Role of atrophic changes in proximal tubular cells in the peritubular deposition of type IV collagen in a rat renal ablation model

Masato Kimura1, Mitsuko Asano1, Katsushige Abe2, Masanobu Miyazaki2, Takayuki Suzuki3 and Akira Hishida3

1University of Shizuoka, School of Nursing, Shizuoka, Shizuoka, Japan, 2Nagasaki University School of Medicine, The Second Department of Medicine, Nagasaki, Nagasaki, Japan and 3Hamamatsu University School of Medicine, The First Department of Medicine, Hamamatsu, Shizuoka, Japan

Abstract

Background. Tubular atrophy, dilation and interstitial fibrosis are common in tubulointerstitial lesions, but the precise roles and inter-relationships of these components in the development of interstitial lesions have not been determined. This study focused on the origin and roles of atrophic tubules in the peritubular deposition of type IV collagen in a rat renal ablation model.

Methods. Male Wistar rats underwent 5/6 nephrectomy or sham operation, and then were sacrificed at 4, 8 or 12 weeks, their remaining kidneys removed for histological and immuno-histochemical studies as well as in situ hybridization for type IV collagen mRNA.

Results. Immmuno-histochemistry demonstrated the positive staining of atrophic tubules to vimentin, platelet-derived growth factor-B chain (PDGF) and heat shock protein 47 (HSP47). Cells positive to one or more of PDGF receptor β, α-smooth muscle actin (α-SMA), and HSP47 accumulated around atrophic tubules. Type IV collagen was also increased in the proximity of the atrophic tubules. These intimate relationships were more clearly demonstrated in ‘mosaic tubules’, which are composed of both intact and atrophic proximal tubular epithelial cells, and which had a mixed pattern of staining with vimentin, PDGF and HSP47. The interstitial cells positive to α-SMA or HSP47, or both, were in close contact with atrophic but not with intact epithelial cells. Type IV collagen was exclusively deposited between atrophic tubules and HSP47-positive interstitial cells. In situ hybridization of type IV collagen mRNA demonstrated predominant expression in atrophic tubular epithelial cells, but not in surrounding interstitial cells.

Conclusions. These findings suggest that atrophic proximal tubular cells are active in the development of collagen deposition in the peritubular space, i.e. in this model type IV collagen in the interstitial fibrotic area may be produced mainly by atrophic proximal tubules.

Keywords: atrophic tubule; α-smooth muscle actin (α-SMA); fibroblast; interstitial fibrosis; platelet-derived growth factor (PDGF); renal ablation

Introduction

It is recognized that tubulointerstitial rather than glomerular scarring is the more representative lesion in patients with progressive renal disease [1–3]. Although the pathogenesis of interstitial fibrosis has been studied extensively, many unsolved questions remain about the initial cause of fibrosis, the stimuli responsible for its perpetuation and the identity of profibrogenic cells [4–10].

Multiple stimuli arising from renal interstitial and tubular epithelial cells along with systemic stimuli seem to converge on the interstitial fibroblasts to promote proliferation and collagen production. Thus, many investigators have suggested that fibroblasts, especially myofibroblasts (which are thought to originate from interstitial fibroblasts), are key cells in bringing about renal interstitial fibrosis [4,5,7–9]. The marked proliferation of interstitial myofibroblasts and their distribution, which parallels that of interstitial transforming growth factor-β and type IV collagen, are observed in both experimental and clinical nephropathies [8]. However, the exact role of renal interstitial myofibroblasts in renal interstitial collagen deposition remains to be determined. In addition to myofibroblasts, other cells, such as macrophages, endothelial cells, adventitial...
cells and tubular epithelial cells, have been implicated in the production and deposition of extracellular matrix during scarring [4,5].

That epithelial cells may play a role in the development of interstitial fibrosis has recently attracted considerable attention. This concept is intriguing, since tubular epithelial cells are of mesodermal origin, with the metanephric mesenchyme giving rise to both epithelial and interstitial cells. Both in vitro and in vivo studies have provided evidence suggesting that tubular cells themselves may transdifferentiate and acquire myofibroblast-like properties, and may secrete matrix proteins, including types I, III, IV and V collagen, into the interstitial space [4,8,10,11]. However, no direct evidence has been demonstrated for in vivo collagen production by tubular cells in interstitial fibrosis.

In the present study, we evaluated interstitial lesions in a rat model of renal ablation, focusing on atrophic tubules and their interaction with interstitial cells in the development of peritubular fibrosis. To investigate the mechanism(s) of tubular basement membrane thickening and the fibrosis of the surrounding interstitium, several antigens were examined in the tubulointerstitium [12]. Vimentin, which is expressed ubiquitously in mesenchymal cells but not expressed by normal tubular epithelial cells, is known to be expressed by degenerating and regenerating tubular cells. Platelet-derived growth factor (PDGF) has been reported to be an important mediator of tubulointerstitial hyperplasia and fibrosis (due to its chemotactic and mitogenic effects on renal interstitial fibroblasts), transforming these cells into myofibroblasts [6]. The PDGF receptor (PDGFR), which is the presumed target for PDGF, has been known to be absent or expressed weakly in either or both of distal tubules and collecting ducts in normal rats. In diseased kidneys, strong staining in interstitial cells (most likely fibroblasts) was observed around the atrophic tubules. α-Smooth muscle actin (α-SMA), which is normally synthesized by vascular smooth muscle cells, has been known to be expressed in contractile fibroblasts, i.e. myofibroblasts. Collagen-synthesizing cells were identified by staining for heat shock protein (HSP)47 and by in situ hybridization of type IV collagen mRNA. This HSP is recognized as a collagen-specific molecular chaperone that plays a pivotal role in the biosynthesis, processing and secretion of procollagen from the endoplasmic reticulum.

In the conclusion, we propose that the atrophic proximal tubular cells are the principal cells involved in the deposition of type IV collagen in the peritubular area.

Subjects and methods

Animal model

Male Wistar rats (n = 19) (SLC, Hamamatsu, Japan) aged 12 weeks and weighing 270–290 g were subjected to right nephrectomy and the ligation of 2 of 3 branches of the left renal artery in order to cause infarction of two-thirds of the left renal mass. Before permanent ligation, we confirmed that the ischemic surface area was approximately two-thirds of the total area of the left kidney. Control rats (n = 6) underwent a sham operation without nephrectomy or arterial ligation. Five to eight rats of the experimental group were sacrificed before and 4, 8 and 12 weeks after surgery. Control animals were sacrificed only 12 weeks after surgery (n = 6). The left kidney from each sacrificed rat was removed and weighed after perfusion with 10 ml of cold saline, then fixed in 10% buffered formalin or methyl Carnoy’s solution. The Ethics Review Committees for Animal Experimentation of the participating universities approved the experimental protocol.

Histochemical and immuno-histochemical examinations

For histochemical and immuno-histochemical examinations, 4μm thick sections were prepared from kidneys fixed in methyl Carnoy’s solution and stained by an avidin-biotin-horseradish peroxidase method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). We tested for several antigens in tubular epithelial cells and the interstitium using the following antibodies: mouse monoclonal antibodies against PDGF (monoclonal antibody to PDGF-B chain, PGF-007: a gift from Mochida Pharmaceutical, Tokyo) [13], human α-SMA (DAKO, Glostrup, Denmark), vimentin (from porcine ocular lens; Sigma, St Louis, MO), rabbit polyclonal antibodies against the PDGF-R (Santa Cruz Biotechnology, Santa Cruz, CA) and type IV collagen (LSL, Tokyo). The secondary antibodies used were biotin-labelled donkey sera against mouse, rabbit or sheep IgG (Chemicon International, Temecula, CA). Collagen-synthesizing cells were identified by staining for HSP47 using a mouse monoclonal antibody (Stressgen; Victoria, BC, Canada).

Negative controls consisted of the substitution of each primary antibody with an irrelevant murine monoclonal antibody or saline. None of the controls exhibited staining. To determine the origin of atrophic tubules, biotin-labelled lectin from Phaseolus vulgaris erythro-agglutinin (PHA-E; Sigma) was used for the proximal tubule [14], sheep polyclonal antibody against human Tamm-Horsfall glycoprotein (THP; Chemicon International) for the thick ascending limb of the loop of Henle [14] and biotin-labelled peanut agglutinin (PNA; Biomeda, Foster City, CA) for the distal tubule [15].

In situ hybridization

For in situ hybridization of type IV rat collagen, the oligonucleotides of the 35mer sequence of mRNA for α-1 type IV rat collagen were selected. The sequence of sense oligonucleotides, corresponding to bases 922–956, was 5’-ctctggcggtattttcagggtttgccacactatccg-3’. The selected sequence showed no significant similarity with known sequences deposited in the gene bank data (GenBank, Release 123, 2001). The oligonucleotide probe was labelled using a digoxigenin (DIG) oligonucleotide tailing kit, following the instructions provided by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). In situ hybridization was performed according to a modified method developed in our laboratory [16]. Briefly, frozen
Atrophic changes in peritubular deposition of type IV collagen

tissue sections (4μm) were fixed with 4% paraformaldehyde in phosphate-buffered saline buffer (PBS) and washed with PBS. To expose the RNA, sections were deproteinized using HCl and proteinase K (Sigma). Sections were then prehybridized in 4× standard saline citrate (SCC), 3× Denhardt’s and 20% formamide. After prehybridization, the sections were hybridized with a DIG-labelled oligonucleotide probe in the prehybridization buffer. After washing with 0.075% BRIJ (Sigma) in 2× and 0.5× SCC at room temperature, sections were stained immuno-histochemically to detect the presence of the hybridized DIG-labelled probe using, successively, mouse monoclonal anti-DIG antibody (Roche Molecular Biochemicals), horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (DAKO) and HRP-conjugated swine anti-rabbit antibody (DAKO). Colour was developed by reaction with H2O2 and diaminobenzidine tetrahydrochloride. Finally, sections were counterstained with methyl green and mounted. Cells whose cytoplasm was clearly stained in or those stained with a perinuclear pattern were identified as type IV collagen mRNA-positive cells. On the other hand, cells with nuclei stained with methyl green alone were considered negative for type IV collagen mRNA. To evaluate the specificity of the signals for type IV collagen mRNA, we performed three control experiments including pre-treatment of RNase, a study with a sense probe and a competitive study, as described previously [17].

Morphometric study

A point-counting method was employed for the morphometric evaluation of interstitial fibrosis, tubular basement membrane thickening and dilation of the tubular lumen, as previously described [18]. For each kidney, 20 photographs with a final magnification of x100 were analysed. The interstitial fibrotic area was calculated by counting the number of crossing points overlapping with the cortical area stained green by trichrome, except in the area immediately surrounding large vessels. The tubular basement membrane was included in the area measured. Tubular basement membrane thickening was assessed by counting these areas, again separately from areas of interstitial fibrosis. The area of the tubular lumen was measured in a similar manner. All areas were expressed as percentages of the total cortical area.

To evaluate the distribution of glomeruli, atrophic tubules and dilated tubules in the cortex and the outer zone of the outer medulla, photographs with a final magnification of x100 were used. Specimens for photography were prepared from eight rats at 12 weeks after surgery. To examine the distribution of glomeruli and atrophic tubules, longitudinal kidney sections stained with Masson’s trichrome were divided into 10 equally wide zones from the renal surface to the outer border of the inner zone of the outer medulla. The numbers of glomeruli and atrophic tubules located in each zone were counted separately and expressed as a percentage of the total number. We defined an atrophic tubule as one that has a pale cell body, lacks brush borders and is surrounded by a cuff-like, thickened basement membrane. Dilated tubules with large lumina and relatively flattened epithelial cells, often having huge proteinaceous casts, are not associated with basement membrane thickening. However, since it is difficult to exactly distinguish abnormally dilated tubules from normal ones, we used the percent area of tubular lumen as an index of tubular dilation. This was derived by calculating the sum of the width of tubular lumina crossing each zonal line: nine zonal border lines were drawn, and the lengths of the lines crossing the tubular lumina were measured and summed up at each line, and expressed as a percentage of the length of each zonal line.

Statistical analysis

Data are expressed as the mean±SEM. Statistical significance was determined by one-way analysis of variance (ANOVA), with Scheffe’s correction for multiple comparisons. A P-value of <0.05 was considered statistically significant.

Results

Histologic and morphometric findings

The sole abnormal histological finding at 4 weeks was dilated distal tubules, scattered predominantly in the superficial cortex. At 8 weeks, we noted additional tubulointerstitial lesions: atrophic tubules with cuff-like thickening of the basement membrane and interstitial fibrosis. Tubulointerstitial lesions progressed thereafter, and 12 weeks after surgery we found severe destruction of renal architecture. The morphometric study confirmed the temporal course of histologic progression (Figure 1). An increase in the area of tubular lumen was the first pathological finding (becoming prevalent by 4 weeks after surgery) followed by the thickening of the tubular basement membrane and interstitial fibrosis, the latter two becoming significant by 8 weeks after surgery. The distribution of atrophic tubules and the tubular lumen in the

Fig. 1. Morphometry of renal tubulointerstitial tissues of 5/6 nephrectomized rats. Areas of tubular basement membrane (A), interstitial fibrosis (B) and tubular lumina (C) were measured by the point-counting method and expressed as a percentage of total cortical area. *P<0.05, **P<0.01, compared with values at surgery.
cortex and the outer zone of the outer medulla are shown in Figure 2. On the Masson’s trichrome-stained specimens, the boundary between OSOM and ISOM is not difficult to define. Most arcuate arteries were located in zone 7. We supposed that the boundary between the cortex and the OSOM is around zones 7 and 8, because some arcuate arteries were found in zone 6 and the number of glomeruli decreased abruptly inside zone 6. The irregularity among rats of the boundary may be related to hypertrophy following ablation. Thus, about 80% of the atrophic tubules were found in the deep half of the cortex and the outer stripe of the outer medulla. In contrast, the increase in the tubular lumen area was noted mostly in the cortex, especially in its superficial portion. Although the morphometric scores of tubular luminal areas do not necessarily indicate the dilation on each tubule, they seem to reflect the increase in the number of dilated tubules or the severity of dilatation, or both. In control animals, the percent areas of tubular lumen were equal on all nine contour lines.

Fig. 2. Distribution of glomeruli, atrophic tubules and dilated tubules located between the renal surface (left margin:0) and the outer border of the inner zone of the outer medulla (right margin:10) of the kidneys of rats sacrificed at 12 weeks after 5/6 nephrectomy. (A) The number of glomeruli located in each zone is expressed as a percentage of the total number of glomeruli. (B) The number of atrophic tubules in each zone is expressed as a percentage of the total number of atrophic tubules. (C) The length of each zonal border line crossing the tubular lumina was summed and expressed as a percentage of the length of each zonal line. Closed circles: values of control rats before surgery.

Histochemical and immuno-histochemical findings

Groups of atrophic tubules with tubular basement membrane thickening were scattered among intact tubules. Interstitial fibrosis was restricted to almost the same area as tubular atrophy. These areas are easily distinguished from the intact area by immuno-histochemical staining with anti-vimentin antibody. The relationships between atrophic tubules and the staining of some antigens were distinctly observable in sequential sections. Masson’s trichrome stain demonstrated surrounding atrophic tubules express vimentin, PDGF-B chain and HSP47 in their cytoplasm, and intact proximal tubules were strongly positive for all of them. Distal tubules (*) do not express any antigens but the PDGF-B chain. Many interstitial cells surrounding atrophic tubules express vimentin, PDGF-R, α-SMA and HSP47. α-SMA-positive cells appear to be the same cells expressing PDGF-R. A massive accumulation of type IV collagen is demonstrated surrounding atrophic tubules, but not between intact proximal tubules. PHA-E are demonstrated in intact proximal tubules. Atrophic tubules and distal tubules (*) do not express these molecules. Black bars represent 100 μm.
in close proximity to atrophic tubules, and seemed to comprise a major part of the cuff-like basement membrane thickening or peritubular fibrosis; however, it was sparsely distributed between intact tubules (Figure 3G), with a distribution similar to the area stained green by Masson’s trichrome method (Figure 3A). Typical atrophic tubules were not stained by any of the markers for the tubular segments: PHA-E (Figure 3H), PNA or anti-THP antibody (data not shown).

Along with atrophic tubules, especially in the periphery of the cluster of atrophic tubules, we found some peculiar tubules that in cross section were composed of two groups of cells: intact proximal tubular epithelial cells with brush borders and atrophic epithelial cells with short cell bodies and no brush border (Figure 4A). The same types of tubules were found in the microembolic model, which were referred to as ‘mosaic tubules’ in our previous report [19]. In these ‘mosaic’ tubules, vimentin, PDGF and HSP47 stained atrophic cells, but not normal cells (Figure 4B, C and E). Using serial sections, we confirmed that most atrophic cells were positive for all three antigens (vimentin, PDGF and HSP47). α-SMA-positive interstitial cells were in close contact with the atrophic epithelial cells, but not with the intact ones (Figure 4D). The HSP47-positive interstitial cells were distributed in a similar pattern and were spatially restricted to atrophic cells in the mosaic tubules (Figure 4E). Masson trichrome’s stain showed collagen to be accumulated between HSP47-positive tubular and interstitial cells (Figure 4A and E).

Though these close relationships between atrophic tubular cells and peritubular collagen deposition were found on almost all sections, a few tubules were positive for vimentin and HSP47, but did not show collagen deposition (data not shown). These findings suggest that the expression of vimentin and HSP47 in atrophic tubules precedes the deposition of collagen in the interstitial space.

When atrophic tubules adjoined intact tubules, collagen deposition was noted only between atrophic tubular cells and either or both of HSP47-positive and α-SMA-positive interstitial cells; but they were never observed at the opposite side of the interstitial cells, that facing intact tubules (Figure 5A and B, double arrows). This relationship between collagen deposition and HSP47- or α-SMA-positive interstitial
cells was also observed in the case of two adjoining mosaic tubules (Figure 5C).

In situ hybridization of type IV collagen mRNA

To identify the cells that produced collagen in the peritubular space, we performed in situ hybridization of type IV collagen mRNA using frozen tissue sections from rats sacrificed 12 weeks after nephrectomy. Type IV collagen mRNA was predominantly expressed in the atrophic tubular epithelial cells with peritubular fibrosis (Figure 5D). Apparently, this expression was strong also in the atrophic cells of mosaic tubules, whereas it was trace or weak in the intact cells of those tubules (Figure 5E). Interstitial cells accumulating around atrophic tubules expressed much less type IV collagen mRNA than tubular cells.

Discussion

In this study, we focused on the role of atrophic tubules in the deposition of type IV collagen in the peritubular area in 5/6th nephrectomized rats. Atrophic tubules appeared 8 weeks after surgery, following tubular dilation at 4 weeks. These atrophic tubules were predominantly distributed in the deep cortex and the outer stripe of the outer medulla. The epithelial cells of atrophic tubules demonstrated a strong expression of vimentin and PDGF. PDGF-R-positive cells, which are most likely fibroblasts [19], accumulated densely around atrophic tubules. Many cells positive for α-SMA, the expression of which indicates myofibroblast transformation [20], were also noted in this area. The collagen-specific molecular chaperone HSP47 was strongly expressed in both the atrophic tubular cells and the interstitial cells bordering the atrophic tubules. These findings suggest a close relationship between atrophic tubules, myofibroblasts and collagen deposition in this model.

Lectin, PHA-E (a proximal tubule marker) [14] or peanut agglutinin (a distal tubule marker) [14,15] did not attach to the atrophic tubules. In addition, the tubules did not stain with antibody against THP, which is a marker for the thick ascending limb of the loop of Henle [15]. These findings do not provide conclusive evidence for the way the atrophy of these atrophic tubules comes about, since the lack of these antigens does not necessarily indicate they were never expressed. In this respect, the observations made on mosaic tubules may be quite instructive. Mosaic tubules in the rat model of renal failure were first recognized by us in the microembolism model [12]. Among typical atrophic tubules, some had a mosaic appearance in cross sections that contained intact proximal tubular cells (which have brush borders and stain positive for PHA-E) as well as atrophic cells (with no brush border and with an immunostaining pattern characteristic of atrophic tubules). The proportions of these two cell types varied in each cross section of mosaic tubules. This finding suggests that atrophic tubules were of proximal tubular origin, at least in part. We were recently able to trace the course of some nephrons using sequential sections, and demonstrated atrophic and mosaic tubules scattered along the course of the proximal tubule, especially in its straight portion (unpublished data). This is compatible with the finding that atrophic tubules were mainly distributed in the deep cortex and the outer zone of the outer medulla.

The above-mentioned relationships between the appearance of atrophic tubules, peritubular accumulation of myofibroblasts and collagen deposition were further confirmed by the analysis of mosaic tubules. In mosaic tubules, atrophic cells positive to vimentin and PDGF expressed HSP47. Peritubular collagen deposition was restricted to the area around atrophic epithelial cells. α-SMA-positive myofibroblasts and matrix protein accumulation around mosaic tubules were also spatially restricted, mostly to the area adjacent to atrophic epithelial cells. These findings suggest that atrophic tubular epithelial cells, more than being just coexistent, are directly involved in the development of peritubular fibrosis.

In the present study, HSP47-positive interstitial cells, which are presumed to actively produce collagen, were also distributed in a pattern similar to that observed in α-SMA-positive interstitial cells. However, in the spaces between atrophic and intact epithelial cells, neither excessive matrix protein nor collagen fibre were present between interstitial cells and intact tubular cells. Type IV collagen deposited around HSP47-positive interstitial cells was found exclusively between them and atrophic epithelial cells. Therefore, there appeared to be a distinct polarity for the collagen deposition around interstitial cells. In addition, with the in situ hybridization of type IV collagen mRNA, we found that mRNA strongly expressed in atrophic tubular epithelial cells whereas interstitial cells around atrophic or mosaic tubules showed only weak and occasional expression. Based on these findings, we propose that tubular epithelial cells, not interstitial cells, are the ones actually responsible for the production and deposition of matrix proteins in the peritubular space.

Although there is indirect evidence for the contribution of interstitial fibroblasts in the development of renal fibrosis, the precise role of fibroblasts or myofibroblasts in the development of interstitial fibrosis has not been established [4,5,7,9]. Increased expression of α-SMA in tubulointerstitial areas has been noted in several models of progressive renal injury [5–7,12,20]. In the microembolism model, we found that α-SMA expression correlated closely with PDGF-R expression and that the number of cells staining positive for α-SMA increased in parallel with the degree of interstitial fibrosis [12]. In an in vitro experiment, Johnson et al. [9] reported that proximal tubular cells, through a paracrine mechanism, modulate the behaviour of neighbouring cortical fibroblasts—cell proliferation, collagen synthesis and IGF-1 binding protein-3 secretion. Thus, we cannot
excluded the possibility that communication between atrophic tubular cells and interstitial cells influences the pattern of collagen production by epithelial cells, its deposition in peritubular spaces, or both.

Ng et al. [10] suggested the active involvement of tubular epithelial cells in renal interstitial fibrosis, presenting evidence of tubular epithelial-myofibroblast transdifferentiation in 5/6 nephrectomized rats. They proposed that epithelial-myofibroblast transdifferentiation may be an important inflammatory process in the development of progressive tubulointerstitial fibrosis. However, we could not find such α-SMA-positive tubular epithelial cells except in a small number of dilated tubules lined by flat epithelial cells expressing α-SMA. The reason for the discrepancy between their findings and those of the present study is not clear at the present. One clue to the difference is that the histological changes they observed were at a rather advanced-stage compared with the ones observed in the present experiment. The use of different strains of rats may be another cause, for it is well known that the course or severity of nephropathy varies between strains of rats.

In this study we demonstrated that the significant increase in dilated tubules follows a chronologically significant increase in atrophic tubules. This finding may point to the contribution of tubular dilation to tubular atrophy. However, the increase in dilated tubules was detected in the superficial cortex, and increased tubular atrophy was observed predominantly in the deep cortex and the outer stripe of the outer medulla. Therefore, it is unlikely that the dilation of the tubular lumina is the process that precedes and causes tubular atrophy.

In conclusion, in the rat renal ablation model, atrophic tubular cells are actively involved in the development of collagen deposition in the peritubular space, that is, type IV collagen in interstitial fibrotic area may be produced mainly by atrophic proximal tubules. Although the spatially close relationship between atrophic tubular cells, interstitial cells and collagen deposition suggests a link between these cells in causing fibrosis, a direct role for interstitial cells in the development of peritubular fibrosis was not demonstrated in this study.

Acknowledgements. The authors thank Misses Yuko Fukumoto, Azusa Kimata and Yukino Nagakura for their excellent technical assistance.

Conflict of interest statement. None declared.

References


Received for publication: 17.11.03
Accepted in revised form: 16.2.05