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Demonstration of collagen type VI and alpha-smooth muscle actin in renal fibrotic injury in man

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Abstract

Background. Overproduction of collagenous fibres types I and III is a common finding of fibrotic injury. Collagen type VI is generally associated with type I. Appearance of fibroblasts expressing alpha-smooth muscle actin (ASMA) and their role in fibrogenesis has been partly defined. However, correlation between renal fibroblasts and accumulation of microfibrillar collagen type VI, as well as its exact distribution, is not fully delineated. This study was undertaken to investigate these issues using a complex morphological approach.

Methods. Morphological examination included immunohistochemical detection of the collagen type VI and ASMA, relying on a streptavidin-biotin-peroxidase-based technique, and electron microscopy.

Results. Collagen type VI was strongly expressed in areas of fibrotic injury, although mild expression was always revealed in renal interstitium. Glomerular immunoreactivity with the anti-collagen type VI antibody was almost nil excepting cases of diabetic glomerulosclerosis and amyloid nephrosis. Glomerular nodules in cases of diabetes displayed intense reactivity. Mesangial, as well as discontinuous peripheral deposition of collagen along the glomerular basement membrane, was noticed in case of amyloidosis. Ultrastructurally, cross-banded collagen microfibrils were found in renal interstitium in close association with the fibroblast membrane. Moreover, fibrillar elements revealing tubular structure and fine filamentous material were observed between cross-banded microfibrils. Some of fibroblasts exhibited bundles of microfilaments in their cytoplasm. An increased number of ASMA-positive cells was detected in fibrotic interstitium. An intense concentric network made up of actin-bearing cells surrounded glomerular capillaries in the case of crescentic glomerular lesions.

Conclusions. Markedly increased deposition of collagen type VI takes place in renal fibrotic lesions. Simultaneously, interstitial fibrotic areas appeared to contain a great number of fibroblasts sharing morphological characteristics of classic fibroblasts and smooth muscle cells. Detailed examination of coexistence of these two interstitial phenomena should further clarify the cellular mechanisms involved in renal interstitial fibrosis.

Key words: alpha-smooth muscle actin; biopsies; collagen type VI; immunohistochemistry; renal fibrosis

Introduction

Increased collagen accumulation with progressive organ scarring is the final common pathway to chronic renal failure in many renal diseases. Several investigators have reported changes in the amount, distribution and composition of collagen fibres as well as their role in the progression of glomerular sclerosis and renal fibrosis [1–4]. These papers provided morphological evidence of striking deposition of collagens type I and III in the glomerulus and interstitium in the case of progressive kidney fibrosis. Collagen type VI is a protein that exists widely in the interstitial connective tissue. It is usually found in close association with the surface of striated collagen fibrils [5,6]. Although recent work [7] suggests that collagen type VI may be involved in the regulation of collagen fibril formation and is important in modifying the interactions of fibrils inducing specificity of connective tissues, almost no data exists on the occurrence of collagen VI in human kidneys in the case of kidney pathology.

The aim of this study was to define the pattern of distribution of collagen type VI in the kidneys of patients with primary glomerular disorders, diabetic glomerulosclerosis, amyloid nephrosis, inflammatory interstitial renal lesions and some vascular diseases, using immunohistochemical analysis. Additional ultrastructural analysis of microfibrillar collagen at sites of interstitial fibrotic injury was also performed.

Contractile cells bearing cytoplasmic actin microfilaments have been suggested to play a role in retractile phenomena and extracellular matrix accumulation observed in fibrotic diseases. These persist in fibrotic lesions of many organs. Thus another aim of this study

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was to examine the correlation between the presence of these cells and collagen accumulation. Myofibroblasts were identified by electron microscopy and immunolabelling for alpha-smooth muscle actin (ASMA).

Subjects and methods

Subjects

The subjects included normal subjects (3), patients with minimal change disease (3), mesangial proliferative (5), endocapillary (9) and membranoproliferative (9) glomerulonephritis, interstitial nephritis (3), diabetes mellitus (3) and amyloidosis (7), rapidly progressive glomerulonephritis (4), atherosclerosis (4).

Methods

Kidney biopsies were processed for conventional light microscopy, immunohistochemistry and electron microscopy. For conventional light microscopy and immunohistochemistry tissues were fixed overnight in 10% formalin, processed through absolute ethanol and xylene, and embedded in Paraplast Plus wax (58°C). For diagnostic purposes, sections (4 μm) were stained routinely with haematoxylin and eosin, periodic acid Schiff and Masson's trichrome stain. Additional paraffin sections were used for immunohistochemistry. These sections were processed according to an immunoperoxidase ABS (avidin-biotin-peroxidase complex) modified method [8]. According to this method, two conventional reagents, avidin and biotin-peroxidase, are substituted with a preformed complex in which streptavidin is present instead of avidin. Sections were dewaxed in xylene (three changes, 7 min each), immersed in absolute ethanol (three changes, 3 min each) and then traditionally in graded alcohols (two changes, 2 min each), transferred to a methanol/0.3% hydrogen peroxide solution for 20 min in order to abolish endogenous peroxidase activity. After quenching of endogenous peroxidase activity sections were washed three times in double distilled water, immersed in 0.01 M phosphate-buffered saline (PBS), pH 7.2–7.4, for 10 min and then incubated with normal horse serum 1 : 50 dilution for 30 min at room temperature and with primary antibodies overnight at 4°C in a humid atmosphere. Thereafter, sections were incubated with anti-mouse Ig biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) 1 : 500 dilution for 30 min and streptavidin-biotin-peroxidase preformed complex (BioGenex Laboratories, San Ramon, CA, USA) 1 : 250 dilution for 30 min. The immunological reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (50 mg in 100 ml of PBS with 0.03% v/v hydrogen peroxide). Sections were counterstained with Harrys haematoxylin and mounted in Kaiser's glycerol gelatin. Negative controls were performed by omitting the primary antibodies on one of the two sections per slide.

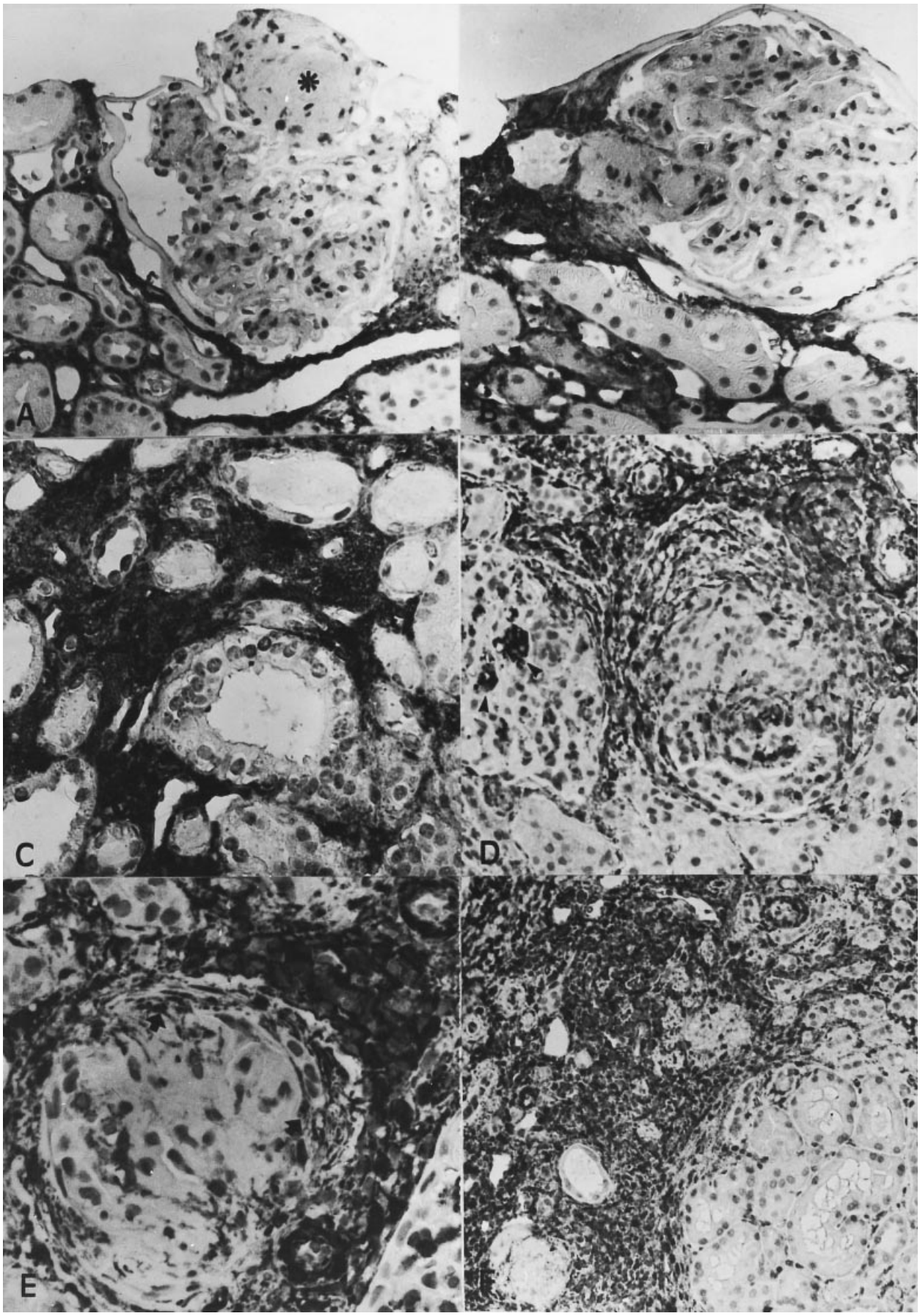
The primary antibodies used in this study were: monoclonal anti-ASMA antibody (clone 1A4) with specificity for only the ASMA isoform (1 : 100, DAKO A/S, Glostrup, Denmark) [9]; monoclonal anti-desmin antibody (1 : 100, DAKO A/S, Glostrup, Denmark), an antibody to intermediate filament protein commonly expressed by smooth muscle cells [10]. The monoclonal antibody to type VI collagen was kindly provided by Prof. Rupert Timpl and Dr Takako Sasaki (1 : 1000, Max-Planck-Institut für Biochemie, Martinsried, Germany) [11].

For electron microscopic studies, material was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h at 4°C, rinsed in three changes of the same buffer for 15 min, post-fixed in 1% osmium tetroxide at 4°C for 1 h, rinsed in buffer for 15 min and placed in 2% uranyl acetate overnight. Thereafter, the samples were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were cut with a diamond knife on an LKB microtome, III. The sections were stained with uranyl acetate and lead citrate and observed using a JEM 100S electron microscope.

Results

In normal kidneys and all cases of non-severe glomerulopathies, as well as diffuse proliferative glomerulonephritides, immunohistochemically detected glomerular deposition of collagen VI was almost nil. End-stage, globally sclerotic glomeruli from cases of crescentic glomerulonephritis showed fine, very mild deposition of collagen VI at the periphery of some sclerotic glomeruli. Collagen type VI was also slightly expressed in mesangial areas in cases of amyloidosis. Besides mesangial, discontinuous deposition of collagen along the glomerular basement membrane was also noticed. Surprisingly, expression of collagen type VI was markedly increased in diabetic glomeruli. Both diffuse, as well as nodular lesions, revealed increased collagen immunoreactivity. Nodules consisting of a central region without cells, surrounded by a rim of cell nuclei

Fig. 1. Immunohistochemical reaction with the anti-collagen type VI antibody. **A.** Diabetes. Glomerulus displays a severe diffuse glomerulosclerosis and a big nodule (*) at the top. Small unchanged portion of glomerulus at 3 o'clock reveals less pronounced immunoreactivity than the rest of the tuft. Magnification: 200×. **B.** Diabetes. Glomerulus showing strong expression of collagen at the nodular lesions to the left. Glomerulus is directly connected with periglomerular area through these protruding nodules. The immunostaining of the nodules is less evident than the one in the periglomerular area revealing slight fibrotic changes. Magnification: 200×. **C.** Membranoproliferative glomerulonephritis. Fibrotic interstitium displays marked collagen expression. Immunohistochemical reaction with the anti-ASMA. Magnification: 250×. **D.** Crescentic glomerulonephritis. Actin-bearing cells are localized between tubules and glomerular crescent cells. These become accumulated in a concentric way in periglomerular area. Glomerular actin-positive cells are very few to the right but in the next, better preserved glomerulus (to the left) these are more numerous (arrowheads). Some inflammatory cells are closely associated with crescent. Magnification: 150×. **E.** Crescentic glomerulonephritis. Cellular crescent displays cells expressing ASMA. These are also present in arteriolar wall. Spindle-like periglomerular actin-positive cells constitute a network in which inflammatory cells are inclosed. Some periglomerular actin-positive cells appear migrating into the crescent (arrows). Magnification: 250×. **F.** Atherosclerosis. Nephrosclerosis. Low power magnification of renal cortex showing strong positivity in fibrous area. Numerous inflammatory cells are also present. Partly visible sclerotic glomerulus reveals only traces of ASMA expression. Adjacent tubules have normal as well as atrophic appearance. Magnification: 150×.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usually displayed more intense reactivity (Figure 1A, B). The extent of glomerular immunostaining was always less pronounced compared with the adjacent areas of periglomerular fibrosis (Figure 1B). Diffuse interstitial fibrosis, as evident in Figure 1C, was regularly present in advanced stage and in most types of glomerulonephritis studied. In cases of atherosclerosis and arteriolosclerosis, the most marked fibrosis was in areas containing obsolescent glomeruli and atrophic tubules. Severe interstitial fibrosis with inflammatory cell infiltration and tubular atrophy was present in interstitial nephritis.

Extraglomerular vessels also showed positive immunoreactivity with the anti-collagen VI antibody. Staining of vascular elements varied in intensity. In arteries, strong immunoreaction was revealed at the lower border of the intima where elastic fibres aggregate to form internal elastic lamina. Moreover, staining of similar extent appeared again in the adventitia. In veins where three layers are less clearly defined, collagen VI-positive fibres crossed practically the whole wall. The pattern of collagen expression in the vasculature did not differ greatly between normal and diseased kidneys. Data on the distribution of collagen type VI are summarized in Table 1.

ASMA-positive cells were revealed by light microscopic examination in the media of all renal blood vessels. This phenomenon served as an internal control of immunoreaction. A greatly increased number of glomerular cells expressing ASMA was detected in all the categories of glomerulonephritis examined in this study. This finding was documented in a previous paper and therefore is outside the scope of the present study.

Table 1. Localization of collagen type VI in the human kidney

Components of the renal tissue	Presence of immunoreactivity
Renal corpuscle	
Glomerular basement membrane	+
Mesangium	+
Bowman's capsule	−
Tubules	
Tubular basement membrane	−
Interstitium	
Cortical peritubular interstitium	+
Cortical periglomerular interstitium	+
Cortical periarterial interstitium	+
Medullary connective tissue	+
Renal vasculature	
Intimal layer	+
Media layer	−
Adventitial layer	+

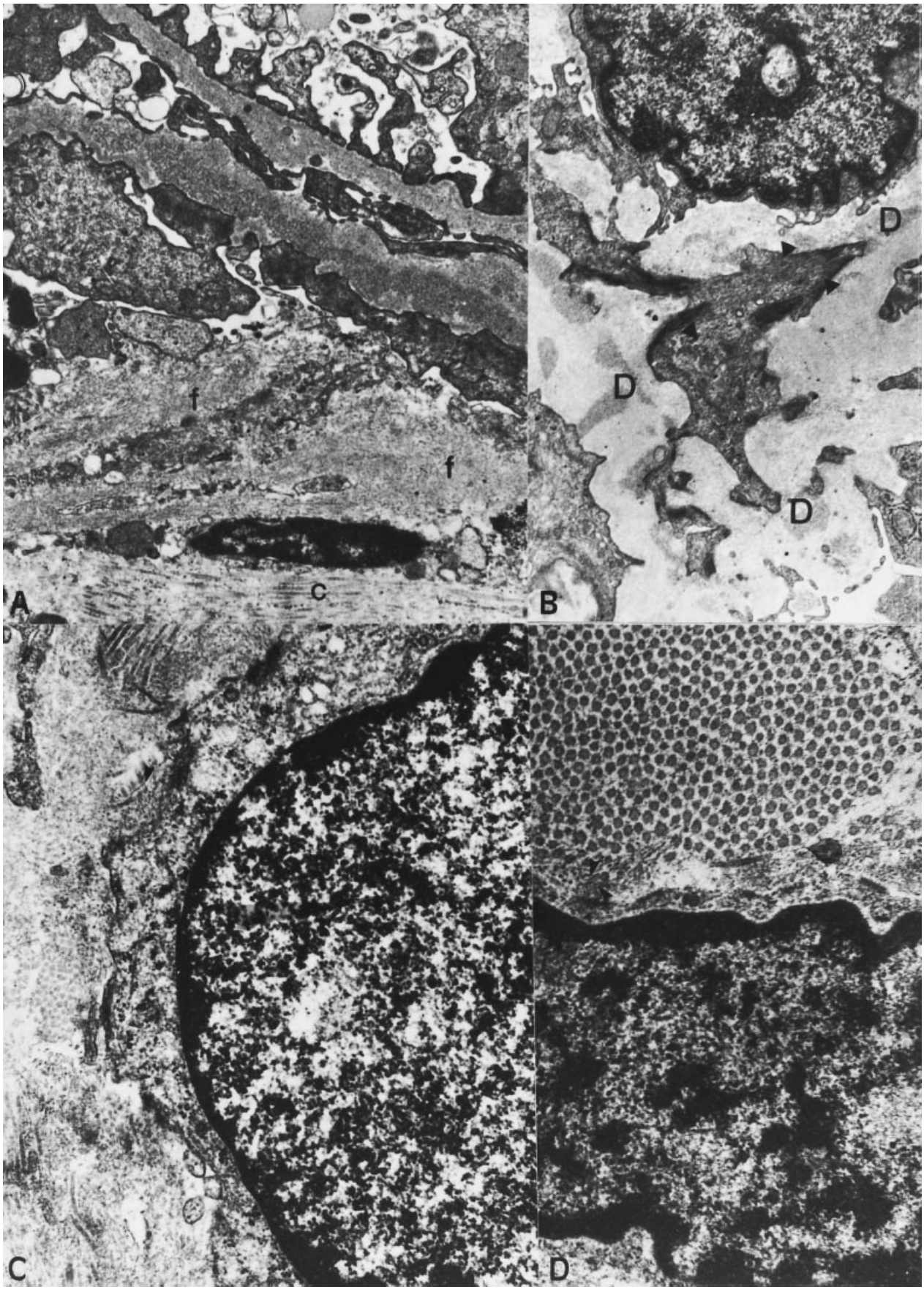
In all cases of diseased kidneys, uninjured portions of interstitium were generally indistinguishable from the interstitial patterns of actin and desmin expression observed in normal kidneys. These uninjured interstitial areas contained readily identifiable populations of spindle-shaped peritubular cells that were reactive with antibody to ASMA but not to desmin. Actin-bearing cells were localized between the peritubular capillary network and tubular basement membranes. Staining for ASMA was markedly increased in fibrotic interstitium which revealed elevated numbers of interstitial actin-expressing cells. There was no up-regulation of desmin expression detectable in these areas. An increased number of actin-positive cells was also identified in areas of interstitial nephritis accompanying some glomerulonephritis such as crescentic glomerulonephritis (Figure 1D). In this form of glomerulonephritis actin-bearing cells constituted the concentric network that surrounded the glomerulus. In the enclosed glomerulus the number of actin-expressing cells was greatly diminished compared with the concentric periglomerular area. Some actin-positive cells appeared to have migrated from the periglomerular rim into the crescent (Figure 1E). In the advanced stage of globally sclerotic glomerulus only traces of ASMA were identified (Figure 1F).

At the ultrastructural level, glomerular collagen could be demonstrated extremely rarely. There was no difference, concerning this finding, between the forms of glomerular disorders studied. Only a very few typical collagen microfibrils were found in the mesangium. More pronounced formation of collagen has been noticed between proliferated capsular epithelial cells and podocytes in the late phase of crescent formation (Figure 2A).

Ultrastructurally, in all cases of mesangiopathic glomerulonephritis, there were abundant microfilaments in the perinuclear cytoplasm and, even more abundantly, in processes of the mesangial cells. These tongue-like processes emerged from primary mesangial processes and cell bodies. Microfilament bundles have been noticed running transversely and terminating within microprojections. Bundles of microfilaments often revealed local densities (Figure 2B).

Normal interstitial cells appeared as inactive fibroblasts having a Golgi apparatus in the perinuclear region, a scant amount of rough endoplasmic reticulum (RER), and long cytoplasmic processes (Figure 2C). The cytoplasmic processes occasionally contained

Fig. 2. Fragment of electron micrograph. **A.** Some typical cross-banded collagen microfibrils (c) and thinner rope-like filamentous (f) material between degenerating parietal capsular cell and podocyte. Podocyte shows foot processes fusion. Magnification: 14 625 ×. **B.** Part of the mesangial area revealing terminal of a cell process. Microfilament bundles are visible in its cytoplasm. Scattered deposits (D) as well as parts of two collapsed capillaries are also evident. Magnification: 14 625 ×. **C.** Part of interstitial fibroblast containing elements of Golgi complex, endoplasmic reticulum, and ribosomes. Some typical cross-banded collagen microfibrils and thinner filamentous units of different appearance surround the fibroblast and are closely associated with the cell membrane. Some units reveal tubular structure (arrowhead). Magnification: 25 500 ×. **D.** Part of a fibroblast containing a portion of nucleus with wavy contour nuclear envelope. Adjacent cross-sectioned collagen fibril consists of numerous microfibrils. Very gentle and thin filamentous units are closely associated with them. Some of them reveal a hollow appearance (arrowheads). Magnification: 34 500 ×.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filamentous bundles just beneath the plasma membrane and the processes of neighbouring cells were closely related and separated by a constant gap.

In the case of glomerulonephritis, interstitial cells revealed elongated processes that frequently made contacts with processes from neighbouring cells. The peripheral cytoplasm of these fibroblasts often contained bundles of microfilaments ranging from 5 to 7 nm in diameter. The perinuclear regions revealed a prominent Golgi apparatus and profiles of RER. In areas of pronounced fibrotic injury, the interstitial fibroblasts were separated by increased amounts of collagen (Figure 2D). The longitudinally sectioned collagenous microfibrils were seen to form parallel collagenous fibrils. Sometimes some tube-shaped structures appeared among the microfibrils. Besides them, some other thin extracellular microfibrils appeared to be closely associated with the typical collagen microfibrils. Cytoplasmic processes could be seen coursing through the collagen fibrils and surrounding the basement membranes of the atrophic tubules. The RER, as well as the Golgi apparatus, was still present.

Discussion

Renal fibrosis is the final common pathway for nearly all forms of kidney disease that progress toward end-stage renal failure. It is believed that overproduction of extracellular matrix molecules, like type I and III collagens, fibronectin, and proteoglycans, generates fibrosis, leading to the permanent loss of normal structure and function in the kidney. There are clear differences between the organization of collagen in various organs as well as their compositional changes in pathological conditions. For example, it has been reported that thick collagen fibres in keloid lack the appropriate orientation. This results in their inability to participate in scar contracture [12]. It has been shown recently that unique microfibrillar type VI collagen, usually present in sites with dominant distribution of type I collagen, may contribute to specific organization of fibrillar components in interstitial tissue [7].

For the first time, the author has examined the localization of collagen type VI in the normal kidneys and in a wide range of kidney biopsies obtained from patients with different primary glomerular disorders, metabolic as well as vascular and inflammatory renal injuries, by immunohistochemistry. Additional information on microfibrillar collagen was obtained applying electron microscopy. Presented data indicate that collagen type VI has a universal character and always participates in fibrotic lesions developing in cases of severe renal injury of different aetiology and pathogenesis. Accumulation of type I along with type III collagen in case of fibrotic injury and progression of glomerular sclerosis has been reported [2,3,13]. Moreover, ubiquitous type VI collagen found in close association with cross-banded collagens [5] has been suggested to be an important modifier of the interactions of fibrillar components of connective tissue [7]. Ultrastructural findings partly supported this possibility; the finest material, filamentous by its nature, was observed between microfibrils. Another explanation could be that fine filamentous structures, located between collagenous microfibrils, present fibril-forming glycoproteins accumulated in the extracellular matrix.

It appears from this study that pathomorphological evaluation of vascular changes including the precise detection of amount and composition of the collagen could also have a high predictive value for allograft survival. Interstitial cellular infiltration, which is the classical sign of acute allograft rejection, is common to all allografts and may in fact be present to a mild or moderate degree in the well-functioning graft, too. The same could also be applied to oedema and lymphocyte infiltration of the arterial intima. It is known that in contrast to early acute rejection, in chronic vascular rejection there will also be found collagen and fibromyocytes, and in cases of longer duration (several months or years), the intimal thickening is severe and due mainly to fibrosis.

Among the series of biopsies examined here, the glomerular expression of type VI collagen was remarkably elevated only in the case of diabetes. This data was in accordance with some previously published papers [11]. These authors proposed the idea of a gradual substitution of collagen type IV by collagen type VI during the transition from the diffuse to the nodular form of glomerulosclerosis. Others examining different types of collagen participating in diabetic nephropathy have found that mesangial cells exposed to periodic high glucose express increased levels of mRNA for collagens types I, III and IV [14]. Moreover, it has been reported that interstitial collagens (types I and III) appear adjacent to Bowman's capsules, especially at the adhesion sites or crescents, and in the outer parts of sclerotic glomeruli [2]. The present immunohistochemical findings suggest that the hyperproduction of collagen type VI is closely linked to the progression of diabetic glomerulosclerosis toward nodular form. It is possible that this progression is guided not by collagen VI itself but, more likely, by collagen I, with which the former is associated. As shown by some micrographs, nodules are directly connected with periglomerular areas expressing VI collagen and, therefore, may be invaded by this through ruptures in the Bowman's capsule. Thereby, morphologically revealed periglomerular fibrosis induced by excessive accumulation of interstitial collagen types I and III associated with type VI could be seen as a warning prior to overt progression to the end-stage of disease.

Glomerular immunostaining for collagen was also noticed in the case of amyloidosis but had a different pattern. There is quite logical implication of this finding. In the injured glomeruli, large amounts of amyloid substance usually accumulate in the mesangial regions contributing to mesangial widening, leaving no space for deposition of other material. Collagen VI appearing in this disease along the basement membrane of glom-

erular capillaries makes an additional contribution to the organization of typical type IV collagen fibrils.

The results of this study showed that glomerular hyperproduction of collagen type VI is a rather rare finding. It makes it possible to extend data on negligible glomerular collagen synthesis, reported previously, to type VI.

It is clear that up-regulation of collagen VI expression detected in areas of interstitial fibrosis should be analysed through the prism of cells responsible for the production of most connective tissue components. *In vivo*, fibroblastic stromal cells synthesize and secrete the various collagen molecules, proteoglycans participating in intercellular adhesion, fibronectin which regulates the spatial organization of the tissue, proteolytic enzymes, such as collagenases and serine-proteases, as well as some of their specific inhibitors, thereby exerting control on the composition and renewal of the extracellular matrix [15]. The concept of the fibroblast being one of the pivotal participants in tissue fibrosis has attracted increasing attention over the last few years but the first studies on renal interstitial fibroblasts appeared a long time ago [16]. Some fibroblasts acquire the morphological and biochemical features of smooth muscle cells, some produce abundant extracellular matrix molecules, and consequently disappear leaving organ fibrosis. Renal myofibroblasts are presumed to be a separate phenotype of fibroblasts, and are detected surrounding arterioles, tubules, and glomeruli in the diseased kidney [17–19]. The amount of myofibroblasts is very negligible in the normal renal tissue but appears to be greatly increased in disease. The detection of such cells can predict subsequent renal fibrosis [17,19]. Moreover, as it has been shown by Bukovsky *et al.* [20], gradual decrease of glomerular mesangial ASMA expression, accompanied by up-regulation of actin expression detectable in periglomerular and intertubular stroma takes place in poorly functioning grafts, and can be used in complex evaluation of function of allografts.

Among cytoskeletal elements examined here, the expression of ASMA was a remarkable phenomenon in renal fibroblasts as fibrosis developed. Moreover, as evidenced by immunohistochemistry, ASMA-bearing fibroblastic cells were localized in interstitial areas with an excessive accumulation of collagen. These findings suggest that phenotypic change of renal fibroblasts may be an event associated with fibrogenesis. It has been shown that fibroblasts obtained from kidneys with interstitial fibrotic injury exhibit significant abnormal hyperproliferative growth and collagen biosynthesis when cultured [21]. Ultrastructural findings also support this possibility [16]. As evidenced by the present electron microscopic study, interstitial fibroblasts exhibited well-developed Golgi complex and RER. These were surrounded by collagen microfibrils and often contacted each other through their peripheral extensions. Some of the fibroblasts showed bundles of thin microfilaments in peripheral cytoplasm. Sometimes these bundles of cytoplasmic microfilaments revealed local densities, which are believed to be characteristic morphological features of myofibroblasts, although these cells were different from typical myofibroblasts in their lack of basement membrane and indented nuclei. These renal fibroblasts may be in the course of differentiation toward myofibroblasts, since ASMA is a characteristic cytoskeleton of myofibroblasts.

Present immunohistochemical and ultrastructural findings were found to be in agreement with results obtained by Hewitson and Becker [3] in the case of IgA glomerulonephritis. It has been shown by them that ASMA expression in areas of interstitial fibrosis correlated with fractional volume of tubular atrophy/dilatation and collagen III. Moreover, actin immunostaining correlated with renal function at the time of biopsy and after 2 years of follow-up. This study extended previously received results to the broad range of renal diseases. These findings are undoubtedly of clinical relevance. Moreover, physicians should take into consideration that myofibroblast-containing tissues respond to various smooth muscle stimulating drugs [22]. This study may be important both in terms of its relevance to the biology of myofibroblasts and mechanisms of fibrogenesis as well as to the evolution of renal interstitial fibrosis.

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