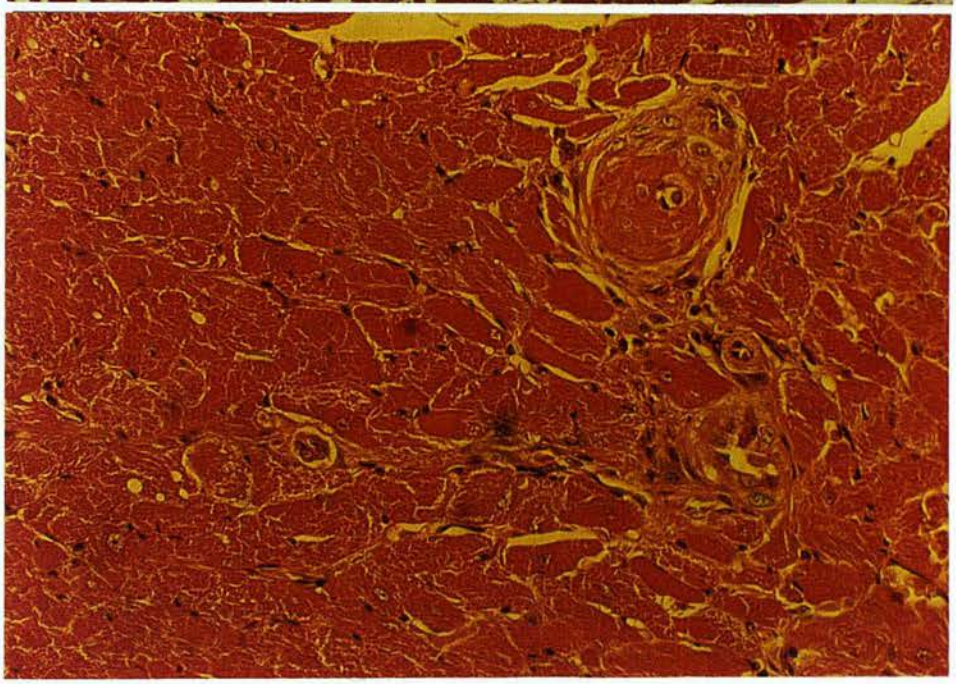
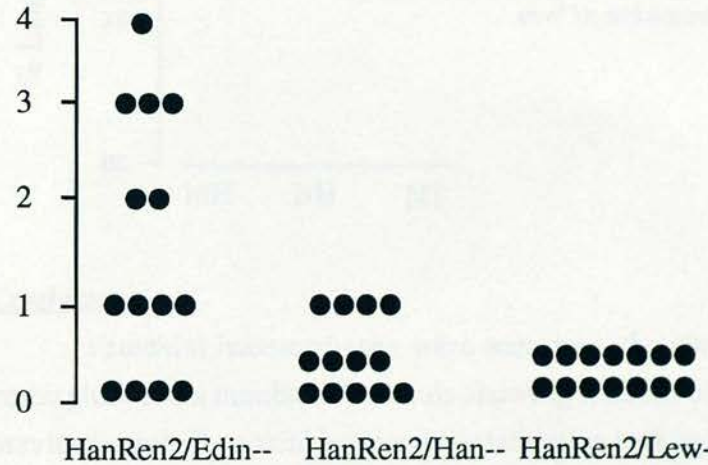


G Normal cardiac arteriole from a non-transgenic Lewis rat (H&E X100).

H Cardiac arteriole, from a rat who had malignant hypertension, showing features typical of fibrinoid necrosis (H&E X250). In some cases myo-intimal proliferation (not shown here) was also observed.

In both HanRen2/Edin-- and HanRen2/Han--, microscopic infarcts were found in LV myocardium (Fig 3.3.3 A to D). Acute lesions appeared as groups of infarcted cells with pale cytoplasm, vacuolation and indistinct cell membranes. Small haemorrhages were occasionally noted within infarcted areas. Lesions of varying age were seen with both foci of granulation tissue and fibrosis existing in the same slide as an acute microinfarct (Fig 3.3.3 C). This suggested that such lesions were not a terminal event, but underwent a healing response with inflammatory cell infiltration, the appearance of granulation tissue and ultimately healed leaving focal areas of scar tissue (Fig 3.3.3 D). On occasion, but not in all cases, microinfarcts appeared to be associated with fibrinoid necrosis of small cardiac arterioles (Fig 3.3.3 H). They were sited throughout the thickness of the LV and septal myocardium, but were not clearly seen in the free wall of the RV. Microinfarcts were found in sick animals but also in apparently healthy heterozygotes. No such lesions were observed in HanRen2/Lew-- or in non-transgenic strains.

Fig 3.3.4



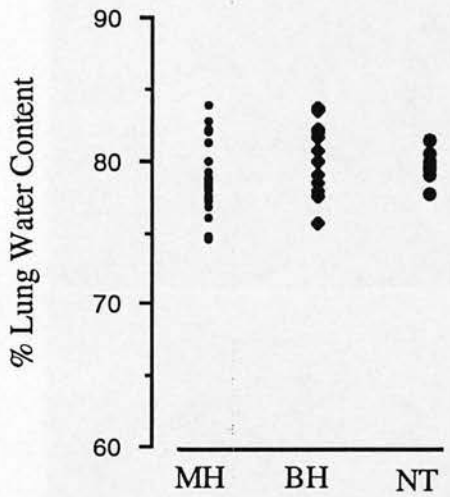
Number of individual microinfarcts seen in one 3 μm section through mid-LV of hearts from age-matched HanRen2/Edin-- (n=14), HanRen2/Han-- (n=13) and HanRen/Lew-- (n=14) males. Each point represents the score for an individual animal.

The incidence of microscopic myocardial infarcts was quantified by looking at H&E sections (3 μm) from randomly selected animals taken from the three transgenic groups aged eight weeks; HanRen2/Edin-- (n=14); HanRen2/Han-- (n=13) and HanRen2/Lew-- (n=14). One 3 μm section, taken through the long axis of the LV including the apex was examined under light microscopy (X100) in a systematic fashion while blinded to the origin of the slide. Looking at all fields, the number of distinct micro-infarcts of any stage was counted, and the results are depicted in Fig 3.3.4. This showed that myocardial microinfarcts were more common in HanRen2/Edin--, with individual animals tending to show more frequent lesions, but

lesions were found both in animals exhibiting the described clinical features but also in apparently healthy animals.

Using a crude index of pulmonary oedema as measured by water content as a percentage of lung wet tissue weight, affected animals were evaluated for evidence of LV failure and the results are depicted in Fig 3.3.5. No significant difference was seen in the lung water content between normotensive SD controls, healthy hypertensive transgenic rats with benign hypertension and rats with clinical and pathological features of MH.

Fig 3.3.5



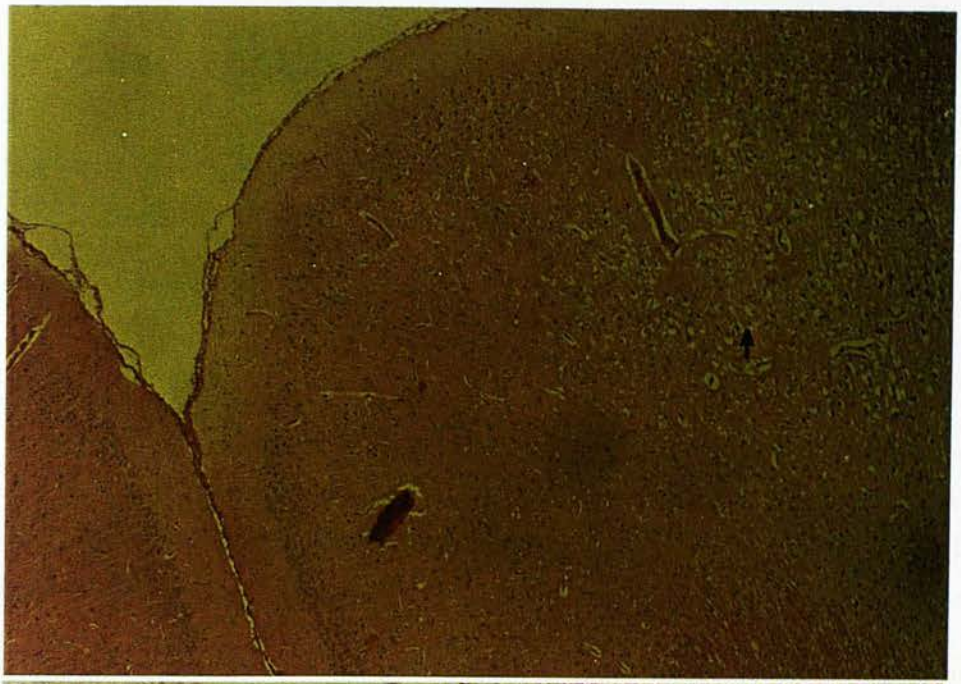
Lung water content as a percentage of total lung tissue (wet) weight from age-matched (8-10 week) transgenic heterozygotes exhibiting clinical features of MH (n=21) confirmed on renal histology; healthy, hypertensive, transgenic heterozygotes with benign hypertension (BH) (n=15); and normotensive SD (NT) (n=8).

Cerebrum:

Petechial haemorrhages were seen over the cortical surface of the cerebral hemispheres in a number of animals showing features of MH. In one rat, evidence of previous cerebral cortical ischaemia or infarction was observed with neuronal loss and gliosis (Fig 3.3.6 A). Coronal sectioning at the level of the mid-brain revealed evidence of haemorrhage around the basal ganglia in the occasional animal (Fig 3.3.6 B).

Fig 3.3.6

A



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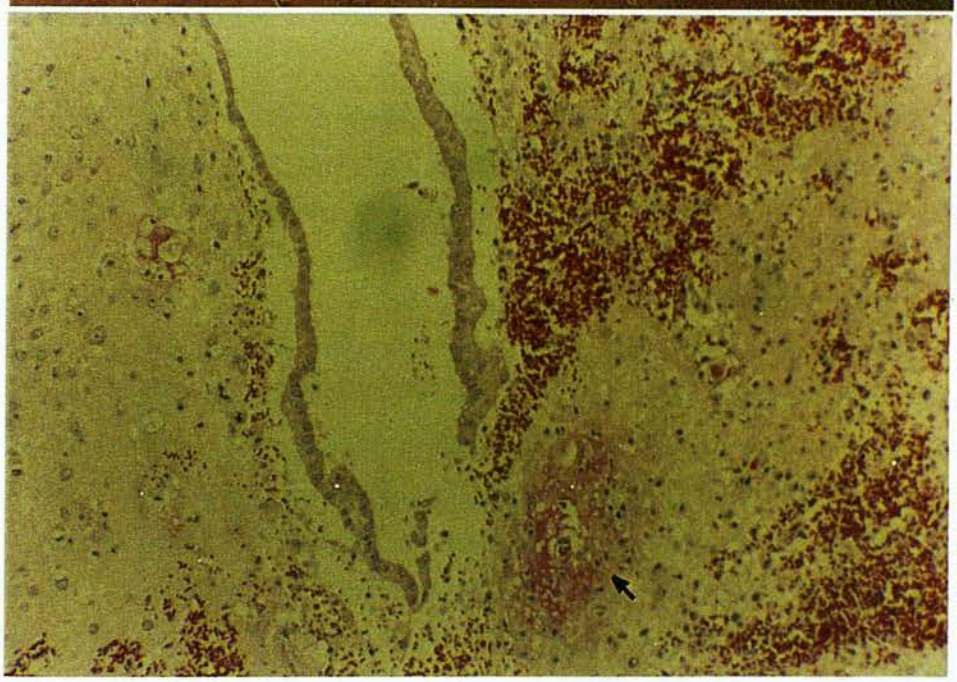


A Section taken through the cerebral cortex of a HanRen2/Edin-- rat which showed evidence of previous cortical ischaemia (arrowed) where there was a marked loss of neuronal tissue and a fibrillary gliosis (H&E X40).

B Areas of cerebral haemorrhage within the thalamus adjacent to the IIIrd ventricle. Area of recent infarction (arrowed) (H&E X100).

Mesenteric vessels:

No evidence of mesenteric ischaemia or infarction was apparent at the macroscopic level and on sectioning of superior mesenteric artery, no clear evidence of any characteristic pathology was seen in rats with MH. Though hypertensive transgenic heterozygotes showed evidence of some medial wall thickening compared to non-transgenics, this was not marked within the age range of rats studied (up to eight weeks), but in a series of sixteen week old transgenic heterozygotes medial hypertrophy was more apparent. This was not increased in rats showing features of MH, nor was there any evidence of fibrinoid necrosis or myo-intimal proliferation.

Aorta:

Within the age range of animals studied there were no specific features observed to be characteristic of the malignant phase. With increasing age, transgenic heterozygotes of all three crosses showed some increased reduplication of the elastic laminae (not shown).

3.4 Blood Pressure

The above findings have described a phenotypic change with mortality of affected animals associated with clear pathological features of MH on light microscopy of renal tissue. It was therefore of interest to look at changes in BP over the course of development of the syndrome.

Established methods of BP measurement in rats have usually relied on measures that require either anaesthesia, tethering or restraint and even with training and acclimatization to conditions, such methods clearly induce stress and elevation of BP[422]. In the context of studying a phenotype which might involve an accelerated phase rise in BP it was important to avoid inducing this prematurely or artificially. It was therefore decided to employ a method that required minimal handling of rats, did not require anaesthesia or restraint and allowed continuous recording of BP. Telemetric BP recording (Data Sciences International, St Paul, MN, USA) had many of these advantages and after recovery from surgical implantation (see section 2.2), data from unstressed animals could be collected on a continuous basis.

Telemetric implants (TA11PA-C40) were implanted by aseptic technique under halothane anaesthesia as described previously, into male transgenic heterozygotes HanRen2/Edin-- (n=10), HanRen2/Han-- (n=6), HanRen2/Lew-- (n=4), and non-transgenic control strains EdinSD (n=3), HanSD (n=3) and Lewis (n=4). Data was collected from 46 to 95 days of age, commencing from three days after surgical

implantation, with SBP, MBP, DBP and HR recordings made every 10 minutes and 30 minute averages stored for later analysis as described in section 2.2. It was observed that variable damping of pulse pressure occurred in some cases and therefore for the purposes of this study, only MBP values were analysed. Animals were housed singly in regular plastic cages with free access to a normal diet (0.32% sodium) and tap water.

MBP over time in both the transgenic stains and non-transgenic controls is shown in Fig 3.4.1 depicted as mean and standard error of the mean (SEM).

Fig 3.4.1

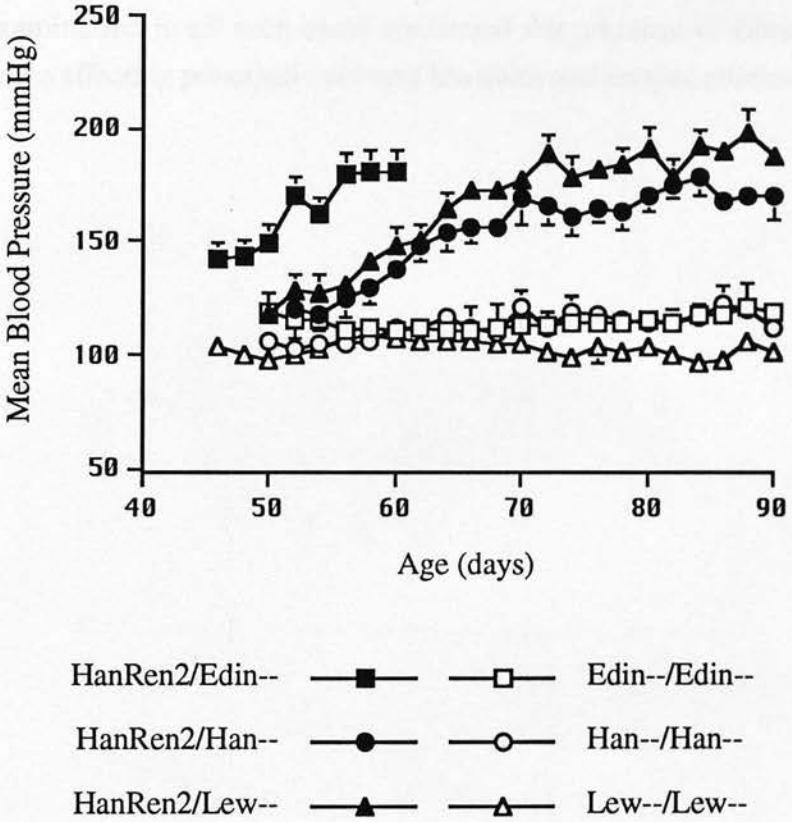


Fig 3.4.1 shows MBP recorded by telemetry. Group mean and SEM are shown every three days for clarity. Where error bars are not seen, they were smaller than the symbol used. Closed symbols represent transgenic heterozygotes, open symbols represent non-TG strains

HanRen2/Edin-- males showed significantly higher MBP from 52 to 60 days of age compared to the other two heterozygous *Ren*-2 crosses HanRen2/Han-- and HanRen2/Lew-- ($p<0.01$), but on attaining the plateau phase, MBP in both HanRen2/Han-- (169 ± 11.9 mmHg) and HanRen2/Lew-- (177.3 ± 4.1 mmHg) were not significantly different to the MBP found in 60 day old HanRen2/Edin-- (180.7 ± 10.0 mmHg) (Fig 3.4.1). Beyond age 60 days, group means for HanRen2/Edin-- are not shown as seven out of ten rats developed features of MH over the ensuing period. Early manifestations of MH in the HanRen2/Edin-- group before 60 days of age may

not have been clinically apparent but may have been associated with the beginning of an accelerated phase rise in MBP. This could result in the average MBP in apparently healthy HanRen/Edin-- to appear artificially elevated. This is supported by observed traces where some (Fig 3.4.2) affected animals exhibited a steeper rise during the period of developing hypertension. In the terminal phase all showed a striking accelerated rise in MBP (Fig 3.4.3). Only a single HanRen2/Han-- rat developed MH on telemetry (Fig 3.4.4).

In five rats on telemetry, who developed MH and where the natural course of events was allowed to progress, the mean rise in MBP over the terminal six hours was 72 mmHg (range 65 to 75 mmHg). It was apparent that those rats which exhibited a steeper rise in MBP had a shorter life expectancy (Fig 3.4.5). Histopathological examination in all such cases confirmed the presence of fibrinoid necrosis in renal tissue affecting principally afferent arterioles and smaller arteries as described.

Fig 3.4.2

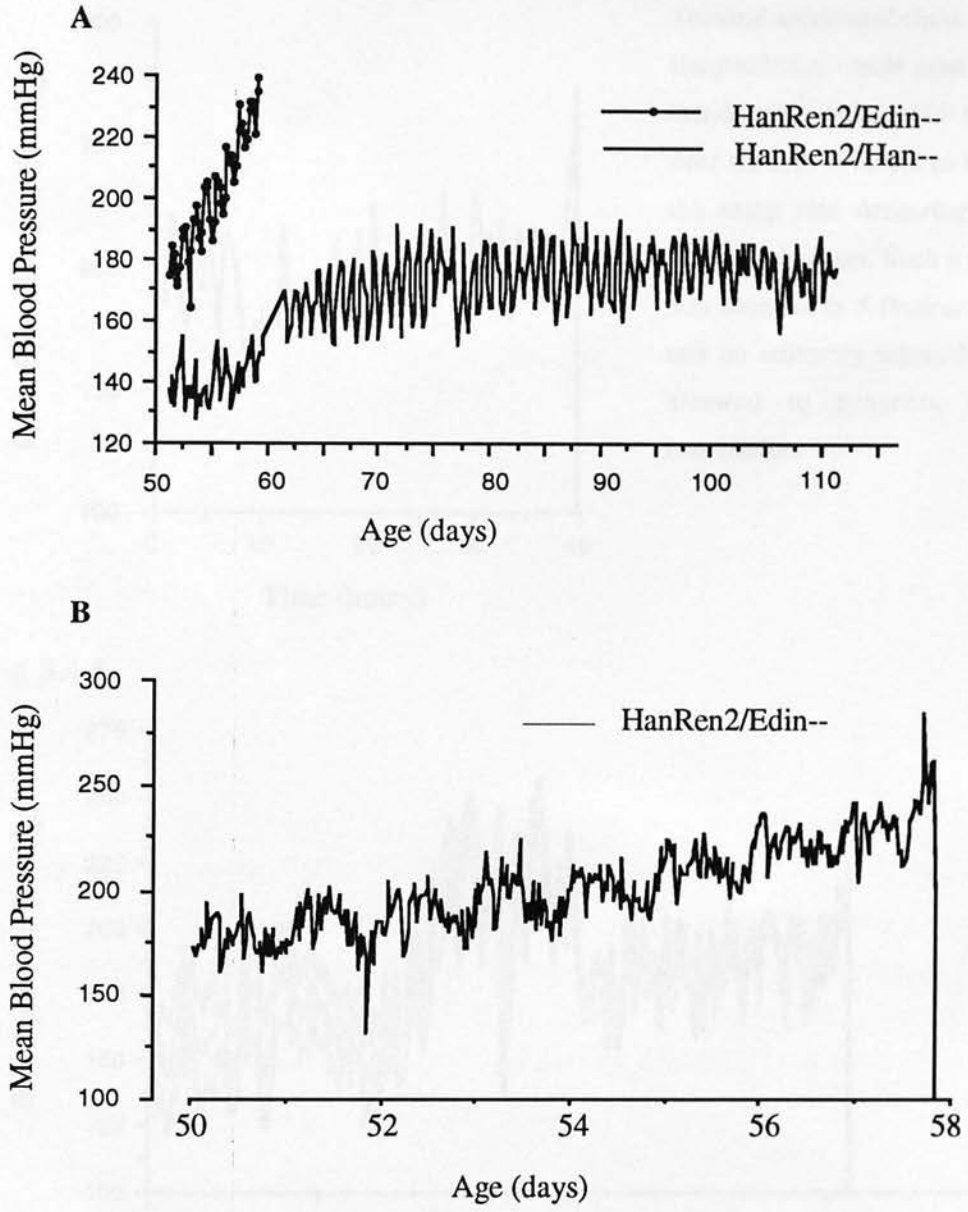
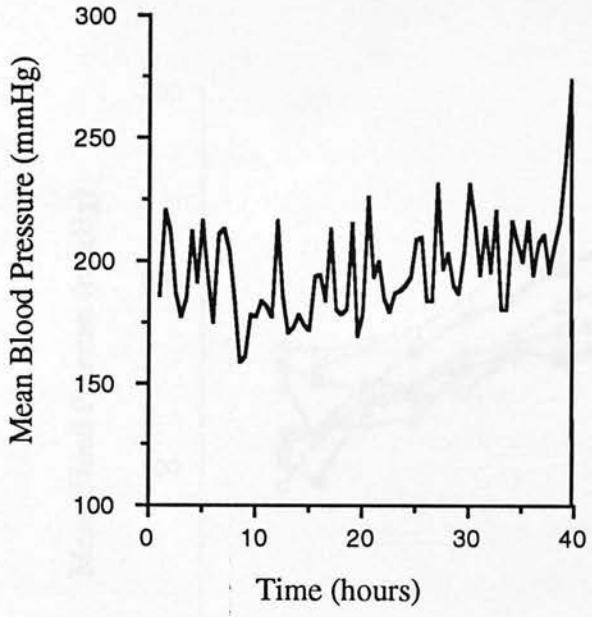


Fig 3.4.2 shows representative traces of MBP recorded by telemetry.

A. HanRen2/Han-- showing development of benign hypertension (BH) characterized by a rise in MBP to a plateau phase by 70 days of age. In contrast a steeper rise in MBP was seen from a HanRen2/Edin- rat who developed features of MH at 58 days.

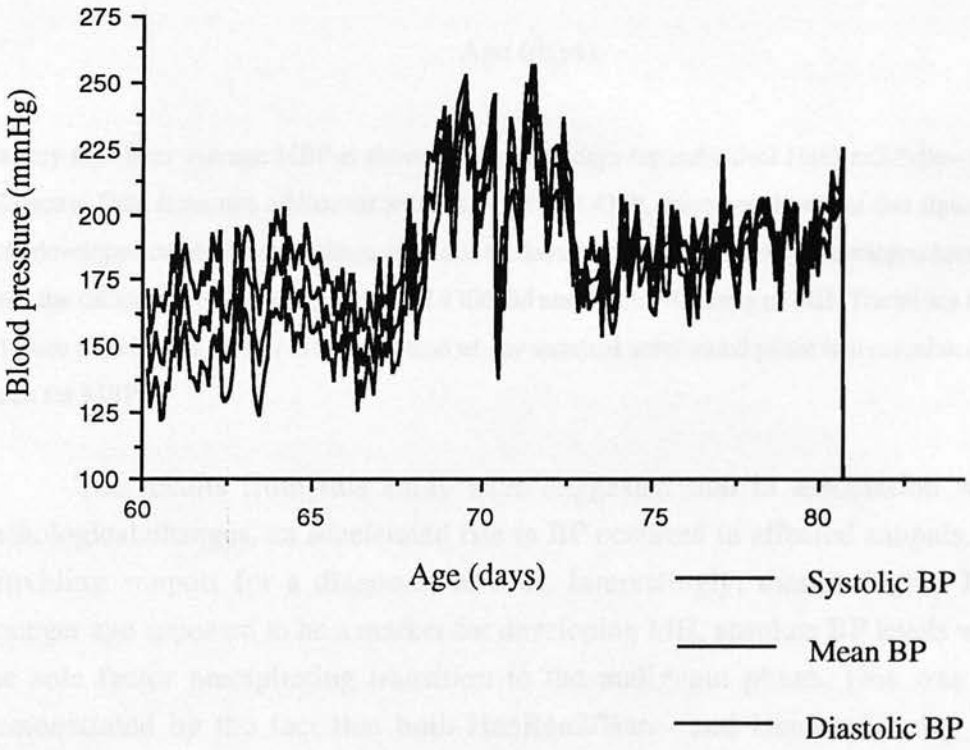
B. shows the same HanRen2/Edin-- recording in more detail.

Fig 3.4.3



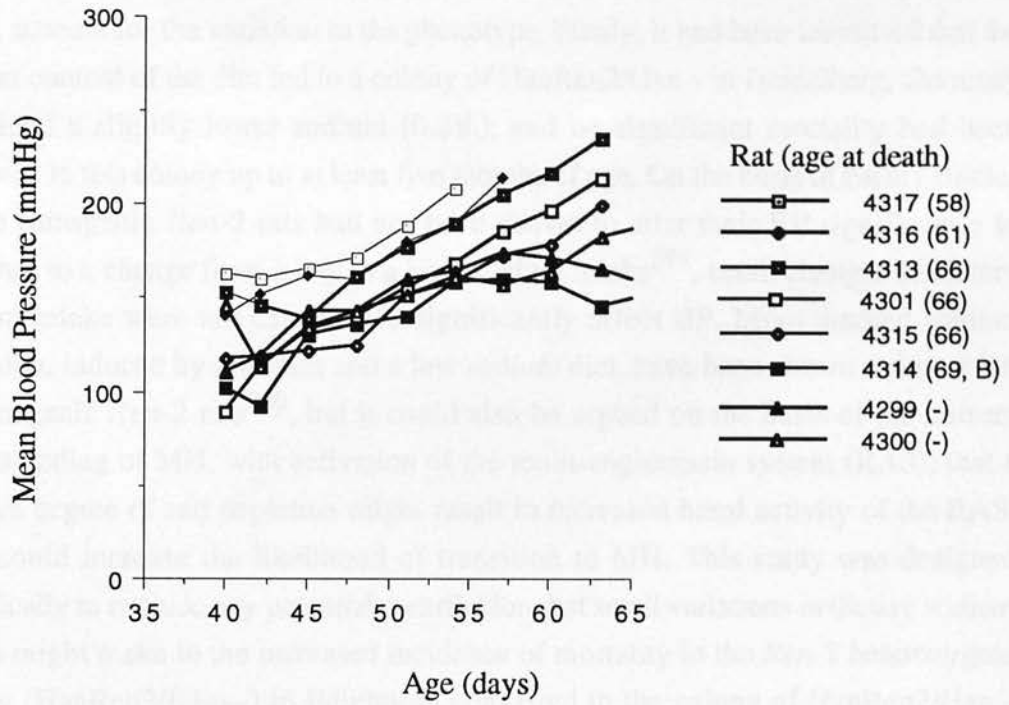
Terminal accelerated phase from the HanRen2/Han-- male aged 81 days that developed MH. MBP is shown over the last 40 hours to illustrate the steep rise occurring in the terminal six hours. Such a rise was also observed in 5 HanRen2/Edin-- rats on telemetry where MH was allowed to progress without intervention.

Fig 3.4.4



Telemetric recording of SBP, MBP and DBP from a HanRen2/Han-- male rat that developed features of MH. Interestingly, this rat showed evidence of an earlier accelerated phase in BP from which it made an apparent recovery before developing a second accelerated phase at 81 days of age. (SBP and DBP are also shown to demonstrate the progressive damping that occurred with time with narrowing of the pulse pressure).

Fig 3.4.5



Twenty-four hour average MBP is shown every three days for individual HanRen2/Edin-- males on telemetry. Data from two additional animals, 4318 and 4319, were not shown in this figure as they both developed catheter haemorrhage at 61 and 62 days respectively. Rat 4314 developed haemorrhage from the catheter at 69 days (B). 4299 and 4300 did not develop features of MH. Traces are shown up till three days before MH to avoid inclusion of any terminal accelerated phase in a calculated 24 hour mean for MBP.

The results from this study have suggested that in association with the pathological changes, an accelerated rise in BP occurred in affected animals, further providing support for a diagnosis of MH. Interestingly, though higher BP at a younger age appeared to be a marker for developing MH, absolute BP levels were not the sole factor precipitating transition to the malignant phase. This was clearly demonstrated by the fact that both HanRen2/Han-- and HanRen2/Lew-- strains developed levels of MBP by 70 days comparable to those found in HanRen2/Edin-- of a younger age who were about to develop MH.

3.5 Effects of small changes in dietary salt intake on the phenotype

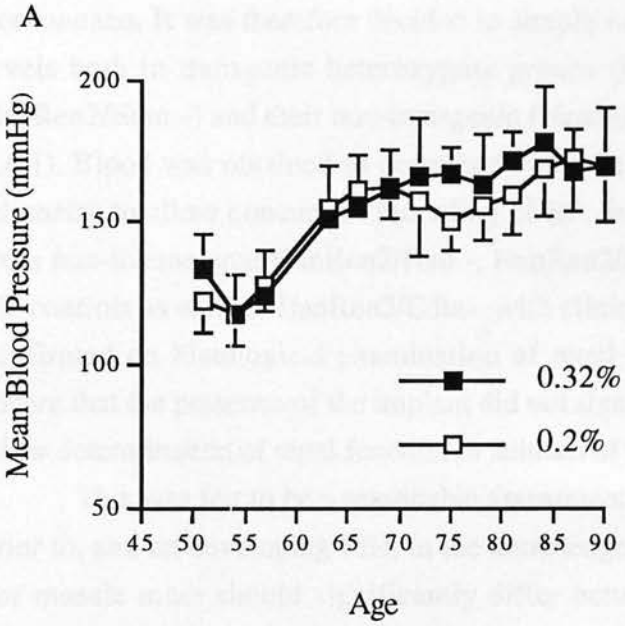
It was of interest to identify whether any small variation in dietary sodium might account for the variation in the phenotype. Firstly, it had been identified that the sodium content of the diet fed to a colony of HanRen2/Han-- in Heidelberg, Germany contained a slightly lower sodium (0.2%), and no significant mortality had been observed in this colony up to at least five months of age. On the basis of earlier studies where transgenic *Ren*-2 rats had not been shown to alter their BP significantly in response to a change from a low to a high sodium intake[291], small changes in dietary sodium intake were not expected to significantly affect BP. More marked sodium depletion, induced by diuretics and a low sodium diet, have been shown to lower BP in transgenic *Ren*-2 rats[280], but it could also be argued on the basis of the current understanding of MH, with activation of the renin-angiotensin system (RAS), that a relative degree of salt depletion might result in increased basal activity of the RAS. This could increase the likelihood of transition to MH. This study was designed specifically to exclude any potential contribution that small variations in dietary sodium intake might make to the increased incidence of mortality in the *Ren*-2 heterozygote colony (HanRen2/Edin--) in Edinburgh compared to the colony of HanRen2/Han-- originally bred and maintained in Heidelberg.

Accordingly, a parallel study was performed on HanRen2/Han males maintained on 0.32% (n=6) and 0.2% (n=6) sodium diets from weaning. Progeny from two litters both derived from the same homozygous transgenic father were randomly allocated into two groups. They had free access to diet and drinking water and were housed singly. HanRen2/Lew male progeny were similarly obtained from the same father and given 0.32% (n=4) and 0.2% (n=5) sodium diets in parallel. MBP was recorded continuously from 45 days by radiotelemetry.

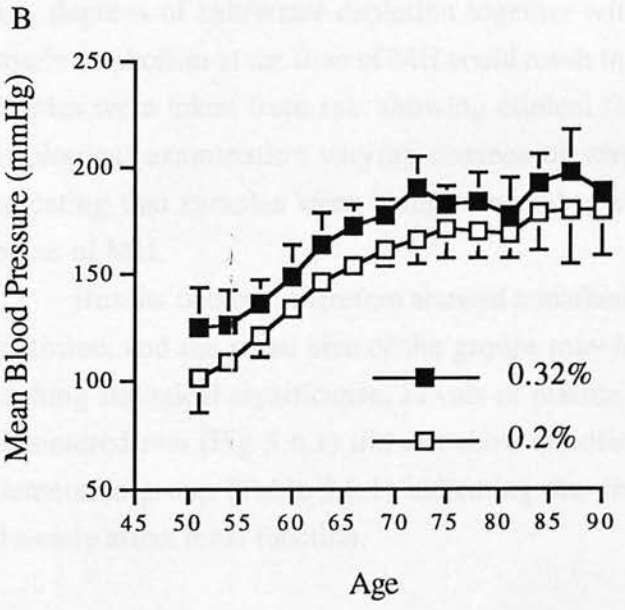
The incidence of MH, confirmed by histopathological examination in the four groups was recorded. Twenty-four hour MBP for each rat was derived as described previously from values obtained every 30 minutes, and group means on the two sodium diets were compared by unpaired t test (Fig 3.5.1). Though small but statistically significant differences were seen between the two sodium diets, at age 75 days in HanRen2/Han-- and at age 66, 69 and 72 days in HanRen2/Lew--, this did not appear to be responsible for the change in phenotype. Only one HanRen2/Han-- rat developed MH while on a 0.32% Na diet. As regards HanRen2/Edin-- rats, weaning onto the lower sodium diet was not protective. Though blood pressure was not measured in this group, it was observed that of a litter of eight animals on a low salt diet, six developed features of MH. In conclusion, after excluding other potential

environmental contributions to the change in phenotype on breeding HanRen2/Edin-- at the Centre for Genome Research, results have suggested that dietary differences were unlikely to be responsible. In the light of the striking differences in the incidence of the phenotype when all animals have been maintained under identical conditions, even within the same room, the evidence would point to genetic factors conferring altered susceptibility to developing MH.

Fig 3.5.1



A. Mean blood pressure in HanRen2/Han-- males on a 0.32% (n=6) and 0.2% (n=5) sodium diet. Results are shown as group means and error bars represent SD. A statistically significant difference in MBP was observed on Day 75 alone. 1/6 HanRen2/Han-- on 0.32% and 0/5 on 0.2% diets developed MH.



B. Mean blood pressure in HanRen2/Lew-- males on 0.32% and 0.2% sodium diets. There was a small but significant increase in MBP at age 66, 69 and 72 days of age. No rats from either group developed MH.

3.6 Renal function

In untreated MH progressive uraemia and renal failure is a common terminal event. This may be a consequence of the afferent vascular pathology leading to a reduced renal blood flow and glomerular filtration rate as discussed in Chapter 1. More accurate methods of determining renal plasma flow in rats have usually employed clearance studies using inulin or para-aminohippuric acid (PAH), but in this model performing such studies in rats exhibiting features of MH was neither practical nor humane. It was therefore decided to simply measure plasma urea and creatinine levels both in transgenic heterozygote groups (HanRen2/Han--, HanRen2/Lew--, HanRen2/Edin--) and their non-transgenic (HanSD, Lewis and EdinSD) controls (Fig 3.6.1). Blood was obtained as described in the Section 2.3 (1). Animals were all on telemetry to allow concurrent recording of BP. In addition blood was also obtained from non-telemetered HanRen2/Han--, HanRen2/Lew, HanRen2/Edin--, and pooled SD controls as well as HanRen2/Edin-- with clinical features of MH which was later confirmed on histological examination of renal tissue (Table 3.6.1). This was to ensure that the presence of the implant did not significantly alter renal function and to allow determination of renal function in animals of four and six weeks of age.

This was felt to be a reasonable assessment of gross changes in renal function prior to, and on developing MH, in the knowledge that neither dietary protein intake nor muscle mass should significantly differ between groups up until the terminal stages. In the later stages of MH, with the observed diuretic phase and body weight loss, degrees of salt/water depletion together with anorexia were likely. Increased protein catabolism at the time of MH could result in increased plasma creatinine. Blood samples were taken from rats showing clinical features of MH, and on subsequent histological examination varying degrees of severity of pathology were apparent indicating that samples were being obtained at varying stages through the natural course of MH.

Results obtained therefore showed a marked variance in the measured urea and creatinine, and the small size of the groups may have resulted in many changes not reaching statistical significance. Levels of plasma urea and creatine measured in the telemetered rats (Fig 3.6.1) did not show significantly higher levels than the non-telemetered group (Table 3.6.1) indicating that the presence of the implant did not adversely affect renal function.

Fig 3.6.1

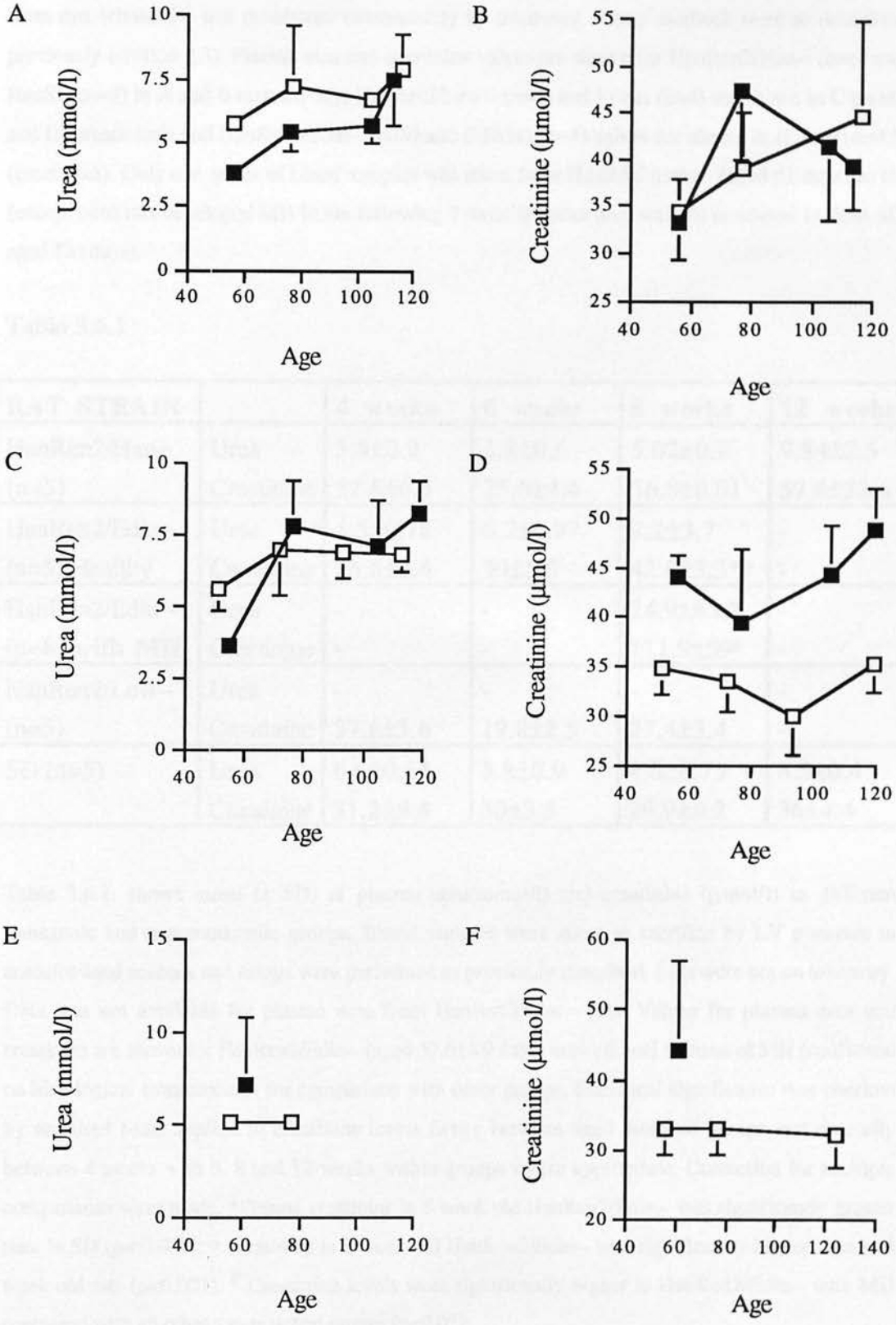

A
Urea (mmol/l)
10
7.5
5
2.5
0
40
60
80
100
120
Age
B
Creatinine (µmol/l)
55
50
45
40
35
30
25
40
60
80
100
120
Age
C
Urea (mmol/l)
10
7.5
5
2.5
0
40
60
80
100
120
Age
D
Creatinine (µmol/l)
55
50
45
40
35
30
25
40
60
80
100
120
Age
E
Urea (mmol/l)
15
10
5
0
40
60
80
100
120
Age
F
Creatinine (µmol/l)
60
50
40
30
20
40
60
80
100
120
140
Age

Fig 3.6.1 shows plasma urea (mmol/l) and creatinine (μmol/l) for transgenic ■ and non-transgenic control strains □. Blood was obtained by retro-orbital bleed into tubes containing lithium heparin, from rats whose BP was monitored continuously by telemetry. Assay methods were as described previously (section 2.3). Plasma urea and creatinine values are shown for HanRen2/Han-- (n=6) and HanSD (n=3) in A and B respectively; HanRen2/Lew-- (n=5) and Lewis (n=4) are shown in C (urea) and D (creatinine); and HanRen2/Edin-- (n=9) and EdinSD (n=4) values are shown in E (urea) and F (creatinine). Only one series of blood samples was taken from HanRen2/Edin-- (aged 61 days) as all (except two) rats developed MH in the following 7 days. (Plasma urea was not measured in Edin SD aged 124 days).

Table 3.6.1

RAT STRAIN		**4 weeks**	**6 weeks**	**8 weeks**	**12 weeks**
HanRen2/Han--	Urea	5.8±0.9	3.8±0.6	5.02±0.7	9.84±2.5
(n=5)	Creatinine	32.6±6.5	25.6±4.4	36.8±6.01	57.4±22.6
HanRen2/Edin--	Urea	4.5±0.78	6.2±0.97	9.2±3.7	-
(n=5) Healthy	Creatinine	26.8±2.6	30±5.5	42.8±3.3*†	-
HanRen2/Edin--	Urea	-	-	24.9±43.7	-
(n=8) **with MH**	Creatinine	-	-	111.9±59#	-
HanRen2/Lew--	Urea	-	-	-	-
(n=5)	Creatinine	27.6±3.6	19.8±2.5	27.4±3.4	-
SD (n=5)	Urea	6.6±0.64	5.8±0.9	4.8±0.75	6.5±0.4
	Creatinine	31.2±9.4	30±3.5	29.9±6.2	36±4.4

Table 3.6.1. shows mean (± SD) of plasma urea(mmol/l) and creatinine (μmol/l) in different transgenic and non-transgenic groups. Blood samples were taken at sacrifice by LV puncture in anaesthetized animals and assays were performed as previously described. Rats were not on telemetry. Data was not available for plasma urea from HanRen2/Lew-- rats. Values for plasma urea and creatinine are shown for HanRen2/Edin-- (aged 57.6±4.9 days) with clinical features of MH (confirmed on histological examination) for comparison with other groups. Statistical significance was checked by unpaired t-test applied to creatinine levels firstly between aged matched groups and secondly between 4 weeks with 6, 8 and 12 weeks within groups where appropriate. Correction for multiple comparisons were made. *Plasma creatinine in 8 week old HanRen2/Edin-- was significantly greater than in SD ($p= 0.008$); † creatinine in 8 week old HanRen2/Edin-- was significantly higher than in 4 week old rats ($p<0.001$). # Creatinine levels were significantly higher in HanRen2/Edin-- with MH compared with all other age-matched groups ($p<0.05$)

A trend of increasing plasma urea and creatinine with age is observed in the three transgenic F1 hybrids, while the non-transgenic strains do not show any significant trend. Over the age range studied, no statistically significant change in creatinine was seen in any of the crosses except for HanRen2/Han-- aged 12 weeks and healthy HanRen2/Edin-- aged 8 weeks. A striking difference is seen for HanRen2/Edin-- with MH (plasma urea 24.9 ± 43.7 mmol/l; plasma creatinine 111.9 ± 59.3 µmol/l) compared both with aged matched healthy HanRen2/Edin-- ($p=0.046$), and with other strains ($p<0.05$). Statistical comparisons were not made between values for urea, as it was felt that interpretation of the values was complicated in MH by concomitant salt/water depletion and a large variance in the measurement was apparent.

In conclusion, in MH varying degrees of renal failure were apparent. The variance in measurements seen in this group may in part be due to the fact that samples were collected from animals showing features of MH but who were inevitably at different stages through the natural course of the condition. Within the age range of animals studied, only HanRen2/Edin-- showed a significant rise in plasma creatinine with age. This rise appeared to occur between 6 and 8 weeks of age and may be relevant to understanding why this hybrid was at increased risk of developing MH. Alternatively, this group of apparently healthy animals may have included a proportion who were already beginning to develop MH and so values measured from an apparently healthy group may have been artefactually increased.

3.7. Renin-angiotensin system

Activation of the kidney RAS has been well described both in humans and animal models of MH[342,340,346,155]. The hypertensive *Ren*-2 transgenic rat line appears to have an Ang II dependent form of hypertension as discussed in Chapter 1. However there may be inappropriate Ang II activity in the kidney of *Ren*-2 rats accounting for an increased pre-glomerular vascular resistance[284], despite reportedly suppressed renal tissue renin content[267,270]. Circulating plasma renin has been thought to be primarily transgene derived, while the majority of kidney renin present may be derived from endogenous rat renin gene expression which is suppressed owing to increased BP[288].

It was therefore interesting to determine what changes in circulating plasma renin, Ang II and aldosterone content occurred on transition to the malignant phase and secondly to observe what response occurred in the JGA cells of transgenic *Ren*-2 rats with MH.

Plasma renin, Ang II and aldosterone were measured by RIA by methods previously described in Chapter 2. Blood samples were obtained either by retro-orbital bleeding or by cardiac puncture from rats which had been briefly anaesthetized with halothane in oxygen, and assay were performed as described in section 2.4. Healthy HanRen2/Edin--, HanRen2/Han--, HanRen2/Lew--, pooled non-transgenic controls (SD and Lewis) and HanRen2/Edin-- with MH were used. All rats were seven to nine weeks of age, male and maintained on the same diet and in the same conditions.

Fig 3.7.1

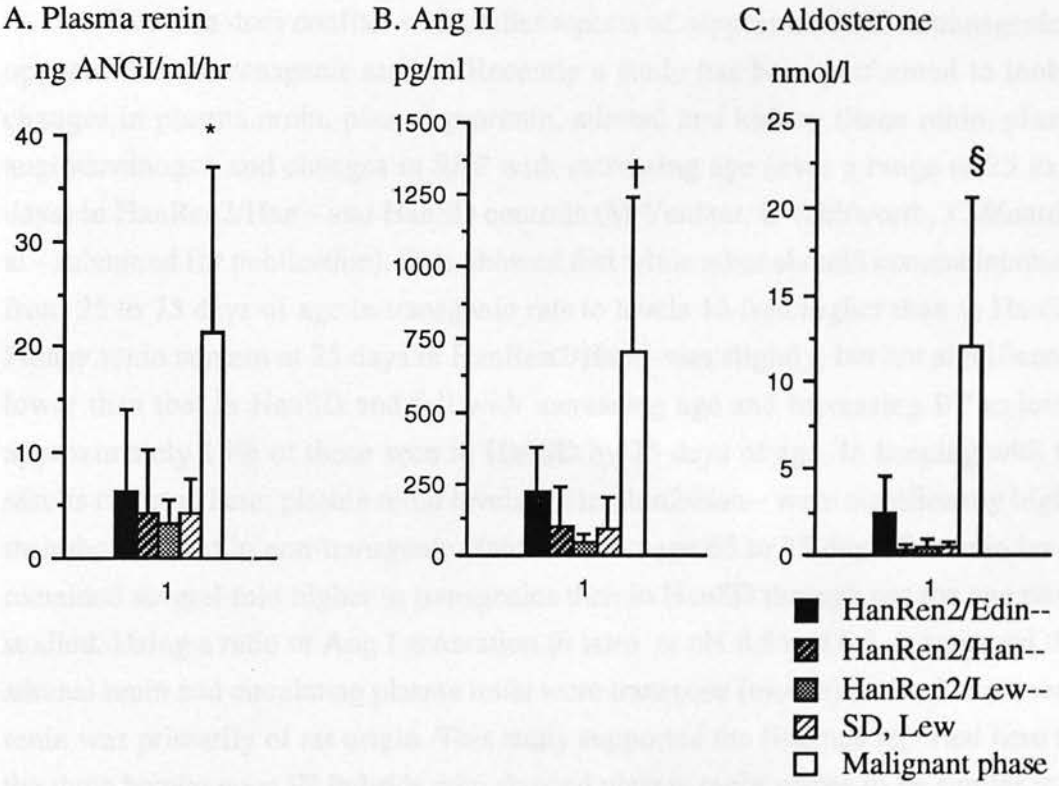


Fig 3.7.1 shows plasma renin concentration (PRC), Ang II and aldosterone, measured in the three hemizygous F1 hybrids, non-transgenic SD/Lewis and in MH. For HanRen2/Edin-- n= 15, 11, 14 for each assay respectively; HanRen2/Han-- n= 19, 11, 30 ; HanRen2/Lew, n= 14,12,19; pooled non-transgenic strains n= 18, 14, 13 (results were pooled having established that there was no significant difference between the strains) and for MH n= 14, 11, and 15 respectively. Results are shown as group means ± SD. Significant increases in PRC* ($p<0.008$), Ang II† ($p<0.04$) and aldosterone§ ($p<0.004$) were seen in the MH group compared to the other 4 non-malignant groups by unpaired t-test with Bonferroni correction for multiple comparison.

Results showed that amongst healthy, non-malignant transgenic male rats of seven to nine weeks of age PRC, Ang II and aldosterone levels were not significantly suppressed in comparison to the non-transgenic, normotensive rats (Fig 3.7.1). A small increase was seen in healthy HanRen2/Edin-- group relative to other transgenic heterozygotes but statistically significant differences were not observed. An increased variance in HanRen2/Edin-- may in part be due to the fact that, of this group a proportion would have been expected to develop MH in the following days. Two animals did develop MH within three days of being classified as being "healthy" while others developed MH at later stages.

This data does conflict with earlier reports of suppressed PRC in transgenic as opposed to non-transgenic strains. Recently a study has been performed to look at changes in plasma renin, plasma prorenin, adrenal and kidney tissue renin, plasma angiotensinogen and changes in SBP with increasing age (over a range of 25 to 75 days) in HanRen2/Han-- and HanSD controls (M Veniant, C Whitworth, J Ménard et al - submitted for publication). Data showed that while adrenal renin content increased from 25 to 75 days of age in transgenic rats to levels 16 fold higher than in HanSD, kidney renin content at 25 days in HanRen2/Han-- was slightly, but not significantly lower than that in HanSD and fell with increasing age and increasing BP to levels approximately 15% of those seen in HanSD by 75 days of age. In keeping with the results reported here, plasma renin levels in HanRen2/Han-- were significantly higher than those found in non-transgenic HanSD rats at age 65 to 75 days. Prorenin levels remained several fold higher in transgenics than in HanSD through out the age range studied. Using a ratio of Ang I generation *in vitro* at pH 8.5 and 6.5, it appeared that adrenal renin and circulating plasma renin were transgene (mouse) derived while renal renin was primarily of rat origin. This study supported the findings reported here for the three hemizygous F2 hybrids who showed plasma renin values to be similar to or slightly higher than those in non-transgenic control strains.

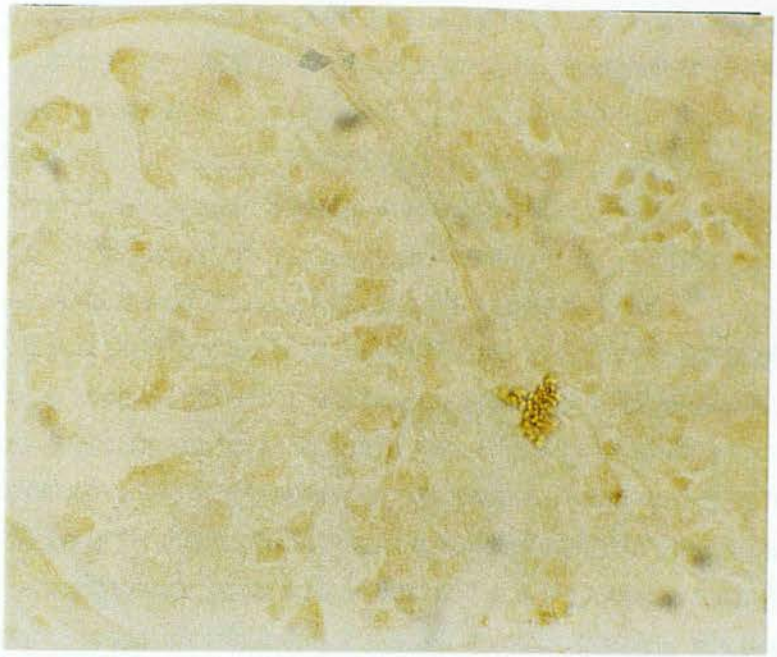
Striking and highly significant increases in circulating PRC, Ang II and aldosterone were observed in the MH group (Fig 3.7.1). The results suggest that in this model on transition from benign hypertension to MH, activation of the RAS occurs. Immunohistochemistry was therefore performed using a polyclonal anti-mouse renin antibody and an avidin-biotin horse radish peroxidase secondary detection system on 3 μm formalin fixed kidney sections. The methods were as described in section 2.4. Primary antibody was omitted to verify that there was no background staining in sections processed in parallel. Sections were taken from healthy HanRen2/Han-- (n=8) HanRen2/Lew-- (n=4), control HanSD and EdinSD (n=4) and rats with MH (n=10). Light microscopy of sections showed staining at the

afferent arterioles in the vicinity of the vascular pole of glomeruli in all sections from non-transgenic strains (Fig 3.7.2). Markedly increased staining was seen in transgenic rats with MH (Fig 3.7.2 B) and this staining was seen to extend to smaller renal arterioles. More frequent and heavier staining of the vascular poles was apparent in the MH compared to healthy hypertensive transgenic heterozygotes. Some sections were obtained from rats that had been on telemetry and it was observed that relatively increased staining was seen in those transgenic heterozygotes that tended to have a lower BP compared to others. Staining in healthy transgenic heterozygotes was scanty (Fig 3.7.2 A) and less intense than that observed in non-transgenic EdinSD and HanSD supporting previous findings of reduced renal renin content in *Ren*-2 transgenic heterozygotes[269].

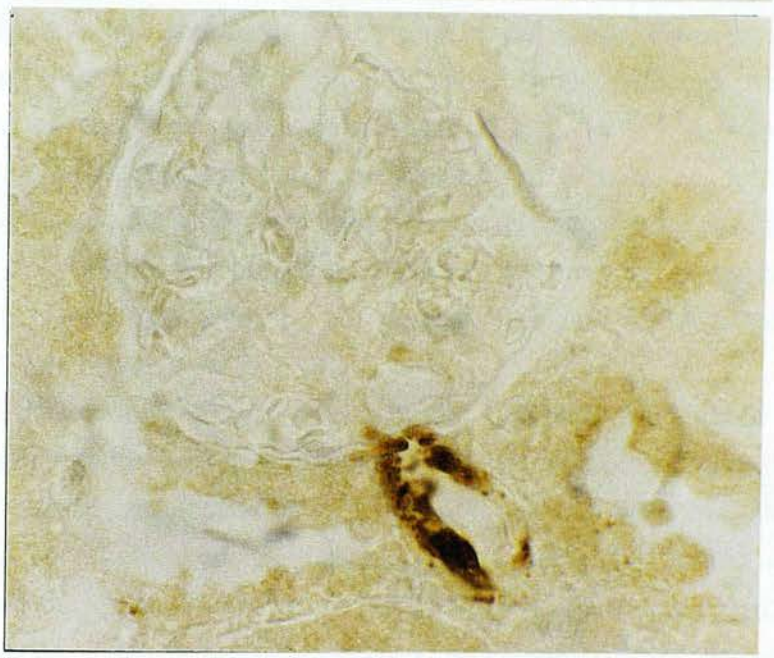
In conclusion in MH there appears to be increased circulating plasma renin, Ang II and aldosterone and this may in part be due to activation of the renal RAS which is normally suppressed in *Ren*-2 transgenic heterozygotes with BH. As the antibody used does not differentiate between mouse and rat renin, it has not been possible from this experiment to differentiate rat from mouse renin. It is possible that both may contribute to the increased staining observed at the vascular poles, but as other experiments have shown by the pH profile of *in vitro* Ang I generation that rat derived renin is the primary renin in *Ren*-2 transgenic kidneys (Veniant, Whitworth, Ménard et al - manuscript submitted), it would appear likely that it is primarily the endogenous renin gene that is being activated in response to the transition to MH. One approach would be to use a specific ribonuclease protection assay that can differentiate and quantify rat *Ren* from mouse *Ren*-2 mRNA on total RNA extracted from transgenic rat kidney with and without MH.

Fig 3.7.2

A



B



Immunohistochemistry was performed using a polyclonal anti-mouse renin antibody with an avidin-biotin horseradish peroxidase secondary detection system on 4% formal saline fixed kidney sections.

A Staining at the vascular pole of glomeruli was scanty in healthy hypertensive transgenic *Ren*-2 rats. Photomicrograph (X 630) shows the vascular pole (not counter-stained) of an individual glomerulus.

B In contrast the staining seen at the vascular poles from transgenic rats with features of MH was frequent and more dense. Photomicrograph (X 400) shows an example of a glomerulus with the arteriole staining for renin.

A process of intravascular haemolysis has been well described in MH and is a common component of the clinical presentation, though it tends to be less florid than that found in conditions such as HUS/TTP (haemolytic-uraemic syndrome/thrombotic thrombocytopenic purpura) which may be accompanied by severe hypertension[363-365]. It has been suggested that the haemolysis may in part be due to the concomitant uraemia[365], while others have suggested that it is a primary process which itself induces vascular wall damage initiating the transition to MH[331].

It was therefore decided to specifically look for evidence of microangiopathic haemolytic anaemia (MAHA) in transgenic rats with MH, in healthy hypertensive age-matched littermates of those with MH who might be considered as pre-malignant and in normotensive SD at age seven to nine weeks. Blood was obtained either by retro-orbital bleed or by direct cardiac puncture under halothane anaesthesia.

A reticulocyte count was determined as a percentage of red cells from all films. The normal range for reticulocyte counts in male Alderley rats (aged 26 weeks) was 0.5 to 2.7%. SD may have a different normal range. Haemolysis was defined as present when there was evidence of spherocytes, microspherocytes and red cell fragments, and films were scored blind on a grade of 0 to +++ (Table 3.8.1). In addition, burr cells were also noted in the films of some rats with MH. Four of the samples were taken from rats who were on telemetry but it was clear that the intra-aortic catheter did not induce intravascular haemolysis. Examples of a blood film (Wrights stain) from a normal SD and one from a rat with MH showing evidence of haemolysis is shown in Fig 3.8.1.

The results clearly demonstrated that with MH, there were features of microangiopathic haemolysis, namely red cell fragmentation with spherocytes and microspherocytes. Reticulocyte counts (%) in rats with MH (mean ± SD) were 11.2±2.5% which were significantly higher ($p=0.003$) than those found in age-matched transgenic littermates (6.22±1.1%). It could not be confirmed that the latter group were truly pre-malignant as blood was obtained at the time of sacrifice, and on pathological examination features of florid fibrinoid necrosis were not seen. However it remains likely that some of them would have developed MH in the subsequent week.

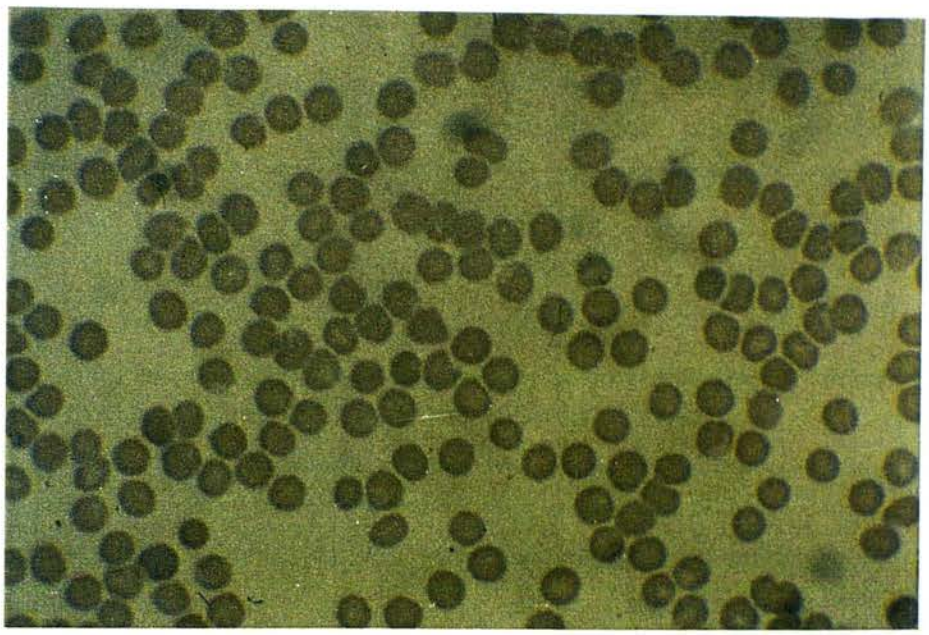
Table 3.8.1

HanRen2/Edin	ID	Comments	Haemolysis	Reticulocyte count
Malignant	4313	on telemetry	+++	13.6%
	4644	-	+++	10.6%
	4666	-	+++	13.2%
	4756	-	++	7.4%
	5061	-	+++	11.2%
Pre-malignant	4299	on telemetry	0	4.7%
	4300	on telemetry	0	6.3%
	4643	on telemetry	0	5.6%
	5067	-	0	7%
	5063	-	0	7.5%
Edin SD	1	-	0	3.5%
	2	-	0	2.4%

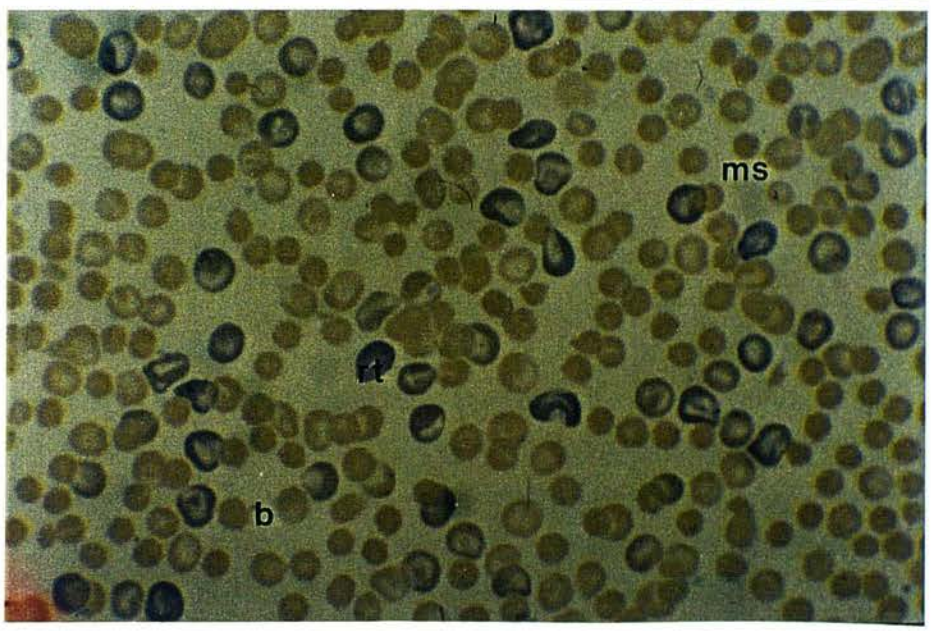
All rats were male. Four were on telemetry as indicated. "Malignant" hypertension was defined as present when affected rats had both clinical features of the syndrome, and evidence of fibrinoid necrosis on histology of renal tissue as described earlier. Potentially "pre-malignant" rats were age-matched litter-mates of HanRen2/Edin-- rats with MH, and blood was taken on the same day as their affected brothers with MH. Two non-transgenic EdinSD rats aged 8 weeks were also included for analysis. Haemolysis was scored blind to the status of the rat on a 0 to +++ scale, with the presence of spherocytes, microspherocytes and red cell fragments indicating haemolysis. Reticulocyte counts are indicated.

Fig 3.8.1

A



B



Blood films were both spread and stained with Wright's stain before photographing at X 630.

A A film, taken from a healthy HanRen2/Edin--, showing normal red blood cell morphology.

B Film taken from blood obtained from a HanRen2/Edin-- rat with clinical and pathological signs of malignant hypertension. It shows an increased reticulocyte count (rt), microspherocytes (ms), red cell fragments and occasional Burr cells (b).

3.9 Conclusions

The results reported here have characterized a rat model of MH where a spontaneous transition from BH to MH has occurred without the need for dietary, pharmacological or surgical manipulation. Features included an accelerated phase rise in MBP with increments of 70 mmHg over the terminal hours. Classical pathological changes affecting the kidney were seen with florid fibrinoid necrosis and myo-intimal proliferation. Surprisingly few secondary ischaemic changes were seen involving glomeruli or tubules suggesting that the duration of severe vascular damage was relatively short. In keeping with the renal pathology, affected rats developed acute renal failure with a rising plasma urea and creatinine.

Evidence of activation of the RAS was seen with increased levels of circulating plasma renin, Ang II and aldosterone in MH compared to BH. Increased staining of afferent arterioles occurred at the vascular poles of glomeruli in affected rats suggesting that activation of the renal RAS occurred in this transgenic rat as seen in other models of MH[155].

Pathological changes were not limited to the kidney. Transgenic heterozygotes developed progressive LVH with statistically significant changes relative to non-transgenic SD present by six weeks of age. In sixteen week old *Ren*-2 heterozygotes, cardiac hypertrophy and reduced LV compliance has been previously reported with evidence of increased peri-vascular fibrosis, but not interstitial fibrosis[290].

Multiple cardiac micro-infarcts were found in HanRen2/Edin-- and in occasional HanRen2/Han--. These were occasionally seen to be associated with fibrinoid necrosis of cardiac arterioles, but were also observed in rats that did not have features of florid renal fibrinoid necrosis. Such lesions have been previously reported in SHR-SP maintained on 1% NaCl drinking solution[362,446]. SHR-SP maintained on water also show an expected increase in myocardial fibrosis with perivascular, interstitial and focal distributions. It has been suggested that perivascular and interstitial fibrosis may be induced by exudation of growth factors, possibly secondary to increased vascular wall permeability[447], but the nature of focal fibrosis has been less well characterized. It would seem likely that focal infarction is responsible. In SHR of over eight months of age, foci of old and new necrosis and fibrosis have been described[448]. The aetiology of these microinfarcts in this strain with their likely sequelae of focal fibrosis is not clear.

Observations from this study have suggested that fibrinoid change in small cardiac arterioles may be contributory. Both Ang II and aldosterone have been implicated as growth factors which, via induction of proto-oncogenes, may be

involved in the process of development of LVH and in remodeling in response to hypertension and endothelial damage[203,194,195,207,208]. Elevated circulating Ang II levels, either of endogenous or exogenous origin, have also been implicated in causing focal cardiac myonecrosis and fibrosis[449], and may be relevant in this model. Continuous ECG recording (two lead) was performed in two HanRen2/Edin-- animals. Implants (TA11CTA-F40) were surgically implanted into 40 day old male rats under halothane anaesthesia with placement of the electrodes at the right shoulder and left groin. Both rats developed MH, and it was observed in one of the two rats that, over the last 24 hours before death, an increasing frequency of ventricular extra-systoles and self-terminating runs of ventricular tachycardia were observed leading to ventricular fibrillation (data not shown). Though preliminary, this suggests that ventricular arrhythmias may well originate from such foci of necrotic and fibrotic myocardium and warrants further investigation. Angina has been reported to be a rare symptom in MH [306,302], and the clinical picture in humans is likely to be strongly influenced by the presence of coronary vascular disease. It is not known however what the incidence of sudden death or documented ventricular tachyarrythmias are in patients with MH.

The reasons why HanRen2/Edin-- are more at risk of developing MH are not clear from these physiological studies. They do appear to have a slightly higher MBP than the other two hemizygous crosses from 52 to 60 days of age, but this finding may be confounded by inclusion of rats in the initial stages of an accelerated phase in this group. Certainly absolute BP levels alone are not crucial to precipitating the change from BH to MH, as HanRen2/Han-- and HanRen2/Lew-- hybrids reached comparable BP levels to HanRen2/Edin-- after a lag phase of approximately 10 days. Transgene dosage effect has been linked to inducing MH, and it has been reported previously that untreated homozygous *Ren*-2 rats have a higher incidence of death[269]. Histopathological examination of tissues from homozygous transgene positive rats, not maintained on a converting enzyme inhibitors, did reveal fibrinoid necrosis of comparable severity. However for the three F1 hybrids examined here who exhibited a clear difference in incidence of the phenotype of MH, no change in either transgene copy number or transgene insertion site can account for the variation as homozygotes from the same transgenic line TGR(mREN2)27 were used for breeding purposes.

Hemizygotes derived from the line TGR(mREN2)26 and EdinSD did not develop MH, but they are less hypertensive compared to TGR(mREN2)27 derived hemizygotes. Hypertensive transgenic rats have been made using a transgene construct (α1AT-REN2), comprising the α1-antitrypsin promoter fused to *Ren*-2 cDNA, introduced into EdinSD rat embryos, and resulted in expression of *Ren*-2 primarily in

liver. MH has occurred in a significant proportion of the progeny and has been confirmed by histopathological examination of renal tissue. These transgenic rats have very elevated circulating prorenin levels, but no significant difference in plasma renin was found in transgene positive rats compared to non-transgenic littermates. Active *Ren*-2 derived renin was found in plasma indicating that activation of transgene derived prorenin was occurring outwith the liver (D Ogg, personal communication) [450,451]. Another group have now reported the early death of some animals from a group of hemizygous *Ren*-2 rats on a SD background associated with severe arterial and arteriolar changes including fibrinoid necrosis. The origins of their SD colony were not published[452].

Thus experiments have suggested that environmental factors are not responsible for the differing incidence between the three transgenic, hemizygous F1 hybrids, but that the EdinSD rat may exhibit an increased predisposition to develop MH. The relatively narrow age range at which these animals develop MH is interesting. Survivors beyond 100 days infrequently develop either clinical or pathological features of MH. This might suggest that the vasculature in younger rats has had less time to develop structural adaptations to the increase in pressure and this may be relevant to understanding the pathogenesis of the transition to MH. It is possible that the reduced incidence seen in HanRen2/Han-- and HanRen2/Lew-- is due to the small lag phase of rise in BP, and therefore that a higher BP at a younger age may be a marker of increased risk of transition to MH.

CHAPTER 4

Transition to Malignant hypertension may have a genetic basis

4.1 Introduction

In Chapter 3 the studies have clearly indicated that genetic factors appeared to be important in increasing the likelihood of transition from benign hypertension to malignant phase in the three heterozygote crosses. Subtle dietary differences did not appear to be important. If a genetic factor or factors were important then this might point towards a putative triggering factor that is acting in conjunction with elevated BP to induce development of the vascular damage and activation of the renin-angiotensin system. In the light of the clear distinction between the HanRen2/Edin-- and HanRen2/Lew-- crosses in terms of incidence of the malignant phenotype it was decided to design an analytical cross to further ascertain whether a genetic factor or factors was important.

4.2 Design of the analytical cross

A breeding programme was instituted with the aim of segregating alleles derived from EdinSD rats. The design of the cross is illustrated in Fig 4.2.1

Fig 4.2.1

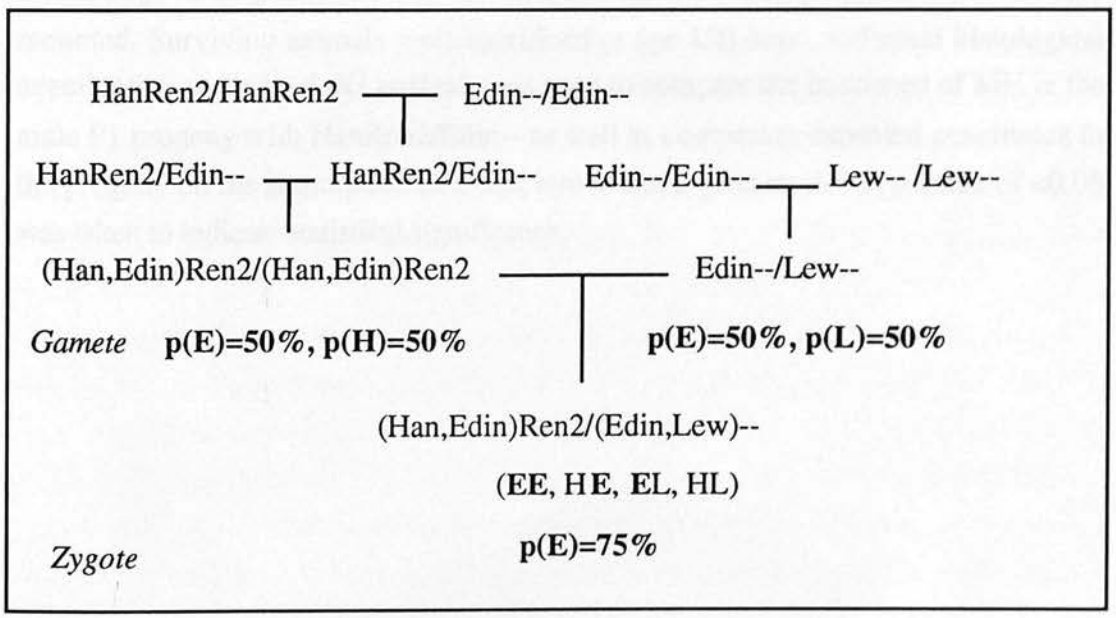


Fig 4.2.1 shows the analytical cross designed to segregate EdinSD derived alleles. Crosses are depicted by their assumed genotype. At the gamete stage from the male side, it can be assumed that there is a 50% probability of an EdinSD (E) and 50% probability of Hannover SD (H) derived allele. On the female side, there is a 50% probability of an EdinSD (E) and a 50% probability of a Lewis (L) allele. Therefore in the progeny depicted (Han,Edin)Ren2/(Edin,Lew)--, the possible alleles at any one locus may be EE, HE, EL or HL giving an overall 75% likelihood of the presence of an EdinSD derived allele. The limited number of crosses reduces the likelihood of recombination events occurring.

4.3. Methods

Male F1 progeny defined as (Han,Edin)Ren2/(Edin,Lew)-- (n=73) were bred from three (Han,Edin)Ren2/(Han,Edin)Ren2 males and eight Edin--/Lew-- females. The (Han,Edin)Ren2/(Han,Edin)Ren2 males in this study were confirmed to be homozygous for the transgene after the progeny of three litters were tested for the presence of the transgene by Southern blotting of tail DNA. Such homozygous males were maintained on Lisinopril (25 mg/l drinking water) to control BP for the purposes of breeding. After mating the females were removed and not exposed further to ACE inhibitors or other drugs. They were raised on a standard diet (sodium 0.32%) with tap water *ad libitum* to drink and housed in groups of three.

Systolic blood pressure (SBP) was measured by tail cuff plethysmography under light halothane anaesthesia in all 73 rats at age 52 days and in a sub-group of 28 rats, SBP was measured at 35 days, 42 days and 52 days. All animals were examined for development of features suggestive of MH and diagnosis was confirmed post-mortem by histological examination of 3 μm sections of kidney stained with H&E and mounted. Surviving animals were sacrificed at age 130 days, and renal histological examination performed. X^2 analysis was used to compare the incidence of MH in the male F1 progeny with HanRen2/Edin-- as well as comparing expected penetrance in the progeny on the assumption of a one, two or three gene model. A p value of <0.05 was taken to indicate statistical significance.

Fig 4.4.1

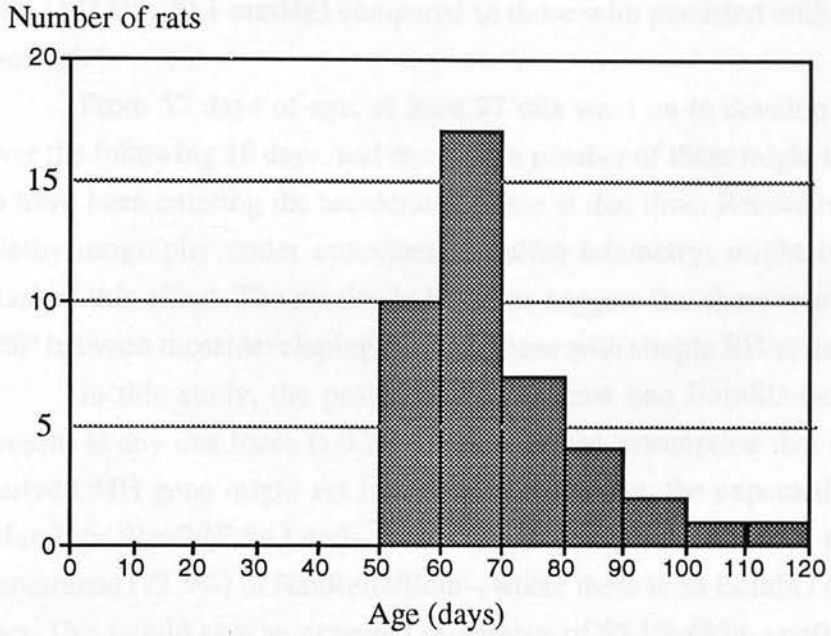


Fig 4.4.1 shows a frequency distribution for the age at death of 43 (Han,Edin)Ren2/(Edin,Lew) -- males raised on a normal sodium diet. Median age was 66 days (range 53 to 117 days). Seventy-three rats were studied.

Forty-three (41 by 100 days of age) of the original seventy-three rats subsequently developed the terminal clinical features suggestive of MH, and on renal histological examination the presence of fibrinoid necrosis was apparent in forty-two of these animals (Table 4.4.1). Those animals that did not develop clinical features of MH and survived to the end of the study period were deemed to have BH. The age range at which rats died from MH is shown in Fig 4.4.1.

The median age was 66 days, range 53 to 117 days which was very comparable to the age range seen for male HanRen2/Edin-- (median 57 days, range 33-103 days). Therefore, the penetrance of the malignant hypertensive phenotype in this cross was taken to be 42/73 or 57.5% (95% confidence limits 45.9 - 69.5%[453]). This was significantly different to the incidence observed in HanRen2/Edin-- 73.5% (95% confidence limits 65.7 - 81.3%) by X^2 analysis ($X^2 = 5.181$, df=1, $p<0.05$).

SBP in those that subsequently went on to develop MH was compared to those with benign hypertension and the results are shown in Fig 4.4.2. SBP was not significantly different in those that developed MH at 52 days of age compared to those

with BH. No significant difference was seen in the 42 day old group (of 28 rats, 13 went on to develop MH, 15 rats had BH). However in the 35 day old group there was a small but significant difference with a higher SBP in those that ultimately developed MH (141.9 ± 20.1 mmHg) compared to those who persisted with BH (124.1 ± 17.8 mmHg).

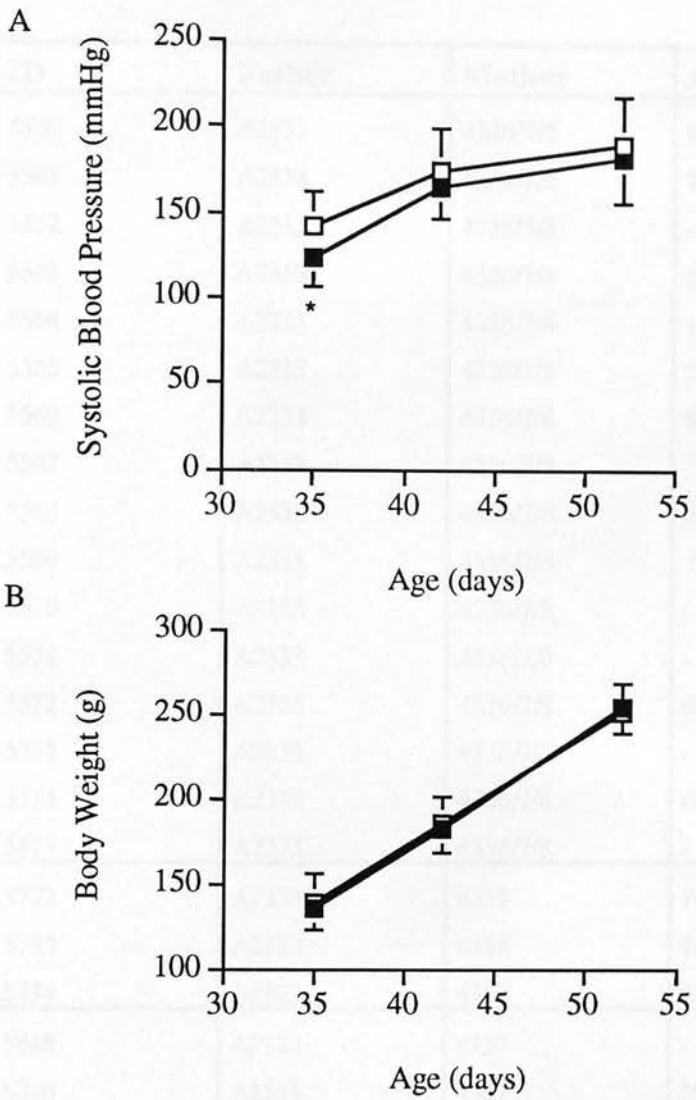
From 52 days of age, at least 27 rats went on to develop and die from MH over the following 18 days, and therefore a number of these might have been expected to have been entering the accelerated phase at that time. Recording SBP by tail cuff plethysmography under anaesthesia, unlike telemetry, might in some way have masked this effect. The results do however suggest that there was little difference in SBP between those developing MH and those with simple BH at that age.

In this study, the probability of at least one EdinSD-derived allele being present at any one locus is 0.75. On making the assumption that a putative EdinSD derived MH gene might act in a dominant fashion, the expected penetrance in the (Han,Edin)Ren2/(Edin,Lew)-- males would be a product of 0.75 times the observed penetrance (73.5%) in HanRen2/Edin-- where there is an EdinSD derived allele at all loci. This would give an expected penetrance of 55.1% (95% confidence limits 49.3 - 61%) which was close to that observed (57.5%). If two genes were involved, the probability of EdinSD derived alleles being present at both loci is 9/16 or 0.562. The expected penetrance would therefore be 41.3% (95% confidence limits 36.9-45.7%).

Two genes could not be excluded with the numbers of rats used in this study. Over the 95% confidence range, X^2 ranged from 2.028 to 6.18 for 1 degree of freedom and therefore p ranged from 0.01 to >0.05. Three genes could be excluded, the expected penetrance being 27/64 or 0.422 of 73.5% which equals 31.0% (95% confidence limits 27.7-34.3%). X^2 ranged from 7.972 to 11.706 giving a p value of <0.01.

In conclusion, these results most strongly supported a monogenic model where the effects of the expression of a single gene acting in conjunction with hypertension leads to transition into MH, but two genes could not be excluded. One potential source of error would be the effects of delayed penetrance. In all groups the majority of affected rats have developed the malignant phenotype by 100 days of age, but in this study two rats developed MH at 103 and 117 days of age and they have been included in the analysis. Secondly some surviving rats (n=8) exhibiting no clinical features suggestive of MH nevertheless had histological signs of myo-intimal proliferation and fibrinoid necrosis at sacrifice. The relevance of this is not clear.

Fig 4.4.2



A shows SBP measured by tail cuff plethysmography in 73 males at 52 days and in 28 of them additionally at 35 and 42 days. □ represents those that ultimately developed MH while ■ represents those with benign hypertension (BH). Unpaired t -test was used to test for any significant difference.

* p=0.02.

B shows mean body weight (error bars represent SD) □ represents MH while ■ represents those with benign hypertension. No significant difference was found.

The penetrance in the HanRen2/Edin-- cross was 73.5%. This therefore raises the question as to why the remainder did not develop MH. Certainly it appeared that some litters of HanRen2/Edin-- tended towards almost 100% penetrance while others had a lower penetrance with the overall incidence of MH being taken to be 73.5% with described confidence limits. The SD rat is an out-bred strain and thus it would be wrong to assume that all rats might carry the responsible gene. This might help to explain the lower penetrance in the HanRen2/Han-- which is also a SD strain together with the finding of MH in other SD strains crossed with transgenic *Ren*-2 homozygotes [452]. In the (Han,Edin)Ren2/(Edin,Lew)-- cross it was important to note that litters derived from all three transgenic homozygotes also gave varying levels of penetrance of the malignant phenotype (see Table 4.4.1).

Table 4.4.1

ID	Father	Mother	Age	Histology
5560	A2333	4336/7/8	66	1
5561	A2333	4336/7/8	73	1
5562	A2333	4336/7/8	-	3
5563	A2333	4336/7/8	62	1
5564	A2333	4336/7/8	117	1+3
5565	A2333	4336/7/8	59	1
5566	A2333	4336/7/8	80	1
5567	A2333	4336/7/8	58	1
5568	A2333	4336/7/8	62	1
5569	A2333	4336/7/8	70	1
5570	A2333	4336/7/8	-	4
5571	A2333	4336/7/8	-	3
5572	A2333	4336/7/8	64	1
5573	A2333	4336/7/8	-	1*
5574	A2333	4336/7/8	66	1
5575	A2333	4336/7/8	-	3
5732	A2333	4338	70	1
5733	A2333	4338	103	1
5734	A2333	4338	53	1
5848	A2333	4337	-	1*
5850	A2333	4337	59	1
5851	A2333	4337	59	1
5852	A2333	4337	-	1*
5853	A2333	4337	55	3†
5854	A2333	4337	-	1*
5855	A2333	4337	62	1
5856	A2333	4337	-	1*
5857	A2333	4337	57	1
5858	A2333	4336	-	2
5859	A2333	4336	68	N/A
5860	A2333	4336	73	1
5861	A2333	4336	-	1*
5862	A2333	4336	-	3

5863	A2333	4639	86	1
5864	A2333	4639	-	2
5865	A2333	4639	-	3
5866	A2333	4639	81	1
5867	A2333	4639	-	4
5868	A2333	4639	-	2
5869	A2333	4639	-	4
5870	A2333	4639	-	2
5576	C2337	4333/4/5	62	N/A
5577	C2337	4333/4/5	61	1
5578	C2337	4333/4/5	66	1
5579	C2337	4333/4/5	82	1
5580	C2337	4333/4/5	67	1
5581	C2337	4333/4/5	70	1
5582	C2337	4333/4/5	68	1
5583	C2337	4333/4/5	70	1
5584	C2337	4333/4/5	-	2/3
5585	C2337	4333/4/5	-	3
5871	C2337	4335	-	2
5872	C2337	4335	56	N/A
5873	C2337	4335	-	1*
5874	C2337	4335	-	3
5875	C2337	4335	92	1
6136	C2337	4334	65	1
6137	C2337	4334	-	1*
6138	C2337	4334	56	1
6139	C2337	4334	-	3
6140	C2337	4334	60	1
6141	C2337	4334	-	2
6142	C2337	4334	-	3
6143	C2337	4333	-	2
6144	C2337	4333	-	1*
6145	C2337	4333	-	3
6146	C2337	4333	91	1
6426	C2339	4333	67	1
6427	C2339	4333	72	1

6428	C2339	4333	67	1
6429	C2339	4333	58	N/A
6430	C2339	4333	73	1
6431	C2339	4333	65	N/A
n=73			**n=42 +1†**	

Table 4.4.1 shows litters born from 3 males A2333, C2337 and C2339 and 8 females 4333-4339, and 4639. Rats (5560-5575) were derived from three litters by A2333 out of the three females (4336, 4337, 4338) and it was not possible to separate the litters. Similarly 5576-5585 were derived from three litters by C2337 out of females (4333, 4334, 4335). Otherwise all litters were maintained separately until ear tagged and weaned and thereafter adults were kept in cages of three. The age at death is shown. Histological grading of kidney sections was performed, while blind to the clinical status of the rat, and is shown as 1. severe, acute fibrinoid necrosis; 2. sub-acute fibrinoid change; 3. chronic hypertensive vascular wall thickening and secondary ischaemic change; 4. no abnormalities detected. *indicates where there was severe acute fibrinoid necrosis, but no clinical evidence of MH. †indicates one animal who died but did not have histological evidence of fibrinoid necrosis or other pathological features of MH on the kidney section studied. The proportion of progeny who derived from A2333 developing MH was (22+1†)/41; from C2337 14/26 and C2339 6/6.

4.5 Preliminary mapping studies of the MH gene.

If it were possible to identify a genetic marker that was in linkage disequilibrium with the phenotype of MH, then by reverse genetics it might be possible to identify the gene responsible, its function and hence the pathophysiology behind the development of MH. Having established an apparent genetic predisposition to MH, it was decided to perform a preliminary study using the above F2 hybrids (Han,Edin)Ren2/(Edin,Lew)-- to determine the feasibility of mapping a putative MH gene or genes. Primers flanking polymorphic microsatellites (mainly CA repeat sequences) were obtained from Dr H Jacob (MapPairs™ (Rat), Cardiovascular Research Centre, Massachusetts General Hospital, Massachusetts, USA) and following PCR and gel electrophoresis (8% sequencing gel) of the PCR products, the F2 hybrids were genotyped.

At the time of sacrifice, either on exhibiting clinical features of MH or at the end of the study period as described above, the spleen was dissected out, snap frozen in liquid nitrogen and stored at -70°C for later DNA extraction. For those rats who were found dead, a small sample of tail was taken post-mortem to obtain DNA. Tail

DNA was also taken from an additional five HanSD, five EdinSD, five Lewis rats and from male A2333 and females 4666, 4667, 4668 and 4639.

This preliminary study was restricted to the 41 male progeny derived from A2333. Informative primers were identified as being able to differentiate Lewis, Edin SD and HanSD alleles. Out of 150 loci tested, 30 primer pairs were found to be informative distinguishing three alleles L, E and H respectively and were therefore used to genotype the F2 hybrids derived from A2333. Examples of results obtained are illustrated in Tables 4.5.1 and 4.5.2 (Yuri Kotelevtsev - unpublished results).

Table 4.5.1

Rat ID	**Phenotype**	**Genotype (A)**			**Genotype (B)**		
Lewis	-	1		3	1 1		
A2333			2	3	1	2	
4336	-	1	2		1		3
4337	-	1	2		1		3
4338	-	1	2		1		3
4639	-	1	2		1		3
5560	M	1		3	1	2	
5561	M		2	3	1 1		
5562	-	1		3	1		3
5563	M		2	3	1	2	
5565	M		2	3		2	3
5566	M		2	3	1		3
5567	M	1		3		2	3
5568	M		2	3	1 1		
5569	M		2 2		1 1		
5570	-	1		3		2	3
5571	-		2 2		1	2	
5572	M	1	2		1 1		
5573	-	1		3	1		3
5574	M		2 2			2	3
5575	-	1	2		1		3
5732	M	1		3	1 1		
5733	M	1		3		2	3
5734	-		2	3		2	3

5848	-	-	-	-		2	3
5850	M	1	2		1 1		
5851	M	1		3	1 1		
5852	-	1		3	1	2	
5853	M		2 2		1	2	
5854	-		2 2		1	2	
5855	M	1		3	1		3
5856	-		2	3	1		3
5857	M	1	2		1		3
5858	-		2	3		2	3
5859	M		2 2		1	2	
5860	M	1		3	1 1		
5861	-	1		3	1		3
5862	-	1	2		1 1		
5863	M	1	2		1 1		
5864	-	1		3	1 1		
5865	-		2 2		1		3
5866	M				1	2	
5867	-	1	2			2	3
5868	-	1	2		1	2	
5870	-		2	3	1 1		

Table 4.5.1 illustrates the results obtained from two informative primer pairs A and B. In each case three alleles have been identified (1, 2 or 3).

Table 4.5.2

Primer Pair	**Genotype**	**Phenotype M/-**
A	1/2	5 M, 4 -
A	2/3	5 M, 4 -
A	2/2	4 M, 3 -
A	1/3	7 M, 6-
B	**1/1**	**9 M, 3 -**
B	1/2	4 M, 4 -
B	2/3	4 M, 5 -

Table 4.5.2 illustrates the analysis of results. Using primer pair A there was random inheritance of the determined genotypes with either the phenotype M of malignant hypertension or - indicating benign hypertension. With primer pair B, there was a deviation from random inheritance with an excess of the alleles 1/1 in the group with MH, but this did not reach a statistically significant LOD score.

It was shown that there were not sufficient animals in this study to obtain statistically significant evidence of linkage disequilibrium, but that further work utilising this technique might potentially demonstrate linkage of a marker with the phenotype. The relatively small number of informative markers available at the time of the study reduced the likelihood of a positive result, but further work will undoubtedly benefit from the increased number of genetic markers available.

4.6 Conclusions

The study described in this chapter has lent further support to the earlier results suggesting that the development of MH in transgenic *Ren*-2 hybrids is in part genetically determined. Previously it was noted that a higher MBP in telemetered HanRen2/Edin-- heterozygotes from 52 - 60 days of age was present relative to the other two heterozygote crosses (Fig 3.3.1), but in this study of (Han,Edin)Ren2/(Edin,Lew)-- hybrids, SBP both at 42 and 52 days of age did not show any clear difference between those destined to develop MH and those who persisted with BH. The method used for measurement of BP may be relevant. Measuring SBP by tail cuff plethysmography under halothane anaesthesia may mask some early changes in BP which are related to the development of MH. There is a suggestion from the results shown in Fig 3.3.1 that HanRen2/Edin-- do have a higher MBP at a younger age and with the results shown here of a significantly higher SBP at 35 days of age in (Han,Edin)Ren2/(Edin,Lew)-- hybrids who developed MH, elevated BP at a younger age may in some way be a marker of a predisposition to develop MH.

The incidence of the MH phenotype in the (Han,Edin)Ren2/(Edin,Lew)-- F2 cross most strongly supports a monogenic model, though with the limitation of the number of rats used two genes could not be excluded. It is possible that a genetic marker might ultimately be found which shows linkage disequilibrium with the trait of malignant hypertension. It would therefore be interesting in the event of such a marker

being found, through reverse genetics, to identify the functional effects. One might hypothesize that altered function or permeability of the endothelium or an altered response of some other component of the arterial wall might be responsible.

CHAPTER 5

The role of endothelin in the transition from benign to malignant phase hypertension

5.1 Introduction

The transition from benign to malignant phase hypertension involves a pressure induced natriuresis and diuresis, an accelerating rise in BP, myointimal proliferation and fibrinoid necrosis particularly affecting afferent arterioles and interlobular arteries in the kidney[306]. In addition as discussed in Chapter 1, there is evidence that activation of the renal renin-angiotensin system (RAS) occurs, which may in turn contribute to the vicious circle of increasing BP and vascular damage[341-343,358]. However the factors which initiate the transition from benign to malignant phase hypertension remain to be identified, together with their relationship with the onset and progression of the resulting pathological damage. Male HanRen2/Edin-- rats derived from crossing homozygote TGR(mREN2)27[267] with EdinSD have been shown to have an increased predisposition to MH, while HanRen2/Lew-- derived from crossing with the Lewis strain developed only benign hypertension (BH). Studies described in Chapter 4 have suggested, on the basis of a genetic cross designed to segregate EdinSD derived genes, that a genetically determined factor acting in addition to but independent of BP, was important in initiating this transition. As discussed in section 1.9, a candidate that might be involved in the endothelial response to hypertension and hence might be a determining factor in the initiation of MH, is endothelin. It was decided to use this transgenic rat model of MH to investigate such a role in the pre-malignant phase and in established MH.

It was decided to look firstly for changes in renal expression of ET -1, -2, and -3 mRNA levels in rats with MH compared to those with benign hypertension or normotension using appropriate transgenic heterozygote crosses and EdinSD controls. To determine whether there was any pathological role for ET, the effects of chronic administration of the non-specific ET receptor antagonist Bosentan (4 -*tert*-butyl - N - (6-(2-hydroxy-ethoxy) - 5 - (2-methoxy-phenoxy) - 2, 2' - bipyrimidin-4-yl) - benzene sulfonamide)[454] on HanRen2/Edin-- was studied. The methods used are described earlier in section 2.5.

5.2 Results

Four groups of 8 to 10 week old rats were studied: (1) HanRen2/Edin-- with malignant phase hypertension (n=11) where malignant hypertension was identified on the basis of the previously described appearances of weight loss, apathy, fitting and confirmed by histopathological examination, (2) age-matched HanRen2/Edin-- with benign hypertension but no signs of malignant phase (n=6), (3) HanRen2/Lew-- with benign hypertension (n=4), and (4) normotensive EdinSD (n=4).

On total RNA extracted from kidney tissue as described earlier, RNase protection assays for ET-1, ET-2 and ET-3 mRNA were performed using human α-globin mRNA as a control for processing and gel loading.

Fig 5.2.1

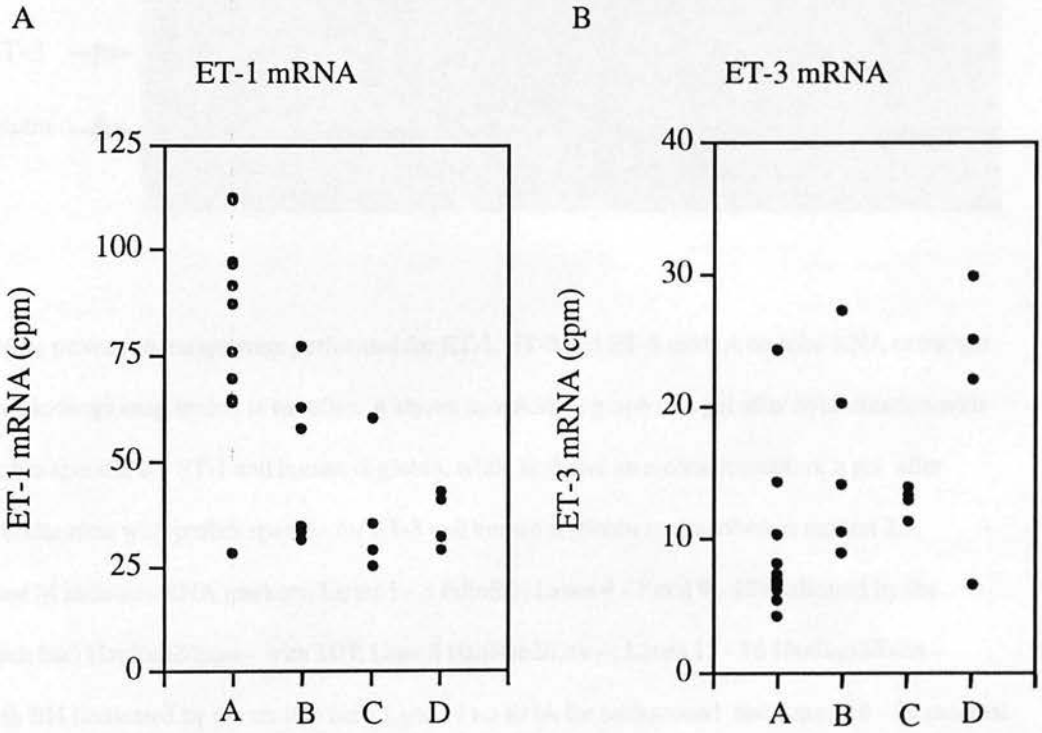
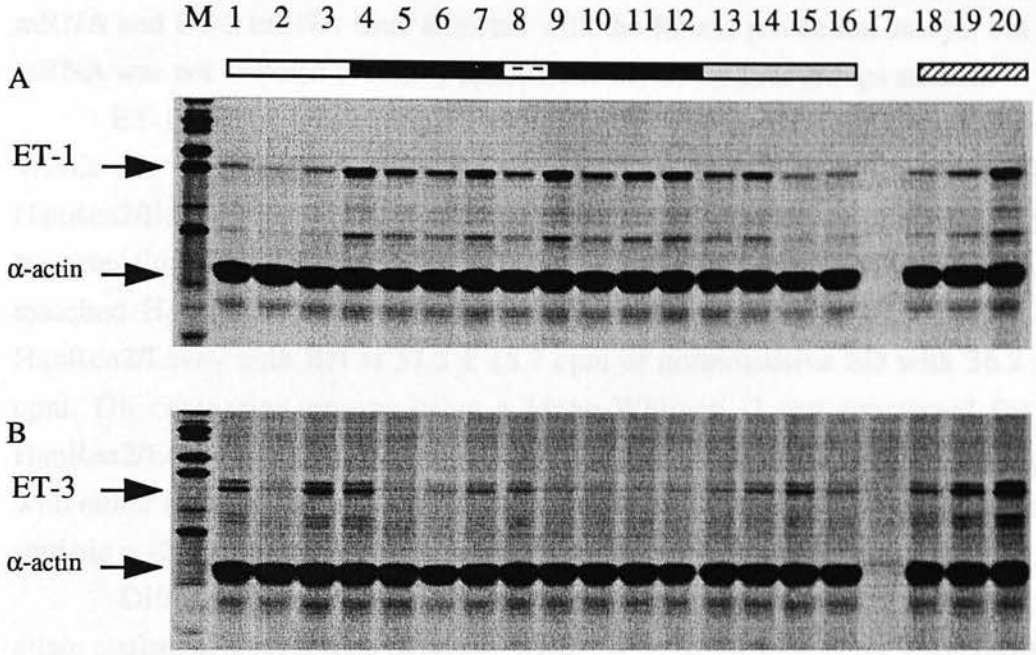


Fig 5.2.1 is a scatter plot of cpm quantifying ET-1 (A) and ET-3 (B) mRNA for individual rats. Four age-matched groups were studied; A - HanRen2/Edin-- with malignant phase hypertension; B - HanRen2/Edin-- with benign hypertension; C - HanRen2/Lew-- with benign hypertension and D-EdinSD.

Fig 5.2.2



RNase protection assays were performed for ET-1, ET-2 and ET-3 mRNA on total RNA extracted from kidneys snap frozen at sacrifice. A shows an autoradiograph of a gel after hybridization with probes specific for ET-1 and human α-globin, while B shows an autoradiograph of a gel after hybridization with probes specific for ET-3 and human α-globin as described in section 2.5. Lane M indicates RNA markers; Lanes 1 - 3 EdinSD; Lanes 4 - 7 and 9 - 12 (indicated by the black bar) HanRen2/Edin-- with MH; Lane 8 HanRen2/Lew--; Lanes 13 - 16 HanRen2/Edin-- with BH (indicated by the shaded bar); Lane 17 no RNA for background and Lanes 18 - 20 external standards using pooled RNA from SD rats.

After excision of the protected fragments, the results were expressed as counts per minute (cpm) after correction for efficiency of processing, gel loading and background activity and results from individual animals are shown in Fig 5.2.1 A and B. Example autoradiographs are shown in Fig 5.2.2 A and B respectively. Both ET-1 mRNA and ET-3 mRNA were detected with the RNase protection assays, but ET-2 mRNA was not detected in kidney tissue from any of the four groups studied.

ET-1 mRNA levels differed significantly between the four groups (Kruskal-Wallis one way ANOVA corrected for ties; $X^2 = 11.757$, df = 3, p = 0.008). For HanRen2/Edin-- with clinical signs and histopathological evidence of malignant phase hypertension (MH), the mean (± SD) was 81.8 ± 24.7 cpm, compared with age-matched HanRen2/Edin-- with benign hypertension (BH) at 49.5 ± 19.1 cpm, HanRen2/Lew-- with BH at 37.2 ± 15.7 cpm or normotensive SD with 36.2 ± 6.8 cpm. On comparing groups using a Mann-Whitney U test (corrected for ties) HanRen2/Edin-- with MH had significantly higher levels of ET-1 mRNA compared with either HanRen2/Edin-- with BH (Z statistic = -2.3, p = 0.02), HanRen2/Lew (Z statistic = -2.49, p = 0.013) or Edin SD (Z statistic = -2.3, p = 0.019).

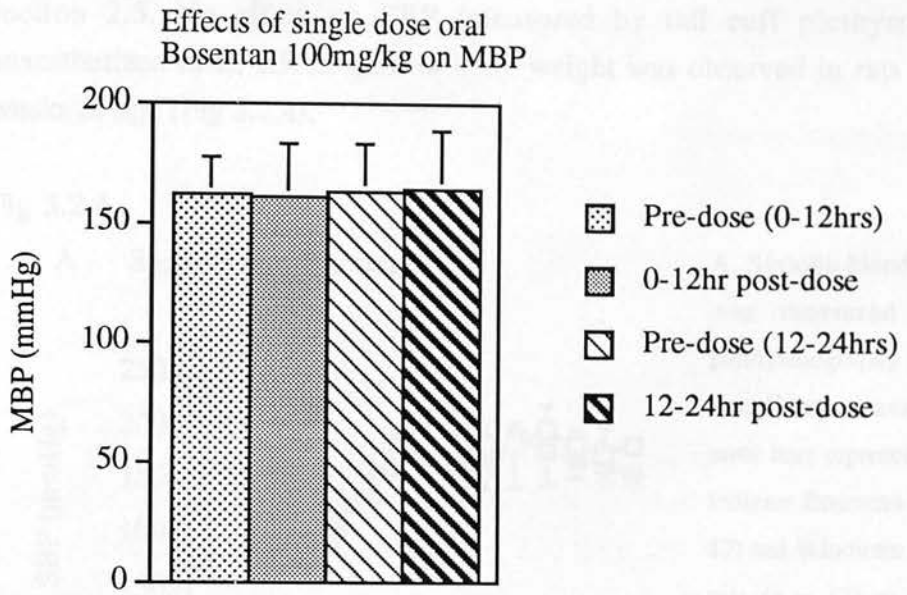
Differences in ET-3 levels detected between the four groups did not quite attain statistical significance (Kruskal-Wallis, $X^2 = 7.810$, df = 3, p= 0.0501), though lower levels were observed to occur in MH kidney (9.15 ± 5.7 cpm) compared with the control groups; HanRen2/Edin-- with BH (16.2 ± 7.5 cpm), HanRen2/Lew-- with BH (12.9 ± 1.1 cpm), or normotensive EdinSD (20.9 ± 10.0 cpm).

It therefore appeared that increased renal ET-1 mRNA levels were present on transition to the malignant phase. In order to determine whether this increase in renal ET-1 mRNA had an important role in the initiation of, or the transition to MH, it was decided to administer a non-specific endothelin antagonist, Bosentan to the susceptible group, HanRen2/Edin--. It was important to exclude any anti-hypertensive effect of the drug which could have also reduced the incidence of MH.

Average MBP, recorded over equivalent 12 hour periods to correct for the effects of diurnal variation, were compared before and after administration of Bosentan by gavage to transgenic *Ren*-2 rats on telemetry. No significant effect on MBP, either within the first 12 hours or from 12 to 24 hours after a single dose was administered, was observed (Fig 5.2.3 A). A repeat dose given one week later to four of the rats also had no effect on MBP.

Fig 5.2.3

A



B

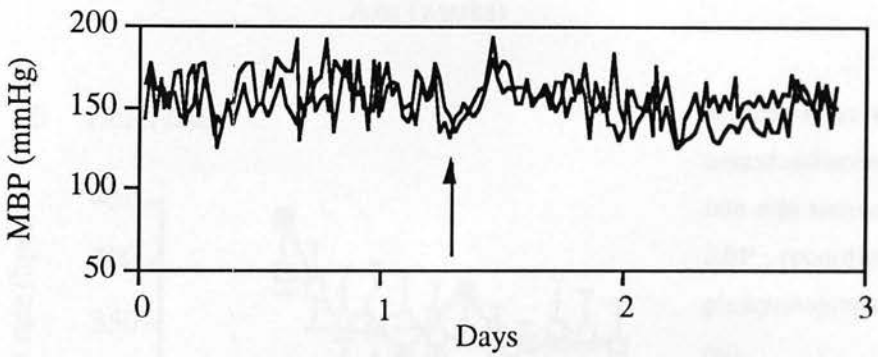


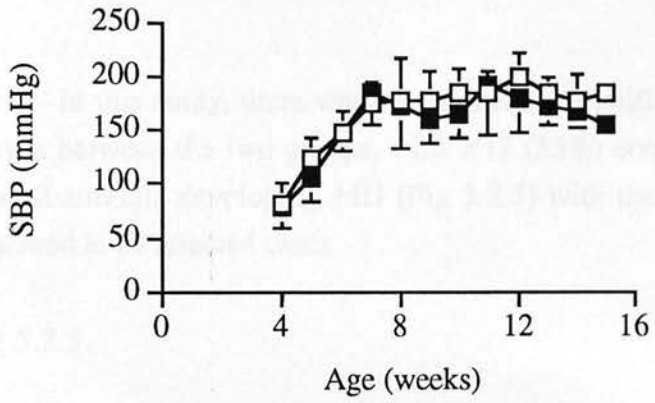
Fig 5.2.3 A shows MBP (mean ± SD) pre-dose and post-dose comparing equivalent periods of the day for male rats (n=11) given Bosentan 100 mg/kg by gavage. MBP was recorded by telemetry in conscious unrestrained rats as previously described. Two example traces are shown in B where recording over a 72 hour period shows no significant hypotensive effect of the non-specific ET antagonist administered at the arrowed time point.

Male HanRen2/ Edin-- rats were therefore housed in groups of three or four and fed a normal sodium diet from weaning at 25 days and randomly assigned to either a treatment group (n = 17), given the non-specific endothelin receptor antagonist Bosentan or a control group (n = 17) given the same diet without Bosentan added. MH was defined as present on development of clear signs of the syndrome, together

with light microscopic examination of renal tissue confirming the presence of fibrinoid necrosis and myointimal proliferation. SBP, heart rates (HR) and body weights were regularly recorded in both groups through-out the treatment period as described in section 2.5. No effect on SBP (measured by tail cuff plethysmography in anaesthetized rats), HR or gain in body weight was observed in rats up to fifteen weeks of age (Fig 5.2.4).

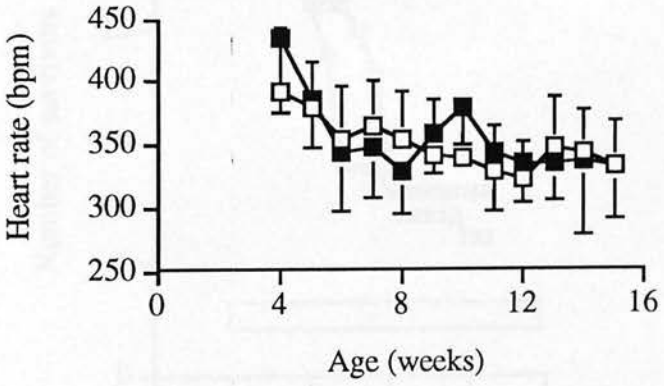
Fig 5.2.4

A Systolic blood pressure

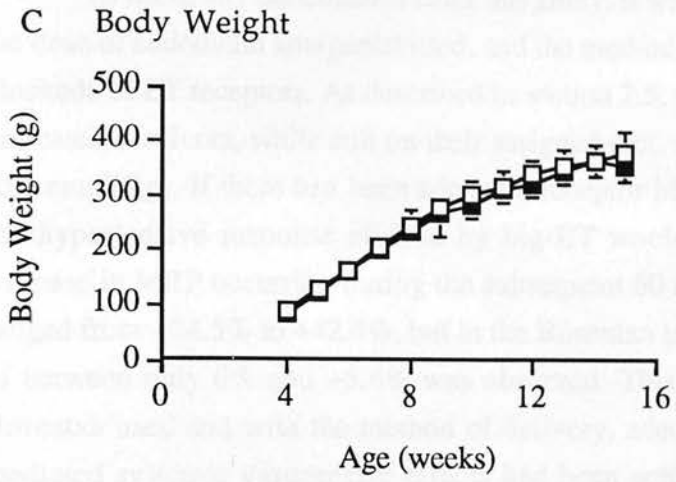


A. Systolic blood pressure (SBP) was measured by tail cuff plethysmography in anaesthetized rats. Group means are shown with error bars representing one SD. □ indicate Bosentan treated rats (n = 17) and ■ indicate control untreated rats (n = 17) at the start of the study.

B Heart rate



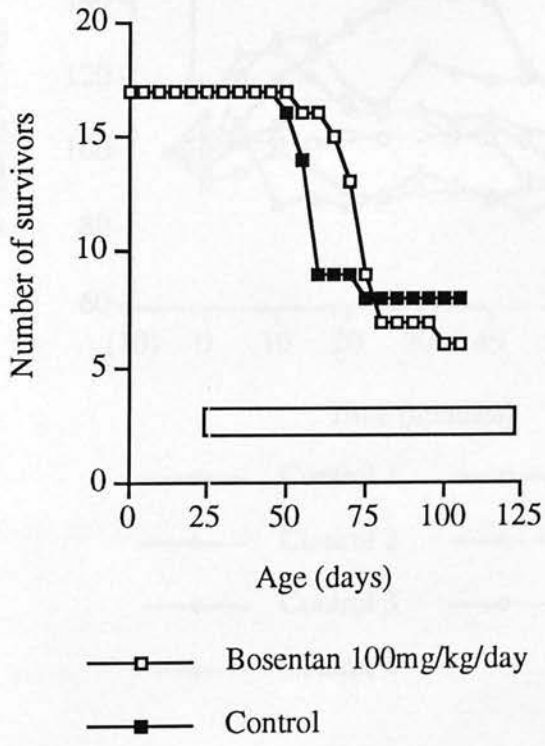
B shows heart rate (mean ± SD) for treated and untreated controls. Heart rate was measured at the time of SBP recording by tail cuff plethysmography in anaesthetized rats.



C shows body weight (mean ± SD). Both treated and untreated groups received the same powdered rat diet. Bosentan was mixed into the diet in the treated group. There was no evidence of a difference in food intake between the two groups

In this study, there was no statistically significant difference in the survival curves between the two groups, with 9/17 (53%) control animals and 11/17 (65%) treated animals developing MH (Fig 5.2.5) with the pathological features of MH observed in all affected cases.

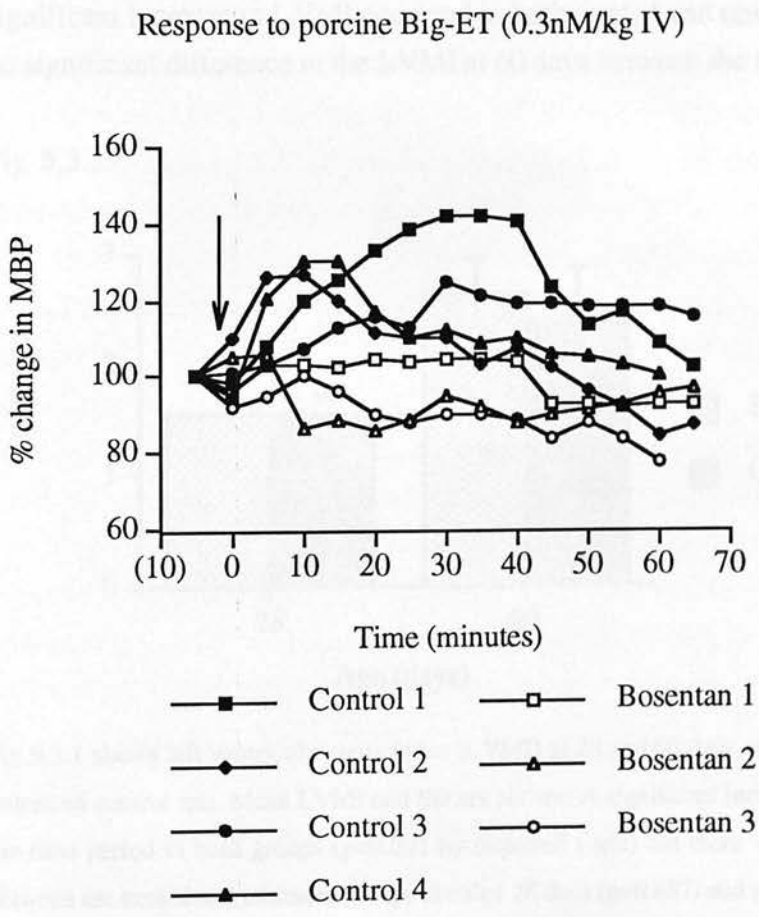
Fig 5.2.5



Survival curve compares Bosentan treated □ and untreated ■ control rats, with the period of treatment indicated by the bar. Necropsy was performed after death and MH confirmed on histopathological examination of kidney tissue.

To make any conclusions from this study, it was important to verify that, both the dose of endothelin antagonist used, and the method of administration had achieved blockade of ET receptors. As described in section 2.5, three treated survivors and four untreated survivors, while still on their assigned diet, were given intravenous big-ET (0.3 nmol/Kg). If there had been adequate receptor blockade, it was anticipated that the hypertensive response elicited by big-ET would be abolished. The maximal increase in MBP occurring during the subsequent 60 minute period in untreated rats ranged from +24.5% to +42.4%, but in the Bosentan treated rats, an increase in MBP of between only 0% and +5.6% was observed. This indicated that, at the dose of Bosentan used and with the method of delivery, adequate blockade of ET-receptor mediated systemic vasopressor effects had been achieved (Fig 5.2.6). In addition, there was no evidence to suggest that tachyphylaxis had occurred during chronic treatment leading to inadequate blockade of the ET receptors.

Fig 5.2.6



Response to porcine big-ET (0.3 nmol/kg IV). Percent change in MBP is shown, with the pre-dosage MBP standardized to 100. MBP was recorded in anaesthetized rats by continuous direct intra-arterial monitoring. Measurements are shown at 5 minute intervals for individual animals; Bosentan treated as open symbols and untreated controls as closed symbols.

5.3 Effects of Endothelin receptor blockade on development of left ventricular hypertrophy

The effects of ET-1 have been shown to be both pro-mitogenic as well as hypertensive as discussed previously[370]. It was therefore of interest to ascertain whether the development of LVH in hypertensive transgenic rats could be diminished by the chronic administration of the endothelin antagonist Bosentan. In the course of the above experiment, male HanRen2/Edin-- rats underwent echocardiographic determination of the left ventricular mass index (LVMI) at 25 days of age, immediately before randomisation to treatment with Bosentan (n=13) or control diet (n=14). This was repeated in healthy, surviving animals at 60 days of age (treated n=12, untreated n=9). Echocardiography was performed as previously described in section 2.3 (II). Three measurements of the left ventricular posterior wall thickness, end-diastolic diameter and interventricular septal thickness were obtained and the means used to determine the LV mass index (LVMI). The results are shown in Fig 5.3.1. Though a significant increase in LVMI occurred in both treated and untreated groups, there was no significant difference in the LVMI at 60 days between the two groups.

Fig 5.3.1

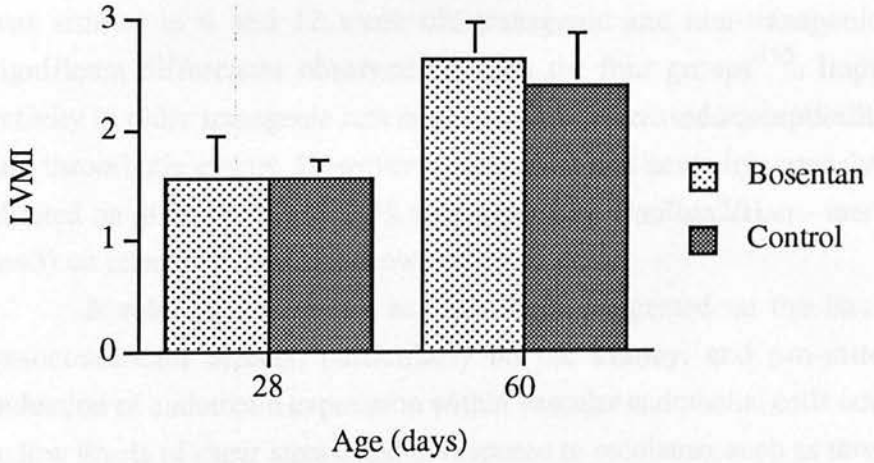


Fig 5.3.1 shows left ventricular mass index (LVMI) at 28 and 60 days of age in Bosentan treated and untreated control rats. Mean LVMI and SD are shown. A significant increase in LVMI occurred over the time period in both groups ($p<0.001$ by unpaired t test) but there was no significant difference between the treated and untreated groups at either 28 days ($p=0.887$) and at 60 days ($p=0.167$).

In conclusion, though ET has been demonstrated to exhibit pro-mitogenic effects *in vitro*, in this *in vivo* situation there was no attenuation in development of

LVH in response to hypertension in the face of chronic endothelin receptor blockade. Because some rats developed MH in both groups before the second echocardiographic determination of LVMI, it is possible that this might have affected the group comparisons but it seems unlikely that the findings would have been significantly altered.

5.4 Discussion

The identification of a genetic susceptibility to malignant phase hypertension in transgenic *Ren*-2 rats led to the question as to what factor(s) additional to high blood pressure might be responsible for initiating the transition to malignant phase hypertension. A few studies have suggested that transgenic *Ren*-2 rats may have altered endothelial function. It has been reported that an *in vitro* ring segment preparation taken from left anterior descending coronary arteries of 12 week old HanRen2/Han-- showed almost no contractile response to the NO synthase inhibitor L-arginine N-nitro-L-arginine methyl ester (L-NAME) compared to the response seen in 6 week old transgenic rats or in 12 week old HanSD and this suggested that there was an attenuated basal release of NO in older transgenic rats. However the endothelial-dependent relaxation response to the nitrovasodilator 3-morpholino-sydnonimine (linsidomine) and the vasoconstrictor response to potassium chloride was similar in 6 and 12 week old transgenic and non-transgenic rats, with no significant differences observed between the four groups[455]. Impaired basal NO activity in older transgenic rats might result in increased susceptibility to vasospasm and thrombotic events. However a significant and acute hypertensive response was elicited on giving L-NAME (75 mg/kg) to male HanRen2/Han-- rats aged 15 weeks (n=3) on telemetry (data not shown).

A role for endothelin has also been suggested on the basis of its potent vasoconstrictor effects, particularly on the kidney, and pro-mitogenic effects. Induction of endothelin expression within vascular endothelial cells occurs in response to low levels of shear stress, and in response to mediators such as thrombin, and Ang II[370] which could be involved in the process of MH. Recently it has been reported that the effects of pressure alone, without inducing shear stress, on cultured human umbilical vein endothelial cells resulted in an increase in ET-1 release via a mechanism involving protein kinase C and phospholipase C[456]. Elevated plasma immunoreactive ET-1 levels have been reported in two experimental rat models of malignant hypertension, following deoxycorticosterone acetate (DOCA)-salt administration to

spontaneously hypertensive rats (SHR) and chronic administration of caffeine to two-kidney, one clip rats[420].

The short half-life of plasma endothelin, low circulating plasma levels and lack of evidence for endothelin storage have supported an autocrine or paracrine role for this peptide and this has made interpretation of plasma endothelin levels difficult[374]. In the DOCA-salt hypertensive rat, increased ET-1 gene expression has been reported, together with evidence of increased ET-1 peptide from acid extracts of thoracic aorta and mesenteric vessel wall tissue. However there was no difference in circulating ir-ET-1 levels between DOCA-salt hypertensive rats and their normotensive controls[391,392]. In contrast, the same authors reported finding a statistically significant increase in plasma ir-ET-1 in 16 week old SHR compared to age-matched normotensive WKY, but a significantly lower total ir-ET-1 content was found in acid extracts of SHR thoracic aorta segments and a small, but statistically insignificant, reduction was found in mesenteric vessel wall compared to those found in WKY[391]. Measurements of tissue endothelin mRNA levels were therefore felt to be a better reflection of the activity of the ET system.

In this study the kidney was specifically studied in view of its known involvement in the pathogenesis of MH. RNase protection assays were used which could clearly differentiate between ET-1, ET-2 and ET-3 mRNA. The significant finding was of an increase in ET-1 mRNA expression in kidneys taken from rats with MH. Interestingly there was a tendency towards a reciprocal relationship between expression of ET-1 and ET-3 which has previously been observed[414], with lowest levels of ET-3 occurring in MH kidneys. However the differences in ET-3 mRNA between groups did not reach statistical significance. ET-2 was not detected in rat kidney, as previously reported[414]. The results here have shown that increased renal expression of ET-1 mRNA is associated with the development of MH in concordance with the findings in other rat models of MH. It was important however to identify an active role for ET in MH, as an increase in ET-1 may simply have been an epiphenomenon reflecting endothelial cell damage.

To examine this possibility, the effect of pharmacological manipulation of the ET system on the development of MH was studied. ET-A receptors preferentially bind ET-1 compared to the other isoforms, have a high efficacy and mediate vasoconstrictor effects, though the relative contribution of the ET-A and ET-B receptors to vasoconstriction may depend on the vascular bed studied. ET-B receptors show equal affinity for all three isoforms and have been linked to the formation of nitric oxide and prostacyclin[374]. It may be that changes in the ratio of ET-1 to ET-3, highest in MH, are relevant as they may reflect an imbalance between local vasoconstrictor and

vasodilator effects. In this study, the effects of the non-specific ET antagonist Bosentan, were examined in the MH model using a dose (100 mg/kg/day) which had previously been demonstrated to effectively block both ET-A and ET-B mediated vasoconstrictor effects[454]. It was important to exclude any anti-hypertensive effects of the antagonist which might reduce the risk of transition to MH. As such, with either acute administration of Bosentan to conscious rats, who had BP monitored by telemetry or on chronic administration to rats where BP was assessed weekly by tail cuff plethysmography, there was no anti-hypertensive response to the ET antagonist. This suggested that maintenance of blood pressure in transgenic *Ren*-2 rats was not mediated in the 'benign' hypertensive state by either ET receptor.

It was also verified that at the end of the eleven week period of chronic oral administration of the antagonist, adequate receptor blockade and hence abolition of a systemic hypertensive effect from exogenous administration of porcine big-ET (0.3 nmol/kg) had been achieved. It may still be possible that intra-renal ET receptor antagonism might not be complete, despite abolition of a systemic hypertensive response to exogenous big-ET.

Chronic administration of the ET receptor antagonist did not significantly alter the incidence of MH, basal SBP, HR or gain in body weight. Though untreated HanRen2/Edin-- rats did develop MH slightly earlier than those in the Bosentan treated group, the age at onset for both groups did not differ significantly from the age range (median age 62 days, range 33-113 days) in which a larger group (92/117) of untreated rats developed the syndrome (see Fig 3.2.1).

In summary, this study has clearly demonstrated increased ET-1 mRNA expression in the kidney on transition to MH in the hypertensive transgenic HanRen2/Edin-- cross. In an age-matched group of healthy HanRen2/Edin-- which might be considered as potentially pre-malignant, there was no significant difference in ET-1 mRNA levels relative to the non-susceptible cross HanRen2/Lew-- with equivalent BP. The non-selective ET receptor antagonist, Bosentan did not significantly reduce the incidence of MH and this suggested that, in this model, the pathophysiological effects of endothelin are not involved in either initiating the transition from BH to MH or in the progression of the natural course of MH. Increased renal ET-1 mRNA expression may occur as a result of endothelial cell damage or ischaemia or simply by the effects of exposure to pressure, shear stress, Ang II or thrombin.

It has also been demonstrated in this hypertensive rat model that endothelin receptor blockade does not attenuate the development of LVH. A similar finding was reported by Li et al (1994) who gave Bosentan (100 mg/kg/day) to DOCA-salt rats for

three weeks commencing from the time of surgery. No difference in heart weight/body weight ratio was seen between the treated and untreated groups despite there being a small but significant reduction in SBP in the Bosentan treated DOCA-salt rats. Interestingly Bosentan treatment did reduce the increment in mesenteric artery medial wall thickness and wall to lumen ratio that occurred over the same period[393]. ET peptides have been shown to have pro-mitogenic effects *in vitro* and it remains possible that a small contribution to the development of LVH is mediated by endothelin but as such it was too small to detect in these *in vivo* experimental models over the time scale used. Alternatively ET peptides may not be involved in either a hypertrophic or hyperplastic response to hypertension.

CHAPTER 6

Conclusions

Essential hypertension remains a major cause of morbidity and mortality in the world. In developed nations, it has recently been estimated that 24 - 30 % of all U.S. citizens over 18 years of age are hypertensive, and of them only 45% are adequately treated[1]. Hypertension remains one of the main reasons for initiating life-long medical therapy with resulting cost implications, in order to reduce both target organ damage and associated morbidity and mortality. Despite the fact that an elevated BP has been recognised as a risk factor for a number of cardiovascular, cerebrovascular and renal conditions, a clear understanding of the causes of high BP in the majority of cases with essential hypertension has not been found. In Chapter 1, a review of the evidence was presented suggesting that genetic factors may be important. By using "reverse genetics", identifying abnormal genes has led to improvement in the understanding of the aetiology of hypertension arising from some single gene defects, but the genetic basis of essential hypertension remains to be described. However, it is important to recognise that the finding of linkage between a polymorphism lying close to a candidate gene and hypertension does not imply causality. Secondly the contribution of environmental influences to elevation of BP, particularly those operating *in utero*, may be hard to quantify.

The ability to study the contribution of individual candidate genes to a phenotype by transgenesis has resulted in some interesting models of monogenic hypertension, and in the case of the TGR(mREN2)27 hypertensive rat, this has led to much interest being focused on extra-renal RAS activity and the role of prorenin in the genesis of hypertension.

Having the ability to recognise which hypertensive individuals are at risk of developing clinically significant target organ damage would be a useful adjunct to the management of hypertensive patients. To date recognised cofactors likely to increase the risk of cardiovascular, cerebrovascular or renal complications include cigarette smoking, diabetes mellitus, hyperlipidaemia and a family history of cardiovascular disease. More recently it has become recognised through using techniques such as linkage analysis, that genetic risk factors for target organ damage arising from hypertension exist. Specifically the ACE DD genotype has been shown to be an independent risk factor for LVH, cardiomyopathies and myocardial infarction.

Malignant phase hypertension, arising as a complication of either essential or secondary hypertension, has undoubtedly reduced in incidence over the last 40 years

probably as a result of improved treatment and control of BP. While the classical pathological findings have been previously described in detail, the reasons why a transition from benign to malignant phase hypertension occurs have remained unknown. Animal models have depended on either surgical, pharmacological or dietary manipulation to induce the transition to the malignant phase.

Once in the malignant phase of hypertension, studies have clearly implicated involvement of the RAS. MH is proportionately a more common complication of high renin hypertension such as renal artery stenosis than of low or normal renin hypertension. The frequent finding of elevated plasma renin, Ang II and aldosterone in affected individuals, even when they had a low renin hypertension prior to developing MH, has led to the suggestion that many of the pathological manifestations may be a consequence of high circulating Ang II. This was supported again by the ability to induce many of the features of MH simply by infusing Ang II into rats[350]. The known physiological actions of Ang II and the often dramatic response to ACE inhibitors would support a pivotal role for this peptide in MH. It would be very interesting to ascertain whether the incidence of MH developing in essential hypertensives with poorly controlled BP differs between those receiving an ACE inhibitor and those on other anti-hypertensive therapy such as calcium channel antagonists. Blockade of Ang II receptors at sub-hypotensive doses has been shown to reduce the development of fibrinod necrosis in SHR-SP[354] and it is likely that inhibition of tissue ACE activity even at doses that do not lower systemic BP may be protective.

It is not clear however, how the transition from a state of hypertension and RAS suppression converts to a state of hypertension and RAS activation with sodium retention, vasoconstriction, increasing BP, renal afferent vascular pathology and progressive renal failure. It has been suggested, mainly on circumstantial evidence, that an initial pressure diuresis and natriuresis might be important in leading to a state of relative sodium depletion and hence RAS stimulation. This is supported by the clinical findings of postural hypotension and extra-cellular fluid depletion in some affected patients together with the ability to lower BP in some patients with MH simply by infusing isotonic saline[360].

It was therefore of considerable interest that a heterozygous cross, transgenic for the mouse *Ren*-2 transgene, (HanRen2/Edin--) exhibited a tendency for the spontaneous transition from benign hypertension to MH with many of the classical described features. By utilising telemetry, an accelerated phase rise in BP in the terminal stages was demonstrated. This was associated in many cases with clinical signs of salt/water depletion associated with a significant loss in body weight in 65% of cases. Behavioural changes included adoption of a hunched and apathetic posture,

seizures or agitation and these may have been indicative of a hypertensive encephalopathy. Biochemical evidence of acute renal failure was present. The classical pathological feature of fibrinoid necrosis was invariably present, though within individual arterial sections, the patchy nature of such vascular pathology was observed. A microangiopathic haemolytic anaemia was seen in the malignant phase.

Classically the TGR(mREN2)27 rat line has been thought of as having hypertension with a low circulating plasma renin and reduced renal renin content relative to non-transgenic controls[267]. Evidence reviewed in Chapter 1 would suggest that the elevation of BP is however mediated by Ang II, derived from extra-renal sources with the adrenal and/or vascular wall being implicated. With development of MH, dramatic increases in circulating plasma renin, Ang II and aldosterone were seen. Immunohistochemistry suggested that there was increased renin protein in the kidney of transgenic rats with MH. It is not clear whether the renin is derived from transgene or endogenous gene expression, as the renin antibody used did not differentiate mouse from rat renin. Treatment of heterozygous *Ren*-2 transgenic rats with Lisinopril has been recently shown to increase renin activity in the kidney in a dose-dependent manner. Using RNase protection assays, the authors were able to show that both endogenous renin gene expression and to a lesser extent transgene expression increased in response to ACE inhibition[457]. In addition expression of the transgene in the adrenal gland was also seen to occur in response to ACE inhibition[457]. Further work is required to clarify this, but one might hypothesize that the increase in kidney renin may be both endogenous and transgene derived in origin. In any case unlike rat models with high renal renin hypertension such as 2K-1C rats, MH appears to be developing on a background of renal RAS suppression leading to the expected changes of renal afferent arteriolar pathology and renin synthesis and release. Changes in *Ren*-2 transgene expression in other tissues including adrenal in rats with MH were not looked for in this study, and it remains possible that a contribution to the increase in plasma renin in MH comes from altered transgene expression in extra-renal sites.

The development of the MH syndrome in affected HanRen2/Edin-- animals occurred within a relatively narrow age range (mean age 62 days ±12.7 days for males, 65 ± 15 days for females). Surviving rats thereafter developed pathological evidence of target organ damage with progressive glomerulosclerosis and left ventricular hypertrophy[269], but beyond 120 days of age there was no significant incidence of MH.

Studies were performed to clarify why HanRen2/Edin-- were at increased risk of MH relative to either HanRen2/Han-- and particularly to HanRen2/Lew--. No environmental agent was identified, with small variations in dietary sodium intake

being specifically excluded as a contributory factor in view of the difference in the diets available to transgenic rats originally maintained in Germany. The influence of additional "hypertensinogenic" genes interacting with the physiological effects of the transgene expression appeared unlikely as there was no clear difference in BP between non-transgenic EdinSD and HanSD. Lewis rats being inbred, were both smaller and had a slightly lower BP. However on comparing the transgenic heterozygote crosses, BP in HanRen2/Edin-- did appear to be significantly higher between 52 and 60 days of age. The rate of rise in BP in the susceptible cross was not steeper than the relatively less susceptible or resistant HanRen2/Han-- and HanRen2/Lew crosses, but it remains possible that a higher BP at a younger age is important. This observation was repeated in the later study looking at (Han,Edin)Ren2/(Edin,Lew)-- crosses where a higher BP at 35 days of age was the only significant difference between rats who ultimately developed MH and those that did not.

Early descriptions of MH prompted Volhard and Fahr and later investigators to suggest that the pathological damage might be initiated by vascular spasm leading to endothelial and sub-endothelial ischaemia leading in turn to extravasation of plasma proteins and the deposition of fibrinoid[316,317]. One might hypothesize that younger rats exposed to higher arterial pressures have had a relatively shorter time to allow vascular remodeling and that structural adaptation may be important in protecting the blood vessel wall from an insult that precipitates transition to MH. This is interesting in the light of the observation that relatively younger patients present with MH compared to the age distribution for hypertension in the population[306]. It is clear that absolute BP level was not important, both on the basis of the study (Chapter 3) comparing the three heterozygote crosses and more definitively in the later study (Chapter 4) looking at (Han,Edin)Ren2/(Edin,Lew)-- rats where there was no significant difference in BP between affected and unaffected rats beyond 42 days of age. It would therefore be interesting to attempt to delay the rise in BP in the susceptible HanRen2/Edin-- cross, perhaps by a limited period of anti-hypertensive treatment from 30 to 40 days of age. If this then reduced or abolished the tendency to MH, then this would be a clear pointer towards high BP at a relatively young age being of aetiological importance.

No clear differences were elicited between the three heterozygote crosses when studied in the pre-malignant/benign hypertensive state in terms of base-line renal function or plasma RAS activity. Secondly there was no significant difference in the development of another marker of target organ damage, namely LVH.

The study that specifically looked at rats on telemetry, and their subsequent development of MH compared three heterozygote crosses where all F1 progeny were

derived from the same homozygous HanRen2/HanRen2 father. Transgene copy number and insertion sites would not be altered in the three crosses, but it is theoretically possible that other strain dependent factors might alter levels of transgene expression[262] or function and this was not specifically looked at in the course of these studies.

Kincaid-Smith suggested that "malignant hypertension may be determined by some new and unknown factor in addition to high BP"[306] and reported in a later paper of the considerable overlap that exists in actual BP between those with MH and those with BH[368]. In this thesis, a genetic susceptibility to MH in a hypertensive transgenic rat model has been demonstrated and from the data presented in Chapter 4, this appears to be conferred by one or possibly two loci. The number of rats bred in the analytical cross designed to segregate EdinSD alleles together with the number of available genetic microsatellite markers was not adequate to identify significant linkage. With the availability of increasing numbers of markers, this aspect should be re-addressed.

Another group has reported features suggestive of MH in a colony of *Ren*-2 transgenic rats crossed with SD rats[452]. The SD is not inbred and other groups have also found higher death rates from MH than was observed here amongst HanRen2/Han-- heterozygote crosses (H Montgomery, London - personal communication). The responsible loci may not be limited to EdinSD.

It is therefore possible that genetic factors determine the risk of transition to MH from BH in humans and identification of the locus(ci) might lead to an understanding of the trigger factor. Various postulated hypotheses have included altered vascular reactivity leading to vasospasm and ischaemia. This might not be the primary trigger, but could simply be an effect of it and hence identifying a responsible gene would be a major step forward. Even though the relative clinical importance of MH declines with the decreasing incidence of the condition, it is possible that the aetiology may be relevant to a number of related conditions. Examples include scleroderma renal crisis, haemolytic-uraemic syndrome/thrombotic thrombocytopenic purpura, and acute vascular rejection of renal transplants.

On the basis of the known pathophysiology of MH and the evidence in the literature of the role of endothelins in various pathological conditions (reviewed in Chapter 1), it was decided to ascertain whether endothelins might have a role in MH. They had been implicated by previous investigators because of reported increased levels of plasma ir-ET-1 in animal models of MH[420]. It was recognised that increased ET-1 synthesis occurred in response to hypoxia, low levels of shear stress, thrombin and Ang II. In turn, ET-1 acting as a powerful vasoconstrictor with pro-mitogenic

activity could induce some of the physiological and pathological changes seen in MH. The studies described in Chapter 5 found that despite there being evidence of a significant increase in ET-1 mRNA expression in kidney from rats with MH, no clear role for ET-1 could be identified on the basis of the lack of effect of the combined ET-A and ET-B receptor antagonist in preventing transition from BH to MH.

ET-1 has been implicated in a number of pathological states and in many of these cases a clear role needs to be identified for the peptide. There are examples in the literature of situations where measurements of ET-1 peptide have not shown a good correlation with either vascular wall ET-1 content or ET-1 mRNA levels[391]. The short plasma half-life and the lack of evidence of any storage of ET-1 outwith the posterior pituitary has suggested that this peptide is likely to work in a paracrine or autocrine fashion. In conditions such as myocardial infarction and reperfusion injury, pre-eclampsia or cyclosporin-induced nephrotoxicity where endothelial cell ischaemia and damage are likely, it may well be that the appearance of increased circulating levels of ET may be an epi-phenomenon reflecting cell damage rather than being intrinsic to the pathological process. Other mediators of endothelial function and vascular reactivity could be relevant and it would be of interest for example, to look for differences in basal NO activity and NO synthase gene expression in vascular wall tissue between the the relatively susceptible HanRen2/Edin-- and resistant HanRen2/Lew-- crosses.

The HanRen2/Edin-- cross appears to be good model of malignant or accelerated phase hypertension in that it arises spontaneously exhibiting many of the features seen in the human condition. It therefore offers a valuable model to look for the factors that initiate onset of MH, which may be potentially relevant to other conditions with a similar pathology. The work presented has suggested that in this case, there may be a genetic factor operating in addition to high BP which confers an increased susceptibility to MH.

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Appendix 1.

Publications arising from the work presented in this thesis

1. CE Whitworth, JJ Mullins. The role of transgenesis in investigating the cardiovascular system. *Annual of Cardiac Surgery*. 7th Edition. Editors: M Yacoub, J Pepper; Current Science Ltd, London: 1995 pp1-8

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2. CE Whitworth, S Fleming, AD Cumming, JJ Morton, NTJ Burns, BC Williams, JJ Mullins. Spontaneous development of malignant phase hypertension in transgenic Ren-2 rats. *Kidney International*; 46 (1994) pp1528-1532

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The role of transgenesis in investigating the cardiovascular system

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Introduction

An increasing understanding of the genetic factors and of the molecular mechanisms that contribute to cardiovascular disease has led to new avenues for intervention. As a consequence of finding genetic loci linked to phenotypes and disease patterns, attention is being directed towards the regulation and function of candidate genes. This can be done at a physiological level by monitoring, for example, plasma renin levels or blood pressure, and at a molecular level, by identifying the sites and levels of mRNA expression. These two approaches may be effectively combined with the use of transgenesis to investigate the expression of specific genes and their effects on physiological processes.

Within the limits of this review, we propose to concentrate on three areas of transgenic research:

- Investigations into the renin–angiotensin system (RAS) in hypertension
- Lipids and apolipoproteins in atherogenesis
- Approaches towards understanding cardiomyocyte cell biology and cardiac disease

Generation of transgenics

A transgene may be defined as a gene or gene construct which has been introduced into the germ line. This DNA may originate from the same species or may be constructed from a combination of DNA elements from different species. After being integrated into the germ line it will, under normal circumstances, be inherited in a Mendelian fashion. Transgenic animals may be generated through the 'addition' of transgenes by several methods [1], including the direct microinjection of DNA into the pronuclei of one-cell embryos *in vitro* or by using retroviral vectors in the pre-implantation embryo and their subsequent re-introduction into a foster mother (Fig. 1). By such an approach the effects of specific RNA molecules and/or polypeptides may be addressed in the whole animal. In addition, genetic regulatory elements can be studied using 'reporter' gene constructs which are typically designed to express a heterologous and conveniently assayable enzymatic activity [2,3]. Successive series of such gene constructs can yield valuable information concerning the location and interaction of regulatory sequences controlling, for example, cell-specific or developmentally regulated gene expression. In certain cases such reporters can be assayed colorimetrically or fluorimetrically (e.g., the *Escherichia coli* Lac Z gene coding for β-galactosidase which produces a blue colour on staining with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) providing a variety of experimental possibilities.

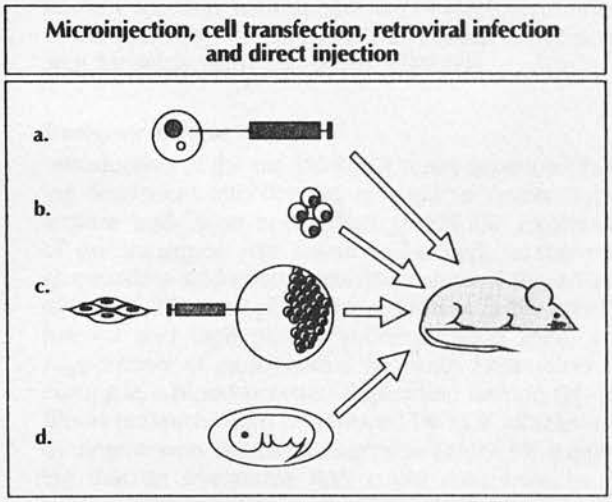

Fig. 1a. Fertilized oocytes from superovulated females. Microinjection of DNA into pronucleus of fertilized oocyte. Returned to pseudopregnant foster mother. **b.** Retroviral infection of 4-cell embryo. **c.** Introduction of DNA constructs into embryonic stem cells in culture results in random integration or homologous recombination. Identification of appropriate transformation is followed by microinjection of cells into a blastocyst and re-implantation into foster mother. **d.** Retroviral infection of embryo.

A second route to the generation of transgenic animals (presently limited to the mouse), involves the use of embryonic stem cells which can be maintained and genetically manipulated *in vitro*, before being used to develop viable animals (Fig. 1). Through the use

Abbreviations

AI—angiotensin I; **AII**—angiotensin II; **ACE**—angiotensin converting enzyme; **apo**—apolipoprotein; **HDL**—high-density lipoprotein; **IDL**—intermediate-density lipoprotein; **LDL**—low-density lipoprotein; **Lp(a)**—lipoprotein (a); **MLC**—myosin light chain; **MT1**—metallothionein promoter; **RAS**—renin–angiotensin system; **SHR**—spontaneously hypertensive rat; **SV40**—simian virus 40; **TGR(hAOGEN)**—transgenic rats carrying human angiotensin gene; **TGR(hREN)**—transgenic rats carrying human renin gene; **VLDL**—very-low-density lipoprotein.

 ISBN 1-859221-43-2 ISSN 0952-0562

of such cells the ablation or modification of a gene is possible. In contrast to the microinjection of single-cell embryos, such an approach provides the ability to target alterations to specific chromosomal sites thereby providing a greater measure of experimental precision. To a large extent, however, the two techniques complement each other and are valuable for addressing different questions. Such methods can be used to verify that a particular gene, implicated with a disease on epidemiological grounds, is actually causative. In cardiovascular medicine, polygenic conditions with environmental influences, for example hypertension and atherosclerosis, contribute largely to the presenting pathology. Transgenesis, using the whole animal as an in-vivo preparation, is a powerful tool for studying the interaction of specific genes with both genetic background and environment.

The RAS in hypertension

Essential hypertension has no defined phenotype but is a quantitative trait. Net blood pressure is a consequence of interactions between an individual's genetic make-up, diet and environment. Early research was based on descriptions of physical and biochemical measurements of the phenotype and of the effects of pharmacological or surgical intervention. Over the past 5 years, molecular studies have opened up the field with animal models of genetic hypertension helping to identify chromosomal loci which might harbour candidate genes [4]. Selective breeding from initially normotensive rat populations has led to the derivation of the spontaneously hypertensive rat (SHR), stroke-prone SHR [5], salt-sensitive Dahl rat, and the Milan hypertensive strain. An inherent disadvantage of these models is that selective breeding causes not only the segregation of genes relevant to the phenotype but also concurrent segregation of the vast majority of irrelevant genes. In addition, molecular divergence of supposed inbred populations complicates the study of genotypic differences between hypertensive and normotensive populations [6–8].

In spite of these caveats, a number of candidate genes have been identified. Linkage studies in crosses between stroke-prone SHRs and normotensive Wistar–Kyoto controls suggested that a locus on rat chromosome 10 close to the angiotensin converting enzyme (ACE) gene may contribute to regulation of blood pressure in salt-loaded stroke-prone [9•,10•] SHRs, but no linkage between human essential hypertension and the human ACE locus has been found [11,12]. A deletion polymorphism of the ACE gene (DD) has been linked with increased risk of myocardial infarction [13•], parental history of myocardial infarction [14], idiopathic and ischaemic dilated cardiomyopathy [15] and hypertrophic cardiomyopathy [16]. Inheritance of a DNA restriction fragment length polymorphism marking the renin gene from SHR, as opposed to the renin gene from normotensive Lewis rats, is associated with a higher blood pressure in F_1 progeny [17]. Similarly, a renin gene restriction fragment length polymorphism from the Dahl salt-sensitive rat cosegregates with higher blood pressure [18]. No overwhelming evidence of linkage of human essential hypertension and renin polymorphisms have been found to date [19,20]. Linkage of a molecular variant of the angiotensinogen gene, associated with increased plasma angiotensinogen levels, has been found in essential hypertensive individuals from two populations [21] and in individuals with pre-eclampsia [22,23]. Such genetic evidence implicating the RAS in both human and animal hypertension, together with the known physiological effects of the RAS (Fig. 2) has made this system an attractive area for study using transgenic methods.

Transgenic models

Introduction of the mouse Ren 2 renin gene and flanking sequences into the rat, resulted in severe hypertension and target-organ damage [24,25]. Expression of the transgene was found to be high in the zona glomerulosa and outer zona fasciculata of the adrenal gland [26,27]. Low plasma and kidney renin concentrations and high plasma prorenin were seen, with suppression of endogenous rat renin expression occurring in a blood-pressure dependent fashion [26–28]. Blood pressure could be reduced by ACE inhibition or by angiotensin II (AII) antagonists [24,29,30] suggesting that an overactive RAS could contribute to the severe hypertension in spite of the apparently low plasma renin concentrations. The role of local tissue and extrarenal RAS activity compared with circulating plasma RAS activity has been an important issue since the efficacy of ACE inhibitors in treating essen-

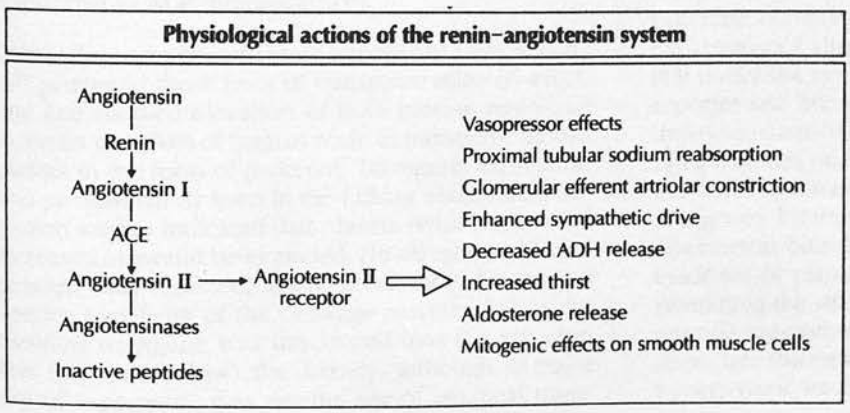

Fig. 2. Physiological actions of the RAS. Genetic polymorphisms have been identified at the angiotensinogen, renin and angiotensin converting enzyme loci. RAS, renin–angiotensin system.

tial hypertensive individuals with low plasma renin concentrations was shown. In this transgenic model, the role of the adrenal gland has been of particular interest because of the high adrenal transgene expression. Adrenalectomy experiments in transgene-positive animals have suggested that the elevated prorenin and hypertension may result from transgene expression in this organ [25], but how this leads to a rise in blood pressure is not clear. There is some evidence of increased adrenal mineralocorticoid production [31,32], but insufficient to account for the rise in blood pressure. Evidence of activation of a vascular RAS in TGR(mREN2)27 came from finding mRNA in vascular tissue [26,33], and from studies of angiotensin I (AI) and AII release from perfused hind-limb preparations where levels were found to be significantly higher in transgenic rats compared with Sprague–Dawley controls [33]. Prior nephrectomy reduced the release of angiotensins from Sprague–Dawley hind limbs but not from TGR(mREN2)27 animals.

A hypertensive phenotype was similarly generated after microinjection of the rat angiotensinogen gene into the mouse germ line [34•]. Three transgenic lines were established with elevated blood pressure, the presence of rat angiotensinogen and increased plasma AII being found in two of the lines. In a third transgenic line (#92) elevated plasma AII levels appeared to be insufficient to cause hypertension. Using a different approach in which the mouse metallothionein promoter (MT1) was fused to the rat angiotensinogen coding region, Ohkubo *et al.* [35•] were able to induce expression of rat angiotensinogen in response to heavy metals, i.e., $ZnSO_4$ treatment. In this study no difference in blood pressure levels was reported; however, plasma levels of angiotensinogen were approximately half those reported above [34•].

Mice carrying a rat renin transgene under the control of MT1 also remained normotensive, in spite of evidence of rat renin production in mouse liver [35•], presumably as a consequence of species differences in substrate specificity as rat renin has been shown not to cleave mouse angiotensinogen *in vitro* [36]. Such biochemical differences can clearly affect the interpretation of transgenic experiments. Hypertension was seen in double transgenic mice carrying both the rat renin and the rat angiotensinogen transgenes, and was responsive to ACE inhibition [35•].

Using a human genomic renin transgene, Takaori *et al.* [37] generated three lines of transgenic mice of which one line showed elevation of both plasma renin and prorenin with 80% of human renin in transgenic mouse plasma in the form of prorenin. Transgene expression was predominantly seen in the kidney and sodium depletion studies indicated that plasma renin levels were increased as would be expected. No elevation of blood pressure was reported, again, presumably because of species specificity of the cleavage reaction. When the identical transgene was introduced into the rat germ line [38] (see below) the kidney, although a major site of expression, was not the site of maximal transgene expression. Some caution must therefore be exercised when interpreting the results of cross-species transgenic experiments.

Transgenic rats carrying both the human renin [TGR(hREN)] and angiotensinogen [TGR(hAOGEN)] genes have been established [38]. In TGR(hREN) plasma, human renin and prorenin were found, although the level of renin was 12-times higher than is normally seen in humans. Parameters of the rat RAS were unaffected by the presence and activity of human transgenes, implying that human renin did not act on rat angiotensinogen *in vivo*. TGR(hAOGEN) animals showed elevated plasma angiotensinogen ranging between two- and 40-fold higher than in normal human plasma with expression of the transgene occurring mainly in liver. As expected, the endogenous rat RAS was unaffected. No animals from either transgenic strain were spontaneously hypertensive, nor were double-transgenic progeny from a preliminary test cross. A hypertensive response was elicited by infusing rat renin or human renin into TGR(hAOGEN); Ro 42-5892, a human renin-specific antagonist, lowered blood pressure specifically in transgenic rats given human renin; while DUP 753, an AII antagonist, lowered blood pressure after infusion of either human or rat renin. Infusion of human renin into transgene-negative animals had no hypertensive effect. In contrast, however, and in a different type of study, Tomita *et al.* [39] observed transient hypertension and evidence of cleavage of rat angiotensinogen after the direct transfer of the human renin gene into rat liver using a Sendai virus (HVJ)-liposome carrier. This elevation in blood pressure was responsive to the human-specific renin inhibitor FK 906.

The vascular smooth cell response to hypertension

The vascular wall response to hypertension, namely smooth muscle cell migration and remodelling, may be secondary to elevated blood pressure or in part a consequence of direct actions of mitogens [40]. The promitogenic effects of AII on smooth muscle cells, independent of vasopressor effects, have implicated the RAS system in pressure overload cardiac hypertrophy, post-myocardial infarction ventricular remodelling and restenosis after balloon angioplasty or endarterectomy [41,42]. Separating the contribution of these two potential components is difficult. Experiments using expression of simian virus 40 (SV40) large T antigen [43] under the control of the Ren 2 promoter as both a reporter and immortalization agent resulted in cellular transformation of renin expressing cells in transgenic mice. Marked proliferation of smooth muscle cells occur within the media of arterial walls resulting in thickening and luminal narrowing. This transgenic model has normal blood pressure, although there was some evidence of plasma volume expansion. In addition to permitting the study of juxta-glomerular-like cells these animals may prove to be a useful model in helping to dissociate the vascular wall hypertrophic responses to hypertension and to RAS perturbation [44,45].

Lipids and atherogenesis

Atherosclerosis, like essential hypertension, is a polygenic disorder with environmental contributions. Familial hypercholesterolaemia can result from defects in the low-density lipoprotein (LDL) receptor gene. The type of defect (deletion or missense mutation) in humans influences both plasma cholesterol and severity of coronary heart disease [46]. Attempts at gene therapy in animal models have included the introduction of functional human LDL receptor genes into Watanabe heritable hyperlipidaemic rabbits, using retrovirus transduced hepatocytes which resulted in a transient reduction in serum cholesterol [47]. Similarly, adenovirus-mediated gene transfer resulted in transient hepatic LDL receptor expression in normal mice lowering plasma cholesterol and the half-life of labelled LDL [48]. Overexpression of the human LDL receptor in transgenic mice, using 3 kb of the mouse transferrin promoter linked to a human LDL receptor minigene, resulted in either a 50% reduction in total plasma cholesterol (normal diet), or in an attenuated elevation of cholesterol levels (high-fat diet) compared with transgene-negative littermates. This was mainly achieved by reducing the rise in intermediate-density lipoprotein (IDL) and LDL fractions [49].

Apolipoprotein (apo)E is a high affinity ligand for the LDL receptor and is found on chylomicrons, IDL, very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) [50]. Three common structural alleles have been identified. ApoE-2, because of an amino acid substitution, has 2% of normal binding affinity for the LDL receptor and may account for altered postprandial lipid clearance in 50% of the population. Type III or remnant hyperlipidaemia, affecting one in 5000 individuals, is caused by homozygosity for the *Apoe* ^{2} gene. In a few patients complete ApoE deficiency has been described. ApoE-deficient mice have been created by two groups using homologous recombination, targeting the murine apoE gene [51••,52••]. Such mice showed a five- to eight-fold increase in plasma cholesterol levels and two-fold rise in triglyceride on a normal mouse diet. On a high-fat diet (42% of calories as fat), a rise in plasma cholesterol to 1821 ± 395 mg/dl occurred, mainly in the VLDL and IDL fractions, compared with 132 ± 18 mg/dl in non-transgenic rats [51]. Early atherosclerotic lesions were seen in both transgenic models from 10–12 weeks of age.

ApoA-I and apoA-II are the major lipoprotein components of HDL, which is thought to have a protective influence. In a susceptible mouse strain (C57BL/6) given a high-fat diet, introduction of a human apoA-I transgene resulted in an increase in the HDL fraction, but no change in non-HDL fractions. Transgenic mice had a smaller fatty streak lesion area on the atherogenic diet compared with transgene negative controls [53•,54•], but introduction of a human apoA-II transgene into the apoA-I transgenics resulted in an increased susceptibility to atherogenesis in spite of similar lipid profiles [55]. Further work is needed to understand the in-vivo mechanisms behind the protective effect of HDL, apoA-I and the metabolic effects of apoA-II.

Lipoprotein (a) [Lp(a)] consists of a particle similar to LDL, namely phospholipids, cholesterol and apoB-100 which is linked to apo(a). Apo(a) is a glycoprotein showing considerable size polymorphism (300–800 kD) and both size and sequence variations at the apo(a) locus may partly determine plasma levels [56]. High levels of Lp(a) have been found to be an independent risk factor for myocardial infarction [57]. Homology of amino acid sequence of 80% between apo(a) and plasminogen has led to the suggestion that binding of apo(a) to fibrin as part of a wound healing response might result in the delivery of excess lipid to sites of damaged endothelium and alter fibrinolysis [58]. *In vitro*, Lp(a) and apo(a) stimulate proliferation of human aortic smooth muscle cells in a dose-dependent fashion [59•]. This is caused by inhibition of plasminogen activation and hence in turn, reduced plasmin mediated-activation of transforming growth factor-β (a potent inhibitor of smooth muscle cell proliferation). The absence of apo(a) from all species, apart from primates (and hedgehogs) has precluded the development of good animal models. Transgenic mice were made by the introduction of human apo(a) complementary DNA fused to the mouse transferrin promoter directing expression principally to the liver which resulted in plasma apo(a) levels comparable with human values [60••]. Mean lipid staining lesion areas in aortas of transgene-positive animals on an atherogenic diet were significantly higher than those found in transgene-negative littermates or in transgene-positive animals on a normal diet. Infusion of human LDL into these transgenic mice resulted in association of human LDL and apo(a) in the plasma to form Lp(a), but such an association did not occur between mouse LDL or human HDL with apo(a) [61].

These experiments have helped to establish that genes for apo(a), apoA-I, apoE and the LDL receptor, for example, can interact with diet to alter predisposition to atherosclerosis and support previous work where these genes were implicated simply on the basis of epidemiological associations. In addition, they offer animal models of a human disease which can be used for developing therapeutic strategies.

Cardiomyocyte cell biology and cardiac disease

Unlike skeletal muscle, cardiac cells do not possess a stem cell system which allows regeneration, so infarction or disease leads to an irreversible loss. When undifferentiated skeletal myoblasts have been grafted by injection into ventricular myocardium of syngeneic mice, they ceased proliferation, adopted a phenotype of differentiated skeletal myocytes and survived for up to 3 months [62]. Whether contractility and appropriate electrical properties would be achieved with larger grafts is not certain. Targeting the SV40 T antigen to cardiac myocytes has enabled the generation of cardiomyocyte cell lines (AT1) which retain a proliferative capacity and a degree of differentiation [63], but the tumorigenic potential of these may preclude extensive transplantation studies.

Understanding the control mechanisms that govern cessation of proliferation and differentiation in muscle cells may allow a means of inducing controlled proliferation of cardiomyocytes. In skeletal muscle, a family of myogenic transcription factors has been identified: myf5, MyoD, and myogenic regulatory factor 4/herculin/myf6. They share a basic helix–loop–helix protein structure and are muscle-specific [64,65]. Informative cell culture experiments include the transfection of fibroblasts by a construct consisting of the *Myo*D gene fused to the hormone binding domain of the human oestrogen receptor gene [66] resulting in the oestrogen-inducible expression of skeletal muscle markers. In separate studies it has been shown that insulin-like growth factor-induced myogenesis in cultured myoblasts could be inhibited by antisense oligomers to myf5 [67]. Transgenic mice with a null mutation of MyoD, following homologous recombination in embryonic stem cells, showed normal muscle cell development, though a possible compensatory increase in myf5 mRNA did occur [68]. In contrast, ablation of the *myf5* gene by homologous recombination resulted in perinatal lethality caused by defect in rib formation and respiratory failure; however, the progeny appeared to have relatively normal skeletal muscle development [69•]. The mild skeletal muscle changes in these two gene knock-out strains perhaps suggest functional redundancy of individual myogenic transcription factors.

Although progress has been made in the search for *cis*-acting transcriptional control elements, cardiac muscle-specific *trans*-acting factors have been difficult to identify. In addition to the relatively well characterized atrial natriuretic factor gene [70], several other genes expressed in the heart have been studied in transgenic animals. A promoter region in the human myoglobin gene, identified using a Lac Z reporter construct in mice, controls the changing spatial pattern of expression of the myoglobin gene in the ventricle during murine embryogenesis [71]. The regulatory myosin light chain (MLC)-2 is normally found in both skeletal slow twitch and cardiac muscle. It is postulated that *trans*-acting factors might bind to a 250 base pair fragment of the MLC-2v (ventricular) gene promoter and determine ventricular specification in the developing heart during murine embryogenesis [72]. Within this fragment a 28 base pair element (HF-1) confers cardiac specificity with point mutations at two sites (HF-1a and HF-1b) significantly reducing MLC-2 promoter-luciferase fusion gene expression [73]. A complementary DNA coding for a C_2H_2 zinc finger protein that binds to the HF-1b site has been isolated, and can activate transcription *in vitro* [74]. Clearly, significant steps still need to be made before a comprehensive understanding of cardiomyocyte development and differentiation is possible.

Single gene defects are more amenable to transgenic investigation and such a candidate would be familial hypertrophic cardiomyopathy, an autosomal dominant condition with several identified mutations, mostly on chromosome 14q1, affecting the β-cardiac myosin heavy chain gene [75–77]. While β-cardiac myosin heavy chain protein is found in both left and right ventricles and skeletal muscle, the pathology manifests principally in the left ventricle and interventricular septum. The reasons for this are not clear, but cloning of the mutations and generation of transgenic models might shed light on this. The incidental finding of a dilated cardiomyopathy occurring in transgenic mice expressing an Epstein–Barr virus protein (EBNA-LP), has resulted in a potential animal model of dilated cardiomyopathy [78]. The v-*fps* transgene (encoding a cytoplasmic protein — tyrosine kinase) in mice causes progressive cardiac fibrosis and variable hypertrophy with some secondary myocyte damage [79]. Targeting and knock-out of the transforming growth factor-β_1 gene by homologous recombination in mouse resulted in a pancarditis and perivasculitis, but normal cardiac development [80].

Conclusions and future approaches

Although technically possible, the production of transgenic animal models of polygenic diseases remains difficult without further information concerning the genes involved. The above examples illustrate the value of the techniques for investigation of individual genes and their interactions with the environment. Breeding of transgenic animals with various inbred or outbred strains can introduce additional genetic factors which can modify a phenotype. Appropriate experiments such cross-breeding will enable complex genetic and physiological interactions to be studied.

Gene ablation strategies are at present limited within the field of cardiovascular research because of the requirement for embryonic stem cell lines, which to date are only available for the mouse. Although some elegant studies have been done, physiological investigations in this species are difficult because of size constraints. The development of embryonic stem cell technology in larger species will open up new possibilities including elucidation of abnormalities in genetic models of hypertension (e.g., the SHR) which may help in understanding human essential hypertension.

Approaches using direct gene transfer in larger animals e.g., dogs, pigs [3,81] are increasing the scope for development of therapeutic strategies. In the future, genetic material, including gene constructs, oligonucleotides etc., might be introduced directly into cardiomyocytes or endothelial cells to improve contractility, promote cellular regeneration or stimulate development of collateral blood flow to ischaemic regions [82]. In certain situations transplantation of modified cells may be practical [83]; however within the cardiovascular field, antisense methodology is the most likely technology to first reach clinical practice as promising results have been obtained in experimental systems. Local delivery of antisense c-*myb* oligonucleotides has been shown to suppress intimal accumulation of smooth muscle cells after balloon angioplasty of carotid arteries in rats [84••] and it is thought that this protooncogene is involved in the common pathway of mitogen-induced smooth muscle cell proliferation. Practical problems with oligonucleotide delivery

and stability may limit applications but much effort is being placed at present to circumvent such difficulties.

One exciting area of research which is likely to become increasingly important is the development of transgenic animals with a view to facilitating xenotransplantation. In this area larger animals species such as the pig will certainly feature more prominently. So, the contributions of transgenic technology range from basic gene expression studies through to dissection of multigenic traits. It is safe to say that transgenic technology is still going through rapid development, and over the course of the next few years ever-more useful methods for altering gene expression will become standard experimental and therapeutic tools.

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- •• of outstanding interest

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A hint of the future regarding therapeutic strategies. Local delivery of antisense oligonucleotides to the proto-oncogene *c-myb* suppresses intimal accumulation of smooth muscle cells after balloon angioplasty of rat carotid arteries.

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Kidney International, Vol. 46 (1994), pp. 1528–1532

Spontaneous development of malignant phase hypertension in transgenic Ren-2 rats

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Spontaneous development of malignant phase hypertension in transgenic Ren-2 rats. Spontaneous development of malignant phase hypertension in TGR(mREN2)27 heterozygotes occurs as a consequence of crossing TGR(mREN2)27 homozygotes with Edinburgh Sprague-Dawley rats. Similarities to human malignant phase hypertension are seen with an accelerated rise in blood pressure, fibrinoid necrosis of renal afferent arterioles, renal failure and evidence of renin-angiotensin system activation. It appears that introduction of an additional genetic factor or factors into a monogenic model of hypertension results in malignant phase hypertension.

Malignant hypertension (MH) is a rare but important complication of human essential hypertension [1, 2]. Clinical markers of transformation to the accelerated phase include a rising blood pressure, presumed pressure diuresis, renal failure and development of grade III or IV retinopathy. Characteristic pathological changes in the renal vasculature occur including myointimal proliferation or 'onion skinning' with endothelial swelling and fibrinoid necrosis [3, 4]. Activation of the renal renin-angiotensin system (RAS) is a presumed consequence of salt and water loss combined with afferent renal vascular pathology. This may serve to further increase blood pressure resulting in a vicious circle of progressive renal damage and rising blood pressure.

Most animal models of MH to date have been limited by the need for dietary, surgical or pharmacological intervention to precipitate onset and this has made the study of both primary or initiating factors and secondary events operating in malignant hypertension difficult to study [5–7].

The transgenic rat line TGR(mREN2)27 develops hypertension as a consequence of introduction and expression of the mouse Ren-2 renin gene into the Sprague-Dawley (SD) rat [8]. Blood pressure rises to a plateau at 10 weeks of age and development of progressive left ventricular hypertrophy, arterial medial thickening and glomerulosclerosis occurs with increasing age [9]. Fibrinoid necrosis was not described in transgenic heterozygotes. However, the underlying pathogenesis of the hypertension remains unclear.

The breeding of a colony of TGR(mREN2)27 heterozygotes in Edinburgh using SD rats (Centre for Genome Research, University of Edinburgh) was associated with the spontaneous onset of a phenotypic change with the occurence of a fatal syndrome at 50 to 90 days of age. Features included a one to three day history of polyuria, weight loss, dehydration, apathy, piloerection, seizures and hemiplegia. Some similarities to human MH prompted this study to characterize this as a potential animal model and to identify whether environmental or genetic factors may be responsible.

Methods

Initially three transgenic heterozygote crosses were bred. Homozygote TGR(mREN2)27 rats (TGR#27), derived from a Hannover SD colony (Central Institute for Laboratory Animal Breeding, Hannover, Germany) were crossed with (1) SD (Edinburgh) obtained from the colony in the Centre for Genome Research, (2) SD (Hannover) from the Central Institute for Laboratory Animal Breeding (Hannover, Germany) and (3) Lewis rats from Harlan-Olac (Bicester, Oxford).

All animals were housed in the same room with a 12:12 hour light-dark cycle, controlled temperature (18 to 20°C), humidity (45 to 65%), diet (0.32% sodium CRM diet, SDS, Witham, Essex, UK) and *ad libitum* tap water to drink. An alternative standard rat diet containing 0.2% sodium (CRM X) was prepared, and when used was given from weaning.

Direct blood pressure monitoring using telemetry (Data Sciences International, St. Paul, Minnesota, USA) allowed continuous recording of mean blood pressure (MBP) in conscious, unrestrained male rats starting from 46 to 50 days of age following recovery from surgical implantation under halothane anaesthesia [10, 11]. Indirect measurement of systolic blood pressures (SBP) used a tail cuff plethysmography method (IITC Life Sciences) under light halothane anaesthaesia. Student's t-test was used for statistical analysis with a P value of less than 0.05 taken to be significant.

Light microscopic examination of kidney, heart, brain and mesenteric artery was performed after fixation of tissues in 4% formal saline, wax embedding, sectioning (3 μm) and staining (hematoxylin and eosin, Martius Scarlett Blue, and periodic acid schiff stains). Renal function was assessed by plasma creatinine (Electro-nucleonics®). Plasma renin activity (PRA), angiotensin II (Ang II) and aldosterone were assayed by specific radioimmunoassay [12–14]. Immunohistochemistry of fixed kidney sections

Table 1. The incidence of the MH phenotype in male and female transgenic heterozygotes maintained under the same environmental conditions and on the same diet

Heterozygote cross	Name	Incidence of MH phenotype	
		Male	Female
TGR#27-Edin SD	TGR/Edin	86/117 (73.5%)	83/158 (52.5%)
TGR#27-Han SD	TGR/Han	7/39 (18%)	2/44 (4%)
TGR#27-Lewis	TGR/Lew	0/35 (0%)	0/31 (0%)

was performed using a polyclonal rabbit anti-mouse renin antibody, which cross reacts with rat renin (provided by Dr. D.J. Campbell, Melbourne, Australia).

Results

The occurrence of the MH phenotype in male and female transgenic heterozygotes is given in Table 1, which shows a significantly higher incidence in TGR/Edin heterozygotes than either TGR/Han or TGR/Lew heterozygotes. The median age at the time of death was 59 days (range 46 to 102 days).

Continuous recording of blood pressure in conscious TGR/Han and TGR/Lew heterozygotes by telemetry showed development of hypertension with a characteristic rise to a plateau by 70 days of age with a MBP of 170 mm Hg (Fig. 1A). In contrast, those TGR/Edin (7 out of 10) and TGR/Han (1 out of 12) that developed MH while on telemetry showed a continuing increase in blood pressure, which culminated in a terminal accelerated rise of 67 to 75 mm Hg (mean 72 mm Hg) over six hours (Fig. 1B). MBP in pre-malignant phase TGR/Edin was significantly higher than either TGR/Han or TGR/Lew at 52 to 57 days of age, but by 75
days of age the MBP attained by TGR/Han and TGR/Lew heterozygotes was not significantly different to pre-malignant phase TGR/Edin (data not shown).

All animals that exhibited signs of the MH phenotype were found to have pathological changes in the kidney. Figure 1C shows a kidney section from an 8-week-old hypertensive, but not malignant phase, transgenic rat kidney. In contrast, Figure 1D shows changes of MH. Fibrinoid necrosis was seen to principally affect afferent arterioles, interlobular arteries and occasional portions of glomerular tufts. Secondary ischemic changes were relatively rare, implying that such changes were acute. In addition proliferative myointimal changes with endothelial swelling and luminal thrombi were observed. More chronic hypertensive vascular and glomerular changes were not seen in the age range of the animals developing malignant phase hypertension. Small myocardial microinfarcts were seen in many TGR/Edin that developed MH, some in relation to fibrinoid changes within small cardiac arterioles. Cerebrovascular pathology was infrequent, with occasional infarction and hemorrhage. Minor thickening was seen in mesenteric artery wall, but there was no difference between malignant and non-malignant phenotypes.

The MH phenotype was associated with varying degrees of renal failure with a mean creatinine of 111.9 μmol/liter in malignant TGR/Edin compared to 55 healthy age-matched hypertensive heterozygotes (range 22.4 to 60.4 μmol/liter, 95% confidence limits). No significant difference in renal function was observed between the transgenic heterozygote crosses prior to the development of MH.

Significant elevation of PRA (29.1 $\pm$ 11.0 vs. 9.2 $\pm$ 8.3 ng Ang I/ml/hr), Ang II (829.5 $\pm$ 653.4 vs. 28.0 $\pm$ 25.2 pg/ml) and aldosterone (11.4 $\pm$ 7.7 vs. 0.71 $\pm$ 0.97 nmol/liter) were seen in MH rats compared with age matched, non-malignant phase, hypertensive transgenic heterozygotes (Results shown as mean $\pm$ SD, $N = 6$ to 24 per group; $P < 0.001$). Immunohistochemical labeling of kidney sections using a rabbit anti-mouse renin antibody demonstrated a marked increase in renin staining at the vascular poles in malignant hypertensives (not shown). Staining of other renal structures was not found.

A small reduction in dietary sodium (0.2% vs. 0.32%) within the 'normal' range was given from weaning to transgenic heterozygotes to mimic rat chow fed to original TGR/Han heterozygotes when first established and studied in Department of Pharmacology, University of Heidelberg, Germany [9]. There was a small reduction in MBP in telemetered TGR/Han and TGR/Lewis (Fig. 2 A, B) which was only significant at 75 days in TGR/Han and 75 to 81 days in TGR/Lew. No significant differences in the occurrence of MH occurred, with one case in the TGR/Han group on 0.32% sodium diet, but none in either TGR/Lew group.

SBP measured by tail cuff plethysmography under light halothane anaesthesia in non-transgenic Edinburgh SD and Hannover SD ($N = 6$ to 14 per group) on a 0.32% Na diet from 4 to 12 weeks of age showed a lower SBP in Edinburgh SD from six weeks of age and slightly lower body weight (Fig. 2 C, D)

Discussion

The spontaneous occurrence of the MH phenotype in the transgenic rat line TGR(mREN2)27 is associated with evidence of classical pathological changes of fibrinoid necrosis and myointimal proliferation, renal failure, evidence of renal RAS activation and an accelerated rise in BP. Furthermore, the high percentage of male TGR/Edin affected offers a potentially valuable animal model in which to study MH, in particular the initiating and subsequent events.

In this case transgenesis has created a monogenic model of hypertension in which additional interactions, either environmental or genetic, have resulted in MH. What factors determine susceptibility in humans are poorly understood. Cigarette smoking [15, 16], oral contraceptive use [17] and HLA type have all been implicated [18]. Environmental factors were questioned in view of the change in phenotype on breeding TGR#27 heterozygotes in Edinburgh as compared to Heidelberg. Analysis of rat chow revealed a slightly higher sodium content in 'Scottish' rat chow, but a comparison of the two diets did not significantly affect either blood pressure or survival. No other environmental differences such as temperature, humidity profiles or lighting times were found to explain the phenotypic change. Furthermore, the high incidence of MH in TGR/Edin compared with TGR/Han and TGR/Lew when maintained in identical conditions and on the same diet suggested that environmental factors were not crucial. It is likely that genetic diversity within the SD strain, which is outbred, explains the difference between Hannover and Edinburgh strains. The finding of a lower SBP in Edinburgh SD compared with Hannover SD rats argues against additional 'hypertensive' genes contributing to the MH phenotype.

Neither the rate of rise in blood pressure nor absolute BP appear to be important in determining the risk of onset of malignant phase hypertension, but higher BP at a younger age may be important in these rats. The continuing rise in BP during

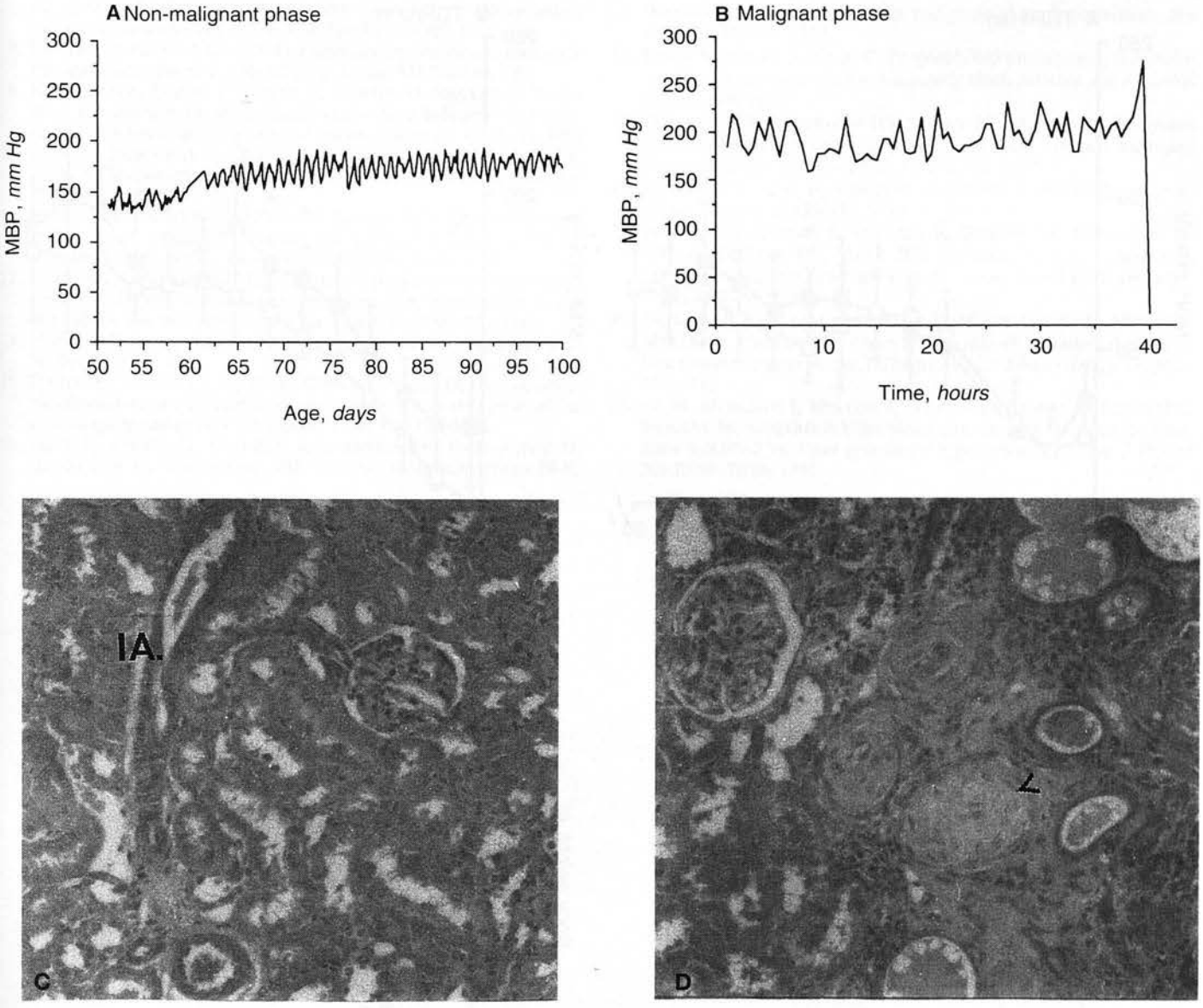

Fig. 1. *Two examples of continuous recording of MBP by telemetry show in* (**A**)*, 'normal' hypertension reaching a plateau phase of 170 mm Hg in a TGR/Han, and in* (**B**)*, the terminal 40 hours of a 79 day old TGR/Edin with MH demonstrating the accelerated phase with a rise of 75 mm Hg in six hours.* (**C**) A light micrograph showing a normal glomerulus, afferent arteriole and interlobular artery (IA) from a non-malignant phase transgenic heterozygote. (**D**) Myointimal proliferative changes (arrowed) and fibrinoid necrosis of interlobular arteries from a TGR/Edin with MH. (H&E stain ×100)

the accelerated phase may be due to loss of autoregulatory mechanisms and the development of a high renin hypertension. Initial characterization of the TGR(mREN2)27 rat line has suggested that the hypertension may be Ang II dependent [19], though there is evidence that altered adrenal steroid metabolism may be important [20, 21]. Certainly transgenic rats with established hypertension have evidence of a suppressed kidney RAS [8]. In the malignant phase, activation of the renal RAS was seen with increased immunohistochemical staining of the afferent arteriole at the vascular pole. By the method used, we have not differentiated mouse from rat renin, but would expect that as a consequence of the relatively low level of transgene expression in the kidney [8], that it is predominantly endogenous rat renin which is expressed in the malignant hypertensive transgenic rat kidney.

In conclusion, we believe that this may be a valuable animal model in which it may be possible to identify factors, either genetic or environmental, that contribute to the development of malignant phase hypertension. It suggests that the target organ damage resulting from hypertension in this case may in part be dependent on the interaction of additional genetic factors.

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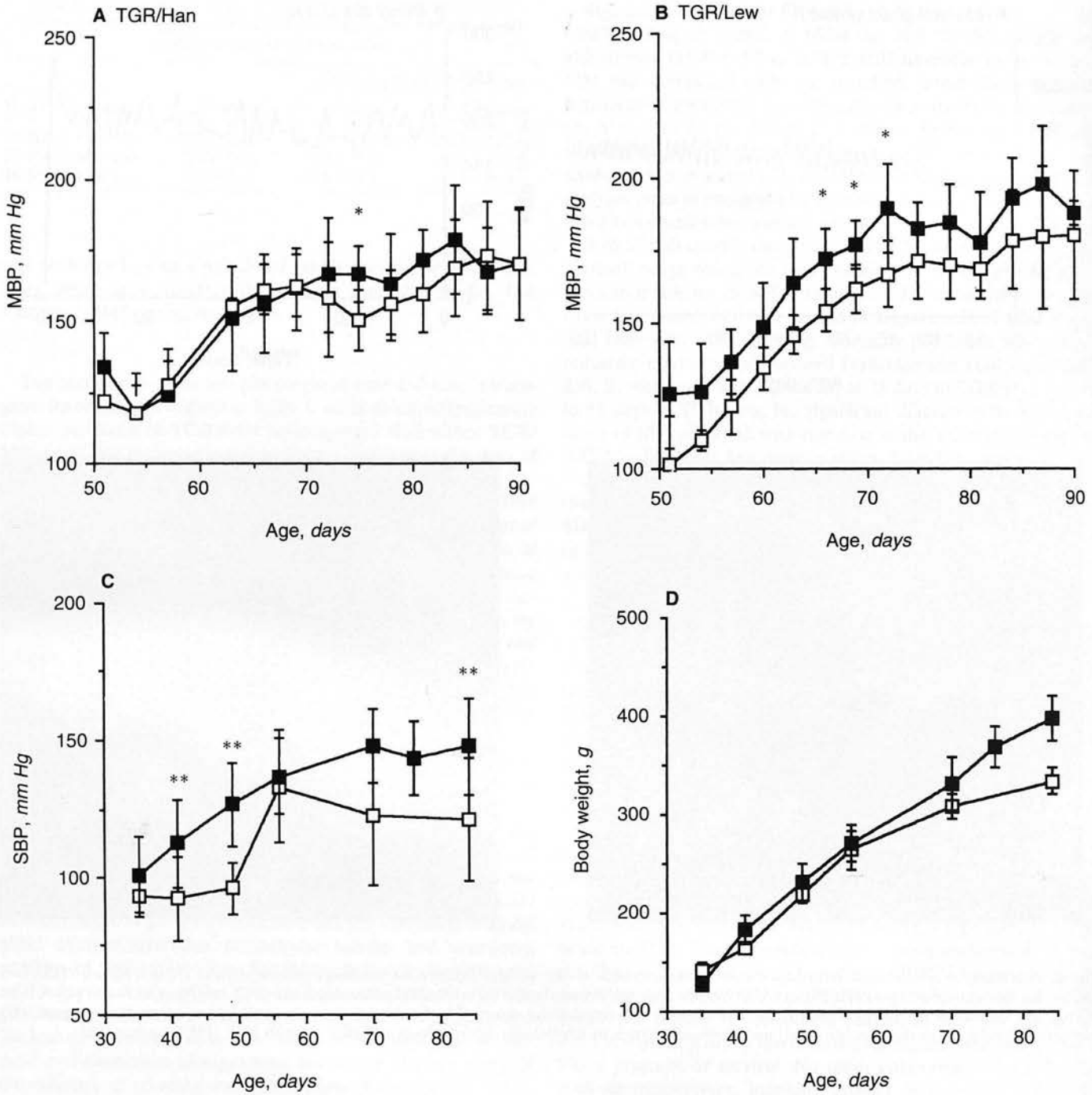

Fig. 2. *MBP (mm Hg) measured by telemetry in* (**A**) *TGR/Han* (N = 6, 5) *and* (**B**) *TGR/Lew heterozygotes* (N = 4, 5) *on 0.32%* (■) *and 0.2%* (□) *sodium diets, respectively.* Results are shown as mean ± SD for each group. MBP for each animal is taken as the mean over the 24 hour period (recordings at 10 min intervals). *Significant difference ($P < 0.05$) in MBP arising between 0.32% and 0.2% sodium diets. (**C**) SBP (mm Hg) of Edinburgh SD (□) and Hannover SD (■) rats from 4 to 12 weeks of age measured weekly by tail cuff plethysmography under light halothane anaesthesia; **SBP for Han SD > Edin SD ($P < 0.05$); (**D**) body weight.

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Kidney International, Vol. 47 (1995), pp. 529–535

A genetic model of malignant phase hypertension in rats

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A genetic model of malignant phase hypertension in rats. A genetic model of malignant phase hypertension in rats is described which closely parallels the natural history of untreated human malignant phase hypertension. Although the factors initiating transition from essential hypertension to the accelerated phase in humans remain unknown, we report the characteristics of a genetically determined and reproducible phenotype which was found to result from a cross between hypertensive transgenic Ren-2 rats and normotensive Sprague-Dawley (Edinburgh) rats. Male F1 hybrids developed malignant phase hypertension with a penetrance of 73.5% (95% confidence limits 65.7 to 81.3%) by 100 days of age. Phenotypic features included an accelerated rise in blood pressure, fibrinoid necrosis, activation of the renal renin-angiotensin system and microangiopathic hemolytic anemia. In an analytical cross no significant difference in blood pressure was observed between malignant phase and non-malignant phase animals prior to transition, implying that a factor in addition to hypertension appears necessary for inducing transition to the malignant phase phenotype. Segregation of the malignant phenotype suggested that susceptibility is determined by at most two genetic loci.

It is estimated that between 1 and 2% of essential hypertensives develop malignant phase hypertension precipitated by a sudden rise in blood pressure [1]. This incidence has been reduced from previous levels by improved management of essential hypertension [2], but if untreated the mortality from this complication remains extremely high with a one year survival of less than 10% [3]. The pathological changes affecting the vasculature in malignant hypertension have been well described and include a myointimal proliferative response with endothelial swelling, areas of fibrin deposition, fibrinoid and cellular necrosis predominantly affecting small renal arteries and afferent arterioles [4–8]. Activation of the renal renin-angiotensin system (RAS) occurs in response to the afferent vascular pathology and a pressure induced diuresis and natriuresis [9, 10], and may serve to further increase blood pressure producing a vicious circle of accelerating blood pressure (BP) and RAS activation [2]. A microangiopathic hemolytic anemia results from intravascular hemolysis leading to the appearance of red cell fragments, spherocytes, microspherocytes and a reticulocytosis [11]. How environmental and genetic factors might determine susceptibility or induce transition to the malignant phase are poorly understood, but cigarette smoking [12], the oral combined contraceptive [13] and HLA type [14] have all been implicated. Since there has been no available genetic model for this disease, animal models of malignant hypertension have only been made possible by dietary, surgical or pharmacological manipulation [15–17].

The transgenic rat line TGR(mRen2)27 exhibits sustained hypertension as a consequence of expression of the mouse Ren2 renin gene in a Hannover Sprague-Dawley (HanSD) rat [18]. The exact mechanism of the hypertension is not clear but overexpression of the transgene in adrenal cortex resulting in activation of the adrenal RAS [19–21], a high circulating prorenin [22], increased vascular wall renin activity [23] and the response to converting enzyme inhibiton [24] suggest that blood pressure may well be Ang II dependent. Expected target organ damage is seen in the kidney and heart of heterozygotes from four to six months of age [25], but no hallmarks of malignant phase hypertension have been reported to date. In this study we have used the presence and function of the Ren2 transgene as a method of inducing hypertension in three rat strains and have observed a strain-dependent difference in the incidence of a phenotype analogous to malignant hypertension which was invariably terminal. Observed features included the adoption of a hunched posture with extreme lethargy, piloerection, a diuresis with a 5 to 10% loss in body weight occurring over one to three days, and on occasion unilateral or bilateral seizures or hemiparesis. A second analytical cross demonstrated that segregation of the phenotype occurred independent of BP prior to onset of malignant phase, and was due to one or at most two genetic loci.

Methods

Animals

All animals were housed in identical conditions with a 12:12 hour light-dark cycle, controlled temperature (18 to 20°C) and humidity (45 to 65%). A normal sodium diet (0.32% sodium CRM diet, SDS, Witham, Essex, UK) was used with tap water ad *libitum* to drink.

F1 hybrids, hemizygous for the Ren2 transgene (Ren2/—) were derived from crossing male TGR(mRen2)27 homozygotes on a Hannover Sprague-Dawley (HanSD) background with: (i) female Edinburgh Sprague-Dawley (EdinSD) (Centre for Genome Research, University of Edinburgh, UK) to give hybrids of genotype HanRen2/Edin—; (ii) HanSD (Central Institute of Laboratory Animal Breeding, Hannover, Germany) to give HanRen2/Han—; and (iii) Lewis rats (Harlan-Olac, Oxford, UK) to give HanRen2/Lew—. Animals were closely observed for the appearance of terminal features of the phenotype as described, and were put

Received for publication July 11, 1994
and in revised form September 12, 1994
Accepted for publication September 13, 1994

down for humane reasons in accordance with the Animals (Scientific Procedures) Act 1986. Where possible kidney, heart, descending thoracic aorta, superior mesenteric artery and brain were removed, fixed in 4% formal saline and paraffin embedded. Three μm sections were stained with hematoxylin and eosin (H&E) according to standard methods [26]. Control tissues were obtained from age- and sex-matched healthy transgenic heterozygotes and non-transgenic HanSD and Edin SD. Blood, obtained by cardiac puncture from malignant phase male HanRen2/Edin ($N = 5$), and from healthy, hypertensive, age-matched male HanRen2/Edin littermates ($N = 5$) under halothane anesthesia was collected into tubes containing EDTA (Sarstedt Monovette 2.7 ml tubes) before spreading and staining with Wright's stain. Plasma creatinine was assayed by a colorimetric method (Gemeni, Electro-nucleonics, Inc., NJ, USA) from blood obtained as above and collected into lithium heparin (1 ml tube, Teklab M.L. Ltd, Durham, UK).

Blood pressure

Continuous monitoring of BP in conscious, unrestrained male rats was performed using telemetry (Data Sciences International, St. Paul, MN, USA). Implantation of transmitters (TA11PA-C40) was performed at 37 to 49 days of age by aseptic technique, under halothane anesthesia (2 to 4% in oxygen), with placement of the catheter tip in the aorta distal to the origin of the renal arteries. Rats were housed singly in cages above a RA 1010 receiver pad. Receivers were connected to a BCM100 consolidation matrix and signals transmitted to a Dataquest IV acquisition system. BP was corrected for atmospheric pressure. Systolic (SBP), mean (MBP) and diastolic (DBP) pressures were collected at 10 minute intervals after recovery from surgery. Twenty-four hour MBP (group mean and SEM) for male HanRen2/Edin— ($N = 10$), HanRen2/Han— ($N = 6$), HanRen2/Lew— ($N = 4$), EdinSD ($N = 3$), HanSD ($N = 3$) and Lewis ($N = 4$) was recorded from 46 to 90 days of age.

Renin-angiotensin system parameters

Plasma renin activity (PRA), angiotensin II (Ang II) and aldosterone were measured by radioimmunoassay, by methods described elsewhere [27–29], in healthy seven to nine-week- old HanRen2/Edin—, HanRen2/Han—, HanRen2/Lew—, non-transgenic controls (EdinSD, HanSD, Lewis) and malignant phase Ren2 hemizygotes. Blood, obtained by retro-orbital bleeding or cardiac puncture under halothane anaesthesia, was collected into pre-chilled tubes on ice: in lithium heparin (1 ml tube, Teklab M.L. Ltd, Durham UK) for PRA; in inhibitor (1 μl/ml) 1,10 phenanthroline monohydrate (Sigma, UK) in EDTA (BDH Laboratory Supplies, UK) for Ang II; or for aldosterone determination, with no anticoagulant, spun at 4000 g at 4°C for 10 minutes to obtain plasma or serum and samples were stored at −70°C before assay. Immunostaining for renin was performed on 3 μm de-paraffinized sections of kidney (fixed in 4% formal saline). A rabbit anti-mouse renin primary antibody (cross reacts with rat) was applied, with an avidin-biotin horseradish peroxidase secondary detection system (Vectastain, Vector Laboratories, Inc., Burlingame, CA, USA). Primary antibody was omitted as a negative control.

Analytical cross

Seventy-three male F1 progeny (Edin,Han)Ren2/(Edin,Lew)— were bred from three (Han,Edin)Ren2/(Han,Edin)Ren2 males (derived from a HanRen2/Edin intercross) and seven Edin—/Lewis— females and housed and maintained as described above. Animals were examined for the terminal features suggestive of malignant phase hypertension as described, and diagnosis was confirmed postmortem by the presence of fibrinoid necrosis on light microscopy of 3 μm kidney sections (H&E stain). SBP was measured by tail cuff plethysmography (Model 179 blood pressure analyzer, Model 27 cuff pump, IITC Life Science Instruments, Norfolk, UK) under light halothane anaesthesia (2% in oxygen) on a heating pad (33°C), in all 73 rats at 52 days and measured weekly in a subgroup of 28 rats from 35 days.

Statistics

Blood pressures are shown as group means and SEM. Student's t-test was used for unpaired comparisons of group means at each time point with the Bonferroni correction for multiple comparisons where appropriate. Statistical comparisons made between malignant phase and each non-malignant phase group for PRA, Ang II and aldosterone levels were performed using unpaired t-tests with Bonferroni correction. Penetrance of the phenotype in crosses is shown with 95% confidence limits. The χ^2 test was used to compare penetrance of the malignant phenotype in the progeny of the analytical cross, (Edin,Han)Ren2/(Edin,Lew)— with that in HanRen2/Edin—. Observed penetrance and expected levels of penetrance (with 95% confidence intervals) if one, two or three genes were operating were also compared using χ^2 test. The level of statistical significance was taken to be $P < 0.05$.

Results

Characterization of the phenotype

The spontaneous development of an altered phenotype in TGR(mRen2)27 heterozygotes was observed on crossing HanSD males homozygous for the Ren2 transgene (genotype HanRen2/HanRen2) with EdinSD (genotype Edin—/Edin—). Animals were noted to have a diuretic phase resulting in up to 10% loss in body weight over one to three days. They adopted a hunched apathetic posture, with evidence of unilateral seizures or hemiparesis in a minority. Death occurred between 33 and 103 days (median 57 days) of age in males and between 46 and 103 days (median 62 days) in females.

Three different F1 hybrid groups resulting from crossing homozygote HanRen2/HanRen2 with EdinSD, HanSD, or Lewis were compared for incidence of the phenotype in males and females on a 0.32% sodium diet, and maintained in identical conditions (Table 1). HanRen2/Edin— showed the highest incidence with the phenotype affecting 73.5% of males (65.7 to 81.3%, 95% confidence interval), and 52.5% (44.7 to 60.3%) of females. For HanRen2/Han—, 18% (6 to 30%) of males and 4% (0 to 10%) of females were affected. No HanRen2/Lew— males or females developed the phenotype.

Histopathology of kidney from affected animals demonstrated both myointimal proliferation and fibrinoid necrosis, particularly affecting afferent arterioles, interlobular arteries and occasional glomerular tufts (Fig. 1, A-D). Changes indicative of chronic ischemic damage or long standing hypertension were not a feature in transgenic heterozygotes up to 12 weeks of age. Microscopic

myocardial infarcts were found, occasionally seen to be related to fibrinoid necrosis of cardiac arterioles. Early lesions appeared as collections of cardiomyocytes showing evidence of focal necrosis, but granulation tissue and focal fibrotic scars suggested that a healing response did occur. Cerebral hemorrhage and infarction occurred infrequently and no significant pathology of either aorta or superior mesenteric artery was seen in the malignant phase (data not shown). All blood films from malignant phase HanRen2/Edin— ($N = 5$) exhibited microangiopathic hemolytic anemia with red cell fragments, spherocytes, microspherocytes and reticulocytosis, while films from age-matched controls (non-malignant HanRen/Edin— littermates ($N = 5$) showed no features of hemolysis (Fig. 1 E, F). Renal failure accompanied the clinical features with elevation of plasma creatinine (measured in 8 affected animals) to a mean of 111.9 μmol/liter (range 59 to 239 μmol/liter) compared with a mean of 41.4 μmol/liter (22.8 to 60.0 μmol/liter, 95% confidence limits) for healthy but hypertensive age-matched transgenic heterozygotes ($N = 55$).

An accelerated rise in MBP occurred in heterozygotes who died from malignant phase hypertension ($N = 5$) while on telemetry with a mean increase of 72 mm Hg (range 65 to 75mm Hg) occurring over the terminal six hours (Fig. 2A). MBP measured in the three F1 hybrids and in non-transgenic strains, EdinSD, HanSD and Lewis is illustrated in Figure 2B. HanRen2/Edin— showed a significantly higher MBP, from 52 to 60 days compared to either HanRen2/Han— or HanRen2/Lew— ($P < 0.01$), but on attaining the plateau phase at 70 days the MBP ± SEM for HanRen2/Han— and HanRen2/Lew— were 169 ± 11.9 and 177.3 ± 4.1 mm Hg respectively, which was not significantly different to the MBP ± SEM of HanRen2/Edin— at 60 days (180.7 ± 10.0 mm Hg), around the median age for onset of malignant phase. No significant difference in MBP was seen between non-transgenic strains.

PRA, Ang II and aldosterone levels in plasma were significantly elevated in the malignant phase compared to both age-matched non-malignant transgenic heterozygotes and non-transgenic controls (Fig. 3A). Immunostaining of kidney sections using an anti-mouse renin antibody which cross reacts with rat renin showed marked, but selective, staining of afferent arterioles at the vascular poles of glomeruli from malignant phase kidneys (Fig. 3B). Little renin staining was seen in healthy hypertensive age/sex matched hybrids, relative to both non-transgenics (not shown) as previously described [25], and malignant phase transgenic rats.

Analytical cross

A segregating cross for EdinSD alleles was designed (Fig. 4A). Seventy-three hybrid males of genotype (Han,Edin)Ren2/ (Edin, Lew)— were bred. Forty-two out of seventy-three (57.5%, 95% confidence limits 45.9 to 69.5%) developed signs of malignant phase hypertension, verified by the presence of fibrinoid necrosis on light microscopy of H&E stained kidney sections, and died between 53 and 103 days (median 66 days). This incidence was significantly different to that observed in HanRen2/Edin— ($\chi^2 = 5.181$, df = 1, $P < 0.05$). Expected levels of penetrance if one, two or three genetic loci were involved were calculated, as discussed below, and compared with the observed penetrance in the cross. The results most closely fit a monogenic model where expected penetrance would be 55.1% (95% confidence interval 49.3 to 61%).

SBP was measured in all 73 rats by tail cuff plethysmography at 52 days of age and weekly in a subset of 28 rats from 35 days of age (Fig. 4B). At 52 days there was no significant difference in SBP between those rats destined to develop malignant hypertension and those who did not (mean ± SEM, 193.0 ± 4.8 mm Hg and 182.8 ± 4.8 mm Hg, respectively, $P = 0.278$), but at 35 days a small but significant difference did exist (malignant phase 141.9 ± 5.6 mm Hg and non-malignant phase 124.2 ± 4.6 mm Hg, $P = 0.02$).

Table 1. F1 hybrids, hemizygous for the Ren2 transgene were derived from crossing male TGR(mRen2)27 homozygotes on a HanSD background with female EdinSD, HanSD and Lewis rats giving the genotypes shown

Heterozygote cross	Genotype	Incidence of Malignant hypertension	
		Male	Female
HanRen2 × Edin SD	HanRen2/Edin—	86/117 (73.5%)	83/158 (52.5%)
HanRen2 × Han SD	HanRen2/Han—	7/39 (18%)	2/44 (4%)
HanRen2 × Lewis	HanRen2/Lew—	0/35 (0%)	0/31 (0%)

The incidence (percentage) of male and female progeny developing malignant phase hypertension for each cross on a normal diet is shown.

Discussion

The phenotype described above appears to be an excellent experimental model exhibiting within a defined age range all the main clinical characteristics of malignant phase hypertension, namely an accelerated rise in blood pressure, diuresis, impaired renal function, activation of the renal RAS, microangiopathic hemolytic anemia and the classical pathological changes of myointimal proliferation and fibrinoid necrosis. Some variation in the severity of pathological, biochemical and immunological change measured is to be expected due to the necessity for intervention for humane reasons when animals were showing signs of terminal malignant phase, as samples were therefore obtained at varying stages during the natural history of the established phenotype.

Malignant phase hypertension is arising in a model where expression of the Ren-2 transgene has been used as a tool to elevate blood pressure, but without requiring dietary, surgical or pharmacological manipulation or selective breeding, an increased tendency for spontaneous transition to malignant phase is exhibited in certain animals. Although the underlying hypertension in this rat model may well be Ang II dependent, activation of the renal RAS system is seen during malignant phase. Elevated plasma renin, increasing Ang II and resulting hyperaldosteronism is thought to be central to the process of MH providing the drive for further BP elevation [30–32]. In animal models, infusion of renin and aldosterone induced typical pathological changes [33], though while Ang II infusion in rabbits did cause acute renal failure and tubular necrosis, arteriolar changes were not observed [34]. In salt loaded SHR-SP, both ACE inhibition and the specific Ang II antagonist, Losartan, slowed both the rise in BP and prevented or delayed the development of renal arterial necrosis [35, 36].

Factor(s) provided by the genetic background appear to be acting in concert with "benign hypertenion" to induce the change in phenotype. Environmental factors do not play a major role as shown by the differing incidence of the malignant phenotype in the three transgenic hemizygote hybrids tested (HanRen2/Edin—, HanRen2/Han— and HanRen2/Lew—), despite maintenance in identical conditions. The reason for the difference in incidence

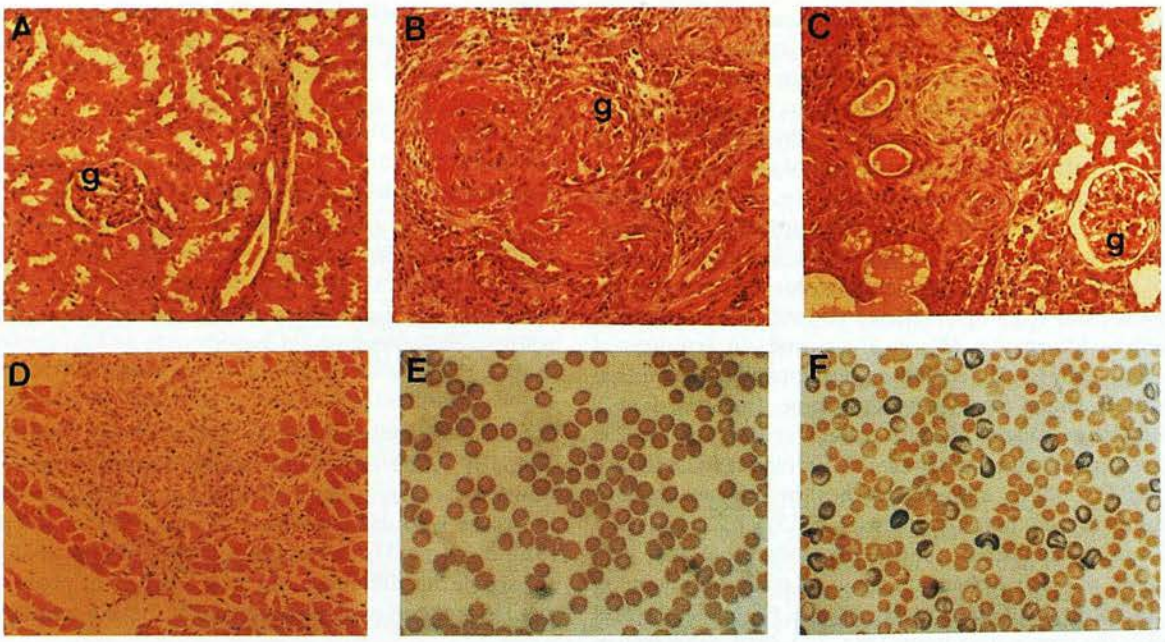

Fig. 1. *Kidney, heart, brain, aorta and mesenteric artery was fixed in 4% formal saline, paraffin-embedded and 3 μm sections were stained with hematoxylin and eosin.* **A.** Normal kidney from SD (× 100) showing glomerulus (g). **B.** Fibrinoid necrosis and **C.** myointimal proliferation in HanRen2/Edin— kidney (× 100). **D.** Illustrates a microscopic infarct in heart (× 100). Blood films (Wright's stain, × 630) **E** and **F** show a normal film from a healthy hypertensive HanRen2/Edin—, and microangiopathic hemolytic anemia with reticulocytosis, red cell fragments, and spherocytes from the malignant phase, respectively.

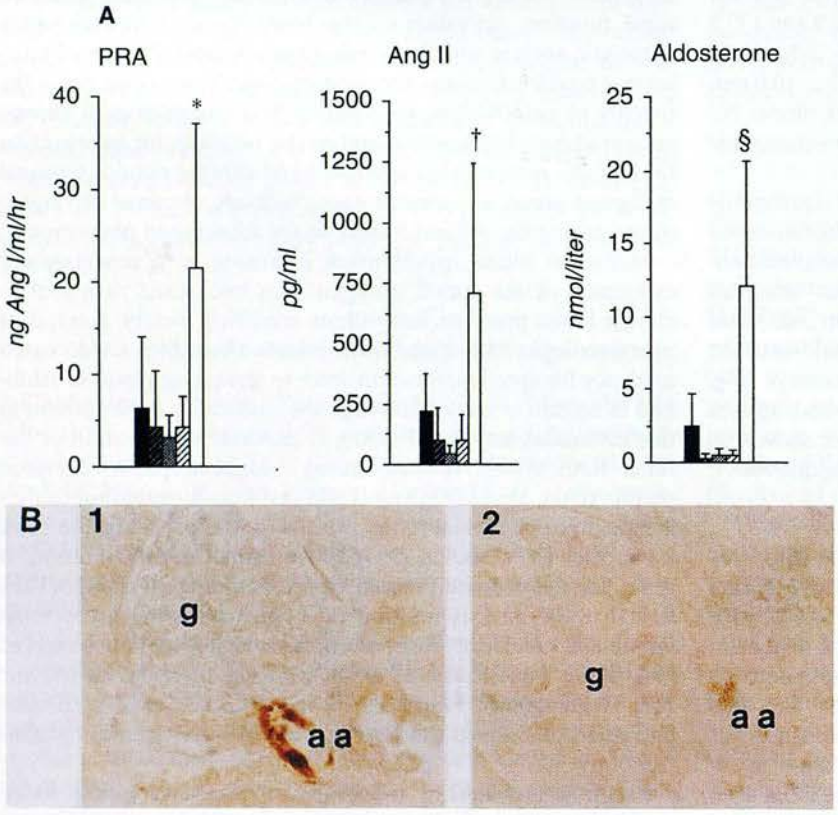

Fig. 3. *PRA, Ang II and aldosterone were measured by radioimmunoassay in 7- to 9-week-old healthy (■) HanRen2/Edin— (N = 15,11,14 for each assay respectively); ( ) HanRen2/Han— (N = 19,11,30); (▩) HanRen2/Lew— (N = 14,12,19); (▨) pooled non-transgenic EdinSD/HanSD/Lewis (N = 18,14,13); (□) and malignant phase Ren2 hemizygotes (N = 14,11,15). Results are shown as group means ± SD. Significantly higher values were seen for PRA(*P < 0.008), Ang II (†P < 0.04) and aldosterone (§P < 0.004) in the malignant phase compared to the other 4 non-malignant groups (unpaired t-test with Bonferroni correction).* **B.** *Immunostaining of 3 μm kidney sections using an anti-mouse renin antibody which cross reacts with rat shows (1) rat glomerulus (g) (× 400) from malignant phase Ren2 hemizygote demonstrating dense immunostaining of the afferent arteriole (aa) at the vascular pole, but (2) little staining of the vascular pole (aa) is seen in the non-malignant phase transgenic littermate (× 630).*

between male and female strains has not been addressed in this study. Small variations in dietary sodium (0.32% vs. 0.2%) did not influence either MBP or survival in telemetered transgenic heterozygotes [37]. Therefore, if genetic factors are important these might take the form of either loci leading to an increase in baseline BP or act by some other mechanism independent of BP. There was no significant difference in MBP between telemetered EdinSD and HanSD as shown. In addition, a larger group of

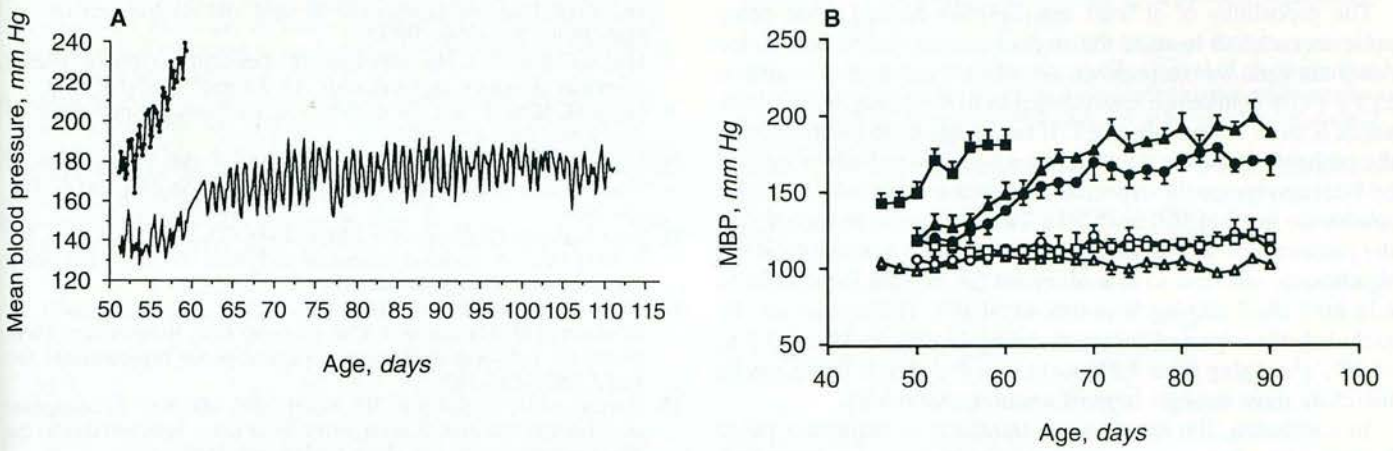

Fig. 2. A. *Examples of recordings of MBP by telemetry from an individual HanRen2/Han— (——) with chronic hypertension and a HanRen2/Edin— (-●-) with malignant phase hypertension.* **B.** MBP (24 hr average) as recorded by telemetry was plotted every 2 days for clarity as group mean ± SEM starting from 46 to 52 days of age for: (■) HanRen2/Edin— (N = 10); (●) HanRen2/Han— (N = 6); (▲) HanRen2/Lew (N = 4); (□) EdinSD (N = 3); (○) HanSD (N = 3); and (△) Lewis (N = 3).

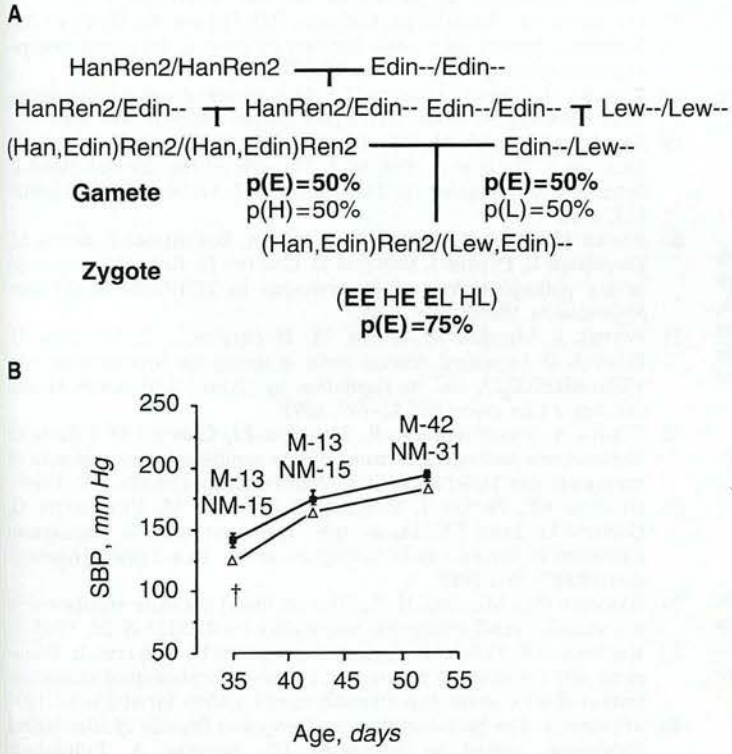

Fig. 4. A. *Seventy-three male F1 progeny, genotype (Edin,Han)Ren2/(Edin, Lew)— were bred from three (Han,Edin)Ren2/(Han,Edin)Ren2 males (derived from a HanRen2/Edin— intercross) and seven Edin—/Lew— females.* At the gamete stage the probabilities of carrying EdinSD (pE), HanSD (pH) and Lewis (pL) alleles are shown. The probability of at least one allele for any locus being derived from EdinSD (pE) in the (Edin,Han)Ren2/ (Edin, Lew)— progeny is therefore 3/4 or 75%. **B.** SBP was measured weekly by tail cuff plethysmography under light halothane anethesia in 28 of the progeny from 35 days of age and in all 73 rats at 52 days. The number of those destined to develop malignant (M, ■) and non-malignant phase (NM, △) is shown for each time point. At 35 days, there was a small but significant difference (*P = 0.02).

EdinSD and HanSD rats (N = 11 to 15 per group) had weekly tail cuff plethysmography performed under light halothane anesthesia from 35 to 90 days of age, and a slightly higher SBP was observed in HanSD compared to EdinSD [37].

HanRen2/Edin— showed the highest incidence of the malignant phenotype with a penetrance of 73.5% (65.7 to 81.3%), and did show a significantly higher MBP at 57 days (median age of death) compared to MBP in the other two groups at that age, but it was not significantly different to MBP of 70-day-old HanRen2/Han— or HanRen2/Lew—. Therefore absolute MBP may not be the critical factor precipitating the transition, but a higher blood pressure in younger rats may well be a marker of predisposition.

To test for the possibility of genetic susceptibility to malignant phase hypertension, a segregating cross for EdinSD alleles was designed. The incidence of the malignant phenotype, as defined by the onset of the described signs and confirmed by the presence of fibrinoid necrosis was 42 of 73 or 57.5% (45.9 to 69.5%) which was significantly different to the incidence seen in HanRen2/Edin—, but the age range (53 to 103 days) and median age (66 days) of affected animals was similar. SBP measured just before the expected time of onset of malignant phase (52 days) did not differ between malignant and non-malignant rats, but significantly higher SBP was observed in those destined to develop malignant phase hypertension at 35 days old.

The probability of at least one EdinSD derived allele being present at a given locus in the studied progeny is 3/4, thus if one dominant gene was responsible an expected penetrance would be 55.1% (95% confidence interval 49.3 to 61%), being 3/4 of 73.5% which is close to that observed. If two genes were involved, then the probability of EdinSD alleles being present at both loci would be 9/16, and hence the expected incidence would be 41.3% (95% confidence interval 36.9 to 45.7%). Two loci cannot be excluded at the present time as the upper limit of expected penetrance is not significantly different to that observed (χ^2 ranging from 2.028 to 6.18 for 1 df; P ranging from 0.01 to >0.05). Three genes can be excluded, the expected incidence being 22.6/73 or 31% (27.7 to 34.3%, χ^2 ranging from 7.972 to 11.706; $P < 0.01$). These results therefore most strongly support a monogenic model.

In conclusion, the spontaneous transition to malignant phase hypertension depends on either one or at most, two loci which confer susceptibility. A phenotypic marker of predisposition may be higher BP at a younger age, but absolute BP does not appear to be the single critical factor in precipitating transition. We speculate that an additional genetic factor is interacting with a rising BP, perhaps at the level of the endothelium, and is responsible for the stimulus initiating the transition into MH and allowing RAS activation. We believe that the HanRen2/Edin—cross offers a useful and clinically relevent model to study factors responsible for initiating malignant phase hypertension and its pathological sequelae, and will facilitate genetic mapping of the locus(ci) responsible.

Acknowledgments

This work was made possible with financial help from the Scottish Hospitals Endowment Research Trust and the A.F.R.C. The rabbit anti-mouse renin antibody was kindly given by Dr. D. Campbell, Melbourne, Australia. RIA of PRA and Ang II was done by Dr. J.J. Morton, MRC Blood Pressure Unit, Western Infirmary, Glasgow, Scotland, UK, and aldosterone measurements by N.J.T. Burns and Dr. B.C. Williams, Department of Medicine, Western General Hospital, Edinburgh, Scotland, UK.

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Appendix. *Abbreviations*

Ren2 - Mouse Ren2 renin transgene; BP - blood pressure; SBP - systolic blood pressure; MBP - mean blood pressure; RAS - renin-angiotensin system; PRA - plasma renin activity; Ang II - angiotensin II; EdinSD - Sprague-Dawley (Edinburgh strain); HanSD - Sprague-Dawley (Hannover strain); df - degrees of freedom.

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