Mechanisms underlying progressive nephron injury in chronic kidney disease

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Declaration

I, Maarten Willem Taal, hereby declare that the research described herein was performed by me with assistance as indicated in the acknowledgements. The dissertation was written by me and reviewed by my supervisor. Neither the whole thesis nor any part of it has been, is being or will be submitted by me for any other degree at this or any other University.

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4. Taal MW, Nenov VD, Satyal SR, Wong W, Sakharova O, Choi Jeong Hoon, Troy JL and Brenner BM. Vasopeptidase inhibition affords greater renoprotection than ACE inhibition alone. *J Am Soc Nephrol* 12:2051-2059, 2001.

Abstract

It has been appreciated for several decades that when renal disease of diverse aetiology results in substantial renal injury, a common clinicopathological syndrome ensues characterised by hypertension, proteinuria and a progressive decline in renal function due to focal and segmental glomerulosclerosis. These observations suggest that common mechanisms may contribute to progressive renal injury and that therapeutic interventions that inhibit these common pathways may result in renal protection.

Early studies of glomerular haemodynamic adaptations to nephron loss identified increased glomerular capillary hydraulic pressure (P_{GC}) as a critical factor. Subsequent experiments showed that treatment with angiotensin-converting enzyme inhibitors (ACEI) normalized P_{GC} and afforded substantial renal protection. Angiotensin II (Ang II) also appears to exert non-haemodynamic effects that may contribute to progressive renal injury. In addition proteinuria, recognized as a marker of glomerular injury, has recently been proposed to contribute directly to renal damage. Finally, a major role for proinflammatory cytokines and profibrotic growth factors in renal scarring has been proposed. The importance of ACEI in renal protective strategies has been confirmed in several experimental and clinical studies, indicating a central role for angiotensin II in the pathogenesis of chronic kidney disease (CKD). The development of the angiotensin subtype 1 receptor antagonists (AT_1RA), a new class of drug that inhibits the renin-angiotensin system by blocking the AT_1 receptor has made it possible to compare the effects on renal protection of inhibiting the renin-angiotensin system by two different means.

The first two studies presented in this dissertation examined the relative effects of ACEI and AT_1RA treatment in the 5/6 nephrectomy rodent model of CKD. In the first experiment drug treatment was started immediately after 5/6 nephrectomy. ACEI and AT_1RA treatment were equally effective in controlling hypertension and proteinuria as well as preventing renal injury over a period of 12 weeks. Reverse transcriptase PCR and immunohistology showed an increase in expression of several proinflammatory genes including TGF-β1, MCP-1, ICAM-1, VCAM-1, IL-1β and TNF-α as well as mesangial and interstitial infiltration by macrophages in untreated rats. Both drugs abrogated the proinflammatory gene expression and macrophage infiltration observed in untreated rats. In the second study treatment with either an ACEI or AT_1RA was delayed for 5 weeks after 5/6 nephrectomy, when evidence of renal injury was already

present. This strategy would potentially allow detection of more subtle differences in renal protective efficacy of ACEI vs. AT_1RA as both drugs reduced but did not prevent renal injury. Treatment with either ACEI or AT_1RA did not suppress remnant kidney expression of TGF-β1 or MCP-1 to normal levels at 12 or 24 weeks. Strong correlation was shown between TGF-β1, MCP-1 or IL-1β mRNA levels and the extent of renal injury at 24 weeks. In addition linear regression analysis showed that blood pressure and the level of proteinuria were major determinants of renal injury at 24 weeks. There was no difference in the extent of renal injury between rats receiving ACEI or AT_1RA treatment nor was there a difference in the relationship between renal injury, blood pressure and proteinuria between the groups. We conclude that in this model, ACEI and AT_1RA treatment afforded equivalent renal protection and exerted their beneficial effects predominantly by inhibiting the actions of Ang II mediated by AT_1 receptors. In addition our results lend support to the notion that coordinated upregulation of proinflammatory molecules and associated macrophage infiltration play an important role in CKD progression.

A third set of experiments revisited the concept of lowering P_{GC} with vasopeptidase inhibitors (VPI), a novel class of antihypertensive agents that inhibit neutral endopeptidase in addition to angiotensin-converting enzyme. A preliminary study showed that ACEI and VPI treatment both abrogated renal injury when started shortly after 5/6 nephrectomy. A subsequent study utilizing the delayed treatment model described above showed that VPI treatment delayed the progression of renal injury significantly more than ACEI treatment. In addition micropuncture studies revealed that VPI treatment reduced P_{GC} to significantly lower levels than ACEI treatment. We suggest that this greater lowering of P_{GC} may be one of the mechanisms whereby VPI treatment affords more effective renal protection.

Together these data shed new light on some of the many mechanisms implicated in the progression of CKD. This improved understanding should lead to more effective strategies for limiting renal injury in a wide variety of kidney diseases.

1. Literature Review

1.1. Introduction

Despite substantial advances in our understanding of the pathogenesis and pathophysiology of many renal disorders over the past 50 years there are as yet few effective treatments for specific renal diseases and many cases progress towards chronic renal failure. Moreover the rapid rise in the prevalence of type 2 diabetes mellitus in many parts of the world can be predicted to result in a large increase in the number of patients with diabetic nephropathy, the single most common cause of end-stage renal disease (ESRD) in many parts of the world. Consequently the worldwide population of patients requiring renal replacement therapy is projected to grow rapidly. In the U.S. alone approximately 300,000 people currently require chronic dialysis and 80,000 are living with renal transplants [1]. This "epidemic" of chronic kidney disease (CKD) has serious implications for health care providers and patients alike. Whereas first-world countries are struggling to come to terms with the high cost of dialysis, many third-world countries are simply unable to fund adequate dialysis provision. From a patient's perspective chronic dialysis is associated with an annual mortality rate of 20-25% and substantial morbidity. Whereas renal transplantation represents optimal renal replacement therapy there is a worldwide shortage of donor organs. Transplanted kidneys also tend to suffer a progressive decline in function over time. The development of more effective treatment for slowing the rate of progression of CKD therefore represents one of the most urgent priorities facing Renal Physicians today.

In an attempt to address this problem, much attention has been focused on mechanisms whereby CKD progresses to end-stage renal failure (ESRF). It has been appreciated for several decades that when diverse renal diseases result in substantial loss of functioning nephrons a common clinicopathological syndrome ensues, characterized by systemic hypertension, proteinuria and a progressive decline in glomerular filtration rate (GFR). The rate of decline in renal function seems to depend more upon individual patient characteristics than specific disease aetiology [2, 3]. These observations suggest that CKD progresses via a common pathway of mechanisms and that therapeutic interventions inhibiting these mechanisms may be successful in slowing the rate of progression of CKD irrespective of the initiating cause. Central to this hypothesis is the notion that the loss of functioning nephrons provokes adaptive changes in remaining nephrons ultimately resulting in injury to those nephrons and establishing a vicious

cycle of progressive nephron loss. Considerable progress has been made in understanding the central role of glomerular haemodynamic adaptations and the renin-angiotensin system in CKD progression. In this dissertation we review previously published work in this field describing the adaptive changes observed after nephron loss and discuss how these may contribute to progressive renal injury. We then present a series of experiments designed to define the role of selected factors and the impact of different treatment strategies in CKD progression.

1.2. Structural and functional adaptation of the kidney to nephron loss

1.2.1. Alterations in Glomerular Physiology

Glomerular haemodynamic responses to nephron loss have largely been studied in animals subjected to surgical ablation of renal mass. As early as 1947 it was recognized that unilateral nephrectomy in rats resulted in a rapid increase in function of the remaining kidney such that the GFR eventually achieved 70-85% of the previous 2-kidney value [4]. An increase in GFR is detectable 3 days after nephrectomy and is maximal 2-3 weeks later [5]. As no new nephrons are formed in mature rodents [6-11] this rise in GFR represents an increase in the filtration rate of remaining nephrons.

Detailed investigation of glomerular haemodynamics was made possible by the study of Munich-Wistar rats that are unique in bearing glomeruli on the kidney surface. Surface glomeruli are accessible for micropuncture studies that allow direct measurement of intraglomerular pressures as well as sampling of blood from afferent and efferent arterioles. These techniques made it possible to study the mechanisms underlying observed compensatory increases in GFR after renal mass ablation. Early studies found that increases in whole kidney GFR at 2-4 weeks after unilateral nephrectomy resulted from an in increase in single nephron GFR (SNGFR) averaging 83%, an effect achieved in large part by an augmented glomerular plasma flow rate (Q_A) that in turn resulted from dilation of both afferent and efferent arterioles. Although systemic blood pressure was not elevated, glomerular capillary hydraulic pressure (P_{GC}) and the glomerular transcapillary pressure difference (ΔP) were increased significantly post uninephrectomy, accounting for an estimated 25% of the rise in SNGFR [12]. The glomerular ultrafiltration coefficient K_f (the product of glomerular hydraulic permeability and surface area available for filtration) was unaltered at this stage but may become elevated later [13].

With more extensive nephron loss, even greater compensatory increases in SNGFR were observed [14]. In Munich-Wistar rats studied 7 days after unilateral nephrectomy and infarction of 5/6 of the contralateral kidney, SNGFR in the remnant was more than double that of 2-kidney controls. This increment was again attributable to large increases in Q_A, and a substantial rise in P_{GC}. Efferent and afferent arteriolar resistances were reduced to half or less of control values [15]. Changes in K_f after extensive renal mass ablation appear to be time-dependent, with a decrease reported at 2 weeks after surgery [16], and an increase at 4 weeks [17]. Further studies indicated that glomerular haemodynamic responses to nephron loss seem to be similar between the superficial cortical and juxtamedullary nephrons [18]. The rise in SNGFR associated with renal mass ablation is generally referred to as *glomerular hyperfiltration* and the elevated P_{GC} is termed *glomerular hypertension.* Together these terms summarise the central concepts underlying haemodynamic adaptations in the remnant kidney.

Glomerular haemodynamic adaptations to nephron loss may show interspecies variation. In dogs, increases in SNGFR observed 4 weeks after 3/4 or 7/8 nephrectomy were attributable largely to increases in Q_A and K_f. In contrast to the findings in rodents, ΔP was only modestly elevated. After ablation of 7/8 of their renal mass, dogs developed a significant rise in P_{GC} independent of arterial pressure, as a result of relatively greater relaxation of afferent versus efferent arterioles [19].

In humans, the effects of nephron loss on the physiology of the remnant kidney have been studied mainly in healthy individuals undergoing donor nephrectomy for kidney transplantation. Inulin clearance studies of the earliest kidney donors revealed that GFR in the donor's remaining kidney had increased to 65-70% of the previous 2-kidney value by 1 week post nephrectomy [20, 21]. A meta-analysis of data from 48 studies that included 2,988 living kidney donors estimated that GFR decreased on average by only 17ml/min after uninephrectomy [22]. These observations imply that single kidney GFR (and therefore also the average SNGFR) increases by 30%-40% after uninephrectomy in humans. There is currently no non-invasive method for estimating SNGFR or P_{GC} in humans and more detailed assessments of glomerular haemodynamics have thus not yet been possible.

1.2.2. Mediators of the glomerular haemodynamic responses to nephron loss

The factors that serve as signals to initiate the changes in glomerular haemodynamics after renal mass ablation remain ill defined. Nevertheless, the effector mechanisms have been studied extensively and haemodynamic changes can be attributed to the net effects of complex interactions of multiple factors each having specific and sometimes opposing actions on the various determinants of glomerular ultrafiltration. Several vasoactive substances, including angiotensin II (Ang II), natriuretic peptides (NP), endothelins (ET) and prostaglandins (PG) have been implicated in this process. Moreover, sustained increases in SNGFR also require resetting of the autoregulatory mechanisms that normally govern GFR and renal plasma flow (RPF).

1.2.2.1. Renin-Angiotensin System

Angiotensin (Ang) II appears to play a central role in the development of glomerular capillary hypertension following renal ablation and may also contribute to changes in K_f. Acute infusion of Ang II in normal rats results in a rise in P_{GC}, due to a greater increase in efferent than afferent arteriolar resistance, and reductions in Q_A and K_f [23-25]. Chronic administration of Ang II for 8 weeks resulted in systemic hypertension, lowered single kidney GFR and with the exception of K_f, elicited similar glomerular haemodynamic changes to those observed after acute infusion in both normal and uninephrectomized rats [13]. The importance of the influence of endogenous Ang II on glomerular haemodynamics in remnant kidneys was further revealed by studies with pharmacological inhibitors of the renin-angiotensin system (RAS). Chronic treatment of 5/6 nephrectomized rats with either angiotensin-converting enzyme inhibitors (ACEI) [26, 27] or angiotensin II (subtype 1) receptor antagonists (AT_1RA) [28, 29] resulted in normalization of P_{GC} through reduction in systemic blood pressure and dilatation of both afferent and efferent arterioles. SNGFR, however, remained elevated due to an increase in K_f. Furthermore, acute infusion of an ACEI or saralasin, a peptide analogue receptor antagonist of Ang II, was found to normalize P_{GC} in 5/6 nephrectomized rats through efferent arteriolar dilatation, without affecting mean arterial pressure (MAP) [16, 30]. For reasons that are not clear these changes were not duplicated by infusion of the AT_1RA, losartan, [31].

These effects of RAS inhibition imply that there is increased local activity of endogenous Ang II even though plasma renin levels are reduced following 5/6 nephrectomy [26, 32]. This suggests a differential regulation of the systemic versus intrarenal RAS and implies that Ang II is formed locally. Renin mRNA and protein levels are both increased in glomeruli adjacent to the infarction scar in 5/6 nephrectomized rats [33-35]. These findings suggest that ablating renal mass by infarction activates the RAS by creating a margin of ischaemic tissue around the organizing infarct, explaining the greater severity of hypertension and glomerulosclerosis obtained than that observed when renal mass is excised surgically [36]. Detailed studies of intrarenal Ang II levels following 5/6 nephrectomy achieved by infarction have confirmed these findings by showing higher Ang II levels in the peri-infarct portion of the kidney than the intact portion at all time points [32]. By contrast the rise in intrarenal Ang II following 5/6 nephrectomy was transient. Whereas Ang II levels in the peri-infarct portion were elevated compared to sham-operated controls 2 weeks after surgery, they were not statistically different at 5 or 7 weeks. In the intact portion of the remnant kidney Ang II levels were similar to controls at 2 and 5 weeks and were lower at 7 weeks [32]. Sustained increases in intrarenal Ang II levels are therefore not required to maintain the hypertension and progressive renal injury characteristic of this model.

Subsequent studies have shown that the renal protective effects of ACEI and AT_1RA treatment are associated with a reduction in intrarenal Ang II levels in both the peri-infarct and intact portions of the remnant kidney [37]. By contrast, treatment with the dihydropyridine calcium antagonist, nifedipine, did not reduce proteinuria despite lowering blood pressure to the same levels as the RAS antagonists and was associated with an increase in intrarenal Ang II [37]. Thus intrarenal Ang II appears to play a central role in the pathogenesis of hypertension and renal injury in this model even in the absence of sustained increases in Ang II levels. Further research is required to fully explain these findings. It is possible that apparently normal intrarenal Ang II levels are inappropriately high in the context of hypertension and ECF volume expansion in these animals or alternatively average intrarenal Ang II levels measured may have failed to detect important local elevations of Ang II.

1.2.2.2. Endothelins

Renal production of endothelins is increased after 5/6 nephrectomy, raising the possibility that these potent vasoconstrictor peptides may also contribute to the characteristic adjustments in glomerular haemodynamics described above [38, 39]. Acute infusion of endothelin consistently elicits dose dependent reductions in RPF and GFR in normal rats and dogs [40-43]. Chronic infusion of endothelin-3 in normal rats resulted in sustained reductions in RPF and GFR, likely due to constriction of both afferent and efferent arterioles, with resultant reductions in Q_A and SNGFR [44]. Inconsistencies in the observed effects of endothelins on P_{GC} may reflect differences in experimental conditions that influenced the relative effects on afferent and efferent arterioles [43, 45-47]. Most studies have however reported a reduction in K_f after endothelin infusion [43, 45-47].

1.2.2.3. Natriuretic peptides

Atrial natriuretic peptide (ANP) and other structurally related natriuretic peptides (NP) mediate to a large extent the functional adaptations in tubular sodium reabsorption that maintain sodium excretion in 5/6 nephrectomized rats [48, 49]. NPs also appear to be important mediators of observed increases in GFR and RPF. Circulating ANP levels are elevated in 5/6 nephrectomized rats [49, 50] and acute administration of a NP antagonist elicited profound decreases in GFR and RPF in rats on high salt diet but not in those subjected to sodium restriction [51]. When pharmacological doses of exogenous ANP were administered to normal rats, increases in baseline GFR were observed that were attributable to a rise in P_{GC} resulting from significant afferent arteriolar dilatation [52]. Nevertheless, baseline remnant kidney GFR was not decreased when the NP system was suppressed by sodium restriction suggesting that factors other than NPs contribute to the afferent arteriolar vasodilatation associated with glomerular hyperfiltration in this model [51].

1.2.2.4. Eicosanoids

Renal prostaglandins, another family of potent vasoactive molecules present in abundance in the kidney, may also play a role in mediating glomerular hyperfiltration. Infusion of PGE_2, PGI_2 or 6-keto PGE_1 into the renal artery elicits significant renal vasodilatation in normal kidneys [53]. After renal mass ablation urinary excretion per nephron of both vasodilator and vasoconstrictor prostaglandins is increased in rats

and rabbits [54-57] and acute inhibition of prostaglandin synthesis by infusion of indomethacin after 3/4 or 5/6 nephrectomy lowers both SNGFR and Q_A [55, 56]. The relative effects of prostaglandin synthesis inhibitors on afferent and efferent arterioles may vary with time post nephrectomy. Afferent arteriolar constriction was the predominant finding reported at 24 hours post surgery, whereas constriction of both afferent and efferent arterioles was observed at 3-4 weeks [55, 56]. Some contribution of thromboxanes to glomerular haemodynamic adjustments in 5/6 nephrectomized rats is suggested by the increase in GFR seen after acute infusion of a selective thromboxane synthesis inhibitor [57]. Nevertheless the general impression, although not entirely uniform [58], is that the combined effects of vasodilator prostaglandins outweigh those of the vasoconstrictors.

1.2.2.5. Nitric oxide

Intravenous infusion of nitric oxide synthase (NOS) inhibitors results in systemic and renal vasoconstriction in rats [59, 60], dogs [61, 62] and rabbits [63], and a reduction in GFR in rats and dogs. Chronic NOS inhibition in 5/6 nephrectomized rats produced elevations in systemic blood pressure and P_{GC} without affecting GFR [64]. Thus NO appears to exert a tonic effect on the physiological maintenance of systemic blood pressure and renal perfusion under resting conditions. It is unclear, however, whether NO plays a specific role in the adaptive haemodynamic changes that follow renal mass ablation. MAP, renal vascular resistance, RBF and GFR all changed to a similar extent after acute infusion of a NOS inhibitor irrespective of whether given to normal, uninephrectomized or 5/6 nephrectomized rats. It therefore appears that NO retains a tonic influence on systemic and renal haemodynamics in the context of renal mass ablation but is not a specific determinant of the adaptive changes in glomerular haemodynamics [60]. This view is further supported by a recent study, which found that renal expression of all three isoforms of NOS is decreased after 5/6 nephrectomy. Furthermore, inhibition of the inducible isoform of NOS with aminoguanidine had no effect on renal haemodynamic variables including P_{GC}. Interestingly, aminoguanidine treatment did attenuate the extent of glomerular and interstitial fibrosis, possibly through anti-inflammatory mechanisms [65].

Table 1.1. Haemodynamic effects of vasoactive molecules mediating glomerular haemodynamic adaptations after partial renal mass ablation

	R_A	R_E	P_{GC}	Q_A	K_f	SNGFR	RPF	GFR
angiotensin II [26-29]	↑	↑↑	↑	↓	↓↔	↓↔	↓	↔
endothelins [40-47]	↑↔	↑	↑↔	↓	↓↔	↓↔	↓	↓↔
natriuretic peptides [51, 52]	↓	↑(?)	↑	↔	↔	↑	↑↔	↑
prostaglandins [55, 56, 58]	↓	↓	↔	↑	↑	↑	↑	↑
observed changes after partial renal ablation [14-18]	↓↓	↓	↑	↑	↑↓	↑	–	↓

1.2.2.6. Adjustments in renal autoregulatory mechanisms

There is a marked readjustment of the autoregulatory mechanisms that control RPF and GFR after extensive renal mass ablation [66, 67]. The tubuloglomerular feedback system is reset to permit and sustain the elevations in SNGFR and P_{GC} described above [68, 69]. Resetting appears to occur as early as 20 minutes after unilateral nephrectomy [70] and the magnitude of the change is in proportion to the extent of renal ablation [68].

The adjustments in glomerular haemodynamics seen after renal mass ablation represent the net effect of several of the endogenous vasoactive factors discussed above (Table 1.1.). Ang II, vasoconstrictor prostaglandins and possibly endothelins constrict both afferent and efferent arterioles whereas NPs and vasodilator prostaglandins dilate predominantly the preglomerular vessels. A net fall in preglomerular vascular resistance may therefore be expected, whereas efferent arteriolar resistance may decrease to a lesser degree. The role of bradykinin, a potent vasodilator that is elevated in the remnant kidney [32], remains to be defined. Together with transmission of a greater proportion of the raised systemic blood pressure to the glomerular capillary network, these alterations in microvascular resistances account for the observed elevations in Q_A, P_{GC}, ΔP and SNGFR.

1.2.3. Renal Hypertrophic Responses to Nephron Loss

The notion that a single kidney enlarges to compensate for the loss of its partner has been entertained since antiquity. Aristotle (384-322 BC) noted the sufficiency of a single kidney to sustain life in animals and that such kidneys were enlarged [71, 72]. In preparation for the first human nephrectomy in 1869 a German surgeon, Gustav Simon, uninephrectomized dogs and noted a 1.5 fold increase in the size of the remaining kidney at 20 days [71, 72]. Compensatory renal hypertrophy has been studied in a variety of species including rats, mice, guinea pigs, rabbits, cats, dogs, pigs, baboons and toads [73]. In recent years the majority of experimental work has been conducted on rodents subjected to uninephrectomy. Hypertrophic responses have also been studied in response to unilateral ureteric obstruction [74-78] or after nephrotoxin administration [79], presumably reflecting hypertrophy of the least injured nephrons.

1.2.3.1. Whole - Kidney Hypertrophic Responses

Among the earliest responses to unilateral nephrectomy are biochemical changes that precede cell growth. Increased incorporation of choline, a precursor of cell membrane phospholipid, has been detected as early as 5 minutes [80, 81] and increased choline kinase activity at 2 hours after nephrectomy [82]. Activity of ornithine decarboxylase, the enzyme catalyzing the first step of polyamine synthesis, is elevated at 45-120 minutes and polyamine levels peak 1-2 days post nephrectomy [83-85]. Early alterations in mRNA metabolism have also been observed. Although there is no change in the half-life or cytoplasmic distribution of mRNA, a near 25% increase in the fraction of newly-synthesized poly (A)-deficient mRNA occurs within 1 hour of uninephrectomy and total RNA synthesis in the kidney increases by 25%-100% relative to that in the liver [86-88]. Ribosomal RNA synthesis is increased by 40%-50% at 6 hours [89]. The rate of protein synthesis is increased at 2 hours and is nearly doubled at 3 hours [90]. Data on cyclic nucleotide levels, which are thought to affect cell growth and proliferation, are conflicting. Some studies report elevated levels of cGMP in the remaining kidney as early as 10 minutes after surgery [91-93] whereas others have found no consistent changes in cAMP or cGMP levels [94].

Early biochemical changes are followed by a period of rapid growth. DNA synthesis is increased at 24 hours and increased numbers of mitotic figures are evident at 28-36 hours. Both reach a maximum increase of 5-10 fold at 40-72 hours [73, 95-98]. Kidney weight is increased at 48-72 hours and on

average achieves a 35% gain at 2-3 weeks [73, 99]. As nephron number is fixed shortly after birth in most species, this gain in kidney weight is attributable to increased nephron size. Growth is thought to occur largely through cell hypertrophy, accounting for 80% of the increase in renal mass seen in adult animals and, to a lesser extent, through hyperplasia [90]. Renal mass continues to rise for 1-2 months until a 40-50% increase is achieved [73]. The degree of compensatory growth is related to the extent of renal ablation. Uninephrectomy has been shown to provoke an 81% increase of residual renal mass at 4 weeks compared to an increase of 168% after 70% renal ablation. Controls gained 31% in kidney weight during normal growth over the same period [100]. Hypertrophic responses in the kidney appear to diminish with age. After uninephrectomy, greater increases in kidney weight and more extensive hyperplasia were observed in 5-day vs. 55-day old rats [73, 101] and aging rats exhibited gains in kidney weight of only 1/3 to 3/4 of those seen in younger controls [73, 102-104].

In humans, radiological studies estimating gain in renal size after uninephrectomy based on tomography or intravenous urograms reported average increases of 3.3%-9% in renal length [105-108], 18%-23% in renal size "index" (product of length and width) [105, 109], or 20% in renal cross sectional area, as assessed by planimetry [110]. One study, however, was unable to detect any increase in average renal length [111]. Ultrasound estimates report inceases of 19%-100% in kidney volume [112-114] and CT studies show an increase of 30%-53% in renal cross-sectional area [115, 116]. Interpretation of these data is complicated by the small numbers of subjects, wide variation in the time intervals between nephrectomy and assessment of renal size as well as differing indications for nephrectomy.

1.2.3.2. Glomerular Enlargement

The principal morphometric change observed in glomeruli after *uninephrectomy* is an increase in volume [117-121]. Glomerular enlargement appears to parallel whole-kidney growth and has been detected as early as 4 days after surgery [120]. The degree of enlargement of superficial and juxtamedullary glomeruli is similar. Proportionally similar increases in number and size of all cell types occur with preservation of the relative volumes of different glomerular components [118, 119]. There is consensus that glomerular capillaries increase in length and number (i.e. more branching) but most studies report that the diameter or cross sectional surface area of the glomerular capillaries remains constant or increases only minimally

[118, 119, 122, 123]. Transplantation of hypertrophied kidneys into uninephrectomized recipients has demonstrated regression of glomerular hypertrophy within 3 weeks, yet the increase in capillary length was maintained [123].

Glomerular hypertrophy, as evidenced by elevated RNA/DNA and protein/DNA ratios and by increased glomerular volume (V_G) on electron microscopy, has been detected at 2 days after 5/6 nephrectomy [124]. The initial increase in V_G was almost entirely due to increases in visceral epithelial cell volume, whereas at 14 days the increase in V_G was largely accounted for by mesangial matrix expansion. Several studies report glomerular capillary lengthening after 5/6 nephrectomy but few have detected any increase in cross sectional area or diameter of the glomerular capillaries [125-128]. These observations should, however, be considered in the light of important technical considerations. In vitro perfusion of isolated glomeruli demonstrates that V_G increases as perfusion pressure is raised through physiological and pathophysiological ranges. Moreover, glomerular capillary "compliance" in these studies was a function of the baseline V_G such that glomeruli obtained from remnant kidneys post 5/6 nephrectomy had a higher compliance than those from control animals [129]. These findings have two important implications. Firstly, glomerular pressures are only minimally elevated after uninephrectomy yet the glomerular capillary hypertension associated with more extensive renal ablation is likely to contribute significantly to the increase in V_G. Secondly, estimates of V_G in tissues that have not been perfusion-fixed at the appropriate blood pressure should be interpreted with caution. Direct comparison of V_G in perfusion-fixed versus immersion-fixed kidney from the same rats yielded estimates of V_G in immersion-fixed samples that were 61% lower than those from perfusion-fixed kidneys [130].

1.2.3.3. Mechanisms of Renal Hypertrophy

Despite more than a century of research that has identified a large number of mediators or modulators of renal hypertrophy, the identities of the specific factors that regulate hypertrophy and the stimuli to which these factors respond remain elusive. Several hypotheses have been advanced to account for the observed changes that are associated with renal hypertrophy but none is able to explain satisfactorily all of the reported observations. A detailed discussion of these hypotheses is beyond the scope of this dissertation and here they are summarised briefly. The "solute load" hypothesis proposes that renal

hypertrophy is a response to the increased demand for water and solute reabsorption imposed by increased SNGFR [72, 131]. In contrast, others have proposed that renal growth is under the control of specific growth and/or inhibitory factors and that the primary stimulus for renal hypertrophy is a change in renal mass. Whereas a substantial body of evidence supports a role for a putative "renotropin", such a molecule has to date not been identified [132]. Several of the major endocrine systems influence renal growth but each lacks selective effects on the kidney. At least three growth factors, insulin-like growth factor-I (IGF-I) [133-137], epidermal growth factor (EGF) [138, 139] and hepatocyte growth factor (HGF) [140, 141] appear to be associated with renal hypertrophy [142, 143]. Nevertheless, the timing of changes in growth factor levels in relation to hypertrophy remains unclear. Whereas some investigators report early increases [135, 138], several others report changes only at time points when significant hypertrophy is already present, thus failing to provide convincing evidence that they represent the proximal effectors in a renotropic system [133, 134, 136, 137, 139].

1.3. Adverse long-term consequences of adaptations to nephron loss

The above haemodynamic and hypertrophic responses to renal mass ablation may readily be regarded as appropriate adaptations that allow the remnant kidney to partially compensate for nephron loss. However, a considerable body of evidence suggests that in the long-term, these adaptations may result in injury to remaining nephrons and thus provoke a vicious cycle of progressive renal injury. The adverse effects of extensive renal mass reduction have been appreciated since 1932, when rats subjected to partial nephrectomy were observed to develop hypertension, albuminuria, remnant kidney hypertrophy and azotemia [144]. Detailed histopathological studies performed several decades later revealed that early hypertrophy in rat remnant kidneys after 5/6 nephrectomy was followed by mesangial accumulation of hyaline material that progressively encroached on capillary lumina, obliterating Bowman's space and finally resulting in global sclerosis of the glomerulus [145]. These findings, together with the observation that sclerosed glomeruli are a common finding in human CKD of diverse aetiology, led the authors to speculate that the pathological findings described were a consequence of glomerular hyperfiltration resulting from a reduction in nephron number [145]. The 5/6 nephrectomy model has been extensively studied over several decades and considerable progress has been made in elucidating how the physiological adaptations of remaining nephrons that initially permit greatly augmented function per

nephron, ultimately produce a complex series of adverse effects that eventuate in progressive renal injury and an inexorable decline in function.

1.3.1. Haemodynamic Factors

As early as 1 week after extensive renal mass ablation, glomerular hyperfiltration and glomerular capillary hypertension are associated with morphological changes including cytoplasmic attenuation, protein reabsorption droplets and foot process fusion in visceral epithelial cells, mesangial expansion and focal lifting of endothelial cells from the basement membrane [15]. Evidence that these morphological changes were indeed a consequence of the glomerular haemodynamic alterations was provided by studies in rats subjected to extensive renal mass ablation and fed a low protein diet. This intervention prevented the haemodynamic changes, effectively normalizing Q_A, P_{GC} and SNGFR and also largely attenuated the structural lesions observed in rats on a standard diet. Dietary protein restriction also prevented the proteinuria and glomerulosclerosis that subsequently developed in rats on a standard diet [15]. Similar findings were subsequently described in a variety of animal models of CKD including diabetic nephropathy [146, 147] and deoxycorticosterone (DOCA)-salt hypertension [148]. Together, these observations led Brenner et al. to propose that the haemodynamic adaptations following renal mass ablation ultimately prove injurious to glomeruli and initiate processes that result in glomerulosclerosis. Loss of further glomeruli would induce hyperfiltration in remaining, less affected glomeruli, thereby establishing a vicious cycle of progressive nephron loss. This vicious cycle therefore constitutes a "common pathway" for renal damage that could account for the inexorable progression of CKD, independent of the cause of the initial renal injury (Figure 1.1.) [149]. The hypothesis also explained the typical finding of both atrophic and hypertrophic nephrons in chronically diseased kidneys. Further evidence supportive of the "hyperfiltration hypothesis" was gleaned from the study of experimental diabetic nephropathy in which glomerular hyperfiltration was also found to be a forerunner of glomerular pathology [15, 147]. Manoeuvres such as unilateral nephrectomy, which exacerbates hyperfiltration in the remaining kidney, were found to exacerbate diabetic renal injury in the remaining kidney [150]. When a kidney was shielded from hyperfiltration and glomerular capillary hypertension by creating unilateral renal artery stenosis, the ipsilateral kidney was protected against development of diabetic injury, which progressed unabated in the contralateral kidney [151]. In addition, when glomerular hyperfiltration was reversed in 5/6

nephrectomized rats by transplantation of an isogeneic kidney, hypertension and proteinuria were ameliorated and glomerular injury was limited [152]. Similarly, augmenting renal mass in the Fisher→Lewis rat transplantation model normalized P_{GC} and greatly reduced the development of chronic renal allograft injury [153, 154]. Direct evidence that similar mechanisms may operate in human kidneys is derived from a study of 14 patients with solitary kidneys who had undergone varying degrees of partial nephrectomy of the remaining kidney for malignancy [155]. Before surgery proteinuria was absent in all patients. Although serum creatinine remained stable after an initial rise of 50% in 12 patients, 2 patients subjected to the most extensive nephrectomy (75% and 67%, respectively) developed progressive renal failure and required long-term dialysis. Moreover, among the remaining patients, 7 developed proteinuria, the levels of which were inversely related to the amount of renal tissue preserved. Renal biopsy specimens in 4 patients with moderate to severe proteinuria showed FSGS [155], which later morphometric analysis revealed to involve virtually all glomeruli examined [156].

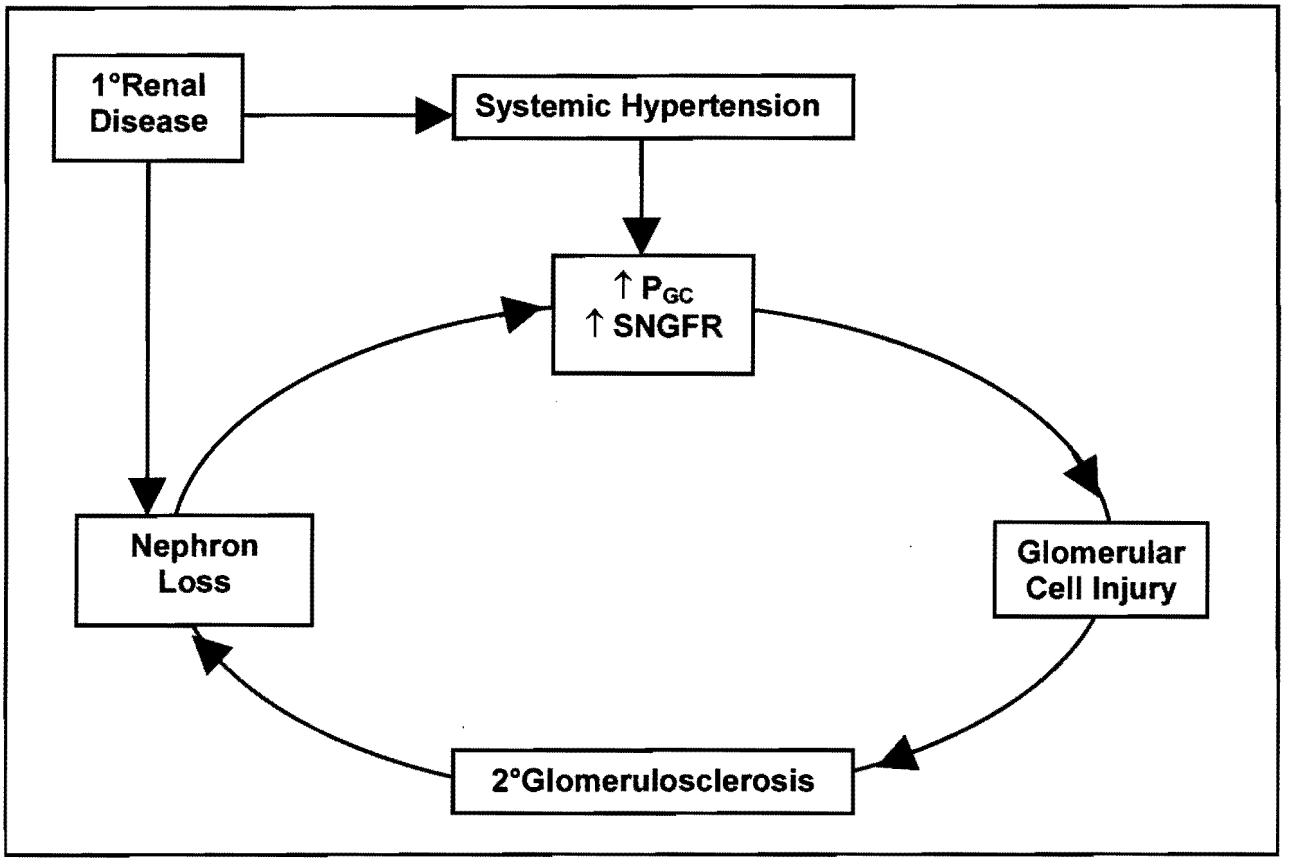


Figure 1.1. Schema illustrating the "haemodynamic hypothesis" proposed by Brenner et al. to explain the mechanisms underlying progressive nephron loss in CKD [149].

The importance of glomerular haemodynamic factors in the development of progressive renal injury was further illustrated by studies that reported dramatic protective effects against the development of glomerulosclerosis after chronic inhibition of the RAS with either ACEI or AT_1RA in 5/6 nephrectomized rats [26-29]. Micropuncture studies showed that, as with low protein diet, the renal protective effects of RAS inhibition were associated with near normalization of the P_{GC}. In contrast to the effects of dietary protein restriction, SNGFR remained elevated [26]. This suggested that glomerular capillary hypertension, rather that hyperfiltration *per se* was the key factor in the initiation and progression of glomerular injury. Confirmation of this view came from an experiment in which rats were treated with a combination of reserpine, hydralazine and hydrochlorothiazide ("triple therapy") to lower arterial pressure to levels similar to those obtained with an ACEI. In contrast to the glomerular haemodynamic effects of the ACEI, triple therapy did not alleviate glomerular hypertension or proteinuria and glomerular injury progressed unabated [27, 28]. The effectiveness of both ACEI and AT_1RA in lowering glomerular pressure and ameliorating glomerular injury has since been observed in several other animal models of chronic renal disease. These include models of diabetic nephropathy [146, 157, 158], hypertensive renal disease [159, 160], experimental chronic renal allograft failure (a model that lacks systemic hypertension but exhibits glomerular capillary hypertension) [161-163]; age-related glomerulosclerosis [164, 165] and obesity-related glomerulosclerosis [166]. It is noteworthy that the phase of transition from an acute, nonhypertensive experimental injury induced by puromycin aminonucleoside (PAN) administration, to a chronic nephropathy characterized by proteinuria and glomerulosclerosis, is also associated with the development of glomerular capillary hypertension [167]. Clinical trials showing substantial renal protective effects with ACEI and AT_1RA treatment strongly suggest that similar mechanisms are relevant in human CKD progression [168-171]. In contrast to the RAS, the role of endothelins in mediating glomerular capillary hypertension and experimental renal disease progression is less well defined. Pharmacological blockade of endothelin receptors has been shown to ameliorate disease progression in some studies but glomerular haemodynamics were not investigated and the mechanisms involved therefore require further elucidation [172-174].

1.3.2. Mechanisms of haemodynamically – induced injury

1.3.2.1. Mechanical Stress

Whereas the importance of glomerular haemodynamic factors in the pathogenesis of glomerular injury has been appreciated for two decades, the specific mechanisms whereby elevated P_{GC} may result in glomerular cell injury have only recently been elucidated. According to the Law of Laplace (which states that wall tension of a cylinder is proportional to the product of its radius and the intraluminal pressure), a rise in P_{GC} produces an increase in capillary wall tension that results in stretching of the glomerular capillary wall [129]. Owing to the pulsatile nature of glomerular perfusion, glomerular cells are exposed to cyclical stretching. In addition, endothelial cells are exposed to barostress and shear stress that result from increased glomerular pressure and flow. Experimental evidence suggests that excessive mechanical forces may have adverse consequences for all three major cell types in the glomerulus. Furthermore, advances in the study of cellular responses to mechanical stress raise the possibility that glomerular hyperperfusion may promote the development of glomerulosclerosis through more subtle and complex pathways that induce profibrotic phenotypic alterations in glomerular cells [175].

1.3.2.2. Endothelial Cells

The vascular endothelium serves multiple complex functions including acting as a dynamic barrier to leukocytes [176] and plasma proteins, secretion of vasoactive molecules (prostacyclin, nitric oxide (NO) and endothelin), conversion of Ang I to Ang II [177] and expression of cell adhesion molecules. It is also the first cellular structure in the kidney that encounters the mechanical forces resulting from glomerular hyperperfusion. After extensive renal mass ablation endothelial cells are activated or injured, resulting in detachment and exposure of the basement membrane. This in turn may induce platelet aggregation, deposition of fibrin and intracapillary microthrombus formation [15, 178, 179]. In addition to direct cellular injury, exposure of endothelial cells to the shear stress, cyclic stretch or pulsatile barostress that results from glomerular hyperperfusion may also increase expression of cell surface adhesion molecules, cytokines, MHC molecules, increase platelet adhesiveness and alter endothelial barrier functions [180-182]. In vitro, shear stress can stimulate expression of the adhesion molecule, intercellular adhesion molecule (ICAM)-1, on endothelial cell surfaces [183]. Ambient barometric pressure has been shown to enhance expression of endothelin [184] and basic fibroblast growth factor bFGF [185] by endothelial cells.

Exposure to cyclic stretch induced cell proliferation, increased cAMP generation [186] and enhanced endothelin release in endothelial cell cultures [187, 188]. These effects may be mediated by calcium entry into endothelial cells via mechanosensitive ion channels in the cell surface membrane [189, 190]. Mechanical stress - induced alterations in endothelial cell synthesis of vasoactive and growth promoting factors may shift the local balance to favour the vasoconstrictive and growth-promoting effects of factors such as endothelins and Ang II over the vasodilating and growth-inhibitory properties of molecules such as prostacyclin, heparan sulphate and NO [191]. Transforming growth factor (TGF)-β, another product of activated endothelial cells that is upregulated in the glomerular endothelium of 5/6 nephrectomized rats [192], may have a dual role, having the potential to act as a growth promoter at low levels and a suppresser at higher levels [193]. The complexity of endothelial responses to mechanical stress is underscored by the finding that the supernatant of cells cultured under sustained shear stress conditions has an antiproliferative effect on cultured mesangial cells [194]. Macrophage chemoattractant protein (MCP)-1, as well as being responsive to its classic cytokine inducers, interleukin (IL)-1, tumour necrosis factor (TNF)-α interferon (IFN)-γ and lipopolysaccharide [195], is also upregulated in endothelial cells exposed to laminar shear stress [196] and is overexpressed in the arteries of rats with hypertension [197]. Furthermore, endothelial cells may contribute autocrine and paracrine effects via classic cytokine signalling mechanisms. For example, activated endothelial cells may release TNF-α, an autocrine inducer of endothelial cell adhesion molecule expression [198-200]. Expression of endothelial cell adhesion molecules could in turn lead to indirect amplification of endothelial cell activation via TNF-α or IL-1 derived from adjacent glomerular cells or monocytes tethered to the endothelium [201]. It is clear from the above why biomechanical activation has been described as an "emerging paradigm" in endothelial cell biology [202] and there is now considerable support for the notion of endothelial activation as a mechanism for transducing the adverse effects of glomerular hyperperfusion.

Recent studies have shed further light on the potential importance of endothelial cell injury in progressive renal injury. It has been recognized for some time, that segmental glomerulosclerosis is associated with focal obliteration of capillary loops [203] and that interstitial fibrosis is associated with loss of peritubular capillaries [204]. This loss of capillaries in the remnant kidney is associated with a decrease in endothelial cell proliferation and reduced constitutive expression of vascular endothelial growth factor (VEGF) by

podocytes and renal tubule cells as well as increased expression of the anti-angiogenic factor, thrombospondin-1, by the renal interstitium [205]. Since VEGF is an important endothelial cell angiogenic, survival and trophic factor, these findings suggest that capillary loss may be in part due to failure of recovery from haemodynamically mediated endothelial cell injury. Furthermore, short-term treatment of rats with VEGF reduced both glomerular and peritubular capillary loss after 5/6 nephrectomy [206]. This preservation of capillaries was associated with a trend towards less glomerulosclerosis and significantly less interstitial deposition of type III collagen as well as better preservation of renal function. Long-term studies are required to evaluate further the potential benefit of improving renal angiogenesis in the setting of progressive renal injury.

1.3.2.3. Mesangial Cells

Subjecting mesangial cells to cyclical stretch or strain in vitro induces proliferation [207] and synthesis of matrix constituents including collagen [208, 209], or switches mesangial cell phenotype to overexpress extracellular matrix constituents and promote fibrogenesis [210]. Transduction of mechanical forces by mesangial cells has been associated with tyrosine phosphorylation [211] and protein kinase C-induced increases in S-6 kinase activity [212]. Mesangial cells cultured at ambient pressures of 50-60mmHg (levels corresponding to glomerular capillary hypertension) show enhanced synthesis and secretion of extracellular matrix when compared with cells grown at "normal" pressures of 40-50mmHg [213]. When this intervention was applied to cultured macrophages, the higher pressure was associated with increased secretion of factors that induced mesangial cell proliferation [213]. Cyclic stretch stimulates mesangial cell synthesis of the profibrotic growth factors TGF-β [214] and connective tissue growth factor (CTGF) [215] as well as increased expression of TGF-β receptors [216]. Since TGF-β is closely associated with fibrotic states, it has been suggested that TGF-β may represent an important link between glomerular capillary hypertension and glomerulosclerosis [217]. Cyclical stretch also activates the RAS within cultured mesangial cells [218] and Ang II in turn may induce TGF-β synthesis [219]. Exposure of mesangial cells to barostress, achieved by culture under increased barometric pressure, also stimulates expression of cytokines including platelet derived growth factor (PDGF)-B [220] and MCP-1 [221].

1.3.2.4. Podocytes

As mentioned above, podocytes display morphological evidence of injury as early as 1 week after extensive renal mass ablation [15]. Subsequent studies have identified similar evidence of podocyte injury at six months after uninephrectomy [222] and after renal ablation in rats with adriamycin-induced nephropathy [223]. Detailed in vitro studies have recently revealed that podocytes respond to cyclical stretching by undergoing reversible Ca^{2+} influx- and Rho kinase-dependent reorganization of the actin cytoskeleton, a response not seen in fibroblast or epithelial cell lines [224]. This specific mechanosensitivity suggests that podocytes may also respond to mechanical forces resulting from glomerular hypertension, but further studies are required to elucidate the nature and relevance of this response.

1.3.2.5. Inflammatory cell infiltration

A cellular infiltrate composed predominantly of macrophages and smaller numbers of lymphocytes accompanies clinical and experimental FSGS [225-229]. The presence of glomerular macrophages has led the development of secondary focal glomerulosclerosis following extensive renal mass ablation to be compared to the pathogenesis of atherosclerosis [230]. In the latter setting, over expression of endothelial adhesion molecules [231] is associated with passage of leukocytes across the endothelium, where monocytic infiltrates are thought to contribute to subintimal proliferation by releasing cytokines and growth factors [232]. Similarly, the observed upregulation of renal endothelial adhesion molecules may facilitate egress of leukocytes from the circulation into the mesangium, where they may participate in further renal injury. The recruited cellular infiltrate represents an abundant source of potent pleiotropic cytokine products that may influence other infiltrating leukocytes and kidney cells, further stimulating cell proliferation, elaboration of extracellular matrix components and increased endothelial adhesiveness as well as alterations in renal haemodynamics. Evidence is now emerging that these proposed mechanisms, based largely on in vitro observations, are indeed relevant in vivo. In the 2-kidney 1-clip model of renovascular hypertension, upregulated expression of adhesion molecules and TGF-β as well as cell infiltration is observed only in the non-clipped kidney that is exposed to the hypertensive perfusion pressure [233, 234]. In the 5/6 nephrectomy model a prominent monocyte/macrophage infiltrate has been

reported by several authors [235, 236] and in one study these macrophages showed enhanced TGF-β expression [237].

Several lines of evidence suggest that this cellular infiltrate does contribute to renal injury and is not merely a consequence of it. In one study multiple linear regression analysis identified glomerular macrophage infiltration in the remnant kidney as a major determinant of mesangial matrix expansion and adhesion formation between Bowman's capsule and glomerular tufts [238]. Furthermore, depletion of leukocytes in rats by irradiation delayed the onset of glomerular injury after renal ablative surgery [226]. Several studies have reported amelioration of the cellular infiltrate and renal injury in the 5/6 nephrectomy model following treatment with the immunosuppressive agent, mycophenolate mofetil [239-242]. Together, these data are consistent with the notion that chronic inflammatory processes are activated in the remnant kidney and contribute to progressive renal injury.

Infiltrating cells, although present in the glomeruli of remnant kidney, are chiefly distributed in the tubulointerstitial regions [225-227]. Their role in the development of tubulointerstitial fibrosis that accompanies glomerulosclerosis is unclear. It is possible that interstitial infiltrates are recruited as the result of tubulointerstitial cell activation by the downstream effects of cytokines released in the glomeruli. More recently, it has been proposed that the proinflammatory phenotype of tubule epithelial cells that take up filtered proteins may account for the expression of cell adhesion and chemoattractant molecules that recruit macrophages and other monocytic cells to tubulointerstitial areas [243]. The finding that renal tubule cells in the remnant kidney of 5/6 nephrectomized rats expressed α-smooth muscle actin, a myofibroblast marker not usually expressed by epithelial cells [244], suggests that transdifferentiation of renal tubule epithelial cells to a myofibroblast phenotype may have an important role in the development of tubulointerstitial fibrosis [245]. Interstitial myofibroblasts are thought to contribute to tubulointerstitial fibrosis in association with increased expression of platelet derived growth factor (PDGF) by distal tubule and collecting duct epithelial cells [246]. The significance of these observations remains to be determined.

The importance of inflammatory factors acting "downstream" from the haemodynamic changes in the common pathway mechanisms of CKD progression has recently been demonstrated by studies using a peroxisome proliferator-activated receptor γ (PPARγ) receptor agonist [247]. These compounds have recently been introduced as antidiabetic agents that improve insulin resistance in type 2 diabetes mellitus. The PPARγ receptor is a member of the nuclear receptor superfamily of transcriptional factors and in vitro studies suggest that PPARγ receptor agonists may have a wide range of effects including modulation of adipocyte differentiation, macrophage function and activation of other transcription factors [247]. Rats treated with a PPARγ receptor agonist after 5/6 nephrectomy showed significant attenuation of the proteinuria and glomerulosclerosis observed in untreated rats, despite the failure of treatment to lower blood pressure. This renal protection was observed in association with marked reductions in glomerular cell proliferation, glomerular macrophage infiltration, and renal expression of plasminogen activation inhibitor (PAI)-1 and TGF-β [247]. The authors speculate that some of these effects may have resulted from the known actions of PPARγ receptor activation to antagonize the activities of the transcription factors AP-1 and NF-κB. These findings strongly support the hypothesis that downstream cellular and molecular mediators of the effects of glomerular haemodynamic adaptations are critical to progressive renal injury after nephron loss. Treatments that antagonize these mediators may therefore be of benefit in slowing the rate of progression of CKD.

1.3.3. Non-haemodynamic factors in the development of nephron injury following extensive renal mass ablation

The weight of evidence in support of the hypothesis that glomerular haemodynamic adaptations are central to progressive renal injury does not exclude the possibility that the kidney may also be affected by a variety of factors not directly attributable to haemodynamic changes. These non-haemodynamic factors have been extensively studied in recent years and may offer new therapeutic targets for future renal protective interventions.

1.3.3.1. Hypertrophy

The consistent observation of renal and in particular glomerular hypertrophy after renal mass ablation, has prompted investigators to propose that processes involved in, or resulting from hypertrophy may

contribute to progressive renal injury in CKD [248, 249]. The well documented observation that renal and glomerular hypertrophy precede the development of diabetic nephropathy [250] and the finding of a positive association between glomerular size and early sclerosis in rats subjected to renal mass ablation [251] suggested that hypertrophy may play a direct role in pathogenesis of glomerulosclerosis. This notion was further supported by the observation that PVG/c rats, which show the same increase in whole kidney GFR as Wistar rats after unilateral nephrectomy, do not develop the glomerular hypertrophy or the lesions of FSGS seen in the remaining kidney of Wistar rats at 1 year after nephrectomy [252]. On the other hand, the same study reported greater numbers of nephrons per kidney in PVG/c than Wistar rats and since glomerular haemodynamics were not studied, these data should be interpreted with caution.

Several clinical observations also support an association between glomerular hypertrophy and renal injury. Oligomeganephronia is a rare congenital condition in which nephron number is 25% of normal or less. Such kidneys are characterized by marked hypertrophy of the glomeruli and development of proteinuria and renal failure in adolescence, with FSGS as the typical renal biopsy finding [253-255]. In children with minimal change disease, a glomerulopathy generally associated with spontaneous remission and lack of progression to renal failure, investigators noted an association between glomerular size and the risk of developing FSGS and renal failure [256].

Two forms of intervention have been employed in an attempt to interrupt the development of glomerular hypertrophy after renal mass ablation and thereby assess its role in renal disease progression. Rats subjected to 5/6 nephrectomy were compared to rats in which 2/3 of the left kidney was infarcted and the right ureter drained into the peritoneal cavity (an intervention that apparently results in decreased renal clearance without compensatory renal hypertrophy). Micropuncture studies confirmed similar degrees of elevation of P_{GC} and SNGFR in both models. At 4 weeks the maximal planar area of the glomerulus was, however, significantly less and glomerular injury as assessed by sclerosis index, significantly reduced in ureteroperitoneostomized rats vs. 5/6 nephrectomized controls. Accordingly the authors concluded that glomerular hypertrophy was more important than glomerular capillary hypertension in the progression of glomerular injury in this model [257]. Dietary sodium restriction has also been utilized to inhibit renal hypertrophy after 5/6 nephrectomy. Although sodium restriction had no effect on glomerular

haemodynamics, glomerular volume was significantly reduced in 5/6 nephrectomized rats fed low versus normal sodium diets. Moreover, urinary protein excretion was lower and glomerulosclerosis was less severe in rats on restricted sodium intake [127]. These findings were extended by another study in which the effect of sodium restriction in preventing glomerular hypertrophy and ameliorating glomerular injury was confirmed, but which also found that these benefits were overcome by administration of an androgen that stimulated glomerular hypertrophy despite sodium restriction. Glomerular haemodynamics were similar among the groups [258].

Glomerular hypertrophy may contribute to glomerulosclerosis through a number of different mechanisms. According to the Law of Laplace, the increase in glomerular volume could result in an increase in capillary wall tension only if the capillary wall diameter was also increased. Cyclic stretch would then exert degrees of stress capable of damaging epithelial, mesangial and endothelial cells as described above. Alternatively, glomerulosclerosis may be viewed as a maladaptive growth response following loss of renal mass and resulting in excessive mesangial proliferation and extracellular matrix production [248]. In the past there has tended to be a dichotomy of viewpoints regarding the relative importance of haemodynamic factors or hypertrophy in the pathogenesis of glomerulosclerosis [248, 249, 259]. Proponents of the "hypertrophy hypothesis" have pointed out that in some experiments a disassociation between glomerular haemodynamic changes and glomerulosclerosis has been observed [260-262] and that in one study, antihypertensive therapy was renal protective without lowering P_{GC} [251]. On the other hand, those favouring the "haemodynamic hypothesis" have noted that treatment with ACEI [27] or AT_1RA [29] in 5/6 nephrectomized rats resulted in renal protection without preventing glomerular enlargement [27]. Furthermore, in the experiment of Yoshida et al. rats subjected to ureteroperitoneostomy developed significantly more glomerulosclerosis than sham-operated controls despite a lack of increase in glomerular size [257]. Moreover, many studies purporting to show a positive association between glomerular hypertrophy and sclerosis failed to report glomerular haemodynamic data [259]. In recent years there has developed a growing consensus that the above views are not necessarily contradictory. Whereas raised glomerular capillary pressure is still regarded as the critical factor in initiating glomerulosclerosis, it is also acknowledged that glomerular hypertrophy and other pathogenetic mechanisms may act in concert with haemodynamic factors in a complex interplay that eventuates in progressive renal injury [175, 263].

1.3.3.2. Transforming Growth Factor-β

TGF-β is increasingly being associated with chronic fibrotic states including chronic renal disease [264]. In vitro TGF-β elicits overproduction of extracellular matrix constituents by mesangial cells and its expression is increased in several experimental models of renal disease including diabetic nephropathy [265, 266], anti-Thy-1 glomerulonephritis [217], adriamycin-induced nephropathy [267] and chronic allograft nephropathy [268] as well as in human glomerulonephritis [269, 270], HIV nephropathy [271], diabetic nephropathy [265] and chronic allograft nephropathy [272]. The role of TGF-β in renal fibrosis is further illustrated by experiments in which transfection of the gene for TGF-β into one renal artery resulted in ipsilateral renal fibrosis [273]. Moreover, transfection of the gene for decorin, a naturally occurring inhibitor of TGF-β, into skeletal muscle limited progression of renal injury in anti Thy-1 glomerulonephritis [274]. In 5/6 nephrectomized rats a 2-3 fold increase in remnant kidney mRNA levels for TGF-β was observed and *in situ* hybridization revealed elevations in TGF-β mRNA throughout glomeruli, tubules and interstitium [237]. Recently, another fibrogenic molecule, connective tissue growth factor (CTGF), that has been associated with tissue fibrosis, has also been observed to be overexpressed in kidney biopsies from patients with a variety of renal diseases [275]. The specific induction of CTGF expression by exogenous TGF-β in mesangial cells [215, 276] and fibroblasts [277], together with the finding that blocking antibodies to TGF-β inhibited increased CTGF expression in mesangial cells exposed to high glucose concentrations [276], suggests that CTGF may serve as a downstream mediator of the profibrotic effects of TGF-β [278].

1.3.3.3. Angiotensin II

As discussed above, Ang II plays a central role in glomerular haemodynamic adaptations observed after renal mass ablation. Nevertheless, experimental studies have revealed several non-haemodynamic effects of Ang II that may also be important in CKD progression (Figure 1.2.). In isolated, perfused kidneys, infusion of Ang II results in loss of glomerular size permselectivity and proteinuria, an effect that has been attributed to both haemodynamic effects of Ang II resulting in elevations in P_{GC}, and a direct effect of Ang II on glomerular permselectivity [279]. In vitro, Ang II has been shown to stimulate mesangial cell proliferation and induce expression of TGF-β, resulting in increased synthesis of

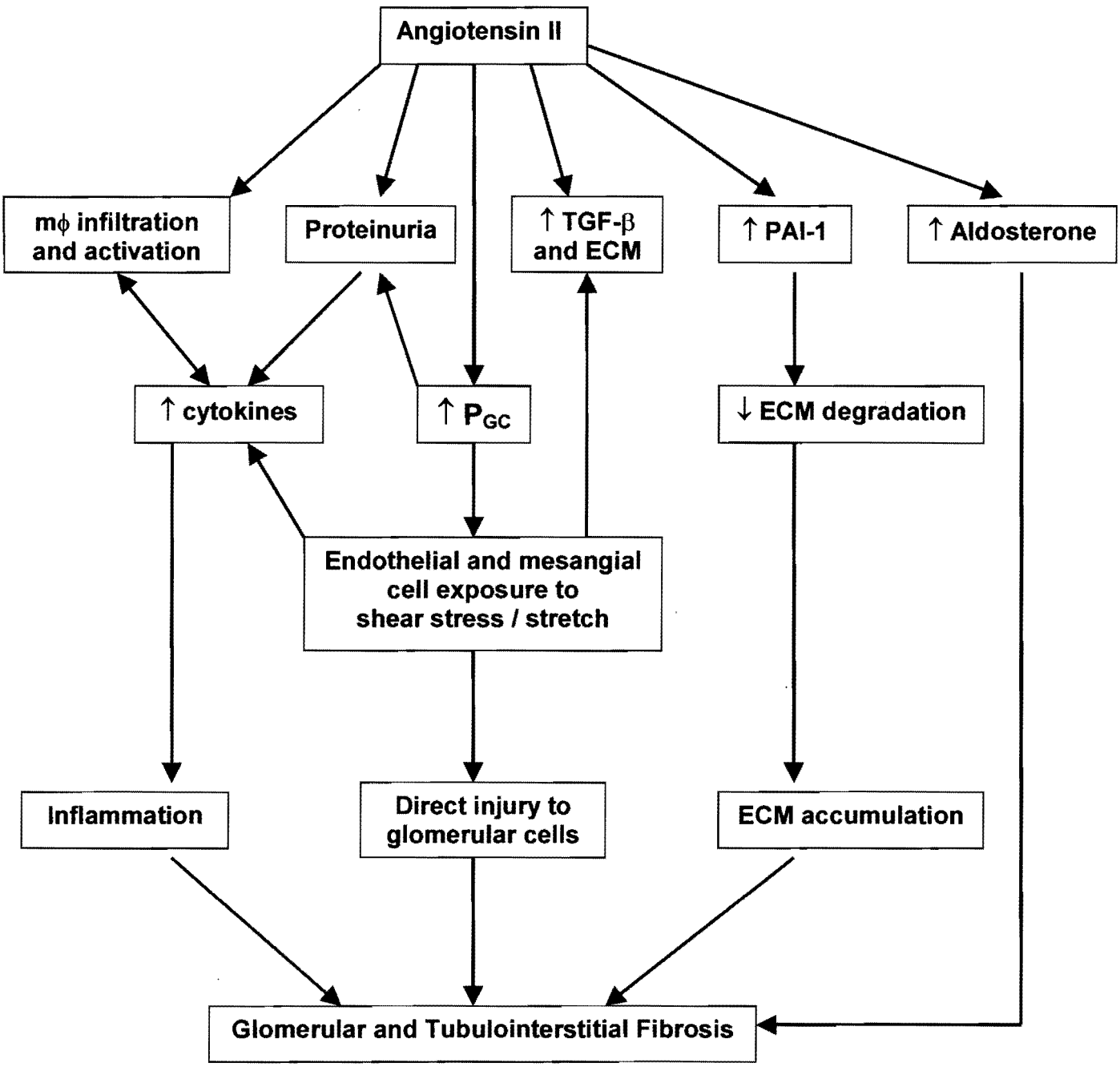


Figure 1.2. Schema depicting the central role of angiotensin II, through hemodynamic and non-hemodynamic effects, in the pathogenesis of progressive renal injury and fibrosis following nephron loss. (ECM: extracellular matrix; mϕ: macrophage; PAI-1: plasminogen activator inhibitor-1; P_{GC}: glomerular capillary hydraulic pressure; TGF-β: transforming growth factor-β)

extracellular matrix (ECM) [219]. In vivo, transfection of rat kidneys with human genes for renin and angiotensinogen, resulted in glomerular ECM expansion within 7 days [280]. Ang II also stimulates production of PAI-1 by endothelial cells and vascular smooth muscle cells [281-283] and may therefore further increase accumulation of ECM through inhibition of ECM breakdown by matrix metalloproteinases that require conversion to an active form by plasmin. Recent reports indicate that Ang II may directly induce transcription of a variety of cell adhesion molecules and cytokines via activation of the transcription factor, NF-κB [284] and may also directly stimulate monocyte activation [285]. Finally, Ang II may have fibrogenic effects via mineralocorticoids (see below).

1.3.3.4. Aldosterone

Aldosterone has been shown to stimulate collagen synthesis in the myocardium and myocardial fibrosis may be inhibited by spironolactone [286, 287]. Such observations provided the rationale for clinical studies that reported a significant improvement in survival of patients with cardiac failure who received an ACEI plus spironolactone, versus those receiving ACEI alone [288]. These findings in turn gave impetus to studies that investigated the potential role of aldosterone in renal fibrosis. In the remnant kidney model, adrenal hypertrophy and markedly elevated plasma aldosterone levels have been reported. Furthermore, administration of exogenous aldosterone during inhibition of the RAS with combination ACEI and AT_1RA therapy in the 5/6 nephrectomy model negates the renal protective effects of the latter [289]. Further evidence of the role of aldosterone in progressive renal injury is provided by experiments in which rats subjected to adrenelectomy after 5/6 nephrectomy received replacement glucocorticoid but not mineralocorticoid therapy, resulting in less severe renal injury than rats with intact adrenal glands [290]. To date the experimental use of aldosterone receptor blockers has yielded only modest renal protective effects, likely due to a further compensatory rise in aldosterone levels [289, 291]. Nevertheless one preliminary clinical study has reported a similar reduction in proteinuria following treatment with the spironolactone receptor blocker, eplerenone, or an ACEI, among patients with type 2 diabetes and microalbuminuria [292]. It has been proposed that aldosterone contributes to renal injury through a combination of haemodynamic (maintenance of hypertension) non-haemodynamic effects, including induction of TGF-β and PAI-1 expression [293].

1.3.3.5. Hepatocyte Growth Factor

Recent investigations have shed light on the role of HGF as a potential anti-fibrotic factor in CKD. Initial studies focused on the property of HGF to ameliorate tubule cell injury in models of renal ischaemia [294, 295] but studies in models of CKD suggest that HGF may also ameliorate chronic renal injury through its mitogenic and anti-apoptotic actions [296]. As discussed above, HGF is upregulated in the remaining kidney after uninephrectomy and may play a role in compensatory renal hypertrophy [141]. A recent study has confirmed that HGF and its receptor, c-met, are also upregulated in the remnant kidney after 5/6 nephrectomy [297]. Furthermore, blockade of HGF action with anti-HGF antibodies resulted in a more rapid decline in GFR and more severe renal fibrosis that was associated with increased extracellular matrix accumulation and a greater number of myofibroblasts in the interstitium and tubules. Moreover, in vitro studies revealed that HGF decreased ECM accumulation in proximal tubule cell cultures by increasing expression of collagenases such as matrix metalloproteinase-9 (MMP-9) and decreasing expression of the endogenous inhibitors of MMPs, tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 [297]. Further experiments provide support for a possible renal protective of HGF: the renal protective effects of ACEI and AT_1RA are associated with increased renal expression on HGF mRNA [298]; treatment with anti-HGF antibodies resulted in increased TGF-β levels in a mouse model of chronic glomerulonephritis [299]; HGF treatment ameliorated the progression of chronic allograft nephropathy in a renal transplant model [300]; HGF blocked the TGF-β induced transdifferentiation of tubule epithelial cells to myofibroblasts [301]; exogenous HGF administration [301] or HGF over-expression [302] blocked myofibroblast activation and prevented interstitial fibrosis in the unilateral ureteric obstruction model. In contrast, other studies have reported adverse renal effects associated with excess HGF exposure: Transgenic mice that over-expressed HGF developed progressive renal disease characterized by tubular hypertrophy, glomerulosclerosis and cyst formation [303] and HGF administration resulted in more rapid deterioration of creatinine clearance as well as increased albuminuria in obese db diabetic mice [304]. Available evidence thus suggests that HGF may play a role in ameliorating chronic renal injury but that inappropriate or excessive exposure to HGF may have adverse renal effects.

1.3.3.6. Altered glomerular permselectivity to proteins

Abnormal excretion of protein in the urine has long been regarded as the hallmark of glomerular disease. Whereas immune complex deposition and resulting inflammation account for abnormal permeability of the glomerular filtration barrier to proteins in glomerulonephritis, studies in rats subjected to extensive renal ablation have shown loss of glomerular barrier function to proteins of similar molecular size in the absence of primary immune-mediated renal injury or inflammatory response. Sieving studies using dextrans and other macromolecules in rats 7 or 14 days after 5/6 nephrectomy revealed loss of both size and charge-selectivity of the glomerular filtration barrier. Ultrastructural examination of the remnant kidneys revealed detachment of glomerular endothelial cells and visceral epithelial cells from the glomerular basement membrane. In addition, protein reabsorption droplets and attenuation of cytoplasm resulting in bleb formation was observed in podocytes. The authors concluded that altered permselectivity may be due, in part, to separation of endothelial cells from the glomerular basement membrane allowing access of macromolecules and, in part, to loss of anionic sites in the lamina rara externa resulting in both loss of charge-selectivity and detachment of podocytes [305]. A direct role for Ang II in modulating glomerular capillary permselectivity is suggested by the observation of marked increases in urinary protein excretion during infusion of Ang II in normal rats [306, 307]. While one group of investigators has attributed this to a direct effect of Ang II on the cellular components of the glomerular filtration barrier, resulting in opening of interendothelial junctions and epithelial cell disruption [306], others have shown that the increase in proteinuria may be accounted for almost completely by the associated haemodynamic changes, principally a reduction in Q_A and an increase in filtration fraction [307]. On the other hand, the notion that Ang II may mediate changes in glomerular permselectivity independent of its effects on glomerular haemodynamics is supported by studies in an isolated perfused rat kidney preparation in which infusion of Ang II augmented urinary protein excretion and enhanced the clearance of tracer macromolecules independent of any change in filtration fraction [279].

Proteinuria has recently been implicated in experimental studies as an important effector of injury processes involved in renal disease progression, especially those resulting in tubulointerstitial fibrosis. In rats with aminonucleoside-induced nephrotic syndrome the proteinuric phase of the disease was associated with an acute interstitial nephritis, the intensity of which correlated closely with the severity of

the proteinuria [308, 309]. Furthermore, in an overload proteinuria model induced by daily intraperitoneal administration of bovine serum albumin to uninephrectomized rats, proximal tubule cell injury and interstitial infiltration of macrophages and lymphocytes were evident after 1 week [310]. The severity of proteinuria showed a positive correlation with the intensity of the infiltrate. At 4 weeks, focal areas of chronic interstitial inflammation were noted [310]. A causative association between excessive proteinuria and interstitial inflammation has been suggested by in vitro studies of proximal tubule epithelial cells cultured in media supplemented with high concentrations of albumin, immunoglobulin G (IgG) or transferrin. Cellular uptake of these proteins was observed to increase secretion of endothelin-1 [311], MCP-1 [312] and RANTES [313]. Electrophoretic mobility shift assay of cell nucleus extracts in the latter study revealed intense activation of the transcription factor NF-κB that was dependent on the concentration of protein in the medium. Furthermore, the liberation of these molecules was noted to be predominantly from the basolateral aspect of the cells. This would be in keeping with secretion into the renal interstitium in vivo, thereby contributing to the development of tubulo-interstitial inflammation and fibrosis. It has been proposed that the mechanism for cytokine induction in renal tubule epithelial cells that ingest excessive amounts of protein resembles that operating in virus-infected cells, where accumulation of viral protein in the endoplasmic reticulum activates the transcription factor NF-κB [243, 314]. Alternatively, increased lysosomal enzyme activity associated with protein ingestion may result in leakage of lysosomal enzymes into cell cytoplasm causing cell injury which could then provoke reactive inflammation and scarring [315-318].

The relevance of these findings to the processes occurring in vivo has been borne out by studies in rats. In the protein-overload model, the development of proteinuria at 1 week was associated with significant increases in TGF-β at both protein and mRNA levels, in interstitial as well as proximal tubule cells [319]. Similarly, renal cortical mRNA levels encoding the macrophage chemoattractant, osteopontin, were increased on day 4 and immunofluorescence localized increased osteopontin staining to cortical tubules at day 7. MCP-1 and osteopontin mRNA and protein levels were elevated at 2 and 3 weeks. Furthermore, a significant effect of proteinuria on molecules involved in extracellular matrix protein turnover was observed. Although mRNA levels for renal matrix proteins were variable, staining for the proteins in the cortical interstitium increased progressively. Levels of mRNA for the protease inhibitors PAI-1 and tissue

inhibitor of metalloproteinases-1 (TIMP-1) were elevated at 2 weeks, at which time significant renal fibrosis was present [319]. In other models of proteinuric renal disease including 5/6 nephrectomy and passive Heymann nephritis, accumulation of albumin and IgG by proximal tubule cells occurred before infiltration of the interstitium by macrophages and MHC-II positive mononuclear cells. The infiltrates localized to areas where proximal tubule cells stained positive for intracellular IgG, or where luminal casts were present. Furthermore, proximal tubule cells that stained positive for IgG also showed evidence of increased osteopontin production [320]. Studies in the 5/6 nephrectomy model have suggested that tubulo-interstitial injury may play an important role in the decline of GFR, especially in the late stages of progressive renal injury [321]. By examining serial sections of remnant kidneys, investigators were able to show that in association with a doubling in serum creatinine, there was a substantial increase in the proportion of glomeruli no longer connected to glomeruli (atubular glomeruli) or connected to atrophic tubules. The majority of these glomeruli were not globally sclerosed, implying that tubular injury was responsible for the final loss of function in these glomeruli. The authors speculate that absorption of excess filtered protein may play an important role in this tubular injury [321]. Earlier findings that renal function is associated more closely with tubulointerstitial injury than glomerulosclerosis in human renal disease suggest that similar mechanisms may operate in human renal disease [322, 323].

Proteins other than albumin or immunoglobulin may also play a role in the progression of chronic nephropathies. Although normally absent from tubular fluid, complement components C3 and C5b-9 neoantigen were observed along the luminal border of tubule epithelial cells in the protein overload proteinuria model [310]. To examine the role of filtered complement in renal injury, rats with puromycin aminonucleoside nephrosis were subjected to complement depletion with cobra venom factor or inhibition of complement activation by administration of soluble recombinant human complement receptor type 1, before the onset of proteinuria. In control rats proximal tubular degeneration, interstitial leukocyte infiltrate and renal impairment (as assessed by inulin and *para*-aminohippurate (PAH) clearances) occurred at 7 days, together with positive staining for C3 and C5b-9 along the proximal tubule brush border. Both interventions were associated with significantly less tubulointerstitial pathology and greater clearance of PAH but not inulin, whereas the severity of proteinuria was unaffected, suggesting that filtered complement plays a significant role in tubulointerstitial injury associated with proteinuria [324]. The

lipoproteins HDL and LDL have been identified in the urine, renal interstitium and tubule cells in renal biopsies of patients with nephrotic syndrome [325] and may also have a role in renal injury. In vitro, cultured human proximal tubule epithelial cells take up LDL and HDL [326]. Oxidized LDL may cause tubule cell injury and exposure of tubule epithelial cells to HDL is associated with increased synthesis of endothelin-1 [326, 327]. Finally, a role has also been proposed for compounds bound to filtered proteins such as IGF-1, which has been detected in increased amounts in the proximal tubular fluid of rats with adriamycin nephrosis. Proximal tubule cells cultured in the presence of proximal tubular fluid from nephrotic rats exhibit enhanced cell proliferation and increased secretion of Type I and Type IV collagen. Both effects were inhibited by neutralizing IGF-1 receptor antibodies [328].

In experimental models of proteinuric renal disease, filtered proteins have also been found to accumulate in the glomerular mesangium [305, 329, 330] and may contribute to glomerular as well as tubulointerstitial injury. Further support for this notion is derived from a meta-analysis of 57 studies of experimental CKD that found a consistent positive correlation between the severity of proteinuria and extent of glomerulosclerosis [331]. Lipoproteins, in particular, accumulate in the glomeruli of patients with glomerulonephritis [332, 333]. Furthermore, low density lipoprotein (LDL) stimulates mesangial cells to proliferate in vitro [334, 335] and enhances mesangial cell synthesis of the extracellular matrix protein fibronectin [336]. LDL exposure is also associated with increased mesangial cell mRNA levels for MCP-1 [336] and PDGF [335]. Oxidation of LDL by mesangial cells or macrophages may enhance its toxicity [334]. Thus, accumulation of proteins in the mesangium may stimulate a number of different mechanisms that contribute to glomerulosclerosis.

While establishing cause-effect relationship between proteinuria and renal injury in humans is difficult, several recent clinical studies provide strong evidence in support of this notion. A meta-analysis of 17 clinical studies of chronic renal disease revealed a positive correlation between the severity of proteinuria and extent of biopsy-proven glomerulosclerosis [331]. Observations from the Modification of Diet in Renal Disease (MDRD) trial also suggest that proteinuria is an independent determinant of CKD progression: Greater levels of baseline proteinuria were strongly associated with more rapid declines in GFR; reduction of proteinuria, independent of reduction in blood pressure, was associated with lesser rates of decline in

GFR. Furthermore, the degree of benefit achieved by lowering blood pressure below usual target levels, was highly dependent on the level of baseline proteinuria [337]. Similar findings were obtained in the Ramipril Efficacy In Nephropathy (REIN) trial where higher baseline proteinuria was associated with more rapid rates of decline in GFR. In patients with pretreatment urinary protein excretion in excess of 3g/day, treatment with ramipril reduced proteinuria to an extent that correlated inversely with the subsequent rate of decline in GFR. Treatment with other antihypertensives to achieve equivalent levels of blood pressure control did not decrease proteinuria and was associated with a higher rate of decline in GFR and an increased risk of reaching the combined end-point of doubling of the baseline serum creatinine or end-stage renal failure [169]. Reanalysis of data from both strata of the REIN study found that the percentage reduction in proteinuria over the first 3 months as well as the absolute level of proteinuria at 3 months were strong independent predictors of the subsequent rate of decline in GFR [338]. A meta-analysis that included data from 1860 patients with non-diabetic CKD confirmed these findings and showed that during antihypertensive treatment, the current level of proteinuria was a powerful predictor of the combined end-point of doubling of baseline serum creatinine or onset of ESRD (relative risk 5.56 for each 1.0g/day of proteinuria) [339]. Analysis of data from the RENAAL study reported that among type 2 diabetics with nephropathy, baseline urinary albumin:creatinine ratio was the strongest predictor of the risk of reaching the combined end-point of doubling of baseline serum creatinine or onset of ESRD [340]. Taken together, the evidence from experimental and clinical studies provides strong support for the hypothesis that excessive filtration of proteins due to impaired glomerular permselectivity directly damages the kidney. Whether or not this is so, the close association between the severity of proteinuria and renal prognosis implies that reduction of proteinuria should be regarded as an important independent therapeutic goal in clinical strategies seeking to slow the rate of progression of CKD.

1.4. Strategies for Achieving Renal Protection

1.4.1. Control of Systemic Hypertension

There is clear evidence that hypertension accelerates the rate of progression of pre-existing renal disease, most likely through transmission of raised blood pressure to the glomerulus, resulting in exacerbation the glomerular capillary hypertension associated with progressive nephron loss [149]. The treatment of systemic hypertension was the first intervention shown to significantly slow the rate of CKD progression

and it remains fundamental to renal protective strategies. Among insulin-dependent diabetic patients with diabetic nephropathy, initiation of antihypertensive therapy resulted in marked reductions in the rates of GFR decline [341, 342] implying that hypertension, an almost universal consequence of impaired renal function, also contributes to the progression of CKD. Similar observations were subsequently reported among patients with non-diabetic forms of CKD [343-345]. Uncertainty remained, however, as to what level of blood pressure lowering was required to achieve optimal renal protection. The Modification of Diet in Renal Disease (MDRD) Study sought to resolve this issue by directly evaluating whether lower than previously recommended blood pressure targets afforded greater renal protection than "usual" blood pressure control among patients with predominantly non-diabetic CKD. In addition to the dietary interventions described below, patients were randomized to a target mean arterial pressure (MAP) of 107mmHg or 92mmHg. Whereas the primary analysis did not show any overall difference in rate of GFR decline between these groups, patients randomized to the low blood pressure group showed an early rapid decrease in GFR, probably due to associated renal haemodynamic effects, that obscured a later significantly slower rate of GFR decline than observed in the "usual" blood pressure target group. Furthermore, a higher level of baseline proteinuria was associated with a greater difference in GFR decline between "usual" and low blood pressure groups [346]. Secondary analysis revealed significant correlations between the rate of GFR decline and achieved blood pressure, an effect that was also more marked among those with greater baseline proteinuria. In study 1 (patients with GFR of 25-55ml/min/1.73m^2), rates of GFR decline increased above a MAP of 98mmHg among patients with baseline proteinuria of 0.25-3.0g/day, and above 92mmHg in those with baseline proteinuria >3.0g/day. In study 2 (patients with GFR of 13-24ml/min/m^2), among patients with baseline proteinuria >1g/day, higher achieved blood pressure was associated with greater rates of GFR decline at all levels. The authors concluded by recommending a blood pressure goal of <125/75 (MAP=92mmHg) for CKD patients with >1g/day of proteinuria, and a goal of <130/80 (MAP=98mmHg) for those with proteinuria of 0.25-1.0g/d [337]. A second major study has recently investigated the potential benefit of a lower blood pressure goal on the rate of GFR decline among 1094 African American patients with hypertensive renal disease, randomized in a 2 x 3 factorial design to a MAP target of 102-107mmHg or ≤92mmHg and treatment with ramipril, amlodipine or metoprolol. Similar to the primary findings in the MDRD study, no significant difference in the rate of decline in GFR was observed between the two blood pressure target groups [347].

Since not all the patients in the MDRD Study received ACEI, it remains unclear to what extent the level of blood pressure attained remains important in CKD patients receiving ACEI or angiotensin receptor antagonists (AT_1RA). At least one experimental study has found systolic blood pressure to be a major determinant of glomerular injury in rats receiving ACEI treatment [348]. Moreover among patients with type 1 diabetes and established nephropathy receiving ACEI, randomisation to a low (MAP=92mmHg) vs. "usual" (MAP=100-107mmHg) target blood pressure was associated with significantly lower levels of proteinuria after 2 years, although there was no significant difference in the rate of GFR decline [349]. A recent finding that intensive blood pressure control was not associated with significantly improved renal function among patients with autosomal dominant polycystic kidney disease appears to contradict the above findings but, by the authors' own admission, the study may not have had adequate statistical power to detect such a difference [350]. Furthermore not all patients in this study received an ACEI (approximately 70% did).

There has been some controversy over the role of calcium channel blockers (CCB) as antihypertensive agents in patients with CKD. There is concern that the dihydropyridine class of CCB may in fact have adverse effects on the progression of CKD. In experimental studies dihydropyridine CCB allowed greater transmission of systemic blood pressure to the renal microcirculation and were associated with more rapid progression of renal injury than ACEI treatment in the 5/6 nephrectomy model [351]. Whereas one relatively small study found no difference between the renal protective effects of the dihydropyridine CCB, nifedipine, and the ACEI, captopril [352], two larger studies have reported adverse outcomes associated with the use of dihydropyridine CCB. A secondary analysis of data from the Ramipril Efficacy In Nephropathy (REIN) Study found that treatment with a dihydropyridine CCB (nifedipine or amlodipine) was associated with higher levels of proteinuria and more rapid GFR decline than other antihypertensives in those patients who failed to achieve a MAP of <100mmHg and who were not receiving an ACEI [353]. In the African American Study of Kidney Disease and Hypertension (AASK) [354] patients with CKD and hypertension were randomized to treatment with an ACEI or amlodipine or a β-blocker and diuretic in combination. The amlodipine arm of the study was stopped prematurely due to a more rapid decline in GFR among these patients versus those receiving the β-blocker or ACEI, particularly among those with

>1g/day of proteinuria. The AASK Study implies that this is particularly true for African American patients. On the other hand available data suggest that the non-dihydropyridine CCB do contribute to renal protection. Experimental data have shown that non-dihydropyridine CCB lower P_{GC}, reduce proteinuria and afford renal protection. Furthermore, in one non-randomized study non-dihydropyridine CCB reduced proteinuria in patients with type 2 diabetes and overt nephropathy, an effect that was additive to that of ACEI alone [355].

1.4.2. Pharmacological inhibition of the Renin-Angiotensin System

Following the observation of dramatic renal protective effects associated with ACEI treatment in experimental models, investigators sought to establish whether similar effects could be achieved in human CKD. Over the past decade several of clinical studies have been published that together provide strong support for the use of pharmacological inhibitors of the RAS as an essential component of any strategy aiming to achieve maximal renal protection in patients with CKD (summarized in table 1.2.).

1.4.2.1. Angiotensin-Converting Enzyme Inhibitors

1.4.2.1.1. Diabetic Nephropathy

Following experimental evidence of the specific renal protective effects of ACEI, several reports and one small prospective study suggested that ACEI afforded renal protection in type 1 diabetics with established nephropathy [356]. In 1993, the Captopril Collaborative Study Group published the first large prospective randomized controlled trial to clearly show specific renal protection attributable to ACEI treatment in human CKD [168]. Four hundred and nine patients with type 1 diabetes and established nephropathy (proteinuria >0.5g/day; serum creatinine <2.5mg/dl) were randomized to receive captopril or placebo with a blood pressure goal of <140/90mmHg. After a median follow-of 3 years, captopril treatment was associated with a 50% reduction in the risk of the combined end-point of death, dialysis and renal transplantation and a 48% reduction in the risk of a doubling of serum creatinine. Moreover, this additional renal protection was not attributable simply to the antihypertensive effects of ACEI as blood pressure control was not statistically different between the groups. This landmark study prompted several further studies to investigate whether ACEI may also benefit type 1 diabetic patients with the increased

risk of developing nephropathy associated with microalbuminuria. A meta-analysis of 12 such studies including 689 patients with type 1 diabetes that were followed for at least a year found that ACEI treatment was associated with a significant reduction in the risk of progression to overt nephropathy (odds ratio 0.38) and three times the incidence of complete normalization of the microalbuminuria [357]. In another study, the effect of ACEI in preventing progression to overt nephropathy was sustained over 8 years and was associated with preservation of a normal GFR [358]. Finally, subgroup analysis of the EUCLID Study found that ACEI treatment reduced albuminuria by 12.7% among normotensive, normoalbuminuric type 1 diabetics. However, this trend was not statistically significant and was associated with a statistically lower blood pressure in the ACEI-treated group [359].

Data on the renal protective effects of ACEI in patients with type 2 diabetes are, to some extent, conflicting. Studies comparing ACEI and other antihypertensives among type 2 diabetics with overt nephropathy have included relatively small numbers of patients and only one [360] was able to show a greater reduction in GFR decline associated with ACEI versus other antihypertensives [361-363]. In contrast, several studies including the diabetic subgroup analysis of the Heart Outcomes Prevention Evaluation (HOPE) Study, have reported beneficial effects of ACEI treatment in decreasing microalbuminuria [364-367] or reducing the number of patients progressing from microalbuminuria to overt proteinuria among type 2 diabetics (risk reduction 24-67%) [368-370]. In addition, the HOPE Study reported a 25% reduction in the combined primary endpoint of myocardial infarction, stroke or cardiovascular death in Ramipril-treated type 2 diabetics with risk factors for cardiovascular disease. Finally, at least one study has reported a beneficial role for ACEI in primary prevention of nephropathy among 156 normotensive, normoalbuminuric type 2 diabetics. Patients receiving enalapril over a 6-year period had an absolute risk reduction of 12.5% versus placebo for developing microalbuminuria [371]. On the other hand, one relatively large study found no renal protective benefit of ACEI over β-blocker treatment among hypertensive type 2 diabetics with normo- or microalbuminuria [372].

1.4.2.1.2. Non-Diabetic CKD

Following the publication of studies showing the renal protective effects of ACEI in diabetics, investigators sought to study the potential of ACEI to afford renal protection in non-diabetic forms of CKD. Maschio et

al. randomized 583 patients with CKD of diverse aetiology to treatment with benazepril or placebo. After 3 years of follow-up, the study found a 53% reduction in the risk of reaching the combined end point of doubling of base-line serum creatinine or the need for dialysis associated with ACEI treatment. However, a significantly lower blood pressure among patients receiving ACEI vs. placebo made it impossible to separate the beneficial effects of lowering blood pressure from any unique effects of ACEI treatment [373]. By contrast, in the REIN Study, 352 patients with non-diabetic CKD randomized to either ACEI or placebo achieved similar control of blood pressure. Among patients with ≥3g/day of proteinuria at base line, the study was stopped early due to a significantly lower rate of decline in GFR in patients receiving the ACEI (0.53 vs. 0.88ml/min/month) [169]. Further analysis showed a significant reduction in the risk of the combined end-point of a doubling of serum creatinine or ESRD in the ACEI group (risk ratio = 1.91 for the placebo group). In the next phase of this study, patients who had received placebo were switched to ACEI and those already on ACEI continued treatment. Consistent with the findings of the first phase of the study, there was a significant reduction in the rate of decline in GFR of patients switched to ACEI. In addition, patients continuing on ACEI treatment showed a further reduction in the rate of GFR decline, to levels similar to those associated with normal aging. Patients who received ACEI from the start of the REIN study had a significantly lower risk of reaching end-stage renal failure (ESRF) than those switched to ACEI after the initial phase of the study (relative risk for placebo group = 1.86). Indeed from 36 to 54 months of follow up, no further patients in the former group reached ESRF [374]. Interestingly, a small number of patients continued on ACEI actually showed an increase in GFR after prolonged treatment [375]. REIN Study patients with <3g/day of proteinuria (n=186) were followed for a median of 31 months after randomisation. Similar to the findings among those with more severe proteinuria, ACEI treatment significantly reduced the incidence of ESRF (relative risk for placebo group = 2.72), particularly among those with a GFR of <45ml/min at baseline [376]. The findings of these individual studies have recently been confirmed by a meta-analysis of 11 studies that included 1860 patients with non-diabetic CKD [377]. Antihypertensive regimens that included ACEI resulted in significantly greater reductions in blood pressure and proteinuria, but even after statistical adjustment for these factors, ACEI treatment was associated with significantly lower risks of reaching ESRD (relative risk = 0.69; CI 0.51-0.94) and the combined end-point of a doubling in baseline serum creatinine or ESRD (relative risk = 0.70; CI = 0.55-0.88). These data indicate that the renal protective effects of ACEI are mediated by factors in addition to their

antihypertensive and antiproteinuric effects. Moreover, further analysis showed that the benefits of ACEI treatments were greater in patients with higher levels of baseline proteinuria. The recent AASK study has shown that the renal protective benefits of ACEI therapy also apply to African American patients, a group who tend to be resistant to the antihypertensive effects of ACEI. Patients with hypertensive renal disease randomized to ACEI treatment showed a significant risk reduction for the combined outcome of reduction of baseline GFR by ≥50%, ESRD or death versus patients treated with a β-blocker (22% risk reduction) or a non-dihydropyridine CCB (38% risk reduction) [347].

In addition to the renal protective benefits of ACEI treatment, the HOPE Study reported substantial reductions in overall (relative risk = 0.84) and cardiovascular mortality (relative risk = 0.74) as well as reductions in myocardial infarction (relative risk = 0.80) and stroke (relative risk = 0.68) in patients receiving an ACEI vs. placebo among 9297 who were at increased risk of cardiovascular disease [378]. Although the HOPE Study did not include large numbers of patients with non-diabetic CKD, cardiovascular disease remains the single largest cause of morbidity and mortality among these patients and the data therefore provide a further rationale for the use of ACEI therapy as the single most important intervention in patients with CKD.

1.4.2.2. Angiotensin Receptor Antagonists

Angiotensin (subtype 1) receptor antagonists inhibit the RAS by blocking angiotensin II subtype 1 (AT_1) receptors. Thus ACEI and AT_1RA differ significantly in their effects on the RAS in ways that may be therapeutically relevant (discussed in detail in section 1.5.2.). In small preliminary clinical studies, AT_1RA and ACEI produced similar antihypertensive and antiproteinuric effects among patients with essential hypertension [379], non-diabetic CKD [380] or type 2 diabetes and early nephropathy [381]. The publication of 3 large randomized studies has established a clear role for AT_1RA therapy in achieving renal protection for patients with type 2 diabetes. In the Reduction of Endpoints in NIDDM with Angiotensin II Antagonist Losartan (RENAAL) Trial, 1513 patients with established diabetic nephropathy were randomized to losartan or placebo and followed for a mean of 3.4 years [171]. Losartan treatment was associated with significant reductions in the incidence of a doubling of baseline serum creatinine (risk reduction = 25%) and ESRD (risk reduction = 28%) as well as a 35% reduction in proteinuria. In the

Irbesartan Diabetic Nephropathy Trial (IDNT) 1715 patients with hypertension and established diabetic nephropathy were randomized to treatment with irbesartan, amlodipine or placebo [170]. After a mean of 2.6 years, irbesartan was associated with a 33% lower risk of a doubling of baseline serum creatinine vs. placebo and a 37% reduction vs. amlodipine. Although not statistically significant, irbesartan was associated with a 23% reduction in the risk of ESRD vs. placebo and amlodipine. Importantly, close matching of achieved blood pressure between groups in both these trials implies that, as with the ACEI studies, the additional renal protective effects of AT_1RA treatment could not be attributed merely to their antihypertensive effects. A third study examined the renal protective effects of irbesartan in 590 type 2 diabetics with hypertension and microalbuminuria [382]. Patients were randomized to irbesartan at 2 different doses (300 or 150mg/day) or placebo and followed up for 2 years. During this period significant differences emerged in the incidence of overt proteinuria (5.2% vs. 9.7% vs. 14.9%) and the higher dose of irbesartan was associated with a substantial reduction in the risk of developing overt nephropathy (hazard ratio = 0.30; CI 0.14-0.61 vs. placebo). Although significantly lower blood pressures were achieved with irbesartan, the risk reduction was similar after adjustment for the baseline level of microalbuminuria and blood pressure. In summary, whereas clear evidence of the renal protective effects of ACEI in type 2 diabetics with overt nephropathy is lacking, there are now 2 large studies showing the benefits of AT_1RA in this population. Second, a single large study supports the notion that AT_1RA treatment is similarly effective as ACEI treatment in preventing progression from microalbuminuria to overt nephropathy in type 2 diabetes mellitus.

Although large randomized clinical trials of the renal protective effects of AT_1RA in type 1 diabetics or non-diabetic CKD are not yet available the above data suggest that the AT_1RA will duplicate the renal protection achieved by ACEI in these populations. In addition, one important advantage of AT_1RA over ACEI is their more favourable side effect profile. In clinical trials AT_1RA have been reported to have side effect profiles similar to placebo [383, 384]. Importantly, AT_1RA are not associated with the cough that may occur in up to 20% of patients receiving ACEI. Among patients converted from ACEI to AT_1RA therapy, recurrence of a cough was significantly lower than in patients rechallenged with an ACEI [385, 386]. Thus even in the absence of clinical trials in type 1 diabetes or non-diabetic CKD, available evidence supports the use of AT_1RA as an alternative in patients groups who are unable to tolerate ACEI

due to side effects. Furthermore, the favourable side effect profile of AT_1RA suggests that it may be possible to employ higher doses. Preliminary data have shown that doubling the dose of an AT_1RA may result in greater lowering of proteinuria without a further reduction in blood pressure [380]. Finally, the differing effects of ACEI and AT_1RA on the RAS imply that in combination they may have additive or even synergistic effects. Among 8 patients with IgA nephropathy treated consecutively with an ACEI, AT_1RA and combination therapy, the combination was associated with greater antiproteinuric effects than either treatment alone, without significant additional antihypertensive effects [387]. Addition of an AT_1RA to prior ACEI therapy in 11 patients with CKD was associated with a 6mmHg fall in MAP and a further 30% reduction in proteinuria [388]. In the largest study to date, combination ACEI and AT_1RA therapy afforded greater reductions in blood pressure and albuminuria than either treatment alone among 199 type 2 diabetic patients with hypertension and microalbuminuria [389]. Thus, although more data are required before firm recommendations can be made, evidence is accumulating that combination ACEI and AT_1RA therapy may offer additional renal protection to that afforded by either agent alone.

Table 1.2. Summary of studies showing the renal protective effects of ACEI and AT_1RA in diabetic and non-diabetic CKD.

Angiotensin-Converting Enzyme Inhibitors

CRD Type	Trial Outcome	Reference
Type 1 DM + CKD	50% ↓ risk of dialysis, Tx or death	168
Type 1 DM + μA	↓ risk of overt nephropathy (OR=0.38)	357, 358
Type 1 DM + NA	12.7% ↓ in albuminuria (NS)	359
Type 2 DM + CKD	Benefit in only 1 study	360-363
Type 2 DM + μA	24-67% ↓ risk of overt nephropathy	364-370
Type 2 DM + NA	12.5% ↓ risk of developing μA	371
Non-diabetic CKD	↓ creatinine doubling / ESRD (RR=0.52)	169, 347, 374, 376, 377

Angiotensin Receptor Antagonists

CKD Type	Trial Outcome	Reference
Type 2 DM + μA	↓ risk of overt nephropathy (HR=0.30)	382
Type 2 DM + CKD	25-37% ↓ risk of creatinine doubling	170, 171
	23-28% ↓ risk of ESRD	

Abbreviations: HR – hazard ratio; OR – odds ratio; μA – microalbuminuria; NA – normoalbuminuria; RR – risk ratio; Tx - transplant

1.4.3. Dietary Protein Restriction

Increases dietary protein intake or intravenous protein loading in animals or humans with intact kidneys are associated with increases in renal mass, renal blood flow and GFR, as well as a decrease in renal vascular resistance [390-393], changes similar to those observed after renal mass ablation. It has been proposed that the augmented renal function induced by dietary protein may be an evolutionary adaptation of the kidney to the intermittent heavy protein intake of the hunter-gatherer [149]. Thus renal hyperfunction following a protein load would allow excretion of the waste products of protein catabolism, thereby achieving homeostasis in the face of an abrupt increase in protein intake. The subsequent decline of GFR to baseline during the intervals between meals would facilitate conservation of fluid and electrolytes in times of scarcity. Continuous excessive protein intake that results in persistent renal hyperfunction, however, leads to renal injury in experimental models. Laboratory animals with intact kidneys and ingesting food *ad libitum* become proteinuric and develop glomerulosclerosis with age [149, 394, 395]. Furthermore, dietary protein intake has been shown to influence the rate of progression of established renal disease in experimental models. This progression was significantly accelerated by a high protein diet and attenuated by dietary protein restriction or alternate day feeding [394]. Similarly, in diabetic rats, progression of nephropathy was markedly accelerated in the setting of a high protein diet and substantially attenuated by a low protein diet [147]. Similar renal protection was observed after dietary protein restriction in the 5/6 nephrectomy model [15]. In both the diabetic nephropathy and 5/6 nephrectomy models, the renal protective effects of dietary protein restriction in experimental animals are associated with virtual normalization of P_{GC} and SNGFR.

Despite unambiguous evidence from experimental studies, confirmation of a beneficial effect of protein restriction in clinical trials has proved elusive. Following the publication of several smaller studies that generally suggested a beneficial effect from protein restriction but that suffered from deficiencies in design or patient compliance, a large, multicenter, randomized study, the MDRD study, was conducted to resolve the issue [346]. 585 patients with moderate chronic renal failure (GFR=25-55ml/min/1.73m2) were randomized to "usual" (1.3g/kg/day) or "low" (0.58g/kg/day) protein diet (study 1) and 255 patients with severe chronic renal failure (GFR=13-24ml/min/1.73m2) to "low" (0.58g/kg/day) or "very low" (0.28g/kg/day) protein diet. All causes of CKD were included but patients with diabetes mellitus requiring insulin therapy were excluded. Patients were also assigned to different levels of blood pressure control. After a mean of 2.2 years follow-up, the primary analysis revealed no difference in the mean rate of GFR decline in study 1, and only a trend towards a slower rate of decline in the "very low" protein group in study 2. Secondary analyses of the MDRD data, however, revealed that dietary protein restriction probably did achieve beneficial effects. In study 1 "low" protein diet was associated with an initial reduction in GFR that likely resulted from the functional effects of decreased protein intake and not from loss of nephrons. This initial reduction in GFR obscured a later reduction in the rate of GFR decline that was evident after 4 months in the "low" protein group and that may have resulted in more robust evidence of renal protection had follow-up been continued for a longer period [396]. Further evidence to support a beneficial effect of dietary protein restriction in CKD is derived from a meta-analysis of five randomized, controlled trials (including MDRD Study 1). Despite inconclusive findings in several of the individual studies, Pedrini, et al. [397] determined that the overall relative risk for renal failure or death was reduced to 0.67 (CI 0.50-0.89) with protein restriction, an effect that was not attributable to differences in blood pressure control between the groups.

1.4.4. Treatment of Hyperlipidaemia

CKD is commonly associated with abnormalities of plasma lipids characterized by elevated levels of the triglyceride-rich lipoproteins VLDL and LDL, and reduced levels of HDL [398]. In addition to contributing to the increased risk of cardiovascular disease associated with CKD, these lipid abnormalities may also accelerate the progression of CKD. In the MDRD Study, low serum HDL cholesterol was an independent predictor of more rapid decline in GFR [399] and in another study elevated triglyceride-rich apo-B containing lipoprotein levels correlated significantly with the rate of deterioration of renal function [400]. Hypercholesterolemia has been associated with more rapid progression of renal disease among patients with diabetic [401-403] and non-diabetic forms of CKD [404]. Analysis of data from 12,728 patients without significant renal disease found that elevated baseline serum triglyceride levels and low HDL cholesterol levels were independent predictors of a rise in serum creatinine over the following 2.9 years, suggesting that dyslipemias may also be involved in the initiation of CKD [405]. The mechanisms whereby hyperlipidemia my contribute to renal injury are the subject of ongoing research, but studies to date have identified several different mechanisms, including stimulation of mesangial cell proliferation, cytokine expression and extracellular matrix synthesis [335, 336], oxidation of LDL to form reactive oxygen species [406] and elevations in P_{GC} [407]. In experimental studies treatment of hyperlipidemia has resulted in attenuation of renal injury in a variety of models of CKD [17, 408]. Although large randomized clinical trials of lipid-lowering therapy in CKD are awaited, a recent meta-analysis of 12 small studies that included both diabetic and non-diabetic renal disease found that lipid-lowering therapy significantly reduced the rate of decline in GFR (mean reduction 1.9ml/min/year) [409]. Further studies are required to confirm this finding in larger numbers of patients and to identify appropriate levels for intervention as well as therapeutic targets.

1.4.5. Smoking Cessation

Smoking has been identified as a risk factor for the development of microalbuminuria, overt proteinuria and renal disease progression in type 1 and 2 diabetics [410-412]. Similarly, smoking is a risk factor for progression in a variety of forms of non-diabetic CKD: Among patients with adult polycystic kidney disease or IgA nephropathy, smokers had a 10-fold increased risk of progression to ESRD vs. non-smokers [413]; the median time to ESRD was almost halved in smokers vs. non-smokers in patients with lupus nephritis

[414]; patients with a primary glomerulonephritis and serum creatinine >1.7mg/dl were significantly more likely to be smokers than those with normal creatinine [415]; smoking was the most powerful predictor of a rise in serum creatinine among patients with severe essential hypertension [416]. Moreover, smoking has been associated with increased albuminuria and both increased and decreased GFR in a large population-based study of non-diabetic subjects [417]. Proposed mechanisms whereby smoking may exacerbate renal injury include glomerular hyperfiltation, endothelial dysfunction and increased albuminuria. Though prospective studies showing renal benefit from smoking cessation are lacking, the above evidence suggests that the kidney is another organ that may be adversely affected by smoking.

1.5. Unanswered Questions

Two decades of research has shed considerable light on the multiplicity of factors that may contribute to the common pathway of mechanisms that drive the relentless decline in renal function characteristic of CKD. Following the land-mark studies that identified glomerular capillary haemodynamic adaptations as the primary driving force behind CKD progression, detailed in-vitro studies have suggested mechanisms whereby haemodynamic forces may provoke pro-inflammatory and fibrotic responses in glomerular cells. Considerable progress has also been made in understanding the potential role of non-haemodynamic factors such as hypertrophy, proteinuria and the non-haemodynamic effects of angiotensin II. Nevertheless, several areas require further elucidation and many questions remain unanswered. The work presented in the following chapters is an attempt to contribute to our understanding of CKD progression in some of these areas.

1.5.1. In vivo expression of pro-inflammatory and fibrotic growth factors

Several studies have reported the induction of cell adhesion molecules, proinflammatory cytokines and profibrotic growth factors in glomerular cells exposed to mechanical forces such as shear stress or barostress in vitro. Few studies have, however, attempted to confirm that these molecules are also upregulated in vivo in models of CKD. Whereas some studies have focused on individual cytokines or growth factors, none has previously attempted to examine the expression of multiple molecules over time in a single model. In Chapter 2 we present a series of experiments that examine the expression of six different molecules over time in the 5/6 nephrectomy model. In Chapter 3 we investigate the expression

of cytokines in the 5/6 nephrectomy model with prolonged follow-up and show correlations between the level of expression and the extent of renal injury.

1.5.2. Pharmacological inhibition of the Renin-Angiotensin System

Experimental and clinical studies have clearly established the central role of angiotensin II in the pathogenesis of CKD progression. Most of these studies are based on observations after the inhibition of Ang II synthesis by treatment with an ACEI. Nevertheless, ACEI do have effects in addition to the inhibition of ACE. The development of pharmacological inhibitors of the AT_1 receptor provided the opportunity to examine the effects of inhibiting the RAS by a different mechanism that does not affect the metabolism of other vasoactive molecules. Inhibition of angiotensin-converting enzyme results in reduced conversion of angiotensin I to angiotensin II and a compensatory rise in renin levels due to loss of negative feedback inhibition of renin production by juxtaglomerular apparatus (JGA) cells [418]. By contrast AT_1RAs produce elevations in both renin and Ang II because normal feedback inhibition of JGA cells through stimulation of AT_1 receptors is blocked [419]. These differences in the level of inhibition of the RAS may have important implications for the therapeutic efficacy of AT_1RA vs. ACEI in at least two respects.

First, ACEIs are able to reduce only ACE-dependent Ang II production whereas AT_1RAs block the effect of Ang II from any source at the receptor level. In the presence of ACE inhibition, studies have shown that Ang II may be produced by other proteases including chymase and other serine proteases [420-422]. Indeed, despite reduced plasma concentrations of Ang II, normal tissue levels of Ang II were found in mice following homozygous deletion of the ACE gene [423]. These normal tissue levels of Ang II were associated with a 14 fold increase in chymase activity in the kidneys of the knock-out mice.

Second, it is now known that there are at least two subtypes of AT receptor. Thus blockade of subtype 1 (AT_1) receptors in the presence of elevated Ang II levels can be expected to result in stimulation of subtype 2 (AT_2) receptors. AT_1 receptors mediate most of the known effects of Ang II including vasoconstriction, stimulation of aldosterone synthesis and release, and renal tubule sodium and water reabsorption [424-426]. The role of AT_2 receptors on the other hand, is not clearly defined. Although they

do not appear to be involved in any of the main effects of Ang II, AT_2 receptors are important in foetal kidney development [427, 428] modulate pressure-natriuresis [429, 430], mediate Ang II-induced renal production of nitric oxide [431] and renal conversion of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ [432]. In addition, experimental evidence suggests that AT_2 receptors may counterbalance some of the effects mediated by AT_1 receptors. In cell culture, Ang II suppresses the proliferation of fibroblasts selectively expressing AT_2 receptors [433]. Furthermore, vascular smooth muscle cells transfected with an AT_2 receptor expression vector attenuate neointimal proliferation following balloon injury [434]. Pharmacogical inhibition of AT_2 receptors and deletion of the gene for the AT_2 receptor have each been associated with enhanced interstitial fibrosis in models of unilateral ureteric obstruction [435, 436]. Disruption of the AT_2 receptor gene in mice results in a significant increase in blood pressure and an enhanced pressor response to exogenous Ang II [437]. In the isolated microperfused rabbit afferent arteriole, pretreatment with an AT_2 receptor antagonist completely abolishes the vasodilation associated with the administration of an AT_1RA [438]. Taken together, these data suggest that stimulation of AT_2 receptors may produce antiproliferative, anti-fibrotic and antihypertensive effects that might prove beneficial in the context of CKD. In one relatively short-term study, treatment with an AT_2 receptor antagonist (AT_2RA) had no effect on the renal protective effects of losartan in rats subjected to partial renal ablation [439]. On the other hand, a recent study has reported partial attenuation of proteinuria with AT_2RA treatment in the 5/6 nephrectomy model, together with a similar reduction in expression of osteopontin and proliferating cell nuclear antigen (PCNA) as well as macrophage infiltration to that achieved with an AT_1RA, but no effect on blood pressure or histological markers of renal injury. As reported in the earlier study combination AT_1RA and AT_2RA therapy conferred no additional renal protection to the already complete protection afforded by the AT_1RA alone, although the combination did produce a greater lowering of PCNA expression than either monotherapy [440].

Differences between ACEIs and AT_1RAs have also been observed with respect to their effects on aldosterone levels. Among normotensive human subjects during low sodium intake, both ACEI and AT_1RA treatment lowered serum aldosterone levels, but this effect was significantly greater for ACEI, despite equivalent hypotensive effects between the groups [441]. The same study also found that ACEI treatment lowered plasma PAI-1 antigen levels and activity whereas no effect on PAI-1 was observed with

AT_1RA. However, both ACEI and AT_1RA lowered PAI-1 expression in an experimental model of glomerulonephritis [442]. Since both aldosterone [289] and the fibrinolytic system may be involved in the pathogenesis of renal fibrosis, these differences may have significant implications for the relative efficacy of these agents as renal protective drugs.

ACEI effects on hormonal systems other than the RAS may also contribute to some of their therapeutic effects. Thus angiotensin-converting enzyme, also termed kininase II, is responsible for the breakdown of bradykinin; ACE inhibition therefore results in elevated bradykinin levels [418, 443]. AT_1RAs on the other hand, have no direct effect on bradykinin levels [419]. In several experimental models evidence suggests that elevated kinins are responsible for at least some of the effects of ACEIs. Neointima formation following balloon injury to the carotid artery is inhibited by ACEI but the effect is lost in animals cotreated with a kinin receptor antagonist [444]. Similarly, in an animal model of cardiac failure the combination of bradykinin receptor blockade with ACE inhibition significantly reduces the benefit of the latter [445]. Furthermore, several of the effects of ACEI which may contribute to renal protection have been attributed to the associated rise in kinins, including: antihypertensive effects in normal and hypertensive patients [446-449]; renal vasodilation resulting in increased renal blood flow [450-453]; dilation of the efferent arteriole and the associated fall in P_{GC} in normal rats subjected to intravascular volume depletion [454] and salt-depleted rats with 1-kidney, 1-clip hypertension [455]; antiproteinuric effects in rats with passive Heymann nephritis [456] and puromycin aminonucleoside (PAN) nephrosis [457]; tissue plasminogen activator release with resultant stimulation of ECM degradation [458, 459]; and inhibition of monocyte/macrophage infiltration in unilateral ureteric obstruction [460]. It is important to note, however, that the above kinin-mediated effects of ACEI have been observed only in specific experimental models and are therefore not necessarily relevant to the mechanisms involved in the progression of clinical CKD. On the contrary, the observation that chronic administration of a kinin receptor blocker did not decrease the renal protective effects of an ACEI in rats after 5/6 nephrectomy [461, 462] or in streptozotocin-induced diabetes [463] suggests that kinin-mediated effects are not necessary for renal protection.

Despite the above theoretical considerations, many studies have detected no significant differences in the renal protective effects of ACEI vs. AT_1RA in experimental models of CKD, when treatment was initiated

before the onset of substantial renal injury [28, 160, 163, 164, 266, 463-467]. A single study however, purports to show an advantage of AT_1RA over ACEI in 5/6 nephrectomized rats [468]. In small preliminary clinical studies, AT_1RA and ACEI produced similar antihypertensive and antiproteinuric effects among patients with essential hypertension [379], non-diabetic CKD [380] or type 2 diabetes and early nephropathy [381]. The experiments presented in Chapter 2 examine the comparative effects of ACEI and AT_1RA treatment on the renal expression of pro-inflammatory and fibrotic cytokines in the 5/6 nephrectomy model in order to identify possible differences on this level. In Chapter 3 we compare the renal protective effects of ACEI and AT_1RA treatment after 5/6 nephrectomy in a study in which treatment is delayed until after renal injury is established, a model more likely to identify subtle differences between the efficacy of ACEI and AT_1RA treatment.

1.5.3. Extent of Blood Pressure and Proteinuria Reduction

The importance of blood pressure control in achieving renal protection has been appreciated for several decades. Nevertheless the optimum level of blood pressure remains to be determined. Moreover, the importance of the level of blood pressure in the setting of treatment with ACEI or AT_1RA is uncertain. In the studies presented in Chapter 3 we exploited the relatively large number of subjects and long duration of follow-up to examine the effect of achieved blood pressure during ACEI and AT_1RA treatment on renal injury. In the light of evidence suggesting that proteinuria may contribute directly to progressive renal injury we also examine the association between proteinuria and renal injury in this model.

1.5.4. New Pharmacological Agents for lowering Glomerular Capillary Pressure

To date ACEIs and AT_1RAs are the only pharmacological agents that have been found to lower glomerular capillary pressure and afford effective renal protection. Nevertheless, ACEI or AT_1RA treatment slows but does not arrest the progression of CKD. A novel class of antihypertensive agents, the vasopeptidase inhibitors (VPI), shows promise as a renal protective agent. VPIs are single molecules that simultaneously inhibit both ACE and neutral endopeptidase, an ecto-enzyme that catabolizes several vasoactive molecules. In Chapter 4 we present studies in which we compared VPI treatment and the current "gold standard" of ACEI treatment with respect to glomerular haemodynamic and renal protective effects.

2. Proinflammatory gene expression and macrophage infiltration in the rat remnant kidney.

2.1. Introduction

Land-mark research into mechanisms contributing to the progression of CKD provided compelling evidence of the importance of glomerular haemodynamic adaptations to nephron loss, in particular, glomerular hyperperfusion and glomerular capillary hypertension [15] in the development of a vicious cycle of progressive nephron loss. Nevertheless, subsequent studies have identified non-haemodynamic factors that may also contribute to progressive renal injury. Prominent among these are proinflammatory and fibrotic cytokines. Further evidence of the importance of inflammatory factors is provided by the observation of macrophage (mϕ) infiltration in the remnant kidneys of rats subjected to extensive renal mass ablation [229, 238, 469-471].

A growing body of evidence suggests that mϕ may participate in chronic renal injury processes not conventionally considered inflammatory in nature. A possible link between altered glomerular haemodynamics and mϕ recruitment is suggested by in vitro studies showing that exposure of glomerular cells to mechanical stresses results in the induction of cell adhesion molecule (CAM) and proinflammatory cytokine expression [183, 196, 214, 472]. Angiotensin II, an important mediator of glomerular capillary hypertension in CKD progression, may also contribute directly to the development of a proinflammatory microenvironment in the remnant kidney by activating monocytes [285], stimulating TGF-β1 expression (in mesangial cells) [219] and activating the proinflammatory transcription factor, nuclear factor-kappa B (NF-κB) [473]. Tubular absorption of the increased amounts of filtered protein resulting from impaired glomerular barrier function to macromolecules may induce proinflammatory gene expression in renal tubule cells [312, 313, 319, 471], providing a link between augmented glomerular haemodynamics and the activation of chronic low grade tubulointerstitial inflammation.

Thus, a substantial body of in vitro data suggests that the expression of CAM and chemotactic molecules, necessary for monocyte recruitment and activation, may be induced in vivo in response to glomerular

capillary hypertension in the absence of classic "immune" or acute inflammatory stimuli. Previous in vivo studies have examined the expression of only 1 or 2 cytokines in temporal relation to macrophage infiltration in the remnant kidney model. In this study we sought to test the hypothesis that coordinated upregulation of several pro-inflammatory factors is a fundamental response to renal mass ablation and that inhibition of the RAS, which leads to virtually complete protection from progressive renal injury after 5/6 nephrectomy [27-29], suppresses this coordinated response. Our aims therefore were:

1. To describe changes in the expression of a selection of CAM and cytokines in relation to mϕ infiltration in the remnant kidney following 5/6 nephrectomy.
2. To examine the effect of renal protective treatment with inhibitors of the RAS on the expression of CAM and cytokines in the remnant kidney.
3. To compare the effect of RAS inhibition with ACEI vs. AT_1RA on the expression of CAM and cytokines in the remnant kidney.

2.2. Methods

2.2.1. Animals

Male Munich-Wistar rats (weight 230-310g) were obtained from Simonsen Laboratories (Gilroy, California, USA), housed under standard conditions and given unrestricted access to standard rodent chow and water. Rats were subjected either to renal mass ablation by right nephrectomy and ligation of two or three branches of the left renal artery, producing infarction of approximately two-thirds of the left kidney (n=60), or to sham operation by laparotomy and mobilization of the renal vessels (*SHM*; n=18). All surgical procedures were performed under pentobarbital anaesthesia (Nembutal 50mg/kg by intraperitoneal injection; Abbott Laboratories, Chicago, Illinois, USA). On day 4 after renal mass ablation, rats were started on candesartan cilexetil (Takeda Chemical Industries, Osaka, Japan) 70mg/l (4-10 mg/kg/day) in the drinking water with vehicle [ethanol (0.1%v/v), polyethylene glycol (0.1%v/v) and sodium bicarbonate (1 mmol/l)] added to solubilize candesartan (*CSN*; n=24) or enalapril (Merck Research Laboratories, Rahway, New Jersey, USA) 100mg/l (6-14 mg/kg/day) in drinking water (*ENA*; n=18). Controls received vehicle alone (*VEH*; n=18). Drug doses were determined during a pilot study and were chosen to achieve equivalent control of blood pressure in the ACEI and AT_1RA groups. At 4-week intervals, systolic blood pressure (SBP) was measured in conscious rats by the tail-cuff method. The mean of 5 readings was

taken to represent the SBP. Daily urinary protein excretion rate ($U_{pr}V$) determined by spectrophotometry after precipitation with 3% sulfosalicylic acid on urine collected from rats individually housed in metabolic cages for 24 hours. Rats from each group were sacrificed at 4, 8 and 12 weeks after surgery. Before sacrifice, rats were anaesthetized and portions of renal cortex from areas that were distant from the infarct scar were excised and snap-frozen in liquid nitrogen for subsequent extraction of RNA. The rest of the remnant kidney was then removed and fixed in 10% phosphate buffered formalin.

The above study protocols and those described in Chapters 3 and 4 were approved by the Standing Committee on Animals at Harvard Medical School.

2.2.2. Competitive reverse transcription polymerase chain reaction (RT-PCR).

Total RNA was extracted from frozen portions of renal cortex by the cesium chloride ultracentrifugation method [474]. RNA was quantitated by determination of ultraviolet absorbance at 260 nm, and its purity was assessed by measuring the optical density ratio at 260 and 280 nm. For preparation of cDNAs, 4 μg of heat-denatured RNA was used in the reverse transcription (RT) reaction. The entire sample in a total volume of 20 μl contained 4 μg of RNA; 0.5 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia Biotech Inc., Piscataway, NJ); 0.5 μg oligo-d(T)$_{12-18}$ (Pharmacia Biotech Inc.); 40U RNasin ribonuclease inhibitor (Promega, Madison, WI); 200U M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD) in a buffer of 50 mM Tris-HCl (pH 8.3); 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol. The solution was incubated for 60 minutes at 37 °C, and then held at 95 °C for 5 minutes to arrest the reaction.

Preparations of cDNA were then used as substrate for competitive PCR reaction using competitive DNA mimics and oligonucleotide primer sets (Genosys Biotechnologies, Inc., Woodlands, TX). Competitive DNA mimics for each factor, comprising a segment of neutral DNA with sequences complementary to the gene specific primers attached to each end, were constructed using a PCR MIMIC Construction Kit (CLONTECH Laboratories, Inc., Palo Alto, CA). Primer sets were designed for rat VCAM-1, ICAM-1, MCP-1, IL-1β, TNF-α, TGF-β1 and GAPDH based on published cDNA sequences [266, 475]. An equal volume of each cDNA solution was used for amplification in 20 μl of reaction mixture containing competitive DNA mimic, 0.5 μM primer sets; 0.5 U *Taq* DNA polymerase (Pharmacia Biotech Inc.); 250

μM each of dATP, dCTP, dGTP and dTTP (Pharmacia Biotech Inc.) in a buffer of 10 mM Tris-HCl (pH 9.0) and optimal concentration of $MgCl_2$. PCR was performed using a Peltier Thermal Cycler (MJ Research Inc, Watertown, MA). Optimal PCR conditions, namely concentration of competitive DNA mimic, annealing temperature and amplification cycles, were determined for each factor in preliminary studies. Amplification was initiated with incubation at 94 °C for 2 minutes followed by amplification cycles as follows: 94 °C for 15 seconds, annealing temperature for 30 seconds, 72 °C for 1 minute. Sequences of oligonucleotide primer sets and optimal conditions are listed in Table 2.1. PCR products (7 μl) were subjected to gel electrophoresis (5 % polyacrylamide) and then DNA bands were visualized under ultraviolet light after ethidium bromide staining (0.05 μg/ml for 10 minutes) and photographed (Fig. 2.1A). Densities of competitive mimic and target gene DNA bands were measured by scanning densitometry using ScanJet 4c (Hewlett Packard, Corvallis, OR) with NIH Image software. The ratios of the densities of the respective bands were plotted to establish a linear relationship of the ratios over serial dilutions of template (Fig. 2.1B). Thus, absolute amounts of mRNA from unknown samples were calculated as previously described [266, 476], from the known amount of the mimic in the starting reaction using the formula:

$$[\text{target gene}] = [\text{mimic}] \times ([\text{target gene product}] \div [\text{mimic product}])^{1/\alpha}$$

where α = gradient of the log plot of [target gene product]:[mimic product] vs. serial dilutions of starting cDNA (Fig 2.1B). Specimens were run in duplicate and the average value used. In addition, for 1 specimen, extra tubes were added containing half and double the standard amount of cDNA. Figure 2.1C confirms the linear relationship between the amount of cDNA added to the reaction and the estimated target gene concentration and shows that the assay is consistently capable of detecting a two-fold difference in target gene concentration [266]. As the number of specimens exceeded the capacity of the thermal cycler (n=96), all specimens from the study could not be included in a single PCR reaction. Specimens from *VEH* and *SHM* groups at each time point were initially run together to determine the time course of gene expression after 5/6 nephrectomy. To assess the effect of treatment group on gene expression, all specimens from a given time point were then assayed together in single PCR reactions. To provide uniformity in these latter 3 cross-group data sets, data were expressed as ratios to the mean value for the *SHM* group at each time point. Levels of mRNA for glyceraldehyde phosphate

dehydrogenase (GAPDH), a gene constitutively expressed at constant levels in cells, were used to confirm that starting amounts of cDNA were similar among groups.

2.2.3. Immunohistochemistry

Macrophage infiltration and expression of ICAM-1, MCP-1, IL-1β, TNF-α and TGF-β1 proteins was assessed by immunohistochemistry. For this purpose, 4 μm thick paraffin sections of formalin-fixed tissues were used for immunoperoxidase analysis after baking at 60 °C for one hour, deparaffinization and rehydration (100% xylene X 4 for three minutes each, 100% ethanol X 4 for three minutes each and running water for five minutes). The sections were then microwave treated at 93°C for 30 minutes in preheated 10 mM citrate buffer, pH 6.0, cooled for 15 minutes and transferred to phosphate buffered saline (PBS). Sections were then blocked with a 1.5% solution of serum of the species from which the secondary antibody was derived at room temperature for 15 minutes. Next, sections were incubated with mouse monoclonal antibodies against *rat* ICAM-1 (clone CD54, 1:150 dilution, Biosurface International, Camarilla, CA), hamster antimouse MCP-1 which cross-reacts with rat MCP-1 (clone 2H5, 1:50 dilution, Biosurface International), rabbit polyclonal antibodies against rat IL-1β (1:100 dilution, Endogen, Woburn, MA), TNF-α (1:50 dilution, Endogen), TGF-β1(V) (1:150 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mouse monoclonal antibodies to the rat monocyte/macrophage marker ED1 (clone ED1, 1:150 dilution, Biosurface International) for 1 hour in a humid chamber at room temperature. The secondary antibody (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlington, CA) was used according to manufacturer's instructions. Slides were rinsed with PBS between each incubation. Sections were developed using 3,3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO) as substrate and counter stained with Gill's Hematoxylin (Fisher Scientific, Pittsburgh, PA). Specimens in which the above procedure was repeated but with the primary antibody omitted or substituted with pre-immune serum from the respective species of the primary antibody, served as negative controls.

Macrophage infiltration was assessed by counting the number of ED1-positive cells in 30 glomerular profiles and in 20 randomly chosen 0.25 X 0.25mm areas of tubulointerstitium for each kidney.

2.2.4. Morphology

Renal tissue was embedded in paraffin and processed for light microscopy. The frequency of FSGS was estimated by examining all glomeruli seen in one or two coronal sections from each kidney stained by the periodic acid-Schiff method. Segmental sclerosis was defined as glomerular capillary collapse with hyaline deposition and/or adhesion to the parietal layer of Bowman's capsule. A glomerulosclerosis score was determined by expressing the number of glomeruli with segmental or global sclerosis as a percentage of the total number of glomeruli counted for each rat (>70 per rat). Tubulointerstitial injury was assessed at medium power on the same sections. Each of 3 aspects of tubulointerstitial injury (tubule proteinaceous casts and dilation; interstitial inflammation; interstitial fibrosis) was assigned a score from 0 to 3 according to severity (0=no abnormality; 1=mild; 2=moderate; 3=severe), and these scores added to yield an overall Tubulointerstitial Score (TIS) from 0 to 9 (arbitrary units). The pathologist was unaware of the group assignment of individual rats.

2.2.5. Statistical Analysis

Continuous variables are expressed as mean(SEM). Differences among multiple groups were assessed using the Kruskal-Wallis test; between two groups, with the Mann-Whitney test. The null hypothesis was rejected at $P<0.05$. Statistical analyses were conducted using Statview 4.01 (Abacus Concepts Inc., Berkley, California, USA).

2.3. Results

2.3.1. General Data

Before surgery, there were no differences among groups with respect to body weight, SBP or $U_{pr}V$ (Table 2.2.). Body weight increased in all groups throughout the study but was marginally lower in *ENA* and *CSN* rats than *SHM* rats at 12 weeks. Following 5/6 NPX, SBP increased in *VEH*, but not in *ENA* or *CSN* rats. Indeed, treatment with enalapril or candesartan lowered SBP to levels below those observed in *SHM* rats. A progressive rise in $U_{pr}V$ was observed in *VEH*, but not in *SHM*, *ENA* or *CSN* rats. Although $U_{pr}V$ levels at 4 weeks were lower in *ENA* and *CSN* than *SHM* rats, similar tendencies were not significant at 8 and 12 weeks. Severe, progressive FSGS and tubulointerstitial fibrosis developed in the remnant kidneys of *VEH*

rats over time whereas treatment with enalapril or candesartan markedly limited renal injury to levels not significantly different to those observed in *SHM* rats (Table 2.3.).

2.3.2. Competitive RT-PCR

Mean values for GAPDH mRNA levels, determined by competitive RT-PCR, were similar among the groups in each set of PCR reactions, confirming that the starting concentrations of cDNA were not significantly different. Data for each gene are presented here as absolute values (and not as a ratio to GAPDH) because it is not possible to determine GAPDH and target gene mRNA levels on the same aliquot of cDNA with competitive RT-PCR. Nevertheless, when calculated as a ratio to GAPDH, the patterns of expression obtained were very similar to the patterns seen with the absolute values. Although no differences in mRNA levels for VCAM-1, ICAM-1, IL-1β or TNF-α between *VEH* and *SHM* rats were observed at 4 weeks, approximately 2 fold statistically significant increases became evident for each of these factors in *VEH* rats at 8 and 12 weeks (Figure 2.2.). By contrast, marked increases in renal cortical mRNA levels for TGF-β1 (approximately 3 fold vs. *SHM*) and MCP-1 (approximately 4 fold) were observed at 4 weeks after surgery in *VEH* rats, elevations that were sustained at 8 and 12 weeks (Figure 2.2.). Treatment with enalapril or candesartan virtually abrogated the respective elevations in mRNA levels for VCAM-1, ICAM-1, MCP-1, IL-1β and TGF-β1 at 12 weeks, such that each remained similar to the values observed in *SHM* rats (Figure 2.3.). mRNA levels for MCP-1 and TGF-β1 were marginally higher in *CSN* vs. *SHM* rats at 12 weeks, but values in both treated groups were markedly lower than those of *VEH*. The tendency for TNF-α mRNA levels to be lower in both *ENA* and *CSN* rats vs. *VEH* did not achieve statistical significance. Indeed, TNF-α levels remained significantly greater in *CSN* than *SHM* rats. When mRNA levels for each molecule were expressed as a ratio to the respective mean *SHM* value, trends among the groups at earlier time points were similar to those observed at 12 weeks (Table 2.4.). The upregulation of TGF-β1 at 4 weeks was effectively prevented by enalapril and candesartan, whereas the increase in mRNA levels for MCP-1 was only partially inhibited by enalapril and candesartan. At 8 weeks after surgery the upregulation of VCAM-1, ICAM-1 and MCP-1 was largely blocked in both *ENA* and *CSN* groups. Increases in TGF-β1, IL-1β and TNF-α mRNA levels at 8 weeks were significantly inhibited in

ENA but not in *CSN* rats. Similar patterns of expression were obtained without factoring gene expression for the mean *SHM* value (data not shown).

2.3.3. Immunohistochemistry

Immunohistology confirmed that the increases in mRNA levels described above were accompanied by corresponding increases in protein expression in *VEH* rats and allowed localization of the enhanced expression within the remnant kidney (Figure 2.4.). TGF-β1 staining was strongly positive in the glomeruli, tubules and interstitium of remnant kidneys in *VEH* rats at all time points. Staining for MCP-1 was present in tubule cells and interstitial cells within areas of cellular infiltration in *VEH* rats at 4, 8 and 12 weeks. Staining for IL-1β and TNF-α also was largely localized to tubule epithelial cells. For TNF-α, occasional positive-staining cells were also present in glomeruli and areas of interstitial inflammation. By contrast, staining for ICAM-1 was localized to the glomeruli of *VEH* rats, in particular to the glomerular capillary loops, and was strongly positive at all time points. Peritubular capillaries were also positive for ICAM-1. Weak staining for ICAM-1 was accompanied, in some tubules, by positive staining of the epithelial cell brush border. Staining for TGF-β1, MCP-1, ICAM-1, and IL-1β appeared much weaker in kidneys from *SHM* or those from *ENA* and *CSN* rats. For TGF-β1, glomerular staining was absent in *SHM* and treated rats and tubule staining was only weakly positive. Glomeruli of *SHM* rats were generally negative for ICAM-1 and although some stained positive in *ENA* and *CSN* rats, staining was of lesser intensity than in *VEH* rats. By contrast, immunostaining of tubules for TNF-α did not appear to be reduced either in *ENA* or *CSN* rats. Controls in which the primary antibody was omitted, were negative apart from some staining of erythrocytes. Controls in which pre-immune serum from the appropriate species was substituted for the primary antibody showed only minimal background staining. The use of pre-immune mouse serum was associated with positive staining of intraluminal tubular proteinaceous casts (Figure 2.4.).

2.3.4. Macrophage infiltration

Macrophage infiltration was evident in both the glomeruli and interstitium of remnant kidneys in *VEH* rats. At 4 weeks after surgery the number of macrophages was markedly increased in *VEH* vs. *SHM* rats, by approximately 10 fold in glomeruli and 20 fold in the interstitium. These increases were sustained at 8 and 12 weeks. Treatment with enalapril or candesartan largely prevented glomerular and tubulointerstitial mϕ

infiltration although mϕ numbers were marginally but significantly higher in the treated groups *vs. SHM.* There were no differences in mϕ numbers between *ENA* and *CSN* rats (Table 2.3.).

Table 2.I. Summary of primer sequences and PCR conditions.

Primer	Sense sequence	Antisense sequence	Size (bp)	Annealing Temp. (°C)
MCP-1	ATG CAG GTC TCT GTC ACG	CTA GTT CTC TGT CAT ACT	447	55
TGF-β1	CTT CAG CTC CAC AGA GAA GAA CTG C	CAC GAT CAT GTT GGA CAA CTG CTC C	298	64
TNF-α	TAC TGA ACT TCG GGG TGA TTG GTC C	CAG CCT TGT CCC TTG AAG AGA ACC	295	65
IL-1β	TGA TGT TCC CAT TAG ACA GC	GAG GTG CTG ATG TAC CAG TT	378	55
ICAM-1	AGA AGG ACT GCT TGG GGA A	CCT CTG GCG GTA ATA GGT G	332	60
VCAM-1	CTG ACC TGC TCA AGT GAT GG	GTG TCT CCC TCT TTG ACG CT	260	60
GAPDH	AAT GCA TCC TGC ACC ACC AA	GTA GCC ATA TTC ATT GTC ATA	516	55

Table 2.2. Physiological data; mean(SEM)

		n	**Baseline**	*n*	**4 weeks**	*n*	**8 weeks**	*n*	**12 weeks**
Weight (g)	SHM	*18*	267(2)	*6*	273(11)	*6*	322(14)	*6*	360(4)
	VEH	*18*	272(4)	*6*	264(16)	*6*	317(10)	*6*	342(18)
	ENA	*18*	272(3)	*6*	249(13)	*6*	317(4)	*6*	338(7)[b]
	CSN	*24*	267(3)	*9*	282(5)	*6*	301(7)	*9*	334(6)[b]
SBP (mmHg)	SHM	*18*	130(4)	*6*	123(3)[a]	*6*	123(3)[a]	*6*	109(2)[a]
	VEH	*18*	131(3)	*6*	173(13)	*6*	156(9)	*6*	172(8)
	ENA	*18*	124(3)	*5*	100(5)[a,b]	*6*	105(3)[a,b]	*6*	105(6)[a]
	CSN	*24*	128(2)	*9*	96(7)[a,b]	*6*	100(5)[a,b]	*9*	97(3)[a,b]
UprV (mg/day)	SHM	*18*	4.7(0.4)	*6*	6.0(1.2)[a]	*6*	7.4(2.4)[a]	*6*	9.4(1.3)[a]
	VEH	*15*	4.9(0.5)	*6*	58(14)	*6*	44(2.9)	*6*	93(12)
	ENA	*18*	5.4(0.5)	*6*	2.0(0.3)[a,b,c]	*6*	4.6(1.0)[a]	*6*	8.4(0.9)[a,c]
	CSN	*24*	4.5(0.4)	*9*	3.4(0.3)[a,b]	*6*	6.1(3.4)[a]	*9*	5.7(0.8)[a]

[a] $P<0.05$ versus VEH; [b] $P<0.05$ versus SHM; [c] $P<0.05$ versus CSN by Kruskal-Wallis and Mann-Whitney tests.

SHM: sham-operated rats; VEH: rats treated with vehicle after 5/6 nephrectomy; ENA: rats treated with enalapril after 5/6 nephrectomy; CSN: rats treated with candesartan after 5/6 nephrectomy

Table 2.3. Histological data; mean(SEM)

		4 weeks	8 weeks	12 weeks
GS (%)	SHM	0.1(0.06)[a]	1.1(0.6)[a]	2.1(0.5)[a]
	VEH	27.1(5.6)	34.6(5.4)	62.3(4.1)
	ENA	1.7(1.0)[a,b]	1.7(0.6)[a]	3.3(0.9)[a]
	CSN	1.0(0.2)[a,b]	4.9(2.1)[a]	1.3(0.4)[a]
TIS (0-9)	SHM	0.0(0.0)[a]	0.0(0.0)[a]	0.0(0.0)[a]
	VEH	1.3(0.4)	1.1(0.3)	3.6(0.7)
	ENA	0.0(0.0)[a]	0.0(0.0)[a]	0.0(0.0)[a]
	CSN	0.3(0.1)	0.0(0.0)[a]	0.0(0.0)[a]
glomerular mϕ	SHM	0.3(0.1)[a]	0.3(0.1)[a]	0.3(0.04)[a]
(cells/glomerular profile)	VEH	3.2(0.4)	3.3(0.5)	3.7(0.3)
	ENA	0.8(0.1)[a,b]	0.7(0.1)[a,b]	0.5(0.2)[a]
	CSN	0.6(0.1)[a,b]	1.0(0.1)[a,b]	0.7(0.1)[a,b]
interstitial mϕ	SHM	0.2(0.1)[a]	0.5(0.2)[a]	0.2(0.04)[a]
(cells/0.0625 mm^2)	VEH	3.9(0.5)	4.7(0.9)	4.2(1.2)
	ENA	0.9(0.2)[a,b]	0.7(0.1)[a]	0.6(0.1)[a,b]
	CSN	0.9(0.2)[a,b]	1.2(0.2)[a,b]	0.6(0.07)[a,b]

[a] $P<0.05$ versus VEH; [b] $P<0.05$ versus SHM by Kruskal-Wallis and Mann-Whitney tests.

GS: glomerulosclerosis expressed as percentage of glomeruli with segmental or global sclerosis; TIS: tubulointerstitial score indicating tubulointerstitial damage on a scale from 0-9; mϕ: macrophage.
SHM: sham-operated rats; VEH: rats treated with vehicle after 5/6 nephrectomy; ENA: rats treated with enalapril after 5/6 nephrectomy; CSN: rats treated with candesartan after 5/6 nephrectomy.

Table 2.4. Renal cortical mRNA levels for CAMs and cytokines in rats after 5/6 nephrectomy expressed as a ratio to levels in sham-operated rats; data=mean(SEM).

		4 weeks	8 weeks	12 weeks
VCAM-1	VEH	1.85(0.18)	1.36(0.08)	2.15(0.43)
	ENA	0.88(0.10)[a]	1.03(0.1)[a]	1.39(0.11)
	CSN	1.30(0.19)	1.14(0.13)	1.28(0.20)
ICAM-1	VEH	1.41(0.20)	1.50(0.14)	2.14(0.29)
	ENA	0.88(0.09)[a]	0.66(0.06)[a]	1.33(0.21)
	CSN	1.02(0.14)	0.85(0.14)[a]	1.31(0.24)[a]
MCP-1	VEH	11.9(3.53)	2.64(0.18)	2.67(0.23)
	ENA	7.25(1.60)	1.29(0.12)[a]	1.00(0.11)[a,b]
	CSN	5.12(1.06)[a]	1.39(0.31)[a]	1.41(0.16)[a]
IL-1β	VEH	2.08(0.45)	3.44(0.58)	1.80(0.20)
	ENA	2.53(0.57)	0.75(0.13)[a,b]	1.02(0.17)[a]
	CSN	3.04(0.72)	2.66(0.85)	1.10(0.13)[a]
TNF-α	VEH	2.02(0.27)	1.44(0.17)	3.40(0.52)
	ENA	1.33(0.26)	0.55(0.08)[a,b]	1.82(0.35)[a]
	CSN	1.24(0.07)[a]	1.04(0.16)	2.46(0.40)
TGF-β1	VEH	2.87(0.35	2.78(0.42	3.76(0.63
	ENA	0.73(0.16)[a,b]	0.55(0.08)[a,b]	1.27(0.18)[a]
	CSN	1.34(0.20)[a]	2.11(0.32)	1.63(0.18)[a]
GAPDH	VEH	1.23(0.13)	0.96(0.16)	1.19(0.19)
	ENA	1.11(0.17)	1.03(0.14)	0.90(0.06)
	CSN	1.24(0.14)	1.22(0.28)	1.04(0.15)

[a] $P<0.05$ versus VEH; [b] $P<0.05$ versus CSN by Kruskal-Wallis and Mann-Whitney tests.

VEH: rats treated with vehicle after 5/6 nephrectomy; ENA: rats treated with enalapril after 5/6 nephrectomy; CSN: rats treated with candesartan after 5/6 nephrectomy.

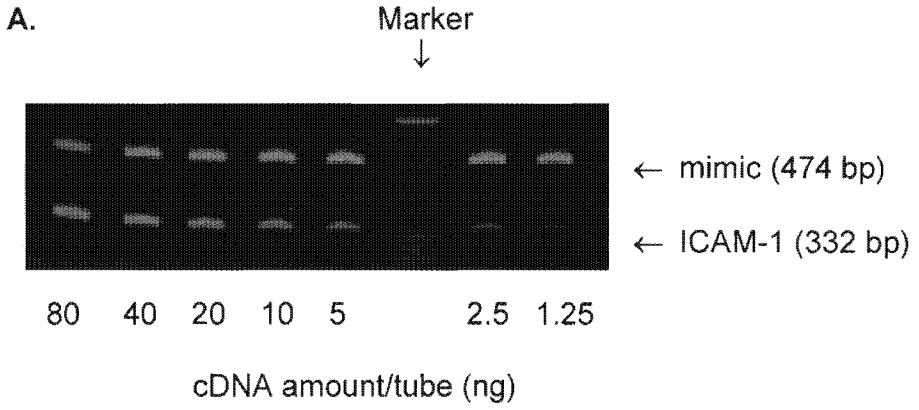
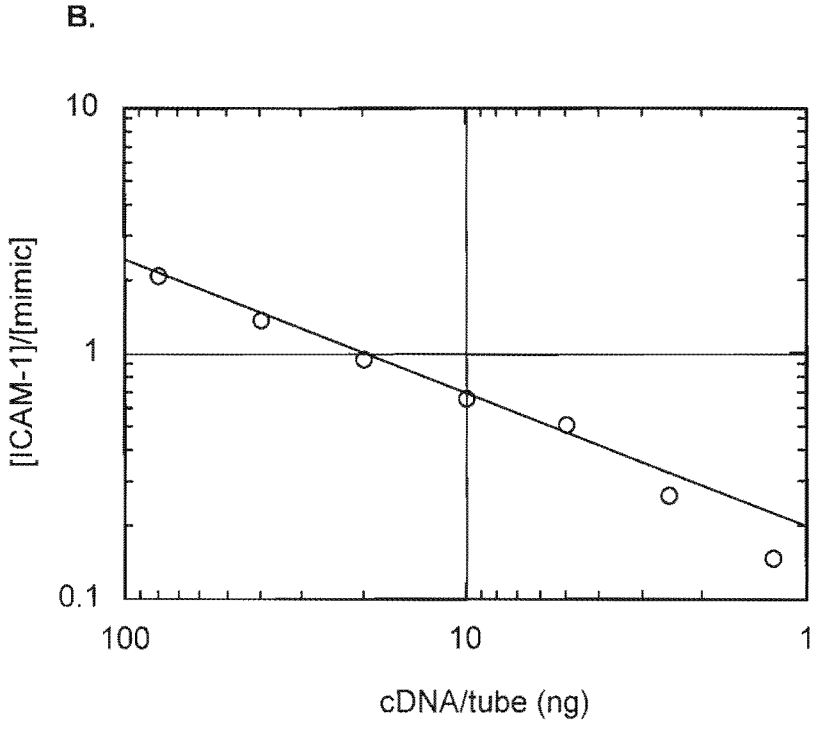
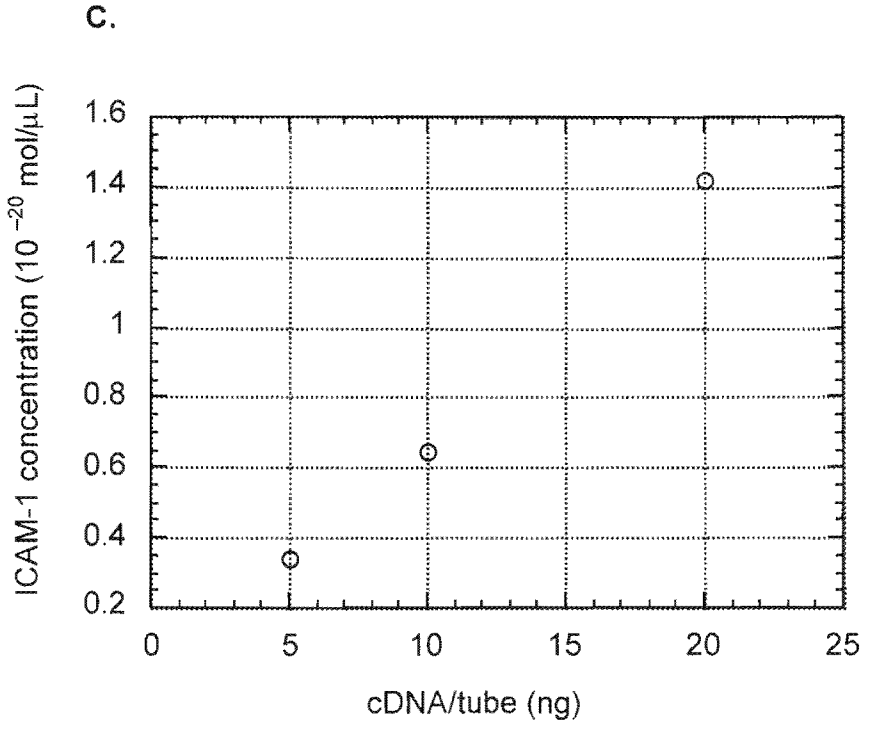


Figure 2.1. A: Polyacrylamide gel showing electrophoretic bands for mimic and gene-specific (ICAM-1) cDNA at different amounts of starting cDNA. **B:** Log-log plot of starting cDNA amount versus the ratio of gene specific (ICAM-1):mimic PCR product concentration showing a linear relationship over serial dilutions of starting cDNA. **C:** Linear plot of starting cDNA amount versus ICAM-1 concentration determined by competitive RT-PCR illustrating the feasibility of detecting a 2-fold difference in gene expression with this assay.





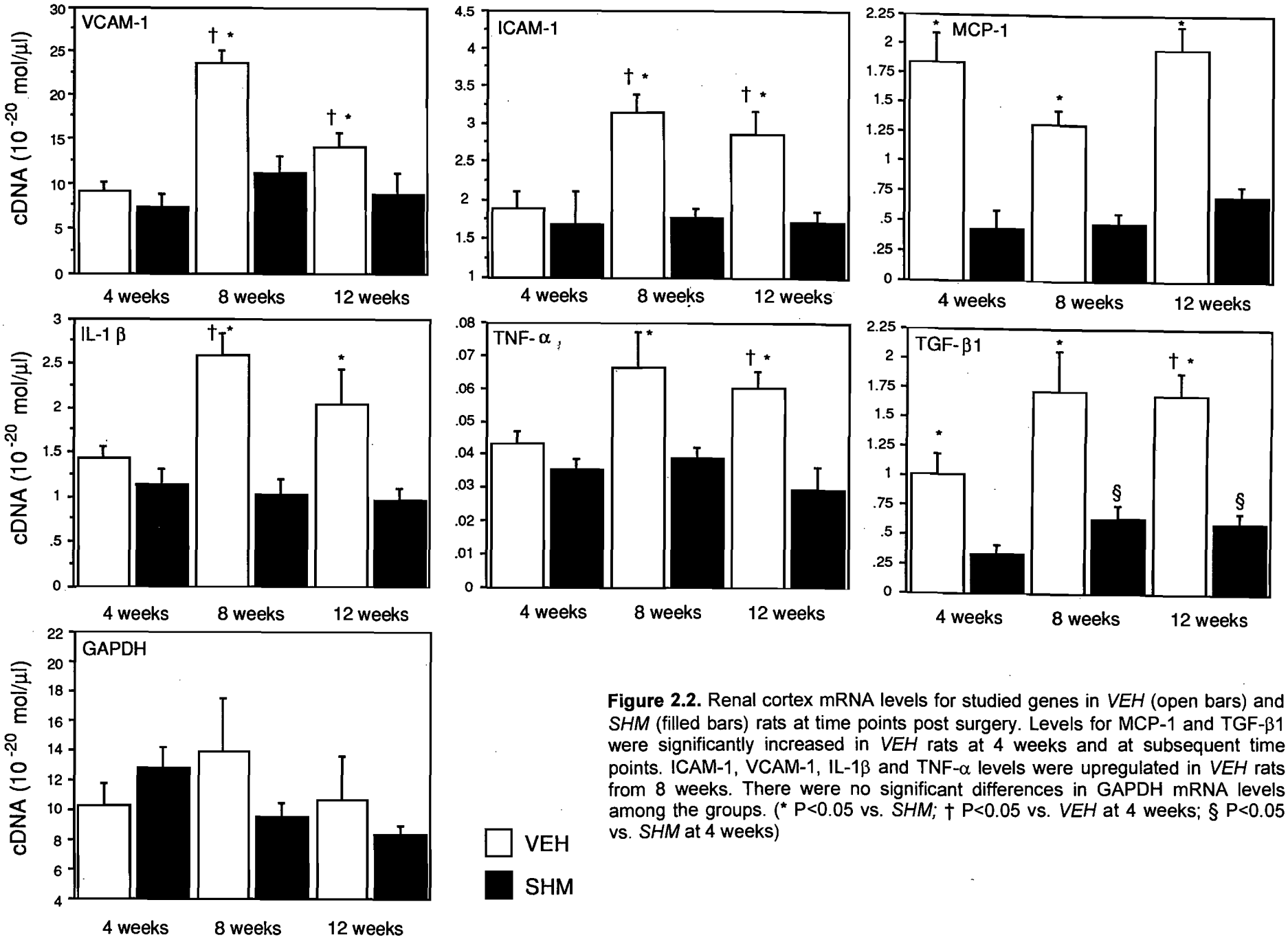


Figure 2.2. Renal cortex mRNA levels for studied genes in *VEH* (open bars) and *SHM* (filled bars) rats at time points post surgery. Levels for MCP-1 and TGF-β1 were significantly increased in *VEH* rats at 4 weeks and at subsequent time points. ICAM-1, VCAM-1, IL-1β and TNF-α levels were upregulated in *VEH* rats from 8 weeks. There were no significant differences in GAPDH mRNA levels among the groups. (* P<0.05 vs. *SHM;* † P<0.05 vs. *VEH* at 4 weeks; § P<0.05 vs. *SHM* at 4 weeks)

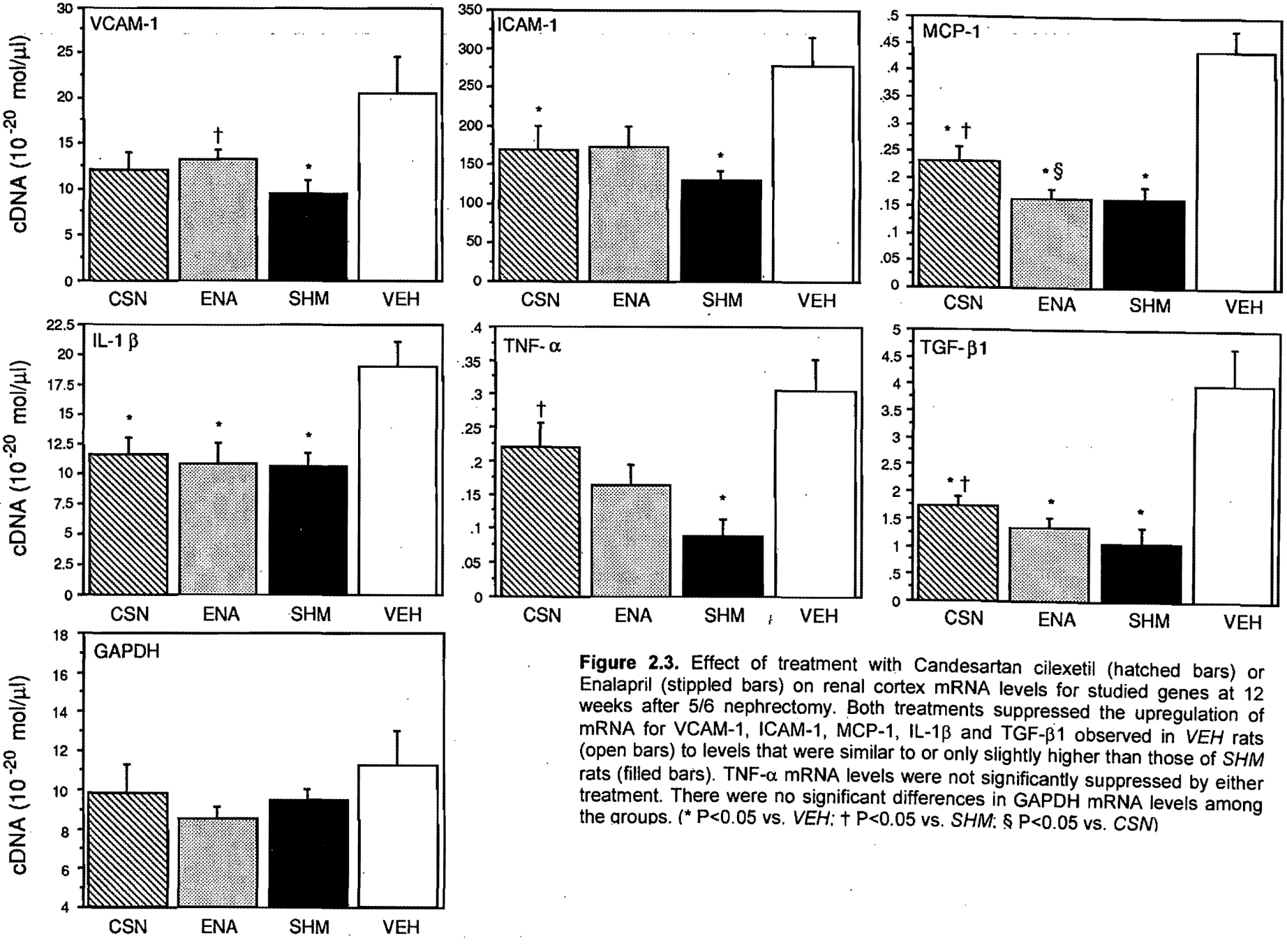


Figure 2.3. Effect of treatment with Candesartan cilexetil (hatched bars) or Enalapril (stippled bars) on renal cortex mRNA levels for studied genes at 12 weeks after 5/6 nephrectomy. Both treatments suppressed the upregulation of mRNA for VCAM-1, ICAM-1, MCP-1, IL-1β and TGF-β1 observed in *VEH* rats (open bars) to levels that were similar to or only slightly higher than those of *SHM* rats (filled bars). TNF-α mRNA levels were not significantly suppressed by either treatment. There were no significant differences in GAPDH mRNA levels among the groups. (* P<0.05 vs. *VEH*; † P<0.05 vs. *SHM*; § P<0.05 vs. *CSN*)

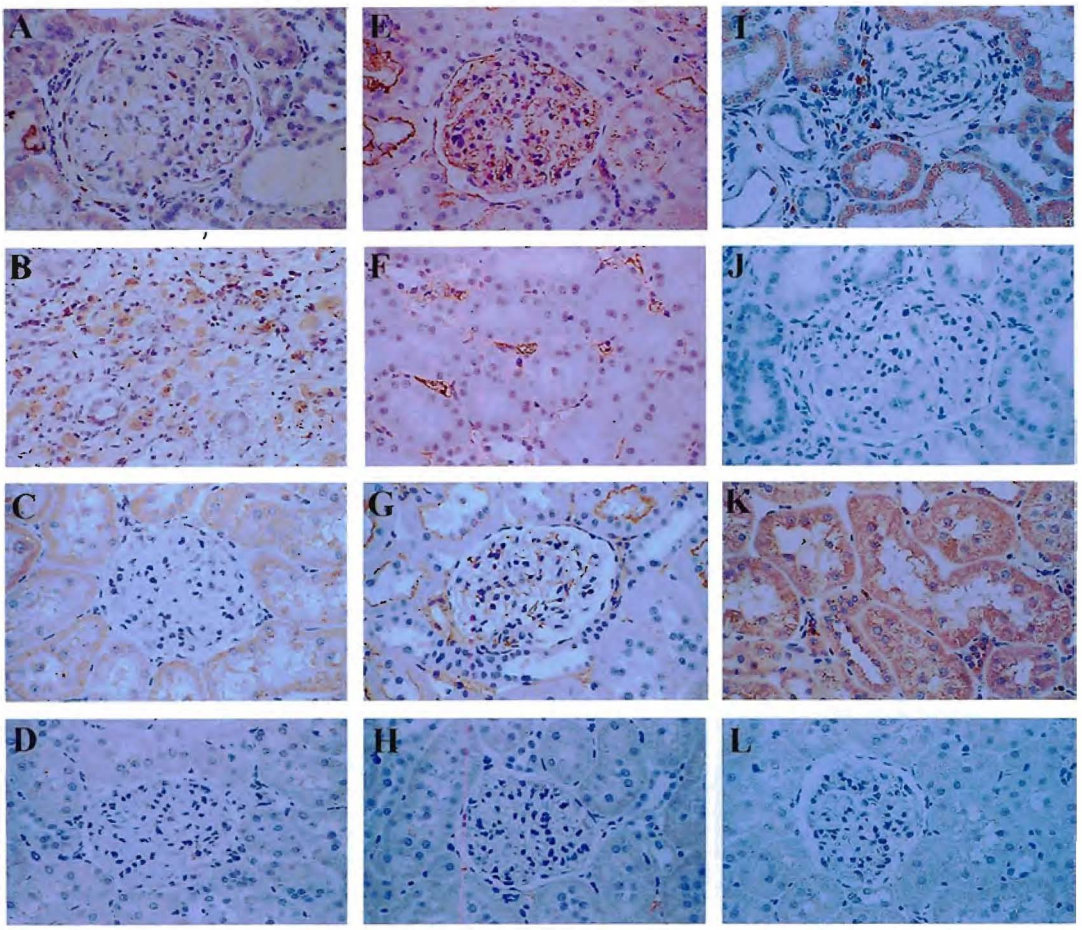


Figure 2.4. Immunohistology:

A-D: MCP-1immunostaining was evident in tubule cells (A) and infiltrating interstitial cells (B) but was weakly positive or negative in glomeruli (A) of remnant kidneys from *VEH* rats at 12 weeks after 5/6 NPX. Weak tubule staining was observed in *CSN* rats (C) and negative staining, in sham-operated *SHM* rats (D).
E-H: ICAM-1immunostaining was strongly positive in glomeruli of *VEH* rats at 4 weeks, particularly in endothelial cells of glomerular capillaries (E). Positive staining was also observed along the brush border of tubule cells (E) and in endothelial cells of peritubular capillaries (F). *CSN* rats displayed only weak glomerular and tubule brush border staining (G) and kidneys from *SHM* rats were negative for ICAM-1(H).
I, K, L: TNF-α immunostaining was positive in tubule cells and infiltrating cells in *VEH* rats (I) and tubule cells in CSN rats (K) at 12 weeks after 5/6 NPX. Staining was negative in kidneys from *SHM* rats (L).
Panel J shows a negative control in which the primary antibody was omitted to exclude non-specific binding of the secondary antibody to renal tissue.
(All panels 250X magnification)

2.5. Discussion

Macrophages represent a prominent component of inflammatory cell infiltrates in several forms of experimental and human glomerulonephritis and are thought to contribute directly to glomerular injury through secretion of reactive oxygen species, proteases, eicosanoids and cytokines [477, 478]. Our data confirm previous observations that mϕ infiltrate the remnant kidney in response to extensive renal mass ablation [229, 238, 469-471], attaining near-maximum levels at 4 weeks after surgery, a time point at which renal injury is relatively mild [238, 469]. The infiltration of mϕ in advance of renal injury in this model suggests that mϕ recruitment is a response to nephron loss *per se* and not merely part of the healing response to renal injury. When taken together with observations that short-term depletion of mϕ by irradiation results in a transient reduction in mesangial matrix expansion in partially nephrectomized rats [226], our findings are consistent with the hypothesis that the recruitment of mϕ plays an important role in the pathogenesis of chronic progressive renal injury resulting from extensive nephron loss.

Recruitment of mϕ in acute inflammatory settings is thought to involve cytokine-induced endothelial CAM expression, margination, diapedesis and migration of mϕ into tissues down a chemotactic gradient (reviewed in [478, 479]). In the remnant kidney, alternative mechanisms may operate to initiate mϕ accumulation in the absence of major acute inflammatory or immune stimuli. As discussed in more detail in Chapter 1, these include induction of CAMs and chemoattractants in glomerular cells exposed to mechanical forces [183, 196], *in situ* proliferation of mϕ [229, 470] and renal tubule cell complement activation as well as expression of CAMs and chemoattractants following their uptake of abnormal amounts of protein [319, 320, 471].

We have observed approximately 2 fold increases in renal cortex mRNA levels for VCAM-1 and ICAM-1, adhesion molecules critical for monocyte recruitment, in untreated rats at 8 and 12 weeks after 5/6 nephrectomy. Increases in ICAM-1 and VCAM-1 mRNA were observed also in kidneys from rats subjected to unilateral ureteric obstruction (UUO) [480], a model characterized by interstitial mϕ infiltration and fibrosis [460]. Immunohistochemistry revealed positive staining for ICAM-1 in glomeruli and to a

lesser extent in tubules, a pattern similar to that observed in immune-complex mediated nephropathies [481-483]. ICAM-1 expression may be induced in endothelial cells exposed in vitro to shear stress [183]. In vivo, shear stress due to altered glomerular haemodynamics in the remnant kidney may conceivably therefore account for the induction ICAM-1 in the glomerular endothelium. Although we anticipated that ICAM-1 would be upregulated at 4 weeks after 5/6 nephrectomy in *VEH* rats due to glomerular hyperperfusion, we did not detect a significant increase in ICAM-1 mRNA levels in renal cortex. However, increased glomerular ICAM-1 immunostaining was observed at 4 weeks. This apparent contradiction may be explained by difficulty in detecting relatively small increments in glomerular ICAM-1 mRNA due to "dilution" in the face of stable levels of ICAM-1 mRNA in the non-glomerular portions of the cortex. An alternative explanation is that post-transcriptional regulation of glomerular ICAM-1 expression may occur.

Ingestion by renal tubule cells of grossly elevated amounts of filtered protein, as occurs in the protein overload model, results in interstitial macrophage infiltration associated with increased tubule expression of both VCAM-1 and ICAM-1 [319]. Similar mechanisms may play a role in the 5/6 nephrectomy model after 4 weeks, in response to the cumulative effects of significant proteinuria [320, 471].

MCP-1 is a specific monocyte chemoattractant associated with renal mϕ infiltration in several experimental models of glomerulonephritis [484-486], UUO [480] and human glomerulonephritis [487-489]. In vitro observations suggest that MCP-1 may also contribute to tissue injury by stimulating monocyte activation [490] and IL-1 production [491] as well as production of TGF-β and collagen by fibroblasts [492]. Our data confirm and extend the findings of previous investigators who have detected increases in renal MCP-1 mRNA levels at 2 weeks after 5/6 nephrectomy by northern blot analysis [469]. We observed a 4 fold increase in renal cortex MCP-1 mRNA levels at 4 weeks which was sustained at 8 and 12 weeks. Like ICAM-1, expression of MCP-1 is induced in endothelial cells exposed to shear stress [196]. Elevated MCP-1 mRNA levels have also been observed in glomeruli from DOCA-salt hypertensive rats [493] and from aortas of hypertensive rats [494], strongly suggesting that mechanical forces also induce MCP-1 expression in vivo. Renal tubule cells cultured with abnormally high extracellular protein concentrations synthesize MCP-1 [312], suggesting that MCP-1 expression by tubule epithelial cells and interstitial mϕ accumulation in the remnant kidney may also occur in response to proteinuria. Although immunostaining

was limited to tubule cells in our study, previous studies in diabetic rats [266] and 5/6 nephrectomized rats [469] have observed positive staining for MCP-1 in glomeruli.

TNF-α and IL-1β are important inflammatory cytokines that may be produced by infiltrating mϕ or intrinsic renal cells (reviewed in [495, 496]). Both TNF-α and IL-1β may play a role in sustaining inflammation through autocrine and paracrine induction of CAMs [479], chemotactic factors [487, 497, 498] and cytokines [499, 500]. A previous study was unable to detect TNF-α or IL-1β expression in the remnant kidney using northern analysis of whole kidney RNA [469], a technique that may fail to detect genes with very low levels of expression. Using RT-PCR, we have observed approximately 2 fold increases in renal cortex mRNA levels for TNF-α and IL-1β at 8 and 12 weeks after 5/6 nephrectomy, at which time points mϕ infiltration and renal injury were well established. Confirmatory immunohistology revealed positive staining for TNF-α and IL-1β localized mainly to tubule and interstitial cells with occasional positive glomerular cells. These findings are consistent with TNF-α and IL-1β playing a "down-stream" role in maintaining mϕ infiltration and activation in the remnant kidney, but perhaps not a critical role in initiating mϕ recruitment. The apparent discrepancy between mϕ infiltration and expression of the mϕ products, TNF-α and IL-1β at 4 weeks may be due to difficulty in detecting increases in mϕ gene expression in the presence of unchanged expression in the much larger mass of renal parenchyma. Later increases in TNF-α and IL-1β mRNA likely reflect expression by intrinsic renal cells as well as mϕ.

A large body of experimental and clinical evidence supports the view that the profibrotic cytokine, TGF-β1, plays a major role in renal fibrosis resulting from a wide range of causes (reviewed in [501]). On the other hand, evidence suggests that this cytokine may also play a counter-regulatory, anti-inflammatory role in the kidney and may suppress cytokine production by glomerular mϕ [502-504]. Increased TGF-β1 expression has been observed in the remnant kidney using northern blot analysis [237, 505], in situ hybridization [237], immunohistology [237, 506] and ELISA [505]. Notably, Wu et al. localized TGF-β1 expression to infiltrating mϕ, using combined *in situ* hybridization and immunohistology techniques [237]. TGF-β1 mRNA has also been localized to endothelial cells and mesangial cells early in the development of glomerulosclerosis [192]. Our competitive RT-PCR data confirm that TGF-β1 mRNA levels were

increased approximately 3 fold at 4 weeks and sustained at 12 weeks. Immunostaining revealed TGF-β1 expression in glomeruli, tubules and interstitial cells. Several mechanisms may account for the induction of TGF-β1 expression in this model: exposure of endothelial cells to shear stress [472] and mesangial cells to cyclical stretching [214]; exposure of tubule cells to excess filtered protein [319]; direct effects of Ang II on mesangial cells [219]. Although TGF-β1 production has often been associated with primary immune-complex mediated injury, the interplay of several antigen-independent mechanisms may account for upregulation of TGF-β1 in the 5/6 nephrectomy model.

As reported in previous studies, we found that treatment with an ACEI or AT_1RA resulted in almost complete protection of the remnant kidney from progressive injury when started soon after 5/6 nephrectomy [27-29]. Further, the renal protection achieved by RAS inhibition was associated with significantly fewer infiltrating mϕ and less proinflammatory gene expression, suggesting that these latter processes are also dependent, directly or indirectly on the actions of Ang II. As discussed in Chapter 1, Ang II occupies a central role in many pathways thought to contribute to the pathogenesis of chronic injury in the remnant kidney. Renal protection with either ACEI or AT_1RA is therefore likely to be achieved through the consequences of reduced effects of Ang II at multiple sites. Since enalapril and candesartan achieved similar effects on all variables studied, we may also conclude that despite differences in the pharmacology of ACEIs and AT_1RAs, both agents exert their renal protective effects primarily through inhibition of the AT_1 receptor-mediated effects of Ang II. Findings of differences between ACEI and AT_1RA treatment on mϕ infiltration and VCAM-1 expression in the UUO model, but lack of effect of either treatment on ICAM-1 expression, suggest that AT_1 receptor independent mechanisms play a role in mϕ recruitment in this model of interstitial fibrosis [460, 480].

Our results extend the findings of other investigators who observed inhibition of renal TGF-β1 expression [505] and mϕ infiltration [237, Abbate, 1999 #368] with ACEI or AT_1RA treatment. Interestingly, MCP-1 upregulation is also inhibited by dietary protein restriction [469], which inhibits the RAS by decreasing renin mRNA levels [507]. Furthermore, inhibition of MCP-1 expression by low protein diet was only partial at 2 weeks after 5/6 nephrectomy. This is reminiscent of our current observation of partial inhibition of MCP-1

at 4 weeks by both ACEI and AT_1RA, suggesting that significant RAS-independent stimuli may also contribute to MCP-1 expression, at least in the early phases after 5/6 nephrectomy.

The lack of suppression of IL-1β and TGF-β1 with candesartan treatment at 8 weeks after surgery appears to be transient and of questionable significance since inhibition of TGF-β1 was evident at 4 and 12 weeks and inhibition of IL-1β, at 12 weeks. The absence of consistent suppression of TNF-α by either treatment on the other hand, strongly suggests that TNF-α expression is largely independent of both haemodynamic factors and the RAS in this setting and further implies that TNF-α alone is insufficient to promote the development of chronic injury in 5/6 nephrectomized rats receiving RAS inhibitors. While the concordance of RAS-inhibitor-associated suppression of proinflammatory gene expression and mϕ infiltration supports the notion that the group of factors studied are involved in the pathogenesis of progressive renal injury, data from this study cannot address the issue of the roles of specific cytokines. More direct evidence of the role of individual factors awaits experiments in which the effects of individual molecules or transcription factors are specifically inhibited or enhanced.

Our data provide further support for the hypothesis that following extensive renal mass ablation, RAS-dependent adaptive changes in the remnant kidney provoke the coordinated upregulation of various proinflammatory genes, resulting in recruitment and activation of mϕ. Further, the data suggest that mϕ may play an important contributory role in the pathogenesis of progressive FSGS and TIF. Although both ACEI and AT_1RA were effective in preventing or attenuating upregulation of several proinflammatory genes in this model, additional interventions that specifically inhibit proinflammatory gene expression may lead to increased therapeutic options for patients with established CKD progression. Indeed, in a recent study of rats with diabetic nephropathy the addition of treatment with monoclonal anti-TGF-β antibodies to ACEI treatment tended to produce more effective renal protection than either treatment alone [508].

3. Renal Protection in the Setting of Established Renal Injury

3.1. Introduction

The majority of experimental studies into mechanisms contributing to CKD progression have employed protocols in which renal protective strategies were introduced prior to the development of renal injury. This approach provides a useful experimental model but differs critically from clinical CKD because in patients, treatment is typically started only after renal injury is established. Early treatment of rats subjected to extensive renal mass ablation with ACEI effectively prevents the focal and segmental glomerulosclerosis and tubulointerstitial fibrosis that ensues in untreated rats [26, 27]. AT_1RAs, a novel class of antihypertensive drugs, inhibit the RAS distal to angiotensin-converting enzyme (ACE) and may offer therapeutic advantages over ACEI. Nevertheless, previous studies from this and other laboratories detected no significant differences in the renal protective effects of ACEI vs. AT_1RA in experimental models of CKD, when treatment was initiated before the onset of substantial renal injury [28, 160, 163, 164, 266, 463-467]. A single study however, purports to show an advantage of AT_1RA over ACEI in 5/6 nephrectomized rats [468]. In this study treatment was initiated only after renal injury was evident and was of considerably longer duration than previous studies, raising the possibility that subtle benefits of AT_1RA over ACEI may become evident only over an extended time period in a model where RAS blockade results in slowing but not arrest of CKD progression. This is particularly important because clinical trials of ACEI treatment in CKD have also observed slowing rather than arrest of CKD progression [168, 169, 373] implying that there is a need for more effective renal protective therapies.

Since the therapeutic ideal is to arrest or even reverse CKD progression, it is important to examine all factors that may contribute to slow progression of CKD during RAS inhibition. Systemic blood pressure has been shown in experimental [509] and clinical [337, 341, 342, 346, 510, 511] studies to be an important determinant of chronic renal injury but the role of blood pressure in the context of ACEI treatment requires further elucidation. Proteinuria, long regarded as a marker of glomerular injury, has recently been proposed as an important factor contributing to the pathogenesis of CKD progression [243]. Finally, as shown in the studies presented in Chapter 2 and others [237, 469], extensive renal mass ablation results in an apparently coordinated induction of several proinflammatory genes and infiltration of the remnant kidney by macrophages (mϕ). These chronic inflammatory responses are prevented by early

treatment with ACEI or AT_1RA, suggesting that mϕ and a variety of proinflammatory molecules may contribute to the pathogenesis of progressive renal fibrosis [237]. If this is indeed so, we hypothesized that the slow progression of renal injury that can be predicted to ensue when ACEI or AT_1RA therapy is started only after renal injury is established, would be associated with persistent upregulation of proinflammatory and fibrotic gene expression.

In this study we utilized relatively large numbers of rats in a delayed treatment protocol with prolonged follow up with the following objectives:

1. To determine whether, at doses that produce equivalent antihypertensive effects, the AT_1RA, candesartan and the ACEI, enalapril, share equivalent renal protective effects.
2. To examine the relationships between systemic blood pressure, proteinuria and renal injury during RAS inhibition in this model.
3. To examine the expression of selected inflammatory and profibrotic genes in the remnant kidney and the relationship between expression of these genes and renal injury.

3.2. Methods

3.2.1. Animals

Adult male Munich-Wistar rats (weight 218-278g) were obtained from Simonsen Laboratories (Gilroy, California, USA), housed under standard conditions and given unrestricted access to standard rodent chow and water. Rats were subjected either to 5/6 nephrectomy as described in Chapter 2 (n=63), or to sham operation (*SHM* rats; n=15). At 2-week intervals, systolic blood pressure (SBP) was measured by the tail-cuff method and daily urinary protein excretion rate ($U_{pr}V$) determined on urine collected from rats individually housed in metabolic cages for 24 hours. At five weeks after renal mass ablation, rats were stratified according to SBP and $U_{pr}V$ and allocated to the following groups: *CSN* rats (n=30) received candesartan cilexetil (TCV-116; Takeda Chemical Industries, Osaka, Japan) 50mg/l (3-7 mg/kg/day) in the drinking water. Vehicle comprising ethanol (0.1%v/v), polyethylene glycol (0.1%v/v) and sodium bicarbonate (5 mmol/l) was added to achieve water solubility of candesartan; *ENA* rats (n=27) received enalapril (Merck Research Laboratories, Rahway, New Jersey, USA) 110mg/l (7-15 mg/kg/day) in the drinking water with sodium bicarbonate (5 mmol/l). Some dosage adjustments were made initially to

achieve equivalent blood pressure control in the treatment groups. Six rats were sacrificed at 5 weeks after renal ablation and the remnant kidneys harvested to provide pretreatment data (*5WK* rats; n=6). The remaining rats were studied for a total of 12 weeks (Set A: CSN_A n=11; ENA_A n=11; SHM_A n=6) or 24 weeks (Set B: CSN_B n=19; ENA_B n=16; SHM_B n=9). At the end of the observation period, rats were anaesthetized with pentobarbital and portions of renal cortex distant from the infarct scar were excised and snap-frozen in liquid nitrogen for subsequent RNA extraction and immunohistology. The remnant kidney was then perfusion-fixed with 1.25% glutaraldehyde in 0.1mmol/l sodium cacodylate buffer (pH7.4), delivered through a catheter in the abdominal aorta at the measured systolic blood pressure of each rat. Kidneys were weighed after perfusion fixation. In order to evaluate renal hypertrophy, final remnant kidney weight, corrected for the increase in weight associated with perfusion fixation, was compared to an estimate of baseline remnant kidney weight taken as 1/3 of the weight of the right kidney removed at the time of renal mass ablation. The correction factor (1.38) was derived by comparing the weights of the unfixed right kidney with those of the perfusion-fixed left kidneys removed contemporaneously from 15 sham-operated rats.

Untreated controls were not included in the study because, based on previous experience in our laboratory, survival to 24 weeks was expected to be close to zero. For purposes of comparison, data from untreated control rats after 5/6 nephrectomy were pooled from previous 12-week studies recently performed in this laboratory. The percentage of glomeruli affected by sclerosis in 22 untreated rats at 12 weeks after 5/6 nephrectomy was 43(4)% (mean(SEM)) [29, 465].

3.2.2. Morphology

Renal tissue was postfixed in 10% phosphate buffered formalin, embedded in paraffin and processed for light microscopy. The frequency of FSGS was estimated by examining all glomeruli seen in one or two coronal sections from each kidney stained by the periodic acid-Schiff method. Segmental sclerosis was defined as glomerular capillary collapse with hyaline deposition and/or adhesion to the parietal layer of Bowman's capsule. A glomerulosclerosis score (GS) was determined by expressing the number of glomeruli with segmental or global sclerosis as a percentage of the total number of glomeruli counted for each rat (mean 132; range 73-274 per rat). Tubulointerstitial injury, as evidenced by dilated tubules

containing protein casts and interstitial inflammation or fibrosis, was assessed at medium power on the same sections prior to evaluation of glomerulosclerosis. A scoring system (Tubulointerstitial Score - TIS) was used to grade the injury from 0 to 3 based on the percentage of abnormal tissue (0%, <20%, 20-50% and >50% respectively). The pathologist was unaware of the group assignment of individual rats.

3.2.3. Chemical Analysis

The concentration of protein in the urine was determined by spectophotometry after precipitation with 3% sulphosalicylic acid.

3.2.4. Competitive reverse transcription polymerase chain reaction (RT-PCR).

Total RNA was extracted from frozen portions of renal cortex by the cesium chloride ultracentrifugation method [474] and RT-PCR performed according to the protocol described in Chapter 2. Sequences of oligonucleotide primer sets and optimal conditions are listed in Table 3.1. As the number of specimens exceeded the capacity of the thermal cycler (n=96) all specimens from the study could not be included in a single PCR reaction. Specimens from *5WK*, CSN_A, ENA_A and SHM_A were therefore included in one set of PCR reactions and specimens from *5WK*, CSN_B and ENA_B, in a separate set of PCR reactions. To allow direct comparison of results from different PCR reactions data were expressed as ratios to the mean value for the *5WK* group. β-actin mRNA levels were used to confirm that starting amounts of cDNA were similar among groups.

3.2.5. Immunohistochemistry

Expression of TGF-β1, MCP-1 and IL-1β proteins and macrophage infiltration was assessed by immunohistochemistry. For macrophage staining, 4 μm paraffin sections of fixed tissues were used for immunoperoxidase analysis after baking at 60 °C for one hour, deparaffinization and rehydration (xylene X 4 for three minutes each, 100% ethanol X 4 for three minutes each and running water for five minutes). Sections were then microwave treated at 93 °C for 30 minutes in preheated 10 mM citrate buffer, pH 6.0, cooled for 15 minutes and transferred to phosphate buffered saline (PBS). They were then blocked (for 15 min) with a 1.5% solution of serum from the animal source of the secondary antibody at room temperature. Next, sections were incubated with mouse monoclonal antibodies to the

monocyte/macrophage marker ED1 (clone ED1, 1:150 dilution, Biosurface International, Camarilla, CA) for 1 hour in a humid chamber at room temperature. The secondary antibody (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlington, CA) was used according to manufacturer's instructions. Slides were rinsed with PBS between each incubation. Sections were developed using 3,3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO) as substrate and counter stained with Gill's Hematoxylin (Fisher Scientific, Pittsburgh, PA). Macrophage infiltration was assessed by counting the number of ED1-positive cells in 10 glomerular profiles and in 10 randomly chosen 0.25 X 0.25mm areas of tubulointerstitium for each kidney. Owing to a shortage of tissue not all kidneys in the 12 week set could be assessed for mϕ infiltration (n=5 for CSN_A and ENA_A). All kidneys were assessed for CSN_B (n=19) and ENA_B (n=16).

For TGF-β1, MCP-1 and IL-1β staining, 4 μm sections of frozen tissues were fixed in acetone for 10 min at -20°C and then rinsed with PBS. Sections were then blocked with a 1.5% solution of serum and incubated with primary antibodies (hamster anti-mouse antibody against MCP-1 (clone 2H5, 1:50 dilution, Biosurface International), rabbit polyclonal antibodies against IL-1β (1:100 dilution, Endogen, Woburn, MA) and TGF-β1(V) (1:150 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA)) and then secondary antibody as described above. Methyl Green was used as a counterstain.

3.2.6. Statistical Analysis

Continuous variables, expressed as mean(SEM), were compared with analysis of variance (ANOVA) derived from general linear models. Pairwise comparisons of physiological data from weeks 4, 12 and 24 were performed using the Student-Neuman-Keuls procedure. Determinants of proteinuria, glomerulosclerosis and tubulointerstitial injury were analyzed using multivariable linear regression with stepwise variable selection. Multiplicative interaction terms were tested to evaluate whether estimated effects of blood pressure and degree of proteinuria on glomerulosclerosis were uniform across treatment modality. Dependent variable distributions approximated the normal, and regression diagnostics showed no outliers. Repeated measures ANOVA, factorial ANOVA and paired t-tests were employed for other comparisons. For PCR data, which were not normally distributed, differences among multiple groups were assessed using the Kruskal-Wallis test; between two groups, with the Mann-Whitney test. P-values less than 0.05 were considered significant. Statistical analyses were conducted using Statview 4.01

(Abacus Concepts Inc., Berkley, California, USA) and SAS 6.08 (SAS Institute, Cary, North Carolina, USA)

3.3. Results

3.3.1. Chronic Studies

Mean body weight increased in all groups during the study and no statistical differences in body weight developed between candesartan- and enalapril-treated rats over time in either the 12 or 24 week sets. Sham-operated rats attained significantly greater body weight than partially nephrectomized rats in the pre-treatment period and continued to maintain significantly higher average body weight than enalapril-treated rats in the 24 week set. In the 12 week set only the body weight difference between sham-operated and enalapril-treated rats in the pre-treatment period was statistically significant. (Table 3.2.).

Mean SBP increased in all groups after 5/6 nephrectomy and did not differ statistically among the groups before initiation of therapy at week 5. Treatment with either candesartan or enalapril resulted in an initial fall in SBP to levels similar to those of sham-operated rats. SBP remained similar among treated groups and sham-operated rats over weeks 6 to 12 in both 12 and 24 week sets. Thereafter, there was a gradual increase in SBP such that from 18 weeks, SBP levels were statistically higher than the lowest values, observed at 8 weeks, in both CSN_B and ENA_B. There were no statistically significant differences in SBP between treatment groups over time, in either 12 or 24 week sets (mean differences: CSN_A vs. ENA_A = 11mmHg, P=0.26; CSN_B vs. ENA_B = 7mmHg, P=0.41). Sham-operated rats remained normotensive throughout the study (Figure 3.2A and 3.3A). $U_{pr}V$ increased after 5/6 nephrectomy to levels 7 to 8 fold higher than in sham operated rats and was similar among the groups before initiation of therapy at week 5. In both candesartan- and enalapril-treated rats $U_{pr}V$ declined at first, but later increased progressively to reach levels approximately 2 fold those of pretreatment values and 8 to 9 fold those of sham-operated rats at 24 weeks. No statistically significant differences were observed in $U_{pr}V$ between treatment groups over time, in either the 12 or 24 week sets (mean differences: CSN_A vs. ENA_A = 8.8 mg/day, P=0.16; CSN_B vs. ENA_B = 8.4 mg/day, P=0.31). In sham-operated rats, mean $U_{pr}V$ remained low, although a small increase was evident over time (Figure 3.2B and 3.3B). $U_{pr}V$ was directly correlated with SBP in combined data from CSN_B and ENA_B rats at 12 and 24 weeks (r=0.60 and 0.73, respectively; P<0.0001

for both). There were still no differences in $U_{pr}V$ between the treatment groups after adjusting for the effects of SBP (P=0.50 and P=0.70 at 12 and 24 weeks, respectively). Furthermore, there was no effect of treatment group on the relationship between $U_{pr}V$ and SBP (interactive terms: P=0.78 and P=0.30 at 12 and 24 weeks, respectively).

Remnant kidneys hypertrophied considerably in all groups such that the weight increased 3 to 4 fold over baseline. There were no statistically significant differences in final remnant kidney weight among the groups. Analysis of remnant kidney weights, expressed as kidney:body weight ratio, yielded similar results (Table 3.3.).

3.3.2. Morphology

Histological data are summarized in Table 4. Five weeks after partial nephrectomy and before initiation of therapy, glomerulosclerosis was evident in a mean of 26(6)% of glomeruli (*5WK* rats). At 12 weeks post surgery, the extent of glomerulosclerosis in candesartan- and enalapril-treated rats was similar to that observed before treatment in the *5WK* group; moreover there was no statistical difference in mean GS between CSN_A and ENA_A. At 24 weeks after surgery, there was again no statistically significant difference in the mean GS of CSN_B vs. ENA_B rats. Comparison of combined CSN_B and ENA_B data with combined CSN_A and ENA_A data, revealed a trend towards more extensive glomerulosclerosis at 24 weeks vs. 12 weeks (P=0.06 by ANOVA). When these data are viewed in the context of data for untreated 5/6 nephrectomized controls at 12 weeks, it is evident that both treatments slowed the progression of secondary FSGS such that the extent of glomerulosclerosis previously observed at 12 weeks after 5/6 nephrectomy (GS=43(4)%; mean(SEM)), was attained only after 24 weeks in CSN_B and ENA_B rats (Figure 3.4.). Minimal glomerulosclerosis was noted in sham-operated rats.

Tubulointerstitial injury showed similar patterns of change to those of glomerulosclerosis and was also not statistically different between *CSN* and *ENA* rats at 12 or 24 weeks. There was a direct and highly significant correlation between GS and TIS in pooled data from CSN_B and ENA_B rats (r=0.85; P<0.001). As with glomerulosclerosis minimal tubulointerstitial injury developed in sham-operated rats.

3.3.3. Multivariable Analysis

Analysis of data from rats sacrificed at 5 weeks after surgery revealed statistically significant correlations between $U_{pr}V$ and GS (r=0.87; P=0.02) or TIS (r=0.87; P=0.02). There were no statistically significant correlations between SBP and GS or TIS in this group.

Since there were no significant differences between enalapril and candesartan treated rats with respect to the relationship between $U_{pr}V$ and SBP or extent of histological injury, data from these two groups were combined to allow analysis of relationships between SBP, $U_{pr}V$ and histological injury during RAS inhibition. At 24 weeks direct and highly significant correlations were evident between SBP and GS (r=0.81; P<0.0001) (Figure 3.5A). Similarly, $U_{pr}V$ was highly correlated with GS (r=0.86; P<0.0001) (Figure 3.5B). By contrast, there was no statistical effect of ACEI vs. AT_1RA treatment on GS at 24 weeks (P=0.9). Stepwise multiple linear regression analysis with GS as the dependent variable and SBP, $U_{pr}V$, and remnant kidney/body weight ratio as independent variables, entered only SBP and $U_{pr}V$ into the model. These variables together accounted for 72% of the variance in glomerulosclerosis observed at 24 weeks. The magnitude of the effects of SBP and $U_{pr}V$ as determinants of glomerulosclerosis was such that a 10 mmHg change in SBP or a 10mg/day change in $U_{pr}V$ were each associated with a change of 3 percentage points in GS at 24 weeks.

TIS at 24 weeks also correlated with SBP and $U_{pr}V$ (r=0.78 and r=0.69 respectively; P<0.0001 for both). Stepwise multiple linear regression analysis with TIS as the dependent variable and the same independent variables as above entered only SBP into the model.

3.3.4. Competitive RT PCR

Mean levels of β-actin mRNA were similar among groups for each set of PCR reactions, confirming that starting concentrations of cDNA were similar and not subject to systematic error. Renal cortex mRNA levels for TGF-β1 and MCP-1 in 5/6 nephrectomized rats before treatment (i.e. at 5 weeks) were approximately 2 fold higher than those of sham-operated rats. At 12 weeks after surgery, TGF-β1 and MCP-1 mRNA levels were similar to pretreatment *5WK* values in both CSN_A, and ENA_A rats and were significantly higher than in SHM_A rats. For IL-1β, mRNA levels were similar in *5WK* and SHM_A rats. At 12

weeks, IL-1β mRNA exhibited a trend towards higher levels in CSN_A and ENA_A vs. SHM_A rats that was not statistically significant. At 24 weeks after 5/6 nephrectomy, TGF-β1 mRNA levels were significantly lower than pretreatment values in CSN_B and ENA_B rats, but were still significantly higher than those of sham-operated rats. By contrast, MCP-1 mRNA levels were not different from pretreatment values in CSN_B and ENA_B rats and remained higher than SHM_A levels. IL-1β mRNA levels in both CSN_B and ENA_B rats were similar to those of SHM_A rats (Figure 3.6.). There were no statistically significant differences in mRNA levels for any of the genes examined between *CSN* and *ENA* rats at either 12 or 24 weeks.

Since there were no differences between candesartan- and enalapril-treated rats with respect to any of the variables determined in this study, data from both groups were pooled for further analysis. In this combined group, strong and highly statistically significant correlations were evident between 24-week renal cortex mRNA levels for TGF-β1, MCP-1 and IL-1β, and the extent of FSGS (Figure 3.7.) or TIF (Table3.5.). Somewhat weaker but nevertheless statistically significant correlations were evident among TGF-β1, MCP-1 and IL-1β, and SBP or $U_{pr}V$ (Table 3.5.). Analysis of pooled CSN_A, and ENA_A data revealed only weak or absent correlations among these parameters at 12 weeks after surgery (data not shown).

3.3.5. Immunohistology

Immunohistology confirmed that the increases in gene expression detected by competitive RT-PCR were accompanied by qualitative increases in expression of the gene product and localized the protein expression within the renal cortex. Negative controls, in which no primary antibody was used, showed minimal staining of tubules and no glomerular staining. For TGF-β1, kidneys from *SHM* rats exhibited moderate staining of tubules and negative staining of glomeruli. Among *5 WK* rats, positive TGF-β1 staining of both tubules and glomeruli was observed. Patterns of staining similar to those of *5 WK* rats were observed among rats in both treatment groups with particularly strong staining seen in areas of segmental glomerulosclerosis (Figures 3.8. A, B, C). MCP-1 staining was limited to minimal positivity of tubule cells in *SHM* rats. Positive MCP-1 staining of tubules and glomeruli was observed in *5WK* rats and

among rats from both treatment groups (Figures 3.8. D, E, F). Positive staining for IL-1β was localized mainly to tubule cells in specimens from all groups. However, among *5 WK* rats and rats from the treatment groups, focal areas of positive staining for IL-1β were observed in some glomeruli (Figures 3.8. G, H, I). (This observation is consistent with staining of individual cells within glomeruli but due to the limitations of histology performed on frozen tissue sections, this could not be confirmed.).

3.3.6. Macrophage Infiltration

Extensive infiltration of glomeruli and remnant kidney interstitium by mϕ was evident prior to initiation of therapy at 5 weeks after surgery. Whereas mϕ were virtually absent from the kidneys of sham-operated rats, a mean of 5.7(0.22) mϕ per glomerular profile and 6.6(0.19) mϕ per 0.0625mm^2 area of interstitium were observed in *5WK* rats. Treatment with candesartan or enalapril was associated with 2 to 5 fold reductions in glomerular mϕ infiltration and an approximately 4-fold reductions in interstitial mϕ at 12 weeks after surgery. Nevertheless the extent of mϕ infiltration of both glomeruli and interstitium remained significantly higher in treated rats vs. sham-operated rats at 12 and 24 weeks. Glomerular mϕ counts were slightly, albeit significantly, higher in enalapril- vs. candesartan- treated rats at 12 and 24 weeks. There was no difference in interstitial mϕ counts between treatment groups at either time point (Figure 3.9.).

Table 3.1. Summary of primer sequences and PCR conditions.

Primer	Sense sequence	Antisense sequence	Size (bp)	Temp (°C)*
TGF-β1	CTTCAGCTCCACAGAGAAGAACTGC	CACGATCATGTTGGACAACTGCTCC	298	64
MCP-1	ATGCAGGTCTCTGTCACG	CTAGTTCTCTGTCATACT	447	55
IL-1β	TGATGTTCCCATTAGACAGC	GAGGTGCTGATGTACCAGTT	378	55
β-Actin	TTGTAACCAACTGGGACGATATGG	GATCTTGATCTTCATGGTGCTAGG	764	60

* Annealing temperature

Table 3.2. Body weights (g); data=mean(SEM)

	weeks post surgery						
Group	**0**	**4**	**8**	**12**	**16**	**20**	**24**
WK5 (n=6)	259(2)	272(7)					
CSN_A (n=11)	260(3)	282(7)	306(4)	330(5)			
ENA_A (n=11)	253(5)	270(7)*	312(9)	328(7)			
SHM_A (n=6)	261(3)	301(6)	322(10)	331(15)			
CSN_B (n=19)	262(2)	282(3)†	309(4)	331(5)	347(4)	360(4)	360(7)
ENA_B (n=16)	256(2)	278(3)†	309(4)	327(4)	344(5)	350(6)	359(6)‡
SHM_B (n=9)	266(2)	298(7)	332(5)	350(4)	359(5)	369(4)	373(5)

* P<0.05 vs. SHM_A over weeks 0-4; † P<0.05 vs. SHM_B over weeks 0-4; ‡ P<0.05 vs. SHM_B over weeks 6-24.

WK5: untreated rats sacrificed at 5 weeks after 5/6 nephrectomy; CSN_A: rats treated with candesartan after 5/6 nephrectomy and sacrificed at 12 weeks; ENA_A: rats treated with enalapril after 5/6 nephrectomy and sacrificed at 12 weeks; SHM_A: sham-operated rats sacrificed at 12 weeks; CSN_B: rats treated with candesartan after 5/6 nephrectomy and sacrificed at 24 weeks; ENA_B: rats treated with enalapril after 5/6 nephrectomy and sacrificed at 24 weeks; SHM_B: sham-operated rats sacrificed at 24 weeks.

Table 3.3. Remnant kidney weight and kidney:body weight ratio at initial surgery and at sacrifice; data=mean(SEM)

	Remnant kidney weight (g)		Remnant kidney:body weight (g/kg)	
Group	**baseline**	**final**	**baseline**	**final**
WK5 (n=6)	0.29(0.01)*	1.16(0.08)	1.12(0.04)*	4.25(0.24)
CSN_A (n=11)	0.33(0.01)	1.28(0.03)	1.28(0.03)	3.87(0.09)
ENA_A (n=11)	0.35(0.01)	1.25(0.07)	1.37(0.03)	3.80(0.15)
CSN_B (n=19)	0.36(0.01)	1.43(0.06)	1.37(0.02)	3.95(0.11)
ENA_B (n=16)	0.35(0.01)	1.39(0.07)	1.37(0.04)	3.85(0.17)

Final remnant kidney weights (or kidney weight : body weight ratios) were significantly greater than baseline in all groups ($P<0.0001$). Baseline kidney weights are an estimate calculated as one third of the weight of the kidney resected at the time of initial surgery.

There were no statistically significant differences in final remnant kidney weight (or kidney weight : body weight ratio) among the groups.

* $P<0.05$ vs. all treatment groups

WK5: untreated rats sacrificed at 5 weeks after 5/6 nephrectomy; CSN_A: rats treated with candesartan after 5/6 nephrectomy and sacrificed at 12 weeks; ENA_A: rats treated with enalapril after 5/6 nephrectomy and sacrificed at 12 weeks; SHM_A: sham-operated rats sacrificed at 12 weeks; CSN_B: rats treated with candesartan after 5/6 nephrectomy and sacrificed at 24 weeks; ENA_B: rats treated with enalapril after 5/6 nephrectomy and sacrificed at 24 weeks; SHM_B: sham-operated rats sacrificed at 24 weeks.

Table 3.4. Histological analysis of remnant kidney tissue; data=mean(SEM)

Group	Time post surgery (weeks)	Glomerulosclerosis Score (%)	Tubulointerstitial Score (0-3)*
WK5 (n=6)	5	25.5(6.4)	0.6(0.3)
CSN_A (n=11)	12	32.1(4.2)	0.9(0.1)
CSN_B(n=19)	24	41.5(6.5)	1.3(0.3)
ENA_A (n=11)	12	29.2(4.6)	0.9(0.2)
ENA_B (n=16)	24	41.8(4.4)	1.2(0.2)
SHM_A (n=6)	12	0.2(0.1)	0.0(0.0)
SHM_B (n=9)	24	2.8(0.4)	0.0(0.0)

There were no statistically significant differences in GS or TIS between *CSN* and *ENA* rats in either the 12 or 24 week set. A trend towards an increase in GS from 12 weeks to 24 weeks in combined data from *CSN* and *ENA* rats was not statistically significant (P=0.06).

*Score from 0 to 3 based on estimated percentage abnormal tissue: 0%, <20%, 20-50% or >50%

WK5: untreated rats sacrificed at 5 weeks after 5/6 nephrectomy; CSN_A: rats treated with candesartan after 5/6 nephrectomy and sacrificed at 12 weeks; ENA_A: rats treated with enalapril after 5/6 nephrectomy and sacrificed at 12 weeks; SHM_A: sham-operated rats sacrificed at 12 weeks; CSN_B: rats treated with candesartan after 5/6 nephrectomy and sacrificed at 24 weeks; ENA_B: rats treated with enalapril after 5/6 nephrectomy and sacrificed at 24 weeks; SHM_B: sham-operated rats sacrificed at 24 weeks.

Table 3.5: Correlation coefficients (R) for renal cortex cytokine mRNA levels vs. measures of renal injury in a combined group of *CSN* and *ENA* rats at 24 weeks after 5/6 nephrectomy.

	SBP	UprV	GS	TIS
TGF-β1	0.67*	0.77*	0.81*	0.78*
MCP-1	0.57*	0.46†	0.57*	0.56*
IL-1β	0.65*	0.57*	0.70*	0.73*

* P<0.0005; † P<0.01

GS: glomerulosclerosis score; TIS: tubulointerstitial score indicating tubulointerstitial damage

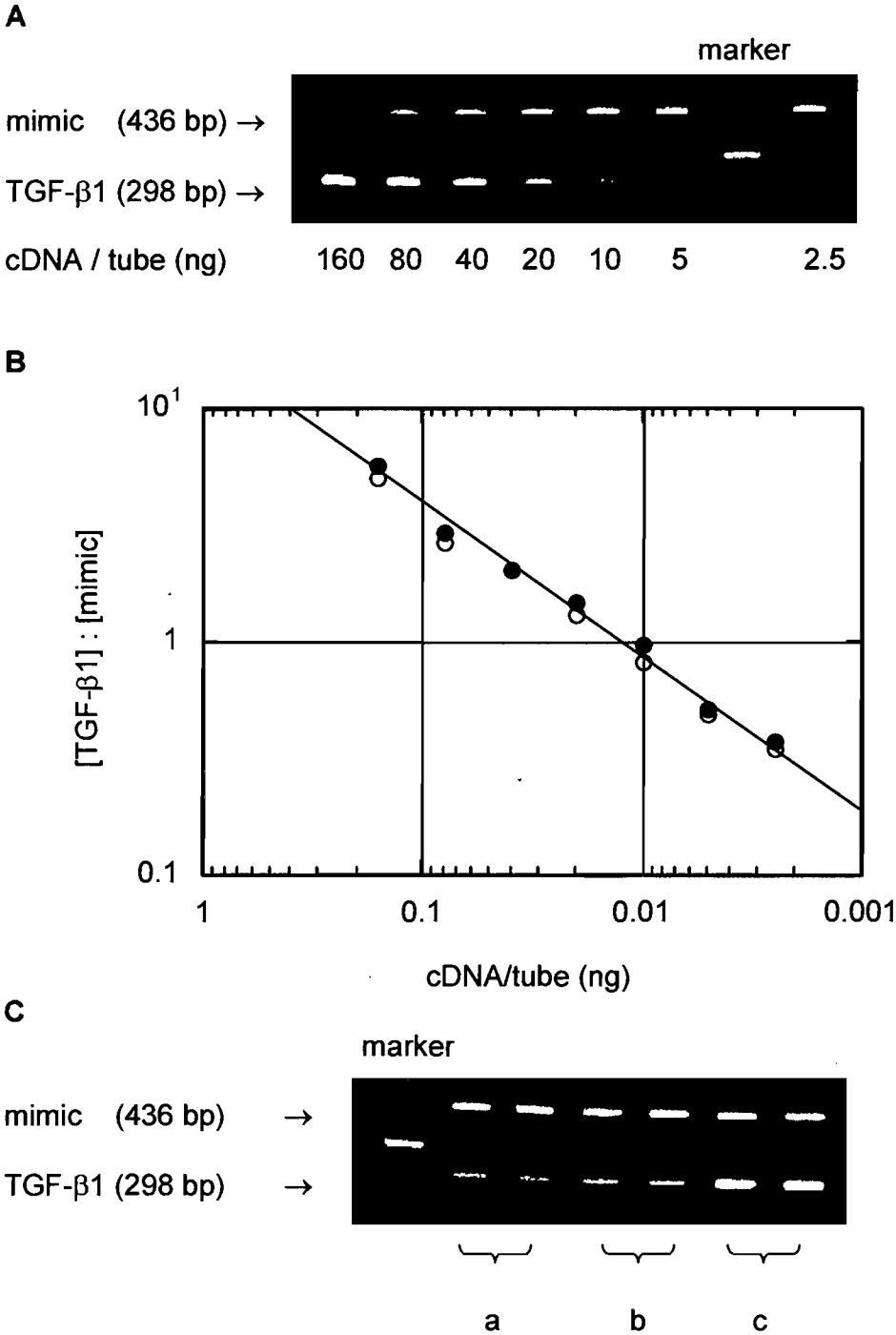


Figure 3.1. A: Polyacrylamide gel showing electrophoretic bands for mimic and gene-specific (TGF-β1) PCR products at different amounts of starting cDNA. **B:** Log-log plot of starting cDNA amount versus the ratio of gene specific (TGF-β1):mimic PCR product concentration (in duplicate) showing a linear relationship over serial dilutions of starting cDNA. **C:** Representative portion of a gel showing electrophoretic bands for mimic and gene-specific (TGF-β1) PCR products for different samples of renal cortex mRNA. Specimens a and b were from rats who had low levels of glomerular injury after 24 weeks (GS= 24.2% and 18.0%, respectively) whereas specimen c was from a rat with severe glomerular injury at 24 weeks (GS=75.6%). Higher levels of TGF-β1 mRNA expression can be appreciated in the latter even on visual inspection.

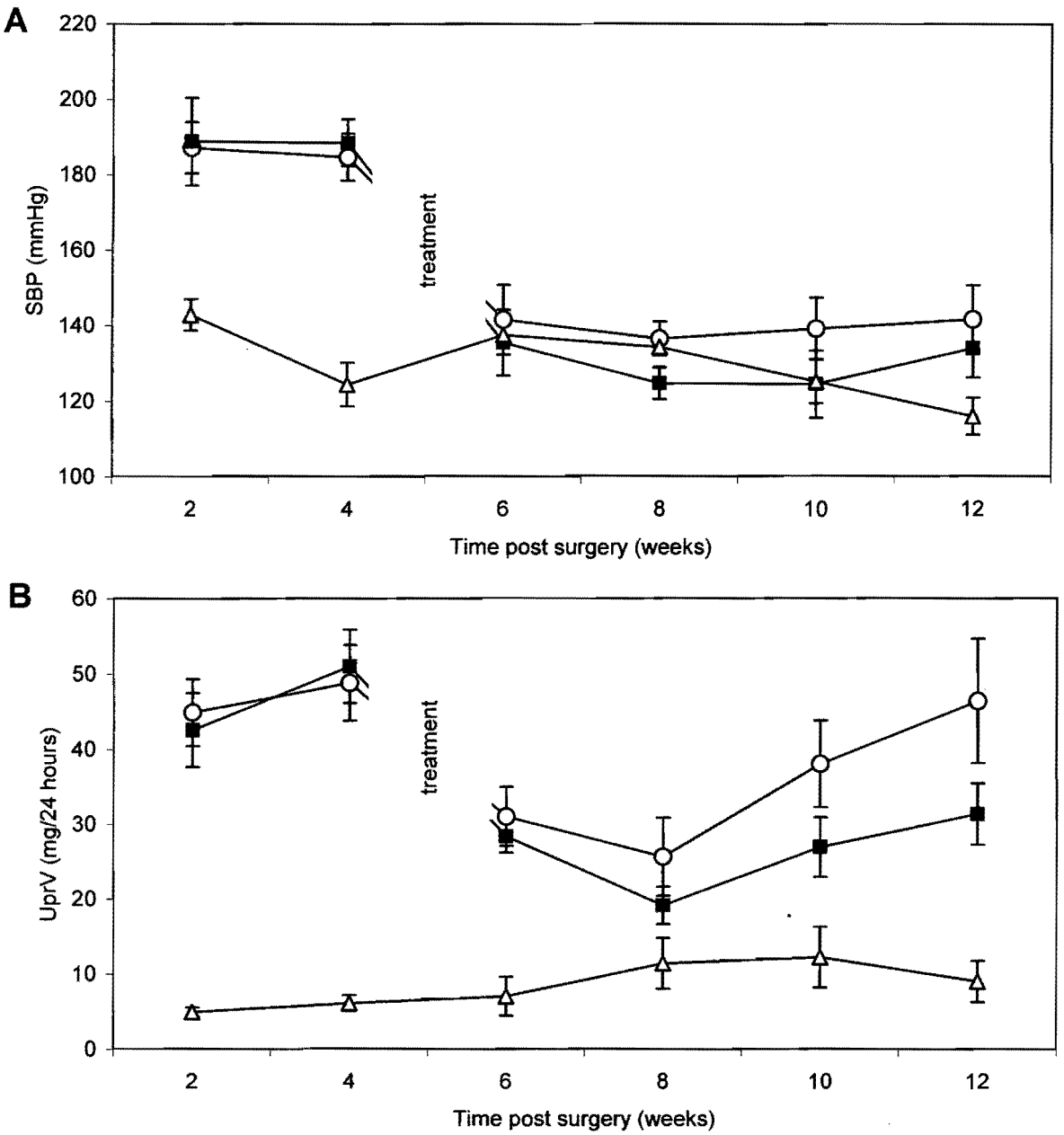


Figure 3.2. A: Systolic blood pressures (SBP) (mean(SEM)) of rats in the 12 week set over time. SBP increased in all groups after 5/6 nephrectomy and did not differ statistically among the groups before initiation of therapy at week 5. Treatment with either candesartan (CSN_A - filled squares) or enalapril (ENA_A - open circles) resulted a fall in SBP to levels similar to those of sham-operated rats (SHM_A - open triangles). There were no statistically significant differences in SBP between CSN_A and ENA_A over weeks 6-12 (on treatment). Sham-operated rats remained normotensive over 12 weeks.

B: Urinary protein excretion rate ($U_{pr}V$) (mean(SEM)) of rats in the 12 week set over time. $U_{pr}V$ increased in all groups after 5/6 nephrectomy and did not differ statistically among the groups before initiation of therapy at week 5. Treatment with either candesartan (CSN_A - filled squares) or enalapril (ENA_A - open circles) was associated with an initial decline in UprV which later increased from week 8 to week12. There were no statistically significant differences in $U_{pr}V$ between CSN_A and ENA_A over weeks 6-12 (on treatment). $U_{pr}V$ remained at normal levels in sham-operated rats over 12 weeks (SHM_A - open triangles).

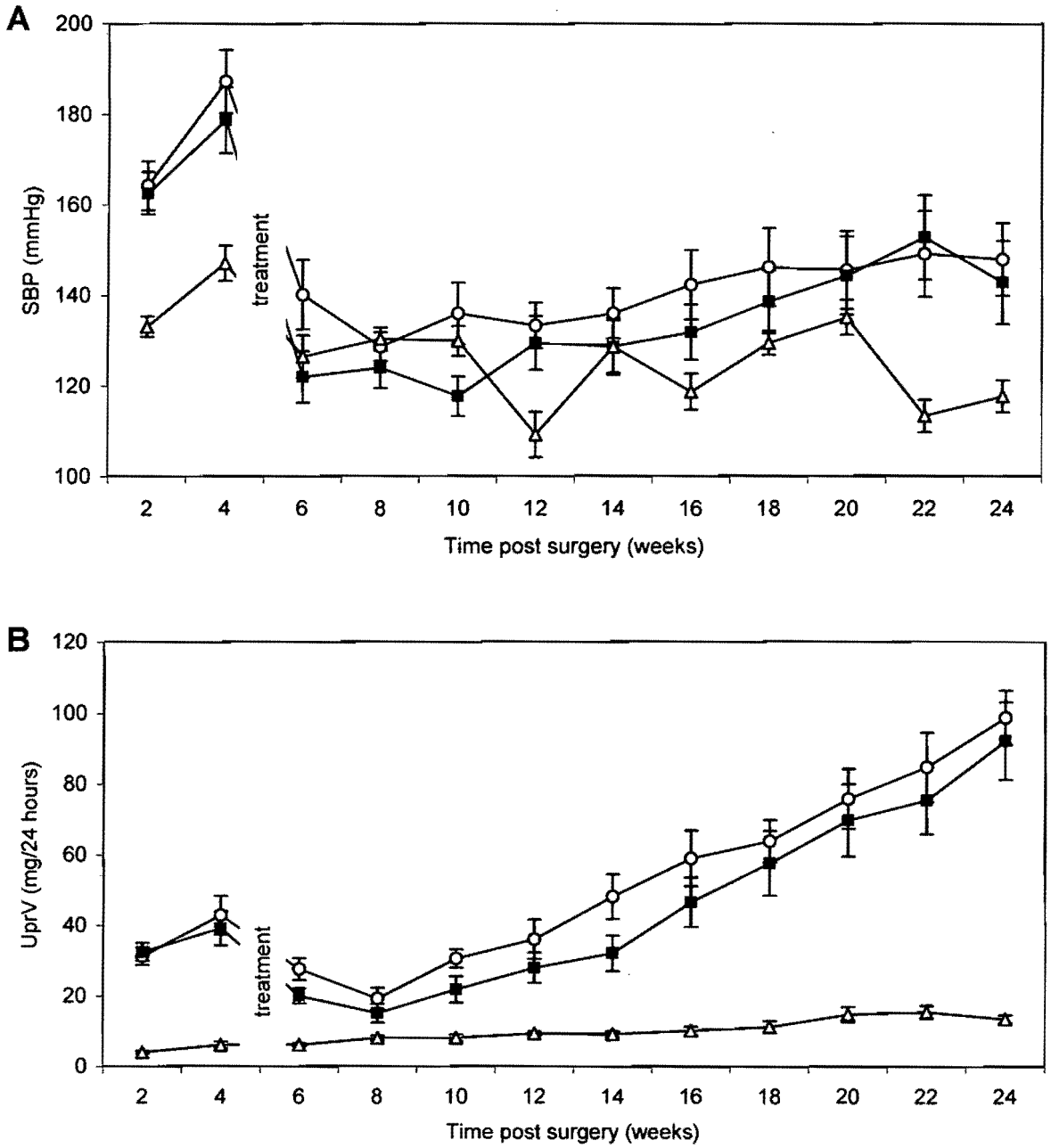


Figure 3.3. A: Systolic blood pressures (SBP) (mean(SEM)) of rats in the 24 week set over time. SBP increased in all groups after 5/6 nephrectomy and did not differ statistically among the groups before initiation of therapy at week 5. Treatment with either candesartan (CSN_B - filled squares) or enalapril (ENA_B - open circles) resulted in an initial fall in SBP to levels similar to those of sham-operated rats (SHM_B - open triangles). However, there was a gradual increase in SBP over time such that from 18 weeks, levels were signficantly higher than the lowest values, observed at 8 weeks in both CSN_B and ENA_B. There were no statistically significant differences in SBP between CSN_B and ENA_B over weeks 6-24 (on treatment). Sham-operated rats remained normotensive over 24 weeks.
B: Urinary protein excretion rate ($U_{pr}V$) (mean(SEM))of rats in the 24 week set over time. $U_{pr}V$ increased in all groups after 5/6 nephrectomy and did not differ statistically among the groups before initiation of therapy at week 5. Treatment with either candesartan (CSN_B - filled squares) or enalapril (ENA_B - open circles) was associated with an initial decline in $U_{pr}V$ followed by a progressive increase over weeks 8-24. There were no statistically significant differences in $U_{pr}V$ between CSN_B and ENA_B over weeks 6-24 (on treatment). $U_{pr}V$ remained at normal levels in sham-operated rats over 24 weeks (SHM_A - open triangles).

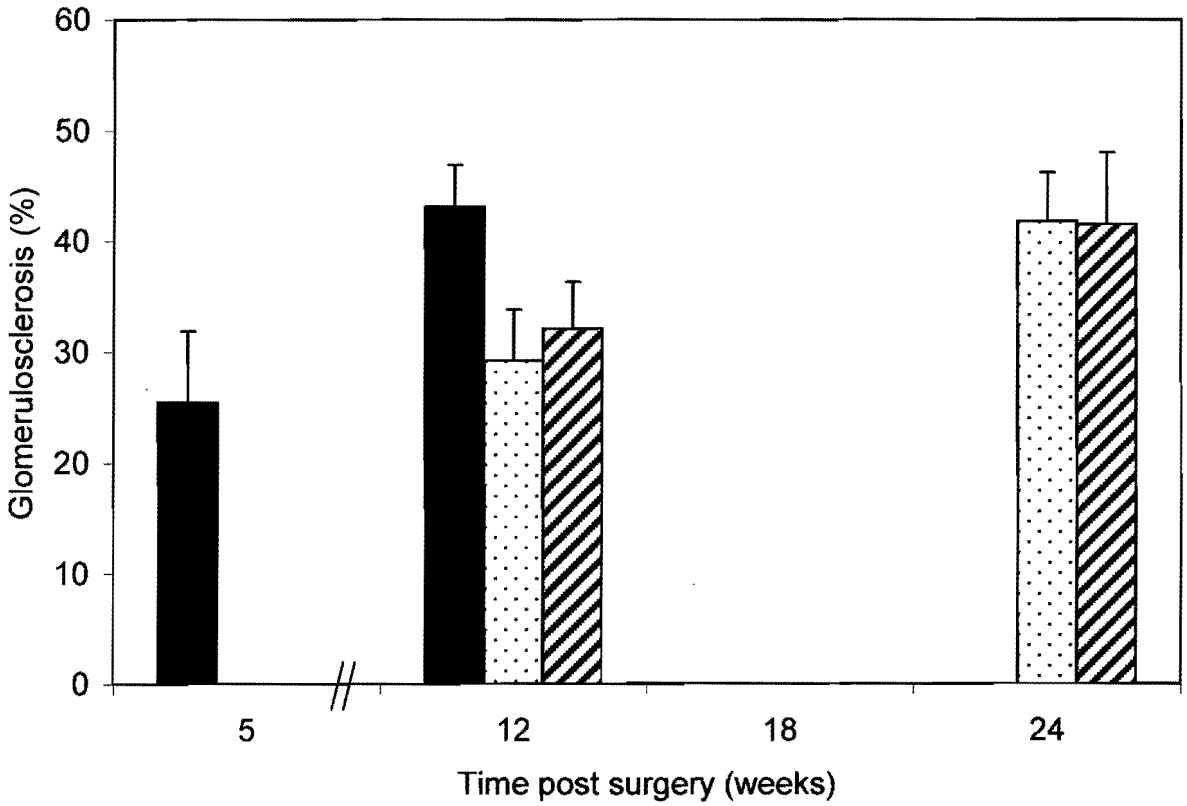


Figure 3.4. Glomerulosclerosis score (mean(SEM)) at different time points in untreated rats (5WK: filled bar) and rats receiving candesartan (CSN: hatched bars) or enalapril (ENA: stippled bars). Filled bar at 12 weeks indicates GS at 12 weeks after 5/6 nephrectomy in 22 untreated control rats from previous studies.

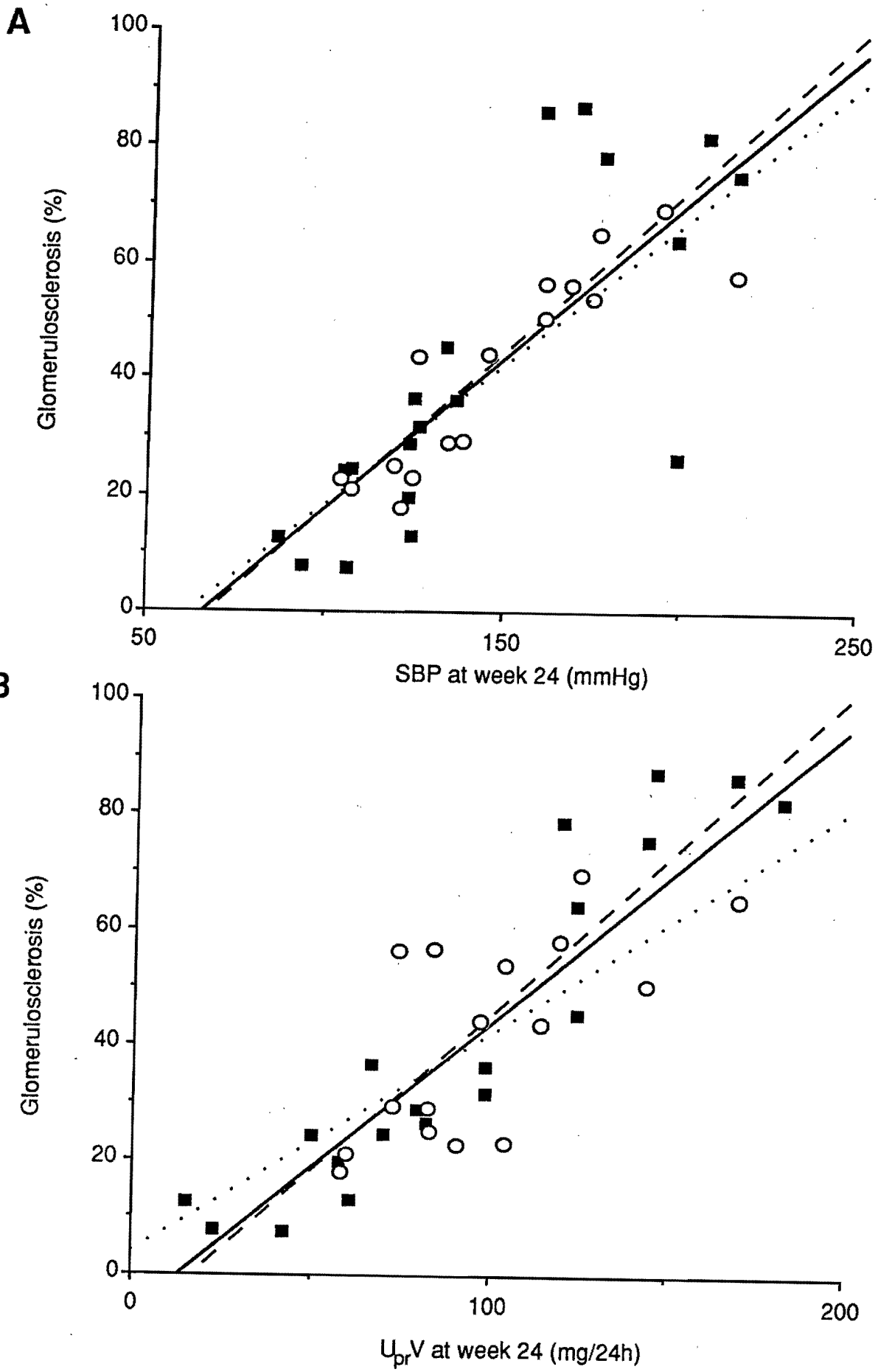


Figure 3.5. A: Scatter plot for GS vs. SBP at 24 weeks in CSN_B (filled squares, regression line in dashes) and ENA_B (open circles, regression line in dots) rats. Linear regression equation for combined data (solid line): y = - 34.5 + 0.52x; $r^2 = 0.65$

B: Scatter plot for GS vs. $U_{pr}V$ at 24 weeks in in CSN_B (filled squares, regression line in dashes) and ENA_B (open circles, regression line in dots) rats. Linear regression equation for combined data (solid line): y = - 6.5 + 0.51x; $r^2 = 0.74$

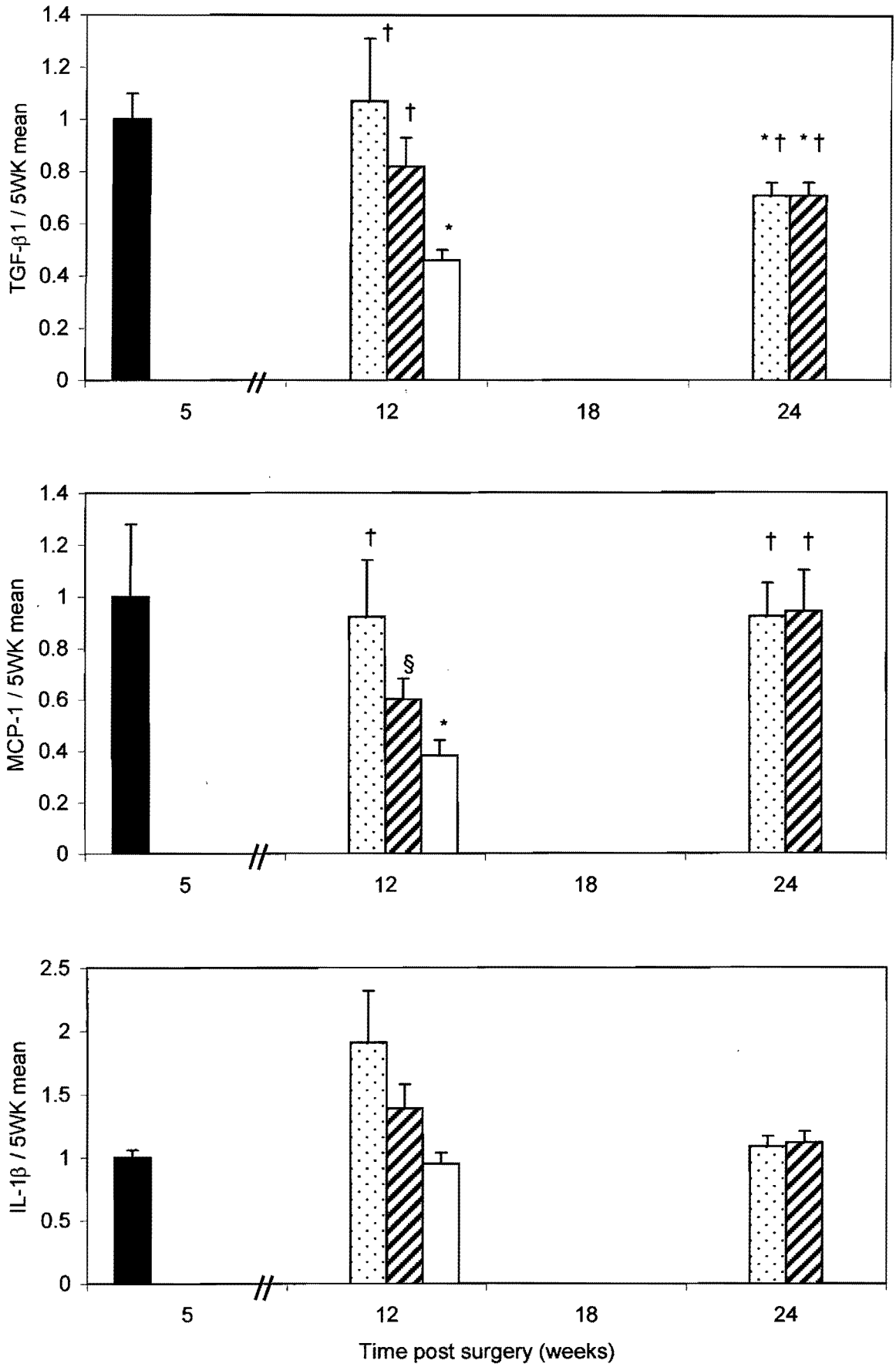


Figure 3.6. Renal cortex mRNA levels (mean(SEM)) for TGF-β1, MCP-1 and IL-1β (expressed as a ratio to the mean value for untreated rats at 5 weeks) in 5/6 nephrectomized rats receiving no treatment (5WK: filled bars), candesartan (CSN: hatched bars) or enalapril (ENA: stippled bars) and sham-operated rats (SHM: open bars). (* $P<0.05$ vs. 5WK; † $P<0.05$ vs. SHM; § $P=0.05$ vs. SHM)

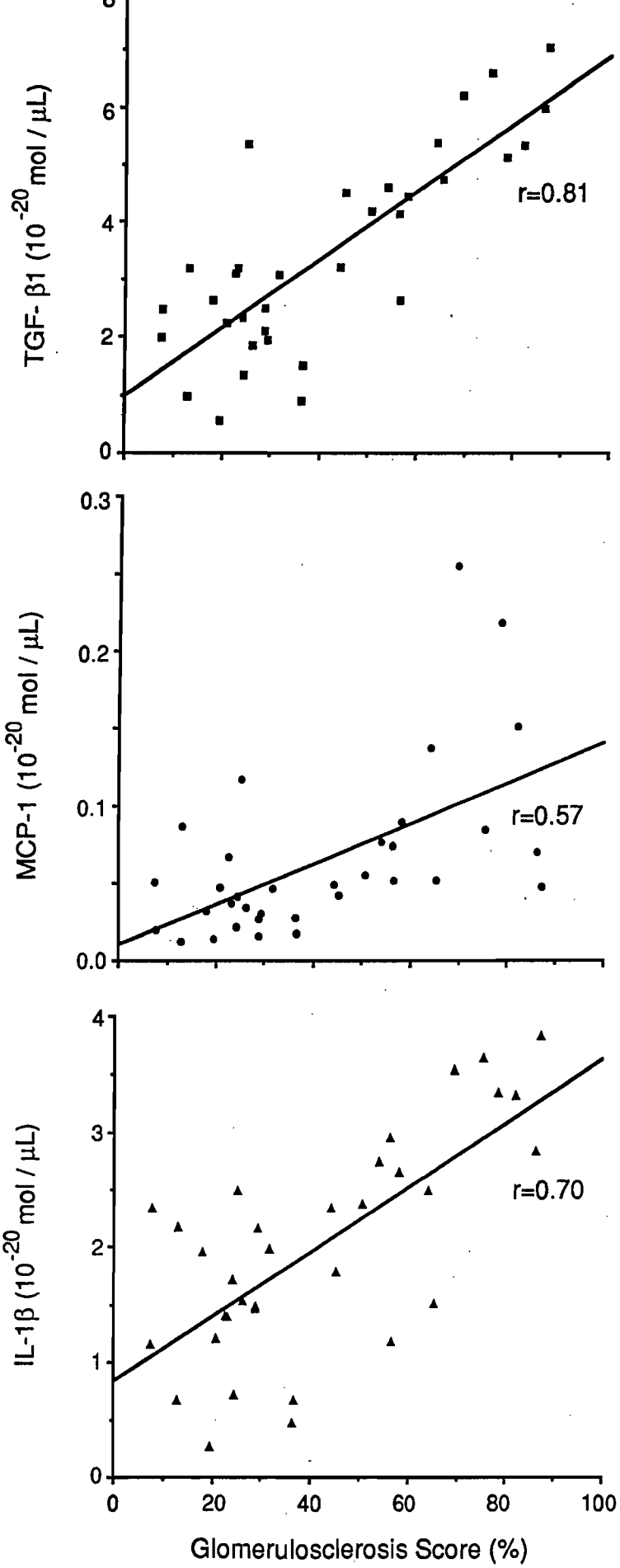


Figure 3.7. Scatter plots of renal cortex mRNA levels for TGF-β1, MCP-1 and IL-1β vs. glomerulosclerosis score for pooled data from candesartan- and enalapril-treated rats (n=34) at 24 weeks. Regression lines and correlation coefficients are also shown.

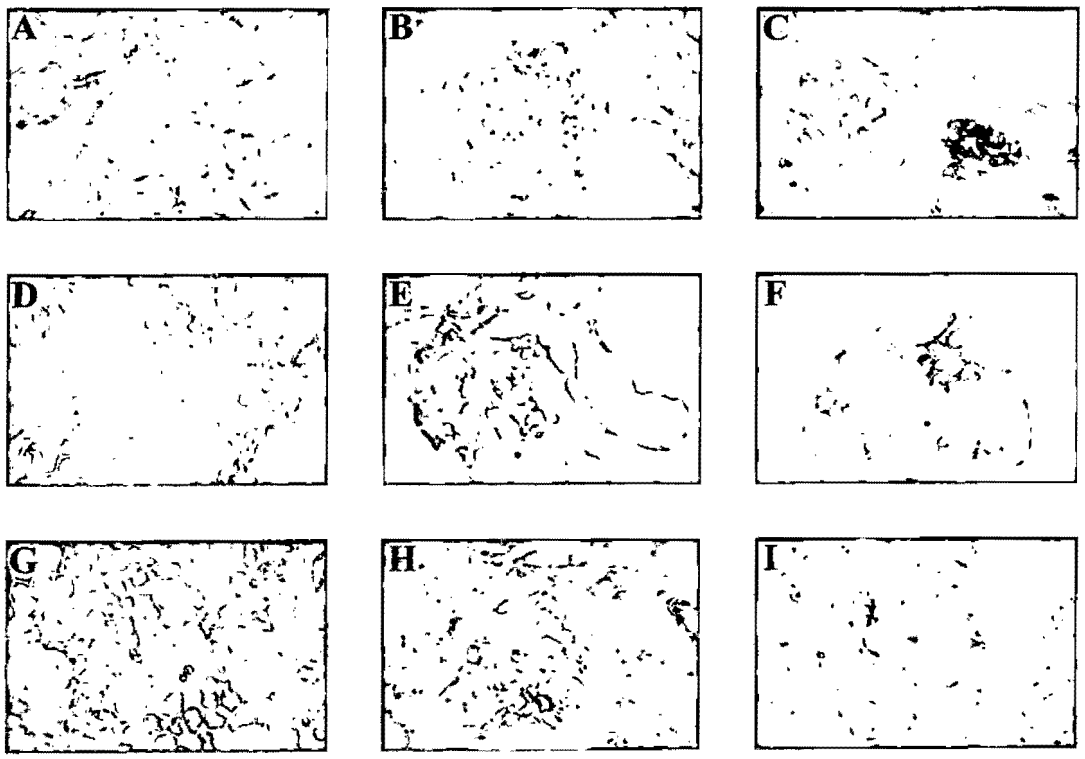


Figure 3.8. Immunohistology:
A-C: TGF-β1 immunostaining was positive only in tubule cells of *SHM* rats (A, 100X magnification) whereas tubule and glomerular staining was evident in *5WK* rats (B, 100X magnification) and in rats from both treatment groups (C - subject from CSN_B shown, 100X magnification). Particularly strong staining was observed in glomeruli exhibiting extensive sclerosis.
D-F: MCP-1 immunostaining was minimal in tubules and negative in glomeruli from *SHM* rats (D, 40X magnification). Among *5WK* rats (E, 100X magnification) and rats from both treatment groups (F - subject from CSN_B shown, 100X magnification), positive staining of tubules and glomeruli was observed.
G-I: IL-1β immunostaining was localized mainly to tubule cells among *SHM* (G, 100X magnification), *5WK* (H, 100X magnification) and treated rats (I - subject from CSN_B shown, 100X magnification). However, among *5WK* and treated rats, focal areas of positive staining, consistent with staining of individual cells, were observed in some glomeruli (H and I).

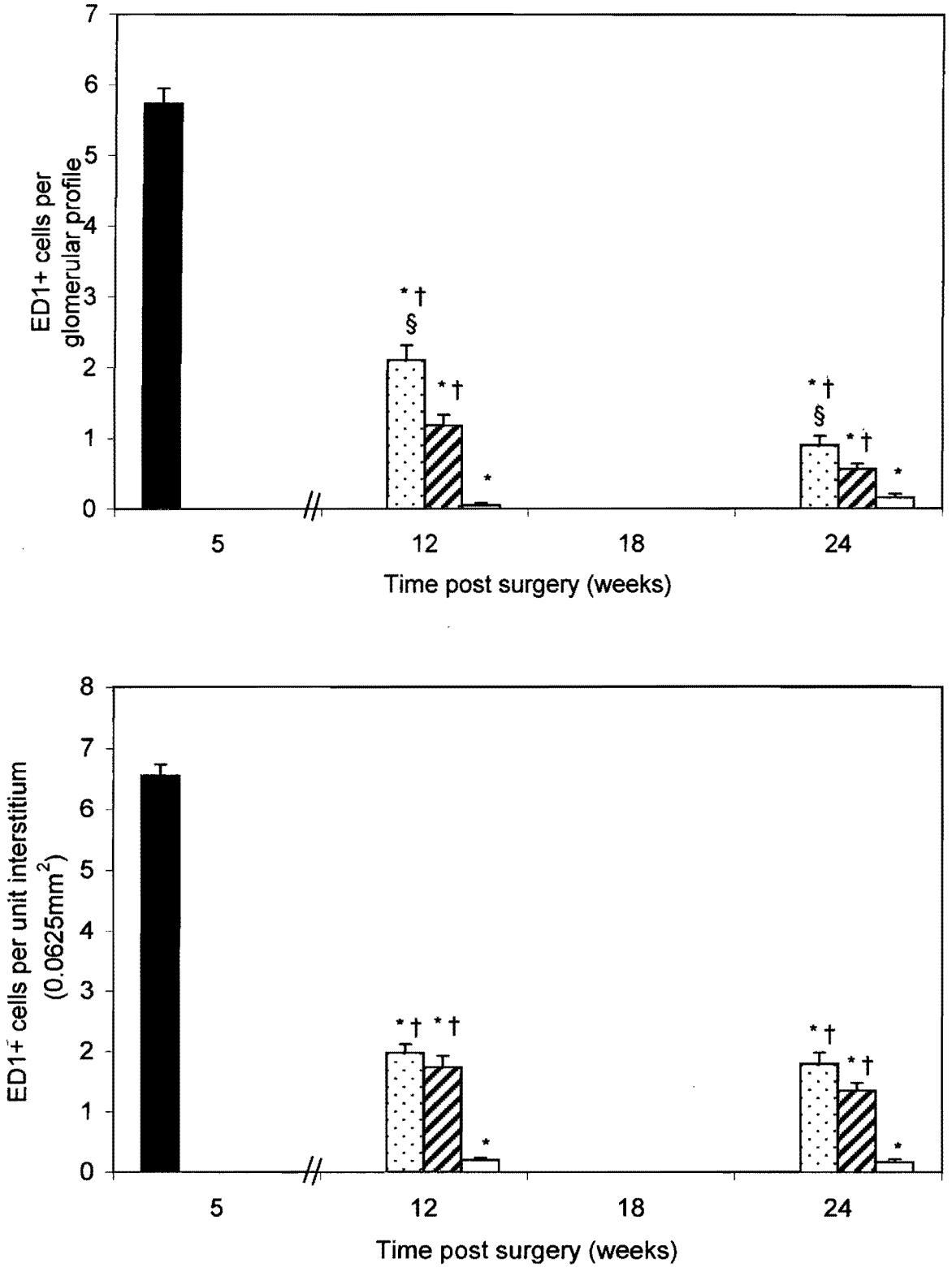


Figure 3.9. Glomerular and interstitial ED1 positive cell (macrophage) counts (mean(SEM)) in 5/6 nephrectomized rats receiving no treatment (5WK: filled bars), candesartan (CSN: hatched bars) or enalapril (ENA: stippled bars) and sham-operated rats (SHM: open bars). * $P<0.05$ vs. 5WK; † $P<0.05$ vs. SHM; § $P<0.05$ vs. CSN

3.4. Discussion

These results show that even when started after the onset of renal injury, the renal protective effects of candesartan are equivalent to those of enalapril when dosing is adjusted to achieve similar levels of SBP control. Comparison with data for untreated rats from previous studies in this model shows that delayed treatment with either ACEI or AT_1RA, appears to slow the rate of progression of glomerulosclerosis by about half, an effect reminiscent of that achieved in clinical trials [168, 169, 373]. Our study differs from the previous study comparing delayed therapy with ACEI and AT_1RA in the 5/6 nephrectomy model by virtue of its longer duration and increased statistical power [468]. Furthermore, 5/6 nephrectomy was achieved by surgical excision of renal mass in the former study whereas we performed infarction of 2/3 of the left kidney, a model resulting in more severe renal injury as well as greater activation of the RAS [33, 512] and therefore more likely to expose subtle differences in efficacy between ACEI and AT_1RA. Nevertheless, we detected no differences between the treatment groups with respect to any of the markers of renal injury examined in this study ($U_{pr}V$, GS and TIS) or in the remnant kidney levels of cytokine gene expression. Moreover, no tendencies for differences to emerge were detected even over a prolonged observation period. The extent of hypertrophy of the remnant kidney also was similar between ACEI- and AT_1RA-treated rats. Finally, the correlations among SBP, $U_{pr}V$ and GS as assessed by linear regression techniques were not affected by treatment group. These data therefore provide the most conclusive evidence to date that ACEI and AT_1RA are equivalent in their renal protective effects in this model of progressive CKD.

Based on the differences in the mechanisms whereby ACEI and AT_1RA inhibit the RAS, it has been suggested that AT_1RA may have therapeutic benefits over ACEI because they inhibit the actions of Ang II formed by other serine proteases in the presence of ACEI, or because of the antihypertensive and anti-proliferative effects that may result from stimulation of AT_2 receptors by elevated Ang II levels during blockade of AT_1 receptors. On the other hand, it has been suggested that at least some of the therapeutic benefit of ACEIs results from elevated bradykinin levels, which are not present during AT_1RA treatment [419]. Our findings strongly suggest that although they inhibit the RAS at different levels, both ACEI and AT_1RA exert their beneficial effects predominantly by inhibiting the effects of Ang II mediated by AT_1

receptors. This conclusion is consistent with findings of previous studies showing that the elevated bradykinin levels associated with ACEI therapy do not appear to play a significant renal protective role [461-463]. Moreover, the AT_2 receptor stimulation that results indirectly from blockade of AT_1 receptors does not appear to afford AT_1RA therapy any renal protective advantage or disadvantage over ACEI therapy.

The success of RAS inhibitors in reducing renal injury in non-hypertensive models of renal disease [460] suggests that an unopposed RAS may itself be regarded as a risk factor for CKD progression. On the other hand, RAS inhibitors are highly effective antihypertensive and antiproteinuric agents. It is not yet clear to what extent blood pressure control retains its importance as a renal protective measure in the context of RAS inhibition. Nor is it clear whether reduction of proteinuria merely reflects blood pressure reduction or if lower levels of protein excretion at a given level of systemic blood pressure are associated with additive renal protective effects. The second major finding of this study is therefore the demonstration by linear regression techniques that systolic blood pressure and urinary protein excretion are independent determinants of glomerulosclerosis in rats receiving inhibitors of the RAS after extensive renal ablation. Together, SBP and $U_{pr}V$ accounted for 72% of the variance in GS, implying that they represent major determinants of glomerulosclerosis in this model.

While it is not possible from these data to exclude the possibility that higher levels of blood pressure merely reflect greater severity of renal injury, it is true to say that failure to control blood pressure optimally in this model was associated with failure to achieve renal protection. Furthermore, the lack of any correlation between the level of SBP and severity of renal injury at an early time-point, before initiation of treatment, argues in favour of a direct effect of blood pressure on renal disease progression over time. Using radiotelemetry, Bidani et al. also found a strong correlation (r=0.88) between mean SBP and glomerulosclerosis in untreated 5/6 nephrectomized rats. In keeping with our observations, the correlation between SBP and glomerular injury was much weaker during the first 2 weeks after injury [509]. The renal protective effect of lowering blood pressure has been clearly established in clinical studies of CKD [337, 341, 342, 346, 510, 511]. In diabetic patients treated with ACEI, lower target levels of blood pressure control have been shown to result in greater reduction of proteinuria in one randomized trial [349]. Our

data are therefore consistent with this clinical evidence that the level of blood pressure control remains an important determinant of progressive renal injury in CKD during treatment with inhibitors of the RAS. It should be remembered that micropuncture studies in 5/6 nephrectomized rats suggest that glomerular capillary hydraulic pressure (P_{GC}) rather than systemic blood pressure *per se*, is the critical determinant of renal injury [27, 513] and that ACEIs [27] and AT_1RAs [28, 29] reduce both systemic and glomerular capillary pressures. Nevertheless, systemic blood pressure is partially transmitted to the glomerular capillaries and it is probably for this reason that it remains a significant determinant of renal injury in the setting of RAS inhibition.

Proteinuria has traditionally been regarded merely as a marker of glomerular injury. Recent clinical studies, however, report that proteinuria may also be an independent epidemiological risk factor of CKD progression [169, 337, 514]. Furthermore, treatments which reduce proteinuria also slow the progression of CKD [168, 169, 373] and a reduction in proteinuria, independent of blood pressure, was associated with slower progression of CKD in the MDRD study [337]. As discussed in Chapter 1, these and other experimental findings raise the possibility that proteinuria *per se* exacerbates renal injury. A meta-analysis of 57 animal studies including various models of renal disease reported consistent positive associations between the level of protein- or albuminuria and the severity of glomerulosclerosis (mean weighted correlation coefficients r=0.82 and 0.76) [331]. We have confirmed that a direct correlation exists between proteinuria and glomerular injury in 5/6 nephrectomized rats receiving RAS inhibitors (r=0.86), independent of the level of blood pressure. This implies that at a given level of blood pressure, rats with higher levels of proteinuria can be expected to have more severe renal injury, a conclusion similar to those supported by clinical studies [169, 337]. Although these data do not prove that proteinuria contributes directly to renal injury, they do reveal the extent to which the renal protective effects of ACEI and AT_1RA are related to their antiproteinuric effects.

The detection of proinflammatory and fibrotic gene induction and mϕ infiltration in the remnant kidney supports the notion that inflammatory processes may contribute to the progressive renal injury and fibrosis that follows 5/6 nephrectomy. As shown in Chapter 2 the protection from progressive renal injury afforded by ACEI or AT_1RA treatment initiated early after 5/6 nephrectomy is associated with suppression of

proinflammatory gene induction to levels similar to those of sham-operated rats and inhibition of renal mϕ infiltration to levels only slightly greater than sham. In this study we observed that when treatment was delayed until 5 weeks after 5/6 nephrectomy, a time point when remnant kidney mRNA levels for TGF-β1 and MCP-1 are known to be upregulated, ACEI or AT_1RA did not suppress expression of these two cytokines to normal levels. At 12 weeks post surgery mRNA levels for TGF-β1 and MCP-1 were similar to those observed before initiation of treatment, and remained significantly higher than those of sham-operated rats. At 24 weeks after surgery, TGF-β1 mRNA levels were significantly lower than pretreatment values but remained significantly higher than sham levels and MCP-1 mRNA levels remained at pretreatment values. Failure of suppression of the TGF-β1 and MCP-1 responses at 12-weeks, when renal injury had not yet progressed beyond that observed before the initiation of treatment, was associated with slow progression of renal injury despite probable amelioration of adverse glomerular haemodynamic factors. IL-1β mRNA levels were not elevated in pretreatment vs. sham-operated rats. This is consistent with findings presented in Chapter 2, that IL-1β induction was not apparent until 8 weeks after 5/6 nephrectomy. The trend towards higher IL-1β mRNA levels in both treatment groups vs. sham at 12-weeks suggests that failure of suppression of this gene, a product of activated mϕ, may also be associated with subsequent progression of injury. Strong correlations observed between the extent of renal injury at 24 weeks (as measured by either GS or TIS) and mRNA levels for TGF-β1, MCP-1 and IL-1β further support the hypothesis that upregulation of these proinflammatory and profibrotic genes contributes to progressive renal injury.

Together, these observations in remnant kidneys indicate that incomplete suppression of proinflammatory gene expression with ACEI or AT_1RA treatment is associated with failure to arrest progression of renal injury and secondly, that the extent of progression is directly correlated with the level of gene expression. It should be stressed that these observations were made in rats receiving chronic treatment at doses of ACEI or AT_1RA with documented success in normalizing P_{GC} even when initiated after the onset of renal injury in this model [28, 515, 516]. This implies that the process of renal injury initiated by glomerular capillary hypertension and the direct or indirect effects of Ang II may eventually be sustained more by autonomous cellular and molecular factors and become less dependent on the initiating factors. This

notion is consistent with the observations of Ichikawa and others, that in glomeruli with severe established injury, treatment with enalapril did not prevent further progression to global sclerosis [515]. It is also possible that in the face of existing renal injury, treatment with ACEI or AT_1RA did not completely normalize P_{GC} in the long term or achieve total blockade of the RAS. In keeping with suggestions by other authors [517], these findings suggest that patients in whom progression of chronic renal injury persists during RAS blockade may benefit from additional therapy targeting the effects of proinflammatory and fibrotic cytokine gene expression [508].

4. Glomerular Capillary Hydraulic Pressure Lowering Revisited with Novel Therapeutic Agents

4.1. Introduction

As shown in the preceding chapters, treatment with ACEI significantly retards the rate of progression of CKD and represents the standard with which novel renal protective therapies must be compared. As ACEI treatment in clinical studies slows rather than arrests the progression of CKD [168, 169, 374, 376], there is however a need for further research to identify more effective renal protective therapies.

Vasopeptidase inhibitors (VPI) are a novel class of drugs comprising single molecules that simultaneously inhibit both angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP). This latter ecto-enzyme is localized principally in the brush border membrane of renal tubule cells and catabolizes several vasodilator molecules including the natriuretic peptides, adrenomedullin and bradykinin [518, 519]. Thus VPI treatment is associated with reduced production of the vasoconstrictor, Ang II, and accumulation of the above vasodilators. In experimental [520, 521] and clinical studies [518] VPIs have been shown to be effective antihypertensive agents in both low and high renin states. In addition, VPI treatment was associated with greater survival than ACEI treatment alone in an experimental model of cardiac failure [522]. In clinical studies of cardiac failure, VPI treatment has been associated with better clinical outcomes than ACEI treatment in some [523] but not all studies [524].

Although they appear to act at multiple levels to slow CKD progression the renal protective effects of ACEI are closely associated with their ability to normalize the elevated glomerular capillary hydraulic pressures (P_{GC}) observed in remaining nephrons after loss of more than half of the total renal mass [27]. Since VPIs alter the levels of multiple vasoactive peptides, it is difficult to predict the effects of VPI treatment on glomerular haemodynamics. Short-term studies in experimental models of CKD suggest that VPI treatment may afford renal protection in addition to that achievable with ACEI alone [525, 526]. We hypothesized that VPI treatment would afford more effective renal protection than ACEI treatment in the 5/6 nephrectomy model but that this would be evident only in a protocol in which ACEI treatment did not confer complete renal protection. Second, we hypothesized that the improved renal protection with VPI

treatment would be associated with greater lowering of P_{GC} than that produced by ACEI. The aims of this study were therefore:

1. To compare the glomerular haemodynamic effects of the VPI, omapatrilat, with that of an ACEI, enalapril, in the 5/6 nephrectomy model.
2. To compare the renal protective effects of omapatrilat vs. enalapril in rats after 5/6 nephrectomy in a short-term experiment in which treatment is started before the onset of renal injury.
3. To compare the renal protective effects of omapatrilat vs. enalapril in rats after 5/6 nephrectomy in an experiment in which treatment is started only after the development of renal injury and follow up is continued for a prolonged period.

4.2. Methods

4.2.1. Short-term treatment protocol

Twenty seven male Munich-Wistar rats obtained from Simonsen Laboratories (Gilroy, California, USA) were subjected to 5/6 nephrectomy as described in the preceding chapters. Rats were housed under standard conditions and given unrestricted access to standard rodent chow and water. On day 2 after surgery, rats were started on omapatrilat (Bristol-Myers Squibb, Princeton, New Jersey, USA) 150 mg/L (*OMA*; n=6) or enalapril (Sigma Chemical Company, St Louis, Missouri, USA) 100mg/L in drinking water (*ENA*; n=6). Sodium bicarbonate (1.5 mmol/L) was added to solubilize omapatrilat and was also added to the enalapril solution. The doses of omapatrilat and enalapril were adjusted to achieve equivalent normalization of blood pressure in each group such that final doses were: omapatrilat 133mg/L (8-12 mg/kg/day) and enalapril 120mg/L (7-11 mg/kg/day). Controls received no treatment and were used for comparison in both short and long-term treatment protocols (*CON*; n=15). At 2-week intervals, systolic blood pressure (SBP) was measured by the tail-cuff method and daily urinary protein excretion rate ($U_{pr}V$) determined by spectrophotometry after precipitation with 3% sulfosalicylic acid on urine collected from rats individually housed in metabolic cages for 24 hours. All rats were sacrificed at 12 weeks after surgery. The remnant kidney was perfusion-fixed with 10% phosphate buffered formalin delivered through a catheter in the abdominal aorta at the measured systolic blood pressure of each rat.

4.2.2. Micropuncture studies

Forty male Munich-Wistar rats underwent 5/6 nephrectomy and at 2-5 days after surgery, were assigned to treatment with omapatrilat 25mg/L (n=8) or 150mg/L (n=8), enalapril 25mg/L (n=8) or 150mg/L (n=8), or received no therapy (n=8). Micropuncture studies were performed at 4-6 weeks after surgery. Rats were anaesthetized with Inactin (100mg/kg body weight, i.p.; Byk Gulden, Konstnaz, Germany) and prepared for micropuncture as previously described [27]. A PE-50 tubing catheter was placed in a femoral artery for blood sampling and monitoring of arterial blood pressure. The euvolemic state was maintained by infusion of bovine serum albumin (4% in Ringer's Lactate; 0.8% of body weight over the first 25 minutes and 0.6mL/hour thereafter). Solutions of inulin (3%) and *para*-aminohippurate (PAH) (0.2%) in 0.9% saline were infused at 27 µL/min throughout the experiment.

Micropuncture experiments were conducted during two clearance periods of 30-40 minutes. During each period an arterial blood sample was obtained for determination of haematocrit as well as plasma inulin, PAH and protein concentrations. A timed collection of urine was obtained for determination of urine PAH and inulin concentrations to allow calculation of whole kidney plasma flow rate and glomerular filtration rate (GFR). Timed collections (1-1.5 minutes) of tubule fluid were obtained from 4 surface proximal tubules for determination of single nephron GFR (SNGFR). Time averaged hydraulic pressures were measured in surface glomerular capillaries, efferent arterioles and proximal tubules using a continuous recording servo-null micropipette transducer system (Instrumentation for Physiology and Medicine, San Diego, California, USA).

Urine volumes were determined gravimetrically. Plasma protein concentration was determined using a refractometer. The concentration of inulin in plasma and urine was determined by the macro-anthrone method [527] and in tubule fluid specimens, by a micro-immunofluorescence method [528]. PAH concentrations in plasma and urine were determined using a colorimetric method [529]. Variables derived from the above direct readings were calculated using established formulae [530].

4.2.3. Long-term treatment protocol

Forty one male Munich-Wistar rats were subjected to 5/6 nephrectomy as described above. At 4 weeks after renal mass ablation, rats were assigned to two groups matched for SBP and $U_{pr}V$, and started on omapatrilat (*OMA*; n=22) or enalapril (*ENA*; n=19) in drinking water. Doses were chosen to achieve equivalent control of SBP in the treatment groups and after some initial adjustment, 100mg/L (6-9 mg/kg/day) of omapatrilat and 100mg/L of enalapril was used. SBP and $U_{pr}V$ were measured at 2-week intervals. At 20 weeks after surgery several rats from each group were sacrificed (20 week set: *OMA*, n=15; *ENA*, n=11). These rats were identified at the start of treatment at which time they were matched for SBP and $U_{pr}V$. The remaining rats were continued on treatment until $U_{pr}V$ showed a sustained increase to levels above pretreatment values (long-term set: *OMA*, n=7; *ENA*, n=8). *ENA* rats were sacrificed at 32 weeks after surgery, when rapidly increasing $U_{pr}V$ levels suggested that they would soon die due to rapid progression of their renal injury. Among *OMA* rats, the dose of omapatrilat was increased to 120mg/L, 150mg/L and 200mg/L at 38, 39 and 44 weeks after surgery, respectively, due to a rising trend in SBP and the rats were sacrificed at 50 weeks after surgery. At sacrifice, remnant kidneys were perfusion-fixed as described above.

4.2.4. Morphology

Renal tissue was embedded in paraffin and processed for light microscopy. The frequency of glomerulosclerosis was estimated by examining all glomeruli seen in one or two coronal sections from each kidney (mean=208(9) glomeruli) stained by the periodic acid-Schiff method. A glomerulosclerosis score (GS) was determined by expressing the number of glomeruli with segmental or global sclerosis as a percentage of the total number of glomeruli counted for each rat. Tubulointerstitial injury was assessed at medium power on the same sections. Each of 3 aspects of tubulointerstitial injury (tubule proteinaceous casts and dilation; interstitial inflammation; interstitial fibrosis) was assigned a score from 0 to 3 according to severity (0=no abnormality; 1=mild; 2=moderate; 3=severe), and these scores added to yield an overall Tubulointerstitial Score (TIS) from 0 to 9, which was then expressed as a percentage. The histological assessment was performed without knowledge of the treatment assignment of individual rats.

4.2.5. Statistical Analysis

Continuous variables are expressed as mean(SEM). Differences among multiple groups were assessed using analysis of variance (ANOVA) and a post hoc Scheffe's test. Variables measured repeatedly over time were compared among groups using repeated measures ANOVA. A paired t-test was used to compare values at different time points in the same subjects. P-values less than 0.05 were considered significant. Statistical analyses were conducted using Statview 4.01 (Abacus Concepts Inc., Berkley, California, USA).

4.3. Results

4.3.1. Short-term treatment protocol

Rats from all groups gained weight over 12 weeks, although body weights among *CON* rats tended to be moderately higher than *OMA* or *ENA* rats at each time point. Only the difference between *CON* and *OMA* was statistically significant. There was no difference in body weight between *OMA* and *ENA* over time (mean difference=15g; P=0.3). *CON* rats developed sustained hypertension after 5/6 nephrectomy. Treatment with either omapatrilat or enalapril prevented this rise in blood pressure and maintained SBP at levels generally from 90 to 100 mmHg (Figure 4.1A). There was no significant difference in SBP over time in *OMA* vs. *ENA* rats. A progressive rise in proteinuria was observed in *CON* rats after 5/6 nephrectomy whereas proteinuria was almost completely prevented by both treatments (Figure 4.1B). There was no difference in $U_{pr}V$ between *OMA* vs. *ENA* rats over time. Remnant kidney histology revealed substantial glomerulosclerosis and tubulointerstitial injury in *CON* rats whereas histological injury was virtually absent in *OMA* and *ENA* rats. There was no difference in GS or TIS in *OMA* vs. *ENA* rats (Figure 4.1C).

4.3.2. Micropuncture studies

The results of micropuncture studies are shown in Table 4.1. Body weight was similar among groups. Both treatments were associated with small but significant reductions in haematocrit. Mean arterial pressures (MAP) displayed similar patterns to those of SBP in the early treatment protocol. Untreated rats evidenced severe hypertension whereas both enalapril and omapatrilat treatment resulted in normalization of MAP. Although there was a tendency for MAP to be lower at the higher dose of each drug, these differences were not statistically significant. As shown in the Table, all other variables measured did not

differ between the higher and lower dose of each treatment and data for each treatment were therefore pooled for comparison between treatments. Despite lower perfusion pressures, both treatments were associated with significant increases in renal plasma flow vs. untreated controls, findings in keeping with renal vasodilation. Whole kidney GFR on the other hand, was not different among the groups. As has previously been observed, untreated rats evidenced substantial elevations in glomerular capillary hydraulic pressure (P_{GC}) when compared to values reported for normal rats [15]. Treatment with either enalapril or omapatrilat was associated with reductions in P_{GC} to values similar to those of normal rats. At both dose levels P_{GC} tended to be lower in *OMA* vs. *ENA* rats and when data from all *OMA* and *ENA* rats were combined, omapatrilat treatment was associated with significantly lower P_{GC} than enalapril. Glomerular transcapillary hydraulic pressure difference (ΔP) showed similar trends although the difference between *OMA* and *ENA* was not statistically significant. In keeping with the reductions in ΔP, whole kidney filtration fraction was significantly lower in both treatment groups. SNGFR and glomerular plasma flow rate were substantially higher than previously reported normal values in untreated controls [15] and were similarly elevated in all treatment groups. Renal mass ablation was associated with substantial decreases in afferent (R_A) and efferent (R_E) arteriolar resistances in untreated rats compared to previously reported normal values [15]. Treatment with omapatrilat or enalapril resulted in significantly greater reductions in both R_A and R_E than 5/6 nephrectomy alone. There were no significant differences in glomerular capillary ultrafiltration coefficient, although values tended to be higher in the *OMA* and *ENA* groups.

4.3.3. Long-term treatment protocol

At the initiation of treatment (4 weeks) body weights were similar among the groups (P=0.98). Body weight increased steadily throughout the study in *CON* rats and in rats from the 20 week set of both treatment groups. Among rats followed beyond 20 weeks, body weight stopped increasing after 26 weeks and remained stable thereafter. No statistically significant differences in body weight emerged over time between the treatment groups in either the 20 week set or long-term sets.

Following 5/6 nephrectomy, rats in all groups developed hypertension and SBP was similar among groups before treatment (P=0.98) (Figure 4.2.). *CON* rats remained hypertensive until sacrifice. Treatment with either omapatrilat or enalapril lowered blood pressures to normal levels and maintained SBP at mean

levels that were generally below 120mmHg throughout the study. No statistically significant difference in SBP developed over time between *OMA* and *ENA* rats either over weeks 6-20 (mean difference=4 mmHg, P=0.3) or weeks 22-32 (mean difference=2 mmHg, P=0.7).

Rats developed substantial proteinuria over the first 4 weeks after 5/6 nephrectomy and $U_{pr}V$ was similar among the groups before treatment (P=0.7) (Figure 4.3.). *CON* rats continued to show a progressive increase in $U_{pr}V$ over the subsequent 8 weeks. Both omapatrilat and enalapril treatment initially reduced $U_{pr}V$ to below 20mg/day. However, from 12 weeks there was a progressive rise in $U_{pr}V$ among *ENA* rats such that mean values at 20 weeks had reached 0.81 of pretreatment values (P=0.2 for week 20 vs. week 4 values among *ENA* rats). By contrast $U_{pr}V$ increased more slowly among *OMA* rats such that at 20 weeks the mean value was only 0.44 of pretreatment values (P<0.0001 for week 20 vs. week 4 values among *OMA* rats). Over weeks 6-20, $U_{pr}V$ was significantly higher in *ENA* vs. *OMA* rats (P=0.04). Beyond 20 weeks, $U_{pr}V$ continued to rise among *ENA* rats, reaching a mean value 1.8 times that of pretreatment levels at 32 weeks (P=0.03 for week 32 vs. week 4 values among *ENA* rats). Among *OMA* rats $U_{pr}V$ rose more slowly such that 50 week levels were similar (1.2 times) to pretreatment values (P=0.3 for week 50 vs. week 4 values). Over weeks 22-32 $U_{pr}V$ was significantly higher in *ENA* vs. *OMA* rats (P=0.03). In the long-term set, 6 of 8 rats in the *ENA* group and 5 of 7 rats in the *OMA* group had $U_{pr}V$ levels greater than pretreatment values at the time of sacrifice. However, the mean time to reach a $U_{pr}V$ greater than the pretreatment level on two readings was significantly shorter among *ENA* than *OMA* rats (20 weeks vs. 34 weeks, respectively; P=0.002).

Histological assessment of remnant kidneys revealed a mean glomerulosclerosis score (GS) of 36.1% among *CON* rats, which were all sacrificed at 12 weeks post 5/6 nephrectomy (Figure 4.4.). Among *OMA* rats at 20 weeks, GS was significantly lower than this value (GS=13.9%; P=0.001 vs. *CON*). A similar trend was observed in *ENA* rats at 20 weeks, although the difference was not statistically significant (GS=22.1%; P=0.3 vs. *CON*). GS tended to be lower in *OMA* vs. *ENA* rats at 20 weeks but this difference also was not significant (P=0.8). In the long-term set, *ENA* rats sacrificed at 32 weeks (GS=34±5%) and *OMA* rats sacrificed at 50 weeks (GS=38±8%) had mean GS values very similar to those of untreated *CON* rats at 12 weeks. Tubulointerstitial injury exhibited a similar pattern to that of glomerulosclerosis. At

20 weeks the TIS was significantly lower in *OMA* vs. *CON* rats and tended to be lower in *ENA* vs. *CON* rats. In the long-term set, *OMA* rats at 50 weeks had a mean TIS similar to that of *CON* rats at 12 weeks. The TIS among *ENA* rats at 32 weeks tended to be lower than that of *OMA* rats at 50 weeks but this difference was not statistically significant (Figure 4.4.). Overall there was a highly significant correlation between GS and TIS in individual rats ($r=0.82$; $P<0.0001$).

Table 4.1: Micropuncture data at 4-6 weeks after 5/6 nephrectomy; data=mean(SEM).

	Body Wt *g*	**Hct** *%*	**MAP** *mmHg*	**RPF** *ml/min*	**GFR** *ml/min*	**FF**	**C_A** *g/dl*	**P_{GC}** *mmHg*
CON	279(7)	45(1)	143(5)	1.4(0.2)	0.44(0.05)	0.33(0.03)	4.8(0.1)	62(3)
ENA (25mg/l)	300(7)	40(1)[a]	97(6)[a]	2.7(0.4)[a]	0.62(0.06)	0.26(0.02)[a]	4.6(0.1)	53(3)
OMA (25 mg/l)	306(6)	40(1)	93(3)[a]	2.7(0.3)[a]	0.61(0.07)	0.22(0.00)[a]	4.7(0.1)	47(3)[a]
ENA (150 mg/l)	284(7)	40(1)[a, b]	85(2)[a, b]	2.6(0.3)[b]	0.54(0.07)	0.21(0.01)[a, b]	4.6(0.1)	49(2)[a, b]
OMA (150 mg/l)	283(5)	40(1)[a, b]	85(3)[a, b]	2.3(0.2)[b]	0.49(0.03)	0.21(0.01)[a, b]	4.5(0.1)	42(1)[a, b, c]
	P_T *mmHg*	**P_E** *mmHg*	**ΔP** *mmHg*	**SNGFR** *nl/min*	**Q_A** *nl/min*	**$R_A \times 10^{10}$** *dyne·s·cm^{-5}*	**$R_E \times 10^{10}$** *dyne·s·cm^{-5}*	**K_f** *nl/(s·mmHg)*
CON	14(1)	25(2)	48(4)	76(8)	237(26)	1.62(0.20)	1.20(0.15)	0.048(0.006)
ENA (25mg/l)	12(1)	19(1)	41(2)	84(6)	334(23)	0.63(0.06)[a]	0.73(0.09)	0.065(0.009)
OMA (25 mg/l)	11(1)	18(1)[a]	36(3)[a]	61(7)	275(28)	0.84(0.10)[a]	0.79(0.12)	0.063(0.011)
ENA (150 mg/l)	11(1)	20(1)[b]	38(2)[b]	56(6)	275(30)	0.69(0.11)[a, b]	0.82(0.11)[b]	0.047(0.006)
OMA (150 mg/l)	11(0)[b]	18(2)[a, b]	31(1)[a, b]	54(8)	264(40)	0.93(0.17)[a, b]	0.74(0.10)[b]	0.068(0.012)

[a] $P<0.05$ vs. CON

[b] $P<0.05$ for combined ENA 25mg/l and 150mg/l data or combined OMA 25mg/l and 150mg/l data vs. CON

[c] $P<0.05$ for combined OMA 25mg/l and 150mg/l data vs. combined ENA 25mg/l and 150mg/l data

Body Wt: body weight; Hct: hematocrit; MAP: mean arterial pressure; RPF: renal plasma flow; GFR: glomerular filtration rate; FF: filtration fraction; C_A: afferent arteriolar protein concentration; P_{GC}: glomerular capillary hydraulic pressure; P_T: proximal tubule hydraulic pressure; P_E: efferent arteriolar hydraulic pressure; ΔP: glomerular transcapillary hydraulic pressure difference; SNGFR: single nephron GFR; Q_A: glomerular plasma flow rate; R_A: afferent arteriolar resistance; R_E: efferent arteriolar resistance; K_f: glomerular capillary ultrafiltration coefficient

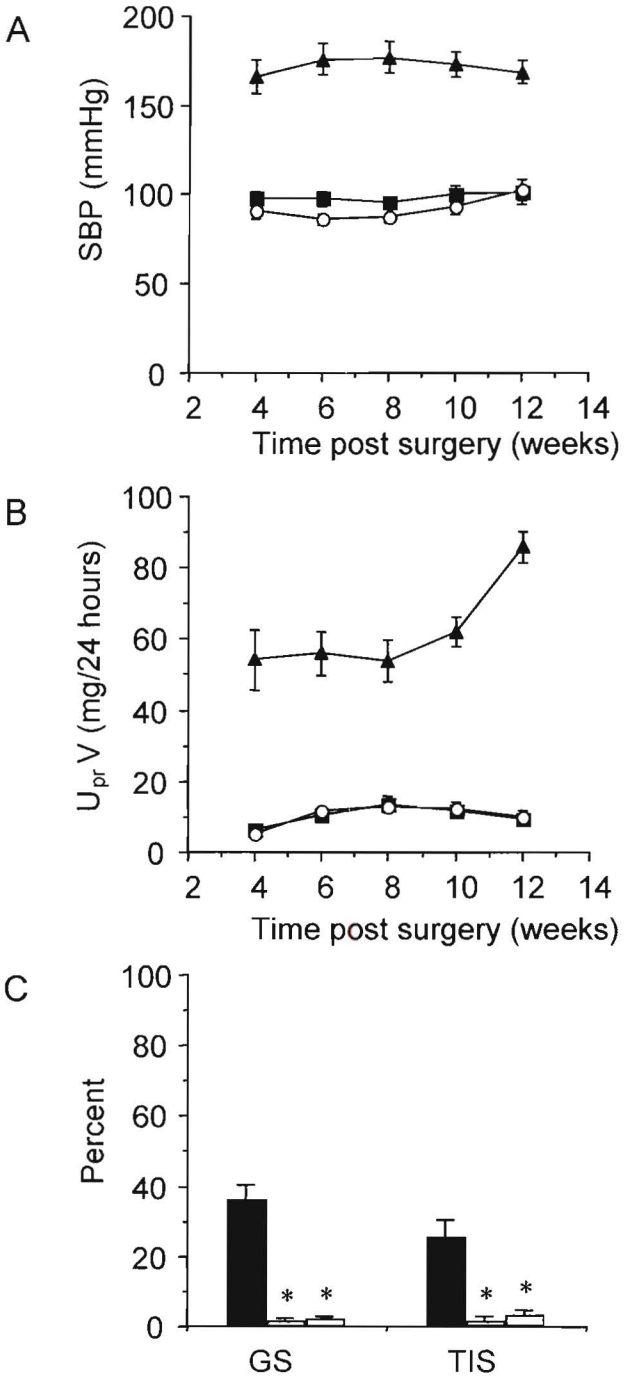


Figure 4.1. Early Treatment Protocol: **(A)** Systolic blood pressure (SBP) and **(B)** urinary protein excretion rate ($U_{pr}V$) over time among rats subjected to 5/6 nephrectomy and started on omapatrilat (*OMA*-open circles), enalapril (*ENA*-filled squares) or no treatment (*CON*-filled triangles) on day 2 post surgery. SBP and $U_{pr}V$ were normalized by both treatments and were significantly lower in treated vs. untreated rats ($P<0.0001$ vs. *CON* for both systolic blood pressure and proteinuria over week 6-12).

C: Glomerulosclerosis (GS) and Tubulointerstitial Injury Scores (TIS) from kidneys removed at 12 weeks after 5/6 nephrectomy revealed that omapatrilat (*OMA*-open bars) and enalapril (*ENA*-shaded bars) effectively prevented the substantial glomerulosclerosis and tubulointerstitial injury observed in untreated rats (*CON*-filled bars) when started on day 2 post surgery (* $P<0.005$ vs. *CON*).

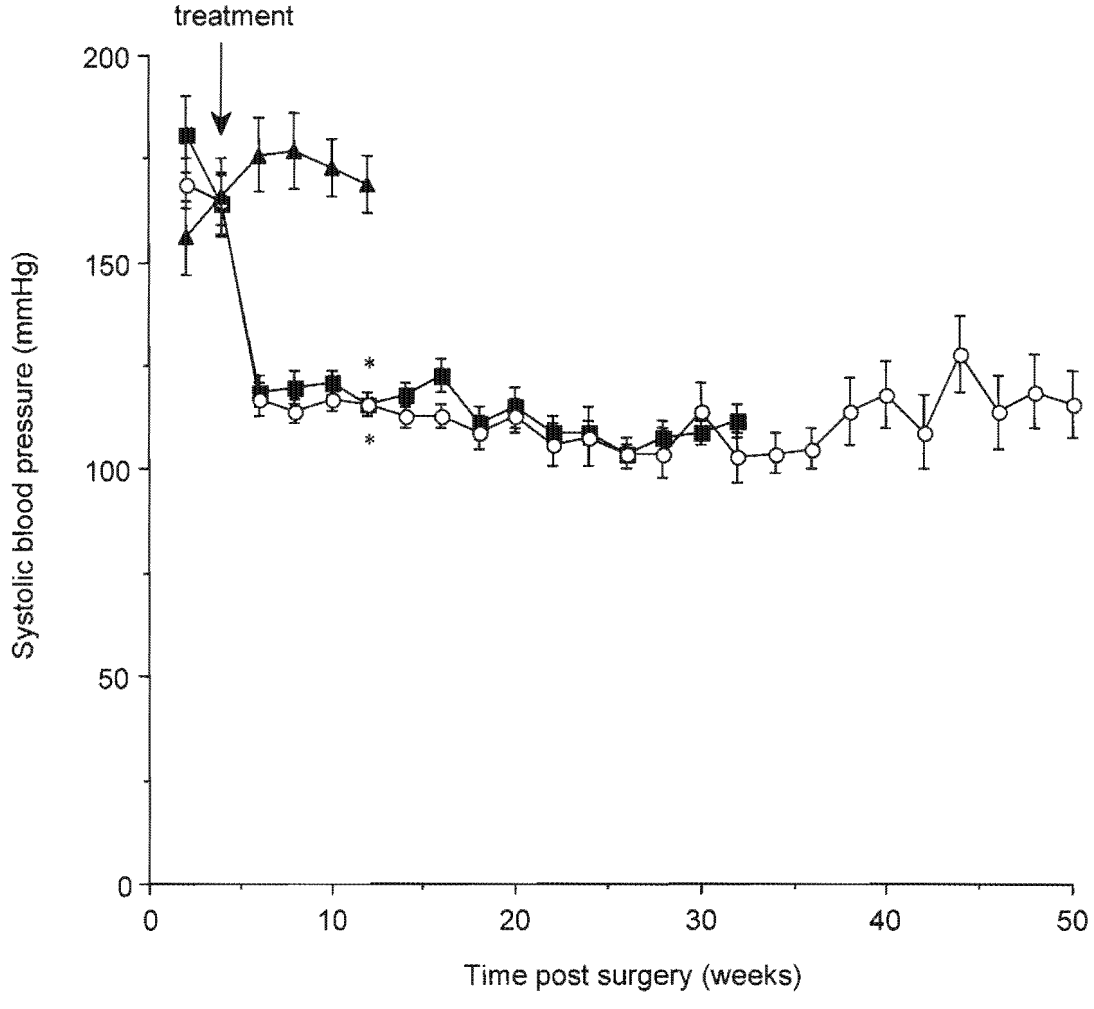


Figure 4.2. Delayed Treatment Protocol: Systolic blood pressure (SBP) over time among rats subjected to 5/6 nephrectomy and started on omapatrilat (*OMA*-open circles), enalapril (*ENA*-filled squares) or no treatment (*CON*-filled triangles) at 4 weeks post surgery. Rats from all groups developed hypertension prior to therapy and were closely matched for SBP at 4 weeks post surgery. Both treatments resulted in normalization of SBP and blood pressure levels were similar for omapatrilat vs. enalapril-treated rats throughout the treatment period (mean differences over weeks 6-20 = 4 mmHg and over weeks 22-32 = 2mmHg; P=0.3 and 0.7 respectively by repeated measures ANOVA). Untreated rats remained hypertensive and SBP among *OMA* and *ENA* rats was significantly lower than untreated rats over weeks 6-12 (* P<0.0001 vs. *CON*).

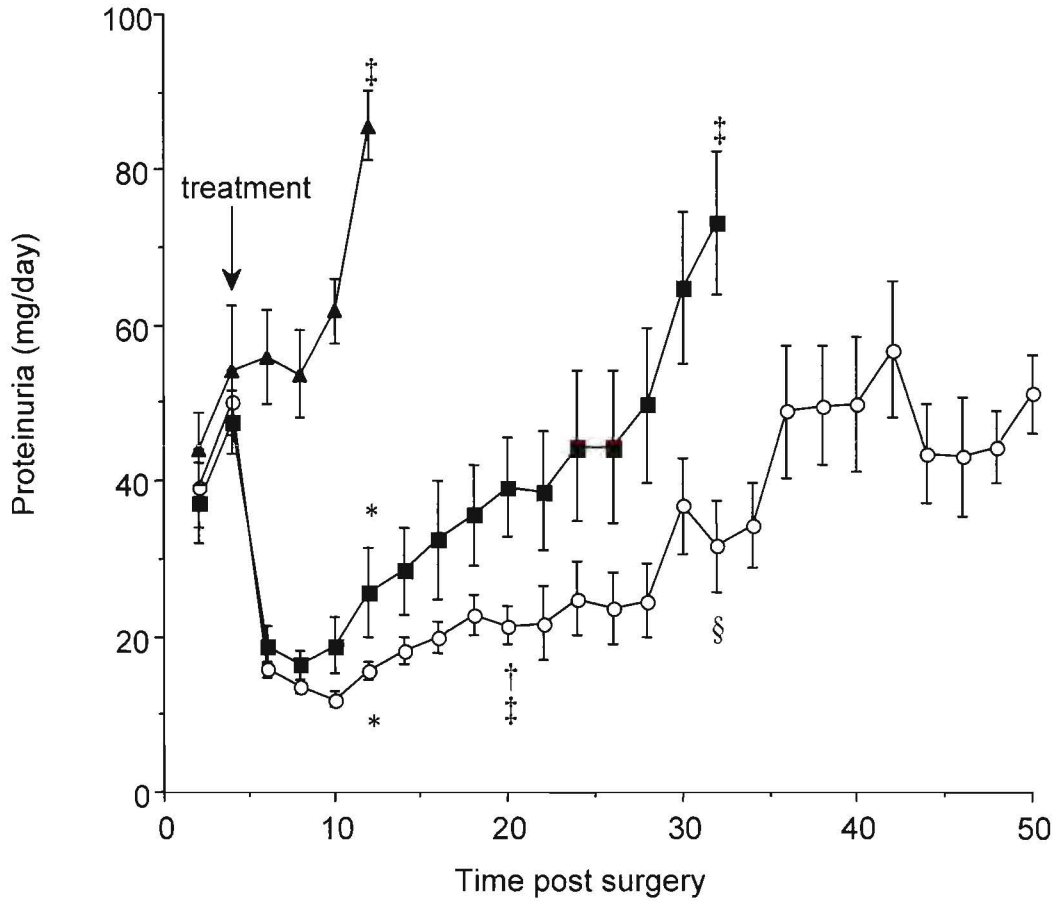


Figure 4.3. Delayed Treatment Protocol: Urinary protein excretion rate ($U_{pr}V$) over time among rats subjected to 5/6 nephrectomy and started on omapatrilat (*OMA*-open circles), enalapril (*ENA*-filled squares) or no treatment (*CON*-filled triangles) at 4 weeks post surgery. Prior to treatment rats from all groups developed substantial proteinuria and were closely matched for $U_{pr}V$ at 4 weeks post surgery. Treatment with either omapatrilat or enalapril resulted in an initial reduction in proteinuria such that $U_{pr}V$ was significantly lower among *OMA* and *ENA* rats than untreated rats over weeks 6-12 ($P<0.0001$). $U_{pr}V$ increased after week 10 among *ENA* rats such that levels at week 20 were no longer significantly lower than pretreatment values and at week 32, $U_{pr}V$ was significantly higher than pretreatment values. Among *OMA* rats $U_{pr}V$ increased more slowly, such that levels at week 20 remained significantly lower than pretreatment values and 50 week levels were similar to pretreatment values. $U_{pr}V$ was significantly lower in *OMA* vs. *ENA* rats over week 6-20 and over weeks 22-32. At week 32, mean $U_{pr}V$ among *ENA* rats was more than double that of *OMA* rats.
(* $P<0.05$ vs. *CON* over week 6-12; ‡ $P<0.05$ vs. pretreatment value; † $P<0.05$ vs. *ENA* over week 6-20; § $P<0.05$ vs. *ENA* over week 22-32)

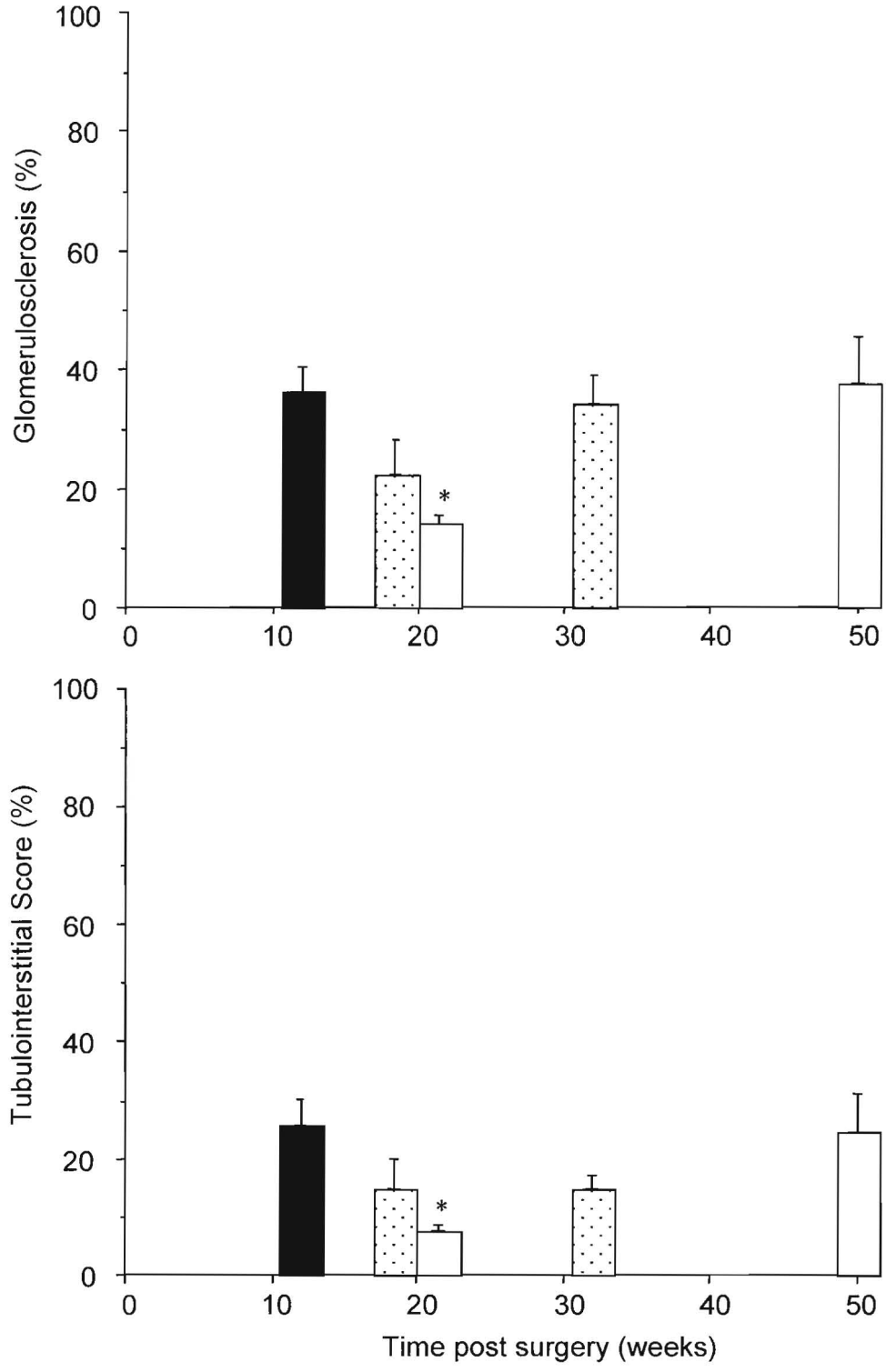


Figure 4.4. Delayed Treatment Protocol: Glomerulosclerosis (GS) and Tubulointerstitial Injury Scores (TIS) for rats subjected to 5/6 nephrectomy and started on omapatrilat (*OMA*-open bars), enalapril (*ENA*-stippled bars) or no treatment (*CON*-filled bars) at 4 weeks post surgery. Both treatments resulted in protection from histological injury such that GS and TIS were lower in *OMA* and *ENA* rats at week 20 than in *CON* rats at week 12, although only the difference between *OMA* and *CON* rats was statistically significant. GS among *CON* rats at week 12, *ENA* rats at week 32 and *OMA* rats at week 50 were similar, implying that enalapril delayed the progression of glomerular injury by 20 weeks whereas omapatrilat delayed it by 38 weeks. TIS showed a similar trend, although *ENA* rats at week 32 tended to have less tubulointerstitial injury. (* $P<0.005$ vs. *CON*)

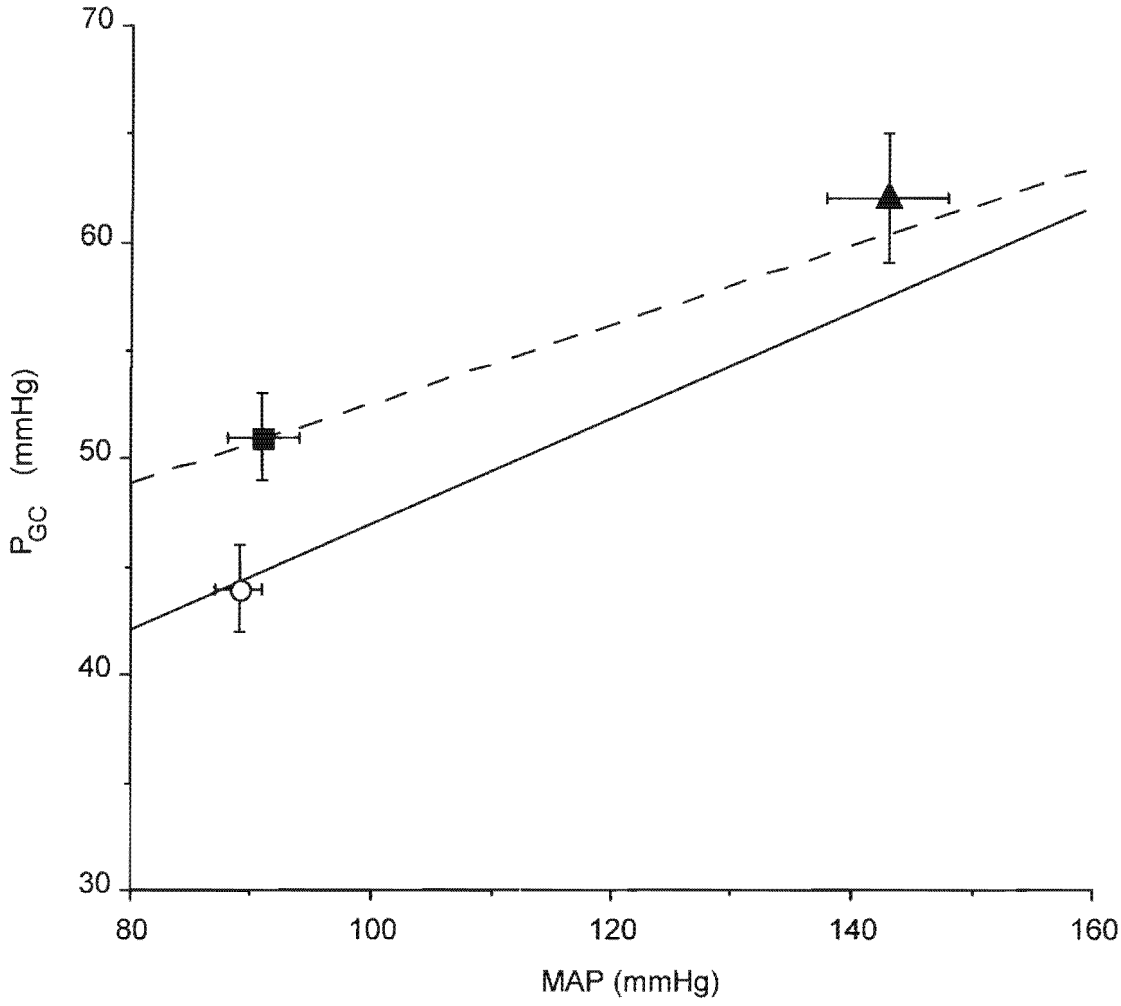


Figure 4.5. Micropuncture: Means for the plot of glomerular capillary hydraulic pressure (P_{GC}) vs. mean arterial pressure (MAP) among rats subjected to 5/6 nephrectomy and started on omapatrilat (*OMA*-open circle), enalapril (*ENA*-filled square) or no treatment (*CON*-filled triangle) on day 2-5 post surgery. Values were obtained during micropuncture studies at 4-6 weeks after 5/6 nephrectomy. The broken and solid lines represent regression lines for P_{GC} vs. MAP among *ENA* and *OMA* rats, respectively.

4.4. Discussion

4.4.1. Short-term treatment protocol

We have shown that when treatment was initiated early after 5/6 nephrectomy at doses sufficient to normalize blood pressure, both omapatrilat and enalapril almost completely prevented the progressive rise in proteinuria and histological renal injury observed in untreated controls over 12 weeks. Since enalapril treatment has previously been shown to result in almost complete renal protection in this model [27], we did not expect to observe any greater benefit with omapatrilat than enalapril. Other studies comparing VPI and ACEI treatment in an early treatment protocol after 5/6 nephrectomy have reported greater renal protective effects with the VPI [525, 526]. However, in one of these studies blood pressure was substantially different between the treatment groups at the lower dose studied and was not normalized in either group, even at a higher dose [525]. In the other, SBP was also significantly higher in ACEI-treated than VPI-treated rats [526]. These previous data therefore were unable to distinguish additional renal protective effects attributable to greater systemic blood pressure reduction from effects related to unique renal actions of VPIs.

4.4.2. Micropuncture Studies

Previous micropuncture studies in the partial nephrectomy model have shown that elevated glomerular capillary pressure is a critical factor in the pathogenesis of progressive renal injury and the renal protective effects of low protein diet, ACEI or AT_1RA treatment were closely associated with normalization of P_{GC} [15, 26-28]. In the present study enalapril and omapatrilat produced similar effects on most of the haemodynamic parameters studied. Importantly MAP was lowered to the same extent with both treatments. The observed increases in renal plasma flow together with a reduction in filtration fraction suggest renal vasodilation and a lowering of P_{GC}. Measurements of single nephron haemodynamics confirmed that both afferent and efferent arteriolar resistances were reduced and P_{GC} was significantly lowered by both treatments. The P_{GC} values observed in *ENA* rats were very similar to those reported previously in enalapril-treated rats [26-28]. In contrast, omapatrilat treatment was associated with greater lowering of P_{GC} than enalapril. As expected, P_{GC} was significantly correlated with MAP when data from all rats were pooled ($r=0.69$; $P<0.0001$). As shown in Figure 4.5. however, regression lines for P_{GC} vs. MAP

in the treatment groups show that at each level of MAP, omapatrilat lowered P_{GC} to a greater extent than enalapril. We suggest that this greater lowering of P_{GC} at equivalent levels of MAP represents one of the mechanisms whereby omapatrilat produces greater renal protective efficacy. The mechanism whereby greater lowering of P_{GC} with omapatrilat was achieved is not revealed by our data since both treatments had equivalent effects on afferent and efferent arteriolar resistances. There was, however, a trend towards a higher R_A in omapatrilat-treated rats that would tend to lower P_{GC}. The precise mechanism responsible for this higher value of R_A with omapatrilat, whether myogenic or neurohumoral, remains to be determined. Two different doses of each drug were studied in order to investigate whether potential differences in the haemodynamic effects may be dose-related. However, no significant differences were observed between the two doses for each drug studied, suggesting that even at the lower dose, near-maximal haemodynamic effects were achieved.

Whereas the additional lowering of P_{GC} achieved with omapatrilat was not associated with discernable additional renal protection in the short-term protocol, these findings suggested that additional renal protection might be achieved with omapatrilat in longer-term studies.

4.4.3. Long-term treatment protocol

As described in Chapter 3 we have observed that when treatment with ACEI or AT_1RA is delayed until after the onset of renal injury in 5/6 nephrectomized rats, an initial decline in proteinuria is followed by a slow but progressive increase. This model of slow progression of CKD during inhibition of the renin-angiotensin system more closely resembles the findings of clinical trials that have reported slowing rather than arrest of CKD progression with ACEI treatment [168, 169, 374, 376]. We therefore believe that this is an appropriate model in which to investigate the possibility that VPI may offer clinically relevant renal protective advantages over ACEI alone. Although both treatments initially lowered proteinuria to the same extent, we found that omapatrilat maintained significantly lower levels of proteinuria over time than enalapril. Moreover, the mean time for rats to reach pretreatment levels of proteinuria was extended by an additional 14 weeks in omapatrilat vs. enalapril treated rats (34 weeks vs. 20 weeks). Although this study was not designed to assess survival, the fact that *ENA* rats were sacrificed at 32 weeks due to rapidly rising proteinuria whereas *OMA* rats survived to 50 weeks, suggests that *OMA* rats would have

survived longer than *ENA* rats. The histological findings also attest to more effective renal protection with omapatrilat. Among rats sacrificed at 20 weeks after 5/6 nephrectomy, both treatment groups exhibited glomerulosclerosis and tubulointerstitial injury scores that tended to be lower than those of untreated controls at 12 weeks, implying that both treatments delayed the progression of renal injury. Injury scores tended to be lower among *OMA* rats vs. *ENA* rats suggesting a possible advantage of omapatrilat over enalapril at this relatively early time point. *ENA* rats sacrificed at 32 weeks had glomerulosclerosis scores very similar to those of untreated controls at 12 weeks, indicating that enalapril delayed the progression of CKD by 20 weeks. Among *OMA* rats sacrificed at 50 weeks, GS was also similar to 12 week *CON* levels, implying a 38 week delay in progression to this level. Thus long-term treatment with omapatrilat resulted in almost double the delay in CKD progression achieved with enalapril. The dose of enalapril used in this study was the same or higher than that used to achieve renal protection in previous studies in this model [465, 516], implying that the observed differences between enalapril and omapatrilat treatment are not attributable to inadequate dosing of enalapril. Finally, it should be noted that these differences were observed between groups of rats in which blood pressures were very closely matched. We are thus able to conclude that the additional benefit observed with omapatrilat was due to unique renal actions of VPI and not to differences in systemic blood pressure control. The micropuncture studies discussed above suggest that one of these differences may be more effective lowering of P_{GC} by omapatrilat. Micropuncture studies performed at later times after 5/6 nephrectomy would further test this hypothesis but are complicated by the heterogeneity of the glomerular lesions typical of this model, making it doubtful that measurements obtained from the few available surface glomeruli would be representative of the remnant kidney as a whole.

Further studies will be required to elucidate the specific roles of different biochemical mediators, particularly the natriuretic peptides and bradykinin, in the enhanced renal protective efficacy observed with omapatrilat. The natriuretic peptides are known to mediate some of the haemodynamic changes after 5/6 nephrectomy [51] but also exert non-haemodynamic effects that may contribute to renal protection [531-535]. Bradykinin is a potent vasodilator that may mediate some of the antihypertensive effects of ACEI [448], but does not appear to contribute significantly to the renal protective effects of ACEI in rats after 5/6 nephrectomy [461, 462] or induction of diabetes [463]. Nevertheless in other models, bradykinin may

mediate effects that are potentially beneficial in renal protection [454, 456, 457, 460]. Moreover, renal bradykinin levels are likely to be higher with VPI than ACEI treatment because bradykinin is catabolized by both ACE and NEP [536] and NEP is the major route of bradykinin catabolism in the kidney [537]. The effects of NEP inhibition on other vasoactive molecules such as endothelin-1 may also be important [538]. It is possible that the additional renal protective efficacy of omapatrilat results from a combination of all of the above and other as yet unidentified effects.

5. Discussion and Conclusions

The experiments described above were designed to answer some of the many questions that remain regarding mechanisms that contribute to progressive renal injury in CKD. The data presented have added important insights, yet many aspects require further elucidation. In this final chapter we place the findings of our studies in context and discuss future potential developments in each aspect covered.

5.1. ACEI versus AT_1RA in renal protection

ACEIs and AT_1RAs both inhibit the RAS but differences in their mechanism of action make it possible that these agents differ in their renal protective efficacy. Early experiments suggested that ACEIs and AT_1RAs afforded similar degrees of renal protection, but the majority may not have been able to detect relatively subtle differences as they employed models in which ACEI treatment had already been shown to afford almost complete protection from renal injury. In the experiments described in Chapters 2 and 3 we examined multiple aspects of renal protection during ACEI or AT_1RA treatment to investigate whether subtle differences may emerge. Since blood pressure is known to be an important determinant of CKD progression, every effort was made to eliminate differences in blood pressure control as a possible explanation for any observed differences between the treatments. In all experiments, drug dose was titrated to achieve close matching of blood pressure control.

We were unable to identify any significant differences between ACEI and AT_1RA treatment with respect to their effect on urinary protein excretion, renal hypertrophy or histological injury. This was true whether treatment was started soon after 5/6 nephrectomy (Chapter 2) or delayed until renal injury was established (Chapter 3). Even when follow up was continued for considerably longer than is the norm in studies employing the 5/6 nephrectomy model, no differences were observed. Minor differences may have emerged if follow up had been continued for even longer, but as there was no trend towards a difference at 24 weeks, this seems unlikely.

In both sets of experiments we examined the effect of RAS inhibition on the expression of proinflammatory and fibrotic molecules. We selected molecules that are widely recognized as important mediators of inflammation or fibrosis. In Chapter 2 we were unable to detect any clear difference in the

effect of ACEI vs. AT_1RA treatment on the expression of any of the genes studied. In Chapter 3 we noted that when RAS inhibitor treatment was started after the onset of renal injury, suppression of proinflammatory cytokines was at best incomplete. Nevertheless, the level of expression of these molecules at different time points was similar with ACEI and AT_1RA treatment.

These experiments provide the most conclusive evidence to date that ACEIs and AT_1RAs are equivalent with respect to their renal protective effects, at least in the 5/6 nephrectomy model. We examined physiological, histological and molecular biological variables in two different study protocols and were unable to identify any significant differences between enalapril and candesartan treatment. Clinical studies have shown that ACEI treatment results in effective renal protection in type 1 diabetics [168] and in patients with non-diabetic CKD [169] whereas AT_1RAs are of proven renal protective efficacy in patients with type 2 diabetes [170, 171, 382]. Large randomized studies that directly compare the renal protective effects of ACEI and AT_1RA treatment in human CKD have not yet been published. This is an important clinical question because up to 20% of patients are intolerant of ACEI treatment due to a cough [539] and would benefit from conversion to an AT_1RA. In the absence of further clinical evidence these experimental studies do provide at least some support for the option of converting patients intolerant of ACEIs to AT_1RA treatment.

The pharmacodynamic differences between ACEIs and AT_1RAs raise the possibility that they may be useful as combination therapy. This is question beyond the scope of the present investigation but preliminary evidence suggests that combination ACEI and AT_1RA therapy may afford more effective renal protection than that achieved by either agent alone [389, 540].

5.2. Proinflammatory and fibrotic cytokines in CKD progression

The experiments in Chapters 2 and 3 examined the expression of a group of CAMs (ICAM-1 and VCAM-1), proinflammatory cytokines (MCP-1, IL-1β and TNF-α) and profibrotic growth factors (TGF-β) in the 5/6 nephrectomy model. Previous investigations into the potential role of these molecules relied largely on in vitro experiments or studied only 1 or 2 factors in vivo. Our goal in studying a group of factors was to show that they are upregulated in concert after 5/6 nephrectomy. In this model immunological stimuli for

the induction of proinflammatory molecules are absent. Our observations therefore provide strong support for the notion that the observed responses of cells in culture to stimuli such as physical stresses, Ang II and excessive protein absorption, play a role in establishing a proinflammatory environment in the remnant kidney. The data also provide evidence that mϕ recruitment in the remnant kidney is an active process mediated by the expression of specific genes. The normalization of proinflammatory gene expression associated with renal protection during ACEI or AT_1RA treatment demonstrates a close association between prevention of gene expression and renal protection but does not prove a causal relationship between the two. In Chapter 3, we showed that failure to completely suppress the upregulation of MCP-1 and TGF-β with RAS inhibiting treatments was associated with slowly progressive renal injury despite effective control of blood pressure. These observations extend the findings in Chapter 2 by showing that failure to suppress proinflammatory gene expression at 12 weeks (when renal injury was only moderate and blood pressure as well as proteinuria were well controlled) was associated with a subsequent progressive rise in proteinuria and more severe renal injury at 24 weeks, despite ongoing ACEI or AT_1RA treatment. Furthermore, we observed strong correlations between the level of MCP-1 or TGF-β expression and the severity of histological injury. Together these data provide further support for the notion that uncontrolled upregulation of proinflammatory molecules and recruitment of macrophages contributes to renal injury in the remnant kidney. Studies reporting renal protective effects in the 5/6 nephrectomy model following treatment with the immunosuppressant mycophenolate [239-242] or the PPAR-γ agonist troglitazone (which has mϕ antiproliferative effects) [247], provide further support for this hypothesis.

We have examined relatively few of the large number of proinflammatory molecules that may be involved in CKD progression. In order to examine a larger number of molecules in a single sample, newer molecular biology techniques such as RNAase protection assays and oligonucleotide micro-arrays will be required. The latter technique in particular allows simultaneous assessment of the expression of several thousand genes and will doubtless lead to a more comprehensive picture of the molecular changes induced by nephron loss. Such studies will also lead to new challenges because it may be difficult to identify which of the many observations are central to renal injury mechanisms.

Our data do not allow us to draw conclusions regarding the relative importance of different proinflammatory molecules in CKD progression. To address this issue, experiments will be required in which specific molecules or genes are selectively inhibited by techniques such as monoclonal antibodies, antisense oligonucleotides and gene deletion. These techniques have already been applied in experiments investigating the role of TGF-β in renal fibrosis [274, 508, 541, 542] and will hopefully be applied to other molecules. Such experiments may identify specific molecular targets and suggest novel therapeutic strategies for attenuating the rate of progression of renal injury. Given the high degree of redundancy identified within the inflammatory response it is likely that extensive research will be required before safe and effective new treatments are developed.

5.3. Hypertension and CKD progression

The data presented in Chapter 3 illustrate the extent to which the level of blood pressure control is a major determinant of renal injury, even in the setting of treatment with RAS inhibitors. These observations have been confirmed and extended by another study that reported strong correlations ($r=0.81$, $P<0.0001$) between achieved blood pressure and glomerulosclerosis among rats assigned to different doses of ACEI or AT1RA following 5/6 nephrectomy [348]. This is important because previous clinical studies that have examined the renal protective effects of ACEI or AT_1RA have employed relatively loose blood pressure targets of <140-135/85-90. If the level of blood pressure is indeed important, even greater renal protection may have been achieved if lower blood pressure targets had been employed. This issue has unfortunately proved difficult to address in clinical studies because of the difficulty in achieving significant differences in blood pressure levels between randomized groups [346, 347, 349]. As a result only one major clinical study has shown clear benefit associated with a lower blood pressure target. In this study of type 1 diabetics receiving ACEI treatment, a lower blood pressure target was associated with lower levels of proteinuria but there was no difference in the rate of decline in GFR over 2 years [349]. These practical difficulties do not diminish the weight of experimental and clinical evidence suggesting a central role for lower blood pressure targets in renal protective strategies. Conclusive proof of benefit will, however, require further prospective randomized studies.

5.4. Proteinuria and CKD progression

The data presented in Chapter 3 do not allow us to draw conclusions regarding the potential role of proteinuria in the pathogenesis of progressive renal injury. Despite compelling in vitro evidence of the proinflammatory effects of exposing tubule cells to plasma proteins, in vivo observations have been restricted largely to associations between proteinuria and the severity of renal injury. Since proteinuria has long been accepted as a marker of renal injury, further in vivo studies are required in order to strengthen the case for a causative role of filtered protein in tubulointerstitial injury. The most useful data would be derived from studies in which proteinuria reduction was isolated from other interventions or in studies where tubular absorption of filtered protein was selectively inhibited. Such strategies have not yet been employed. Currently available interventions for reducing proteinuria (ACEI or AT_1RA treatment) also lower blood pressure and P_{GC}, making it difficult to identify benefit specifically attributable to reduction of proteinuria. Our data do, however, confirm the close association between the amount of proteinuria and the severity of renal injury in this model of CKD progression. Similar observations have been made in clinical studies and one recent meta-analysis found that each 1g/day increase in proteinuria was associated with a relative risk 5.56 for the combined end-point of doubling of baseline serum creatinine or onset of ESRD [339]. These data allow us to conclude that CKD patients with persistent proteinuria despite RAS inhibitor therapy are at increased risk for further deterioration in renal function and require further intervention to optimize their renal protective treatment.

5.5. Glomerular capillary hypertension in CKD progression

Glomerular capillary hypertension was proposed two decades ago as a critical factor in the development of the vicious cycle of nephron loss associated with CKD. This hypothesis was based on experiments in which normalization of P_{GC} achieved by dietary protein restriction [15] or ACEI treatment [26, 27] was associated with dramatic protection of the remnant kidney from subsequent injury. There is to date no technique for assessing glomerular haemodynamics in human subjects and it has therefore not been possible to examine the relevance of these findings in clinical CKD. Nevertheless, in vitro experiments showing the proinflammatory effects of exposing glomerular cells to mechanical stresses such as shear stress, cyclical stretching and barostress, do provide further support for the hypothesis by suggesting one mechanism whereby glomerular capillary hypertension may contribute to renal injury. Our observations in

Chapter 4 provide further support for the notion that increases in P_{GC} ultimately prove detrimental to the glomerulus. We found that despite equal effects of ACEI and VPI treatment on systemic blood pressure, treatment with a VPI was associated with greater lowering of P_{GC}. In subsequent chronic studies, VPI treatment was associated with more effective renal protection than ACEI treatment, suggesting that this greater lowering of P_{GC} may have afforded additional renal protection. In order to substantiate this hypothesis, micropuncture studies at later time points would be required to show that VPI treatment was associated with a lower P_{GC} in the long term. Unfortunately there are generally only 2-4 surface glomeruli available for micropuncture in a Munich Wistar rat making it technically difficult at later time points when significant glomerulosclerosis is known to be present. Since glomerulosclerosis tends to occur in a patchy distribution, it would also be difficult to determine whether or not the measurements obtained were representative of remnant kidney glomeruli as a whole.

5.6. Vasopeptidase inhibitors in renal protection

The vasopeptidase inhibitors are a relatively novel class of agent with potent haemodynamic effects. We have shown in Chapter 4 that treatment with a VPI was associated with more effective long-term renal protection than current "gold standard" treatment with an ACEI, despite equivalent control of blood pressure. We proposed that greater lowering of P_{GC} is one mechanism whereby VPI treatment may confer more effective renal protection. In view of the multiple effects of VPIs resulting from the simultaneous inhibition of two different enzymes that together effect the metabolism of a multiplicity of vasoactive molecules, it must be conceded that there are several other mechanisms that may account for our observations. A recent finding that VPI treatment was associated with less effective renal protection than ACEI treatment in rats with adriamycin nephrosis, a non-hypertensive model of proteinuric renal disease, supports the notion that the potential renal protective benefits of VPIs are related to their haemodynamic effects [543]. Further studies are required to determine whether differences in the effects of VPIs on bradykinin or natriuretic peptide catabolism may also be relevant. Regardless of their mechanisms of action, these initial studies suggest that the VPIs may prove a useful addition to our renal protective armamentarium. Clinical studies of CKD have shown at best a halving of the rate of decline in GFR with RAS inhibitor therapy and there is therefore a place for more effective renal protective interventions. The clinical licensing of some of the VPIs has unfortunately been delayed due to safety

concerns, particularly over the incidence of angio-oedema. It is hoped that these problems will be overcome and that clinical trials of the renal protective effects of VPI therapy will follow.

5.7. Conclusion

We have employed an integrated approach to study several aspects of mechanisms that contribute to progressive renal injury in the 5/6 nephrectomy model of CKD as well as the effects of treatment with ACEIs, AT_1RAs and VPIs. These studies investigated physiological, histological and molecular biological variables in an attempt to provide new insights into mechanisms whereby hypertension, glomerular capillary pressure, proteinuria and the renal expression of proinflammatory and profibrotic molecules may interact to sustain a vicious cycle of nephron loss that results in end-stage renal failure. In Figure 5.1 we present a schema that draws together findings from our studies and those of others to represent a comprehensive model incorporating many of the mechanisms currently thought to be involved. Though our understanding of the pathogenesis of progressive renal injury has advanced considerably over the past two decades, many aspects require further investigation. It is hoped that the development of new molecular biology techniques will allow further elucidation of the mechanisms whereby the observed responses to nephron loss occur. This would make possible the development of novel therapeutic interventions aimed at arresting CKD progression in the majority of patients. It may also become possible to address the even more interesting possibility of reversing renal injury in CKD. While the outcome of such research is awaited, current clinical emphasis should be on applying strategies already proven to afford renal protection to all patients and thereby diminish the burden imposed by CKD on individuals and health-care systems.

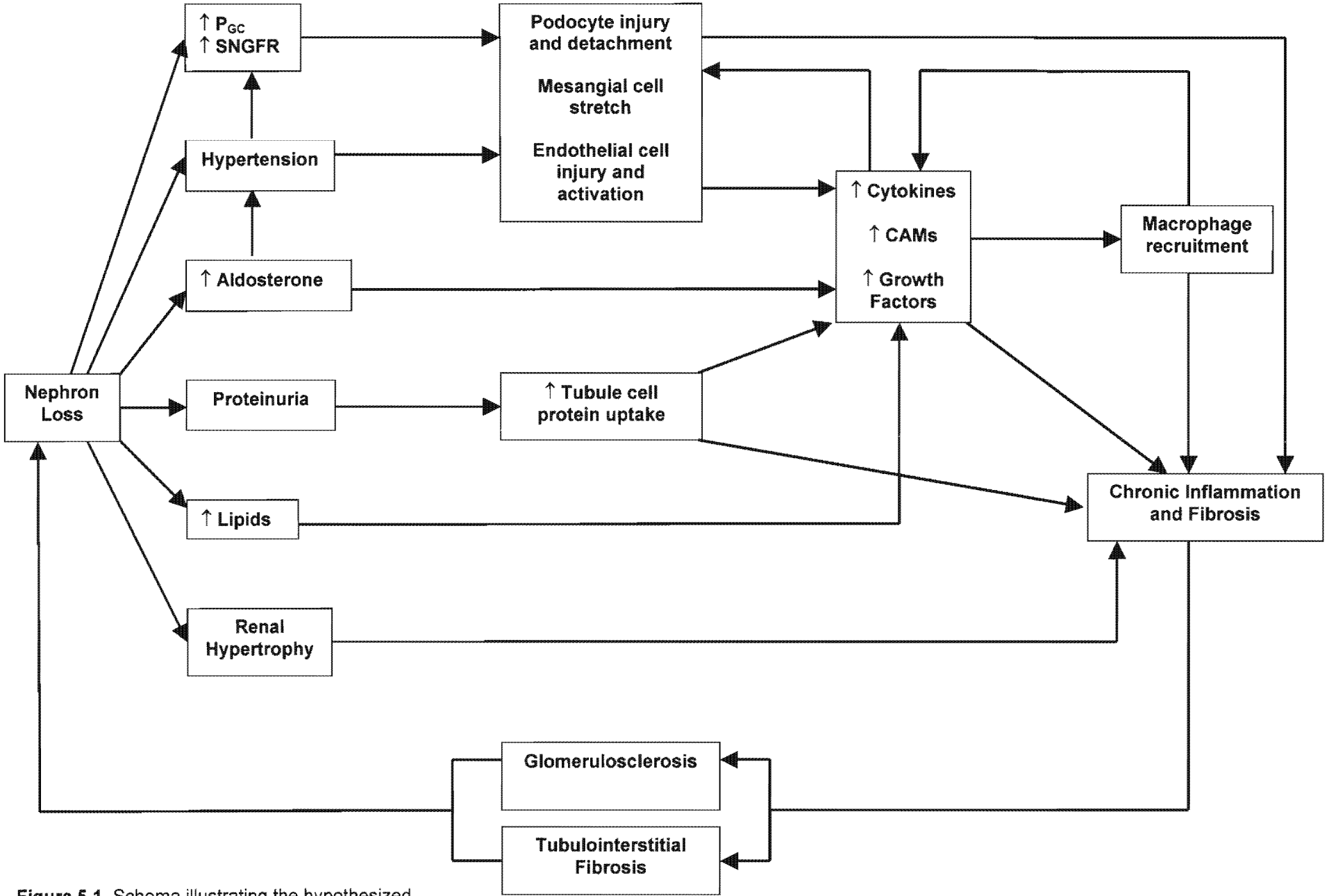


Figure 5.1. Schema illustrating the hypothesized interaction of multiple haemodynamic and non-haemodynamic factors in the pathogenesis of progressive nephron injury in chronic kidney disease

(P_{GC} – glomerular capillary hydraulic pressure; SNGFR – single nephron GFR; CAMs – cell adhesion molecules)

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