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Effects of TGF-β1 in ischemia / reperfusion injury and chronic allograft nephropathy

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Contents

1. Introduction

1.1. Present status and problems of renal transplantation

In the past few years, the short-term success of kidney transplantation has substantially improved, primarily due to the advancement in the techniques of tissue typing, organ preservation, operation, and the advent of more effective immunosuppressive agents. One year survival of cadaveric kidneys has increased from approximately 50% by the end of the 1960s, to about 85% nowadays (Gjertson DW. 1991; Koo DDH. 1999), and the one of living-related kidneys from 80% to 90-95% (Terasaki PI et al. 1993).

Despite the profound improvements of early results, the rate of long-term graft failure in the period beyond one year has remained constant (Hostetter TH. 1994). The half-life of cadaveric kidney allografts has been consistent at 7.5-9.5 years, and 50-80% of the patients ultimately return to dialysis after kidney transplantation (Ponticelli C. 2000).

It has now become clear that chronic allograft nephropathy (CAN) is the most important cause of late renal graft deterioration and failure. Around 35-58% of kidney graft loss is due to CAN (Paul LC. 1999). There is still no effective treatment to inhibit or prevent CAN, and a conclusive therapeutic strategy will not be available until the etiology and pathophysiology of CAN are fully understood.

1.2. The concept of chronic rejection or chronic allograft nephropathy

In 1955, Hume et al. first described a case in which rejection developed within 5 and half months, with obliteration of the arteries (Hume DM. 1955). Systematic investigation of late rejection by Porter et al. (Porter KA. 1963) and Jeannet et al. revealed that arterial intimal fibrosis was frequent and probably represented a reaction to immune injury, perhaps due to alloantibody (Porter KA. 1963; Jeannet M. 1970). By the late 1960s and early 1970s, transplant glomerulopathy distinct from recurrent glomerulonephritis was recognized, and was attributed as a variable feature of CAN (Zollinger HU. 1973).

The term chronic rejection is avoided because it implies an ongoing immune response that cannot be proven. The extent of immune involvement in CAN still cannot be determined, and the risk factors include a large number of nonimmune components. Previous efforts to define chronic rejection as a distinct disease often excluded kidneys with poor but stable function, while they included kidneys with better function that have experienced recent deterioration (Halloran PF. 1999).

Hence, chronic allograft nephropathy is accepted as a more accurate term describing the process. It is defined as a state of impaired renal allograft function at least 3 months after transplantation, independent of acute rejection, overt drug toxicity, and recurrent or de novo specific disease entities (Halloran PF. 1999). CAN is characterized by functional impairment with non-specific pathology: tubular atrophy, interstitial fibrosis, and fibrous intimal

thickening in the arteries, with variable glomerular lesions (Solez K. 1993). However, the term chronic rejection is still in use in some cases, in order to emphasize the importance of immunologic mechanisms, even in events triggered by alloantigen-independent factors.

1.3. The characteristics of CAN

Chronic allograft nephropathy displays a gradual deterioration of graft function months to years after transplantation, eventually leading to graft failure, which is accompanied by characteristic histological features (Hostetter TH. 1994).

Clinically, chronic transplant dysfunction in renal allografts manifests as a slow progressive decline in glomerular filtration rate, usually accompanied by proteinuria and arterial hypertension (Modena FM. 1991). The onset of proteinuria serves as an early predictor for CAN, and proteinuria parallels the severity of the disease (Cosio FG. 1999). Additionally, patients with CAN usually demonstrate arterial hypertension, and the severity of hypertension is correlated with the degree of histologic damage of allografts (Hostetter TH. 1994). Renal insufficiency develops at late stages of CAN, with elevated serum creatinine and lower creatinine clearance (Kasiske BL. 1991).

The pathology of CAN is non-specific and requires exclusion of specific entities. The cardinal histomorphological features of CAN are fibroproliferative vascular lesions (Hostetter TH. 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, foam cell accumulation, disruption of the internal elastic lamina, hyalinosis and medial thickening, and presence of subendothelial T-lymphocytes and macrophages. In addition, a persistent focal perivascular inflammation is often seen. Fibrous intimal thickening in arteries involves smooth muscle cell proliferation and increased lipid- and glycosaminoglycan-rich matrix in the intima, narrowing the lumen. But part of the loss of lumen in diseased vessels is due to failure of the vessel wall to dilate in response to decreased flow, and represents exhaustion of the normal remodelling 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 with duplication of the capillary walls and mesangial matrix increase has been identified as a highly specific feature of CAN. Less specific lesions are glomerular ischemic collapse, tubular atrophy, and interstitial fibrosis. Furthermore, peritubular capillary basement splitting and laminations are associated with late decline of graft function (Ponticelli C. 2000). The criteria for histological diagnosis of CAN are internationally standardised in the BANFF scheme for Renal Allograft Pathology (Cosio FG. 1999).

1.4. Influences of ischemia/reperfusion injury in CAN

Ischemia-reperfusion (I/R) injury is an inevitable pathophysiological alteration to transplanted organs, which is an exacerbation of graft damage incurred by the reestablishment of blood flow. I/R injury of renal allografts is associated with delayed graft function and may predispose an allograft to chronic rejection (Heemann U. 2000; Szabo A. 1998). Clinical data showed that organ preservation for more than 24 hours significantly impaired late kidney graft survival rates as compared to cold ischemic times between 0-24 hours (Ojo AO. 1997). Experimentally, rat kidney isografts develop the same functional and morphological changes as allografts, including vasculopathy, albeit over a much longer time interval (Land W. 1994). These changes were found to be triggered mainly by ischemia. It has also been suggested that in allografts the effect of ischemia on CAN is indirect by predisposing for acute rejection. Organ grafts with prolonged cold ischemia or with delayed graft function experience more often an early acute rejection episode than grafts that functioned immediately (Szabo A. 1998).

It has been suggested that apoptosis is the principle mode of cell death after I/R injury, which is responsible for renal tubular damage and the ensuing inflammatory response [Daemen MC. 2001, Ojo AO. 1997, Troppmann C. 1995]. Electronic and light microscopy of kidney undergoing I/R injury have revealed typical morphological changes of apoptosis in tubular epithelial cells, including chromatin condensation, cell shrinkage with membrane

blebbing, and formation of apoptotic bodies without membrane lysis or accompanying inflammation [Schumer M. 1992, Nogae S. 1998]. Additionally, in-situ TUNEL staining in conjunction with morphological criteria highlighted the presence of apoptotic tubular epithelia [Burns AT. 1998]. Furthermore, endonuclease and caspase activities, characteristic biochemical markers for apoptosis, are markedly elevated in kidney after ischemia-reperfusion injury [Ueda N. 1995, Kaushal GP. 1998]. In addition, treatments that protect renal grafts against reperfusion injury, such as antecedent administration of endotoxin [Heemann U. 2000, Martinez-Mier G. 2000, Shoskes DA. 1998, Daemen MARC. 2000], and single or multiple periods of brief antecedent ischemia [Nakajima T. 1996, Chien CT. 1999], are associated with a reduction of apoptosis in tubular epithelia. On the other hand, anti-apoptotic agents, such as caspase inhibitors and exogenous survival factors, ameliorate renal damages after I/R injury [Daemen MARC. 1999].

The number of apoptotic epithelial cells in kidneys after ischemia and reperfusion increases as early as 12 hours after ischemia probably as a result of ischemic tubular damage [Schumer M. 1992, Shimizu A. 1993]. More interestingly, massive apoptosis was also observed in regenerating tubular cells during the late recovery phase after reperfusion [Shimizu A. 1993]. However, the mechanisms responsible for the epithelial apoptosis at this period remain obscure.

During the recovery phase after ischemia/reperfusion, a variety of autocrine and paracrine growth factors, including EGF, HB-EGF, TGF-β, IGF-1, and HGF, participate in the regulation of the concerted cellular events [Harris RC. 1997, Hsing AY. 1996, and Miller SB. 1994]. Among these factors, the effects of TGF-β1 seem to be pleomorphic. It is not only a regulator for cell migration and differentiation [Basile D. 1996], but also influences apoptosis of epithelial cells [Hamasaki K. 2001, Mizuno S. 2000, Rosfjord EC. 1999, Antoshina E. 1997, Alvarez C. 1999, Hsing AY. 1996]. For example, the application of TGF-β_1 induced apoptosis in epithelial cells of liver [Hamasaki K. 2001], renal tubules [Mizuno S. 2000], mammary gland [Rosfjord EC. 1999], trachea [Antoshina E. 1997], pancreatic duct [Alvarez C. 1999], and prostate glands [Hsing AY. 1996], both in vitro and in vivo. On the other hand, treatment with antibodies against TGF-β_1 reduced the apoptosis of epithelial cells [Miyajima A. 2000]. These observations suggest that TGF-β_1 serves as a proapoptotic factor for epithelial cells of different origins.

1.5. Effects of cytokines and growth factors in CAN

Cytokines and growth factors are pleiotropical have biological effects on many cell subpopulations. Furthermore, they are regulated via autocrine, paracrine or systemic pathways. Th2 type cytokines have been associated with the development of CAN in allografts surviving long-term (Azuma H. 1994). Among them, IL-4 and IL-10 promote the

proliferation of fibroblasts in vitro, and, thus, fibrogenesis and artheriosclerosis. TGF-β contributes primarily to the enhancement of collagen production in fibroblasts by promoting collagen synthesis and inhibits its degradation. Transfection of TGF- β to the kidney increased the accumulation of the extracellular matrix and glomerulosclerosis. Treatments, which ameliorate CAN, are usually accompanied by a reduction of TGF-β expression (Hancock WH. 1993).

Cytokines and growth factors released by intragraft inflammatory infiltrates, activate mesangial cells and fibroblasts, and enhance their effects on extracellular matrix synthesis. Additionally, growth factors produced by activated macrophages or T lymphocytes, such as TGF-β, inhibit the activities of matrix degrading enzymes (MMPs), and hinder the degradation of extracellular matrix (ECM) components. Arterial intimal hyperplasia and the proliferation of smooth muscle cell in vessel walls are accompanied by excessive synthesis of connective tissue proteins (Paul LC. 1999).

1.6. The regulation of steroids on the expression of growth factors in CAN

It is widely assumed that glucocorticoids can ameliorate chronic rejection but the pathogenesis is not completely understood [Ribarac-Stepic N. 2001, Briggs WA. 1999, Kokot F. 1996]. The histopathological features of CAN are intimal proliferation of cortical arteries, glomerulosclerosis, interstitial fibrosis and tubular atrophy [Racusen LC. 1999].

Transforming growth factor-beta 1 (TGF-β1) has been implicated in the development of these lesions [Langham RG. 2001, Robertson H. 2001].

It has been reported that steroids regulate the expression and the activity of TGF-β1 in pulmonary fibroblasts [Wen FQ. 2001], dermal fibroblasts [Gras MP. 2001], ovary [Hernandez ER. 1990] and blister fluid [Leivo T. 2000], etc. Although steroids are part of most schemes of immunosuppression, the modulated interaction between steroids and TGF-β1 in CAN has basically not been investigated in vivo so far.

There are two potential sources of steroidal hormones in kidney graft recipients: endogenous production by the adrenal glands and exogenous administration [Oka K. 1990, Oka K. 1993]. Long-term treatment with glucocorticoids suppresses adrenal function via suppression of the hypothalamic-pituitary-adrenal axis, and thus, the endogenous release of glucocorticoids. The resulting glucocorticoid balance may influence the expression of growth factors in renal allografts.

1.7. Aim of present study

Up till now, reports on the localization of TGF-β_1 expression and its correlation to tubular epithelial apoptosis in kidneys subjected to I/R injury were not sufficient. The present study investigated the localization of TGF-β_1 mRNA and protein after ischemia-reperfusion, and the correlation to apoptosis in time and space. Meanwhile animals with transplanted

kidney were treated with a steroid hormone antagonist, Mifepriston [Zhou YF. 2000, Quaia M. 2000, Ghosh D. 1998], to block the action of glucocorticoid and progestogen. And the ensuing histological changes as well as the alternation of growth factor expression in renal allografts were investigated to explore the modulated mechanisms of steroid hormones on the expression of growth factors in chronic allograft nephropathy.

2. Materials and methods

2.1. Part I: Expression of TGFβ_1 and tubular apoptosis in I/R kidney

2.1.1. Animals

Male Sprague-Dawley rats (weight, 250-300 g) were maintained under standard laboratory conditions, and fed with rat chow and water ad libidum. All experiments were approved by a governmental committee on animal welfare.

2.1.2. Surgery and experimental protocol

Operative procedures were performed as previously described [Heemann U. 2000]. Under general anesthesia with sodium pentobartital (50 mg/kg; Butler Co., Columbus, OH, USA) administrated intraperitoneally (i.p.), the animals underwent a midline laparotomy, and the renal arteries and veins of both kidneys were dissected. The vascular pedicle was occluded with a microvascular clamp (Accurate Surgical & Scientific Instruments Co., Westbury, NY, USA) for 30 min. After ischemia, the clamps were withdrawn, the laparectomy incision was closed, and animals were allowed to wake up.

Before ischemia (time 0) or 4, 12, 24 hours, 2, 4 and 8 days after reperfusion, animals were narcotized and bled, and kidneys were promptly removed and stored in 4% buffered paraformaldehyde (pH 7.4) or liquid nitrogen (n=8/time point).

2.1.3. Histology

Paraffin sections of kidneys fixed in 4% neutral buffered formalin were stained with haematoxylin /eosin (HE) and periodic acid-Schiff reagent. Samples were coded and examined in a blinded fashion based on tubular damage and leukocyte infiltration on a scale from 0 to 3 (0 = none, 1 = mild, 2 = moderate, 3 = severe) [Heemann U. 2000].

2.1.4. In situ hybridization

Paraffin sections were hydrated through descending concentrations of alcohol to H_2O and resuspended in PBS (pH 7.4). In situ hybridization was performed as previously described [Amander TC. 2001]. Briefly, sections were treated with proteinase K and triethanolamine/acetic anhydride, followed by hybridization with TGF-β_1 probes (Maxim Biotech Inc. USA) for 15 hours at 54°C. TGF-β1 biotinylated probes were subsequently visualized using the streptavidin-biotin method of probe detection with alkaline-phosphatase, followed by NBT/BCIP chromogen (Boehringer Mannheim) and counterstained with nuclear fast red. Sense probes toTGF-β_1 served as negative controls. To semi-quantify TGF-β_1 mRNA expression, renal tubules with TGF-β_1 mRNA expression on epithelial cells were counted, and the proportion of TGF-β_1 mRNA expression to total tubules was expressed as percentage.

2.1.5. Immunohistology

Labelled Streptoavidin biotin (LSAB) method of immunohistochemistry was used to stain acetone-fixed 4-μm cryostat tissue sections. Endogenous peroxidase activity was quenched by incubation with 3.0% hydrogen peroxide in methanol for 5 min. Sections were washed in PBS and blocked for 1h in wash buffer containing 5% normal goat serum. A mouse monoclonal anti-rat TGF-β_1-specific immunoglobulin G (IgG; Sigma, St. Louis, MO, USA) was added as primary antibody at 1:50 dilution in PBS for overnight incubation at 4^{o}C. After washing with PBS, the sites of primary antibody binding were localized by sequential incubation with biotinylated goat anti-mouse antibody and then streptavidin conjugated with horseradish peroxidase (LSAB detection kit, DAKO Corp., Copenhagen, Denmark). After further washes in PBS, diaminobenzidine (DAB) was used as a chromogen and sections were lightly counterstained with haematoxylin. In the negative control section, the peptide immunogen, to which the antibody was raised, was included at 1μg/ml during primary antibody incubation as a direct, internal competitive control for antibody specificity. Lung carcinoma sections with TGF-β_1 expression were used as positive controls. Renal tubules with positive TGF-β_1 immunostaining on epithelial cells were counted, and the proportion of TGF-β_1 positive to total tubules was expressed as percentage.

2.1.6. TUNEL assay

Apoptosis was examined on paraffin-embedded sections via the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-AP nick end labeling (TUNEL) technique using an Apoptosis Detection Kit (Boehringer-Mannheim, Mannheim, Germany). Briefly, after dewaxing and hydration, sections were treated with 20 mg/mL of proteinase K in PBS (sodium phosphate 50 and sodium chloride 200 mmol/L, pH 7.4) for 10 minutes and incubated at 37°C for 30 minutes in TUNEL complex solution (including TdT enzyme, dUTP conjugated with fluorescence). Sections were then washed in TB buffer (300 mmol/L NaCl, 30 mmol/L sodium citrate) to terminate the reaction, and dUTP- fluorescence was detected by a rabbit anti-fluorescence antibody conjugated with Alkaline Phosphatase (AP). Antibody binding was visualized using fast red chromogene solution, and the sections were counterstained in Harris haematoxylin. Positive controls were treated with DNase I before they were incubated with TUNEL solution and processed as described above. Negative controls were incubated with TUNEL solution without TdT enzyme. All positive tubular epithelial cells in each section were counted and related to the number of fields of view per section.

2.1.7. Statistical analysis

Data are presented as mean ± SEM. Parametric data were compared using one-way analysis of variance, followed by multiple pair-wise comparison according to the Newman-

Keuls test. Nonparametric 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 was considered significant.

2.2. Part II: Expression of TGF-β_1 regulated by steroids in CAN

2.2.1. Animals

Naive inbred male Fisher (F344, RT1v1) and male Lewis (Lew, RT1) rats (Charles River, Sulzfeld, Germany), weighing 200-250 g, were kept under standard conditions and fed with rat chow and water ad libidum. All experiments were approved by a governmental committee on animal welfare.

2.2.2. Kidney transplantation

Under ketamine (Ketamin, 100mg/kg i.p.; CP-Pharma, Burgdorf, Germany) and xylacine (Rompun, 10 mg/kg i.p.; Bayer, Leverkusen, Germany) anesthesia 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. No ureteral stent was used. To overcome infectious complications due to operation rats received Cephtriaxone (Rocephin; 20 mg/kg/day, i.m, Hoffmann-la Roche AG, Grenzach-Wyhlen, Germany) on the first postoperative day. Animals were treated with low-dose Cyclosporine A (1.5 mg/kg/day s.c.; Novartis GmbH, Nürnberg, Germany) over the first 10 days after

transplantation to overcome an initial episode of acute rejection. The contralateral native kidney was removed on the 10th postoperative day.

2.2.3. Experimental design

Transplanted animals were assigned to four experimental groups (n=10/group): Mifepriston, Mifepriston + prednisone (combined treated group), prednisone and vehicle. Mifepriston (other name RU486; 8 mg/kg/day, Hualian Pharmaceutical Corp. Shanghai, China) and prednisone (5mg/kg/day, Ratiopharm, Germany) were dissolved in physiological saline, and administered to recipients by oral gavage. Rats were treated with Mifepriston and / or prednisone or vehicle for 28 weeks after transplantation. Kidney allografts of different groups were harvested at the end of the follow-up period at 28 weeks.

2.2.4. Functional measurements

Every 4 weeks, body weight was measured and 24-hour urine samples were collected using metabolic cages with a urine-cooling system. Quantitative urine protein was nephelometrically determined. Serum and urine creatinine levels were measured and creatinine clearance was calculated at the end of the study.

2.2.5. Histology

For histology, kidney tissues were fixed in 4 % buffered formalin, embedded in paraffin and sections were stained with hematoxylin/eosin, periodic acid-Schiff (PAS) and Trichrome Masson staining. Glomerulosclerosis was defined as a collapse of capillaries, adhesion of the

obsolescent segment of Bowman's capsule and entrapment of hyaline in the mesangium. All glomueruli in each section were counted and the proportion of sclerosis to total glomeruli was expressed as percentage. CAN in terms of glomerulosclerosis, tubular atrophy, interstitial fibrosis and vascular intimal proliferation were quantified according to the Banff´97 classification and graded as follow: 0 = no signs of CAN; grade 1 = mild CAN with mild fibrosis and tubular atrophy; grade 2 = moderate CAN with moderate fibrosis and tubular atrophy; grade 3 = severe CAN with severe fibrosis and tubular atrophy.

2.2.6. Immunohistochemistry

For immunohistology, cryostat sections (4 μm) were fixed in acetone, air dried and stained individually with primary monoclonal mouse derived antibodies against monocytes/macrophages (ED1) and CD5+ T-lymphocytes (OX19) (Serotec Camon Labor-Service GmbH, Wiesbaden, Germany). After incubation with primary antibody, sections were incubated with rabbit anti-mouse IgG and thereafter with the alkaline phosphatase antialkaline phosphatase (APAAP) complex (DAKO A/S, Copenhagen, Denmark). APAAP substrate developed the positive colour and counterstain with hematoxylin was finally proceeded. Cells staining positive were counted and expressed as cells per field of view (cells/fv). At least 20 fields of view per section or per specimen were evaluated at 400x magnification.

2.2.7. RNase protection assay

Total RNA was extracted using Trizol reagent (Gibco) according to manufacturer's instructions. Intragraft mRNA expression specific for TGFβ1 and GAPDH (Riboquant Multi-Probe template set, Pharmingen, Becton Dickinson GmbH, Hamburg, Germany) was determined by RNase protection assay using the In vitro Transcription Kit and RPA Kit (Pharmingen, Hamburg) as described previously [Antus B. 2001]. Briefly, ^{32}P-labeled antisense riboprobes were synthesized with the use of T7 RNA polymerase transcription in the presence of [α^{32}P]UTP. Radiolabelled antisense riboprobes were then hybridized with 10 μg of total RNA extracted from cultured cells at 56°C overnight. After hybridization, RNase A + T1 were added to digest unhybridized RNA and duplex RNA hybrids were separated by electrophoresis on a 5% polyacrylamide gel. Intensities of the protected bands were quantified by a phosphorimager (Fuji-BAS 1500, Düsseldorf, Germany) and the ratios of the investigated genes to GAPDH (internal control) were calculated.

2.2.8. Statistical analysis

Data are presented as mean ± SEM. Parametric data were compared using one-way analysis of variance, followed by multiple pair-wise comparison according to the Newman-Keuls test. Nonparametric 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 was considered significant.

3. Results

3.1. Part I: Expression of TGFβ_1 and tubular apoptosis in I/R kidney

3.1.1. Histology

Four hours after reperfusion, some tubular cells, primary tubules of the outer renal medulla, developed necrosis (Fig.1a), which was more frequent thereafter and peaked on days 4 and 8 post-reperfusion. Leukocytic infiltration of interstitial areas was first identified 4 hours after reperfusion, and peaked on the 8th post-reperfusion day (Fig.1b). Infiltrating leukocytes primarily constituted of lymphocytes, mononuclear cells, eosinophils and neutrophils.

3.1.2. TGF-β_1 expression

To investigate mRNA and protein expression of TGF-β_1 in kidneys undergoing ischemia-reperfusion injury, in situ hybridization and immunohistochemistry were performed. TGF-β_1 mRNA primarily localized in the cytoplasm of tubular epithelial cells, and in an "all-or-none" expression fashion in renal tubules, where all or none of the epithelial cells in the tubules showed positive staining (Fig.2a). In addition, TGF-β_1 mRNA expression was detected at all evaluated time points. The percentage of positive tubules increased time-dependently from 12 hours to 4 days after reperfusion, but dropped at 8 days (Fig.3a). Although TGF-β_1 mRNA expression was observed in a few glomeruli and interstitial infiltrates, the staining in these cells was weak.

Localization of TGF-β_1 expression was further confirmed at the protein level by immunohistochemistry. The distribution of TGF-β_1 protein was in consistency with its mRNA pattern, which was primarily localized in renal tubular epithelial cells with only weak and dispersed staining in glomeruli and interstitial leukocytes (Fig.2b). A similar "all-or-none" pattern of TGF-β_1 expression was observed on the protein level. Furthermore, the percentage of immunohistochemically positive tubules related to the time course was identical to the pattern observed for TGF-β_1 mRNA (Fig.3b).

3.1.3. Apoptosis of tubular epithelia

In order to investigate apoptosis associated with ischemia/reperfusion injury, we evaluated in-situ DNA fragmentation in ischemic kidneys with the TUNEL assay. TUNEL-positive cells primarily distributed in tubular epithelia, and were rare in interstitial infiltrates. Apoptotic cells as identified by TUNEL staining were accompanied by morphological signs of apoptosis, including chromatin condensation, detachment of cytoplasm from the environment, and formation of apoptotic bodies (Fig.2c, Fig.2d).

Apoptosis of tubular epithelia was detected at all time points. However, the percentage of apoptotic tubular epithelia varied over time. With the exception of 24 hours after reperfusion, it constantly increased over the observation period (Fig.4).

To evaluate the contribution of apoptosis to renal ischemia-reperfusion injury, we correlated the number of apoptotic epithelial cells to the histopathological findings. Renal tubules without or with mild necrosis demonstrated a significantly lower percentage of apoptotic epithelia (2.88 ± 0.83 %) in comparison to those with moderate or severe necrosis

(6.63 ± 1.06 %; $p<0.05$). Likewise, the percentage of apoptotic renal epithelia was higher in kidneys with moderate or severe interstitial infiltration (4.13 ± 1.13 %) than in those without or with mild infiltration (1.13 ± 1.13 %; $p<0.05$).

Furthermore, we analyzed the spatial relation between epithelial apoptosis and TGF-β_1 expression in renal tubules by comparing the consecutive sections from all time points. Renal tubules with positive TGF-β_1 mRNA or protein expression demonstrated a higher percentage of apoptotic epithelial cells (5.38 ± 1.92 %) in comparison to those without TGF-β_1 expression (2.88 ± 0.83 %; $p<0.05$).

3.2. Part II: Expression of TGF-β_1 regulated by steroids in CAN

3.2.1. Functional measurements

The blockade of the steroid receptors with Mifepriston resulted in a significantly higher proteinuria as compared to controls from week 16 (28 ± 2.8 vs. 23 ± 1.3 mg/24h, $p<0.05$) after transplantation. By contrast, treatment with prednisone decreased proteinuria as compared to the controls at 28 weeks (19 ± 1.3 vs. 23 ± 1.5 mg/24h, $p<0.05$). Animals treated with Mifepriston and prednisone also revealed a significantly higher proteinuria as compared to controls. No difference of proteinuria was observed between Mifepriston treated animals and the combined treatment group at any time point (Fig.5).

3.2.2. Mifepriston aggravated renal nephropathy in allografts

All transplanted kidneys developed changes of chronic allograft nephropathy such as glomerulosclerosis, interstitial fibrosis, tubular atrophy and intimal proliferation of graft arteries. However, Banff score and glomerulosclerosis differed between the groups.

3.2.2.1. Glomerulosclerosis

PAS staining revealed varied degrees of structureless hyaline material accumulating within the sclerotic glomeruli. In severe cases, the glomeruli were completely replaced by this material. Mifepriston alone and in combination with prednisone increased the percentage of sclerotic glomeruli (Fig.6a). In animals treated with prednisone alone, however, glomerulosclerosis was lower than in controls ($p<0.05$).

3.2.2.2. Banff score of nephropathy

In most sections, only mild or medium interstitial fibrosis was revealed by Trichrome Masson staining (Fig.6b). The tubular epithelium of atrophic tubules appeared shronken and the cytoplasm of deep colour (Fig.6c). Intimal proliferation of vessels protruded into the lumen, which was obstructed to various degrees (Fig.6d). There was a trend towards more severe nephropathy in Mifepriston treated animals as compared to controls.

3.2.2.3. Mifepriston enhanced intragraft macrophage infiltration

Animals treated with prednisone tended to have fewer ED1 positive infiltrates in their graft tissue as compared to controls ($p>0.05$) while Mifepriston resulted in extensive macrophage infiltration (Fig.7a, Fig.7b, $p<0.05$).

The number of OX-19 positive lymphocytes, on the other hand, did not significantly differ between the groups (Fig.7c, d).

3.2.3. Mifepriston increased intragraft mRNA expression of TGF-β_1

RNase protection assay showed that treatment with prednisone significantly decreased TGF-β1 mRNA expression (Fig.8, $p<0.05$). By sharp contrast, Mifepriston treatment increased TGF-β1 mRNA expression in allografts by about two folds as compared to controls ($p<0.01$).

3.2.4. Mean arterial blood pressure

Mean arterial blood pressure was significantly higher in Mifepriston treated animals than in controls (96.3 ± 11.5 vs. 71.4 ± 10.7 mm Hg, $p<0.05$) at 28 weeks, while blood pressure was similar in the other groups ($p>0.05$).

3.2.5. Body weight

Body weight tended to be lower in Mifepriston treated animals (473 ± 47.3 g) and Mifepriston + prednisone treated group (448 ± 41.5 g) as compared with controls (498 ± 53.7 g) at 28th week after transplantation but there was no any significant difference between these groups ($p>0.05$).

4. Discussion

Overexpression of TGF-β_1 as documented in various chronic renal diseases is accompanied by diffuse fibrosis [Border W. 1997]. Our present study demonstrated that TGF-β_1 was upregulated in kidneys subjected to ischemia-reperfusion injury at both, mRNA and protein level, and localized primarily in tubular epithelia. The peak of TGF-β_1 expression preceded the surge of apoptosis in tubular epithelial cells, and the distribution of the growth factor spatially correlated to apoptotic epithelial cells.

The potential source of TGF-β_1 production in the insulted kidneys includes tubular epithelial cells and the infiltrating lymphocytes and macrophages present in interstitial areas [Border W. 1997]. In chronic renal fibrotic diseases, such as chronic allograft nephropathy, TGF-β_1 derives primarily from the activated infiltrates, and, in concert with other mitogenic growth factors, contributes to the enhancement of collagen production in fibroblasts by promotion of collagen synthesis and inhibition of its degradation [Inigo P. 1999, Eddy AA. 1996]. Treatments that inhibit the recruitment and activation of renal infiltrates reduce the production of TGF-β_1, and thus hinder the development of renal fibrogenesis [Song E. 2002].

In contrast to fibrogenic renal diseases, our present findings suggest that TGF-β_1 is principally produced by tubular epithelia in kidneys subjected to ischemia-reperfusion injury, as both TGF-β_1 mRNA and protein localized primarily in tubular epithelial cells, while only weak staining was observed in glomeruli and interstitial infiltrates. This is in agreement with

recent immunohistochemical findings on renal allograft biopsies, which showed that TGF-β_1 was predominantly expressed in renal tubules of acutely rejected grafts [Inigo P. 1999, Robertson H. 2001]. In addition, expression of other growth factors, such as EGF, IGF and HGF, has been identified in tubular epithelial cells of kidneys in response to ischemia-reperfusion injury and is suggested to be associated with the regeneration of damaged tubules. Likewise, TGF-β_1 upregulation in tubular epithelia may also be a reperfusion-related response of the insulted tubules [Basile D. 1996]. Indeed, ischemia-reperfusion results in an excessive production of reactive oxygen species, which induce the activation of transcription factors, such as NFκB [Sun Y. 1996], and thus promote the synthesis of proinflammatory cytokines and related growth factors, including TGF-β_1 [Ishibashi N. 1999, Li N. 1999].

Unlike other growth factors, which contribute to the repair and regeneration of renal tubules [Harris RC. 1997, Humes HD. 1989, Miller SB. 1994], TGF-β_1 expression, herein, was associated with epithelial apoptosis. Temporally, up-regulation of TGF-β_1 preceded the second peak of apoptosis in our present study. We observed overexpression of TGF-β_1 mRNA and protein 12 hours after reperfusion, which peaked at day 4. In parallel, the percentage of apoptotic epithelial cells started to increase after 24 hours post-reperfusion, and peaked at day 8. This may imply that the expression of TGF-β_1 in tubular epithelial cells triggered their subsequent apoptosis. To provide further support for this hypothesis, we evaluated whether tubular epithelial cells expressing TGF-β_1 were apt to undergo apoptosis.

Analyzing the spatial correlated between TGF-β_1 expression and apoptosis in consecutive sections. Tubular epithelial cells, which expressed TGF-β_1 mRNA and protein, demonstrated a significantly higher percentage of apoptosis. These data suggested that most cells expressing TGF-β_1 in response to reperfusion are undergoing apoptosis. In agreement, during the regression of cyproterone acetate induced liver hyperplasia, TGF-β_1 was detected in apoptotic bodies of hepatocytes, and almost all apoptotic hepatocytes contained the precusor form of TGF-β_1 [Bursch W. 1993]. Moreover, the presence of TGF-β_1 in hepatocytes prone to apoptosis may last approximately 3 hours before chromatin condensation starts, consistent to the asynchronous appearance of TGF-β_1 expression and tubular epithelial apoptosis in our present study. Additionally, mechanical stretch increased TGF-β_1 expression and induced apoptosis of renal tubular epithelial cells [Miyajima A. 2000, Miyajima A. 2001]. Our data are further supported by numerous in-vitro studies demonstrating that treatment with TGF-β_1 induces apoptosis in cultured epithelial cells [Mizuno S. 2000, Rosfjord EC. 1999, antoshina E. 1997, Alvarez C. 1999, Hsing AY. 1996, Miyajima A. 2000]. The apoptosis-inducing effect of TGF-β_1 is associated with its up-regulation of pro-apoptotic Bax and p53 protein, and a down-regulation of anti-apoptotic Bcl-2 protein [Motyl T. 1998, Nass SJ. 1996]. Thus, the overexpression of TGF-β_1 in the present study may have induced epithelial apoptosis.

The TGF-β_1 increase in tubular epithelial apoptosis occurs during the repair phase after renal ischemia reperfusion injury, and may predispose for the ensuing inflammation [Shimizu

A. 1993]. Correspondingly, we observed a higher percentage of apoptotic tubular epithelial cells, more aggravated tubular necrosis, and more intense leukocytic infiltration. These data suggest that TGF-β_1 produced early after renal ischemia reperfusion may damage the kidney, and antagonize the regeneration-promoting effects of other growth factors. The balance between TGF-β_1 and other growth factors seems to determine the outcome of the repair process [Harris RC. 1997, Basile D. 1996].

Furthermore TGF-β_1 expression, either at mRNA or at protein level, exhibited an "all-or-none" fashion in renal tubules. Some tubules demonstrated positive staining for TGF-β_1 in all epithelial cells, while others were completely negative. This is in consistence with the pattern of tubular necrosis in kidneys subjected to ischemia / reperfusion injury, where some tubules undergo necrosis, while others remain intact [Heemann U. 2000]. These phenomena were also observed in chronic allograft nephropathy renal tissues in our previous studies [Song E. 2001]. The mechanism of this "all or none" style in renal tubules is still unclear. It may imply that some tubules are more susceptible to reperfusion injury than others in relation to the overexpression of TGF-β_1.

Our present study demonstrated that chronic allograft nephropathy (CAN) was ameliorated by treatment with prednisone, while it was aggravated by it's receptor antagonist. Furthermore prednisone could not protect renal allografts from chronic damages in the presence of receptor antagonist.

Mifepriston was first described as an antiglucocorticoid agent, and later its antiprogestin and abortifacient properties were reported [Garfield RE. 1987, Baulieu EE. 1989, Pinski J. 1993, Crombie DL. 1995]. It inhibits the action of steroid hormones at the receptor level in target tissue [Rauch M. 1985]. It binds with high affinity to the progesterone receptor (PR) and induces PR binding to a progesterone response element [Schuster C. 1989]. Mifepriston also interacts with the glucocorticoid receptor antagonising the effects of glucocorticoids [Zitnik RJ. 1994, Rivadeneira DE. 1999, Torres A. 1995]. Recent studies [Pandit S. 2002, Ichimaru N. 2000] demonstrated that the glucocorticoid receptor (GR) is a DNA-binding protein that regulates the transcription of a variety of genes in a ligand-dependent fashion. It was demonstrated that Mifepriston does not affect the affinity of GR to DNA, but subtly alters the electrophoretic mobility of the GR-DNA complex. At a certain DNA concentration, prednisone-bound GR dissociates from DNA significantly faster than ligand-free GR or Mifepriston-bound GR.

Mifepriston has been used successfully as a medical alternative to early curettage abortion, and as hormone therapy for advanced breast cancer [Baulieu EE. 1996, Catherino WH. 1995], uterine adenomyosis [Zhou YF. 2000], and Cushing's syndrome [Kawai S. 1987, Weiss BD]. In the present study, we applied Mifepriston to explore the effects of steroid hormones on chronic allograft nephropathy.

It has been well documented that profibrogenic growth factors, such as TGF-β1 and PDGF, contribute to the pathogenesis of chronic allograft nephropathy [Heemann UW. 1996]. It has also been reported that steroid hormones regulate the expression and the activity of growth factors in vitro [Wen FQ. 2001, Gras MP. 2001]. In fact, glucocorticoids are routinely applied in post-transplantational therapy. However, the modulated interaction between steroids and growth factors in allograft nephropathy has rarely been evaluated.

Although long-term treatment with glucocorticoid might reduce endogenous glucocorticoids due to suppression of hypothalamic-pituitary-adrenal function [Clodi M. 1998, Luger A. 1987], such influence seemed to be incomplete and unstable. Furthermore, the hypothalamic-pituitary-adrenal function can recover upon withdrawal of glucocorticoid treatment [Rodger RS. 1986]. In this study, we applied the steroid receptor antagonist Mifepriston to eliminate the effects of endogenous steroid hormones so that the negative modulatory effects of steroid hormones to growth factors could be observed.

In the present study the expression of TGF-β1 increased in Mifepriston treated animals, but decreased upon treatment with prednisone. The alterations of intragraft TGF-β1 levels correlated with the histological changes in allograft. These observations support the important role of TGF-β1 in the development of CAN. Obviously, this effect was modulated by steroids.

Although the effect of regulation of circulating hormones on the expression of TGF-β1 was marked, additional complexity was possible because the kidney is sensitive to both glucocorticoids and progestogen receptors, and both are blocked by RU486. It could not be confirmed which of them, glucocorticoid or progestogen receptor, played dominantly role on the CAN in this study.

5. Conclusions

TGF-β_1 expression of tubular epithelia is increased under ischemia / reperfusion injury and it correlates to tubular apoptosis during the repair process of injury. The results suggested a possible role of TGF-β_1 in the development of tubular apoptosis after I/R.

The development of chronic allograft nephropathy was substantially influenced by the treatment of steroid receptor antagonists. The expression of TGF-β1 was increased after blocking the circulating steroid hormones with Mifepriston, but decreased upon treatment with prednisone. The study suggested that steroid hormones are likely to influence the development of chronic allograft nephropathy by regulating the expression of TGF-β1.

6. Illustrations

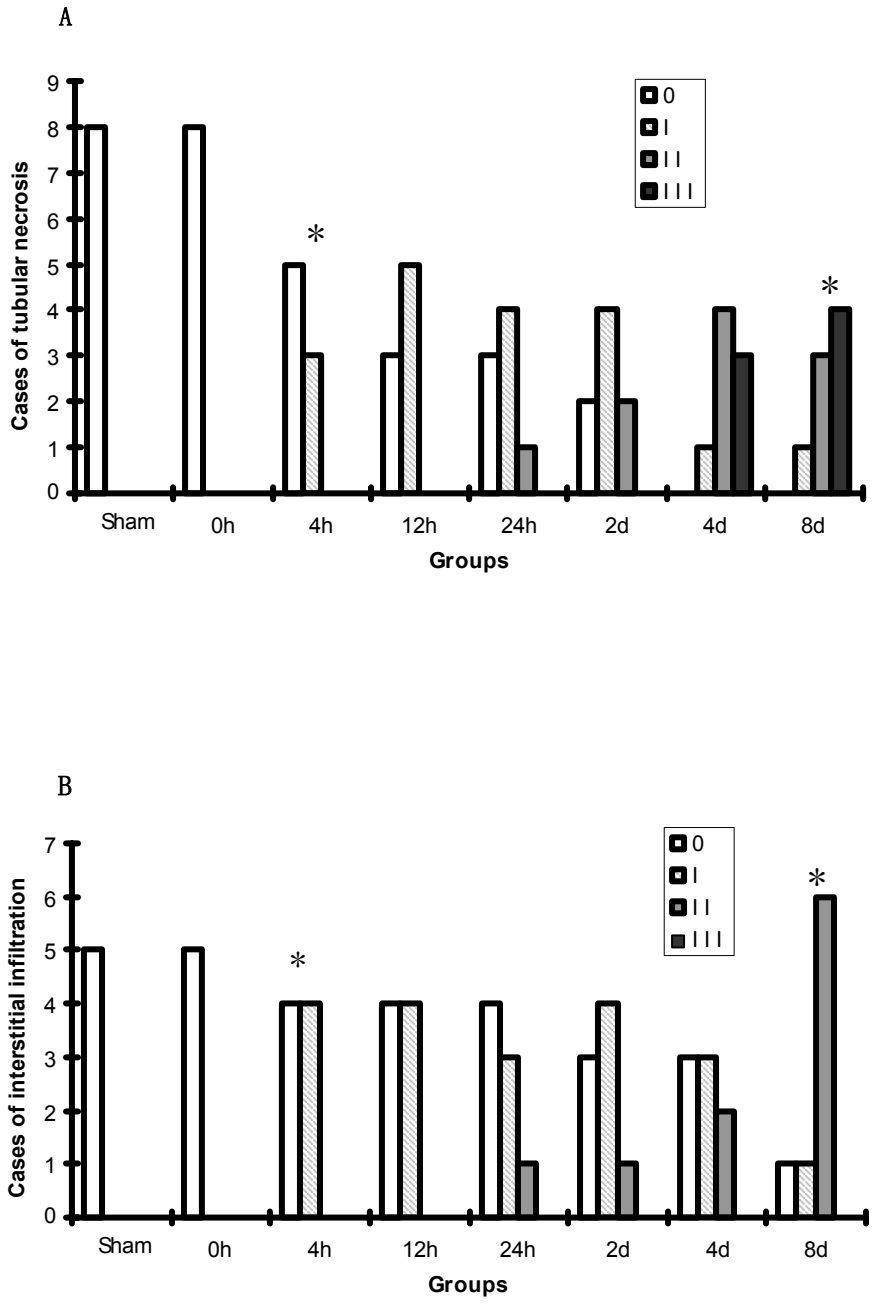

Fig.1 Tubular necrosis (A) and renal interstitial infiltration (B) after renal ischemia reperfusion injury. * denotes $p<0.05$ as compared with the previous time point.

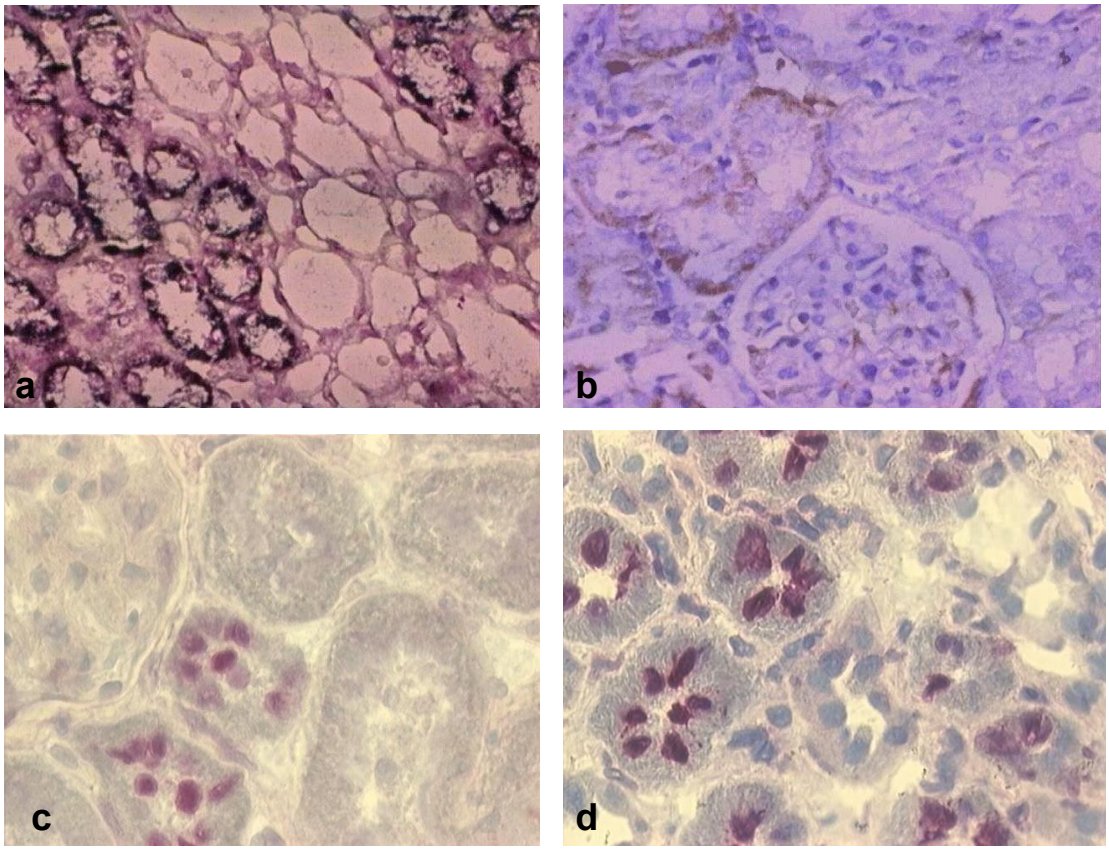

Fig.2 Expression of TGF-β_1 mRNA in tubular epithelial cells (a, ISH staining, ×200), distribution of TGF-β_1 protein in tubular epithelial cells (b, IHC staining, ×200) and apoptosis of tubular epithelial cells (c, d, TUNEL staining, ×400) after renal ischemia reperfusion.

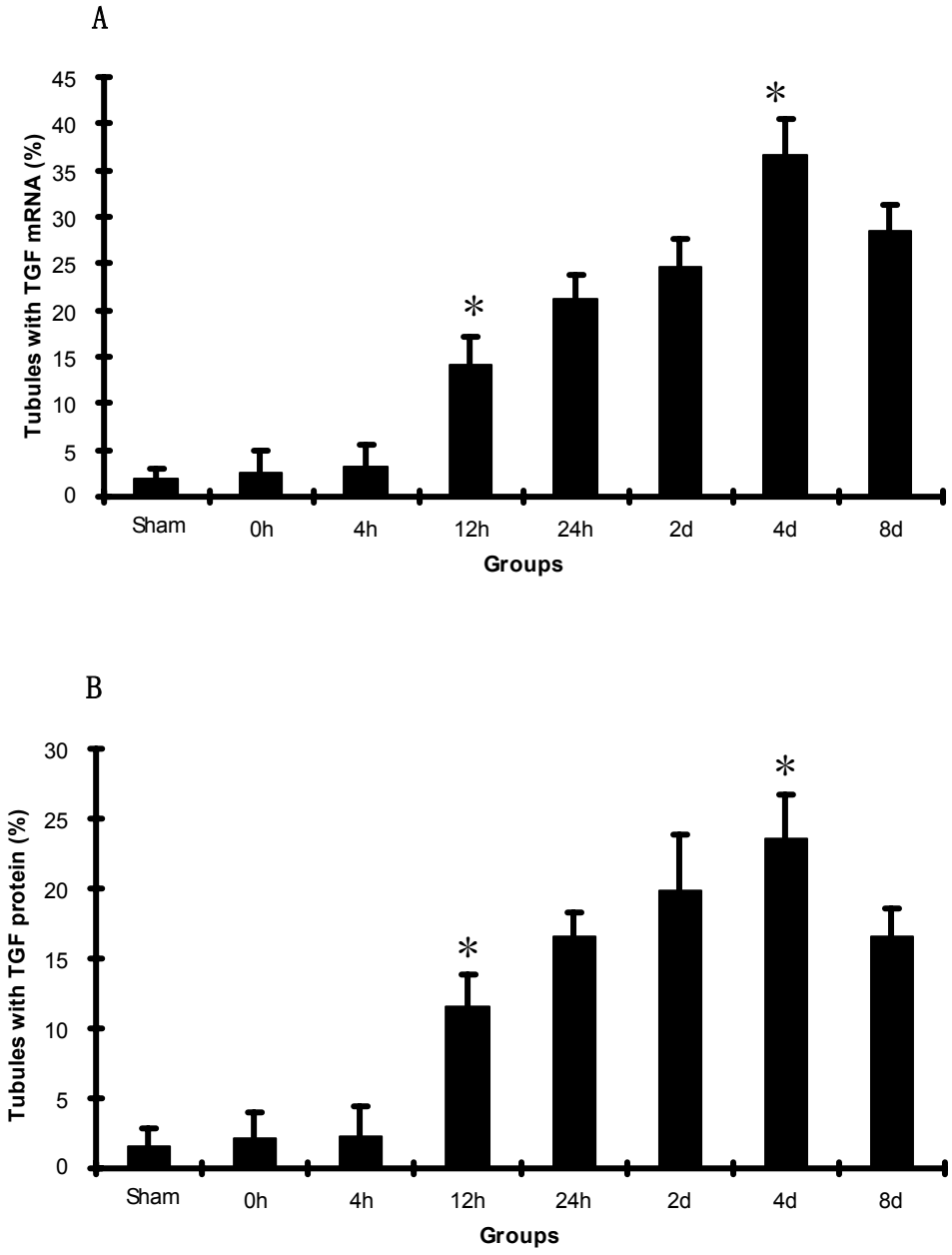

Fig.3 Expression of TGF-β_1 mRNA (A) and TGF-β_1 protein (B) of tubules in different time courses after ischemia reperfusion. "*" denotes $p<0.05$ as compared with the previous time point.

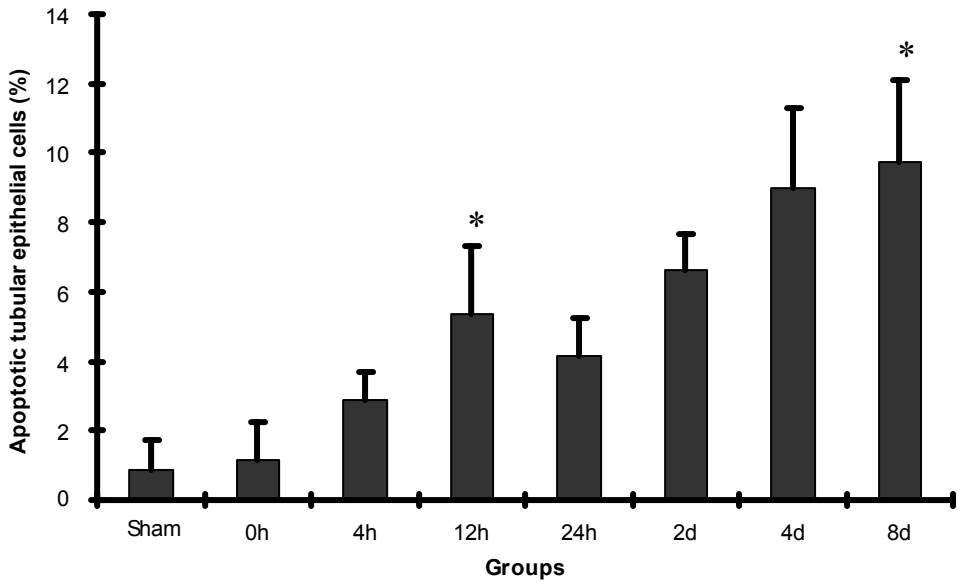

Fig.4 Apoptosis of tubular epithelial cells in different time points after renal ischemia reperfusion. "*" denotes p<0.05 as compared with the previous time point.

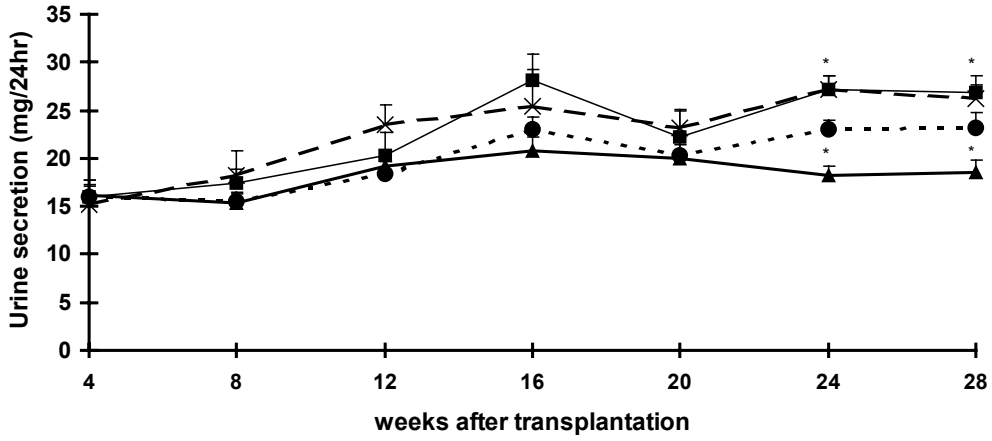

Fig.5. 24-hour urinary protein excretion during the experiment of animals treated with RU486 (■), prednison (▲), prednison + RU486 (×) and vehicle (●) from 1st day to 24th week after transplantation. * denotes p<0.05 as compared to vehicle control at the same time point.

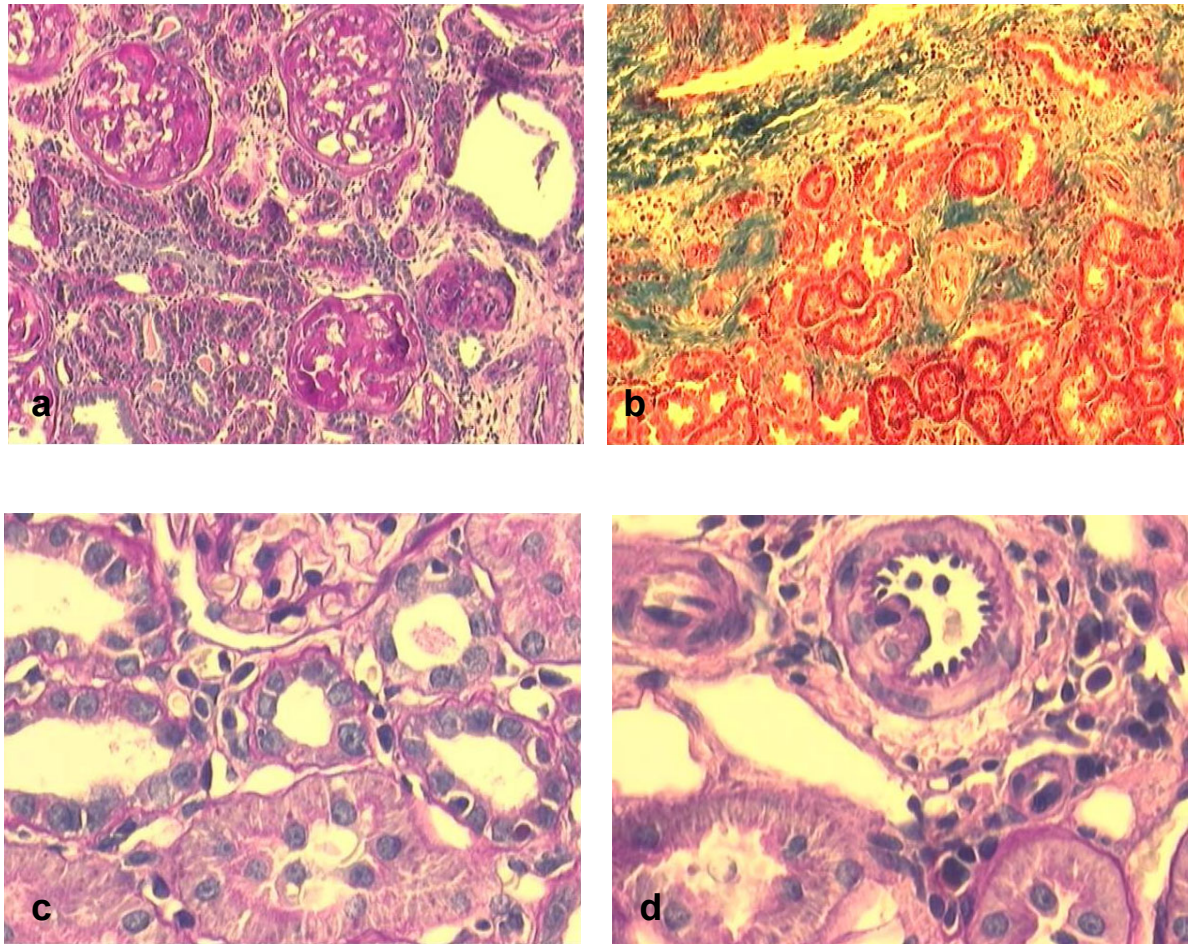

Figure 6. High percentage of glomerulosclerosis (PAS staining) in allograft treated with RU486 (**a**), medium interstitial fibrosis (blue color, Trichrome Masson staining) in renal allograft treated with RU486 and prednison (**b**); mild tubular atrophy (PAS staining) in transplanted kidney treated with prednison (**c**), marked intimal proliferation (HE staining) of artery in renal allograft treated with vehicle (**d**). ×100 (**a**, **b**) and ×400 (**c, d**)

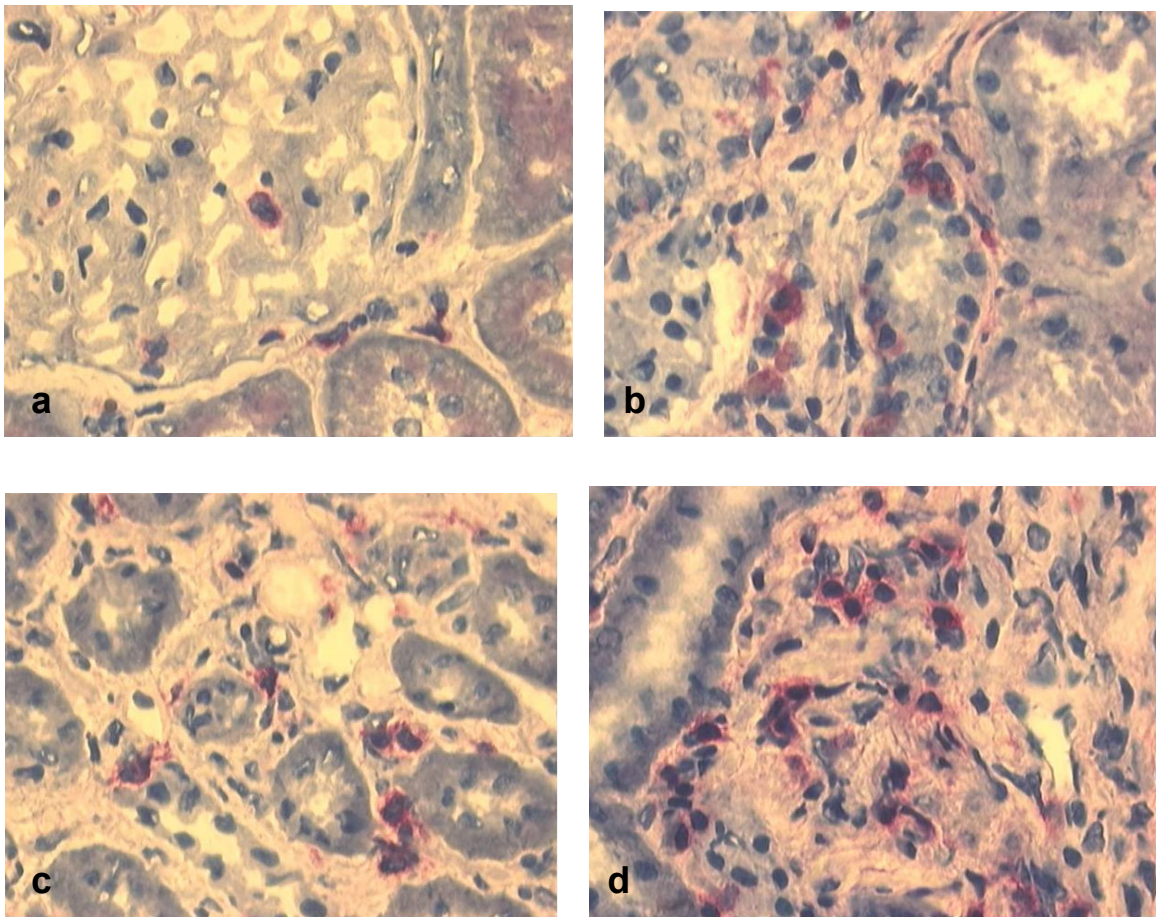

Figure 7. Immunohistochemistric microphotos of ED1+ macrophages in the graft tissue 28 weeks after transplantation in prednisone treated group (**a**) and RU486 treated group (**b**) and infiltrating OX19+ lymphocytes in animals treated with RU486 (**c**) and animals treated with vehicle (**d**). ×400 (**a, b, c, d**).

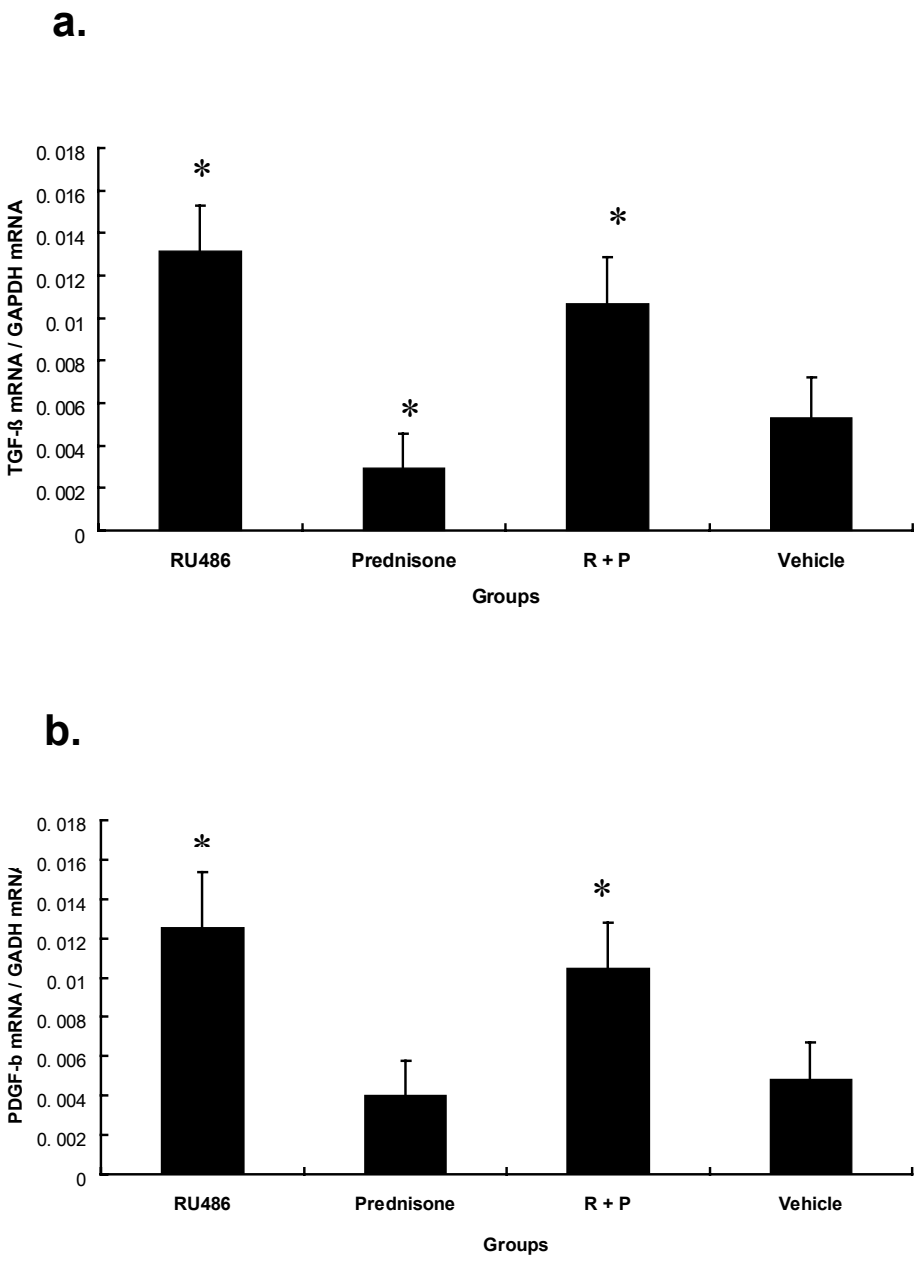

Figure 8. RNase protection assay analysis of TGF-β1 (**a**) and PDGF-b (**b**) mRNA expression in renal allografts treated with RU486, prednisone, combined RU486 and prednisone or vehicle from 1st day to 28th week after transplantation. * denotes $p<0.05$ vs. vehicle.

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8. Abbreviations

AP:	alkaline phosphatase
APAAP:	alkaline phosphatase anti-alkaline phosphatase
BW:	body weight
CAN:	chronic allograft nephropathy
CsA:	cyclosporin A
DAB	diaminobenzidine
DNA:	deoxyribonucleic acid
ECM	extracellular matrix
ED-1:	antibody to CD-68 equivalent macrophage marker
EGF	epithelial growth factor
GAPDH:	glycerinaldehyde-3-phosphate-dehydrogenase
GR:	glucocorticoid receptor
HE	haematoxylin & eosin
I/R:	ischemia/reperfusion
IL:	interleukin
INF:	interferon
i.p.	intraperitoneally
KW:	kidney weight

LSAB	labelled Streptoavidin biotin
MABP:	mean arterial blood pressure
MHC:	major histocompatibility complex
MMP	matrix metalloproteinase
NFκB	nuclear factor κ B
OX19	antibody to CD5+ equivalent pan-T-cell marker
PAS:	periodic acid-schiff
PCR:	polymerase chain reaction
PDGF:	platelet-derived growth factor
RNA:	ribonucleic acid
RPA	RNase protect assay
RT:	reverse transcription
SEM	standard error of mean
TGF-ß:	transforming growth factor-ß
TUNEL:	terminal deoxynucleotidyl transferase mediated dUTP nick end labelling
UTP:	uriddine-triphosphate

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