Angiotensin Receptor Blockade and Apoptosis in Chronic Allograft Nephropathy

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Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Medizin genehmigten Dissertation.

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Die Dissertation wurde am 12.03.2004 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 02.02.2005 angenommen.
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1. Introduction

Kidney transplantation is the treatment of choice for end-stage renal disease. Kidney graft survival has reached 90-95% after one year (Terasaki P.I. 1993), due to improvement in immunosuppression, organ preservation, tissue typing, standardization of operative techniques and better post operative follow-up of the patients. However, long term allograft survival was only marginally prolonged (Hostetter T.H. 1994; Colvin R.B. 2003). Approximately 50% of all grafts that survive for 1 year, develop graft failure within 10 years after transplantation (Kreis H.A. 2001). The half-life of cadaveric kidney allografts has been consistent at 7.5-9.5 years, and 50-80% patients ultimately return to dialysis after kidney transplantation (Ponticelli C. 2000).

Chronic allograft nephropathy (CAN), together with death with functioning graft, are among the most important causes of late renal allograft loss. Around 35-60% of kidney graft loss is due to CAN (Paul L.C. 1990). There is no treatment available so far to inhibit or prevent CAN.

1.1. Chronic allograft nephropathy

In 1955, Hume et al. first described a case in which kidney rejection with obliteration of the arteries, developed within six months after transplantation (Hume D.M. 1955). Systematic investigation of late rejection by Porter et al. and Jeannet et al. revealed that arterial intimal fibrosis was frequent and probably represented a reaction to immune injury, perhaps due to alloantibodies (Porter K.A. 1963; Jeannet M. 1970). By the late 1960s and early 1970s, transplant glomerulopathy distinct from recurrent glomerulonephritis was recognized, and attributed as a variable feature of CAN (Zollinger H.U. 1973).

CAN is defined as a progressive impairment of renal allograft function within months to years after transplantation, eventually leading to graft failure. It is accompanied by characteristic histological features (Hostetter T.H. 1994), independent of acute
rejections, overt drug toxicity, and recurrent or de novo specific renal disease entities (Halloran P.F. 1999).

Alloantigen dependent factors such as frequency of acute rejection, HLA histocompatibility, mismatches, as well as alloantigen independent factors such as arterial hypertension, disorders of lipid metabolism, or age of the recipient/donor are involved in the pathogenesis of CAN.

Clinically, CAN manifests as a slow and progressive decline in glomerular filtration rate (GFR), usually accompanied by proteinuria and arterial hypertension (Modena F.M. 1991). The onset of proteinuria is an early sign of CAN, and parallels the severity of the disease (Cosio F.G. 1999). Renal insufficiency with a progressive decline of creatinine clearance develops at late stages of CAN (Kasiske B.L. 1991).

The pathology of CAN is non-specific and requires exclusion of specific entities such as recurrent glomerular disease. The cardinal histomorphological feature of CAN is a vasculopathy with fibroproliferative vascular lesions (Hostetter T.H. 1994). The vascular lesions affect the whole length of the arteries in a patchy pattern. There is concentric myointimal proliferation resulting in fibrous thickening and the characteristic “onion skin” appearance of the intima in small arteries. Other findings include endothelial swelling, subendothelial foam cell accumulation, disruption of the internal elastic lamina, hyalinosis and medial thickening. In addition, a persistent focal perivascular inflammation can often be seen. Fibrous intimal thickening in arteries involves smooth muscle cell proliferation and increased lipid and glycosaminoglycan-rich matrix in the intima, which ultimately narrows the lumen. However, part of the luminal occlusion in diseased vessels is due to failure of the vessel wall to dilate in response to decreased flow, and represents exhaustion of the normal remodeling process, possibly due to decreased endothelial function (Ponticelli C. 2000).

In addition to vascular changes, allografts undergoing CAN also demonstrate interstitial fibrosis, tubular atrophy and glomerulopathy. Chronic transplant
glomerulopathy presents with duplication of the basement membranes and increased mesangial matrix. Additionally, glomerular collapse, tubular atrophy, and interstitial fibrosis occur. All these findings represent criteria for the histological diagnosis of CAN and are internationally standardized in the BANFF Classification of Renal Allograft Pathology (Cosio F.G. 1999).

Growth factors have been implicated in the development and progression of such changes. One of them, transforming growth factor β (TGF-β), has been identified as a critical mediator in the progression of interstitial fibrosis in renal allografts and high TGF-β levels have been associated with a more rapid decline in renal function (Cuhaci B. 1999). In addition to its fibrogenic effects, TGF-β may also contribute to the progression of renal disease by its potent pro-apoptotic activity (Huang Y. 2000). Moreover, both cyclosporine A and tacrolimus, the mainstays of immunosuppressive therapy in organ transplantation, appear to stimulate transcription of TGF-β (Shin G.T. 1998; Bicknell G. 2000).

Another important mediator of proliferative responses and tissue remodeling in the kidney graft is the renin angiotensin aldosterone system (RAAS) with angiotensin II (AngII). Angiotensinogen is processed into angiotensin I (AngI) by renin. In a second step AngII is formed from AngII by angiotensin-converting enzyme (ACE). It acts through two receptors: the angiotensin II type1 and type2 receptor (AT₁ and AT₂ receptor)

1.2. Renin angiotensin aldosterone system (RAAS) and CAN

1.2.1. Renin angiotensin aldosterone system

Inhibition of the RAAS with ACE inhibitors or AT₁ receptor blockers (AT₁ receptor antagonists) can delay the progression of chronic renal diseases including CAN (Jafar T.H. 2001; Veelken R. 2001; Parving H.H. 2001; Ecder T. 2001). ACE inhibition reduced proteinuria and ameliorated the progression to end-stage renal disease more effectively than conventional antihypertensive drugs independent of blood pressure lowering effects.
in diabetic and non-diabetic chronic nephropathies (Perna A. 2000). ACE inhibitors and AT₁ receptor antagonists also have been shown to reduce tubular injury in inflammatory, metabolic (Gilbert R.E. 1998), hemodynamic (Wu L.L. 1997) and mechanically induced renal injury (Ishidoya S. 1996). However, the exact mechanisms that protect renal function and slow progression are not known in the case of CAN.

Basically, the RAAS contributes to the regulation of arterial blood pressure, renal hemodynamics, renal handling of sodium, as well as proliferative responses. The juxtaglomerular cells of the kidney produce and store renin, which, when released into the blood stream, forms an enzyme-substrate complex with angiotensinogen, generating AngI. AngI is converted by the ACE to AngII. It acts as a potent vasoconstrictor of arterioles, thereby increasing blood pressure and in the kidney, leads to a vasoconstriction of the efferent arteriole, thus, increasing intraglomerular pressure and GFR. Additionally, it increases the reabsorption of sodium and water into tubular cells, thus increasing blood pressure. Moreover, it stimulates the adrenal gland to secrete aldosterone, thereby inducing increased sodium reabsorption in the distal tubule, which contributes to the development of systemic arterial hypertension. AngII has also proliferation promoting effects through induction of the expression of growth factors such as platelet-derived growth factor (PDGF), epithelial growth factor (EGF), and TGF-β (Naftilan A.J. 1989; Gibbons G.H. 1992; Kagami S. 1994), thus, contributing to the development of interstitial fibrosis. Moreover, it induces the expression of extracellular matrix molecules and reduces matrix metalloproteinase activity (Fogo A.B. 2000; Zhuo J.L. 2000), which leads to an increase of extracellular matrix (ECM). Thus, AngII has systemic effects such as hypertension, as well as local effects such as induction of vasoconstriction and proliferation, leading to tissue fibrosis and deterioration of organ function.

AngII binds to two cell surface receptors: AT₁ and AT₂ receptor. Compared with AT₂ receptors, AT₁ receptors dominate by far in the adult (Chansel D. 1992) and rat (Zhuo J. 1993) kidney; only 5% to 10% of the AngII receptors are type2 receptors (AT₂). AT₁ receptor-associated responses include vasoconstriction, renal salt and water retention,
aldosterone and vasopressin release, effects on GFR and renal blood flow, as well as the stimulation of cell growth, with induction of PDGF-A chain and TGF-β (Stouffer G.A. 1992). The AT$_2$ receptor is expressed at high levels during development in embryonic and fetal stages, whereas, in adults, it is mainly expressed in the brain, adrenal medulla, uterus, and the heart. However, its expression can be increased during inflammation and ischemia, and suppresses synthesis of DNA, protein, extracellular matrix proteins, as well as cell proliferation (Matsubara H. 1998). The AT$_2$ receptor influences renal function by controlling pressure natriuresis (Lo M. 1995) and decreasing cGMP production in sodium-depleted rats (Siragy H.M. 1996). Furthermore, in addition to regulating organogenesis in fetal organs (Aguilera G. 1994; Shanmugam S. 1995), the AT$_2$ receptor appears to regulate cell proliferation in experimental models of tissue injury or cells in culture (Liu Y.H. 1997; Nakajima M. 1995).

The AT$_1$ receptor can be blocked by Losartan and its analogues (sartan family). Candesartan belongs to this family, and it is a highly potent AT$_1$ receptor antagonist without agonistic properties (Morsing P. 1999) and does not bind to the AT$_2$ receptor. Candesartan was reported to reduce chronic renal allograft injury in Fisher-Lewis rats kidney transplantation models (Mackenzie H.S. 1997) and decreases poteinuria in patients after kidney transplantation (Omoto K. 2003). The AT$_2$ receptor can be blocked by PD123319.

1.2.2 RAAS blockade in CAN

Experimental studies revealed that AT$_1$ receptor blockade significantly delayed chronic graft injury in a rat model of late renal allograft failure (Mackenzie H.S. 1997; Amuchastegui S.C. 1998). Reduction of glomerular capillary hypertension with AT$_1$ receptor antagonist treatment was paralleled by a reduction in proteinuria and prevented of supervening chronic glomerular and tubulointerstitial injury in rat kidney transplantation model (Mackenzie H.S. 1997; Benediktsson H. 1996; Ziai F. 2000). AT$_1$ receptor antagonists treatment not only reduced proteinuria, but also modulated the generation of
TGF-β (Campistol J.M. 1999). As described above TGF-β plays an important role in the
development of fibrosis during CAN, which could be promoted by activation of the
RAAS, that has been shown to increase expression of fibrogenic cytokines such as TGF-β,
endothelin, and PDGF. Furthermore, in a cross-over study comparing losartan (AT₁
receptor antagonist) and the calcium antagonist amlodipine in renal transplant patients,
administration of losartan resulted in lower levels of serum TGF-β levels and a reduced
estimated glomerular hydrostatic pressure (Inigo P. 2001).

However, the mechanisms responsible for the protective effects of inhibition of
RAAS are not clearly defined. Apart from the effects on the renal microcirculation, the
reduction of proteinuria, or the reduction of the growth factor expression, RAAS
inhibition by AT antagonists might also regulate tissue homeostasis and proliferation
through a direct reduction of apoptosis. Thus, we hypothesize that inhibition of the RAAS
is associated with a reduced number of apoptotic cells, resulting in an improved graft
function through an increased number of functioning nephrons as well as reduced
inflammatory processes within the graft tissue. Interestingly, apoptosis has been shown to
be increased during CAN (Wang J. 1997; Strehlau J. 1996). Moreover, the potential to
reduce the number of apoptotic cells by AT₁ receptor antagonists has been shown in a
model of rat cyclosporine nephropathy (Thomas S.E. 1998).

1.3. Apoptosis and CAN

1.3.1. Apoptosis and apoptotic pathway

Apoptosis was first described by Kerr et al. in 1972, and is characterized by a
particular morphological appearance of the cells including blebbing, chromatin
condensation, nuclear fragmentation, loss of adhesion and rounding (in adherent cells),
respectively, as well as cell shrinkage. Biochemical features associated with apoptosis
include internucleosomal cleavage of DNA, leading to an oligonucleosomal “ladder”
(Cohen G.M. 1994), phosphatidylserine (PS) externalization (Martin S.J. 1995), and
proteolytic cleavage of a number of intracellular substrates (Martin S.J. 1995). It is an
active energy consuming process which in balance with mitosis, regulates cell number and proliferation.

Apoptosis is regulated by extracellular and intracellular molecules, two of the key regulating pathways are the death receptor pathway and the mitochondrial pathway (see Fig. 1). Receptor mediated apoptosis can occur after binding of the respective ligand to the Fas- or the TNF-receptor. Ligation of death receptors causes the rapid formation of a death-inducing signaling complex, through the receptors death domain (DD). This domain is responsible for coupling the death receptor to a cascade of caspases, leading to activation of caspase-3 and following execution of the apoptotic program.

There is accumulating evidence that mitochondria play an essential role in apoptosis (Green D.R. 1998) by releasing apoptosis promoting factors, such as cytochrome c (Kluck R.M. 1997) from the intermembrane space into the cytoplasm, which activates the downstream execution phase of apoptosis through a complex called apoptosome (Fig. 1). In living cells, anti-apoptotic members of the Bcl-2 family proteins predominantly prevent mitochondrial changes.

The mitochondrial pathway is mainly regulated by Bcl-2 family proteins. Bcl-2 was first discovered as a proto-oncogene in follicular B-cell lymphomas and inhibits apoptosis. It resides on the mitochondrial outer membrane. Bax is one of the pro-apoptotic members, that resides either in the cytosol or in the mitochondrial membrane. When an apoptotic signal is detected, such as withdrawal of the growth factors and/or cellular stress, Bax translocates to the mitochondria membrane by formation of homo-oligomers (Gross A. 1998), thus, inducing cytochrome-c release.

The ratio between members of the Bcl-2 family is of pivotal importance in the regulation of apoptosis where Bcl-2 acts as an inhibitor and Bax as a promoter of apoptosis (Brown R. 1996). The ratio between apoptosis inhibiting Bcl-2 and apoptosis promoting Bax is important for the regulation of apoptosis. If the balance is shifted towards apoptosis promoting factors (Bax), the cells undergo apoptosis, while a shift towards apoptosis inhibiting factors (Bcl-2) protects cells from apoptosis (Hengartner
The cysteine aspartate-specific proteases (caspases) play a critical role in the execution of apoptosis and are responsible for many biochemical and morphological changes associated with apoptosis. 14 caspases have been identified so far, which all share a similar structure and substrate specificity. They are divided into subfamilies based on their sequence homology. The caspase-1 subfamily includes caspase-1,-4,-5,-11,-12 and -13; the caspase-2 subfamily includes Caspase-2 and -9; while the Caspase-3 subfamily includes caspase-3,-6,-7,-8 and -10. Caspase-1 belonging to the group of interleukin converting enzyme (ICE)-caspases, it was identified as the protease responsible for the formation of interleukin (IL)-1β and IL-18, two potent pro-inflammatory cytokines (Cerretti D. 1992; Zychlinsky A. 1997). Caspase-3 is of pivotal importance in the caspase cascade reaction. As an executioner caspase, it initiates the final pathway of apoptosis with DNA fragmentation and cleavage of structural proteins, respectively.

Figure 1: Receptor pathway and mitochondrial pathway of apoptosis. Bcl-2 and Bax protein regulate the mitochondrial pathway. Caspase-3 initiates the execution phase of apoptosis. Caspase-1 is responsible for the formation of interleukin (IL)-1β and IL-18, two potent pro-inflammatory cytokine. NF-κB also modulates the apoptotic and inflammatory responses.
1.3.2. Apoptosis and CAN

Under physiological conditions apoptosis participates in tissue homeostasis as it counteracts cell proliferation. Excessive apoptosis may contribute to an imbalance between cell growth and death, leading to the development of deleterious cell depletion. Experimental and clinical evidence has suggested that excessive apoptosis may be responsible for fibrogenesis and the development of renal scarring (Thomas S.E. 1998).

Apoptosis may impair remodeling and tissue repair processes in areas of renal injury, contributing to the formation of tubular atrophy and interstitial fibrosis. A tight correlation between the rate of apoptosis and the degree of interstitial fibrosis and glomerular scarring has been observed in different models of renal injury such as the remnant kidney, glomerulonephritis, unilateral ureteral obstruction, and ischemia-reperfusion injury (Sugiyama H. 1996). Meehan et al. described the pattern of apoptosis occurring in acute cellular rejection with focal lesions including endothelitis, tubulitis and tubular necrosis (Meehan S.M. 1997). Furthermore an excess rate of apoptotic of tubular epithelial cells has been observed in experimental models of chronic tubular atrophy and proteinuric glomerular injury (Schelling J.R. 1998).

More evidence accumulates which suggest that apoptosis could play a role in the pathogenesis of CAN. Increased mRNA expression of Fas or FasL together with increased apoptosis has been demonstrated in experimental models of acute and chronic rejection of kidney allografts in rats (Wang J. 1997) and humans (Strehlau J. 1996). Renal tubular cells express Fas and FasL and infiltration of cytotoxic T lymphocytes, in which Fas-FasL are highly expressed, are often seen in rejected kidney allografts (Olive C. 1999). Furthermore, a shift towards the expression of the apoptosis promoting Bax protein with respect to the expression of Bcl-2 has been observed in renal tubules of grafts with acute rejection (Wever P.C. 1998) and CAN (Nakopoulou L. 1996) as compared to normal functioning allografts.

Interestingly, the administration of an AT1 receptor antagonist resulted in a
significantly decreased number of apoptotic cells and a reduction in fibrosis in a rat cyclosporine nephropathy model, suggesting an important role for angiotensin II in inducing tubular and interstitial cell apoptosis and associated fibrosis (Thomas S.E. 1998).

However, the mechanism how AngII influences or regulates apoptosis, respectively, is not clear so far.

1.4. Angiotensin II and apoptosis

AngII is known to induce apoptosis in various cell types. In vivo studies demonstrated that AngII induces apoptosis in a dose-dependent manner in proximal tubular cells in adult rats (Cao Z. 2000). Several mechanisms have been investigated through with AngII can induce apoptosis.

The protein p53 suppresses cell proliferation and arrests the cell cycle. It integrates multiple stress signals into a series of diverse antiproliferative responses. One of the most important functions of p53 is its ability to activate apoptosis through transcription-dependent and -independent mechanisms that ensure an efficient proceeding of the cell death program. Activation of p53 is considered a gateway to apoptosis (Shankland S.J. 2000). Stretchinduced apoptosis of myocytes has been related to p53 which was increased through stimulation by AT$_1$ receptors (Leri A. 2000). Furthermore, AngII related AT$_1$ receptor stimulation has been reported in vitro to activate p53 DNA binding activity, leading to subsequent upregulation of the pro-apoptotic Bax gene (Pierzchalski P. 1997). However, also AT$_2$ receptor stimulation has also been shown to increase the expression of both p53 and Bax genes (Morrissey J.J. 1999). An initial damage to the graft after transplantation such as ischemia/reperfusion injury could stimulate the production of angiotensinogen and AngII as well as AT receptors. Between expression of p53 and expression of AT receptors as well as angiotensinogen exists positive feed back loops. Thus, a close relation between activation of the RAAS and induction of apoptosis is present. How this relation acts in CAN is presently unknown (see Fig.2)
The nuclear factor-κB (NF-κB) is a transcription factor activated by several stimuli, including cytokines, growth factors and AngII, it was thought to be one of the key proteins that modulate the apoptotic response. It has been demonstrated in vitro that the expression of NF-κB is mediated by both the AT$_1$ and AT$_2$ receptor (Ruiz-Ortega M. 2000) and that activation of NF-κB plays a role in p53-mediated apoptosis (Ryan K.M. 2000).

A number of studies conducted in different types of cell lines have suggested an induction of apoptosis through an inhibition of mitogen-activated protein (MAP) kinases (Horiuchi M. 1998; Horiuchi M. 1997). However, AT$_2$ receptor stimulation can induce apoptosis also by dephosphorylation of the anti-apoptotic protein Bcl-2 (Horiuchi M. 1997). Thus, both AT receptors seem to be involved in the regulation of apoptosis probably depending on the environment of the cells or its particular phenotype. In summary, the role of AT$_1$ and AT$_2$ receptors and their influence on apoptosis in the process of CAN remain need to be analyzed. Moreover, the mechanism how AT receptors participate in the regulation of apoptosis particularly regarding p53 in the circumstance of CAN remain to be established.

![Figure 2: Relation of RAAS and apoptosis via activation of p53 (see text for details)](image-url)
1.5. When should AT receptor antagonist treatment be initiated after transplantation?

Blockade of the RAAS has been proven to have beneficial effects particularly in delaying or preventing chronic renal disease including CAN. However, ACE inhibitors and AngII receptor antagonists are still used with caution in the treatment of renal transplant recipients, because of the fear of adverse outcomes regarding graft function due to a reduction of the renal blood flow and the GFR in the presence of a single kidney, particularly when administered together with calcium inhibitors. However, such functional effects, together with other side effects such as coughing, hyperkalemia, and lower hematocrit are all reversible and should not prevent the use of these agents in view of the long-term beneficial effects on renal function. On the other hand, the use of RAAS interfering compounds should be avoided in patients with hemodynamically significant graft artery stenosis (Curtis J.J. 1983).

It is not known to date, when to start with a treatment interfering with the RAAS such as ACE-inhibitors or AT-receptor antagonists after transplantation.
1.6. Aims of the study

1) Analysis of the effects of an AT$_1$ and/or AT$_2$ receptor antagonist treatment on the progression of CAN.

2) Analysis of mechanisms involved in the protective effects of AT$_1$ receptor antagonist treatment with respect to apoptosis.

3) Analysis of the expression of apoptosis regulating factors such as Bcl-2, Bax, caspase-1 and-3 in relation to AT$_1$ and/or AT$_2$ receptor antagonist treatment and progression of CAN.

4) Analysis of p53 expression in relation to AT$_1$ and/or AT$_2$ receptor antagonist treatment and progression of CAN.

5) Analysis of different treatment periods with AT$_1$ and/or AT$_2$ receptor antagonist during the course of CAN.
2. Materials and methods

2.1. Animals

Male Lewis rats (LEW, RT1) (170 to 210g) (Charles River, Sulzfeld, Germany) and male Fisher rats (F344) (170 to 210g) (Charles River, Sulzfeld, Germany) were maintained under standard laboratory conditions, fed with rat chow and water. All experiments were approved by a governmental committee on animal welfare.

2.2. Surgery and experimental protocol

We orthotopically transplanted kidneys of F344 into male LEW rats as previously described (Szabo A. 2000). Under general anesthesia with Ketamine (Ketamine, 100mg/kg i.p, CP-Pharma, Burgdorf, Germany) and Xylacine (Rompum, 10mg/kg i.p, Leverkusen, Germany), the left donor kidney was removed, cooled and positioned orthotopically into the recipient. Donor and recipient renal artery, vein and ureter were anastomosed end to end with 10-0 Prolene sutures (Resorba, Nürnberg, Germany), and the right recipient kidney was removed at the same time. All animals received daily 1.5 mg/kg/day of cyclosporine A (Novartis S.A., Basel, Switzerland) subcutaneously from day 0 to day 10 after transplantation. We assigned animals to five experimental groups: group AT$_1$ was treated with the AT$_1$ receptor antagonist candesartan (5 mg/kg/day); group AT$_2$ was treated with the AT$_2$ receptor antagonist PD123319 (1.5 mg/kg/day); group AT$_1$+ AT$_2$ was treated with a combination of candesartan and PD123319; control groups were either treated with the calcium channel blocker lacidipine (1 mg/kg/day) (group CCB) or vehicle (group VEH). Group CCB was added to control for the blood pressure lowering effect of candesartan. In order to establish the best treatment period with AT receptor antagonists during the development of CAN, experimental groups were treated for three different time periods: animals with long-term treatment from day -7 to week 24 after transplantation (n=8/group); animals with late treatment from week 12 to week 24 after transplantation.
(n=8/group); and animals with early treatment from day -7 to day 5 after transplantation (n=6/group). The observation period ended 24 weeks after transplantation.

2.3. Functional measurements

Every four weeks, 24-hour urine samples were collected using metabolic cages with a urine-cooling system as previously described. Quantitative urine protein was nephelometrically determined.

2.4. Morphological studies

Histology was based on paraformaldehyde-fixed, paraffin embedded tissue sections stained with hematoxylin and eosin (HE) to evaluate, tubulointerstitial fibrosis, vasculopathy, and tubular atrophy as the morphological characteristics of CAN. These parameters were evaluated according to the BANFF 97 classification as following: 0= no or minor changes; grade 1= mild tubular atrophy, fibrosis, or vasculopathy; grade 2= moderate tubular atrophy, fibrosis or vasculopathy; grade 3= severe tubular atrophy, fibrosis or vasculopathy. Two independent observers examined the slides by light microscopy in a blinded fashion.

Macrophage infiltration, Bcl-2 and Bax were evaluated by immunohistology. For macrophage staining, cryostat sections (4µm) were fixed in acetone, air dried and stained individually with primary monoclonal mouse derived antibodies against monocytes/macrophages (ED1) (Serotec Camon Labor-Service GmbH, Wiesbaden, Germany). After incubation with the primary antibody, sections were incubated with rabbit anti-mouse IgG and thereafter the alkaline phosphatase antialkaline phosphatase (APAAP) complex (DAKO A/S, Hamburg, Germany). ED-1 positive cells were counted and infiltration was graded 0≤ 5 cells/field of view; grade 1= 6-25 cells/field of view; grade 2= 26-50 cells/field of view; grade 3≥ 50 cells/field of view. At least 20 fields of view per section and per specimen were evaluated at 400x magnification by two independent observers in a blinded fashion. Bax was analyzed on paraffin embedded
sections. Sections were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pretreated with microwave (900W, 120sec), then incubated with the first antibody-polyclonal rabbit anti-rat Bax Ab (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and after overnight incubation at 4°C, incubated with the second antibody-monoclonal mouse anti-rabbit IgG (1:50, DAKO A/S, Hamburg, Germany) and rabbit anti-mouse IgG (1:25, DAKO A/S, Hamburg, Germany). Eventually the slides were incubated with the antibody-mouse APAAP monoclonal Ab (1:25, DAKO A/S, Hamburg, Germany). For Bcl-2 sections were treated as for Bax. However we used a polyclonal rabbit anti-rat Bcl-2 Ab (1:800, Pharmingen, San Diego, CA, USA) as the first antibody.

2.5. TUNEL

We performed TUNEL studies on frozen sections fixed in paraformaldehyde 4% as described elsewhere (Cursio R. 1999). Briefly, sections were incubated with Triton 0.1% and sodium citrate 0.1% at 4°C. After washing they were incubated with TUNEL solution. Sections were washed with stop/wash buffer followed by washing and incubation with a rabbit anti digoxigenine antibody. Antibody binding was visualized using fast red chromogene solution. We treated positive controls with DNase I and processed them as described above. Negative controls were incubated with PBS instead of TUNEL solution. Sections were counterstained with hematoxylin. All positive tubular epithelial cells in each section were counted and related to the number fields of view per section. At least 20 fields of view per section and per specimen were evaluated at 400x magnification by two independent observers in a blinded fashion.

2.6. Ribonuclease Protection Assay

Total RNA was prepared as previously described for the analysis of mRNA levels of Bcl-2, Bax, caspase-1, and caspase-3 (Szabo A. 2000). Anti-sense riboprobes (Pharmingen, San Diego, CA) were prepared by in-vitro transcription with the incorporation of 32 P-UTP according to the manufacturers protocol using the RiboQuant TM Kit (RiboQuant
TM Multi-Probe Ribonuclease Protection assay (RPA) System, Pharmingen, San Diego, CA) (Refaeli Y. 1998). We hybridized total RNA samples according to the manufacturer’s protocol with their respective rat anti-sense riboprobes, followed by RNase digestion and separation on a 6% polyacrylamid gel. Dried radioactive blots were scanned on a Fuji-BAS phosphor imager (Fuji, Dusseldorf, Germany). We digitized the blots and measured the density of bands. Data represent a ratio between specific mRNA band density to housekeeping gene mRNA band (GAPDH).

2.7. Western blot of p53

Tissue samples were homogenized in lysis buffer (1mmol/L NaH₂PO₄, 10mmol/L Na₂HPO₄, 154mmol/L NaCl, 1% Triton X-100, 12mmol/L Sodium deoxycholate, 0.2% Sodium azide, 0.1% SDS) then separated by 12% SDS-PAGE under reducing conditions. After electrophoresis, samples were transferred to Hybond™-CExtra membranes (Amersham Pharmacia Biotech, Aylesbury, UK), blocked with 5% skimmed milk in 1xTBS/0.5% Tween 20 overnight at 4°C with shaking.

After washing of membrane with TBS/Tween, they were incubated with the following antibodies: primary antibodies: monoclonal mouse anti human p53 (1:5000, Pharmingen, San Diego, CA, USA), polyclonal rabbit anti β-actin (1:10000, Sigma, St.Louis, MO, USA); secondary antibodies: goat anti mouse IgG-HRP conjugated (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti rabbit IgG-POD conjugated (1:5000, Vector Laboratories, Burlingame, CA, USA). All antibodies were diluted in 5% skimmed dry milk in TBS/Tween buffer. Blots were washed with TBS/Tween and light emission was initiated after incubation with LumiLight working solution (Roche Diagnostics GmbH, Mannheim, Germany).

2.8. Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Parametric data were
compared using one-way analysis of variance, followed by multiple pair-wise comparison according to the Newman-Keuls test. Non-parametric data were tested using the Kruskal-Wallis one-way analysis of ranks. Discrete data were compared using chi square test. A p-value of less than 0.05 (p<0.05) was considered significant.
3. Results

3.1. Long-term treatment with an AT$_1$ receptor antagonist reduced proteinuria and morphological changes of CAN.

3.1.1. Proteinuria 24 weeks after transplantation

Animals with long term AT$_1$ antagonist treatment (AT$_1$) and combined AT$_1$ and AT$_2$ antagonist treatment (AT$_1$+AT$_2$) developed a significantly lower proteinuria as compared to animals with AT$_2$ antagonist treatment (AT$_2$) and controls (groups CCB and VEH) (Fig.3a) (p<0.05). These differences were also observed in animals treated late (Fig.3b). However, this difference did not reach statistical significance (p>0.05). Early treatment resulted in similar proteinuria between the experimental groups (Fig.3c) (p>0.05).

![Fig. 3a](image-url)
Proteinuria 24 weeks after transplantation in animals treated long-term from day –7 prior to transplantation to week 24 after transplantation was significantly lower in animals of groups AT\textsubscript{1} and AT\textsubscript{1}+AT\textsubscript{2} than in the other groups (p<0.05) (a). Proteinuria in animals treated late from week 12 to week 24 after transplantation and early from day –7 prior to transplantation to day 5 after transplantation was not significantly different (b and c).

= AT\textsubscript{1}: treatment with AT\textsubscript{1} receptor antagonist, = AT\textsubscript{2}: treatment with AT\textsubscript{2} receptor antagonist, = AT\textsubscript{1}+AT\textsubscript{2}: treatment with AT\textsubscript{1} and AT\textsubscript{2} receptor antagonist, = CCB: treatment with ca-channel blocker, = VEH: treatment with vehicle.
3.1.2. Histology

Animals receiving long-term and late treatment with the AT\textsubscript{1} antagonist (group AT\textsubscript{1}) or the combination of AT\textsubscript{1} and AT\textsubscript{2} antagonist (group AT\textsubscript{1} and AT\textsubscript{2}) developed a significantly lower grade of CAN (p<0.05) as compared to the other groups (group AT\textsubscript{2}, CCB, and VEH) (Fig. 4a and 4b), while early treatment resulted in no significant differences (p>0.05) (Fig. 4c). The grade of infiltration in the graft tissues by macrophages is given in Table 1. While long-term treatment with AT\textsubscript{1} antagonist or a combination of AT\textsubscript{1} and AT\textsubscript{2} antagonist reduced the number of ED1 positive macrophages in graft tissues as compared to the other groups (group AT\textsubscript{2}, CCB and VEH) (p<0.05), neither late nor early treated animals differed significantly (Tab. 1).
Fig. 4b

Fig. 4c
Fig. 4d

Mild tubular atrophy and dilation, and interstitial fibrosis in grade 1 chronic allograft nephropathy (HE staining, x 100)

Fig. 4e

Severe tubular atrophy in grade 3 chronic allograft nephropathy (HE staining, x 100)
Fig. 4f
Glomerulosclerosis in chronic allograft nephropathy (HE staining, x 100)

Fig. 4g
Bax positive tubular epithelial cells (x 400)
Bcl-2 positive of tubular epithelial cells (x 400)

**Figure 4 (a-h)**

The grade of CAN in animals treated long-term from day –7 prior to transplantation to week 24 after transplantation and late from week 12 to week 24 after transplantation was significantly lower in animals of groups AT₂ and AT₁+AT₂ as compared to the other groups (p<0.05) (a and b). The grade of CAN in animals treated early from day –7 prior to transplantation to day 5 after transplantation did not significantly differ between the groups (c).

Grade of CAN: □ = mild, □ = moderate, ■ = severe.

Fig 4d, 4e and 4f show the mild to severe changes of CAN under microscopy. Most of the Bax and Bcl-2 expression was localized in tubular epithelial cells (Fig 4g and 4h)
<table>
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Table 1

Grade of infiltration by macrophages (ED-1 positive cells): 0 ≤ 5 cells/field of view; grade 1 = 6-25 cells/field of view; grade 2 = 26-50 cells/field of view; grade 3 ≥ 50 cells/field of view. Numbers represent the number of cases with the respective grade of infiltration. Long-term treatment: day –7 to week 24, late treatment: week 12 to week 24, early treatment: day –7 to day +5.

3.1.3. Mean arterial pressure

Mean arterial pressure did not significantly differ between the experimental groups.

Three animals with long-term treatment died during operation: one on group AT₁ died of anesthesia, respectively, the others, one on group AT₂, one on group VEH, died shortly after right nephrectomy due to surgical complications.
3.2. Long-term treatment with an AT$_1$ receptor antagonist reduced the number of apoptotic cells

3.2.1. Number of apoptotic cells

Animals on long-term treatment with the AT$_1$ antagonist (group AT$_1$) as well as on long-term combination treatment with the AT$_1$ and the AT$_2$ receptor antagonists (group AT$_1$+AT$_2$) had a significantly lower number of apoptotic cells as compared to animals receiving AT$_2$ antagonist (group AT$_2$), calcium channel blocker (group CCB) or vehicle (group VEH) (p<0.05) (Fig.5a), while the number of apoptotic cells in animals receiving late or early treatment was similar (Fig.5b and 5c). Apoptosis primarily affected tubular cells.

![Graph showing the number of cells/field of view for different treatments (AT1, AT2, AT1+AT2, CCB, VEH).](image-url)
Figure 5 (a-c)

TUNEL positive cells in animals treated long-term from day –7 prior to transplantation to week 24 after transplantation were significantly less frequent in animals of group AT\textsubscript{1} (a). TUNEL positive cells in animals treated late from week 12 to week 24 after transplantation and early from day –7 prior to transplantation to day 5 after transplantation did not differ significantly between the groups (b and c).

\(\square\) = AT\textsubscript{1}: treatment with AT\textsubscript{1} receptor antagonist,  
\(\blacksquare\) = AT\textsubscript{2}: treatment with AT\textsubscript{2} receptor antagonist,  
\(\square\) = AT\textsubscript{1}+AT\textsubscript{2}: treatment with AT\textsubscript{1} and AT\textsubscript{2} receptor antagonist,  
\(\blacksquare\) = CCB: treatment with ca-channel blocker,  
\(\blackblacksquare\) = VEH: treatment with vehicle.
3.2.2. Levels of mRNA of apoptosis related factors

3.2.2.1. Bcl-2/Bax mRNA ratio

The lower number of apoptotic cells in animals on long-term AT₁ antagonist treatment (group AT₁) was paralleled by a significant shift of the mRNA ratio of Bcl-2/Bax towards the anti-apoptotic Bcl-2 in AT₁ antagonist treated animals (group AT₁) as compared to animals receiving AT₂ antagonist (AT₂) and controls (groups CCB and VEH) (Fig.6a) (p<0.05). A similar difference was observed in animals that were treated late with the AT₁ receptor antagonist or the combination of an AT₁ and AT₂ receptor antagonist (groups AT₁ and AT₁+AT₂) (p<0.05) (Fig.6b), while early treatment resulted in no significant differences (Fig.6c) (p>0.05).

Fig. 5d
Positive TUNEL staining of apoptotic tubular epithelial cells (x400) (red nuclei)
Fig. 6a

Fig. 6b
The Bcl-2/Bax mRNA ratio in animals receiving long-term (day –7 prior to transplantation to week 24 after transplantation) and late (week 12 to week 24 after transplantation) AT₁ antagonist treatment (group AT₁) was significantly shifted towards bcl-2 mRNA as compared to animals of group AT₂ and animals of group CCB and VEH (a and b) (p <0.05) whereas the Bcl-2/Bax mRNA ratio in animals treated early from day –7 prior to transplantation to day 5 after transplantation did not significantly differ between the groups (c).

regular\[square\] = AT₁: treatment with AT₁ receptor antagonist, \[darkgray\] = AT₂: treatment with AT₂ receptor antagonist, \[lightgray\] = AT₁+AT₂: treatment with AT₁ and AT₂ receptor antagonist, \[black\] = CCB: treatment with ca-channel blocker, \[gray\] = VEH: treatment with vehicle.

3.2.2.2. Capase-3 mRNA levels

Animals receiving long-term or late treatment with the AT₁ antagonist (group AT₁) had significantly lower levels caspase-3 mRNA as compared to animals receiving AT₂ antagonist (group AT₂) and controls (groups CCB and VEH) (p<0.05) (Fig. 7a and 7b). Animals receiving early treatment with vehicle (group VEH) tended to have higher caspase-3 mRNA levels as compared to the other groups (Fig. 7c) (p>0.05).
Fig. 7a

Fig. 7b
3.2.2.3. Expression of p53

Animals with long term AT₁ antagonist treatment (AT₁) expressed p53 significantly lower as compared to animals with AT₂ antagonist treatment (AT₂) and controls (groups CCB and VEH) (Fig.8a) (p<0.05). These differences were also observed in animals treated late (Fig.8b). However, this difference did not reach statistical significance (p>0.05). Early treatment resulted in similar expression of p53 between the experimental groups (Fig.8c) (p>0.05).
Fig. 8a

Fig. 8b
P53 expression in animals receiving long-term (day –7 prior to transplantation to week 24 after transplantation) AT₁ antagonist treatment (group AT₁) were significantly lower as compared to groups AT₂, CCB, and VEH (a). (p<0.05)

P53 expression in animals receiving late (week 12 to week 24 after transplantation) AT₁ and AT₁+ AT₂ antagonist treatment (groups AT₁ and AT₁+AT₂) were lower as compared to animals of groups AT₂, CCB, and VEH, respectively, but the difference was not significant.(b) (p>0.05).

P53 expression in animals receiving early treatment (day –7 prior to transplantation to day 5 after transplantation) were not significantly different between the groups (c).

- AT₁: treatment with AT₁ receptor antagonist,
- AT₂: treatment with AT₂ receptor antagonist,
- AT₁+AT₂: treatment with AT₁ and AT₂ receptor antagonist,
- CCB: treatment with ca-channel blocker,
- VEH: treatment with vehicle.

3.2.2.4. Caspase-1mRNA levels

Animals treated with the AT₁ antagonist (group AT₁) long-term or late had significantly lower caspase-1 mRNA levels as compared to the other groups (Fig.9a and 9b) (p<0.05). Animals treated early with vehicle (group VEH), on the other hand, tended to have higher caspase-1 mRNA levels as compared to the other groups (Fig.9c) (p>0.05).
Caspase-1 mRNA levels in animals receiving long-term (day –7 prior to transplantation to week 24 after transplantation) and late (week 12 to week 24 after transplantation) AT1 and AT1+AT2 antagonist treatment (groups AT1 and AT1+AT2) were significantly lower as compared to groups AT2, CCB, and VEH (a and b) (p < 0.05). Caspase-1 mRNA levels in animals receiving early treatment (day –7 prior to transplantation to day 5 after transplantation) were not significantly different (c).

- = AT1: treatment with AT1 receptor antagonist,
- = AT2: treatment with AT2 receptor antagonist,
- = AT1+AT2: treatment with AT1 and AT2 receptor antagonist,
- = CCB: treatment with Ca-channel blocker,
- = VEH: treatment with vehicle
4. Discussion

In our model of chronic allograft nephropathy (CAN), AT$_1$ receptor blockade significantly reduced proteinuria and the grade of CAN as compared to AT$_2$ receptor antagonist treated animals and control groups. AT$_2$ receptor blockade resulted in a higher proteinuria and more severe grade of CAN as compared to AT$_1$ receptor antagonist treated animals. The beneficial effects of the AT$_1$ receptor blockade were most obvious in animals treated for the entire observation period (from transplantation to week 24). AT$_1$ and AT$_2$ receptor antagonists, when applied long-term in combination, reduced proteinuria and the number of apoptotic cells as well as morphological changes of CAN. However, this effect was lower as compared to AT$_1$ receptor antagonist therapy alone. Thus, the effects of AT$_1$ receptor blockade may outbalance the AT$_2$ receptor blocking effects. In animals with late AT$_1$ receptor antagonist treatment, the differences were still present but markedly reduced.

What were the mechanisms involved in the beneficial effects of AT$_1$ receptor antagonist treatment regarding a delay in the progression of CAN, particularly in the long-term treated animals? The blood pressure lowering effect alone was not responsible for the favourable results of the AT$_1$ receptor antagonist treatment as animals with calcium channel blocker treatment had a similar level of proteinuria as compared to controls. Furthermore, the blood pressure levels were not significantly different between the treatment and control groups. This is in congruence with previously published work that the renoprotective effect of RAAS blocking substances is independent of their blood pressure lowering effect (Benediktsson H. 1996; Perna A. 2000).

As apoptosis, also called programmed cell death, can decrease graft function through a reduction of functioning nephrons as well as stimulating inflammatory processes through an activation of so called inflammatory caspases such as caspase-1, it would be an interesting candidate mechanism contributing to the development and progression of CAN. Moreover, angiotensin II binding to the AT$_1$ as well as the AT$_2$ receptor has been shown to increase apoptosis. The role of the two angiotensin receptor subtypes is still under
discussion with most groups reporting apoptosis promoting effects by a stimulation of the AT\textsubscript{2} receptor (Nagashima H. 2001; Siragy H.M. 2000; Dimmler S. 1997), while some report such effects through a stimulation of the AT\textsubscript{1} receptor (Cigola E. 1997; Diep Q.N. 1997). In our experiments the lower proteinuria and grade of CAN in animals treated with the AT\textsubscript{1} receptor antagonist candesartan over the long-term was paralleled by a lower number of apoptotic tubular cells and a shift of the ratio of bcl-2/bax mRNA levels in favour of the anti-apoptotic Bcl-2 as compared to groups with AT\textsubscript{2} receptor antagonist treatment and controls. Thus, blockade of the AT\textsubscript{1} receptor resulted in a reduced number of tubular apoptotic cells, while blockade of the AT\textsubscript{2} receptor with PD123319 resulted in an increased number of apoptotic cells, indicating that the AT\textsubscript{1} receptor mediates apoptotic signals in our model of CAN. Interestingly, Cao et al. observed an inhibition of apoptosis, together with a protective effect, also by the AT\textsubscript{2} receptor antagonist PD123319 in a model of subtotal nephrectomy (Cao Z. 2002). In another experiment, apoptosis of tubular cells was found to be inhibited by an AT\textsubscript{2} receptor blocker in a short term model of obstructive nephropathy (Morrissey J.J. 1999). Such results might be due to a different model with a particular environment that could lead to a changed expression of receptor subtypes on the cells and, thus, different responses regarding apoptosis of tubular cells.

Bcl-2 is thought to prevent apoptosis by inhibiting the formation of Bax homodimers. Thus, the formation of particular channels in the membrane of mitochondria is inhibited with impaired translocation of apoptosis promoting molecules into the cytoplasm followed by a reduced caspase-3 activation. Here, the lower number of apoptotic cells in the long-term AT\textsubscript{1} receptor antagonist treated animals was paralleled by reduced caspase-3 mRNA levels supporting our hypothesis that AT\textsubscript{1} receptor antagonist treatment delays CAN also by reducing apoptosis in the graft.

What were the mechanisms involved in the reduction of apoptosis through an inhibition of the AT\textsubscript{1} receptor? It has already been shown that AngII can induce apoptosis through an up-regulation of p53 that in turn can increase the number of AT\textsubscript{1} receptor, and, moreover, shifts the Bcl-2/Bax ratio towards the pro-apoptotic Bax (Leri A. 1998;
Pierzchalski 1997). This is supported by our findings in animals with long-term AT₁ receptor antagonist treatment, where we observed a reduced expression of p53 in AT₁ antagonist treated animals as compared to controls and AT₂ receptor antagonist treated ones. Thus, the AT₁ receptor antagonist candesartan could reduce the number of apoptosis cells through a reduction of p53. This mechanism could have contributed to the delay of the progression and/or development of CAN in our experimental setting.

However, apart from a direct effect with respect to a reduction of apoptosis in the grafts, also indirect effects may have accounted for the beneficial effects of RAAS blockade by the AT₁ receptor antagonist in CAN. Here, the reduction of proteinuric could also be involved in the reduction of apoptosis of tubular cells in AT₁ receptor antagonist treated animals. Increased loss of protein in the urine is a marker of glomerular injury. However, abnormal amounts of filtered protein may contribute directly to the pathogenesis of progressive renal injury and CAN, by increasing the production of proinflammatory cytokines and inducing hypoxia through an increased metabolic rate of tubular cells. Moreover, albumin overload has been shown to induce apoptosis of cultured proximal tubular epithelial cells (Erkan E. 2001). Thus, the number of apoptotic cells could have been directly and/or indirectly affected through a reduction of proteinuric by AT₁ receptor antagonist treatment.

As stated above, apoptosis could deteriorate graft function, respectively, by promoting inflammatory processes through the activation of pro-inflammatory caspases such as caspase-1. Thus, inhibition of apoptosis together with an inhibition of caspase-1 could reduce the progression of CAN. This is supported in our experiment by a reduction of caspase-1 mRNA levels, together with a reduced macrophage infiltration, in animals receiving long-term AT₁ receptor antagonist treatment in our experiments.

Thus, in our experimental setting of CAN the AT₁ receptors could mediate apoptotic signals which can be blocked by an AT₁ receptor antagonist, resulting in improved graft function and a reduction of apoptotic cells, particularly tubular epithelial cells.

In this study we also addressed the question which treatment period would be best in
order to delay CAN. Animals receiving long-term AT₁ receptor antagonist treatment had a significantly lower proteinuria, grade of CAN and number of apoptotic cells as compared to animals with AT₁ receptor antagonist treatment from week 12-24 (late treatment) or those with treatment around transplantation (early treatment). Recently, it was shown that ACE inhibition limits CAN even when treatment was started at a time point when lesions were already established (Noris M. 2003). A direct comparison of our results with the results of Noris et al. is difficult as they used an ACE inhibitor, while we investigated AT receptor blockers. As Noris et al., we also observed a reduction in proteinuria and grade of CAN in animals with delayed treatment (late treatment group), however, this effect was less pronounced in our experiment. This could be related to the shorter follow up time of 24 weeks as compared to 48 weeks in the work of Noris et al. Moreover, they used a higher cyclosporine A induction dose of 5mg/kg/day for the first 10 days as compared to our induction dose of 1.5mg/kg/day for the first 10 days after transplantation. Thus, our results suggest that AT₁ receptor antagonist treatment should be started as soon as possible after transplantation in order to active the maximum protective therapeutic effect regarding the progression and development of CAN.
5. Conclusions

AT\textsubscript{1} receptor antagonist treatment with candesartan given on a long-term regimen delayed CAN. This protective effect could have been mediated by a reduction of the number of apoptotic tubular epithelial cells in the graft together with a reduction in inflammation of the graft tissue. The reduction of the expression of p53 by the AT\textsubscript{1} receptor antagonist could be involved in the mechanism through which the AT\textsubscript{1} receptor antagonist candesartan reduced the number of apoptotic tubular cells in the graft. On the other hand, long-term treatment with the AT\textsubscript{2} receptor antagonist PD123319 did not delay CAN as compared to controls. AT\textsubscript{2} receptor antagonist treatment was associated with an increased number of apoptotic cells with apoptosis regulating factors shifted towards apoptosis induction.

Later treatment with the AT\textsubscript{1} receptor antagonist candesartan improved morphological outcome, but did not significantly lower proteinuria, while perioperative AT\textsubscript{1} receptor antagonist treatment did not delay CAN as compared to controls. Thus, treatment with the AT\textsubscript{1} receptor antagonist candesartan given over the long-term started at the time of transplantation resulted in the most pronounced delay of CAN.

More research is needed to determine the role of AT\textsubscript{1} and AT\textsubscript{2} receptors and apoptosis in different disease models in order to further elucidate their mechanism in delaying and preventing renal disease progression.
6. Summary

To explore whether blockade of AT\textsubscript{1} and AT\textsubscript{2} receptors had an effect on apoptosis in the graft and the development and progression of chronic allograft nephropathy (CAN), we orthotopically transplanted kidneys of Fisher (F344) rats into Lewis rats. Animals were treated with the AT\textsubscript{1} and AT\textsubscript{2} receptor antagonist and appropriate vehicles either from day –7 before transplantation to week 24 after transplantation, from week 12 to week 24 after transplantation, or from day –7 to day +5. 24-hour protein excretion and number of apoptotic cells were significantly lower in AT\textsubscript{1} antagonist treated grafts as compared to AT\textsubscript{2} antagonist ones and controls. Apoptosis affected primarily tubular cells. mRNA levels of bax, caspase-1, and caspase 3 were lower and bcl-2 increased in AT\textsubscript{1} antagonist treated animals as compared to AT\textsubscript{2} antagonist treated and vehicle treated animals. However, no differences were observed between experimental groups if treatment was started at later time points. AT\textsubscript{1} receptor mediated signals reduce the number of apoptotic cells during CAN with beneficial effects on protein excretion as well as the grade of CAN. Thus, AT\textsubscript{1} receptor antagonist could be an additional tool to prevent CAN, if treatment is started early after transplantation.
7. References


phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 182: 1545-1556


57. Nakopoulou L., Stefananki K., Papadakis J., Boletis J., Zeis P.M., Kostakis A.,


Biochemical mechanisms of IL-2 regulated Fas-mediated T cell apoptosis. Immunity 8: 615-623


77. Strehlau J., Pavlakis M., Lipman M., Maslinski W., Shapiro M., Strom.T.B.


8. Abbreviations

Ab antibody
ACE angiotensin-converting enzyme
AngII angiotensin II
APAAP alkaline phosphatase antialkaline phosphatase
AT\textsubscript{1} angiotensin type 1
AT\textsubscript{2} angiotensin type 2
CAN chronic allograft nephropathy
Caspase cysteine aspartate-specific protease
DD death domain
ECM extracellular matrix
ED1 antibody to CD68 equivalent macrophage marker
EGF epithelial growth factor
GFR glomerular filtration rate
HE hematoxylin and eosin
IL interleukin
i.p. intraperitoneally
NF-\kappa B nuclear factor- \kappa B
PBS phosphate-buffered saline
PDGF platelet-derived growth factor
PFA paraformaldehyde
PS phosphatidylserine
RAAS renin angiotensin aldosterone system
RPA ribonuclease protection assay
SEM standard error of mean
TGF-\beta transforming growth factor \beta
TUNEL terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
9. Acknowledgements

I would like to extend my heartfelt appreciation to Univ. Prof. Dr. med. Uwe Heemann, he invited me from China two years ago, and following him, during these two years of research work in Essen and in Munich, I have learned a lot. His knowledge, his sharp scientific eyes and his excellent organization ability will always be my model in my future clinic and research work.

I would like to extend my heartfelt appreciation to Dr. med. Jens Lutz for his instruction to my study in Munich, for his critical correction of my manuscripts, and for his help to my life in Germany.

I would like to extend my heartfelt appreciation to Dr. med. Christoph Schmaderer. He gave me so many selfless help in my work and my life. I feel proud that I have a friend like him. Friendship has no nation.

I would like to express my special thanks to Matthias Strobl PhD, Miss Sandra Haderer, Dr. med. Patrick Wiese, Miss Judith Gschwandtl, Nengtai Ouyang MD, Hai Huang MD and Meihong Deng MD. Thanks for your excellent cooperation, and thanks for you giving me family warm.

Finally, I would like to express my special thanks to my parents, my sisters, my girlfriend Miss Hui Yang, and my tutor Prof. Jisheng Chen. Their support in China is my motivity to study in advanced, friendly, beautiful but distant Germany.