Possible mechanisms explaining the tendency towards interstitial fibrosis in aristolochic acid-induced acute tubular necrosis

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Abstract

Background. We explored possible mechanisms responsible for the inability of plerosis and the tendency towards fibrosis in aristolochic acid-induced acute tubular necrosis (AA-ATN).

Methods. Renal biopsy tissues from eight AA-ATN cases were examined. Tubulointerstitial injury was semiquantitatively assessed. Immunohistochemical steptavidin-peroxide (SP) methods were used to determine the expressions of proliferating cell nuclear antigen (PCNA), epidermal growth factor (EGF), α-smooth muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), fibronectin (FN), collagen III (Col-III), collagen IV (Col-IV), factor VIII-related antigen (VIII-Ag) and vascular endothelial growth factor (VEGF). Ultramicrostructure of endothelial cells and basement membrane of peritubular capillaries (PTC) and glomerular capillaries was detected by electron microscopy. These data were compared with that of 9 cases of antibiotic-induced ATN (a-ATN) and 10 cases of minor mesangioproliferative non-IgA glomerulonephritis, which served as a control group.

Results. In AA-ATN, almost no renal tubular epithelial cells (RTEC) were PCNA-positive (0.01 ± 0.02%), and EGF expression was considerably decreased (9.55 ± 7.22%). This was in contrast with the highly active tubular proliferation (PCNA-positive RTEC 47.25 ± 19.33%, P < 0.05) and increased EGF expression in a-ATN (64.38 ± 19.22%, P < 0.05). The expression of α-SMA in the tubulointerstitium, the number of interstitial TGF-β1-positive cells and the CTGF-positive interstitial area were all increased in both a-ATN and AA-ATN, with no obvious differences between the two groups. With respect to extracellular matrix (ECM) deposition, FN, Col-III and Col-IV were detected only in the interstitium of AA-ATN. PTC lumina were decreased in size and misshapen in the AA-ATN group. Also in AA-ATN, the luminal wall was partially disrupted, endothelial cells were swollen and vacuoles and granules were found in the cell plasma. Parts of the endothelial cells were detached from the tubular basement membrane.

Conclusion. The strong ability for RTEC repair after acute injury was severely diminished in AA-ATN, and this effect may be partly due to reduced EGF expression. Anti-fibrosis mechanisms may also be impaired in AA-ATN, since both a-ATN and AA-ATN had increased expression of TGF-β1 and CTGF, whereas only the latter group showed ECM deposition. Injury and loss of PTC occurred in AA-ATN, and this may contribute to tubulointerstitial damage, the inability of plerosis and the tendency towards fibrosis in this disease.

Keywords: acute tubular necrosis; aristolochic acid; chinese herb; fibrosis; PTC

Introduction

Aristolochic acid-associated nephropathy (AAN) was first reported by Belgian nephrologists, who described it as a rapidly progressive interstitial renal fibrosis [1]. Since aristolochic acid (AA) is found in many varieties of Chinese herbs which are commonly used in China, it is important to understand its potential toxicity to the kidney and the mechanisms causing renal damage. Guanmutong (GMT, Aristolochia manshuriensis Kom.), a type of Chinese herb that contains AA (AA-I 0.76 mg/g) [2], is widely used for treatment of urinary and cardiovascular diseases [3]. However, it is the predominant cause of AAN in China. In this work, we study patients who developed AAN after taking medications containing GMT.

AAN cases in China differ from those in Europe by presenting three types of clinical–pathological features, including acute, sub-acute and chronic renal tubulointerstitial injuries [4]. Generally, overdoses of GMT usually cause acute renal failure. The pathological features include epithelial necrosis,
collapse and exfoliation, which resemble the acute renal tubular necrosis caused by antibiotics [4]. The same clinical–pathological features were also seen following AA administration in rats [5,6]. In contrast, AA-induced acute tubular necrosis (AA-ATN) shows exceptional pathology by lacking cell regeneration and having a tendency towards fibrosis, which correspond to its unpleasant clinical outcome. Unlike antibiotic-induced ATN (a-ATN), most AA-ATN cases show chronic renal malfunction after drug withdrawal, and some even develop chronic renal failure within half a year [4]. Thus far, the detailed mechanisms responsible for AA-ATN are not clearly understood.

The present study reports the differences between AA-ATN and a-ATN in promoting renal tubular plerosis and the tendency towards fibrosis. In contrast with a-ATN, the ability to repair tubular epithelium was severely suppressed in AA-ATN, which may be due in part to reduced expression of epidermal growth factor (EGF). Although an increased expression of transforming growth factor-β1 (TGF-β1) and connecting tissue growth factor (CTGF) were found in both AA-ATN and a-ATN, deposition of fibronectin (FN) and collagen-IV (Col-IV) was detected only in AA-ATN. The latter findings may explain the progressive nature of interstitial fibrosis in this disease.

Microvascular injury along with subsequent tissue hypoxia and ischaemia has been considered to play an important role in progressive renal disease. We therefore investigated alterations in the peritubular capillaries (PTC). We found that AA-ATN patients had a severe loss of PTC and misshapen as well as disrupted PTC lumina, together with ultrastructural alterations of endothelial cells and tubular basement membrane (TBM). These findings may partly explain the poor outcome of tubulointerstitial damage in AA-ATN.

Methods

Patients

Eight AA-ATN patients, which included six males and two females, aged 29–43 years, were studied. The diagnosis was made according to the following criteria: (i) subjects older than 45 years, (ii) with history of hypertension or family history of hypertension, (iii) with systemic diseases such as systemic lupus erythematosus, systemic vasculitis, etc. and (iv) those having tubulointerstitial injuries caused by other toxins. For the AA-ATN and a-ATN groups, glomerular diseases were excluded by histological examinations.

Histological parameters

All kidney biopsy sections, each including >10 glomeruli per section, were processed for light microscopy examination (HE, periodic acid–Schiff and Masson’s trichrome stain). Parameters for tubular injury, which included degeneration, necrosis, cell shedding and tubular regeneration, were semiquantitatively assessed according to the proportion of lesion areas taking up the total sections: <25%, Grade 1; 25–50%, Grade 2; 50–75%, Grade 3; >75%, Grade 4. The tubular injury index (TI) was calculated as the sum of the three former parameters. Lesions of the glomeruli, arterioles and interstitial infiltrates were also assessed. Ultrastructural changes of the endothelial cells and the basement membrane of glomeruli and PTC were observed through electron microscopy.

Immunohistochemistry staining

Renal biopsy specimens from the above patients were used for immunohistochemistry staining. The following antibodies were used in the study:

- murine anti-human proliferate nuclear antigen (PCNA) Ab (DAKO, 1:200)—to detect proliferating cells
- murine anti-human α-smooth muscle actin (α-SMA) Ab (Zymed, 1:50)
- rabbit anti-human EGF Ab (Santa Cruz, 1:200)
- rabbit anti-human TGF-β1 Ab (Santa Cruz, 1:200)
- rabbit anti-human CTGF Ab (Torre Pines Biolabs, 1:400)
- murine anti-human FN Ab (DAKO, 1:100)
- rabbit anti-human Col-III Ab (Zymed, 1:100)
- murine anti-human Col-IV Ab (DAKO, 1:100)
- rabbit anti-human factor VIII-related antigen (VIII-Ag, Zymed, 1:150) Ab—to detect the endothelial cells of the glomerular and peritubular capillaries
- murine anti-human vascular endothelial growth factor (VEGF) Ab (Zymed, 1:200).

The staining was performed by the biotin–streptavidin–peroxidase method. Briefly, formalin-fixed paraffin-embedded sections were cut 2 μm thick. Dewaxed, hydrated sections were incubated in H₂O₂ (5%) in 100% methanol for 30 min to inhibit endogenous peroxidase activity, washed twice with phosphate-buffered saline (PBS) and then unmasked either by incubation for 30 min at 37°C in 0.1% trypsin (pH 7.2, for FN, Col-III, Col-IV) or by microwave (for α-SMA, EGF, TGF-β1, CTGF, VIII-Ag and VEGF). After incubation with 10% normal goat/horse serum (as appropriate depending on the secondary antibody used) and dilution in PBS containing 1% w/v bovine serum
albumin (BSA; Sigma, UK), the sections were incubated with the primary antibody for 60 min at 37°C. Thereafter, the sections were washed and incubated with the biotinylated secondary antibody (1:200; Vector, UK) for 30 min, followed by incubation with peroxidase-conjugated streptavidin for 30 min at 37°C. Colour reaction of peroxidase was performed in a solution of 3, 3′-diaminobenzidine (DAB) and 0.005% H2O2 in 50 mmol/l Tris (pH 7.6). Finally, the sections were generally counterstained with diluted haematoxylin (1:10).

Control sections were incubated with non-immune gamma globulin or after the omission of the primary antibody.

**Morphometrics**

The number of PCNA-positive tubular cells was counted at random in five microscopic fields (200×), each field containing at least 500 renal tubular epithelial cells (RTEC). Results are shown as the percentages of PCNA-positive RTEC. EGF or VEGF labelling was assessed by the same methodology.

Positive expression of α-SMA, CTGF, TGF-β1, FN, Col-III and Col-IV in RTEC and interstitial area was quantitated by point counting with a medical colour image analyser (Kodak, USA). Five fields (400×) without glomeruli were counted in each specimen. The results are expressed as a fractional area, which was calculated according to the number of grid intersections with positive staining divided by the total number of grid intersections.

Quantification of PTC was made by counting the number of VIII-Ag-positive interstitial capillary lumina in 10 randomly selected fields (0.065 mm²) under 400× magnifications in each sample. The number of PTC was expressed per mm². For each sample, >10 glomerular cross sections were examined for the number of VIII-Ag-positive capillary lumina. The density of glomerular capillary was expressed per glomerulus.

**Statistical analysis**

All values were expressed as the means ± SD. Comparisons were made using Kruskal–Wallis H tests among the groups. Correlations were calculated using Pearson’s and Spearman’s tests.

**Results**

**Clinical–histological characteristics**

As shown in Table 1, patients in the control group exhibited normal renal function, with no signs of tubulointerstitial injury. Compared with the control group, patients in the AA-ATN and a-ATN groups had elevated but similar blood pressures that were still within normal range. Acute renal failure was observed in all ATN patients. Although Scr was lower in the AA-ATN group than in the a-ATN group, this difference did not reach statistical significance. In addition to the epithelial degeneration, necrosis, collapse and exfoliation that were observed in the a-ATN group, patients with AA-ATN also presented histological characteristics such as areas of tubules with completely naked TBM with no apparent signs of regeneration. Both ATN groups exhibited notable renal interstitial oedema, with rare inflammatory cell infiltration. Interstitial fibrosis was not found, and glomeruli as well as arterioles appeared normal in all ATN patients by light microscopy examination. It is noteworthy that patients in the AA-ATN group showed a wide range in Scr (189.3–628.7 μmol/l), which corresponded to the different levels of acute tubular injury by histological semiquantitative scoring (TI from 1.7 to 3.7). For the patient showing the slightest renal dysfunction, the renal biopsy displayed only diffuse degeneration of the renal tubules, with local necrosis and naked TBM. The more severely diseased kidneys showed diffuse tubule necrosis. However, despite a wide range of tubule injury, signs of regeneration were scarcely seen. The same discrepancy was seen in the a-ATN group with respect to the degree of renal dysfunction and histological tubule injury. However, apparent tubule regeneration was observed in all of the a-ATN patients, which contrasted sharply with the lack of regeneration in AA-ATN patients.

The intervals marking the end of drug taking to the renal biopsy were nearly the same in AA-ATN and a-ATN patients. There was no significant difference in the time course between disease onset and renal biopsy. After 2–6 months of follow-up, none of the AA-ATN patients regained normal renal function, which contrasted with that of a-ATN patients, in whom four cases (80%) showed normal levels of Scr (Table 2).

**Evaluation of RTEC regeneration**

To determine the causes of poor tubular repair after injury by GMT, we observed the expression of PCNA and EGF and compared these in the AA-ATN and a-ATN groups. As shown in Table 3 and

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Age (year)</th>
<th>BP (mmHg)</th>
<th>Scr at renal biopsy (μmol/l)</th>
<th>TI (grade)</th>
<th>Tubule regeneration (grade)</th>
<th>Interstitium infiltrate (grade)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>35.4 ± 9.6</td>
<td>118 ± 10</td>
<td>75 ± 8</td>
<td>46.4–100.3 (78.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>a-ATN</td>
<td>9</td>
<td>38.3 ± 10.4</td>
<td>134 ± 5</td>
<td>84 ± 4*</td>
<td>212.8–769.4 (486.5)*</td>
<td>2.9 ± 0.9*</td>
<td>1.9 ± 0.8*</td>
</tr>
<tr>
<td>AA-ATN</td>
<td>8</td>
<td>38.6 ± 8.5</td>
<td>135 ± 3</td>
<td>84 ± 5*</td>
<td>189.3–628.7 (300.6)*</td>
<td>2.7 ± 1.7*</td>
<td>0.5 ± 0.5*</td>
</tr>
</tbody>
</table>

*P < 0.01 vs control; *P < 0.05 vs a-ATN.
Control 10 0.12 \pm 0.07
/C6
/C6
a-ATN 9 47.25 \pm 19.33^a
/C6
/C6
AA-ATN 8 0.01 \pm 0.02^{ab}

\^P < 0.001 vs control; \^P < 0.001 vs a-ATN; \^P < 0.05 vs control.

Table 3. Expression of PCNA and EGF in RTEC

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Number of PCNA^{a+}-RTEC (%)</th>
<th>Number of EGF^{b+}-RTEC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>0.12 \pm 0.07</td>
<td>0.51 \pm 1.38</td>
</tr>
<tr>
<td>a-ATN</td>
<td>9</td>
<td>47.25 \pm 19.33^a</td>
<td>64.38 \pm 19.22^a</td>
</tr>
<tr>
<td>AA-ATN</td>
<td>8</td>
<td>0.01 \pm 0.02^{ab}</td>
<td>9.55 \pm 7.22^{ab}</td>
</tr>
</tbody>
</table>

There was no difference in the number of VIII-Ag-positive glomerular capillary lumina among the three groups (Table 5). There were no notable morphological alterations in glomerular endothelial cells or GBM by electron microscopic examination.

Evaluation of factors related to interstitial fibrosis

To investigate the factors contributing to the progressive renal damage in AA-ATN, we assessed the regulating effect of cytokines on tissue repair and fibrosis by investigating the expression of TGF-\(\beta_1\) and CTGF in RTEC and renal interstitium. Expression of \(\alpha\)-SMA in RTEC was detected as a marker of tubular epithelium transdifferentiation. Components of extracellular matrix (ECM), such as FN, Col-III and -IV in renal interstitium were measured. All factors were then compared between the AA-ATN and a-ATN groups. As shown in Figure 2 and Table 4, the expressions of TGF-\(\beta_1\), CTGF, \(\alpha\)-SMA and all components of ECM in RTEC and renal interstitium were almost negative in the control group. However, the expressions of TGF-\(\beta_1\), CTGF and \(\alpha\)-SMA were increased in the tubulointerstitium with no obvious differences between AA-ATN and a-ATN. The majority of cells expressing TGF-\(\beta_1\) but not RTEC were mononuclear cells. CTGF in the renal interstitium was distributed around TBM, Bowman’s space and renal arterioles, but was rarely seen in RTEC. With respect to ECM deposition, FN, Col-III and -IV were detected in the interstitium of AA-ATN but not in a-ATN patients.

PTC and glomerular capillary alterations

The density and ultrastructure of PTC and glomerular capillaries were examined for the presence of microvascular injuries in the ATN groups.

There was no difference in the number of VIII-Ag-positive glomerular capillary lumina among the three groups (Table 5). There were no notable morphological alterations in glomerular endothelial cells or GBM by electron microscopic examination.

Kidney sections from the control group exhibited a normal PTC pattern, with a lacy capillary network encircling normal-appearing tubules (Figure 3A). The endothelial cells of PTC were seen tightly conjoined with TBM (Figure 4A).

PTC density was slightly diminished in the a-ATN group due to mild expansion of the interstitium caused by oedema (Table 5). The lumina exhibited dilation, with intact capillary wall (Figure 3B). The ultrastructure of PTC endothelial cells and TBM was normal (Figure 4B).

Reduction in VIII-Ag-positive PTC lumina was clearly apparent and highly significant in the AA-ATN group compared with the a-ATN and control groups (211.08 \pm 56.15 vs 413.54 \pm 66.59 and 536.62 \pm 68.38/mm\(^2\), \(P < 0.01\) and 0.001, respectively, Table 5). PTC lumina were obviously dilated and misshapen, and the luminal walls were partially disrupted (Figure 3C). Electron microscopic analysis revealed that the endothelial cells were swollen and fenestration could not be recognized, and that a number of vacuoles and granules were present in the cell plasma. The basement membrane of PTC was shrunken and thickened, and was multilayered in some parts. Some components of the endothelial cells were detached from the TBM. No apparent signs of apoptosis could be seen in the nuclei. (Figure 4C–E)

VEGF expression and its relationship with tubule regeneration

As shown in Table 5 and Figure 5, the expression of VEGF in renal tubules was significantly increased in all of the ATN patients, and it showed a close correlation with PTC density (\(r = 0.793, P < 0.01\)). However, the magnitude of VEGF increment was significantly lower in AA-ATN than in a-ATN patients (increment of 9.4-fold vs 20.2-fold, \(P < 0.01\)). Furthermore, statistical analysis revealed that both PTC density and VEGF expression in renal tubules were correlated with tubule regeneration (\(r = 0.880\) and 0.802, respectively, \(P < 0.01\)).
Discussion

Since Belgian nephrologists first reported a rapidly progressive interstitial renal fibrosis induced by the Chinese herb Aristolochia fangchi in the early 1990s [1], the renal toxicity of Chinese herbs has received much attention in China. It has been suggested that AA contained in Aristolochia fangchi may be responsible for the pathogenesis of this disease [7,8]. GMT (Aristolochiae manshuriensis Kom.) is a herb commonly used for treating disorders of the urinary and cardiovascular systems. Among herbs that contain AA, GMT may in fact be the most widely used in our country. Besides AAs and their derivatives, GMT has been reported to contain other chemicals including magnoflorine, β-sitosterol, acetyl-β-sitosterol, P-hydroxycinnamic acid, stigmastane-3, 6-dione, 6-hydroxy-stigmas-4-en-3-one, daucosterol and 1-octacosanoyl glyceride [9], none of which has been examined for nephrotoxicity. Since AAs are the main

Fig. 1. PCNA staining and EGF expression. (A, D) Controls: no expression of PCNA and EGF in RTEC; (B, E) PCNA\(^{+/+}\) in most RTEC with increased expression of EGF in α-ATN; (C, F) Almost no PCNA \(^{+/+}\) RTEC with decreased EGF expression in AA-ATN.
consists in GMT, and because GMT-induced rat models of acute and chronic tubulointerstitial nephritis resemble that of AAs-induced renal injury in humans [10], GMT has been recognized as a major herb inducing AAN in China.

In recent years, the clinical–pathological features of AAN (caused mainly by taking GMT) have been studied and clarified by Chinese researchers [4]. It is now known that AA appears to damage mainly RTEC and cause acute, sub-acute and chronic renal

![Fig. 2. Expression of TGF-β1, CTGF, α-SMA, FN, Col-III and Col-IV in RTEC and renal interstitium. (A1–3) Expression of TGF-β1 in interstitium. (B1–3) Expression of CTGF in interstitium. (C1–3) Expression of α-SMA in tubulointerstitium. (D1–3) Expression of FN in interstitium. (E1–3) Expression of Col-III in interstitium. (F1–3) Expression of Col-IV in interstitium. 1, Control; 2, α-ATN, 3, AA-ATN. All factors were close to negative in the control group. Expressions of TGF-β1, CTGF in interstitium and α-SMA in tubulointerstitium were all increased in AA-ATN and α-ATN. CTGF in renal interstitium was distributed around TBM, Bowman’s space and renal arterioles. Deposition of FN, Col-III and -IV was detected in the AA-ATN but not in the α-ATN group.](image-url)
tubulointerstitial injuries. *In vitro* studies have demonstrated that the proximal tubules are the main target of AA, which exhibit necrosis, programmed death, transdifferentiation and dysfunction of protein reabsorption [11,12]. Lebeau *et al.* [13] confirmed *in vivo* that proximal tubular dysfunction can be detected as early as 2 days after AA administration, and a more pronounced intoxication may lead to severe tubular atrophy and interstitial fibrosis. In a previous clinical–pathological study, we showed that acute nephrotoxicity by AA presents a severe epithelial degeneration, necrosis, collapse and exfoliation with completely naked TBM (namely AA-ATN) [4]. Interestingly, this cell injury was not accompanied by regeneration as is usually seen in a-ATN. Prognostic analysis shows that 63.5% of patients with AA-ATN have persistent deterioration of renal function after withdrawal of the drug. Some even develop end-stage renal disease within half a year. These findings indicate that AA-ATN represents a special form of ATN,
characterized by absence of cell regeneration and a tendency towards chronic lesions. However, the mechanisms responsible for these pathologies are not known.

Tubular epithelial cells have the potential ability for repair after ischaemic or toxic injury. When injured, cells undergo necrosis and/or apoptosis, the non-injured cells proliferate actively so that the integrity of tubules can be maintained and renal function can be recovered [14,15]. In our study, RTEC showed a remarkable expression of PCNA in a-ATN patients, indicating an active proliferating status. However, no detectable PCNA-positive RTEC were observed in AA-ATN patients, even though the time course from the end of drug to renal biopsy varied from 5 days to as long as 30 days, which suggested that there was no or inadequate regeneration in these cells. To explain the inability of RTEC regeneration in AA-ATN, we further studied the EGF expression in biopsy specimens from these patients. We found that the EGF

Fig. 2. continued.
response was considerably decreased in AA-ATN, which contrasted with the highly increased EGF expression in a-ATN. EGF is recognized as the most potent promoter of RTEC mitogenesis [16]. Expression of EGF and its receptor is significantly enhanced in renal tissue after acute ischaemic or toxic injury [17–19]. Thus, our results suggest that suppressed expression of EGF may probably contribute to the lack of RTEC regeneration.

For complete regeneration, mature RTEC must first be dedifferentiated, and express proteins that exist during the embryo stage, such as α-SMA [20]. These non-mature cells then enter cell cycles and reproduce themselves. After proliferation, the non-mature cells redifferentiate into mature RTEC so that the integrity of the tubules can be maintained. On the other hand, RTEC also have the potential ability to undergo transdifferentiation to then participate in interstitial fibrosis [15,16,21]. Here again, α-SMA is regarded as the marker of interstitial myofibroblasts. In this study, we found that α-SMA, a phenotypic marker of both dedifferentiation and transdifferentiation in RTEC, was highly expressed in both the a-ATN and AA-ATN groups, whereas RTEC entered the cell cycle and proliferated to rebuild the tubules only in the a-ATN group. Thus, the elevated α-SMA expression in AA-ATN indicates that the RTEC may undergo transdifferentiation to further initiate chronic disease progression.

AAN is well-known to exhibit ‘fast’ fibrosis, which is correlated with its poor prognosis. However, the mechanisms of ‘fast’ fibrosis remain unclear. In this study, our results showed that the expressions of TGF-β1 and CTGF were increased in the tubulointerstitial area in both AA-ATN and a-ATN. In contrast, components of ECM (such as FN, Col-III and -IV) were expressed in the renal interstitium only in the AA-ATN group. As previously reported, FN and Col-III are usually detected in the early stages, and Col-IV appears in the end-stage of renal interstitial fibrosis [22]. Therefore, the expression of FN, Col-III

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>PTC density (mm²)</th>
<th>GC density (glomerulus)</th>
<th>VEGF⁺ RTEC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>549.0 ± 67.7</td>
<td>41.3 ± 9.4</td>
<td>2.1 ± 2.7</td>
</tr>
<tr>
<td>a-ATN</td>
<td>9</td>
<td>422.7 ± 67.4</td>
<td>39.4 ± 7.6</td>
<td>43.3 ± 7.2b</td>
</tr>
<tr>
<td>AA-ATN</td>
<td>8</td>
<td>209.1 ± 68.3b</td>
<td>38.9 ± 7.0</td>
<td>23.2 ± 15.0b</td>
</tr>
</tbody>
</table>

aP < 0.01 vs a-ATN; bP < 0.001 vs control; cP < 0.01 vs control.

Table 4. Expression of factors associated with renal interstitial fibrosis

<table>
<thead>
<tr>
<th>Factors</th>
<th>Control (+/400x)</th>
<th>a-ATN</th>
<th>AA-ATN</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 cells</td>
<td>0.00±0.00</td>
<td>13.00±12.00b</td>
<td>7.00±4.00a</td>
</tr>
<tr>
<td>CTGF area (%)</td>
<td>0.00±0.00</td>
<td>0.75±0.56</td>
<td>0.64±0.67a</td>
</tr>
<tr>
<td>α-SMA area (%)</td>
<td>0.00±0.00</td>
<td>0.63±0.40</td>
<td>0.81±0.62a</td>
</tr>
<tr>
<td>FN area (%)</td>
<td>0.34±0.09</td>
<td>0.38±0.15</td>
<td>3.28±1.11b</td>
</tr>
<tr>
<td>Col-III area (%)</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.64±0.31b</td>
</tr>
<tr>
<td>Col-IV area (%)</td>
<td>0.46±0.11</td>
<td>0.50±0.12</td>
<td>3.46±1.43b</td>
</tr>
</tbody>
</table>

aP < 0.001 vs control; bP < 0.001 vs a-ATN.

Table 5. PTC and glomerular capillary density and VEGF expression in renal tubules

Fig. 3. Expression of VIII-Ag in renal interstitium. (A) Control: kidney sections of the control group exhibited a normal PTC pattern, with a lacy capillary network encircling normal-appearing tubules. (B) a-ATN: PTC density was slightly diminished. (C) AA-ATN: reduction in PTC lumina was clearly apparent and highly significant in the AA-ATN group compared with the a-ATN and control groups. Dilation and misshapen PTC lumina were obvious, and the luminal walls were partially disrupted.
and IV confirmed the early initiation of interstitial fibrosis in AA-ATN, at which time fibrosis was not yet observed by light microscopy. TGF-β1 and CTGF both regulate tissue repair in different diseases [23–25]. Since increased expression of TGFβ1 and CTGF were detected in both a-ATN and AA-ATN, whereas ECM deposition was observed only in the latter, it is possible that anti-fibrotic mechanisms were impaired in AA-ATN.

PTC, a network of interstitial vessels, are thought to be essential in maintaining renal function and haemodynamics. In recent years, PTC injury, with subsequent tissue hypoxia and ischaemia, has been suggested to play a very important role in progressive tubulointerstitial injuries, except for proteinuria and inflammation [26,27]. Since AA-ATN, as mentioned above, is characterized by a poor ability for regeneration and a potent tendency towards fibrosis, and

Fig. 4. Morphological alterations in PTC endothelial cells and lumina. (A) Controls: the endothelial cells of PTC were seen tightly conjoined with TBM. (B) a-ATN: the lumina of PTC exhibited dilation, with intact capillary wall. (C, D, E) AA-ATN: the endothelial cells were swollen, with a number of vacuoles (C) and granules (D) occurring in the cell plasma. The basement membrane of PTC was shrunken and thickened, with multilayers in some parts. (D) Parts of the endothelial cells were detached from the TBM (E).
because neither proteinuria nor inflammatory mechanisms could be incriminated in the tubulointerstitial damage in AA-ATN, we further examined the possibility of microvascular injuries. We found that there was severe loss of PTC in AA-ATN, which could not be simply due to interstitial oedema, as was found in α-ATN patients. The misshapen and disrupted PTC lumina, together with ultrastructural alterations of endothelial cells and TBM, which only occurred in AA-ATN patients, suggest that primary PTC damage, including endothelial cell injury and loss of structure, is an additional characteristic of AA-ATN. In a recent study, we observed the same phenomena in acute AAN rats, together with highly expressed hypoxia-induced factor-1 (HIF-1). Hypoxia and decreased blood flow in the tubulointerstitium were observed in the same model (unpublished data). These findings indicate that microvascular damage in α-ATN could cause hypoxia, which is known to have the ability to induce tubule injury, atrophy and interstitial fibrosis [28]. Since there were no notable alterations in glomerular endothelial cells or GBM in the present study, there appears to be a selective PTC injury in AA-ATN. This selective injury may be caused by condensed AA and its metabolites in the renal tubulointerstitium. Moreover, since VEGF is the most potent factor that stimulates angiogenesis [29], and since we observed a close correlation between VEGF expression in renal tubules and PTC density, the relatively lower VEGF response in renal tubules that we observed may contribute, at least in part, to the loss of PTC in AA-ATN.

In summary, AA-ATN represents a special type of ATN associated with a very poor outcome. We demonstrated that the strong ability of RTEC for repair after acute injury is severely reduced in AA-ATN, which may be due in part to reduced EGF expression. Injury and loss of PTC occur in AA-ATN, and this may contribute to the tubulointerstitial damage.

Acknowledgement. This paper was supported by a Grant (985-2001) from Peking University.

Conflict of interest statement. None declared.

References


Received for publication: 3.12.05
Accepted in revised form: 18.8.06