Role of endothelin in progressive proteinuric nephropathies

Citation for published version (APA):

Benigni, A. (2001). *Role of endothelin in progressive proteinuric nephropathies*. [Doctoral Thesis, Maastricht University]. Universiteit Maastricht. <https://doi.org/10.26481/dis.20011212ab>

Document status and date:

Published: 01/01/2001

DOI:

[10.26481/dis.20011212ab](https://doi.org/10.26481/dis.20011212ab)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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- The final published version features the final layout of the paper including the volume, issue and page numbers.

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**ROLE OF ENDOTHELIN
IN PROGRESSIVE
PROTEINURIC NEPHROPATHIES**

Ariela Benigni

ROLE OF ENDOTHELIN IN PROGRESSIVE PROTEINURIC NEPHROPATHIES

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op woensdag 12 december 2001 om 14.00 uur

door

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geboren op 16 december 1955 te Bergamo, Italie

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CONTENTS

Chapter 1: Introduction and objectives of the thesis	9
Chapter 2: Time course and localization of endothelin-1 gene expression in a model of renal disease progression <i>American Journal of Pathology</i> 1997; 151,5:1241-1247	19
Chapter 3: Blocking both type A and B endothelin receptors in the kidney attenuates renal injury and prolongs survival in rats with remnant kidney <i>American Journal of Kidney Diseases</i> 1996, 37,3:416-423	27
Chapter 4: Unselective inhibition of endothelin receptors reduces renal dysfunction in experimental diabetes <i>Diabetes</i> 1998, 47:450-456	37
Chapter 5: Renoprotective effect of contemporary blocking of angiotensin II and endothelin-1 in rats with membranous nephropathy <i>Kidney International</i> 1998, 54:353-359	45
Chapter 6: Nature and mediators of renal lesions in kidney transplant patients given cyclosporine for more than one year <i>Kidney International</i> 1999, 55:674-685	53
Chapter 7: General Discussion and Conclusions	67
Chapter 8: Summary	81
Curriculum Vitae	85
Acknowledgement	86

CHAPTER 1

Introduction

In part published in the Nephrology Forum entitled:

Endothelin antagonists in renal disease

Principal discussant: Ariela Benigni

Kidney International 2000, 57:1778-1794

Introduction

Progression to end-stage renal failure is the final common pathway of several chronic nephropathies independently from their etiology. Brenner et al. (1) first offered the most consistent unifying hypothesis on the progressive nature of renal disease suggesting that the surviving nephrons initially spared by the disease activate common haemodynamic responses which, while serving to maintain glomerular filtration, cause structural injury in a self-perpetuating cycle (1). More recently, studies have established that an excessive and sustained protein traffic through the nephron, as a consequence of high glomerular capillary pressure, is invariably associated with the progression of the disease and exerts intrinsic renal toxicity per se (2). The proteins filtered in excessive amount accumulate in the lumen of the proximal tubule and are actively reabsorbed by the tubular epithelium causing an intracellular congestion that, on the long term, promotes tubulointerstitial injury and renal scarring. Excessive protein reabsorption would induce functional alterations of tubular cells that overexpress inflammatory and vasoactive molecules mainly secreted towards the interstitium where they trigger an interstitial inflammatory reaction, fibroblast proliferation and extracellular matrix deposition, key features of renal scarring. Among such vasoactive and inflammatory molecules endothelin-1 (ET-1) certainly represents one of the major mediators of renal damage.

The scope of the present thesis is to discuss evidence from experimental and human studies in support of such a view.

Endothelin: discovery and synthetic pathways

In the last 20 years it became more and more clear that the endothelium participates directly to the regulation of vascular tone through the release of a wide variety of vasoactive substances. After the discovery of prostacyclin in 1976 (3), evidence was provided in 1980 that the endothelium regulates vascular smooth muscle tone by elaborating potent endothelial-dependent vasodilators (4). Another important observation derives from Rubanyi and Vanhoutte's report in 1985 of the hypoxia-induced release of a diffusible vasoconstrictor substance from endothelial cells independent of prostaglandin synthesis (5). But it was not before 1988 that Yanagisawa and coworkers isolated from porcine aortic endothelial cells an extremely potent vasoconstrictor subsequently identified as endothelin-1 (ET-1) (6). Endothelin -1 belongs to a family of peptides comprising two other isoforms: endothelin-2 and endothelin-3. Each isoform contains 21-

amino acids, two intrachain disulfide bonds constraining the overall structure, and a conserved C-terminal sequence necessary for biological activity (7). Disulfide bridges are also present in the sequence of sarafotoxins (found in the venom of an Israel burrowing asp), suggesting that ETs and sarafotoxins share a long genealogical history. Three distinct genes encoding a specific precursor for the three mature isoforms are located in different chromosomes. The 5' gene-flanking region of the human ET-1 precursor contains consensus motifs for the binding sites of FOS/JUN complex, activating protein 1 and nuclear factor 1, the latter two possibly mediating angiotensin II and transforming growth factor β (TGF β) induction of ET-1. Sequences for the acute phase reactant regulatory elements probably involved in ET-1 induction under acute physical stress *in vivo* are also expressed. In the 3' region there is a sequence that regulates selective destabilization of the ET-1 precursor mRNA, possibly accounting for its short half-life (7). These sites potentially regulate ET-1 synthesis at transcriptional level. The product of the ET-1 gene transcription is preproendothelin-1 consisting of 212 amino acid residues, that is cleaved at two sites by a neutral endopeptidase forming the biologically inactive precursor big endothelin, ultimately converted to mature peptide by a phosphoramidon-sensitive membrane bound metalloprotease called endothelin converting enzyme (ECE). A recent study has suggested the existence of ET-4, a 31 aminoacid vasoconstrictor peptide highly homologous to ET-1 which is generated by a chimase. Three different ECE-1 isoforms differing in their N-terminal regions derive from a single gene through the use of alternative promoters (8). Immunofluorescence microscopy showed distinct subcellular localizations, ECE-1a and ECE-1c being localized at the cell surface, whereas ECE-1b intracellularly possibly in the trans-Golgi network (8). Transfer experiments of ECE-1 cDNA to chinese hamster ovary cells that normally secrete only bigET-1 revealed that extracellular form of ECE-1 cleaves the bigET-1 supplied from outside the cell while intracellular form of ECE-1 converts endogenously produced ET-1 (9). This possibly explains why ET-1 is generally thought to be a paracrine and autocrine rather than a circulating hormone. Two distinct secretory pathways in the transport and release of ET-1 have been proposed on the basis of immunoelectron microscopical localization of the peptide in human umbilical vein endothelial cells (10) as well as in human coronary artery (11). Endothelin-1 is continuously transported in and released from secretory vesicles by the constitutive pathway through a cAMP-independent mechanism. Endothelin-1 is also stored in Weibel-Palade bodies with other vasoactive compounds and is released at the cell surface after an appropriate stimulus. Endothelin-1 is synthesized not only in the endothelial and smooth muscle cells of blood vessels but also in the kidney, heart, brain, lung, pancreas and spleen (7). Secretion of the peptide by endothelial and epithelial cells is predominantly ab-

luminal (12,13). Angiotensin II, TGF β , interleukin-1, adrenalin, insulin and physicochemical factors such as hypoxia stimulate ET-1 synthesis, while nitric oxide, natriuretic peptides, vasodilator prostanoids and increased shear stress inhibit ET-1 generation (14).

The endothelins' receptor family

Because endothelin is hydrophilic and therefore unable to cross the plasma membrane, it must bind to specific cell surface receptors whose expression actually governs cell response to the peptide. Distinct endothelin isoforms with diverse functions predicted the existence of distinct receptors, two of which have indeed been identified as seven transmembrane domain G protein-coupled receptors (15,16). ET_A has higher affinity for ET-1 and ET-2 and less for ET-3, while ET_B binds the three isopeptides with nearly identical affinity. ET_A receptors reside in vascular smooth muscle cells and mediate vasoconstriction and cell proliferation, whereas ET_B receptors on the endothelial cell do mediate vasodilation via nitric oxide. A variety of additional functions have been attributed to ET_B receptors, however and in certain instances, they can even elicit vessel contraction (14). ET_B receptor has an important role in renal sodium handling as recently documented in ET_B deficient mice, that develop salt-sensitive hypertension due to the lack of inhibition of the collecting duct epithelial sodium channel normally sustained by the ET_B receptor. ET-1 clearance from the circulation appears also linked to ET_B receptors (14). Moreover ET_B receptor serves to initiate a positive autocrine loop by which ET-1 regulates its own gene expression by enhancing preproET-1 transcription and mRNA stability (14). Coupling of activated receptors to diverse effector systems generates second messengers (17) which include inositol phosphates, diacylglycerols and calcium ultimately responsible for the biological effects.

Endothelin-producing tissues all express specific binding sites, suggesting a local rather than circulating hormone-type regulation. Consistently plasma concentration of endothelins is in the picomolar order, far below the pharmacological threshold. Specific binding sites for endothelin have been identified in numerous fetal and adult organs including lung, heart, brain and kidney. Rat cardiac atrium contains ET_A receptors as a major component, but also ET_B receptors have been reported. As for brain, the ET_B receptor appears the predominant subtype. Relative abundance of ET_A and ET_B receptors in the kidney depends on the species (14). ET_A/ET_B ratio approaches one in rats but in humans ET_B largely prevails (14) in renal tubuli and appears related to many physiologic functions including sodium excretion.

Systemic and renal effects of exogenous endothelin

Bolus injections of nanomolar amounts of ET-1, ET-2, and ET-3 initially lower blood pressure due to a transient induction of prostacyclin and nitric oxide released from endothelial cells upon endothelial binding to specific receptors (17). The dose-dependent remarkable increase in systemic blood pressure which follows is prolonged and mostly dependent on renal, mesenteric, and muscular vessel constriction also sustained by an increased cardiac output (direct, since it is not inhibited by adrenergic receptor blockers or indomethacin). Pulmonary circulation instead, far less sensitive to the pressor effect of endothelins (18), plays a marginal role in endothelin-induced increase in systemic blood pressure. Endothelin of central nervous system origin may also enhance arterial pressure (19). Systemic but not renal pressor effect of ET-1 in rats was abolished by a selective ET_A receptor antagonist (20). Both systemic and renal vasoconstrictions were instead prevented by a nonselective ET_A and ET_B receptor antagonist, which would suggest a role for ET_B receptor in the renal response to ET-1 (14). In humans, ET-1 infusion causes profound renal vasoconstriction (21) in healthy volunteers, whose systemic blood pressure was negligibly affected (22). Available data in humans taken together are solid enough for a unique susceptibility of renal vessels to ET-1 vasoconstriction. Endothelin by its major interaction with volume regulatory hormones takes part in volume homeostasis, by enhancing plasma renin activity and stimulating aldosterone secretion (23). Data that ET-1 augments atrial natriuretic peptide and inhibits peripheral nerve norepinephrine release highlight the complex interplay of ET-1 with volume regulatory hormones. To add to the complexity ET-1 affects sodium and water balance, so that its renal effects add to the ones of angiotensin II, aldosterone, vasopressin, and ANP in regulating volume homeostasis (23).

Besides its vasoactive and hormonal properties ET-1 increases c-fos and -myc mRNA levels and proliferation of cultured vascular smooth muscle cells and stimulates mitogenesis in human umbilical vein endothelial cells (23). ET-1 is also a strong mitogen for mesangial cells (23) and fibroblasts, and by favoring cell proliferation also promotes matrix protein gene transcription. ET-1 is also chemotactic for blood monocytes (24), a property which in vivo could contribute to the tubulointerstitial damage that accompanies most renal diseases characterized by a progressive course.

The role of endothelin-1 in progressive renal injury

A number of in vitro and in vivo studies consistently suggested a role of ET-1 as

mediator of progressive renal injury. We documented that exposure of a rabbit proximal tubular cell line to increasing concentrations of albumin induced a significant dose-dependent increase in the generation of ET-1 (13). Since delipidated albumin had the same effect on tubular ET-1 synthesis, the lipid component of the protein appears unlikely to be involved. The stimulatory effect on tubular ET-1 synthesis and release was not specific to albumin but was shared by IgG and transferrin. In vivo tubular epithelial cells are organized as a continuous polarized cell layer that serves to separate apical and basolateral compartment with different specific activities. When proximal tubular cells were grown on filters in bicameral systems became organized in a polarized cell monolayer: they had microvilli on the apical membrane and adjacent cells exhibited marked lateral intercellular digitations and well developed tight junctions in the basolateral domain. Apical exposure of polarized tubular cells to high albumin concentration resulted in an increased ET-1 secretion in the basolateral compartment (13). Should this occur in vivo an excessive amount of ET-1 may accumulate in the interstitial compartment with a number of implications due to the vasoactive and inflammatory properties of the molecule.

Endothelin gene expression and the renal synthesis of ET-1 are up-regulated in animal models of disease progression that follows the loss of a critical number of nephrons due to immune or nonimmune damage to the kidney (25). The remnant kidney model in rats is characterized by a time-dependent increase in renal ET-1 gene expression paralleled by an increase in urinary excretion of the peptide, probably reflecting its renal synthesis (25). Increased renal synthesis of ET-1 was also found in rats with passive Heymann nephritis (PHN), an immune model of glomerular disease resembling human membranous nephropathy (26). Other studies have found an up-regulation of renal ET-1 and ET_B receptor gene expression in NZB/WF1 mice that have an immunological disease reminiscent of human lupus (27). Glomerular ET-1 and ET_B receptor mRNAs were higher than normal within few days after puromycin aminonucleoside injection and normalized when rats were no longer nephrotics (28). Also in experimental diabetes mRNA for ET-1 is over-expressed in the kidney in the face of unchanged ET_A and ET_B receptors (29). The most elegant and direct evidence that ET-1 plays a role in progressive renal damage derives from studies using transgenic animals. Mice overexpressing the human ET-1 promoter generate more ET-1 in their kidney and develop renal lesions despite no increase in systemic blood pressure (30). Furthermore rats transgenic for the human ET-2 gene are normotensive and develop renal lesions reminiscent of those seen in rats with remnant kidney (31). Several lines of evidence using ET receptor antagonists definitely support the possible role of ET-1 in renal diseases and provide important indications for

future clinical use of these compounds to slow or even halt disease progression. Thus, in the rat remnant kidney model, a specific antagonist for the ET_A receptor (32), ameliorated renal function and protected against glomerular and tubulointerstitial structural injury. Interestingly, endothelin receptor antagonists, that invariably reduce renal damage, did not consistently normalize proteinuria, confirming the possibility that excessive ET-1 does not cause but is rather the consequence of increased glomerular protein traffic (2). The renoprotective effect of ET_A receptor antagonists was also evident in mice with experimental lupus nephritis (33). The same molecule was effective in preventing the development of renal lesions and attenuating the increase in serum creatinine in rats with streptozotocin-induced experimental diabetes treated at the moment of induction of the disease (34).

Evidence for a potential role of ET-1 is also available for patients with chronic progressive nephropathies, but data are based solely on plasma and urine immunoreactive ET-1 measurements. Plasma concentration of ET-1 in patients with chronic renal failure are 1-2 fold greater than normal. Increased circulating ET-1 concentration in these subjects may reflect impairment of clearance. However, the fact that urinary excretion of ET-1 is also increased in patients with chronic nephropathies (35) suggest that renal generation of ET-1 in these diseases is increased. Moreover, administration of an ET_A/ET_B receptor antagonist to patients with chronic renal failure reduced blood pressure and renal vascular resistance (36). A very recent study has shown that in patients with chronic glomerulopathy whose urinary protein excretion was higher than 2 g, had an increased expression of ET-1 and ET_B receptor in their kidney as previously observed in rats with progressive renal diseases (37).

Objectives of the thesis

Based on these observations a number of studies were designed and performed with the aim of providing more information as for the role of ET-1 in progressive renal injury in experimental animals by means of molecules that specifically antagonize ET-1 receptors. The identification of multiple mechanisms of injury in progressive nephropathies offered the rationale of combining drugs that simultaneously interrupt different pathways to slow progression of renal disease. Finally, since pioneer studies in the animals have consistently indicated a role for ET-1 in Cyclosporin A (CsA)-induced vasoconstriction and tubulointerstitial damage, the possible contribution of ET-1 in the development of interstitial fibrosis in humans has been investigated.

In the present thesis the following objectives have been addressed:

A number of studies convincingly indicated that proteins filtered through the glomerular capillary display an intrinsic renal toxicity and serve as an early trigger of interstitial inflammation through the tubular synthesis of vasoactive and inflammatory substances, including ET-1, mainly basolaterally. Secreted in excessive amounts in the interstitium, ET-1 could promote recruitment of monocyte/macrophages, stimulate fibroblast proliferation and production of matrix proteins. Excessive formation of ET-1 in the kidney may favor progression of renal lesions to the extent that pharmacological manipulation of the ET-1 pathway has a major positive impact on disease progression. However, which cells in the kidney form excessive ET-1 was not clearly defined. This topic is addressed in **chapter 2** in the remnant kidney, a model that was extensively used to study the progression of renal disease and the role of ET-1 in this inexorable process.

In 1993, our group was the first to demonstrate a renoprotective effect of an ET_A receptor antagonist in a model of progressive proteinuric nephropathy. Selective blocking of the ET_A receptor by a specific antagonist (FR 139317) normalized blood pressure, partially reduced the abnormal permeability to proteins, and limited glomerular injury in rats with remnant kidney. The same beneficial effect of FR139317 on progressive renal disease has been reported in lupus nephritis, confirming that at least part of the deleterious effects of ET-1 in the kidney are exerted through binding to the ET_A receptors in various models of renal disease progression. Beside ET_A, however, ET_B receptors are widely represented in the kidney and data indicate that they mediate a variety of functions. Relevant to the role of ET-1 in renal disease progression were data showing that in chronic nephropathies, including the model of renal ablation in the rat, kidney ET_B receptor gene expression was specifically upregulated, at least early after surgery, while time course data were not available. Thus, we first looked at the time course of ET_A and ET_B receptor gene expression in the kidney of rats with severe ablation of renal mass. We then established whether an unselective receptor antagonist was more renoprotective against the progression of renal disease than selective ET_A blockers and improved survival in animals with renal ablation (**chapter 3**).

Evidence in experimental diabetes of increased renal synthesis of ET-1 became available in the early 90ties and studies with ET receptor antagonists, specifically the one of Nakamura (33) employing an ET_A receptor antagonist, showed prevention of the development of renal disease and amelioration of renal function in diabetic animals when treated at the time of disease induction. However, no data were available on whether an ET receptor antagonist were capable of controlling renal disease progression if given therapeutically to animals that al-

ready have clinical signs of diabetic nephropathy. This lack of information provided the rationale for studying the potential renoprotective properties of an unselective ET receptor antagonist, PD 142,893, chronically administered to streptozotocin diabetic animals starting when animals had developed overt proteinuria. The effect of antagonizing ET receptors was formally compared with that of an ACE inhibitor, the best means available so far to control protein excretion and renal disease progression in experimental and human diabetes. This is described in **chapter 4**.

In experimental and human proteinuric nephropathies drugs that limit the synthesis or the biological activity of angiotensin II (AII) retard the progression of renal damage due to their unique capacity of reducing glomerular capillary hypertension, a feature not necessarily shared by conventional drugs. The beneficial effect of ACE inhibitors is also related to the preservation of glomerular membrane size-selective and hydraulic permeability functions. Thus, blocking AII reduces glomerular protein traffic better than other antihypertensives, limiting the intrinsic renal toxicity of proteins on proximal tubular cells. Although ACE inhibitors and AII receptor antagonists are very powerful agents, they are not completely effective in normalizing urinary proteins nor preventing GFR decline and renal damage in any animal models and, even more importantly, in patients with progressive nephropathies when referred to the hospital at an advanced stage of the disease. This could be due to an only partial blocking of locally formed AII by these drugs or to an exaggerated renal synthesis of other mediators including ET-1 that are not necessarily controlled by ACE inhibition. Findings that the protective effect of an ACE inhibitor in the accelerated passive Heymann nephritis, an immune model of progressive renal injury which mimicks the human membranous nephropathy, was only partial, was instrumental to subsequent studies planned to verify whether combination of treatments were more effective than ACE inhibition alone. The rationale for using an ET-1 receptor antagonist to retard renal disease progression in this model rest on data that kidneys from PHN rats synthesize more ET-1 than normal kidneys. Excessive ET-1 mRNA and protein were localized in proximal tubules and preceded inflammatory cells infiltrating the renal interstitium and interstitial fibrosis. Accelerated passive Heymann nephritis was used to study the effect of combining an ACE inhibitor with an ET receptor antagonist on severe progressive renal injury (**chapter 5**).

Cyclosporine (CsA) has improved patient and organ graft survival rates to unprecedented levels. However, nephrotoxicity of CsA manifests with a wide spectrum of effects ranging from marked and rapidly reversible renal hypoperfusion,

which follows each dosing, to a chronic form of renal injury occurring after one or more years of treatment and with a progressive course in certain instances. Although acute renal hypoperfusion induced by CsA is no longer a problem, chronic CsA nephrotoxicity remains a major challenge for transplant physicians. Lesions of chronic CsA nephropathy consist of vascular and tubulointerstitial changes, including degenerative hyaline alterations extending from preglomerular areas proximally up the afferent arteriole, with progressive narrowing and eventual occlusion of the lumen. The precise cause of CsA renal toxicity is not known. It is possible that glomerular afferent arteriole vasoconstriction eventually promotes structural alterations and occlusion of preglomerular vessels determining an ischemic damage of downstream glomeruli and interstitium that culminates in glomerular obsolescence and interstitial fibrosis. Attention has been focussed in the past on enhanced vasoconstrictors (including thromboxane A₂, ET-1 and Ang II) or reduced nitric oxide production induced by CsA, but no evidence emerged that any of these mediators actually mediated structural damage. It appeared more likely that the tubulointerstitial fibrosis associated with CsA treatment was a clearly separate entity that develops independently of the vasoactive effect of the immunosuppressor. We therefore designed a study aimed at evaluating the relationship between afferent arteriolar arteriopathy and tubulointerstitial fibrosis induced by CsA, and to get more insight on the factor(s) mediating tubulointerstitial injury and possible mechanisms associated with this lesions. This will be analyzed in **chapter 6**.

CHAPTER 2

Time course and localization of endothelin-1 gene expression in a model of renal disease progression

I. Bruzzi, D. Corna, C. Zoja, S. Orisio, E.L. Schiffrin,
D. Cavallotti, G. Remuzzi and A. Benigni published in
American Journal of Pathology 1997; 151, 5 1241-1247

Time Course and Localization of Endothelin-1 Gene Expression in a Model of Renal Disease Progression

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Experimental and human proteinuric glomerulopathies are associated with tubulo-interstitial injury that correlates with the decline of renal function even better than glomerular lesions do. Mechanism(s) leading to tubulo-interstitial damage are unknown. It has been proposed that excessive reabsorption of filtered proteins activates renal cells to produce vasoactive and inflammatory molecules including endothelin-1. The aim of the present study was twofold: we first evaluated the cellular origin of excessive renal endothelin-1 production in the renal mass reduction model and then related endothelin-1 distribution to the development of kidney lesions. Four groups of renal mass reduction ($n = 15$) and four groups of control rats ($n = 5$) were studied at 7, 14, 21, and 28 days after surgery. Urinary proteins in renal mass reduction rats were comparable with controls at day 7 but became significantly higher thereafter. Renal mass reduction rats first developed tubulo-interstitial changes, which were already evident at day 14 in the majority of them. At 28 days, renal mass reduction rats also developed glomerulosclerosis. A parallel increase of renal endothelin-1 gene expression and synthesis of the corresponding peptide in renal mass reduction rats *versus* controls was observed from day 14. Nonradioactive *in situ* hybridization confirmed a pattern of endothelin-1 mRNA consistent with the distribution of lesions. At day 14, endothelin-1 staining was stronger in renal mass reduction than in control kidneys and mainly localized to the cytoplasm of tubular cells, whereas glomeruli were negative. At day 28, endothelin-1 expression further increased in renal mass reduction rats as compared with controls, and the staining was apparent also in glomeruli. Thus, in renal mass reduction, a progressive up-regulation of endothelin-1 occurs during the development of renal injury, that first involves the

tubules and, only in a subsequent phase, the glomeruli. (*Am J Pathol* 1997, 151:1241-1247)

Data are available that experimental and human proteinuric glomerulopathies are accompanied by tubulo-interstitial lesions. Thus, in both immunological and nonimmunological models of glomerular injury, sustained proteinuria is consistently followed by tubulo-interstitial damage.¹ Evidence is also available in which drugs that reduce urinary protein excretion or dietary manipulations also prevent tubulo-interstitial inflammatory reaction.²⁻⁴ The most convincing experimental evidence that proteins leaking into the urinary space trigger tubulo-interstitial inflammation has been provided by models of overload proteinuria.^{5,6} Also in humans, proteinuric glomerulopathies are associated with tubulo-interstitial injury, for example, focal and segmental glomerulosclerosis, IgA nephropathy, membranous and mesangiocapillary glomerulopathy, lupus, and diabetic nephropathy.⁷⁻¹¹ As observed in animals, by reducing urinary proteins, angiotensin-converting enzyme inhibitors limit the extent of tubulo-interstitial changes and slow the rate of progression of renal failure in Heymann nephritis, puromycin aminonucleoside nephrosis, and aging-associated nephritis.¹²⁻¹⁴ However, the precise mechanism(s) that link glomerular protein ultrafiltration with tubulo-interstitial lesions has not been fully clarified.

Recent studies have documented that sustained protein trafficking is toxic to the kidney by a complex interplay of processes triggered by reabsorption of filtered proteins by cells of proximal tubules. As a consequence of protein stress, cells are activated to synthesize vasoactive and proinflammatory mediators.¹⁵ Thus, proximal tubular cells, exposed to protein overload, are stimulated to synthesize and release endothelin-1 (ET-1), a powerful vasoconstrictor peptide, toward the basolateral compartment.¹⁶ Secreted in excessive amounts in the interstitium, ET-1 could promote recruitment of monocytes/macrophages,¹⁷ stimulate fibroblast proliferation, and stimulate production of extracellular matrix proteins.¹⁸ Evidence is now available that an excessive formation of ET-1 in the kidney may favor progression of renal lesions to the ex-

Accepted for publication August 6, 1997.

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A portion of this paper has been presented in form of abstract at the 29th annual meeting of The American Society of Nephrology, November 3-6, 1996, New Orleans, Louisiana.

tent that pharmacological manipulation of the ET-1 pathway has a major positive impact on disease progression.^{19,20} However, which cells in the kidney form ET-1 in excessive amounts in the above setting is not yet clear.

The aims of the present study were to assess, by nonisotopic *in situ* hybridization, the cellular origin of excessive renal ET-1 production in renal mass reduction (RMR), a model of progressive renal injury, and to relate ET-1 distribution pattern to the development of kidney lesions.

Materials and Methods

Eighty male Sprague-Dawley rats (225 to 250 g; Charles River Italia S.p.A., Calco, Italy) were used in this study. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, O.J.L. 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publ. No. 85-23, 1985). All animals had free access to standard rat diet, containing 20% protein by weight, and tap water.

Experimental Design

Renal mass was reduced by removal of right kidney and ligation of two or three branches of the left renal artery according to Olson et al.²¹ Four groups of rats were studied at 7 ($n = 15$), 14 ($n = 15$), 21 ($n = 15$), and 28 ($n = 15$) days. Four groups ($n = 5$) of age-matched sham-operated rats were used for controls. At the end of the experimental periods, 24-hour urines were collected in metabolic cages for determination of protein and ET-1 excretion and blood withdrawn from the tail vein for serum creatinine determination. At sacrifice, kidneys were removed for histological analysis, RNA extraction, and *in situ* hybridization experiments.

Preparation of Rat Kidney mRNA and Northern Analysis

Total RNA from RMR and sham-operated rat kidneys was extracted, loaded for Northern analysis, and hybridized for ET-1, as previously described.²² Membranes were subsequently probed with a glyceraldehyde-3-phosphate dehydrogenase cDNA and taken as internal standard for equal loading of the samples on the membrane.

Preparation of Digoxigenin-Labeled Rat ET-1 Riboprobes

Rat ET-1 antisense and sense probes were prepared and labeled by *in vitro* transcription using digoxigenin-labeled uridine triphosphate. A 319 bp rat ET-1 was subcloned into the *HindIII/EcoRI* sites of the pGEM7Z(+) vector between Sp6 and T7 promoters. The plasmid was linearized with the appropriate restriction enzymes (*EcoRI* or *HindIII*) and purified by phenol/chloroform extraction and

ethanol precipitation. Transcription of 1 μ g linearized plasmid was carried out using a DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Before use, digoxigenin-labeled riboprobes were checked for size, integrity, and labeling efficiency by comparison with 10-fold serial dilution of a digoxigenin-labeled control riboprobe (Boehringer Mannheim) and direct detection with an antidigoxigenin antibody. Riboprobe concentrations were adjusted to be equivalent on the basis of the labeling efficiency before use in the *in situ* hybridization studies.

Nonisotopic *In Situ* Hybridization

Renal tissue fixed in Dubosq-Brazil solution and embedded in paraffin were cut at 4 μ m, sequentially heat fixed (65°C, 30 minutes), and deparaffinized. Sections were then incubated with 5 mmol/L levamisole for 30 minutes at room temperature, briefly washed in phosphate-buffered saline and water, and immersed in 0.2 N HCl. After permeabilization with proteinase K (40 μ g/ml, Sigma, St. Louis, MO), slides were postfixed in 1.5% paraformaldehyde/1.5% glutaraldehyde for 1 minute, dehydrated through a graded ethanol series, and air dried. Sections were then overlaid with a hybridization mixture consisting of 0.5 ng/ μ l of digoxigenin-labeled RNA probe, 2 $\times$ SSC, 10% dextran sulfate, 1 $\times$ Denhardt's solution, and 0.1 mol/L sodium phosphate, covered with sealed coverslips, and incubated overnight in a moist chamber at 42°C. After hybridization, slides were washed in 0.2 $\times$ SSC, treated with a blocking solution (50 mg/ml skimmed dried milk, 150 mmol/L NaCl in 100 mmol/L Tris HCl, pH 7.8) at room temperature for 15 minutes, and finally incubated for 30 minutes at 37°C with anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) diluted 1:750. Color development was performed with freshly prepared substrate solution (nitro blue tetrazolium salt and X-phosphate-5-bromo-4-chloro-3-indolyl phosphate 0.4 mol/L in 0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 50 mmol/L MgCl₂, pH 9.5). Slides were then mounted in 60% glycerol and examined by light microscopy. Negative controls included hybridization step using the sense probe and omission of either the antisense RNA probe or the antidigoxigenin antibody.

Histology

Kidney fragments fixed in Dubosq-Brazil solution and embedded in paraffin were stained with Masson's trichrome, hematoxylin and eosin, and periodic acid-Schiff reagent. Glomerular damage was expressed as percentage in respect to 100 glomeruli examined. Tubular (atrophy, casts, and dilation) and interstitial changes (fibrosis and inflammation) were graded from 0 (no changes) to 1+ (changes affecting less than 25% of the sample). All renal biopsies were blindly analyzed by the same pathologist.

Table 1. Analytical and Histological Findings in Sham and RMR Rats

Time (days)	Proteinuria (mg/day)		Serum creatinine (mg/dl)		Tubulo-interstitial damage score (range)		Focal glomerulosclerosis % (range)	
	Sham	RMR	Sham	RMR	Sham	RMR	Sham	RMR
7	18.1 ± 1.8	24.1 ± 1.9	0.61 ± 0.02	1.43 ± 0.12*	0	0	0	0
14	23.8 ± 2.5	112.4 ± 13.9*	0.71 ± 0.04	1.45 ± 0.09*	0	0.75 (0 to 1)	0	0
21	20.1 ± 1.0	111.6 ± 15.4*	0.66 ± 0.01	1.40 ± 0.12*	0	0.25 (0 to 1)	0	0
28	23.9 ± 2.4	307.5 ± 58.3* [†]	0.65 ± 0.02	1.50 ± 0.03*	0	1	0	5 (0 to 14)

* $P < 0.01$ versus sham at corresponding time.

[†] $P < 0.01$ versus RMR at previous times.

Analysis

Serum creatinine was measured with the alkaline picrate method²³ and urinary protein concentration determined by the Coomassie Blue G dye-binding assay with bovine serum albumin as standard.²⁴

Urinary ET-1 was evaluated by radioimmunoassay after extraction with SepPak C18 (Waters, Millford, Massachusetts), as previously described.²²

Statistical Analysis

All results are expressed as means ± SE. Data were analyzed by analysis of variance and subsequent multiple comparisons were performed using SAS program.²⁵ Excretion of urinary proteins and ET-1 were log-transformed before statistical analysis. Linear regression analysis was used to correlate urinary ET-1 excretion with the degree of proteinuria and Spearman's correlation test to correlate urinary ET-1 excretion with tubulo-interstitial damage. Statistical significance was defined as $P < 0.05$.

Results

Seven days after surgery, urinary protein excretion in RMR rats was comparable with control rats. Proteinuria started to be significantly higher ($P < 0.01$) than in sham-operated animals from day 14 after surgery, remained stable at day 21, and increased further on day 28 (Table 1). At day 7, RMR rats developed renal insufficiency as documented by a significant ($P < 0.01$) increase in serum creatinine that remained elevated for all time points considered (Table 1). RMR rats killed 7 days after surgery as well as all control rats had no renal lesions (Table 1). Starting from day 14, RMR rats showed renal abnormalities consisting in trivial interstitial fibrosis, tubular dilation, and casts in the distal tubules that affected the majority (65%) of animals. At this time, none of glomeruli were affected by sclerotic lesions. A similar degree of renal damage was observed 21 days after surgery, although rats presenting pathological features were less (Table 1). At day 28, RMR kidneys showed a more severe histopathological pattern in that tubulo-interstitial damage was present in all animals, and 38% of them developed focal and segmental glomerulosclerosis affecting on average $5.0 \pm 2.6\%$ of glomeruli.

ET-1 mRNA Expression and Synthesis in Renal Tissue from Control and RMR Rats

As shown in Figure 1A, at 7 days, a slight increase over sham rats was observed in renal RMR ET-1 gene expression, which was quantified by densitometric analysis as 43%. This was not accompanied to any change in ET-1 urinary excretion, which was comparable in RMR and sham rats (32.8 ± 2.12 versus 34.7 ± 2.93 pg/day, Figure 1B). Fourteen and 21 days after surgery ET-1 transcript was further increased, reaching values of 78 and 70% over controls, respectively. Consistently, ET-1 urinary excretion was significantly higher ($P < 0.01$) than in control rats, with a trend to be increased more evidently at day 14 than 21 (day 14, 76.2 ± 7.0 versus 32.8 ± 2.6 and day 21, 65.4 ± 7.9 versus 33.7 ± 1.7 pg/day). In RMR studied at 28 days, ET-1 gene expression further increased to levels that were 140% over sham. A parallel significant increment was observed in urinary ET-1 (149.7 ± 20.8 versus 37.1 ± 2.4 pg/day) whose values were significantly ($P < 0.01$) higher than RMR rats at days 7, 14, and 21. A positive, significant correlation between urinary

Figure 1. A: Densitometric analysis of autoradiographic signals showing ET-1 mRNA expression in kidneys of RMR rats at different times after surgery. The optical density of the autoradiographic signals was quantitated and calculated as the ratio of ET-1 to glyceraldehyde-3-phosphate dehydrogenase mRNA. The mRNA levels of 7, 14, 21, and 28 days were calculated by assuming the optical density of controls of each corresponding time as one. Total RNA was extracted from pooled remnant kidneys of each experimental group. Data are means of two Northern blot experiments. B: Time course of urinary ET-1 excretion in RMR ($n = 15$) and sham-operated rats ($n = 5$), whose range value is depicted by the gray bar. Data are means ± SE. * $P < 0.01$ versus sham at corresponding time; O $P < 0.01$ versus sham at corresponding time and versus RMR at previous times.

ET-1 excretion and proteinuria ($r = 0.82$; $P = 0.0001$) as well as between urinary ET-1 excretion and tubulo-interstitial damage ($\rho = 0.82$; $P = 0.0001$) was found.

Localization of ET-1 mRNA in Control and RMR Kidney

In order to localize renal ET-1 expression, we performed *in situ* hybridization experiments using an antisense rat riboprobe. Seven days after surgery, sham-operated rats showed, in all the experiments, a weak or absent ET-1 mRNA signal throughout the renal cortex (Figure 2A). By contrast, an intense staining for ET-1 mRNA was seen in the outer and inner medulla (Figure 2B), in agreement with previous findings.²⁶ Very prominent staining was also observed in the endothelium of some renal arteries and arterioles. Hybridization signal and distribution was similar in sham-operated animals at all time points examined. Seven days after surgery, RMR kidneys showed an ET-1 mRNA expression comparable with control rats (Figure 2C). In contrast, in RMR kidneys at 14 days, ET-1 mRNA expression was detectable in the cortex of those animals having tubulo-interstitial damage. High intensity signal for ET-1 mRNA was selectively observed in proximal tubular epithelial cells of those tubules located in areas of interstitial fibrosis (Figure 2D), and the number of positive tubules was related to the extent of injury. At this time, no ET-1 mRNA signal was found in glomeruli. No additional change in ET-1 mRNA pattern was observed in RMR kidneys at 21 days (not shown). At 28 days, hybridization signal dramatically increased as compared with the previous time point, involving cytoplasm of proximal tubular cells as well as glomeruli located in the vicinity of damaged areas (Figure 2, E and F). ET-1 mRNA was expressed markedly in parietal epithelial cells and in some visceral epithelial cells (Figure 2G, right arrows). In addition, ET-1 mRNA staining was present in mesangial areas, and some of the stained cells clearly showed shape and location of glomerular endothelial cells (Figure 2G, left arrows). ET-1 mRNA staining was also strong in the endothelium of afferent and efferent arterioles (not shown). Specificity of the *in situ* hybridization was confirmed by probing renal tissues from RMR (Figure 2H) or control with the sense ET-1 cRNA probe in which no hybridization signal was observed.

Discussion

The experimental model of renal mass reduction is characterized by initial adaptive increases in the function of remnant nephrons, hypertension, proteinuria, and tubulo-interstitial and glomerular lesions.

Evidence is now available in animals and humans that in chronic renal diseases tubulo-interstitial damage is functionally important and correlates with declining glomerular filtration rate even better than glomerular lesions do.¹⁵ Mechanism(s) leading to tubulo-interstitial damage are not fully clear but appeared largely due to obliteration of peritubular capillaries ensuing from sclerosed glomer-

uli. Here we found focal tubulo-interstitial lesions in most animals by day 14 after surgery, well before glomerulosclerosis was apparent, possibly indicating that factors not necessarily dependent from glomerular structural lesions can alternatively damage the tubulo-interstitial compartment. One of these is possibly linked to excessive reabsorption of filtered proteins abnormally delivered to proximal tubular loops in diseases of enhanced glomerular protein traffic. Data are accumulating that proximal tubular cell protein overload activates cells to produce vasoactive and inflammatory molecules that are potentially toxic to the kidney.¹⁵ In this context, we have recently shown that rabbit proximal tubular cells exposed to albumin, IgG, and transferrin, at concentrations comparable with those in proximal tubular fluid of proteinuric animals, are induced to synthesize and release ET-1 and that, at least *in vitro*, the polarity of peptide secretion is mostly toward the basolateral compartment.¹⁶ If this occurred *in vivo*, it is not surprising that interstitial events were possibly driven by proximal tubular cell protein congestion. Important, additional evidence of this rests on present findings that concomitant to the onset of proteinuria cortical tubules appeared activated to express ET-1 gene. Tubular ET-1 overexpression was invariably found in the vicinity of interstitial fibrosis, consistent with the possibility that the former contributed at least in part to interstitial injury. With time, RMR rats developed more severe proteinuria, which was invariably accompanied by an additional significant increase in tubular ET-1 together with increasingly severe tubulo-interstitial damage so that a strong linear correlation could be established between these variables.

How ET-1 may exert its inflammatory and vasoactive effect on the interstitium remains, at the moment, open to speculation. It is already known that tubular cells possess ET-1 receptors mainly of the B subtype localized at the basolateral side of cellular membrane.²⁷ Although their function has not been defined so far, it is possible that ET-1, by interacting with its specific receptors, triggers a positive feedback that would stimulate its own synthesis and perpetuate tissue damage. Autoinduction of ET-1 via ET_B receptor has been described in human endothelial cells,²⁸ rat mesangial cells,²⁹ and, more recently, human proximal tubular cells.³⁰ A possible autocrine role of the enhanced ET-1 expression has also been suggested by data that gene expression of the ET_B receptor progressively increases with time in the remnant kidney.³¹ A further amplification loop rests on preliminary data that fibronectin, collagen IV, and laminin added to culture media of tubular cells in turn stimulate ET-1 production.³² Moreover, ET-1 by binding to specific receptors on monocytes/macrophages could induce proinflammatory cytokines.³³ Finally, excessive secretion of tubular ET-1 towards the interstitium may induce constriction of peritubular capillaries and ischemia. It has been demonstrated recently that outer medullary-descending vasa recta that derive from juxtamedullary efferent arterioles are particularly sensitive to the vasoconstrictor effects of endothelins.³⁴

Of great interest, we additionally found that RMR rats studied 14 and 21 days after surgery had a consistent

Figure 2. *In situ* hybridization staining of ET-1 expression in normal and RMR kidneys at different times. No staining was observed in the cortex of controls throughout the experimental period (A). Magnification, $\times 360$. By contrast, an intense staining was observed in outer and inner medulla (B). Magnification, $\times 90$. In RMR kidney at 7 days, ET-1 mRNA expression was comparable with controls (C). Magnification, $\times 180$. Fourteen days after surgery, a strong tubular ET-1 expression was selectively detected in areas of interstitial fibrosis (D). Magnification, $\times 90$. No additional changes in ET-1 mRNA expression pattern were observed at 21 days (not shown). A dramatic increase in the number of cortical tubules expressing ET-1 was seen at 28 days (E and F, magnification, $\times 90$ and $\times 360$) together with *de novo* ET-1 expression in some glomeruli particularly in parietal epithelial cells, in some visceral epithelial cells (right arrows), in mesangial areas, and in endothelial cells (left arrows). (G, magnification, $\times 360$) Negative control of G using the sense ET-1 cRNA probe is shown in H. Magnification, $\times 90$.

up-regulation of tubular ET-1 gene, as compared with sham-operated controls, which consistently preceded any glomerular staining for ET-1.

Despite that glomerular cells in culture produce ET-1,³⁵⁻³⁷ we were unable to detect glomerular ET-1 mRNA *in vivo*, neither during the all-study period in sham-oper-

ated rats or before day 28 in the experimental group. These data are in harmony with previous reports in normal rat³⁶ and human kidney²⁶ on ET-1 mRNA distribution that was confined to tubular cells of the medulla with glomeruli being essentially negative. In RMR rats studied by day 28, ET-1 mRNA staining was found in glomerular cells, ie, some 10 to 12 days after ET-1 gene up-regulation could be found in tubular cells. Mechanism(s) leading to enhanced expression of glomerular ET-1 gene, at later stages after surgery in this model, are far from clear. Soon after surgical ablation of renal mass platelet-derived growth factor and transforming growth factor- β , both potent stimulators of ET-1 synthesis,^{36,39} are formed in excessive amounts in remnant glomeruli.^{40,41} Moreover, in the remnant kidney model there is a time-dependent down-regulation of glomerular nitric oxide,⁴² a potent inhibitor of endothelial ET-1 synthesis.⁴³ Interestingly, chronic administration of a nitric oxide donor, molsidomine, and dietary L-arginine supplementation to RMR rats were renoprotective and lowered renal ET-1 synthesis.^{44,45} That increasing nitric oxide supply to the kidney is renoprotective is also supported by recent studies in remnant kidney and in unilateral ureteral obstruction models using angiotensin-converting enzyme inhibitors or L-arginine.^{46,47}

Finally, whatever the mechanism implicated, peritubular capillary ischemia, which is always associated with interstitial injury, may *per se* act as an additional stimulus to enhance local ET-1 generation.³⁰ It has been documented that in the setting of ischemia, vascular endothelium releases vasoactive mediators including ET-1,⁴³ which serves to paracrinally regulate its own secretion by glomerular epithelial and mesangial cells.

In conclusion, the present data demonstrate that in the remnant kidney model tubulo-interstitial lesions develop before glomerular sclerosis and up-regulation of ET-1 gene in the kidney parallels the sequential development of renal lesions being expressed first by tubules and only in a subsequent phase by glomeruli. It is possible that ET-1, induced by tubular protein overloading, triggers interstitial damage that contributes to subsequent glomerular sclerosis and decline in renal function.

Acknowledgments

We thank Dr. Tullio Bertani for renal morphology analysis and Dr. Annalisa Perna for statistical analysis. We are deeply indebted to Dr. Chiara Foglieni for invaluable help in microscopic investigations.

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CHAPTER 3

Blocking both type A and B endothelin receptors in the kidney attenuates renal injury and prolongs survival in rats with remnant kidney

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published in

American Journal of Kidney Diseases 1996, 37,3:416-423

Blocking Both Type A and B Endothelin Receptors in the Kidney Attenuates Renal Injury and Prolongs Survival in Rats With Remnant Kidney

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● Renal disease progression in the rat is associated with a time-dependent upregulation of renal endothelin-1 (ET-1) gene expression and synthesis. We have previously demonstrated that endothelin A receptor subtype (ET_A) blockade in rats with remnant kidney reduced signs of disease activity, suggesting that ET-1 exerts part of its deleterious effects on the kidney through ET_A. No data are available so far on the role of ET_B receptor in progressive renal injury. We first studied renal ET_A and ET_B receptor gene expression in rats with remnant kidney on days 7, 30, and 120 after the surgical procedure. While renal expression of ET_A was unaffected, ET_B receptor gene was significantly upregulated with time in rats with remnant kidney, being 3.5-fold and sixfold higher than sham-operated rats at days 30 and 120. We also evaluated whether bosentan, a nonpeptidic ET_A and ET_B receptor antagonist, offered better protection against renal disease progression than reported for ET_A-selective blockers and whether it improved survival in animals with renal ablation. Two groups of rats with renal mass reduction (n = 11 each) were given bosentan 100 mg/kg/d orally or its vehicle (carboxymethyl cellulose) beginning day 7 after the surgical procedure and were followed until the death of the vehicle-treated animals. Sham-operated animals comprised the control group. Bosentan partially prevented increases in blood pressure and proteinuria, but had a remarkable protective effect on renal function and significantly prolonged animal survival. These data suggest that blocking both renal ET_A and ET_B receptors might have major implications in the treatment of human progressive nephropathies.

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INDEX WORDS: Endothelin; endothelin antagonist; bosentan; progressive renal injury; renal ablation.

SOON AFTER the discovery of the potent vasoconstrictor endothelin-1 (ET-1), it was clear that renal circulation were uniquely sensitive to the vasoactive action of ET-1,¹ much more than coronary and bronchial vascular districts. Subsequent studies documented that ET-1 stimulated rat mesangial cells to proliferate² and accumulate an abnormal amount of mesangial matrix proteins.³ The effects of ET-1 in the kidney are mediated by specific binding of the peptide to cell surface receptors, which exist in two forms.^{4,5} One, the ET_A receptor, mediates mainly vasoconstriction and smooth muscle proliferation,^{4,6} while the other, ET_B receptor, in most systems is coupled to vasodilatory effects⁷ but also may mediate vasoconstriction.⁸⁻¹⁰ Studies on the localization of ET

receptors in the rat nephron have indicated that ET_B receptors are mainly expressed in cortical collecting ducts and glomeruli, whereas ET_A receptors are present in the vessels.^{11,12}

In previous studies we and others have found that renal disease progression in the rat was associated with a time-dependent upregulation of the kidney ET-1 gene,¹³ which resulted in an excessive synthesis of the corresponding protein that could be measured in the urine.^{14,15} These studies support the possibility that at least in some models ET-1, due to its vasoactive and inflammatory properties,¹⁶ mediated at least in part glomerular and tubulointerstitial injury, which is currently associated with the progression of renal disease in experimental animals and humans.^{17,18} More recent findings in transgenic mice provide strong support for the above possibility. Researchers have identified mice transgenic for human ET-1 promoter.¹⁹ These animals have increased ET-1 levels in the kidney, which is associated with an enhanced rate of glomerulosclerosis. As additional evidence that endothelins participate in the progression of renal damage, transgenic rats that expressed enhanced levels of human ET-2 gene were phenotypically characterized by interstitial fibrosis in the renal cortex and renal blood vessel hypertrophy.²⁰

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Received June 15, 1995; accepted in revised form October 24, 1995.

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0272-6386/96/2703-0016\$3.00/0

Despite the above associations, the definitive evidence for a role of endothelins in causing renal disease to progress could only imply the use of specific endothelin antagonists. We have recently reported²¹ that selective blocking of the ET_A receptor by a specific antagonist (FR 139317) normalized blood pressure, partially reduced the abnormal permeability to proteins, and limited glomerular injury in rats with remnant kidney. The same beneficial effect of FR 139317 on progressive renal disease has been recently confirmed in experimental lupus nephritis.²² In this study, ET_A receptor blocker also reduced extracellular matrix accumulation.²² The above studies showed that at least part of the deleterious effects of ET-1 in the kidney are exerted through binding to ET_A receptors. Beside ET_A, however, ET_B receptors are widely represented in the kidney and data are available which indicate that they may possibly mediate vasoconstriction.⁹ Relevant to the role of ET in renal disease progression are data showing that in chronic nephropathies, including the model of renal mass ablation in the rat, kidney ET_B receptor gene expression is specifically upregulated, at least early after surgery,²³ while time course data are not available.

With the present study we first aimed at evaluating the time course of ET_A and ET_B receptor gene expression in the kidney of rats undergoing reduction of renal mass. Moreover, we looked for the possibility that an ET_A and ET_B receptor antagonist, bosentan, (1) offered better protection against the progression of renal disease than so far reported for selective ET_A blockers and (2) improved survival compared with animals with renal ablation given vehicle.

MATERIALS AND METHODS

Animals

Sixty-three male Sprague-Dawley rats (225 to 250 g; Charles River Italia S.p.A., Calco, Italy) were used in this study. Animal care and treatment were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory animals, NIH Publication No. 85-83, 1985). All animals had free access to standard rat diet containing 20% protein by weight and tap water.

Experimental Design

Renal mass reduction (RMR) was obtained by right nephrectomy and ligation of two or three branches of the left

renal artery (RMR) under ether anesthesia according to Olson et al.²⁴

The first aim of the study was addressed by experiment no. 1. Eighteen rats with RMR were divided into three groups and killed on days 7 (group 1, *n* = 6), 30 (group 2, *n* = 6), and 120 (group 3, *n* = 6). An additional 18 rats underwent sham operation and were divided into groups 4, 5, and 6 (*n* = 6 for each group) and killed at the same time periods as the RMR rats. Kidneys were excised and renal tissue was processed for RNA extraction.

The second aim of the study was pursued in experiment no. 2. Two groups of rats with RMR were used. Group 7 (*n* = 11) was given a daily oral dose of bosentan (100 mg/kg) (RO 47-0203; Hoffman-La Roche Ltd, Basel, Switzerland), a mixed ET_A/ET_B receptor antagonist, by gavage beginning day 7 after RMR. Group 8 (*n* = 11) was given bosentan vehicle (0.5% carboxymethyl cellulose) by gavage beginning day 7 after RMR. An additional group of sham-operated rats (group 9, *n* = 5) was studied at the same time and used as controls. The animals were followed until the death of the vehicle-treated animals.

Bosentan is an orally active, nonpeptidic, mixed ET receptor antagonist obtained by structural optimization of the less potent RO 46-2005 and capable of inhibiting responses due to *in vivo* activation of ET_A and ET_B receptors (those mediating either vasodilation or vasoconstriction).²⁵ The dose of bosentan was chosen on the basis of previous experiments showing a long-lasting inhibition of the pressor effect of Big-ET-1 after a single oral administration of 100 mg/kg bosentan.²⁵ Bosentan was resuspended in 0.5% carboxymethyl cellulose and prepared fresh every day.

In all rats systolic blood pressure, proteinuria, and serum creatinine were assessed at time 0 and 7, 15, 30, 60, 90, and 120 days after the beginning of the experiment.

Type A and B Endothelin Receptor Cloning and Northern Blot Analysis

Oligonucleotides spanning the entire coding regions for rat ET_A and ET_B receptors were synthesized based on the published sequences^{1,26} and carrying Sal I linkers in their 5' ends (ET_A receptor: sense oligo 5'-tacagaatt-cgtcgacaagatgggtgctcttgcctt; antisense oligo 5'-tacagaatt-cgtcgactcggtgcttctgcacagggt. ET_B receptor: sense oligo 5'-gacatcgctgcacaacatgcacatgcgtccgcaagccg; antisense oligo: 5'-gacatcgctgcacgacaatgagatcagtgag). Ten micrograms of rat kidney RNA was reverse-transcribed with oligo d(T) as previously described¹³; the resulting cDNA was then amplified with oligonucleotides for rat ET_A and ET_B receptors, respectively. The amplified products of the expected size were SalI digested and ligated into pTZ19 R vector (Pharmacia, Milano, Italy). The identity of the cloned cDNAs to rat ET_A and ET_B receptors was verified by restriction digestion and partial sequencing.

Messenger RNA was isolated from rat kidneys by isothiocyanate/cesium chloride ultracentrifugation and subsequent oligo(dT)-cellulose column chromatography (Clontech, Palo Alto, CA). Ten micrograms of rat kidney mRNA was fractionated on 1.2% denaturing agarose gel, transferred to nylon membranes (Gene Screen Plus; New England Nuclear, Boston, MA), and hybridized with ³²P-labeled cDNA for rat en-

dothelin receptors. Following optimal exposure, membranes were probed for the housekeeping gene glyceraldehyde phosphate dehydrogenase.²⁷ Differential expression in RMR, compared with sham-operated animals, was calculated using densitometric analysis as previously described.¹³

Systolic Blood Pressure

Systolic blood pressure was recorded by tail plethysmography in the conscious rats.²⁸

Urinary Protein Excretion

Proteinuria was determined in 24-hour urine collections (collected in metabolic cages) by the modified Coomassie blue G dye-binding assay for proteins with bovine serum albumin as standard.²⁹

Serum Creatinine

Blood was collected from the tail vein of anesthetized animals. Serum was obtained after whole blood clotting and kept frozen at -20°C until assayed. Creatinine was measured by the alkaline picrate method.³⁰

Statistical Analysis

Data are presented as mean values $\pm$ SE. Data were analyzed by one-way ANOVA and Tukey test for multiple comparisons.³¹ Differences were considered significant at $P < 0.05$.

RESULTS

Renal Type A and B Endothelin Receptor Gene Expression

Gene expression of ET receptors in the kidneys of rats undergoing renal mass ablation was differentially modulated during the course of the disease. Type A and B endothelin receptor tran-

scripts of 4 and 5 kb, respectively, were expressed in both sham-operated and RMR rats. Densitometric analysis of the autoradiographic signals showed no difference in renal ET_A receptor gene expression with time in rats with remnant kidney and in sham-operated animals (data not shown). In contrast, ET_B receptor transcript levels were comparable in rats with renal mass ablation and in sham-operated animals only at 7 days (Fig 1). The transcriptional rate of ET_B receptor gene progressively increased at 30 and 120 days after renal ablation, the mRNA levels being 3.5-fold and sixfold higher than in the corresponding sham rats, respectively.

Effect of Bosentan on Systolic Blood Pressure

Serial values of systolic blood pressure are reported in Fig 2. In rats with RMR, blood pressure progressively increased with time after surgery, with values being statistically significant from those in the sham-operated rats starting on day 15 (182 ± 12 mm Hg ν 135 ± 3 mm Hg; $P < 0.01$). Bosentan lowered systolic blood pressure, the effect being statistically significant starting at day 30 and persisting until day 90 (30 days: 156 ± 6 mm Hg ν 185 ± 8 mm Hg, $P < 0.01$; 60 days: 168 ± 5 mm Hg ν 197 ± 13 mm Hg, $P < 0.05$; 90 days: 173 ± 6 mm Hg ν 207 ± 12 mm Hg, $P < 0.05$). The lowering of blood pressure by ET_A/ET_B receptor antagonist was no longer significant at day 120 (RMR + bosentan 175 ± 7 mm Hg ν RMR 202 ± 11 mm Hg).

Fig 1. Time course of ET_B receptor gene expression in kidney homogenate from sham-operated and RMR rats. Messenger RNA (10 μg) obtained from the kidneys of six sham-operated and six RMR rats for each time point was blotted onto synthetic membranes, which were hybridized with ET_B receptor and the housekeeping (glyceraldehyde phosphate dehydrogenase [GAPDH]) cDNAs.

Fig 2. Systolic blood pressure in rats with RMR given bosentan (100 mg/kg/d orally; $n = 11$) (solid circles) or its vehicle ($n = 11$) (open circles) and in sham-operated rats ($n = 5$) (open triangles). Data are presented as mean values $\pm$ SE. § $P < 0.01$ versus sham-operated rats at corresponding time; * $P < 0.01$, ** $P < 0.05$ versus RMR + vehicle at corresponding time.

Effect of Bosentan on Urinary Protein Excretion

The time course of 24-hour urinary protein excretion is presented in Fig 3. Proteinuria values were similar in all animals at the beginning of the experiment and 7 days after the surgical procedure. Proteinuria progressively increased with time in RMR animals, and values recorded at 30 days were significantly higher than in the sham-operated animals (161 ± 36 mg/d ν 30 ± 2 mg/d; $P < 0.01$). The maximum value of urinary protein excretion was reached at the end of the

experiment (474 ± 84 mg/d). Bosentan effectively limited the urinary protein excretion with time (Fig 3), with values significantly lower than in the untreated animals on days 15 ($P < 0.01$), 30 ($P < 0.05$), and 60 ($P < 0.05$). On days 90 and 120, proteinuria values of the bosentan group were numerically lower than those of the untreated group, but the differences were not significant (90 days 226 ± 36 mg/d ν 375 ± 56 mg/d; 120 days 355 ± 41 mg/d ν 474 ± 84 mg/d).

Effect of Bosentan on Serum Creatinine

Renal function was impaired 7 days after surgery (1.8 ± 0.1 mg/dL) and remained impaired (15 days, 1.5 ± 0.1 mg/dL; 30 days, 1.7 ± 0.2 mg/dL; 60 days, 1.7 ± 0.2 mg/dL; 90 days, 1.7 ± 0.1 mg/dL; 120 days, 2.4 ± 0.7 mg/dL; $P < 0.01$). Bosentan prevented renal function from declining over time, as documented by a significant reduction in serum creatinine, the levels of which remained stable throughout the study (15 days, 1.3 ± 0.1 mg/dL; 30 days, 1.3 ± 0.1 mg/dL; 60 days, 1.2 ± 0.1 mg/dL; 90 days, 1.3 ± 0.1 mg/dL [$P < 0.05$]; and 120 days, 1.3 ± 0.1 mg/dL [$P < 0.01$]).

Effect of Bosentan on Animal Survival

Figure 4 presents the survival curves. All sham-operated rats were alive at the end of the study. Bosentan prolonged survival in rats with remnant kidney. Animals receiving bosentan were alive at 130 days, whereas all RMR animals

Fig 3. Time course of urinary protein excretion in rats with RMR given bosentan (100 mg/kg/d orally; $n = 11$) or its vehicle ($n = 11$) and in sham-operated rats ($n = 5$). Data are presented as mean values $\pm$ SE. § $P < 0.01$ versus sham-operated rats at corresponding time; * $P < 0.01$, ** $P < 0.05$ versus RMR + vehicle at corresponding time.

Fig 4. Survival curve of sham-operated and RMR rats given vehicle or bosentan. All sham-operated and bosentan-treated animals were alive at the end of the observation period. All vehicle-treated animals died within 130 days.

given bosentan vehicle were dead at this time. Table 1 shows the effect of bosentan on blood pressure, survival, and other parameters of renal damage compared with the previously most-used treatments in the renal ablation model.

DISCUSSION

We found that in the rat, reduction of renal mass did not influence renal expression of ET_A receptor mRNA evaluated at three time intervals during the observation period (120 days). However, such a maneuver significantly upregulated the ET_B receptor gene. In fact, at the end of the experiment rats with RMR had sixfold higher ET_B receptors than the sham-operated animals. Our findings are in good agreement with most recent data obtained in the studies of various chronic renal diseases. Thus, cyclosporine-treated animals had little changes in ET_A and ET_B receptors in isolated glomeruli, but remarkably and selectively increased medullary ET_B mRNA.³² Renal ischemia also promoted a long-lasting upregulation of ET_B receptor mRNA, both in glomeruli and inner medullary collecting ducts, whereas ET_A receptor was not modified.³³ Type B endothelin receptor expression was selectively induced in glomeruli from rats with mesangioproliferative glomerulonephritis³⁴ as well as puromycin aminonucleoside-induced nephrosis, and gene expression was effectively modulated by steroid treatment.³⁵ Thus far, a convincing explanation of why renal disease progression,

quite independently from the initial insult, leads the kidney to upregulate ET_B receptors is lacking. Unlike ET_A , which mediates vasoconstriction and cell proliferation and is mainly expressed in arterioles and glomeruli, ET_B receptors appear to be associated with vasodilation by virtue of their property of inducing nitric oxide and prostacyclin synthesis. Studies have shown that ET_B receptors in the kidney reside in the glomeruli and medulla.¹¹ It is interesting that ET_B receptors coupled to vasodilation are not the only type of ET_B receptors that a rat kidney can express. In fact, some evidence, albeit indirect, tends to support the existence of an additional ET_B receptor subtype related to vasoconstriction.^{9,36} Whether upregulation of ET_B receptor associated with progressive nephropathies is a counterregulatory mechanism to overcome the renal vasoconstriction or actually concurs to maintain it is a matter of current investigation. The issue is even more puzzling in chronic renal insufficiency, a condition consistently associated with less nitric oxide formed by the kidney.³⁷ Thus, mechanisms that may operate in normals of ET_B receptor-linked upregulation of nitric oxide synthase machinery may not actually lead to enhanced nitric oxide formation in rats with RMR.

As a follow-up of previous data indicating that chronic blockade of ET_A receptors reduced renal injury in progressive renal disease, we addressed the *in vivo* relevance of the upregulation of renal ET_B receptor gene in rats with remnant kidney. We took advantage of a mixed antagonist of ET_A and ET_B receptors that consistently blocks ET_B -mediated vasodilatory and vasoconstrictory responses.²⁵ The results demonstrated a partial effect on blood pressure and urinary proteins consistent with those of *ex vivo* studies in isolated perfused kidney.³⁸

The key finding of the present study, however, was the remarkable protective effect of bosentan on renal function and mortality. Results documented that unselective inhibition of both endothelin receptors is renoprotective in experimental animals with an effect rather convincing even for a hard end-point such as mortality. Reducing endothelin binding to its receptors in the kidney may reduce local vasoconstriction, an effect much more relevant for the kidney relative to other organs given the unique sensitivity of renal vessels to the vasoconstrictory effect of ET-1. In

Table 1. Comparison of the Effects of Bosentan and the Most-Used Treatment Modalities on Systolic Blood Pressure and Renal Damage in Rats With Renal Ablation

Treatment	SBP	Proteinuria	Renal Function	Glomerular Damage	Tubulointerstitial Damage	Survival
Thromboxane synthase inhibitor (Purkerson et al, 1985 ⁴⁴)	↓	↓	↑	↓	NA	NA
ACE inhibitor (Brunner et al, 1989 ⁴⁵)	↓	↓	↔	↓	NA	↑
Nondihydropyridinic Ca ²⁺ antagonist (Brunner et al, 1989 ⁴⁵)	↔	↑	↓	↑	NA	↓
Selective ET _A receptor antagonist (Benigni et al, 1993 ²¹)	↓	↓	↑	↓	↓	NA
ET _A /ET _B receptor antagonist (present study)	↓	↓	↑	NA	NA	↑

Abbreviations: SBP, systolic blood pressure; ACE, angiotensin-converting enzyme; NA, not available.

another model of disease progression, endothelin receptor blockade suppressed growth factors,³⁹ a mechanism that may explain the effect of endothelin antagonists of reducing exaggerated expression of the extracellular matrix gene,²² which is common to all progressive nephropathies.^{40,41}

How do the present data compare with the data obtained previously with selective ET_A receptor antagonists? The data obtained with ET receptor blockade, as we have done here by administering the compound daily for 5 months, are notably scarce. In the remnant kidney model, FR 139317 did show renoprotective properties by reducing urinary protein excretion and ameliorating signs of renal injury, but a follow-up period sufficient for determining definitive conclusions concerning its effect on mortality was not available. In the progressive renal disease of mice with lupus nephritis a partial but not significant prolongation of animal survival was observed with the use of the ET_A receptor blocker.²²

In summary, renal disease progression in the remnant kidney model was associated with progressive upregulation of ET_B receptors, the significance of which remains speculative. The administration of an endothelin antagonist that binds to both ET_A and ET_B receptors reduced signs of renal damage and remarkably improved animal survival. The renoprotective properties of this class of compounds have to be tested in humans. Despite a high degree of sequence homology in ET receptor subtypes between experimen-

tal animals and humans, differences exist both in receptor distribution as well as in ligand affinity.^{42,43} Specifically, the possibility that renal expression of ET receptors could be different in humans and rats has to be addressed in future studies. In addition, further investigation of appropriate drug doses and time of administration before animal data can be reliably transferred to humans is warranted.

ACKNOWLEDGMENT

The authors are indebted to Dr Martine Clozel (Hoffman-La Roche, Basel, Switzerland) for providing the bosentan (RO 47-0203). They thank Drs Loredana Bonecchi and Lorenza Longaretti for helping with the molecular biology experiments and Raffaello Maffi for excellent technical assistance.

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CHAPTER 4

Unselective inhibition of endothelin receptors reduces renal dysfunction in experimental diabetes

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published in

Diabetes 1998, 47:450-456

Unselective Inhibition of Endothelin Receptors Reduces Renal Dysfunction in Experimental Diabetes

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Chronic nephropathies are associated with enhanced renal synthesis of endothelin (ET)-1. A recent study demonstrated that an ET_A receptor antagonist given to diabetic rats at the moment of disease induction prevented the development of renal injury. Here we investigated whether an unselective ET_A/ET_B receptor antagonist, PD 142,893, was renoprotective when given to streptozotocin diabetic rats when animals were already proteinuric. The effect of PD 142,893 was compared with that of an ACE inhibitor, lisinopril, known to retard progressive renal disease in experimental and human diabetes. PD 142,893 normalized systemic blood pressure, reduced urinary protein and albumin excretion, and ameliorated renal blood flow in diabetic rats, but it did not affect such parameters in control rats. Lisinopril had a renoprotective effect comparable to PD 142,893, although lisinopril controlled systemic blood pressure better. Northern blot analysis of ET-1 mRNA revealed upregulation of ET-1 gene in the diabetic kidney. Similar results were obtained by *in situ* hybridization in glomeruli and tubuli of diabetic rats. Both treatments remarkably attenuated exaggerated renal ET-1 gene expression. These data suggest that ET-1 is a contributory mediator of kidney damage in diabetes and indicate that ET receptor antagonists may represent a new therapeutic mean for treatment of progressive diabetic nephropathy. *Diabetes* 47:450–456, 1998

A number of recent studies documented that renal synthesis of endothelin (ET)-1 is increased in chronic nephropathies either in experimental animals or in humans (1–4) and that mice and rats transgenic for ET-1 or ET-2 are phenotypically characterized by renal lesions (5,6). The vasoactive and inflammatory potential of ET-1 inspired studies to explore its potential role as a mediator of renal damage. Thus, chronic administration of ET receptor antagonists selectively binding either to ET_A or to both ET_A and ET_B receptors protects animals

from developing renal injury (7,8). In a model of immune nephritis induced by repeated injections of ovalbumin, selective blockade of ET_A and ET_B receptors effectively reduced proteinuria and protected animals from renal function impairment (9). Consistent with findings in nondiabetic models of renal disease progression, Nakamura et al. (10) have recently found that a selective antagonist for ET_A receptor given to diabetic rats at the moment of the induction of the disease effectively prevented the development of renal disease and attenuated the increase in serum creatinine. However, no data are available so far on whether an ET receptor antagonist is capable of controlling renal disease progression if given therapeutically to animals that already have clinical signs of diabetic nephropathy. Here we studied the potential renoprotective properties of an unselective ET receptor antagonist, PD 142,893, chronically administered to streptozotocin diabetic animals starting from 6 months after diabetes induction, when animals had developed overt proteinuria. The effect of antagonizing ET receptors was formally compared with that of ACE inhibitors, the best means available so far to control protein excretion and renal disease progression in experimental and human diabetes (11,12).

RESEARCH DESIGN AND METHODS

Pilot study. A preliminary study was performed to check whether PD 142,893 [AC-(D)-DIP-(L)-LEU-(L)-ASP-(L)-ILE-(L)-ILE-(L)-TRP] (13), a peptidic ET_A/ET_B receptor antagonist, at a dose of 3 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, was effective in preventing ET-1-induced increase in systolic blood pressure. Three groups of male Sprague-Dawley rats (Charles River, Calco, Italy), weighing 225–250 g, were treated intraperitoneally for three consecutive days: group 1 ($n = 6$) received 5% glucose (vehicle) and groups 2 ($n = 6$) and 3 ($n = 6$) received PD 142,893 (3 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$). The experiments described below were performed 3 (group 2) or 24 h (group 3) after the last administration of the active drug. Animals were anesthetized with tiopental sodium (60 mg/kg *i.p.*). A polyethylene (PE) 240 cannula was inserted into the trachea to facilitate breathing; a PE 50 cannula was placed in the left femoral vein for bolus injection of ET-1; and a PE 50 cannula was advanced into the left femoral artery to measure arterial blood pressure with a Statham pressure transducer connected to a carrier amplifier (Battaglia Rangoni, Bologna, Italy). Rats were allowed to equilibrate for 30-min intervals. Increasing doses of ET (70, 140, 300 pmol) were given as bolus injections at 30-min intervals. Systolic blood pressure was monitored thereafter. Percent changes in mean arterial pressure induced by ET-1 were calculated from data obtained 10–20 min after injection of the peptide versus the initial control period.

Experimental design. Thirty-two adult male Sprague-Dawley rats, with initial body weights of 250–275 g, were used. Animal care and treatment were conducted in compliance with institutional guidelines and national and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; *NIH Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 85-83, 1985). All animals were allowed free access to food (standard rat laboratory diet containing 20% protein by weight) and tap water.

Twenty-four rats were made diabetic by a single intravenous tail injection of streptozotocin (Sigma, St. Louis, MO), 60 mg/kg body wt, under light ether anesthesia. The remaining eight rats served as controls. The induction of diabetes was

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Received for publication 5 August 1996 and accepted in revised form 4 November 1997.

ET, endothelin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFR, glomerular filtration rate; PAH, *p*-aminohippurate; PE, polyethylene; RPF, renal plasma flow.

confirmed 2 days later by measurement of tail blood glucose level using a reflectance meter (Miles Laboratories, Elkhart, IN). Diabetic rats received daily evening injections of ultralente insulin (Ultralente MC Novo, Industry, Copenhagen, Denmark) in doses individually adjusted to maintain blood glucose levels between 200 and 400 mg/dl. Blood glucose levels were monitored at least once a week in all diabetic rats and occasionally in nondiabetic rats for comparison purposes. Animals were followed for 6 months, until urinary protein excretion reached the value of 40–60 mg/day. Until this period, diabetic rats received no therapy other than insulin. Then animals were grouped as described below and followed for 4 weeks: group 4 ($n = 8$) received no treatment; group 5 ($n = 8$) received PD 142,893 ($3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) twice a day intraperitoneally; and group 6 ($n = 8$) received the ACE inhibitor lisinopril (40 mg/l) in the drinking water. Normal rats (group 7) with no treatment were also followed for the same time period.

In all groups, blood pressure and urinary protein excretion were measured before the induction of diabetes (time 0), at days 120 and 195 after streptozotocin injection, and every 15 days (days 210 and 225) after the beginning of treatment. At the end of the study period, whole-kidney function was measured through glomerular filtration rate (GFR) and renal plasma flow (RPF). After the rats were killed, their kidneys were removed and processed for *in situ* hybridization for ET-1 and histological analysis as well as for RNA extraction and Northern blot analysis of ET-1.

The effect of PD 142,893 was also evaluated in additional control rats ($n = 6$) given the ET receptor antagonist ($3 \mu\text{mol/kg}$) twice a day intraperitoneally for 6 days. Blood pressure, urinary protein excretion, and renal function were assessed before and after treatment in this latter group.

RNA isolation and Northern blot analysis. Total RNA was isolated from rat kidney by the guanidium isothiocyanate/cesium chloride procedure as previously described (1). For mRNA preparation, total RNA of kidneys of each experimental group was pooled together. Poly(A)⁺ RNA was selected by oligo (dT)-cellulose column chromatography (mRNA separator; Clontech, Palo Alto, CA). Ten micrograms of mRNA were then fractionated on 1.2% formaldehyde agarose gel and blotted onto nylon filters (Zeta-probe; Biorad, Richmond, CA). Rat ET-1 cDNA was labeled with $\alpha^{32}\text{P}$ -dCTP by random primed method (1). Hybridization was performed overnight in 50% formamide, 0.12 mol/L Na₂HPO₄, pH 7.2, 0.25 mol/L NaCl, and 7% SDS at 43°C. Filters were washed 3 times for 15 min with 2× SSC and 0.1% SDS at room temperature, for 15 min with 0.5× SSC and 0.1% SDS at room temperature, and for 15 min with 0.1× SSC and 0.1% SDS at 65°C. Membranes were subsequently probed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) taken as the internal standard of equal loading of the samples on the membrane. ET-1 mRNA optical density was normalized to that of the constitutive GAPDH gene expression. Values were expressed as optical density units relative to the specific mRNA levels in control rats.

Non-isotopic *in situ* hybridization. Rat ET-1 antisense and sense probes were prepared and labeled by *in vitro* transcription of a 319-bp rat ET-1 subcloned into a pGEM7zf (+) vector (kindly provided by Dr. E. Schiffrin, Quebec, Canada) using a digoxigenin RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Before use, digoxigenin-labeled riboprobes were checked for size, integrity, and labeling efficiency.

Renal tissue fixed in 10% neutral formalin and embedded in paraffin was cut at 4 μm and deparaffinized. After incubation with 5 mmol/L Levamisole and permeabilization with HCl 0.2 N and proteinase K (40 $\mu\text{g/ml}$, Sigma), sections ($n = 5$ for each animal) were hybridized overnight with a mixture consisting of 0.5 $\mu\text{g/l}$ of digoxigenin-labeled RNA probe, 2× SSC, 10% dextran sulfate, 1× Denhardt's solution, and 0.1 mol/L sodium phosphate in a moist chamber at 42°C. Slides were then washed in 0.2× SSC, treated with a blocking solution (50 mg/ml skimmed dried milk, 150 mmol/L NaCl in 100 mmol/L Tris HCl, pH 7.8), and incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim). Sections were finally colored with freshly prepared nitroblue tetrazolium and X-phosphate-5-bromo-4-chloro-3-indolyl phosphate. Negative controls were performed using the sense probe.

Other analyses. Systolic blood pressure was evaluated by tail cuff plethysmography (14). Protein concentration in the urine was measured by the Coomassie blue G dye-binding method (15). Urinary albumin concentration was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (16). Fifty microliters of urine sample (1:6,000–1:10,000 dilution) were added to a rabbit anti-rat albumin antibody-coated plate (Cappel-DBH, Milan, Italy). After 60 min incubation, a specific sheep anti-rat albumin antibody conjugated with peroxidase was added (Cappel-DBH, dilution 1:2,500). Substrate reaction was started with the addition of 0.137% o-phenylenediamine and stopped with 1 mol/L H₂SO₄. Absorbance was determined at 490 nm in a microtiter plate reader. Renal function was assessed in anesthetized rats by inulin and p-aminohippurate (PAH) clearances as previously described (17).

Inulin concentration in plasma and urine samples was measured by the thiourea-resorcinol method (18). PAH concentration in plasma and urine was determined by the method of Smith et al. (19). GFR and RPF, measured as inulin and PAH clearance, respectively, were calculated using the standard formula. Histo-

logical evaluation was performed in Dubosq-Brazil-fixed, paraffin-embedded kidney fragments stained with Masson's trichrome, hematoxylin and eosin, and periodic-acid Schiff reagent (PAS stain).

Statistical analysis. Results are expressed as means $\pm$ SD. Data were analyzed by repeated measures analysis of variance using the GLM procedure of the SAS program (20). Multiple comparisons between groups were carried out using the Tukey's studentized range test (21). Values of proteinuria and urinary albumin excretion were log transformed before statistical analysis. Statistical difference for GFR and RPF was assessed by Tukey-Cicchetti test. Statistical significance level was defined as $P < 0.05$.

RESULTS

Pilot study. Of the two groups of animals treated with PD 142,893 for 3 days and then challenged with ET-1 (70, 140, and 300 pmol) 3 or 24 h after the last administration of the drug, the former group had a higher blood pressure reduction than did the latter one, suggesting that the compound has a quite short duration of action (Fig. 1). This prompted us to administer it twice a day in all subsequent experiments.

Chronic administration of PD 142,893 to control rats. To evaluate the possible effect of PD 142,893 on control rats, animals were given the compound for 6 days. Blood pressure and urinary protein excretion rate were not modified by the treatment (blood pressure before, 139 ± 8 ; after, 139 ± 6 mmHg; urinary protein before, 21 ± 6 ; after, 20 ± 3 mg/day). Similarly, PD 142,893 did not affect GFR and RPF (GFR 2.1 ± 0.2 ml/min; RPF 6.8 ± 1.0 ml/min).

Chronic administration of PD 142,893 or lisinopril to diabetic rats. Food and water intake were significantly higher in diabetic than in control rats throughout the study and were not modified by PD 142,893 and lisinopril administration. Stable moderate hyperglycemia was maintained throughout the duration of the experiment. The three groups of diabetic rats had comparable levels of blood glucose during all the observation periods. Values of blood glucose at 120 days averaged 383 ± 34 mg/dl in untreated diabetic animals;

FIG. 1. Prevention of blood pressure increase induced by 70, 140, and 300 pmol ET-1 with PD 142,893. Animals ($n = 6$ for each group) were treated for 3 days with vehicle or $3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ PD 142,893, and experiments were performed 3 or 24 h after the last administration of PD 142,893. Data are means $\pm$ SD.

FIG. 2. Periodic assessments of systolic blood pressure in controls (□) and in diabetic rats given vehicle (▨), PD 142,893 (▤), or lisinopril (■). Data are means $\pm$ SD. Determinations were performed in 8 animals for each group at all time points except at 225 days, when only 7 animals in each diabetic group survived. Δ , $P < 0.01$ vs. controls; *, $P < 0.05$ vs. controls, diabetic + PD 142,893, and diabetic + lisinopril; ○, $P < 0.05$ vs. controls, diabetic + lisinopril.

FIG. 3. Time course of urinary protein excretion in controls (□), diabetic rats given PD 142,893 (▤) or lisinopril (■). Data are means $\pm$ SD. Determinations were performed in 8 animals for each group at all time points except at 225 days, when only 7 animals in each diabetic group survived. Δ , $P < 0.05$ vs. controls; ○, $P < 0.05$ vs. diabetics.

344 $\pm$ 45 and 395 $\pm$ 10 mg/dl in PD 142,893-treated and lisinopril-treated animals; and 91 $\pm$ 12 mg/dl in control rats. At the end of the experimental period, blood glucose levels were not affected either by PD 142,893 (375 $\pm$ 40 mg/dl) or by lisinopril (386 $\pm$ 27 mg/dl) and were comparable to untreated diabetic animals (377 $\pm$ 40 mg/dl). Body weight was significantly lower in diabetic than in control animals at 225 days (540.6 $\pm$ 25.2 vs. 655.5 $\pm$ 43.6 g, $P < 0.01$). Treatment of diabetic rats with PD 142,893 or lisinopril did not ameliorate weight gain (PD 142,893: 576.4 $\pm$ 63.0 g; lisinopril: 569.6 $\pm$ 60.0 g, $P < 0.05$ vs. controls). Kidney weight was higher in diabetic than in control rats (2.51 $\pm$ 0.31 vs. 2.01 $\pm$ 0.16 g, $P < 0.01$) and remained higher than in controls in diabetic rats given ET receptor antagonist (2.42 $\pm$ 0.23 g, $P < 0.05$) and ACE inhibitor (2.41 $\pm$ 0.16 g, $P < 0.05$). One rat in each group of diabetic animals died during the course of the experiment.

Blood pressure. At 120 days, all three groups of diabetic rats developed hypertension (diabetic: 158 $\pm$ 7; diabetic + PD 142,893: 155 $\pm$ 13; diabetic + lisinopril: 159 $\pm$ 8 vs. control: 135 $\pm$ 9 mmHg, $P < 0.01$) (Fig. 1). The increase in blood pressure persisted at 195 days (diabetic: 158 $\pm$ 7; diabetic + PD 142,893: 156 $\pm$ 8; diabetic + lisinopril: 160 $\pm$ 10 vs. control: 135 $\pm$ 5 mmHg, $P < 0.01$) (Fig. 2). Fifteen days after the beginning of treatment, either PD 142,893 or lisinopril significantly lowered systolic blood pressure as compared with untreated diabetic rats (diabetic + PD 142,893: 140 $\pm$ 6; diabetic + lisinopril: 126 $\pm$ 17 vs. diabetic: 156 $\pm$ 4 mmHg, $P < 0.05$) to levels that were not significantly different from those of controls (136 $\pm$ 4 mmHg). Lisinopril controlled blood pressure better than did PD 142,893 ($P < 0.05$). At the end of the study, blood pressure in PD 142,893-treated animals was only numerically but no longer significantly reduced relative to that in untreated diabetic animals (139 $\pm$ 10 vs. 152 $\pm$ 10 mmHg). By contrast, lisinopril better controlled blood pressure, further reducing it to values that were even lower than control values (120 $\pm$ 13 vs. 136 $\pm$ 3 mmHg, $P < 0.05$) (Fig. 2).

Urinary protein excretion. The time course of urinary protein excretion is depicted in Fig. 3. Baseline proteinuria val-

ues were similar in all animals. At 120 days, diabetic animals developed significant proteinuria compared with controls ($P < 0.05$). Urinary protein excretion further increased over control values at 195 days ($P < 0.05$). Fifteen days after treatment, both treatments significantly prevented the further increase in proteinuria that was occurring in diabetic untreated rats (PD 142,893: 30 $\pm$ 4; PD 142,893 + lisinopril: 28 $\pm$ 3 vs. diabetic: 57 $\pm$ 3 mg/day, $P < 0.05$). Values of urinary proteins in diabetics were significantly higher than in controls (24 $\pm$ 3 mg/day, $P < 0.05$). At the end of the study, PD 142,893- and lisinopril-treated diabetic animals were still significantly protected from the development of proteinuria relative to untreated diabetic rats (diabetic + PD 142,893: 31 $\pm$ 4; diabetic + lisinopril: 31 $\pm$ 5 vs. diabetic: 68 $\pm$ 8 mg/day, $P < 0.05$), although values of both groups were still significantly higher than those of control rats (23 $\pm$ 2 mg/day, $P < 0.05$).

The time course of urinary albumin excretion is depicted in Fig. 4. Basal values were similar in all groups. Absolute values of urinary albumin excretion were higher in diabetic animals than in controls at 120 and 195 days, but the trend was statistically significant ($P < 0.05$) only at the latter time. At the end of the study, urinary albumin excretion was significantly higher in diabetic versus control rats (14.5 $\pm$ 4.7 vs. 1.1 $\pm$ 0.32 mg/24 h, $P < 0.05$). Both treatments reduced urinary albumin excretion, although only lisinopril reduced it to a statistically significant extent (lisinopril: 3.9 $\pm$ 2.2 mg/24 h, $P < 0.05$; PD 142,893: 9.9 $\pm$ 2.3 mg/24 h).

Renal ET-1 mRNA expression. ET-1 gene expression in kidneys from diabetic rats and the effect of blocking angiotensin II formation and ET-1 biological activity are depicted in Fig. 5. Densitometric analysis of the autoradiographic signals showed that ET-1 transcript levels in diabetic rats were twofold higher than those in control rats. Both the ACE inhibitor and the ET receptor antagonist attenuated the increase in mRNA levels for ET-1 (0.2 and 0.3, respectively). **Localization of ET-1 mRNA in the kidney.** In situ hybridization was performed to evaluate ET-1 mRNA distri-

FIG. 4. Urinary albumin excretion in controls (□), diabetic rats (▨), and diabetic rats given PD 142,893 (■) or lisinopril (■) during the study. Data are means $\pm$ SE. Determinations were performed in 8 animals for each group at all time points except at 225 days, when only seven animals in each diabetic group survived. Δ , $P < 0.05$ vs. controls; $\circ$, $P < 0.05$ vs. diabetics.

bution in kidneys of untreated or treated diabetic rats with respect to normal rats of the same age. As shown in Fig. 6A, in control rats hybridization signal was detectable mainly in epithelial cells of distal tubules. In diabetic rats, ET-1 mRNA staining increased dramatically over controls, and the signal was now detectable in the glomerulus. Strong cytoplasmic staining was also observed in the distal tubules (Fig. 6B). The pattern of distribution of renal ET-1 mRNA was remarkably reduced by PD 142,893 or lisinopril, particularly at the glomerular level (Figs. 6C and 6D, respectively). No hybridization signal could be observed with the ET-1 sense probe (Fig. 6E).

Renal function. The results of GFR and RPF are reported in Table 1. Untreated diabetic rats had GFR comparable to controls, but a significantly lower RPF ($P < 0.05$). PD 142,893 and lisinopril increased RPF to values that were not significantly different from those of controls.

Renal histology. Because diabetic rats in the present study were evaluated early in the course of the disease, they had no glomerular lesions and mild tubulo-interstitial inflammation. Histological assessment of kidney specimens from rats given the ET receptor antagonist or the ACE inhibitor indicated some degree of reduction in the signs of tubulo-interstitial damage, which could be measured by a semiquantitative scoring of the lesions (data not shown).

DISCUSSION

Available in vitro data indicate that high glucose concentrations enhance ET synthesis (22) and modulate ET-1 receptors, at least in glomerular mesangial cells (23). In vivo (24) ET-1 mRNA levels increased in glomeruli from rats with streptozotocin-induced diabetes, while ET_A and ET_B receptor expression was unchanged. Higher-than-normal expression of the ET-1 gene in diabetes was normalized by insulin, suggesting that the abnormality was caused, at least in part, by hyperglycemia (24). A logical implication of the above find-

FIG. 5. Renal expression of ET-1 mRNA in diabetic and control rats and effect of treatment with lisinopril or PD 142,893. mRNA samples (10 μ g) obtained from pooled kidneys of each experimental group were blotted onto synthetic membranes, which were hybridized sequentially with α^{32} P-labeled rat ET-1 (top) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; bottom) cDNA probes. The optical density of the autoradiographic signals was quantitated and calculated as the ratio of ET-1 to GAPDH mRNA. The mRNA levels were calculated by assuming the optical density of controls as unit. Representative Northern blot analysis of $n = 3$ experiments.

ings is that in diabetic renal disease, ET-1 synthesized in excessive amounts by renal cells may contribute to renal damage by at least two distinct pathways of injury, one mediated by cortical and inner medulla hypoperfusion (given the extraordinary sensitivity of renal vessels [25] to the vasoconstrictor effect of ETs) and the other based on the effect of ET-1 of enhancing extracapillary matrix deposition (26). Data that diabetic rats preventively given an ET_A receptor antagonist, unlike untreated diabetics, were protected from the upregulation of extracellular matrix proteins and growth factor renal genes support a role for ET-1 in renal injury. However, so far there are no true therapeutic studies that could be used to establish whether ET-1 antagonist can cure overt diabetic renal disease. The present study has been designed to clarify the functional relevance of increased ET-1 gene expression in diabetes, with the final aim of establishing whether the consequences of excessive renal ET-1 could possibly be pharmacologically modulated for a long-term benefit on the evolution of the disease. Biological activity of ETs was blocked by unselectively inhibiting the two main receptors, A and B. The effect of inhibiting ET receptors was compared with ACE inhibition, a maneuver that effectively limits proteinuria and prevents renal lesions in diabetic animals and humans as documented consistently by many studies (11,12). Our results indicated that the nonselective ET receptor antagonist PD 142,893, given to diabetic rats with hypertension, overt nephropathy, and proteinuria, normalized systemic blood pressure and reduced urinary proteins. In addition, the ET-1 antagonist ameliorated renal blood flow, which was significantly lower than normal in untreated diabetics. The fact that the ET receptor antagonist also remarkably reduced ET-1 mRNA at both the glomerular and tubular levels supports the possibility of an autoinduction

FIG. 6. Representative photomicrographs of the distribution of ET-1 mRNA by in situ hybridization in kidney sections from a control rat (A) and from diabetic rats given vehicle (B), PD 142,893 (C), or lisinopril (D). Experiment was repeated in three of seven animals in each group. A: Signal present in distal tubules; B: signal detectable in glomeruli and stronger in distal tubules. C and D: Signals reduced particularly in glomeruli; E: negative control (ET-1 sense probe). Original magnification $\times 180$.

of ET-1 via the ET_B receptor that was documented recently in rat mesangial cells (27) as well as in human endothelial and proximal tubular cells (28,29).

Compared with PD 142,893, the ACE inhibitor lisinopril was always superior in lowering systemic blood pressure at both time points considered. As observed in previous studies (11,17), we found here that blood pressure in the lisinopril-treated diabetics was significantly lower than in normal controls of the same age. Results of renal function studies did not reveal changes in GFR (whose values are normal at this stage of diabetes) induced by either treatment. Instead, renal blood

TABLE 1

Effects of a chronic administration of PD 142,893 or lisinopril on GFR and RPF in rats with diabetes

Groups	GFR (ml/min)	RPF (ml/min)
Controls	2.21 $\pm$ 0.17	7.22 $\pm$ 1.17
Diabetic	2.19 $\pm$ 0.14	5.64 $\pm$ 0.36*
Diabetic + PD 142,893	2.09 $\pm$ 0.16	6.24 $\pm$ 0.58
Diabetic + lisinopril	2.32 $\pm$ 0.23	6.10 $\pm$ 0.94

Data are means $\pm$ SD. * $P < 0.05$ vs. control.

flow was comparably ameliorated by lisinopril, which also showed similar antiproteinuric properties.

How can this latter finding be interpreted on the basis of the current theories linking perturbation of barrier function to changes in glomerular hemodynamics (30)? Results from animal experiments have consistently documented that in diabetic nephropathy, the relative loss of glomerular autoregulation of flow exposes the capillary tuft to systemic blood pressure. The consequent increase in glomerular capillary pressure with time imposes a mechanic and possibly inflammatory stress on the membrane so as to perturb barrier function first and ultimately impair the anatomical integrity of the capillary network, eventuating in focal or global sclerosis of the tuft (31). Such negative effects on barrier function can be reversed by ACE inhibitors, a class of molecules that by lowering the tone of the efferent arteriole effectively enhances the glomerular flow and restores to normal glomerular pressure and transcapillary pressure gradient (11). As a consequence, filtered protein load is reduced and, in the long run, structural damage to the glomerulus is remarkably limited (32). Findings that an ET-1 receptor antagonist and an ACE inhibitor share comparable antiproteinuric properties could possibly imply that either drug in this model restores glomerular pressure to normal, despite the superior systemic antihypertensive effect of the ACE inhibitor. The concerted action of the ET-1 antagonist we have used here on glomerular arteriolar tone and the ultrafiltration coefficient associated with the effect on systemic blood pressure are conceivably responsible for the improved glomerular permeability in experimental diabetes. An alternative possibility to explain the comparable effect on proteinuria of the two molecules derives from data that ACE inhibitors restore to normal the upregulated ET-1 gene of diabetic rat glomeruli (33). It is therefore possible that perturbations of glomerular barrier function of experimental diabetes recognize a common ET-1-dependent pathway whose cellular and molecular determinants deserve to be further explored.

In conclusion, we have found that an unselective ET antagonist effectively reduced the clinical expression of diabetic nephropathy and ameliorated renal perfusion even when the treatment was initiated after 6 months of diabetes, when the animals already had clinical nephropathy and overt proteinuria. These results support the possibility, raised by many experimental and human studies, that ET-1 is at least one contributory mediator of kidney damage in diabetes, and they open theoretical perspectives for innovative approaches in the treatment of this disease, possibly based on a new class of renoprotective agents.

ACKNOWLEDGMENTS

This study was supported in part by a grant from Berlex Biosciences (Richmond, CA).

The authors acknowledge Dr. Gabor Rubanyi for kindly providing PD 142,893. The authors are deeply indebted to Dr. Ernesto Schiffrin for the gift of rat ET-1 riboprobes, to Fabio Sangalli for measurements of urinary albumin excretion, and to Dr. Norberto Perico for fruitful discussion. Laura Arioli helped in preparing the manuscript.

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CHAPTER 5

Renoprotective effect of contemporary blocking of angiotensin II and endothelin-1 in rats with membranous nephropathy

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published in

Kidney International 1998, 54:353-359

Renoprotective effect of contemporary blocking of angiotensin II and endothelin-1 in rats with membranous nephropathy¹

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Renoprotective effect of contemporary blocking of angiotensin II and endothelin-1 in rats with membranous nephropathy.

Background. We previously showed that chronic administration of an angiotensin converting enzyme (ACE) inhibitor to rats with passive Heymann nephritis (PHN), a model of membranous nephropathy with proteinuria and increased renal synthesis of endothelin-1 (ET-1), reduces urinary proteins and partially limits the exaggerated ET-1 renal synthesis. Here we compared the effect of an ET_A receptor antagonist and an ACE-inhibitor given as single therapies with a combination of the two drugs in uninephrectomized PHN rats.

Methods. PHN was induced with a single i.v. injection of rabbit anti-Fx1A antibody in 40 male Sprague Dawley rats. To accelerate the onset of renal damage rats underwent uninephrectomy seven days later and were subsequently treated until eight months with the ET_A receptor antagonist LU-135252 (50 mg/kg b.i.d. p.o.) or the ACE-inhibitor trandolapril (1 mg/kg in the drinking water) or the combination of the two drugs.

Results. Either LU-135252 or trandolapril given alone prevented the increase in systolic blood pressure (SBP). Combined therapy was even more effective than single drugs. While LU-135252 and trandolapril reduced proteinuria by 23 to 25%, the drug combination resulted in 45% lowering of urinary proteins. Serum creatinine was significantly decreased by the combination, but not by the single drugs. Glomerulosclerosis and tubulointerstitial damage were more reduced by combined therapy than by LU-135252 or trandolapril alone.

Conclusions. These data suggest that contemporary blocking angiotensin II (Ang II) and ET-1 in an accelerated model of PHN had an additive renoprotective effect than single blocking Ang II or ET-1 and would represent a therapeutic advantage for renal disease patients who do not completely respond to ACE inhibitors.

Experimental and human proteinuric glomerulopathies fatally evolve to end-stage renal failure by a process of progressive parenchymal damage, which may evolve independently from the initial insult. There is ample evidence that in these conditions drugs that limit the synthesis or the biological activity of angiotensin II (Ang II) retard the progression of renal damage [1–3]. One possible explanation for the remarkable effect of these classes of compounds on progressive nephropathies is their unique capacity of reducing glomerular capillary hypertension which is not necessarily shared by more conventional drugs [3]. In addition, blocking Ang II reduces glomerular protein traffic better than other antihypertensives, thus limiting the intrinsic renal toxicity of proteins that once filtered in abnormal amount by the glomerulus are endocytosed by proximal tubuli [4]. This process triggers a series of events culminating in tubular cell dysfunction whose ultimate pathogenicity is related to the up-regulation of genes encoding vasoactive and inflammatory mediators [5–7]. Of this complex process ET-1 formed in excessive amount during the process of protein reabsorption is a major player. Tubular ET-1 is secreted toward the basolateral membrane of the cell and *in vivo* accumulates into the renal interstitium [8]. There are *in vitro* data showing that ET-1 is a potent stimulus to interstitial fibroblast proliferation and extracellular matrix synthesis [9, 10]. That locally formed ET-1 does play a role in the process of progressive renal injury is supported by a number of *in vivo* findings. Enhanced renal ET-1 synthesis that correlates with the severity of renal damage has been observed in many forms of progressive nephropathies in animals or humans [11–15]. Furthermore, selective pharmacological manipulation of the ET-1 pathway had a major positive impact on the progression of the disease in several models of proteinuric nephropathies [16–19].

We have recently observed that in passive Heymann nephritis (PHN), a rat model of membranous nephropathy characterized by proteinuria and increased renal ET-1, chronic administration of an ACE-inhibitor partially reduces proteinuria and attenuates the exaggerated ET-1

¹ See Editorial by Kohan, p. 346.

Key words: angiotensin converting enzyme, endothelin receptor antagonist, passive Heymann nephritis, LU-135252, trandolapril, proteinuria, glomerulosclerosis.

Received for publication June 26, 1997
and in revised form February 27, 1998
Accepted for publication February 27, 1998

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renal synthesis [14]. Although ACE-inhibition is a very powerful therapy, it is not completely effective in normalizing urinary proteins nor does it completely prevent the decline of GFR and renal damage either in animal models and, even more importantly, in patients with progressive nephropathies. This could be due to an only partial blocking of locally formed Ang II by these molecules or to an exaggerated renal synthesis of other mediators including ET-1 that are not necessarily controlled by ACE-inhibition. We undertook the present study with the aim to evaluate the effect of combining an ACE-inhibitor with an endothelin receptor antagonist in a model of PHN in the rat that was not completely responsive to an ACE-inhibitor.

Our results indicated that the combined therapy had better renoprotective properties than each drug alone. This study opens novel therapeutic perspectives for the treatment of progressive renal diseases.

METHODS

Pilot studies

A preliminary study was performed to assess the timing of LU-135252 administration. LU-135252 [(+)-(S)-2-(4,6 dimethoxy-pyrimidin-2-yloxy)-3-methoxy-3,3 diphenyl-propionic acid], the active (+) isomer of LU 127043, is a selective, orally active, nonpeptide ET_A receptor antagonist (Knoll AG, Ludwigshafen, Germany) [20]. Two groups of male Sprague-Dawley rats (275 to 300 g; Charles River, Calco, Italy) were orally treated for three consecutive days: groups 1 (*N* = 3) and 2 (*N* = 3) received LU-135252 (50 mg/kg/day by gavage) and evaluation of systolic blood pressure was performed 6 or 24 hours after the last administration of the drug, respectively. An additional group of rats (*N* = 3) were used as controls. The dose of LU-135252 was chosen on the basis of previous experiments showing that 50 mg/kg/day effectively lowered blood pressure in angiotensin II-induced hypertensive rats [20]. Animals were anesthetized with tiopental sodium (60 mg/kg i.p.) and a polyethylene (PE) 240 cannula was inserted into the trachea to facilitate breathing, one PE 50 cannula was placed in the left femoral vein for bolus injection of ET-1 and a PE 50 cannula was advanced into the left femoral artery to measure arterial blood pressure with a Statham pressure transducer connected to a carrier amplifier (Battaglia Rangoni, Bologna, Italy). Rats were allowed to equilibrate for 30 minute intervals. Increasing doses of ET-1 (70, 140, 300 pmol) were given as a bolus injection at 30 minute intervals. Systolic blood pressure (SBP) was monitored thereafter. Percent changes in mean arterial pressure induced by ET-1 were calculated from data obtained 10 to 20 minutes after injection of the peptide versus the initial control period.

The effectiveness of ET_A blockade and timing of LU-135252 administration were also determined in three additional groups of rats with accelerated PHN (*N* = 3 each

group, 350 to 375 body wt), which two weeks after disease induction were orally treated for three consecutive days with 50 mg/kg once a day or 50 mg/kg b.i.d. LU-135252 or with vehicle. Six hours after the last administration of the drug animals were anesthetized and experiments were performed as described above for normal rats.

Experimental design

Male Sprague-Dawley rats (Charles River) weighing 250 to 300 g at the start of the experiment were used in this study. Animal care and treatment were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985). All animals were allowed free access to standard rat diet containing 20% protein by weight, and tap water. PHN was induced in non-anesthetized rats by a single i.v. injection of 0.4 ml/100 g body wt of rabbit anti-Fx1A antibody. Seven days later, when proteinuria was already present, animals were subjected to unilateral nephrectomy to accelerate the onset of renal damage (C. Zoja, unpublished observation) and divided into four groups.

Group 1 (*N* = 10). PHN rats given daily the endothelin receptor antagonist LU-135252, 50 mg/kg b.i.d. by gavage, from day 7 after PHN induction for eight months.

Group 2 (*N* = 10). PHN rats given the ACE-inhibitor trandolapril daily, 1 mg/kg in the drinking water, from day 7 after PHN induction for eight months.

Group 3 (*N* = 10). PHN rats given LU-135252 (50 mg/kg b.i.d. by gavage) and trandolapril (1 mg/kg in the drinking water) daily from day 7 after PHN induction for eight months.

Group 4 (*N* = 10). PHN rats without any treatment, followed for eight months. An additional group of normal rats (Group 5, *N* = 6) with no treatment was followed for eight months and used as control.

The dose of trandolapril was chosen on the basis of previous reports showing nearly 80% inhibition of ACE activity in serum from adult spontaneously hypertensive rats, and blood pressure and renoprotective effects in diabetic rats [21, 22].

In all rats SBP was recorded on months 0 (baseline), 2, 4 and 8 after PHN induction. Serum creatinine levels were measured at baseline and every two months. Twenty-four hour urines were collected in metabolic cages on days 0 and 7 (before treatment) and on months 2, 4 and 8 for urinary protein excretion determination. Renal biopsies were obtained from ether-anesthetized animals at the end of the study for morphological studies by light microscopy.

Systolic blood pressure

Systolic blood pressure was recorded by tail plethysmography in conscious rats [23].

Serum creatinine

Blood was collected from the tail vein of anesthetized animals. Serum was obtained after whole blood clotting and kept at -20°C until assayed. Creatinine was measured by the alkaline picrate method [24].

Urinary protein excretion

Proteinuria was determined in 24-hour urines by the modified Coomassie blue G dye-binding assay for proteins with bovine serum albumin as the standard [25].

Light microscopy

Kidney fragments were fixed for six hours in Dubosq-Brazil, dehydrated in alcohol, and embedded in paraffin. Sections ($3\text{ }\mu\text{m}$) were stained with hematoxylin and eosin and periodic acid-Schiff reagent (PAS stain). Sections including superficial and juxtamedullary glomeruli were evaluated. At least 100 glomeruli were examined for each animal and the percentage of glomeruli presenting focal or global sclerotic lesions was determined. Tubular (atrophy, casts and dilation) and interstitial changes (fibrosis and inflammation) were graded from 0 to 4+ (0, no changes; 1+, changes affecting less than 25% of the sample; 2+, changes affecting 25 to 50% of the sample; 3+, changes affecting 50 to 75% of the sample; 4+, changes affecting 75 to 100% of the sample). All renal biopsies were analyzed by the same pathologist, who was unaware of the nature of the experimental groups.

Statistical analysis

Results are expressed as mean $\pm$ se. Data were analyzed using the non-parametric Kruskal-Wallis test for multiple comparisons. Differences were considered statistically significant at a value of $P < 0.05$.

RESULTS

Pilot studies

When increasing doses of ET-1 (70, 140 and 300 pmol) were infused in anesthetized normal animals, systolic blood pressure gradually increased from 8 to 38%. Pretreatment with LU-135252 prevented an ET-1 induced blood pressure increase. The effect was more marked when the last administration of the drug was given six hours before ET-1 challenge and inhibition averaged 81 to 97%. This result prompted us to give 50 mg/kg LU-135252 twice a day.

The choice of this schedule treatment was confirmed by the experiments performed in rats with accelerated PHN. Actually, ET-1 (70, 140 and 300 pmol) infusion in PHN rats given vehicle resulted in an increase in systolic blood pressure from 4 to 27%. When rats were pretreated with LU-135252 at the dose of 50 mg/kg once a day, the ET-1 induced blood pressure increase was prevented by 34 to 57%, while administration of the same dose of LU-135252 but twice a day gave an inhibition of 79 to 86%.

Characteristics of accelerated PHN

Animals were subjected to unilateral nephrectomy seven days after disease induction when proteinuria was already present. Rats developed hypertension within four months, similarly to the two kidney model of PHN [14, 26]. Absolute values of proteinuria increased with time and were fairly comparable to those of the two kidney PHN rats, although in the accelerated model, animals of course had half the nephron number. Increased protein traffic in the remnant kidney over the two kidney model resulted in a more severe renal damage. Thus, at month 8, the severity of glomerular as well as tubulointerstitial changes were similar to those observed in the two kidney model at month 12 [14, 26]. Animals showed glomerular hypertrophy associated with a moderate increase of mesangial matrix, glomerular capillary walls were diffusely thickened; with silver stain no spikes were detected. In more than 50% glomeruli well defined areas of sclerosis and hyalinosis with adhesion to Bowman capsule were observed. Tubulointerstitial changes consisted of severe interstitial fibrosis and inflammation associated with tubular atrophy and with the presence of large eosinophil casts in distal tubular lumens.

Chronic study

During the chronic study a similar mortality among the groups of PHN rats was observed, accounting for three rats in the treated groups while only two died among the untreated PHN rats. Food intake was comparable in all PHN and control rats for the entire study period. The average food intake at month 8 was: untreated PHN, 24 ± 0.9 ; PHN + LU-135252, 24 ± 0.4 ; PHN + trandolapril, 21 ± 1 ; PHN + LU-135252 + trandolapril, 24 ± 1 ; control, 23 ± 1 g/day. All PHN rats gained weight in a similar manner and at the end of the study body wt values were comparable (PHN, 579 ± 16 ; PHN + LU-135252, 581 ± 22 ; PHN + trandolapril, 624 ± 28 ; PHN + LU-135252 + trandolapril, 593 ± 23 g). Body weights of control rats were numerically higher than those of PHN rats, although a statistical significance was not reached (676 ± 40 g).

Systolic blood pressure

As shown in Figure 1, in untreated PHN rats SBP significantly increased during time as compared to control rats, with values averaging 156 ± 6 mm Hg at the end of the study (vs. control, 129 ± 1 mm Hg, $P < 0.01$). Treatment of PHN rats with LU-135252 maintained SBP at values that were significantly lower than those of untreated PHN (135 ± 3 mm Hg, $P < 0.05$ vs. untreated PHN). Trandolapril given as a single therapy prevented the increase in SBP of PHN rats with values fairly comparable to those of control rats (125 ± 3 vs. 129 ± 1 mm Hg). Combined therapy maintained SBP at values which were even numerically lower than those of controls (119 ± 5 mm Hg), being much more effective ($P < 0.05$) than LU-135252 alone.

Fig. 1. Systolic blood pressure measured during eight months of observation in untreated PHN rats (▨), in PHN animals given LU-135252 (■), or trandolapril (▤), or the combination of LU-135252 and trandolapril (▥) and controls (□). Data are mean $\pm$ SE. * $P < 0.01$ versus control; † $P < 0.05$; # $P < 0.01$ versus PHN; ‡ $P < 0.05$ versus PHN + LU-135252.

Fig. 2. Time course of urinary protein excretion in PHN animals receiving no treatment (▨), or LU-135252 (■) or trandolapril (▤) or the combination of LU-135252 and trandolapril (▥) and controls (□). Data are mean $\pm$ SE. * $P < 0.01$ versus control; † $P < 0.05$ versus PHN; ‡ $P < 0.05$ versus PHN + LU-135252.

Urinary protein excretion

All groups of PHN rats developed proteinuria to a similar extent as early as seven days after induction of the disease compared to control rats. Proteinuria values before treatment was as follows: untreated PHN, 74 ± 15 ; LU-135252, 79 ± 19 ; trandolapril, 59 ± 9 ; LU-135252 + trandolapril, 64 ± 12 ; versus control, 26 ± 1 mg/day. As shown in Figure 2 in untreated PHN rats proteinuria increased during time averaging 855 ± 67 mg/day at the end of the study. LU-135252 and trandolapril reduced proteinuria only by 23 and 25%, respectively, at month 8 (LU-135252, 659 ± 53 ; trandolapril, 645 ± 59 mg/day). During the experimental period the combined therapy had a marked antiproteinuric effect resulting in a 44%, 39%

and 45% reduction of urinary protein excretion as compared to untreated PHN at months 2, 4 and 8, respectively. Values of proteinuria in the combined therapy group were numerically lower than those of each drug alone in most instances, with a statistically significant difference versus LU-135252 at four months.

Renal function

Two months after disease induction untreated PHN animals developed a mild renal insufficiency that worsened during time, as documented by a progressive increase in serum creatinine values as compared to control rats (Table 1). LU-135252-treated PHN animals had a similar impaired renal function. In PHN rats given trandolapril, serum

Table 1. Time course of serum creatinine (mg/dl)

	Months			
	0	2	4	8
PHN	0.60 ± 0.02	0.92 ± 0.03 ^b	0.85 ± 0.09 ^b	1.80 ± 0.33 ^b
PHN + LU-135252	0.64 ± 0.01	0.79 ± 0.02 ^b	0.91 ± 0.02 ^b	1.80 ± 0.28 ^b
PHN + trandolapril	0.61 ± 0.02	0.84 ± 0.01 ^b	0.92 ± 0.11 ^a	1.40 ± 0.19 ^b
PHN + LU-135252 + trand.	0.63 ± 0.02	0.82 ± 0.02 ^b	0.74 ± 0.05 ^a	0.89 ± 0.11 ^{cd}
Control	0.59 ± 0.02	0.57 ± 0.02	0.61 ± 0.03	0.76 ± 0.02

Data are mean ± SE.

^a $P < 0.05$, ^b $P < 0.01$ vs. control at corresponding time^c $P < 0.01$ vs. PHN at corresponding time^d $P < 0.05$ vs. PHN + LU-135252 and PHN + trandolapril at corresponding time

Table 2. Renal histology of treated or untreated PHN rats and controls

	Glomeruli with sclerotic changes %	Interstitial damage (score)	Tubular damage (score)
PHN	52.5 ± 7.8 ^b	2.4 ± 0.3 ^b	2.4 ± 0.3 ^b
PHN + LU-135252	55.3 ± 5.3 ^b	2.8 ± 0.1 ^b	2.8 ± 0.1 ^b
PHN + trandolapril	31.6 ± 7.1 ^{bd}	2.1 ± 0.3 ^b	2.1 ± 0.3 ^b
PHN + LU-135252 + trandolapril	19.5 ± 7.6 ^{ace}	1.4 ± 0.1 ^d	1.4 ± 0.1 ^d
Control	0.8 ± 0.8	0.3 ± 0.2	0.3 ± 0.2

Data are mean ± SE.

^a $P < 0.05$, ^b $P < 0.01$ vs. control, ^c $P < 0.05$ vs. PHN,^d $P < 0.05$, ^e $P < 0.01$ vs. PHN + LU-135252

creatinine levels were slightly lower than those of untreated PHN. By contrast, in PHN rats given the combined therapy the serum creatinine values remained fairly stable throughout the study, at eight months being significantly lower than those of untreated PHN rats as well as of PHN rats treated with LU-135252 or trandolapril.

Renal histology

The results of renal morphology by light microscopy are reported in Table 2. Untreated PHN rats showed focal and segmental glomerulosclerosis affecting on average 52.5% of glomeruli. Tubulointerstitial changes consisted of interstitial fibrosis and inflammation associated with tubular atrophy and large eosinophilic casts in the tubular lumen (mean score 2.4). In PHN rats given LU-135252 glomerular and tubular changes were similar to those of untreated PHN rats. In rats given trandolapril percentage of glomeruli with sclerotic changes averaged 31.6% and score for tubulointerstitial damage averaged 2.1. By contrast, in PHN rats treated with the combined therapy glomerular and tubular structural integrity was better preserved with respect to untreated PHN rats. Thus, sclerotic changes affected on average 19.5% of glomeruli ($P < 0.05$ vs. untreated PHN) and the score for tubulointerstitial damage averaged 1.4.

DISCUSSION

The results of the present study offer a rationale for combining an ACE-inhibitor with an ET receptor antago-

nist in patients who do not respond completely to ACE-inhibitors.

The present results show that the ACE-inhibitor trandolapril limited tubulointerstitial and glomerular damage. In the present set of experiments trandolapril was less effective in reducing urinary proteins and renal injury than other molecules of the same class studied previously by our own group in PHN [14, 26]. However, formal comparison between the present and previous studies performed with other ACE inhibitors cannot be done, since experiments of this kind performed in the accelerated model of the PHN using different ACE inhibitors have never been done. It is therefore conceivable that the apparent discrepancy between the present study and previous ones are indeed explained by the fact that here we have studied a far more severe disease, obtained by uninephrectomizing animals at day 7 after disease induction to accelerate renal damage. Indeed, despite blood pressure levels that were comparable to the two kidney model, proteinuria was much more severe considering the animals had only one kidney. As a consequence of increased protein traffic renal damage occurred earlier. In theory a more severe disease should have maximized the chances of highlighting relative differences in the various drug regimens employed. This is of special interest in the field of progressive nephropathies since in many experimental models, but not in human renal diseases, ACE-inhibition alone is so powerful as to fully normalize urinary protein [3, 27–29]. In most models it entirely protects animals from renal failure and may prolong life occasionally exceeding the expected survival rate of control animals [2, 30]. To start with, findings that the protective effect of trandolapril in this accelerated model of PHN was only partial, were instrumental to subsequent studies planned to verify whether alternative treatments and/or combination of drugs were more effective than ACE-inhibition alone. The rationale for using an ET-1 antagonist to retard renal disease progression in our model rests on data that kidneys from PHN rats synthesize more ET-1 than normal kidneys [14, 31]. Excessive ET-1 mRNA and protein were localized in proximal tubules and preceded inflammatory cells infiltrating the renal interstitium and interstitial fibrosis [31]. At variance with trandolapril,

the endothelin antagonist LU-135252 given daily as a single agent did not prevent proteinuria, renal function impairment and renal structural damage, despite the fact that it maintained systolic blood pressure within normal values.

Lack of efficacy of the ET-receptor antagonist alone in this accelerated model of PHN would indicate that—at least at an early stage of the disease—the major mediator of renal damage is rather Ang II, than ET-1. If anything, one can speculate that ET-1 may contribute to amplify the damage conceivably in a relatively advanced stage of the disease [32]. This possibility is consistent with findings that excessive ET-1 is formed in the kidney not sooner than eight months after the induction of the disease in PHN, as documented by a previous study of our group [14]. To test formally the hypothesis that following a prolonged enough period of heavy proteinuria ET-1 does play a contributory role in the subsequent renal structural damage, possibly favoring interstitial inflammation and fibrosis, we used both trandolapril and the endothelin antagonist LU-135252 in combination. The effect of combining trandolapril with LU-135252 was accompanied by a reduction in proteinuria that reached significantly different values as compared to PHN alone at all time points examined in the course of the study. At variance, neither trandolapril nor LU-135252 given alone, despite numerically reducing proteinuria at all time points examined, did so to an extent that reached statistical significance. The preventive effect of such a treatment regimen as compared to each molecule alone against deterioration in renal function and structural damage was even more remarkable.

The results of this study confirmed the major effect of inhibiting Ang II in progressive immune nephropathies and at the same time provided the evidence of the better renoprotection of a multidrug approach in a model that is clearly more severe as compared to the ones used so far in similar studies. These findings can in some way be reconciled with previous experimental and human data, which collectively indicated that the process of renal disease progression was linked to the local formation of Ang II, whose effect of enhancing glomerular hydraulic pressure [33] has been considered instrumental to initial injury. In turn, elevation of glomerular capillary pressure impairs glomerular permselectivity and promotes a complex interplay of events recapitulated by an increased expression of vasoactive and inflammatory molecules in proximal tubular cells activated by protein overload [5, 6]. Of these, up-regulation of ET-1 gene expression and enhanced synthesis of the corresponding protein were constant findings of *in vitro* and *in vivo* experiments performed thus far. Given the fact that Ang II and ET-1 both promote extracellular matrix accumulation in various renal cells, one may speculate that both contribute to the process of renal scarring. As for the relative role of enhanced renal ET-1 in progressive renal injury, the subject has been considerably advanced by evidence that renal overexpression of human ET-1 gene in

transgenic mice gave severe tubulointerstitial and glomerular injury, ending with reduced GFR [34].

The above findings help to explain the additive renoprotective effect of simultaneously blocking Ang II and endothelin in our accelerated model of PHN, and support the notion that renal disease progresses by an interaction of different mechanism(s) of which at least two have been identified here as suitable to pharmacological modulation. In addition to ACE-inhibitors, drugs that limit the consequences of protein traffic and reabsorption can theoretically be used in the future as part of the therapeutic armamentarium to cure progressive nephropathies. ET-1 antagonists are probably the first example of this kind. Results of experimental studies in the near future will identify many more factors involved in tubulointerstitial and glomerular damage triggered by protein traffic. A multidrug approach targeting these molecules will probably be the way to limit and hopefully avoid renal failure in this setting. Even now those patients whose disease responds only partially to ACE-inhibitors are candidates to try an endothelin antagonist in addition, within the context of controlled clinical designs.

ACKNOWLEDGMENTS

The authors are indebted with Dr. Tullio Bertani for histological examinations of renal specimens and to Gianfranco Marchetti for excellent technical assistance. LU-135252 and trandolapril were provided by Prof. Alfred W.A. Hahn and Dr. Michael Kirchengast (Knoll AG, Ludwigshafen, Germany).

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CHAPTER 6

Nature and mediators of renal lesions in kidney transplant patients given cyclosporine for more than one year

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published in

Kidney International 1999, 55:674-685

Nature and mediators of renal lesions in kidney transplant patients given cyclosporine for more than one year

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Nature and mediators of renal lesions in kidney transplant patients given cyclosporine for more than one year.

Background. Cyclosporine (CsA) has improved patients and organ-graft survival rates, but its chronic nephrotoxicity is still an issue. Although prolonged vasoconstriction could contribute to chronic CsA tubulointerstitial changes by producing chronic ischemia, this relationship has been difficult to demonstrate thus far, and cellular origin and mediators of these structural alterations remain ill-defined.

Methods. As a part of a clinical trial in kidney transplant recipients on triple immunosuppressive therapy (CsA, azathioprine and steroid), which includes renal biopsy as "per protocol," 22 patients enrolled between 12 and 24 months post-transplantation underwent renal hemodynamic evaluation by measuring glomerular filtration rate and renal plasma flow by the plasma clearance of unlabeled iothexol and the renal clearance of para-aminohippuric acid, respectively. In parallel, the CsA pharmacokinetic profile was also determined. A week later, a protocol biopsy of kidney graft was performed. Light microscopy examination and localization of endothelin-1, RANTES, monocyte chemoattractant protein-1 gene expression by *in situ* hybridization in the graft specimens were evaluated and related to the pattern of histologic lesions.

Results. Ten out of 22 kidney transplant recipients who underwent the protocol biopsy had CsA nephrotoxicity, eight had chronic rejection, and four had no lesions at histological examination. The total daily exposure to CsA was higher in patients with CsA nephrotoxicity than in those with chronic rejection or no lesions at biopsy. Renal function was preserved in the CsA toxicity group as compared with the chronic rejection group, despite some degree of renal hypoperfusion. Tubular atrophy and striped interstitial fibrosis were found in all patients with light microscopical evidence of CsA nephrotoxicity, whereas glomerular and arteriolar lesions were less frequent. Intense staining for endothelin-1, RANTES, and monocyte chemoattractant protein-1 mRNAs selectively localized at tubular epithelial cells was found in biopsies taken from patients with CsA nephrotoxicity, but not in the chronic graft

rejection group, whose tubuli had only minimal staining for RANTES mRNA on a few occasions.

Conclusion. Long-term CsA administration to kidney allograft recipients leads to tubulointerstitial injury independently of its vascular effect. The possible contribution to the development of interstitial fibrosis of inflammatory and growth factors released by tubular cells in which CsA accumulates is proposed.

Cyclosporine (CsA) has improved patient and organ-graft survival rates. Indeed, in the past two decades, short-term success rates of kidney, heart, liver, and kidney/pancreas allografts have reached unprecedented levels, largely attributable to CsA immunosuppression [1-3]. However, nephrotoxicity of CsA continues to be an issue [4-6], with a wide spectrum of effects ranging from marked and rapidly reversible renal hypoperfusion, which follows each dosing, to a chronic form of renal injury that manifests after one or more years of treatment [7-9], and may have a progressive course in certain instances [10]. Although acute renal hypoperfusion induced by CsA is no longer a problem, chronic CsA nephrotoxicity remains a major challenge for transplant physicians. Lesions of chronic CsA nephropathy consist of vascular and tubulointerstitial changes, including degenerative hyaline alterations extending from preglomerular areas proximally up the afferent arteriole [11], with progressive narrowing and eventual occlusion of the lumen. Tubulointerstitial changes are narrow stripes of atrophy and fibrosis, mostly corresponding to areas of cortex with afferent arteriolar lesions [12], but can be diffuse. The precise cause of CsA renal toxicity is not known. It may be that sustained glomerular afferent arteriolar vasoconstriction eventually promotes structural alteration and eventual occlusion of preglomerular vessels [5, 10, 13]. This determines an ischemic damage of downstream glomeruli and interstitium that culminates in glomerular obsolescence and interstitial fibrosis. Although prolonged vasoconstriction could contribute to chronic CsA nephropathy by producing chronic ischemia, this relation-

Key words: nephrotoxicity, vasoconstriction, renal transplantation, interstitial fibrosis, endothelin-1, RANTES, MCP-1

Received for publication June 26, 1998

and in revised form September 2, 1998

Accepted for publication September 3, 1998

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Table 1. Characteristic at enrollment of kidney transplant recipients given cyclosporine for more than one year

Age years	44 ± 12 (24–66)
Gender M/F	16/6
Time of CsA treatment before biopsy months	17 ± 4 (13–24)
Dose of CsA at biopsy mg/kg/day	3.42 ± 0.66 (2.21–4.96)
Serum creatinine mg/dl	1.3 ± 0.3 (0.8–2.0)
Proteinuria g/24 hr	0.39 ± 0.48 (0.80–1.62)

Values are mean ± sd, range. CsA is cyclosporine A.

ship has been difficult to demonstrate so far [14]. Attention has been focused in the past on enhanced vasoconstrictors [such as thromboxane A₂, endothelin (ET) and angiotensin II] [7, 15], reduced nitric oxide production [16], or activation of renal sympathetic nerves [17] as mediators of renal hemodynamic changes induced by CsA, but there is no evidence that any of these actually mediated structural changes, specifically tubulointerstitial injury, which, by the way, is seen in only CsA-treated animals subjected to salt depletion [18, 19]. It appears more likely that tubulointerstitial fibrosis associated with CsA treatment is a clearly separate entity that develops independently of the vasoactive effect of the drug.

We therefore designed a study in humans aimed at evaluating the relationship between afferent arteriolar arteriopathy and tubulointerstitial fibrosis induced by CsA, and to get more insights on the factor(s) mediating tubulointerstitial injury and possible mechanisms associated with this lesion.

This study is a part of a clinical trial in kidney transplant recipients on triple immunosuppressive therapy, which includes renal biopsy as "per protocol" (see **Methods**), and is directed towards establishing whether changing immunosuppressive therapy on the basis of histologic findings obtained 12- to 24-months posttransplant provides any advantage in preventing graft function deterioration as compared with patients who do not undergo renal biopsy and continue their conventional triple anti-rejection regimen.

METHODS

Patient characteristics and treatment

Twenty-two consecutive patients (6 female, 16 male) with end-stage renal disease who underwent kidney transplantation at the Ospedale Riuniti di Bergamo were considered for this study (Table 1). They were outpatients regularly followed at the Unit of Nephrology of the Department of Immunology and Clinics of Organ Transplantation and were enrolled between 12 and 24 months post-transplantation. All patients received a first cadaveric transplant and were on maintenance immunosuppression with triple therapy, which included CsA (Sandimmune), prednisone, and azathioprine. CsA was

given preoperatively and throughout the four-day post-operative period by intravenous infusion at a daily dose of 4 mg/kg/day. Thereafter, CsA was administered orally at full dose and was then progressively tapered to maintenance dose within the next three months. The steroid (methylprednisolone) was given preoperatively at the dose of 500 mg intravenously, then orally (prednisolone) in a single morning dose tapered to 16 mg until the end of the third month, and then progressively to 8 mg/day. Azathioprine was given at the dose of 1 to 1.5 mg/kg/day for the entire period. At the time of this study, enrolled patients had normal renal function or mild to moderate renal insufficiency. The study protocol was described in detail to the patients before admission, and informed consent to perform the study and renal biopsy was obtained in each instance. The trial was approved by the appropriate Institutional Review Committee.

Study protocol

Patients were asked to collect urine for measurement of 24-hour protein excretion, starting the day before renal function evaluation. Then all patients underwent renal hemodynamic evaluation by measuring glomerular filtration rate (GFR) and renal plasma flow (RPF) by the plasma clearance of unlabeled iothexol [20] and the renal clearance of para-aminohippuric acid (PAH) [21], respectively. In parallel, the CsA pharmacokinetic profile was also determined.

On the morning of the study, blood was drawn for estimation of serum creatinine concentration and hematocrit values. Renal clearance of PAH was performed under a steady state of diuresis induced by oral water loading and subsequent water intake adjusted to maintain urine flow relatively constant throughout the study. A priming dose, followed by a constant infusion of 20% PAH, was administered. The sustained dose of PAH was started to maintain constant plasma concentration of 0.015 mg/ml. After a 60-minute equilibration period, CsA was given orally, and then six carefully timed urine collections (two hr each) were made. Urine was collected by spontaneous voiding, and blood samples were drawn at the beginning and at the end of each clearance period.

To measure GFR, 5 ml of iothexol solution (Omnipaque 300; Nycomed, Milan, Italy) were slowly injected into the antecubital vein just before CsA administration. Plasma clearance of this x-ray agent was determined by collecting blood samples from the contralateral arm at 120, 180, 240, 300, 450, and 600 minutes. These time schedules were chosen based on analysis of our previous data showing that GFR values obtained by using these sampling points best correlated with renal inulin clearance [20].

Moreover, before CsA administration, at 30 and 60 minutes after CsA administration, and at hourly intervals thereafter for 12 hours, blood samples were collected

from a peripheral vein for measurement of CsA levels in the blood.

A week after the renal hemodynamic evaluation, patients were again admitted to the Unit of Nephrology to perform biopsy of the kidney allograft. In all cases, the renal biopsy was done not because of renal dysfunction, but as part of the research protocol to assess the possible presence of chronic CsA injury and thus to modify the dosage schedule accordingly.

Renal protocol biopsy

Kidney graft tissue was processed for light microscopy by fixation in Dubosq-Brazil fluid and was embedded in paraffin. Specimens were stained with Masson's trichrome, hematoxylin and eosin, and periodic acid Schiff reagent. Focal and segmental glomerular sclerotic lesions were defined as an increase of mesangial matrix substance associated with capillary wall collapse. Glomerular damage, tubular changes (atrophy, casts, and dilation), interstitial changes (fibrosis and inflammation), and vascular lesions were graded from 0 to 3+ (0 = no changes, 1+ = changes in less than 25% of the sample, 2+ = changes affecting 25% to 50% of the sample, and 3+ = more than 50% changes affecting the sample). All renal biopsies were examined by a pathologist unaware of the clinical and laboratory data.

By light microscopy the patterns were categorized into CsA nephropathy, chronic rejection, and no lesions.

CsA nephropathy. This condition was defined according to the following changes: (a) tubular (isometric vacuolization, inclusion bodies corresponding to giant mitochondria, microcalcification, and irregular foci of tubular atrophy); (b) interstitial (striped or patchy fibrosis); (c) vascular—early lesions (vacuolization and clear cell transformation of smooth muscle cells) and vascular—late lesions (circular nodular hyaline deposits in the afferent arteriolar wall, narrowing or occlusion of vascular lumen); and (d) glomerular (sclerosis or ischemic collapse, juxtaglomerular apparatus hyperplasia).

CsA nephropathy was diagnosed when striped tubular atrophy and interstitial fibrosis were found alone or in association with any of the morphologic lesions described above [22, 23].

Chronic rejection. This condition was defined according to the following changes: (a) tubular (diffuse tubular atrophy, wrinkling and thickening of tubular basement membrane, and simplification of the epithelial cells); (b) interstitial (fibrosis and mononuclear cell inflammation); (c) vascular (arteriosclerosis with intimal proliferation and thickening of vessel wall of interlobular and arcuate arteries, elastic lamina fragmentation); and (d) glomerular (wrinkled, thickened, and collapsed capillary wall and mesangial expansion, sclerosis).

Chronic rejection was diagnosed when diffuse tubular atrophy, diffuse interstitial fibrosis, and interstitial in-

flammation were documented alone or in association with arteriosclerotic lesions or glomerulopathy [22, 24].

No lesions. This condition was defined when normal pattern or borderline tubulointerstitial changes were observed.

Preparation of digoxigenin-labeled, RANTES, and monocyte chemoattractant protein-1 riboprobes

Endothelin-1 antisense and sense probes were prepared and labeled by *in vitro* transcription using digoxigenin-labeled uridine triphosphate as previously described [25].

A 310 bp human RANTES (regulated upon activation, normal T cell expressed and secreted) and a 340 bp human monocyte chemoattractant protein-1 (MCP-1) were subcloned into the Bam HI/Sal I and the Bam HI/Hind III sites, respectively, at the pBlue script II SK vector between T3 and T7 promoters. Labeling of RANTES and MCP-1 probes was performed as for ET-1 probes.

Nonisotopic *in situ* hybridization

Dubosq-Brazil fixed, paraffin embedded renal tissue was cut at 4 μ m, sequentially heat fixed (65°C, 30 minutes), and deparaffinized. Sections were then incubated with 5 mmol/liter levamisole for 30 minutes at room temperature, briefly washed in phosphate-buffered saline and water, and immersed in 0.2 N HCl [23]. After permeabilization with proteinase K (40 μ g/ml; Sigma, St. Louis, MO, USA), slides were postfixed in 1.5% paraformaldehyde/1.5% glutaraldehyde for one minute, dehydrated through a graded ethanol series, and air dried. Sections were then overlaid with a hybridization mixture consisting of 0.5 ng/ μ l of various digoxigenin-labeled RNA probes, 2 $\times$ SSC, 10% dextran sulfate, 1 $\times$ Denhardt's solution, and 0.1 mol/liter sodium phosphate covered with sealed coverslips and incubated overnight in a moist chamber at 45°C for ET-1, 55°C for RANTES, and 50°C for MCP-1. After hybridization, slides were washed in 0.2 $\times$ SSC, were treated with a blocking solution (50 mg/ml skimmed dried milk, 150 mmol/liter NaCl in 100 mmol/liter Tris-HCl, pH 7.8) at room temperature for 25 minutes, and were finally incubated for 45 minutes at 37°C with an antidigoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) diluted 1:1000. Color development was performed with freshly prepared substrate solution (nitro blue tetrazolium salt and X-phosphate-5-bromo-4-chloro-3-indolyl phosphate 0.4 mol/liter in 0.1 mol/liter Tris-HCl, 0.1 mol/liter NaCl, 50 mmol/liter MgCl₂, pH 9.5). Slides were then mounted in 60% glycerol and examined by light microscopy. Negative controls included hybridization step using the sense probe and omission of either the antisense RNA probe or the antidigoxigenin antibody.

To allow a semiquantitative evaluation of ET-1,

RANTES, or MCP-1 mRNAs, the staining intensity was graded as 0 (not detectable), 1 (minimal), 2 (moderate), and 3 (strong).

Analytical procedures

Plasma concentrations of iohexol were determined by high-performance liquid chromatography (HPLC) as described previously [20]. The plasma profiles were analyzed by a one-compartment open model system, and the calculated clearance of iohexol (as an index of GFR) was corrected according to the Brochner-Mortensen formula [26]. Plasma and urine concentrations of PAH were measured by the colorimetric method of Smith et al [27], and renal clearance was calculated according to the standard formula.

Blood CsA concentration was determined by HPLC, as previously described [28]. Results were expressed as ng per ml. The blood concentration-time profile of CsA was recorded for all patients together with trough level (blood concentration before the next administration). The area under the blood concentration curve from the time equals zero to the last sampling point [12 hr; area under the curve (AUC) from 0 to >12] was calculated by the trapezoidal rule.

Statistical analysis

Data are expressed as mean $\pm$ SD and range as specified. Results were analyzed using one-way analysis of variance, and specific comparisons were performed by two-tailed Student's *t*-test for unpaired data using the Bonferroni correction. Statistical analysis was performed using the software package StatView (Abacus Concepts Inc., Berkeley, CA, USA).

RESULTS

Incidence and features of histologic lesions

CsA nephropathy. Ten out of 22 patients had evidence of CsA-induced nephrotoxicity at histological examination, as defined in Methods (Table 2). In all of these patients, irregular foci or stripes of tubular atrophy accompanied by fibrosis were the predominant findings. Areas of atrophic tubules embedded in a fibrotic interstitium were adjacent to areas exhibiting normal tubules. Some tubular cells showed fine vacuolization of the cytoplasm. In five patients, a very mild interstitial inflammation was seen. Glomerular lesions, as focal-segmental sclerosis and ischemic collapse, were documented in four patients. Minimal to mild arteriopathy was seen in two patients. This was characterized by vacuolization of smooth muscle cells and necrosis of individual endothelial and smooth muscle cells associated with nodular protein deposits in place of degenerated smooth muscle cells.

Chronic rejection. At light microscopy of the graft biopsy, eight patients had a diagnosis of chronic rejection

(Table 2). In all patients, diffuse tubular atrophy and interstitial fibrosis were present and scored mild to severe. Tubular atrophy was indicated by the thickened, wrinkled tubular basement membranes and was associated with extensive interstitial fibrosis. In the fibrotic interstitium, a sparse mononuclear cell infiltrate was present in almost all patients. Glomerular lesions were found in two patients and were characterized by focal and segmental sclerosis in addition to wrinkling and thickening or collapse of capillary walls. In six patients, mild to moderate vascular alterations were documented. The vessel walls, particularly of the interlobular and arcuate arteries, were markedly thickened. Intimal hyperplasia, similar to that found in classic arteriosclerosis, was shown in these vessels.

No lesions. Four patients had a normal kidney graft at histological examination (Table 2). In three of them, very minor tubular atrophy and interstitial fibrosis were observed. Interstitial inflammation was also documented in one case. No other histological alterations, including glomerular and vascular lesions, were found in any of these four patients.

Characteristics of CsA pharmacokinetics

Table 3 shows CsA pharmacokinetic parameters, including trough concentration and AUC, in the three groups of patients. Mean trough CsA level was 24% and 20% higher in patients with CsA nephrotoxicity than in those with chronic rejection or no lesions at renal biopsy, respectively. Similarly, the total daily exposure to CsA, measured as AUC, was elevated in patients with CsA nephrotoxicity (12% and 23%) as compared with patients with chronic rejection or no lesions at biopsy. Despite numerically higher trough CsA and AUC, values in patients with CsA nephrotoxicity were not significantly different as compared with values in the two other groups.

Renal function

As shown in Table 3, serum creatinine level was normal in all but two patients with CsA nephrotoxicity, whereas it was elevated in seven out of eight patients with chronic rejection.

In patients with CsA nephrotoxicity, the mean GFR was 44% higher than in those with chronic rejection and was comparable to GFR values in patients with no renal lesions at histological examination (Table 3). This would suggest that these patients were studied at an early stage of CsA toxicity. On the other hand, the mean renal plasma flow (RPF) value was similar in patients with CsA nephrotoxicity and in those with chronic rejection. In both instances, RPF was numerically but not significantly lower than in patients with no lesions at renal biopsy.

Two patients in the CsA nephrotoxicity group and one

Table 2. Individual kidney graft histopathology scores by conventional light microscopy analysis

	Scores				
	Glomerulosclerosis	Tubular atrophy	Interstitial fibrosis	Interstitial inflammation	Vascular lesions
CsA nephrotoxicity					
O.L.	1	2.5	2.5	1	1
M.M.	0	1	1	0.5	0
S.G.	1	1	1	1	0
J.B.	0	1	1	0	0
M.G.	1	1.5	1.5	0	0
M.A.	0	1	1	1	0
C.M.	0	2	2	0.5	1
C.G.	0	1	1	0	0
T.E.	1	1	1	0	0
P.G.	0	1	1	0	nv
Mean	0.4	1.3	1.3 ^a	0.4 ^b	0.2 ^b
Range	(0-1)	(1-2.5)	(1-2.5)	(0-1)	(0-1)
Chronic graft rejection					
P.M.	1	1	1	2	1
P.A.	0	2.5	2.5	1	2
G.S.	0	1.5	1.5	1	1
P.G.	0	1	1	2.5	1.5
Z.G.	0	1	1	1	nv
T.D.	1	2	2	2	1
F.G.	0	0.5	0.5	0.5	0
A.B.	0	0.5	0.5	0.5	2
Mean	0.3	1.3	1.3	1.3 ^a	1.2 ^c
Range	(0-1)	(0.5-2.5)	(0.5-2.5)	(0.5-2.5)	(0-2)
No lesions					
L.R.	0	1	0.5	0	0
B.C.	0	0.5	0.5	0.5	0
G.S.	0	0	0	0	0
M.M.	0	0.5	0.5	0	0
Mean	0.0	0.5	0.4	0.1	0.0
Range	(0-0)	(0-0.5)	(0-1)	(0-0.5)	(0-0)

Abbreviation: n.v. = no vessels in the specimen.

^a $P < 0.05$ vs. no lesions^b $P < 0.05$ vs. chronic graft rejection^c $P < 0.05$ vs. no lesions

Table 3. Cyclosporine A (CsA) pharmacokinetic parameters, renal function and proteinuria in the three groups of patients identified according to kidney graft biopsy examination

	CsA nephrotoxicity	Chronic graft rejection	No lesions
Cmin ng/ml	115 ± 49 (68-221)	93 ± 41 (55-184)	96 ± 26 (57-115)
AUC ng/ml × h	3301 ± 909 (2328-5331)	2948 ± 1529 (1466-6280)	2681 ± 899 (1662-3641)
S _{cr} mg/dl	1.2 ± 0.2 (0.8-1.8)	1.5 ± 0.4 (1.0-2.0)	1.1 ± 0.3 (0.8-1.4)
GFR ml/min/1.73 m ²	74.85 ± 22.47 (38.75-103.01)	51.89 ± 16.24 (24.31-68.55)	77.71 ± 22.45 (49.50-100.73)
RPF ml/min/1.73 m ²	323 ± 91 (137-444)	295 ± 76 (187-382)	329 ± 47 (359-460)
Proteinuria g/24 hr	0.45 ± 0.57 (1.57-0.08)	0.43 ± 0.50 (1.62-0.11)	0.18 ± 0.06 (0.13-0.26)

Abbreviations are: Cmin, CsA blood trough level; AUC, CsA area under the blood concentration-time curve; S_{cr}, serum creatinine concentration; GFR, glomerular filtration rate; RPF, renal plasma flow.

in the chronic rejection group had proteinuria (defined as more than 0.5 g/24 hr protein excretion; Table 3).

In situ hybridization for ET-1, RANTES, and MCP-1 mRNAs of protocol biopsies

Table 4 summarizes the intensity scores of *in situ* hybridization staining for ET-1, RANTES, and MCP-1 mRNAs in the three groups of patients. In patients with CsA nephrotoxicity, intense staining for ET-1 mRNA was found in tubular epithelial cells but not in glomeruli,

which were virtually negative for the ET-1 mRNA signal (Fig. 1). A high-intensity signal for ET-1 mRNA was mainly observed in the epithelial cells of those tubules located in areas of interstitial fibrosis. At variance, no staining for ET-1 mRNA was documented in tubuli and glomeruli of patients with chronic rejection. Very minor ET-1 mRNA staining intensity was seen in some patients with no renal lesions at light microscopy. In order to evaluate the frequency of positive *in situ* hybridization in the different groups, biopsies showing a staining inten-

Table 4. Individual scores of tubular and glomerular staining for ET-1, RANTES, and MCP-1 mRNAs by *in situ* hybridization in the three groups of patients

	ET-1 mRNA		RANTES mRNA		MCP-1 mRNA	
	Score		Score		Score	
	Tubuli	Glomeruli	Tubuli	Glomeruli	Tubuli	Glomeruli
CsA nephrotoxicity						
O.L.	3	0	3	0	1	0
M.M.	2.5	0	0	0	1.5	0
S.G.	0	0	0	0	1	0
J.B.	0	0	0	0	0	0
M.G.	2	0	0.5	0	1.5	0
M.A.	1.5	0	1	0	0.5	0
C.M.	0.5	0	3	2	2	0
C.G.	nd	nd	3	1	1.5	1
T.E.	2	0	2	0	1	0
P.G.	0.5	0	2	0	0	0
Mean	1.3*	0	1.5	0.3	1.0*	0.1
Range	(0-3)	(0-0)	(0-3)	(0-2)	(0-2)	(0-1)
Chronic graft rejection						
P.M.	0	0	1.5	0	0	0
P.A.	0.5	0	1	0	0	0
G.S.	0	0	0.5	0	1	0
P.G.	0	0	0	0	0	0
Z.G.	0	0	0	0	0	0
T.D.	0	0	nd	nd	nd	nd
F.G.	0	0	1	0	0.5	0
A.B.	0	0	0.5	0	0	0
Mean	0.1	0	0.6	0	0.2	0
Range	(0-0.5)	(0-0)	(0-1.5)	(0-0)	(0-1)	(0-0)
No lesions						
L.R.	0.5	0	nd	nd	nd	nd
B.C.	0.5	0	1	0	nd	0
G.S.	0.5	0	0.5	1	1	0
M.M.	0	0	0.5	0	0.5	0
Mean	0.4	0	0.7	0.3	0.8	0
Range	(0-0.5)	(0-0)	(0-1)	(0-1)	(0.5-1)	(0-0)

Abbreviation: nd is not done.

* $P < 0.05$ vs. chronic graft rejection

sity of more than one (minimal) in two or more tubuli were considered positive. As shown in Figure 2, in patients with CsA nephrotoxicity, 55% of biopsies stained positively for ET-1 mRNA at the tubular level, whereas all of those from patients with chronic rejection or no lesions at renal biopsy were negative.

As for RANTES mRNA *in situ* hybridization, an intense staining signal was found in tubuli of patients with CsA nephrotoxicity, whereas glomeruli stained minimally for this chemokine (Table 4 and Fig. 3). Strong RANTES mRNA expression was observed in interstitial inflammatory cells (Fig. 3). Tubular staining of RANTES mRNA was also observed in the biopsies from patients with chronic rejection, but the intensity was lower. No glomerular staining of RANTES was documented in these patients. Minimal tubular and glomerular staining was found in some patients with no renal lesions at light microscopy. The frequency of positive RANTES mRNA staining at the tubular level was 60%, 42%, and 33% in patients with CsA nephrotoxicity, chronic rejection, and no renal biopsy lesions, respectively (Fig. 2).

As shown in Figure 4, most patients with CsA nephro-

toxicity had a mild MCP-1 mRNA signal localized in the tubular epithelial cells. Only one patient stained minimally for MCP-1 mRNA in the glomeruli (Table 4). At variance, MCP-1 mRNA staining was almost negative in the tubuli of patients with chronic rejection at light microscopy, being minimally stained in only one case. None of these patients had staining for MCP-1 mRNA at the glomerular level. Minimal tubular MCP-1 mRNA staining was also found in one patient with no renal lesions at light microscopy. As for the frequency of tubular staining, 70% of biopsies from patients with CsA nephrotoxicity were positive for MCP-1 mRNA, and only 14% of those from patients with chronic rejection (Fig. 2).

DISCUSSION

Here we have shown that 10 out of 22 kidney transplant recipients who underwent protocol biopsy had CsA-induced nephrotoxicity at histological examination. The morphological findings were supported by CsA pharmacokinetic parameters showing higher AUC and

Fig. 1. Photomicrograph showing a representative distribution of endothelin-1 (ET-1) mRNA by *in situ* hybridization in kidney sections from a patient with cyclosporine A (CsA) nephrotoxicity at renal biopsy. Intense staining was observed in tubular epithelial cells (a, magnification $\times 90$; b, $\times 360$; and c, $\times 180$). Negative control using ET-1 sense probe in an adjacent kidney section is shown (d, $\times 180$).

trough CsA blood levels in these patients than in those with histologically diagnosed chronic rejection. Renal function, however, was well preserved, as documented by GFR values comparable to those of patients with normal renal biopsy. Defining the role of CsA on long-term renal structure and function changes after transplantation is a difficult task. Chronic rejection, recurrent renal disease, and other factors, including hypertension, hyperlipidemia, and age, can lead to progressive renal structural and functional deterioration [29]. No studies have attempted to establish the role, if any, of CsA in isolation, and both structure and function were never measured together in the same patients. Here, we combined structural, functional, and pharmacokinetic studies to try to evaluate the impact of chronic CsA administration on kidney transplant recipients.

In all patients with light microscopy evidence of CsA nephrotoxicity, we found tubular atrophy and interstitial fibrosis, whereas glomerular and arteriolar lesions were less frequent. These findings are consistent with previous

data in patients with autoimmune diseases, including insulin-dependent diabetes of recent onset, uveitis, psoriasis, Sjögren's syndrome, or polychondritis, given relatively high doses of CsA [30]. In these categories of patients, tubulointerstitial injury was the most common feature at renal biopsy, and lesions were not related to the number of glomeruli with global or segmental sclerosis nor to the degree of arteriolar injury. On the other hand, the glomerulosclerosis reported in heart transplant recipients given CsA was most likely due to extensive tubulointerstitial and arteriolar injury [8, 13, 31] in patients who were exposed to very high doses of CsA soon after transplantation. Collectively, these findings tend to suggest that unless one uses very high doses of CsA, tubulointerstitial injury appears independently from the acute ischemic insults to afferent arterioles.

Our patients with biopsy evidence of CsA nephrotoxicity probably had some degree of renal hypoperfusion as reflected by a lower than normal mean RPF, which was never found in patients with no lesions at renal

Fig. 2. Frequency of positive tubular staining for (A) endothelin-1 (ET-1), (B) RANTES (regulated upon activation, normal T cell expressed and secreted), and (C) monocyte chemoattractant protein-1 (MCP-1) mRNAs in the kidney allograft biopsies from the three groups of transplanted recipients. Staining was considered positive when the score staining intensity was ≥ 1 in two or more tubuli. The numbers indicate positive staining/total biopsies analyzed.

biopsy. One cannot exclude that vasoconstriction in the absence of structural arteriolar lesions contributes to interstitial damage, but in animals given CsA, even at high doses, hemodynamic changes are never associated with major structural interstitial injury [32].

How tubulointerstitial injury induced by CsA develops remains unknown. In an animal model of chronic CsA nephrotoxicity produced by one week of salt depletion,

which mimics the human renal pathology of chronic CsA nephropathy, interstitial fibrosis is mediated, at least partly, through angiotensin II induction of transforming growth factor- $\beta 1$ (TGF- $\beta 1$) expression [33]. Indeed, in these rats, angiotensin-converting enzyme inhibition, as well as angiotensin II receptor blockade, decreased the expression of TGF- $\beta 1$ and markedly reduced tubulointerstitial fibrosis. However, the structural changes in this model are probably related to salt depletion that turns on angiotensin II-dependent growth factors for interstitial fibroblasts, lymphokines, and cytokines, thus questioning the role of angiotensin II in chronic CsA interstitial injury in humans who are mostly on a normal-sodium diet.

In this study, we found an intense staining for ET-1, RANTES, and MCP-1 mRNAs selectively localized at tubular epithelial cells in biopsies taken from patients with light microscopy evidence of chronic CsA nephrotoxicity. This was never observed in patients with chronic graft rejection whose tubuli did not stain for ET-1 and MCP-1 mRNAs at all, with only minimal staining for RANTES mRNA on a few occasions. It is tempting to speculate that CsA exerts its renal toxicity on tubular epithelial cells by inducing excessive formation of vasoactive and chemotactic mediators that are then secreted toward the basolateral compartment of tubular cells, thus contributing to the inflammatory reaction and subsequent fibrosis of the interstitium. Data are available that human interstitial fibroblasts challenged with ET-1 increase their proliferation rate and actively synthesize extracellular matrix proteins [34]. It also seems that there is an amplification loop that further enhances the ET-1 synthetic rate [35] to the extent that fibronectin, collagen type IV, and laminin added to culture media of tubular cells stimulate ET-1 production. Beside ET, it is now evident that a number of additional chemoattractants, including RANTES and MCP-1, may act to amplify tubulointerstitial injury by recruiting mononuclear cells, which are then activated to secrete TGF- $\beta 1$ that induces fibroblasts to proliferate and secrete matrix components, contributing to interstitial remodeling and scarring [36, 37].

Various reports proposed a link between CsA and ET. Exposure of bovine aortic endothelial cells to CsA was associated with a concentration-dependent increase in ET release in the incubation medium [38]. Similarly, Bunchman and Brookshire showed a direct effect of CsA on ET release from cultured human endothelial cells [39]. Evidence is also available that proximal tubular cells in culture enhanced ET-1 synthesis when exposed to CsA [40]. Moreover, *in vivo* in rats, CsA intake was accompanied by an increase of urinary ET excretion [41]. On the other hand, no data are available on the ability of CsA to stimulate RANTES and MCP-1 generation by tubular epithelial cells. Because it has been shown that CsA accumulates in the tubular cells during chronic administration [42, 43], one can hypothesize that high

Fig. 3. Representative RANTES mRNA distribution by *in situ* hybridization in the kidney from a patient with CsA nephrotoxicity. Intense staining was found in tubuli (a, magnification $\times 360$), whereas weak staining in parietal epithelial cells of the glomerulus was observed (c, $\times 360$). Strong staining was observed in interstitial inflammatory cells (e, $\times 360$). Negative control was obtained by hybridization with a sense probe (b, d, f, $\times 360$).

intracellular concentration of the drug may activate the synthetic machinery for ET-1 and possibly for the other chemokines. This possibility is supported by the demon-

stration that CsA is an inhibitor of the multidrug resistance transporter, p-glycoprotein, which is constitutively expressed on the epithelial cells of renal tubuli [44].

Fig. 4. Staining for MCP-1 mRNA by *in situ* hybridization in the kidney from a patient with CsA nephrotoxicity. Staining for MCP-1 mRNA was mild either in tubuli (*a*, magnification $\times 180$; *b*, magnification $\times 360$) as well as in glomeruli (*d*, magnification $\times 360$). No signal was detected in the negative control hybridized with a sense probe (*c* and *e*, magnification $\times 360$).

Inhibition of this transporter could allow the accumulation of CsA into tubular cells that theoretically would result in enhanced generation of mediators of tubulointerstitial injury. This is reminiscent of what has been

recently and consistently shown in *in vitro* studies in cultured proximal tubular cells exposed to an excessive amount of proteins for a protracted period of time. Indeed, increasing concentration of delipidated albumin,

IgG, or transferrin causes a concentration-dependent increase in the rate of synthesis of ET-1 [45], RANTES [46], and MCP-1 [47], which are predominantly secreted toward the basolateral compartment of the proximal tubular cells [45–47]. Activation of this pathway is recognized as a critical step for subsequent interstitial inflammation and fibrosis, key events for progression of proteinuric renal disease to end-stage renal failure [37].

We conclude that long-term CsA administration to kidney allograft recipients leads to tubulointerstitial injury independently from its vascular effect. The possible contribution to the development of interstitial fibrosis of inflammatory and growth factors released by tubular cells in which CsA accumulates is proposed. Further research should clarify this potential mechanism of CsA-induced chronic nephrotoxicity and may disclose possible strategies for new medical treatments.

ACKNOWLEDGMENTS

We thank Gianfranco Marchetti for his continuous technical assistance. We are also indebted to Dr. Susanna Tomasoni for her invaluable help in *in situ* hybridization studies. The nursing staff of the Clinical Research Center for Rare Disease "Aldo e Cele Daccò," Federica Airolodi and Ilaria Ciccia, are also acknowledged for the management of transplant patients.

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APPENDIX

Abbreviations used in this article are: AUC, area under the blood concentration curve; CsA, cyclosporine A; ET-1, endothelin-1; GFR, glomerular filtration rate; HPLC, high pressure liquid chromatography; MCP-1, monocyte chemoattractant protein-1; PAH, para-aminohippurate; RANTES, regulated upon activation, normal T cell expressed and secreted; RPF, renal plasma flow; TGF- β 1, transforming growth factor- β 1.

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CHAPTER 7

General Discussion and Conclusions

General Discussion

Evidence is available in animals and humans that in chronic renal diseases tubulo-interstitial damage is functionally important and correlates with declining glomerular filtration rate even better than glomerular lesions do. Mechanism(s) leading to tubulo-interstitial damage are not fully elucidated but they appeared largely due to the obliteration of peritubular capillaries ensuing from sclerosed glomeruli. It has been proposed that excessive reabsorption of filtered proteins activates renal cells to produce vasoactive and inflammatory molecules including ET-1. Exaggerated formation of ET-1 in the kidney may favor progression of renal lesions to the extent that pharmacological manipulation of the ET-1 pathway has a major impact on disease progression. In **chapter 2** we investigated which cells in the kidney form excessive amounts of ET-1 in pathological conditions and possible relationship with the development of renal lesions. We observed focal tubulo-interstitial lesions in most animals by day 14 after renal mass reduction, well before glomerulosclerosis was apparent, possibly indicating that factors not necessarily dependent from glomerular structural lesions can alternatively damage the tubulo-interstitial compartment. Additional support of this evidence rests on findings that concomitant to the onset of proteinuria cortical tubules appeared activated to express ET-1 gene. Tubular ET-1 overexpression was invariably found in the vicinity of interstitial fibrosis, consistent with the possibility that the former contributed at least in part to interstitial injury. With time, renal mass reduction rats developed more severe proteinuria which was invariably accompanied by a further significant increase in tubular ET-1 together with increasingly severe tubulo-interstitial damage so that a strong linear correlation could be established between these variables. Of great interest, renal mass reduction rats studied 14 and 21 days after surgery had a consistent up-regulation of tubular ET-1 gene, as compared to sham-operated controls, which consistently preceded any glomerular staining for ET-1. Despite glomerular cells in culture produce ET-1, glomerular ET-1 mRNA was undetectable *in vivo*, neither during the all study period in sham-operated rats or before day 28 in the experimental group. In renal mass reduction rats studied by day 28 ET-1 mRNA staining was found in glomerular cells i.e. some 10 to 12 days after ET-1 gene up-regulation could be found in tubular cells. Mechanism(s) leading to enhanced expression of glomerular ET-1 gene, at later stages after surgery in this model are far from clear. Two possibilities could be considered: 1) excessive synthesis of PDGF or TGF β (major stimuli for ET-1) can occur after surgical ablation of renal mass; 2) reduction of nitric oxide generation, potent inhibitor of ET-1 synthesis. Finally, peritubular capillary ischemia, always associated with interstitial injury, may "per se" act as a further stimulus to enhance local ET-1 generation (38).

In the remnant kidney model, tubulo-interstitial lesions develop before glomerular sclerosis and up-regulation of ET-1 gene in the kidney parallels the sequential development of renal lesions being expressed first by tubules and only in a subsequent phase by glomeruli. It is possible that ET-1 induced by tubular protein overloading triggers interstitial damage that contributes to subsequent glomerular sclerosis and decline in renal function.

Expression of ET_A and ET_B receptor was also assessed in animals after reduction of renal mass (chapter 3). While renal expression of ET_A receptor mRNA was not changed, such surgical maneuver did significantly up-regulate ET_B receptor gene. This is consistent with previous reports in animals chronically given cyclosporin A (39) or after renal ischemia (40). Type B endothelin receptor expression was selectively induced in glomeruli from rats with mesangioproliferative glomerulonephritis (41) as well as puromycin aminonucleoside-induced nephrosis. Unlike ET_A, which mediates vasoconstriction and cell proliferation and is mainly expressed in arterioles and glomeruli, ET_B receptors appear associated with vasodilation by virtue of their property of inducing nitric oxide (NO) and prostacyclin synthesis, despite some evidence, albeit indirect, of the existence of an additional ET_B receptor subtype rather related to vasoconstriction (20,42). Whether up-regulation of ET_B receptor associated with progressive nephropathies is a counter-regulatory mechanism to overcome the renal vasoconstriction or actually concurs to maintain it, is a matter of current investigation. The issue is further more puzzling in chronic renal insufficiency, a condition consistently associated with less NO formed by the kidney (43). Thus, mechanisms that may operate in normals of ET_B receptor-linked up-regulation of NO synthase machinery may not actually lead to enhanced NO formation in rats with renal mass reduction. Finally, selective increase of ET_B receptor gene expression could possibly be a consequence of compensatory renal hypertrophy, as recently documented in ET_B knockout mice (44). As a follow-up of previous data indicating that chronic blockade of ET_A receptors reduced renal injury in progressive renal disease, we addressed the *in vivo* relevance of ET_B receptor gene up-regulation in renal mass reduction rats. A mixed antagonist of ET_A and ET_B receptors, bosentan, displayed a partial effect on blood pressure and urinary proteins. The key finding of the pharmacological manipulation, however, was the remarkable protective effect of bosentan on renal function and a remarkable improvement of animal survival. A recent study performed with bosentan in renal mass reduction rats substantially confirmed the effect of ET receptor blocker in ameliorating renal function, but did not consider the hard endpoint of animal survival (45). Whether blocking ET_A or both ET receptors should be the treatment of choice for progressive renal injury has not been consistently demonstrated so far due to the lack of a formal compari-

son of the two compounds in the same setting. Only one study evaluated the effect of ET_A versus ET_A/ET_B selective antagonism given as a short treatment (2 month) to animals with renal mass reduction and found that both drugs equally interfered with the development of glomerulosclerosis independently from an effect on systemic blood pressure (46). However, given the role of ET_B receptor on water and sodium handling and the capacity of such receptor subtype to act as a clearance receptor, it would seem reasonable to use selective endothelin antagonists sparing ET_B receptor. Differences between species in function and distribution of ET receptors would also be taken into account. In humans the involvement of the ET_A and ET_B receptor subtypes in the vasoconstriction and sodium retention have been documented through the infusion of ET-1, an unselective ET_A and ET_B receptor agonist, and ET-3, a selective ET_B agonist (47). Equimolar amounts of ET-1 and ET-3 capable to induce similar increases in plasma levels of ET isoforms were used in these experiments. ET-1 infusion induced an increase in mean arterial pressure of 6 mmHg accompanied by strong renal vasoconstriction and sodium retention, while ET-3 infusion had neither systemic nor renal effects, suggesting that the renal vasoconstrictor effect in humans is mainly mediated by ET_A receptor. Altogether these observations tend to support that ET_A receptor blockers would be probably the best class of compounds to be used.

Diabetic nephropathy is another disease condition of progressive renal injury for which ET-1 has been proposed to play a pathogenetic role. This suggestion is based on in vitro data indicating that high glucose concentrations enhance endothelin synthesis (48) and modulate ET-1 receptors at least in glomerular mesangial cells (49). In vivo ET-1 mRNA levels increased in glomeruli from rats with streptozotocin-induced diabetes, while ET_A and ET_B receptor expression was unchanged. Higher than normal expression of ET-1 gene in diabetes was normalized by insulin suggesting that the abnormality was caused, at least in part, by hyperglycemia (29). A logical implication of the above findings was that in diabetic renal disease ET-1 synthesized in excessive amounts by renal cells may contribute to renal damage by at least two distinct pathways of injury one mediated by cortical and inner medulla hypoperfusion (given the extraordinary sensitivity of renal vessels (50) to the vasoconstrictor effect of endothelins) and the other based on the effect of ET-1 of enhancing extracapillary matrix deposition. Our results reported in **chapter 4** indicated that the non-selective ET receptor antagonist, PD142,893, given to diabetic rats with hypertension, overt nephropathy and proteinuria normalized systemic blood pressure and reduced urinary proteins. In addition, the ET-1 antagonist ameliorated renal blood flow which was significantly lower than normal in untreated diabetics. The fact that the endothelin receptor antagonist also remarkably reduced ET-1

mRNA both at glomerular and tubular level supports the possibility of ET-1 autoinduction via ET_B receptor which was documented recently in rat mesangial cells as well as in human endothelial and proximal tubular cells (14).

Compared to PD 142,893, the ACE inhibitor lisinopril was always superior in lowering systemic blood pressure at all time points considered. As observed in previous studies, we found here that blood pressure in the lisinopril-treated diabetics was significantly lower than in normal controls of the same age. Results of renal function studies did not reveal changes in glomerular filtration rate (GFR, whose values are comparable to normal at this stage of diabetes) induced by either treatment. Instead renal blood flow was comparably ameliorated by lisinopril which also showed similar antiproteinuric properties to ET receptor antagonist. It is well known that ACE inhibitors are molecules, that by lowering the tone of the afferent arteriole, effectively enhance the glomerular flow and restore to normal the increased glomerular capillary pressure. Findings that an ET-1 receptor antagonist and an ACE-inhibitor share comparable antiproteinuric properties would possibly imply that either drugs in this model restore glomerular pressure to normal despite the superior systemic antihypertensive effect of the ACE-inhibitor. The concerted action of the ET-1 antagonist we have used in the study of chapter 4 on glomerular arteriolar tone and ultrafiltration coefficient associated with the effect on systemic blood pressure is conceivably responsible for the improved glomerular permeability in experimental diabetes. An alternative possibility to explain the comparable effect on proteinuria of the two molecules derive from data that ACE-inhibitors restore to normal the up-regulated ET-1 gene of diabetic rat glomeruli (51). It is therefore possible that perturbations of glomerular barrier function of experimental diabetes recognize a common ET-1-dependent pathway whose cellular and molecular determinants deserve to be further explored.

Although extremely effective in normalizing urinary proteins and preventing GFR decline in diabetic and non-diabetic conditions, ACE inhibitors do not consistently affect the course of progressive renal disease in all experimental models and in patients at an advanced phase of the disease. Taking advantage of accelerated PHN, a model in the rat that does not completely respond to ACE inhibition, we proved the concept that combination of drugs interfering with different pathways responsible for progressive renal damage could offer more renoprotection than single drugs alone (chapter 5). Studying the accelerated PHN, a very severe model obtained by uninephrectomizing animals at day 7 after disease induction to accelerate renal damage, we found that the ACE inhibitor, trandolapril, limited tubulointerstitial and glomerular damage. By contrast, the endothelin antagonist LU-135252 given daily as a single agent did not prevent proteinuria, renal function impairment and renal structural damage,

despite the fact that it maintained systolic blood pressure within normal values. Lack of efficacy of the ET-receptor antagonist alone in this accelerated model of PHN would indicate that - at least at an early stage of the disease - the major mediator of renal damage is rather AII, than ET-1. If anything, one can speculate that ET-1 may contribute to amplify the damage conceivably in a relatively advanced stage of the disease. This possibility is consistent with findings that excessive ET-1 is formed in the kidney not sooner than eight months after the induction of the disease in PHN, as previously documented by our group (26). To test formally the hypothesis that following a prolonged enough period of heavy proteinuria ET-1 does play a contributory role in the subsequent renal structural damage, possibly favoring interstitial inflammation and fibrosis, we used both trandolapril and the endothelin antagonist LU-135252 in combination. The effect of combining trandolapril with LU-135252 was accompanied by a reduction in proteinuria that reached significantly different values as compared to PHN alone at all time points examined in the course of the study. At variance, neither trandolapril nor LU-135252 given alone, despite numerically reducing proteinuria at all time points examined, did so to an extent that reached statistical significance. The preventive effect of such a treatment regimen as compared to each molecule alone against deterioration in renal function and structural damage was even more remarkable.

The results of this study confirmed the major effect of inhibiting AII in progressive immune nephropathies and at the same time provided the evidence of the better renoprotection of a multidrug approach in a model that is clearly more severe as compared to the ones used so far in similar studies. These findings can in some way be reconciled with previous experimental and human data, which collectively indicated that the process of renal disease progression was linked to the local formation of AII, whose effect of enhancing glomerular hydraulic pressure has been considered instrumental to initial injury. In turn, elevation of glomerular capillary pressure impairs glomerular permselectivity and promotes a complex interplay of events recapitulated by an increased expression of vasoactive and inflammatory molecules in proximal tubular cells activated by protein overload. Of these, upregulation of ET-1 gene expression and enhanced synthesis of the corresponding protein were constant findings of *in vitro* and *in vivo* experiments performed thus far. Given the fact that AII and ET-1 both promote extracellular matrix accumulation in various cells, one may speculate that both contribute to the process of renal scarring. The possibility that AII may directly induce the intrarenal synthesis of ET-1 in progressive renal diseases should be considered. It is well known that AII stimulates the expression of ET-1 gene in endothelial and renal cells. Infusion of AII enhances functional ECE activity in vascular smooth muscle and kidney via the ET_A receptor, whose vas-

cular expression is upregulated by AII in vascular smooth muscle cells, cardiomyocytes and mesangial cells (52). Moreover, part of the mitogenic effect of AII is mediated by ET-1 as suggested by studies with monoclonal antibodies against ET-1 or drugs that block the ET_A receptor. Furthermore, a reciprocal regulation of AII and ET-1 on matrix protein synthesis and cell proliferation via ET_A and AT1 receptors has been suggested in rat mesangial cells (53). In vivo, ET-1 is involved in regulation of ACE activity but not ACE expression via ET_A (54). Whether ET-1 also contributes to the effect of AII in impairing glomerular permeability to macromolecules in animal and human proteinuric nephropathies is, however, controversial. The available data that in animals with progressive nephropathies, ACE inhibitors, beside reducing glomerular protein traffic, also inhibit the exaggerated synthesis of ET-1, together with the in vitro observation that protein overload enhances ET-1 generation in proximal tubular cells, would favor the idea that changes in ET synthetic pathway in the tubules is probably just related to the stimulatory effect of enhanced protein traffic rather than a direct effect of AII. The definitive conclusion to this open question would possibly derive from studies with AII knockout mice, although preliminary data have indicated that in rat models a dose of ACE inhibitor high enough to completely inhibit ACE activity when failed to reduce proteinuria in cases of advanced or particularly severe disease also did not halt disease progression. Furthermore, in type II diabetic patients in an advanced phase of the disease, so advanced that ACE inhibitor failed to reduce proteinuria, the disease progressed despite complete ACE inhibition.

A multidrug approach targeting AII and ET-1 will probably be the way to limit and hopefully avoid renal failure. Even now those patients whose disease responds only partially to ACE-inhibitors are candidates to try an endothelin antagonist in addition, within the context of controlled clinical designs.

Among different human pathologies for which ET-1 has been considered to play a role is CsA nephrotoxicity. In **chapter 6** we combined structural, functional and pharmacokinetic studies to try to evaluate the impact of chronic CsA administration on kidney transplant recipients. Ten out of 22 kidney transplant recipients who underwent protocol biopsy had CsA-induced nephrotoxicity at histological examination and we found tubular atrophy and interstitial fibrosis while glomerular and arteriolar lesions were less frequent. These findings are consistent with previous data in patients with autoimmune diseases, including insulin-dependent diabetes of recent onset, uveitis, psoriasis, Sjögren's syndrome or polychondritis, given relatively high dose of CsA. In these categories of patients tubulointerstitial injury was far the most common feature at renal biopsy, and lesions were not related to the number of glomeruli with global or segmental sclerosis nor to the degree of arteriolar injury. On the other hand, the

glomerulosclerosis reported in heart-transplant recipients given CsA was most likely due to extensive tubulointerstitial and arteriolar injury (55) in patients who were exposed to very high doses of CsA soon after transplantation. Collectively these findings tend to suggest that unless one uses very high doses of CsA, tubulointerstitial injury appears independent from the acute ischemic insult to afferent arterioles.

Our patients with biopsy evidence of CsA nephrotoxicity had probably some degree of renal hypoperfusion as reflected by a lower than normal mean RPF which was never found in patients with no lesions at renal biopsy. One cannot exclude that vasoconstriction in the absence of structural arteriolar lesions contributes to interstitial damage, but in animals given CsA, even at high doses, hemodynamic changes are never associated with major structural interstitial injury.

How tubulo-interstitial injury induced by CsA develops remains unknown. In an animal model of chronic CsA nephrotoxicity produced by 1 week of salt depletion, that mimics human renal pathology of chronic CsA nephropathy, interstitial fibrosis is mediated, at least partly, through AII induction of TGF- β 1 expression to the extent that ACE inhibition as well as AII receptor blockade decreased the expression of TGF- β 1 and markedly reduced tubulointerstitial fibrosis. In this model structural changes are probably related to salt depletion that turns on AII-dependent growth factors for interstitial fibroblasts, lymphokines and cytokines, thus questioning the role of AII in chronic CsA interstitial injury in humans who are mostly on normal sodium diet.

In the present study we found an intense staining for ET-1, RANTES and MCP-1 - the latter being chemokine involved in progressive renal injury - mRNAs selectively localized at tubular epithelial cells in biopsies taken from patients with light microscopy evidence of chronic CsA nephrotoxicity. This was never observed in patients with chronic graft rejection whose tubuli did not stain for ET-1 and MCP-1 mRNAs at all, with only minimal staining for RANTES mRNA in few occasions. It is tempting to speculate that CsA exerts its renal toxicity on tubular epithelial cells by inducing excessive formation of vasoactive and chemotactic mediators that are then secreted toward the basolateral compartment of tubular cells thus contributing to the inflammatory reaction and subsequent fibrosis of the interstitium. Data are available that human interstitial fibroblasts challenged with ET-1 increase their proliferation rate and actively synthesize extracellular matrix proteins (38). It seems also that there is an amplification loop that further enhances ET-1 synthetic rate (56) to the extent that fibronectin, collagen type IV, and laminin added to culture media of tubular cells stimulate ET-1 production. Beside ET, it is now evident that a number of additional chemoattractants, including RANTES and MCP-1, may act to amplify tubulo-interstitial in-

jury by recruiting mononuclear cells which are then activated to secrete TGF- β 1 that induces fibroblasts to proliferate and secrete matrix components, contributing to interstitial remodelling and scarring (57).

Various reports proposed a linkage between CsA and ET. Exposure of bovine aortic and human renal endothelial cells to CsA was associated with a concentration-dependent increase in ET release in the incubation medium. Evidence is also available in proximal tubular cells in culture. Moreover, in vivo in rats chronic CsA administration was accompanied by an increase of urinary ET-1 excretion (58). Since CsA accumulates in the tubular cells during chronic administration, one can hypothesize that high intracellular concentration of the drug may activate the synthetic machinery for ET-1 and possibly for the other chemokines. This is reminiscent of what has been recently and consistently shown in vitro in cultured proximal tubular cells exposed to protein overload. Indeed, increasing concentration of delipidated albumin, IgG or transferrin causes concentration-dependent increase in the rate of synthesis of ET-1, but also of RANTES and MCP-1, predominantly secreted toward the basolateral compartment of the proximal tubular cells (57).

We conclude that long-term CsA administration to kidney allograft recipients leads to tubulointerstitial injury independently from its vascular effect. The possible contribution to the development of interstitial fibrosis of inflammatory and growth factors released by tubular cells in which CsA accumulates is proposed. Further research should clarify this potential mechanism of CsA-induced chronic nephrotoxicity, that may disclose possible strategies for new medical treatments.

Conclusions

Intensive experimental research carried out in the last few years has shed light into a possible role for endothelin as a mediator of acute and chronic renal dysfunction. So far a number of clinical studies have indicated that also in patients with progressive proteinuric nephropathies and acute and chronic renal dysfunction due to CsA treatment, the endothelin pathway is activated as already observed in experimental animals. Convincing pre-clinical observations have established that endothelin receptor antagonists are likely to have a promising profile in the near future for treatment of acute renal dysfunction (mainly the form associated with CsA toxicity) and in the prevention of progressive deteriorating renal function in proteinuric nephropathies. There is room for consideration that endothelin receptor antagonists, by suppressing one of the factors with pro-inflammatory and fibrotic potential, may theoretically be renoprotective

even independently of changes in renal hemodynamics. Endothelin antagonists would be good candidates in a multidrug treatment for patients who do not respond or only partially respond to ACE inhibition. Selective ET_A receptor antagonists should be the more suitable compounds to start with given the prominent role of ET_B receptor of regulating salt and water renal handling. One may anticipate that recent developments in cell biology and pharmacology of endothelins will translate in the next few years in a novel treatment for those renal diseases for which evidence of excessive local formation of the hormone has been documented in the most consistent fashion.

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CHAPTER 8

Summary

Experimental and human proteinuric progressive nephropathies are associated with tubulo-interstitial injury whose pathogenesis is still undefined. Studies have established that an excessive and sustained protein traffic through the nephron, as a consequence of high glomerular capillary pressure, is invariably associated with the progress of the disease. Exuberant protein excretion induces forced reabsorption by proximal tubular cell, that changes its phenotype overexpressing vasoactive and inflammatory molecules. Release of such molecules towards the interstitium triggers an interstitial inflammatory reaction that precedes renal scarring. Among mediators challenged by protein overload is endothelin-1 (ET-1), a peptide with vasoconstrictor and proliferative properties, which, by virtue of its chemotactic activity, attracts blood monocytes and stimulates them to produce cytokine, which would further amplify the interstitial inflammatory reaction. These features together the bulk of evidence that ET-1 renal gene expression and synthesis are increased in proteinuric progressive nephropathies has stimulated research on ET-1 and on endothelin receptor antagonists, as novel therapeutic tools to slow renal disease progression (reviewed in **chapter 1**).

In this thesis the definition of kidney cells overexpressing *in vivo* ET-1 during the course of the experimental renal disease is accomplished. Furthermore, attempts to slow progression of renal disease in progressive renal injury with endothelin receptor antagonists alone or in combination with angiotensin converting enzyme inhibitors are pursued. Finally, the possible contribution of ET-1 to the development of interstitial fibrosis and inflammation in renal transplant patients chronically receiving CsA is addressed.

In **chapter 2**, we first evaluated the cellular origin of excessive renal ET-1 production in the renal mass reduction model and then related ET-1 distribution to the development of kidney lesions. Animals with renal mass reduction and control rats were studied early after surgery (day 7,14,21,28). Renal mass reduction rats developed proteinuria accompanied first by tubulo-interstitial changes and subsequently by glomerulosclerosis. A parallel increase of renal endothelin-1 gene expression, evaluated by nonradioactive *in situ* hybridization with a rat ET-1 riboprobe, and synthesis of the corresponding peptide assessed as urinary ET-1 excretion was observed in renal mass reduction rats versus controls from day 14 consistent with the distribution of tubulo-interstitial lesions. At day 14, overexpression of ET-1 mRNA staining was mainly localized to the cytoplasm of tubular cells, whereas glomeruli were negative. At day 28, ET-1 expression further increased in renal mass reduction rats as compared with controls, and the staining was evident also in glomeruli. Thus, in renal mass reduction, a progressive up-regulation of ET-1 occurs during the development of renal injury, that first involves the tubules and, only in a subsequent phase, the glomeruli.

In **chapter 3**, we first studied renal ET_A and ET_B receptor gene expression in rats with remnant kidney on days 7, 30 and 120 after the surgical procedure. While renal expression of ET_A was unaffected, ET_B receptor gene was significantly up-regulated with time in rats with remnant kidney, being 3.5-fold and sixfold higher than sham-operated rats at days 30 and 120. We also evaluated whether bosentan, a nonpeptidic ET_A and ET_B receptor antagonist, offered better protection against renal disease progression than reported for ET_A-selective blockers and whether it improved survival in animals with renal ablation. Rats with renal mass reduction were given bosentan orally or its vehicle (carboxymethyl cellulose) from day 7 after surgery and were followed until the death of the vehicle-treated animals. Bosentan only partially prevented the increases in blood pressure and proteinuria, but had a remarkable protective effect on renal function and significantly prolonged animal survival, suggesting that blocking both renal ET_A and ET_B receptors might have implications in the treatment of human progressive nephropathies.

In **chapter 4**, we investigated whether an unselective ET_A/ET_B receptor antagonist, PD 142,893, was renoprotective when given to streptozotocin diabetic rats when animals were already proteinuric. The effect of PD 142,893 was compared with that of an ACE inhibitor, lisinopril, known to retard progressive renal disease in experimental and human diabetes. PD 142,893 normalized systemic blood pressure, reduced urinary protein and albumin excretion, and ameliorated renal blood flow in diabetic rats, but it did not affect such parameters in control rats. Lisinopril had a renoprotective effect comparable to PD 142,893, although a better control of systemic blood pressure was achieved. Northern blot analysis of ET-1 mRNA revealed upregulation of ET-1 gene in the diabetic kidney. Similar results were obtained by non radioactive in situ hybridization in glomeruli and tubuli of diabetic rats. Both treatments remarkably attenuated exaggerated renal ET-1 gene expression. These data suggest that ET-1 is a contributory mediator of kidney damage in diabetes and indicate that ET receptor antagonists may represent a new therapeutic mean for treatment of progressive diabetic nephropathy.

In **chapter 5**, we compared the effect of an ET_A receptor antagonist and an ACE-inhibitor given as single therapies with a combination of the two drugs in rats with accelerated passive Heymann nephritis, which mimics advanced phases of human membranous nephropathy. Animals were treated from day 7 to 8 months with the ET_A receptor antagonist LU-135252 or the ACE-inhibitor trandolapril or the combination of the two. Either LU-135252 or trandolapril given alone prevented the increase in systolic blood pressure. Com-

bined therapy was even more effective than single drugs. While LU-135252 and trandolapril reduced proteinuria by 23 to 25% the drug combination resulted in 45% lowering of urinary proteins. Serum creatinine was significantly decreased by the combination, but not by the single drugs. Glomerulosclerosis and tubulointerstitial damage were more reduced by combined therapy than by LU-135252 or trandolapril alone. These data suggest that contemporary blocking angiotensin II (AII) and ET-1 in an accelerated model of PHN had an additive renoprotective effect than single blocking AII or ET-1 and would represent a therapeutic advantage for renal disease patients who do not completely respond to ACE inhibitors.

In **chapter 6**, we addressed the issue of the cellular origin and mediators of tubulointerstitial changes after chronic CsA administration in kidney transplant patients. As a part of a clinical trial in kidney transplant recipients on triple immunosuppressive therapy (CsA, azathioprine and steroid) which includes renal biopsy as "per protocol", 22 patients enrolled between 12 and 24 months post-transplantation underwent renal hemodynamic evaluation by measuring glomerular filtration rate and renal plasma flow by the plasma clearance of unlabeled iothelol and the renal clearance of para-aminohippuric acid, respectively. In parallel, the CsA pharmacokinetic profile was also determined. A week later, a protocol biopsy of kidney graft was performed. Light microscopy examination and localization of endothelin-1, and two chemokines, RANTES and monocyte chemoattractant protein-1 gene expression by in situ hybridization in the graft specimens were evaluated and related to the pattern of histologic lesions. Ten out of 22 kidney transplant recipients who underwent the protocol biopsy had CsA nephrotoxicity, eight had chronic rejection, and four had no lesions at histological examination. The total daily exposure to CsA was higher in patients with CsA nephrotoxicity than in those with chronic rejection or no lesions at biopsy. Renal function was preserved in the CsA toxicity group as compared with the chronic rejection group, despite some degree of renal hypoperfusion. Tubular atrophy and striped interstitial fibrosis were found in all patients with light microscopical evidence of CsA nephrotoxicity, whereas glomerular and arteriolar lesions were less frequent. Intense staining for endothelin-1, RANTES, and monocyte chemoattractant protein-1 mRNAs selectively localized at tubular epithelial cells was found in biopsies taken from patients with CsA nephrotoxicity, but not in the chronic graft rejection group, whose tubuli had only minimal staining for RANTES mRNA on a few occasions.

Long-term CsA administration to kidney allograft recipients leads to tubulointerstitial injury independently of its vascular effect. The possible contribution to the development of interstitial fibrosis of inflammatory and growth factors released by tubular cells in which CsA accumulates is proposed.

Curriculum vitae

Ariela Benigni was born in Bergamo, Italy in 1955. She achieved her university degree in Biological Science at Università degli Studi di Milano in 1979. From October 1979 to November 1981 she had a fellowship from the "Associazione Bergamasca per lo Studio delle Malattie Rare" at the Laboratory of Division of Nephrology and Dialysis, Ospedali Riuniti di Bergamo, directed by Prof. Giuliano Mecca, in the research group of Prof. Giuseppe Remuzzi. After a fast - doctoral fellowship at the Centre Regional de Transfusion Sanguigne de Strasbourg, he began her career at the Mario Negri Institute in Bergamo participating to the unique group of basic scientist and clinicians working with Prof. Giuseppe Remuzzi in the field of renal disease progression. She was particularly involved in the research of mediators of progressive renal injury from the early studies on prostaglandins and arachidonic acid metabolites to the more recent interest on endothelin from the prospective of pathophysiology and therapeutic interventions. One of her recent major research interest is to find out effective methods of gene transfer to the kidney and to achieve graft tolerance after manipulation of the kidney with genes encoding immunomodulatory proteins. She was appointed in 1991 as Scientific Secretary of Mario Negri Institute in Bergamo, from 1994 to 1999 she was the Head of the Laboratory of Vasoactive and Inflammatory Mediators of Tissue Damage and from January 2000 she became Head of Department of Molecular Medicine. From 1996 to 1998 she was Associate Editor of the Journal of Nephrology and in 1996 she became Vice President and member of the Council of the "Associazione per la Ricerca sulle Malattie Rare". In her over 130 publications, including original articles, reviews and monographs, she has made major advances in many areas of nephrology, with particular focus on platelet endothelial interactions, vascular prostaglandin biology, coagulation and renal disease, experimental models of glomerular damage, and particularly far-reaching are her contributions to understanding of prostaglandin metabolism in pregnancy, renal vascular biology in uremia, the role of endothelin in renal disease progression and induction of renal graft tolerance by gene transfer approach. She has been invited to give a lecture to 23 international meetings.

Acknowledgments

I am very indebted and grateful to my mentor Prof. Giuseppe Remuzzi. He encouraged me since the beginning to pursue the research work and continuously favored my intellectual growth. He never tired to infuse me his contagious enthusiasm in the day-by-day work. He was always available to discuss the results as well as strategies to achieve a real integration between basic scientist and clinician's competence.

I gratefully acknowledge Prof. Karel Leunissen for the opportunity he gave me to discuss my PhD thesis in the prestigious Universiteit Maastricht.