Delayed graft function in renal transplantation

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CHAPTER 5

THE EXPRESSION OF CASPASE-3 AND MANGANESE SOD IN DISTAL TUBULES PREDICTS POST -TRANS-PLANT ACUTE TUBULAR NECROSIS OR DGF

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Background Acute tubular necrosis (ATN) in renal allograft biopsies correlates poorly with delayed graft function (DGF). Factors involved in the pathogenesis of DGF were evaluated in biopsies in an attempt to refine the recognition of DGF.
Methods Of a total of 85 biopsies taken within the first week after transplantation, 41 biopsies were suitable for this study: ten from patients with DGF, and 31 from patients without DGF. Anti-cubulin and anti-AE-1/AE-3 antibodies were used to identify proximal and distal tubules respectively. The TUNEL technique and staining for active caspase-3 were used to demonstrate apoptosis. Antibodies against three types of super oxide dismutase (SOD) were used as markers of the protective tubular response. Tubular regeneration was evaluated using anti-ki 67 and anti-vimentin antibodies.
Results DGF occurred in 24 % of the transplant recipients. ATN coincided with DGF in 31% of the cases. The predictive value of finding ATN in the biopsy of a graft with DGF was only 50 %. Absence of distal caspase-3 staining predicted the absence of ATN in 78 % of cases. The presence of caspase-3 predicted ATN in 75 % of cases. The detection of Mn-SOD in distal tubules predicts the absence of DGF in 78% of the cases.
Conclusions The use of immuno-histochemical staining on renal biopsies improved its predictive value with respect to ATN and DGF: The absence of active caspase-3 in distal tubular epithelium predicts the absence of ATN in 78% of cases, whereas its presence predicts ATN in 75% of cases. The presence of Mn-SOD in distal tubules predicts the absence of DGF in 78 % of cases.

INTRODUCTION

The relationship between the functional and the morphological manifestations of acute renal failure remains enigmatic. It is generally accepted that acute tubular necrosis (ATN) is the morphological expression of acute renal failure due to ischemia-reperfusion injury. However, extensive tubular necrosis is not a typical feature of ATN (1). The tubular changes in human ATN are subtle and consist of flattening of proximal tubules with loss of brush border and necrosis of individual cells with focal denudation of the tubular basement membrane (2). Acute tubular injury therefore might be a better term to describe the more subtle morphological markers as a substrate of acute renal failure. In clinical practice renal biopsies are rarely performed to confirm ATN, but rather to exclude other causes of ARF.

Delayed graft function of renal transplants (DGF) has renewed the clinical and scientific interest in the histopathological characteristics of ARF, as it has been described as a possible risk factor for acute rejection (3) and for the development of chronic allograft nephropathy (4). Olsen reported, that although there are a lot of similarities between ATN in native kidneys and graft ATN, the latter shows more apoptosis and tubular necrosis, sometimes extending to whole tubular cross sections (5,6). Although circumstances leading to ATN in transplants may differ from those leading to ATN in native kidneys, ischemia/reperfusion injury plays a role in both conditions.

Several mechanisms have been described in the development of acute tubular damage caused by oxygen deprivation such as abnormal calcium homeostasis, reactive oxygen species and activation of enzymes involved in the oxidative stress response. Hypoxia has been found to reduce the cellular pool of adenosine triphosphate (ATP) initially leading to accumulation of adenosine di- and monophosphate and the generation of reactive oxygen species. Moderately elevated levels of reactive oxygen species have been shown to cause apoptosis, whereas higher levels cause necrosis of proximal tubules (7). As a consequence of ATP depletion, cells are no longer able to excrete calcium. The influx of calcium occurs predominantly during reperfusion and reoxygenation. High cytosolic concentration of free calcium has been shown to activate calcium-dependent enzymes such as phospholipases, nucleases, and cysteine proteases (8). Activated cysteine proteases are responsible for the disruption of the microtubular and cytoskeletal network and are involved in apoptosis (9,10) whereas administration of inhibitors of cysteine proteases reduce reperfusion injury in rat kidneys (11). Intact structural polarity of proximal tubules is vital for their function. Disruption of the cytoskeletal network has been reported to be associated with disturbed polarity, apoptosis and delayed graft function (12)

Protection against the toxic effects of reactive oxygen species is mediated by antioxidant enzymes, such as catalase, glutathione peroxidase (GPX) and superoxide dismutases (SODs). Extracellular-SOD (Ec-SOD) is mainly present in the extracellular fluid and matrix. Copper–Zinc SOD (Cu/Zn-SOD) is expressed constitutively throughout the body, whereas Manganese-SOD (Mn-SOD) is located exclusively in mitochondria (13) and is easily induced by oxidant stress and inflammatory conditions (14). The kidney has a remarkable capacity to recover from ischemic injury. Signs of regeneration and proliferation such as mitotic activity are characteristic of previous ischemic injury. Renal regeneration recapitulates certain aspects of renal development. Renal tubules are of mesenchymal origin, but they normally

do not express vimentin, unless they are recovering from ischemia (15).
Renal biopsies are generally not taken from renal allografts to confirm ATN; they are taken to detect acute rejection in patients with DGF. Since DGF is a transient and treatable disorder, prospective studies to investigate the relationship between DGF and ATN are not likely to be performed. It is therefore extremely difficult to study ATN in human kidneys. Therefore we performed a retrospective study in order to determine whether molecular markers for cell death, protection against oxidative stress, regeneration and proliferation provide a better correlation with DGF than the histological features of ATN in human renal transplants.

METHODS

Study Design

DGF was defined as a failure of the graft to reduce serum creatinine concentration by more than 10 % over three consecutive days for more than 1 week after transplantation (3). Using this definition DGF occurred in 24% of the transplantation procedures. Ninety percent of the patients were initially treated with prednisone and cyclosporine (Sandimmune) and 10 % with prednisone and azathioprine. Between 1983 and 1996, 85 biopsies were taken within the first 7 days.
To be included in the study, a renal biopsy taken in the first week after transplantation had to be available. These biopsies were taken for clinical reasons to detect or exclude additional acute rejection or cyclosporin toxicity. No biopsies were done to confirm ATN. When acute rejection was not found in the biopsy and cyclosporin toxicity could be excluded on clinical data and histology, the biopsy was included in the study. Patients who had a renal biopsy taken within the first week and who fulfilled these criteria were divided into a group with DGF and a group with primary function (PF). Forty one biopsies were selected. Eleven wedge biopsies were taken during surgical re-interventions and 30 needle biopsies were taken for diagnostic purposes. These biopsies were subsequently evaluated using light microscopy for the presence of acute tubular necrosis (16).
The pathophysiology of acute tubular necrosis is generally divided in an ischemic phase, characterized by tubular cell apoptosis or necrosis, a protective response against superoxide radicals, and a recovery phase in which tubular cell regeneration is prominent (17). Biopsies were therefore stained for markers of apoptosis, using the TUNEL technique and by immunostaining for active caspase-3, as well as for Ki 67 and vimentin, markers for proliferation and regeneration respectively. Staining for Cu/Zn-SOD, EC-SOD and Mn-SOD was performed to evaluate the protective response of the graft against reactive oxygen species. Differences between the 2 groups were subsequently scored in a blinded protocol.

Histopathological examination and scoring

Biopsies were fixed in 3 % formaldehyde and embedded in paraffin. Tissue sections were cut and stained with hematoxylin and eosin (H+E), Periodic acid-Schiff (PAS) and periodic acid-silver methenamine (PASM). The biopsies were reviewed by a pathologist without

knowledge of the clinical findings (He.Be.). Each biopsy was evaluated for the following attributes: number of glomeruli present, extent of global and segmental glomerulosclerosis, interstitial inflammatory infiltrate, interstitial fibrosis, tubular atrophy, tubular cell shedding, focal denudation of the tubular basement membrane ("non-replacement phenomenon"), presence of necrotic tubular cells in the lumen (tubular necrosis), tubular cross-sectional necrosis, tubular nucleolar prominence (tubular cell "activation"), and tubular mitotic activity. A semiquantitative evaluation was applied and each characteristic scored on a scale of 0 - 3. (16). For a better analysis of the relation between these histological parameters and clinical and immuno-histochemical features, this histological score was simplified into 2 point scale The Banff Schema for transplant pathology was applied (18). A biopsy was classified as having morphologic changes characteristic of ATN, when tubular cells were necrotic or showed cross-sectional necrosis or when at least score 2 was reached for tubular regenerative changes (16).

Immunohistochemical techniques

TUNEL Paraffin sections (4µm) were mounted on Superfrost plus glass slides (Mensel-Glaser, Omnilabo, Breda, The Netherlands) and deparaffinized in xylol and ethanol 96% and dried overnight at 37 *0* C. After inactivation of endogenous peroxidases with 1.2% H*2O2* in methanol and hydration, sections were pretreated with blocking buffer consisting of 0.1 M TRIS, 3% BSA and 20% normal calf serum for 30 minutes at room temperature. After rinsing with PBS, TUNEL mix (Roche Diagnostics, Almere, the Netherlands) was applied for only 30 minutes at 37*0* C. The prescribed incubation for 60 minutes resulted in our hands in unacceptable level of false positives in normal controls. The reaction was stopped with a mixture consisting of 40 cc 0.75 M PBS, 30 cc 0.1 M citrate and 30 cc demineralized water for 15 minutes. After rinsing with phosphate buffered saline (PBS), horse radish peroxidase (HRP) conjugated rabbit anti-FITC was applied in blocking buffer for 30 minutes. After three rinses in PBS, the peroxidase reaction was visualized with Novared (Vector Lab Inc., Burlingame; CA 940100).

Antibodies Rabbit anti-caspase-3 (Idun Pharmaceuticals, Inc., La Jolla, CA, USA), rabbit anti-MnSOD (a gift from dr. H. Verspaget, Dept. of Gastroenterology, LUMC, The Netherlands), rabbit anti Cu/Zn - SOD and rabbit anti Extra - Cellular (EC-) SOD (a gift from Dr. S. Marklund, Umea, Sweden), rabbit anti-vimentin (Euro Diagnostica, Arnhem, The Netherlands), mouse monoclonal anti-Ki 67 (DAKO, Glostrup, Denmark), mouse monoclonal anti cubulin (clone 2A3)(19)) and rabbit/mouse anti - AE1/AE3 (Neomarkers, LabVision, Fremont, CA, USA) were obtained as indicated.

Antigen retrieval Deparaffinized sections were first inactivated in 1, 2% H_2O_2 in methanol. In order to retrieve the antigen in the renal biopsy, sections were either boiled in 0.1 M citrate (pH 6) for 1 minute or in 0.01M citrate (pH6) for 10 minutes.

Double staining Specific antibodies directed against the brush border antigen cubulin of the proximal tubules and against the cytokeratin AE1/AE3 were used to achieve a sharper distinction between proximal and distal tubules. When anti-cubulin was used to localize proximal tubules it was applied first and followed by antigen retrieval with 0.1M citrate and incubation with anti-caspase-3, anti- Mn-SOD or anti Ki 67. Double staining for vimentin did not require antigen retrieval. When anti AE1/AE3 antibodies were used to localize

distal tubules, kidney sections were incubated overnight with anti-vimentin followed by antigen retrieval for 10 minutes with 0.01 M citrate and staining for AE1/AE3. When sections were stained for AE1/AE3 and Mn-SOD, antigen retrieval was performed first in 0.01 M citrate followed by incubation with AE1/AE3 and subsequently with anti-Mn-SOD. When antibody binding was visualized by peroxidation of 3.3 diaminobenzidine tetrahydrochloride (DAB), either mouse or rabbit envision (Dako, Glostrup, Denmark) was used as a secondary antibody conjugated to HRP. When tissue binding was visualized using Fast Red (Klinipath, Duiven; The Netherlands) anti-mouse or anti-rabbit IgG antibodies were used conjugated to alkaline phosphatase (AF, Sigma, Zwijndrecht, The Netherlands). Finally double stained sections were counterstained for 1-2 minutes in hematoxylin.

Immunohistochemical scoring

The intensity of staining in tubular cells was scored semi quantitatively using a 3-point scale; 0: negative or weak intensity; 1+: intermediate intensity; 2+: strong intensity. The extent of staining was similarly scored as negative: 0-10 % of tubules; focal: 10-50% of tubules or diffuse: > 50% of tubules. This scoring system was performed on proximal as well as on distal tubules, which were recognized on their immuno-histochemical or morphological characteristics. Scoring was performed at a magnification of 200 x. At time of scoring the investigators (LEs & HeBo) were blinded for clinical and histological data. When immuno-histochemical markers to identify proximal or distal tubules were absent, their morphological characteristics were taken into account. Proximal tubular epithelial cells in ATN kidneys show a flat cytoplasm with few nuclei ,giving them the impression of distal tubules or "distalisation". However, distal tubules have a high nucleus/cytoplasm ratio and their nuclei are better preserved in ATN than the proximal tubular cells, giving them a bead like appearance.

Statistics

The intensity and extent scores of the immunohistochemical stainings were compared between biopsies with and without histological ATN. The groups were compared with regard to the ordinal semiquantative scale of the scoring system of staining intensity and - extent, using non-parametric tests (Mantel-Haenzel chi-square test for linear association). The Kendall's tau-b coefficient shows the direction of this correlation. A p-value of 0.05 or less was considered significant. Statistical analysis was done using the SPSS software package (Version 10.0; SPSS, Inc., Chicago, IL).

RESULTS

Clinical characteristics of patients with or without delayed graft function (DGF) and with or without acute tubular necrosis (ATN)

A total of 41 biopsies was studied. Ten were taken from patients with DGF (Table 1) The remaining 31 were taken from patients with primary function (PF). Donor age for patients with DGF was significantly higher ($p<0.05$) than for patients without DGF (Table 1). Re-

cipient age and cold ischemia time (CIT) did not differ between the 2 groups. ATN was observed in 16 biopsies (table 2), 5 (50%) coincided with DGF, but 11 did not. The interval between transplantation and the timing of the biopsy did not differ between the two groups. Patients who experienced DGF but were not biopsied were slightly younger, had a younger donor and had lower Panel reactive antibodies (PRA). Other clinical characteristics summarized in table 2, did not differ between the ATN and non-ATN group.

Table 1: Clinical characteristics of recipients with primary function (PF) or delayed graft function (DGF)

	PF	DGF
Number of patients	31	10
Mean recipient age (yrs.)	45 (12)	53 (12)
Mean donor age (yrs.)	35 (14)	48 (11)*
Mean Cold Ischemia Time (hrs.)	23 (12)	28 (11)
Biopsy interval (days)	4.35 (2.2)	3.2 (2.3)
ATN (%)	11 (36)	5 (50)
PRA (%)	29 (28)	57 (43)

Chi-square test *p<0.05; (SD)

Table 2: Clinical characteristics of recipients with or without histological evidence ATN

	Non-ATN	ATN
Number of biopsies	25	16
Mean recipient age (yrs.)	45 (11)	50 (15)
Mean donor age (yrs.)	36 (13)	40 (16)
Mean Cold ischemia time (hrs.)	22 (13)	26 (11)
Biopsy interval (days)	3.9 (2.1)	4.4(2.4)
DGF (%)	5 (20)	5 (31)
PRA (%)	38 (35)	31 (35)

Histological parameters

The morphological features of tubular necrosis in the biopsies with or without DGF are shown in Table 3. No correlation was found between the occurrence of DGF and the histological changes. Evaluation of the biopsies according to the Banff 1997 classification for transplant pathology (18) revealed no differences between the ATN and non-ATN group for the acute tubulitis (t)-score, mononuclear cell interstitial inflammation (i)-score and allograft glomerulitis (g)-score. All biopsies except for 2 biopsies in the non-ATN group and one in the ATN group, had grade 0 t-score. Six biopsies in the ATN group and 5 in the non-ATN group showed a grade 1 interstitial inflammation (i) - score. No biopsy had a positive intimal arteritis (v) – score. No histological differences between needle biopsies and wedge biopsies were present.

Table 3: Histological changes observed in renal biopsies classified as Primary Function (PF) or. Delayed Graft Function (DGF)

	PF (n = 31)	DGF (n = 10)	p
Glomeruli			
Mean number (SD)	18.1 (8.0)	20.4 (6.6)	
Glomerulosclerosis (SD)	0.61 (0.89)	0.59 (0.71)	
Tubules			
Tubular necrosis			
Present	7	4	0.22
Absent	24	6	
Cross sectional necrosis			
Yes	3	3	0.14
No	28	7	
Tubular Shedding			
Present	12	7	0.21
Absent	19	3	
Non-replacement phenomenon			
Present	8	3	0.86
Absent	23	7	
Activated tubular cells			
Present	15	8	0.055
Absent	16	2	
Mitosis			
Present	13	5	0.32
Absent	18	5	

* p- value of the Mantel-Haenzel $\chi 2$ test for linear association

Markers for proximal and distal tubules

In eleven out of 16 biopsies of the ATN group and 18 out of 25 biopsies of the non-ATN group, the cubulin positive brush border was absent, indicating damage to the brush border also in renal allografts without ATN. The presence or absence of cubulin in the brush border of proximal tubules did not differentiate between the presence or absence of ATN or DGF. The same applied to AE-1/AE-3 positivity and ATN or DGF: the AE-1/AE-3 marker for distal tubules was not only present in 23 out of 26 non-ATN biopsies, but also in 14 out of 16 ATN biopsies. However, when AE-1/AE-3 positivity was related to histological scores for ATN, it was correlated with the absence of necrosis or cross sectional necrosis. Twenty eight of the 37 biopsies with a positive staining for AE-1/AE-3, had no signs of necrosis, whereas 2 biopsies with a negative staining for AE-1/AE-3 had signs of necrosis (P: 0.02; : -0.23). Thirty three of the 37 biopsies with a positive staining for AE-1/AE-3, had no signs of cross sectional necrosis, whereas 2 biopsies with a negative staining for AE-1/AE-3 had cross sectional necrosis (P: 0.03; κ: - 0.33)

Active Caspase-3 and TUNEL staining

Of the sixteen biopsies classified as ATN, 5 showed no necrosis in the individual score. In these cases, the diagnosis of ATN was made on other diagnostic criteria: 3 showed "non replacement", 3 showed shedding of tubular epithelial cells in the lumen and in 3 biopsies activated tubular epithelial cells and mitosis were seen. The absence of necrosis prompted us to test markers for apoptosis.

Table 4a shows that ATN was significantly associated with intensive staining for active caspase-3 in the distal tubules ($p = 0.04$). The negative predictive value of a negative caspase-3 staining was calculated to be 78 %. On the other hand the positive predictive value was only 54%. Five biopsies had ATN and DGF, whereas 21 biopsies were categorized as non-ATN without DGF (non-ATN/non-DGF). When these categories were analyzed separately, the active caspase-3 staining is positive in the distal compartment of all 5 biopsies from the ATN/DGF group, whereas 13 out of 20 biopsies in the non-ATN/ non DGF group are negative ($p = 0.010$) (Table 4b). No statistical significant difference in the extent of the caspase-3 staining in these biopsies was found.

Interestingly, in the absence of ATN and subcomponents of ATN like tubular necrosis and cross-sectional necrosis, the distal tubules showed more often apoptosis as assessed by the staining with the TUNEL technique than in the presence of ATN ($p = 0.01$). The presence of TUNEL staining in the distal tubules predicted the absence of ATN in 96 % of cases, whereas the absence of TUNEL predicted the presence of ATN in 60 % of the cases. Furthermore tubular atrophy was positively correlated with the extent of TUNEL positivity (data not shown). In the proximal tubules TUNEL staining correlated with the intensity of the active caspase-3 staining ($p = 0.04$; κ: 0.23) (Table 5). This was not the case in the other compartments.

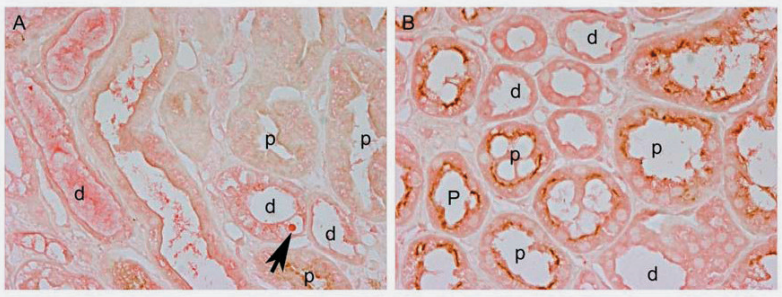


Figure 1
Active caspase-3 staining differentiating between the ATN (a) and the non-ATN group (b), in the distal tubules: Figure a shows the more prominent presence of active caspase-3 positive cells in the ATN group. The arrow points to a caspase-3 positive cell with histological apoptotic characteristics. (→)
Original magnification: **400x**; *proximal tubules:* **p** ; *Distal tubules:* **d**

Table 4a: Expression of active Caspase-3 in distal tubules in relation to ATN

	ATN		p	K
	Absent	Present		
Active Caspase-3 (intensity)				
Negative	14	4		
+	4	4	0.04	0.31
++	6	8		

* p- value of the Mantel-Haenzel $\chi 2$ test for linear association ; ** Kendall's tau β- correlation coefficient

Table 4b: Expression of active Caspase-3 in distal tubules in relation to ATN in 21 biopsies classified as histological non-ATN and clinical non-DGF versus 5 biopsies classified as histological ATN and clinical DGF

	ATN		p	K
	Absent	Present		
Active Caspase-3 (intensity)				
Negative	13	0		
+	3	1	0.010	0.49
++	5	4		

* p- value of the Mantel-Haenzel $\chi 2$ test for linear association ; ** Kendall's tau β- correlation coefficient

Table 5: Correlation between active caspase 3 staining and TUNEL staining in proximal tubules.

	TUNEL		**P**	κ
Active Caspase-3 (intensity)	**<10**	**>10**		
Negative	29	1		
+	5	0	0.04	0.23
++	1	1		

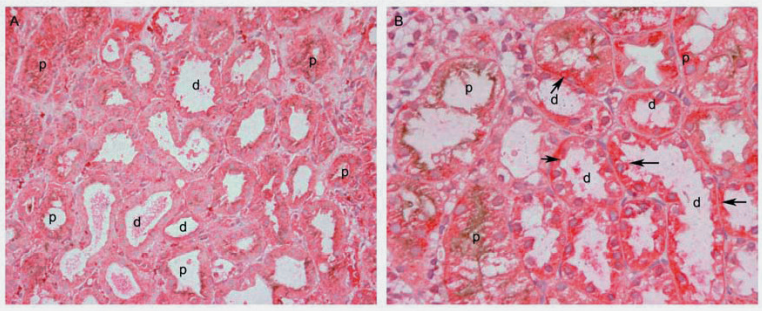
* p- value of the Mantel-Haenzel $\chi 2$ test for linear association ; ** Kendall's tau β- correlation coefficient

ROS deactivating enzymes

As expressions of a protective response against ischemia, tubular localization of Cu/Zn - SOD, Ec-SOD and Mn-SOD were compared between the DGF and ATN groups. Cu/Zn-SOD localization was found in both proximal and distal tubules and the intensity and extent of staining did not differ between the groups. Ec-SOD localization was found in both proximal and distal tubules, but it was slightly more present in the distal tubules. However, there was no difference in intensity and extent of localization between the two groups (data not shown).

The intensity and extent of the Mn-SOD staining in distal tubules were related with the absence of DGF suggesting a protective role of Mn-SOD against the development of DGF (Table 6). This relationship was also found in the proximal tubules, but this relation was not significant. The predictive value for the absence of DGF, of distally located Mn-SOD was calculated to be 76 %, whereas the predictive value of absent Mn-SOD was not associated with the presence of ATN.

Figure 2



Distal tubular localization of Mn SOD in the presence (a) and absence (b) of DGF. Note the increased staining intensity in biopsies with DGF (→).
Original magnification: **400x***; Proximal tubules:* **p** *; Distal tubules:* **d**

Table 6: Relation presence of Mn SOD in distal tubules in relation to the occurrence of DGF.

	DGF		p	K
	Absent	**Present**		
Mn SOD (Intensity)				
Negative	5	3		
+	11	6	0.05	- 0.30
++	15	1		
Mn SOD (Extent)				
Negative	5	3	0.06	-0.31
Focaal	4	4		
Diffuse	22	3		

* p- value of the Mantel-Haenzel $\chi 2$ test for linear association ; ** Kendall's tau β- correlation coefficient

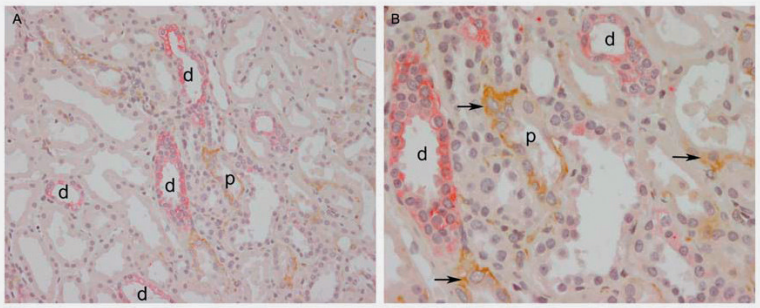
Vimentin staining

Regeneration was assessed by staining for vimentin. Vimentin localization was seen relatively more often in ATN biopsies than in the non-ATN biopsies. The basolateral localization of vimentin was exclusively seen in cubulin positive proximal tubules. However, the difference with non ATN biopsies was not significant (p=0.07).

Ki 67 staining

The proliferation marker Ki 67 was not associated with the occurrence of ATN or DGF. However the extent of the activated Mn-SOD staining in the proximal tubules was associated with a higher extent of staining for Ki 67, but this association was not significant (p 0.09; data not shown). No significant correlation was found between Ki-67 staining and the expression of vimentin.

Figure 3



Magnification 200 x Magnification 400 x

Basolateral vimentin staining in proximal tubules (→), which is present relatively more frequent in the presence of ATN. Note the increased staining intensity in biopsies with ATN.
Original magnification: A :**200x**; B *: 400x. Proximal tubules:* **p** *; Distal tubules:* **d**

DISCUSSION

This study confirms earlier reports documenting the poor correlation between the defined clinical syndrome of delayed graft function (DGF) and the morphological changes of ATN in renal allografts (2,20). The ATN score in this study was performed according to the criteria of Olsen and Solez (16) and did not correlate with the presence of DGF (Table 1). When a functional definition of DGF was used, excluding acute rejection episodes and cyclosporin as a cause of DGF, the presence of ATN in renal allografts predicted DGF, in only 50% of the cases, whereas the absence of ATN predicted the absence of DGF in 80% of our cases (Table 2). This prompted us to study molecular markers allegedly involved in the pathogenesis of DGF hoping to obtain a more reliable histological marker for the presence of DGF and to gain more insight in the pathophysiology of DGF. The markers were selected on the basis of the generally accepted pathogenetic events in ATN, consisting of an initiation phase of ischemic and reperfusion injury, an extension or a maintenance phase in which the renal tubular protective response is important and a recovery phase in which tubular regeneration and proliferation are characteristic (17).
Necrotic tubular cells have been described to undergo the following morphological changes: first proximal tubular cells form "blebs" in their apical membranes and lose their brush border (21). They lose their polarity and the integrity of their tight junctions is disrupted, presumably as a consequence of the redistribution of the Na/K-ATPases, resulting in alterations in the cytoskeletal network and disruption of tubular cells. Dead tubular cells slough into the tubular lumina and therefore are responsible for the denuded aspect of the lumen (non-replacement phenomenon) and the tubular obstruction (20). The denuded tubular basement membrane (TBM) is repopulated by proliferation of adjacent viable cells, under the influence of growth factors and by dedifferentiated stem cells that first spread out over the membrane, giving the proximal tubules the aspect of distal tubules (distalisation of proximal tubules). In contrast to normal distal tubules these recovering proximal tubules show very few nuclei. After the TBM has been repopulated cells will proliferate and dedif-

ferentiate until normal morphology and function is reinstalled.
Since the morphological identification of damaged renal tubular segments during ATN is difficult, we tried to improve the recognition of these segments by introducing proximal and distal specific markers (cubulin and AE-1/AE-3 respectively). These markers enabled us to identify the distal tubules in the presence as well as in the absence of ATN. The presence of AE-1/AE-3 positivity, allowed us to identify proximal tubules, as tubules that were AE-1/AE-3 negative in AE-1/AE-3 positive biopsies. In the four AE-1/AE-3 negative biopsies, distal tubules were identified on morphological grounds.
The paradox of severe reduction of glomerular filtration rate (GFR) and mild tubular necrosis could be explained by cell death via apoptosis instead of necrosis (22). The contribution of apoptosis was evaluated using the TUNEL technique and an immuno-histochemical staining for active caspase-3. In our hands the TUNEL technique had to be modified to obtain a higher specificity at the expense of sensitivity, because of the high percentage of false positive results in biopsies of normal kidneys. TUNEL positivity in the proximal tubules of both groups did not differ. Proximal tubules were by regular light microscopy either vital or necrotic. In the latter case intra-luminal sludging of cubulin positive material was present. Necrosis of proximal tubular cells is probably based on the high sensitivity of the proximal tubules for oxygen deprivation and ATP-depletion (23). Staining for active caspase-3 on the other hand was more prominent in the distal tubules in biopsies with ATN. The presence of apoptosis instead of necrosis in the distal tubules of the ATN group can be explained by a lower susceptibility of the distal tubules to ischemia and reperfusion injury followed by subsequent necrosis. Only when ATP depletion becomes more severe distal tubules will react with necrosis instead of apoptosis (24). Our finding that in selected cases in which extremes of ATN and DGF and non-ATN and non-DGF are compared, the relation of DGF with the extent and intensity of active caspase-3 staining in distal tubules is even stronger, supports this hypothesis. Experimental studies illustrate that various zones within the kidney, are differently susceptible to ischemic damage (25). Since the amount of tissue in human biopsies is very limited in a retrospective study, identification of these separate segments within the nephron was not attempted.
Activated caspases affect the cytoskeletal filaments, including actin. Recent studies have shown that the disruption of cytoskeletal proteins may in itself induce apoptosis and cellular detachment (9). ATN is characterized ultrastructurally by disruption of apical and basolateral membranes of proximal tubules, with redistribution and diminished intensity of cytoskeletal proteins of the apicolateral membrane (21). These mechanisms might contribute to the pathogenesis of ATN. Because of the resemblance between these features of ATN and the mechanisms of action of activated caspases, apoptosis might be the "missing link" in the pathogenesis of ATN. Activation of caspase-3 has been described to be the final step in the apoptotic pathway (27). When biopsies were stained for active caspase-3, cytoplasm of distal tubules stained significantly more intense in the ATN group (Table 4a). This made us conclude that the absence of distal active caspase-3 staining predicted the absence of ATN in 78% of cases, whereas the presence of caspase-3 predicted ATN in 75% of cases. No differences were found in the proximal tubules between the two groups.
TUNEL positivity was more prevalent in the distal tubules, especially in the non-ATN group. This unexpected finding could be explained by experimental evidence that apoptosis is not only an expression of damage, but also of repair(28,29). The number of apoptotic cells

in rat kidneys with experimental ATN were found to peak in the early phase after ischemic injury and in a later stage during regeneration. The first phase of apoptosis occurs early on, between 12 and 48 hours after the acute ischemic or nephrotoxic insult and is a result of the damaging effect of the insult. In contrast, the apoptosis associated with the recovery phase has been postulated to contribute to the remodeling of injured tubules and to facilitate their return to a normal structural and functional state. The discrepancy between the TUNEL and active caspase-3 staining might be explained by the fact that the specificity for apoptosis of the TUNEL staining is not high enough. It is known from experimental models that after treatment with carbon tetrachloride (CCl4) or N-nitrosomorpholine (NNM), rat livers showed a high percentage of TUNEL positive necrotic liver cells (26)

Since reactive oxygen species play an important role in the pathogenesis of ATN, the expression of enzymes with a protective effect against these oxygen radicals was studied. Superoxide dismutases have been found to be involved in the detoxification of reactive oxygen species (30) and have been described to play a role in experimental ischemia reperfusion injury (31). In humans at least three isoforms of SOD have been identified, each with a distinct metal component: Copper/Zinc-SOD (Cu/Zn-SOD), Manganese-SOD (Mn-SOD) and Extracellular -SOD (Ec-SOD). We did not find any difference for the Cu/Zn-SOD or Ec-SOD expression, between the two groups. Mn- SOD was found to be the only inducible form and to be exclusively located in the mitochondria (32). The overexpression of Mn-SOD was reported to correlate with the protection against pro-apoptotic stimuli and ischemic damage whereas decline of Mn-SOD expression has been associated with disease activity, like cancer and transplant rejection and cis-platinum tubulotoxicity (33-35). In biopsies from the non-DGF group, we found a more extensive presence of Mn-SOD in the distal tubules, compared with biopsies from the DGF group. Mn-SOD expression was also present in the proximal tubules, but there was no difference between the groups. These findings suggest that distal tubules are more resistant against Reactive Oxygen Species because they are capable of generating protective upregulation of Mn-SOD. The negative predictive value of the presence of Mn-SOD in the distal tubules predicts the absence of DGF in 76% of cases. This could explain the absence of necrosis but the presence of apoptosis in distal tubules: Proximal tubular cells have been reported to be more vulnerable to necrosis (36) whereas distal tubules may be protected to some extent against necrosis because of their upregulation of Mn-SOD (14). The clinical use of recombinant SOD (rh-SOD), to prevent ischemia reperfusion injury and subsequent DGF was tested in two randomized trials (37,38). Both studies showed that with the pharmacological doses that were used, no effect on the incidence of DGF was found. However one study showed a lower incidence of acute and chronic rejection and a better long-term graft function for patients treated with Rh-SOD (38). We therefore hypothesize that at the time of transplantation, the presence of Mn SOD, either structurally, upregulated or substituted, may be a parameter for the intrinsic potential to recover and may be a surrogate marker for long-term outcome of grafts.

After necrosis and/or apoptosis have occurred, tubular cells will regenerate (39). We found that the vimentin expression in proximal tubular cells was more prominent in biopsies, showing denudation, one of the morphological characteristics of ATN in grafts. However, this did not reach the level of significance. Tubular cells are of mesenchymal origin, but normal tubular cells do not express the mesenchymal intermediate filament protein vimentin. After being damaged, the recovery of the tubular cells is characterized by replace-

ment of irreversibly injured tubular epithelial cells by surviving tubular cells, renal stem cells or cells originating from the bone marrow. These cells differentiate to tubular cells, meanwhile expressing antigens, mimicking renal organogenesis (40,41). Vimentin expression has been described to be increased in damaged tubular cells and in renal carcinomas as a sign of tubular dedifferentiation (42,43). However, we could not confirm their findings in our study, because of the lack of significance. The expression of vimentin that we observed in proximal tubules of the ATN group can be interpreted as a sign of regeneration. Unfortunately no correlation was found between the presence of the proliferation marker Ki 67 in tubular cells and the occurrence of ATN or DGF. However,Ki-67 positivity in proximal tubules was more extensive in biopsies with a high expression of the protective marker Mn-SOD. This means that also the presence of Mn-SOD in proximal tubules correlated with its capacity for regeneration and recovery.

Since biopsies of human kidneys with ATN are difficult to obtain, we studied allograft biopsies taken to detect rejection during the first week after transplantation. Because acute rejection and calcineurine toxicity were excluded, only 41 biopsies were available for this study. This study shows that the use of molecular markers in the evaluation of early post-transplant renal biopsies does not discriminate between patients with DGF and/or ATN. However, in distal tubules correlations were found between the expression of active caspase-3 and the presence of ATN. This could explain why half of our patients with DGF did not show signs of ATN in their biopsies. Furthermore the expression of Mn-SOD in distal tubules is related to a lower occurrence of DGF, suggesting a protective role. Since the absence of distal localization of active caspase-3 is associated with the absence of ATN in 78 % of patients with DGF and the absence of Mn-SOD in the distal tubules is associated with the absence of DGF, these markers should be evaluated in a prospective study with protocolized biopsies.

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