Abnormalities of Extracellular Matrices and Transforming Growth Factor ß1 Localization in the Kidney of the Hereditary Nephrotic Mice (ICGN Strain)

Kozue UCHIO, Noboru MANABE*, Akiko KINOSHITA, Kotaro TAMURA, Makoto MIYAMOTO, Atsuo OGURA[1)], Yoshie YAMAMOTO[1)] and Hajime MIYAMOTO

Unit of Anatomy and Cell Biology, Department of Animal Sciences, Kyoto University, Kyoto 606–8502 and [1)]Department of Veterinary Sciences, National Institute of Infectious Diseases, Tokyo 162–8640, Japan

(Received 28 January 1999/Accepted 9 March 1999)

ABSTRACT. ICR-derived strain with glomerulonephritis (ICGN) is a strain of mice with hereditary nephrotic syndrome with an unidentified cause. Based on histopathological and biochemical data, ICGN mice are considered to be a good experimental model for human idiopathic nephrotic syndrome. In the present study, we histochemically investigated the changes in localization of extracellular matrix (ECM) components and transforming growth factor β1 (TGF-β1). Strong immunohistochemical staining of basal membrane ECM components (collagen IV and laminin) and interstitial ECM components (type III collagen and fibronectin) were demonstrated in glomeruli and tubulointerstitum of ICGN mice as compared with those of sex and age-matched ICR mice, used as normal healthy controls. Marked type I collagen and tenascin deposition, which were not detected in the glomeruli of ICR mice, were seen in the glomeruli of ICGN mice. A remarkable increase in active-TGF-β1 was also detected only in glomeruli of ICGN mice, but not in those of ICR mice. Furthermore, strikingly increased α-smooth muscle actin, a marker of activated glomerular mesangial cells, was demonstrated in the glomeruli, mainly in the mesangial cells, of ICGN mice. These findings indicated that ECM components are increased in the glomerulus and tubulointerstitum of ICGN mice, and that active-TGF-β1 induces such increases in ECM components. The present findings may contribute to elucidation of the pathogenic mechanisms of hereditary nephrotic syndrome in ICGN mice and, in future, human idiopathic nephrotic syndrome.—KEY WORDS: extracellular matrix, hereditary nephrotic syndrome, ICGN mouse, immunohistochemistry, transforming growth factor-β1.

J. Vet. Med. Sci. 61(7): 769–776, 1999

Hereditary spontaneous nephrotic mice, a novel mutant strain of mouse from outbred ICR, were established in the National Institute of Infectious Diseases and named ICGN mice [16, 18]. The ICGN mice develop severe proteinuria, hypoproteinemia and hyperlipidemia, and some of the mice develop systemic oedema [19]. Histopathological analyses demonstrated glomerular lesions consisting of thickened basement membranes of the capillary loops with irregular spike-like protrusions and enlargement of the mesangial area without cellular proliferation [18]. Ultrastructurally, multilaminar splitting of the lamina densa of the thickened glomerular basement membrane (GBM) and fusion of the epithelial foot processes were noted [15, 17, 20]. In our previous study, characteristic changes in the structure of cell-surface carbohydrates, i.e., sugar chains, estimated with 24 different lectins were histochemically demonstrated. Remarkably, in kidney sections of ICGN mice but not in those of ICR mice, peanut agglutinin and *Phaseolus vulgaris* agglutinin-E stained distal and proximal renal tubules, respectively, concanavalin A and *Dolichos biflorus* agglutinin recognized the loop of Henle, wheat germ agglutinin reacted with the whole nephron, and *Bandeiraea simplicifolia* lectin-I stained the endothelial cells of glomerular capillary and vessel walls located between the renal tubules. Based on these previous findings, ICGN mice are considered to be a good experimental model for human idiopathic nephrotic syndrome.

The extracellular matrix (ECM) components play important roles in maintenance of the homeostasis of kidney functions [1, 13, 24]. The relationship between ECM and cells that adhere to it can play important regulatory roles in many basic cellular processes by influencing enzyme activity and phospholipid metabolism and by modifying transcriptional and translational activities of the cell. These regulatory influences can control key events in the life of a cell such as cell motility, proliferation, growth, differentiation and death. Disruption of existing cellular interactions with the ECM, due to experimental, pathological, or normal physiological changes, were also shown in early studies to be closely linked to changes in the functional capacity of cells. Early investigations showed that cell-substratum contact is closely linked to cell growth and cell death, that the relationship between substratum contact and cell growth and differentiation is positive for nonpathological cells, and that abnormalities in ECM-cell interaction plays a key role in the cause of diseases. In the kidney, the structure of the ECM is complex in terms of the multiplicity of different molecules, their spatial organization, and their anatomical distribution. Type IV collagen and laminin are contained as a major component in normal and sclerotic glomeruli of experimental animals [5, 7, 10, 11, 14]. The major characteristic of glomerular injury is increased deposition of ECM, and various studies have examined the components involved [5, 7, 10, 11, 14].

Growth factors, which control ECM production and breakdown, have been demonstrated to be important

* CORRESPONDENCE TO: MANABE, N., Unit of Anatomy and Cell Biology, Department of Animal Sciences, Kyoto University, Kyoto 606–8502, Japan.

mediators of ECM accumulation in glomerulonephritis. Recent studies revealed that transforming growth factor-β1 (TGF-β1) [2], platelet-derived growth factor (PDGF) [8], and basic fibroblast growth factor (bFGF) [23] promote ECM accumulation in model animals with glomerulonephritis, and TGF-β, a family of growth and defferentiation factor, is considered to play a prominent key role in development of glomerulonephritis [9, 12, 22]. TGF-β1 is secreted by virtually all cell types as a biologically inactive molecule. Latent TGF-β1 is composed of an amino-terminal latency-associated peptide (LAP) that remains noncovalently associated with the carboxyl-terminal mature TGF-β1 molecule. The release of mature TGF-β1 from LAP is thought to be necessary for TGF-β1 to contact with its cellular receptors and elicit biological effects. However, the role of growth factors in the progression of hereditary nephritis in ICGN mice remains poorly understood.

The purpose of the present study was to examine the immunohistochemical localization of the ECM components in the tissue sections of the kidney from ICGN mice. Furthermore, we examined whether TGF-β1 is related to progression of hereditary nephritis in ICGN mice by histochemical analysis of latent and activated TGF-β1.

MATERIALS AND METHODS

Animals and tissue preparation: ICGN mice from a specific-pathogen-free colony at the National Institute of Infectious Diseases (Tokyo, Japan) and sex and age-matched ICR mice purchased from Crea Japan (Osaka, Japan) were used in this study. Animals were housed in autoclaved metal cages and provided free access to laboratory diet and water. The room temperature was maintained at about 23 ± 1˚C. Experiments were performed with mice at 5, 10, 15, 30 and 50 weeks after birth. All animals received humane care as outlined in the "Guide for the Care and Use of Animals" (Kyoto University Animal Care Committee according to NIH #86-23; revised 1985). Blood samples were obtained from the cervical vein under ether anesthesia for biochemical analysis, and the left kidney was fixed in 10% phosphate buffered formalin, pH 7.4, for conventional histopathological evaluation. The right kidney was put on a filter paper, mounted in OCT compound (Ames Co., Elkhart, IN, U.S.A.), and then rapidly frozen in dry ice-cooled isopentane for immunohistochemistry. The frozen tissue samples were kept at −80°C until use.

Histopathology: After fixation, the kidney samples were dehydrated through a graded ethanol series and embedded in Histosec (Merck Co., Darmstadt, Germany). Sections 3 μm thick were cut with a microtome, mounted on glass slides, deparaffinized with xylene and rehydrated through a graded ethanol series. For conventional histopathological evaluation, some of the sections were stained with hematoxylin-eosin according to the standard method. As previously reported [10, 11], the extent of glomerulosclerosis was expressed as the degree of collagen deposition, which was assessed on sections stained with Sirius red solution, saturated picric acid in distilled water containing 0.1% (w/v) Sirius red F3B (BDH Chemicals Ltd., Poole, UK). All sections were mounted with Entelan (Merck) and photographed. Light microscopic examination was performed on the kidneys of all mice. In each kidney specimen, approximately 100 glomeruli were selected at random and evaluated by light microscopy as described previously [11]. Briefly, mesangial expansion in the glomeruli was scored according to the extent of the sclerotic lesion in the glomerulus and graded from 0 to 4 (0=normal; 1=change affecting < 25%; 2=change affecting < 25–50%; 3=change affecting < 50–75%; 4=change affecting > 75%). In addition to mesangial expansion, morphological changes of glomeruli (capillary aneurysm and hypercellularity), and tubular (cystic tubular dilation, epithelial cellular atrophy and intraluminal cast formation) and tubulointerstitial (tubulointerstitial expansion and mononuclear cell filtration around arterioles) lesions were also recorded.

Immunohistochemistry: For immunofluorescence staining for ECMs, TGF-β1 and α-smooth muscle actin, fresh frozen sections (5 μm in thickness) were cut on a cryostat, mounted on 3-aminopropyltrimethoxysilane (Aldrich Chemical, Milwaukee, WI, U.S.A.)-precoated slides and then fixed with precooled acetone for 10 min at −80°C. The sections were rinsed with phosphate-buffered saline (PBS, pH 7.4) for 10 min, then incubated with each first antibody (see Table 1) diluted to the optimum concentration with PBS containing 40 mg/m*l* bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, U.S.A.) for 18 hr at 4˚C. After incubation, the sections were washed gently 3 times with PBS for 5 min at room temperature (RT, 20–25˚C), and then incubated with appropriate fluorescein isothiocyanate (FITC)-conjugated second antibody (American Qualex, San Clemente, CA, U.S.A.; see Table 1) for 90 min at RT. Then, the sections were washed 3 times with PBS for 5 min at RT and mounted with glycerol. The sections were examined with a fluorescence microscope (BX-50, Olympus, Tokyo, Japan) and then photographed. The localization of each ECM component was recorded.

Table 1. Antibodies and their dilution rates used in this study

Antigen	First antibody	Dilution
Type I collagen	Rabbit IgG[a)]	1/100
Type III collagen	Rabbit IgG[b)]	1/150
Type IV collagen	Rabbit IgG[a)]	1/100
Fibronectin	Rabbit IgG[c)]	1/200
Laminin	Rabbit IgG[d)]	1/100
Tenascin	Rat IgG[c)]	1/100
Latent TGF-β1	Rabbit IgG[e)]	1/100
Active TGF-β1	Mouse IgG[f)]	1/200
α-SMA	Mouse IgG[c)]	1/200

a) Chemicon, Temecula, CA, U.S.A. b) Rockland, Girbertville, PA, U.S.A., c) Sigma, d) ICN Pharmaceuticals, Aurora, OH, U.S.A. e) Santa Cruz Biotechnology, Santa Cruz, CA. U.S.A. f) Kirin Brewery Co., Tokyo, Japan.

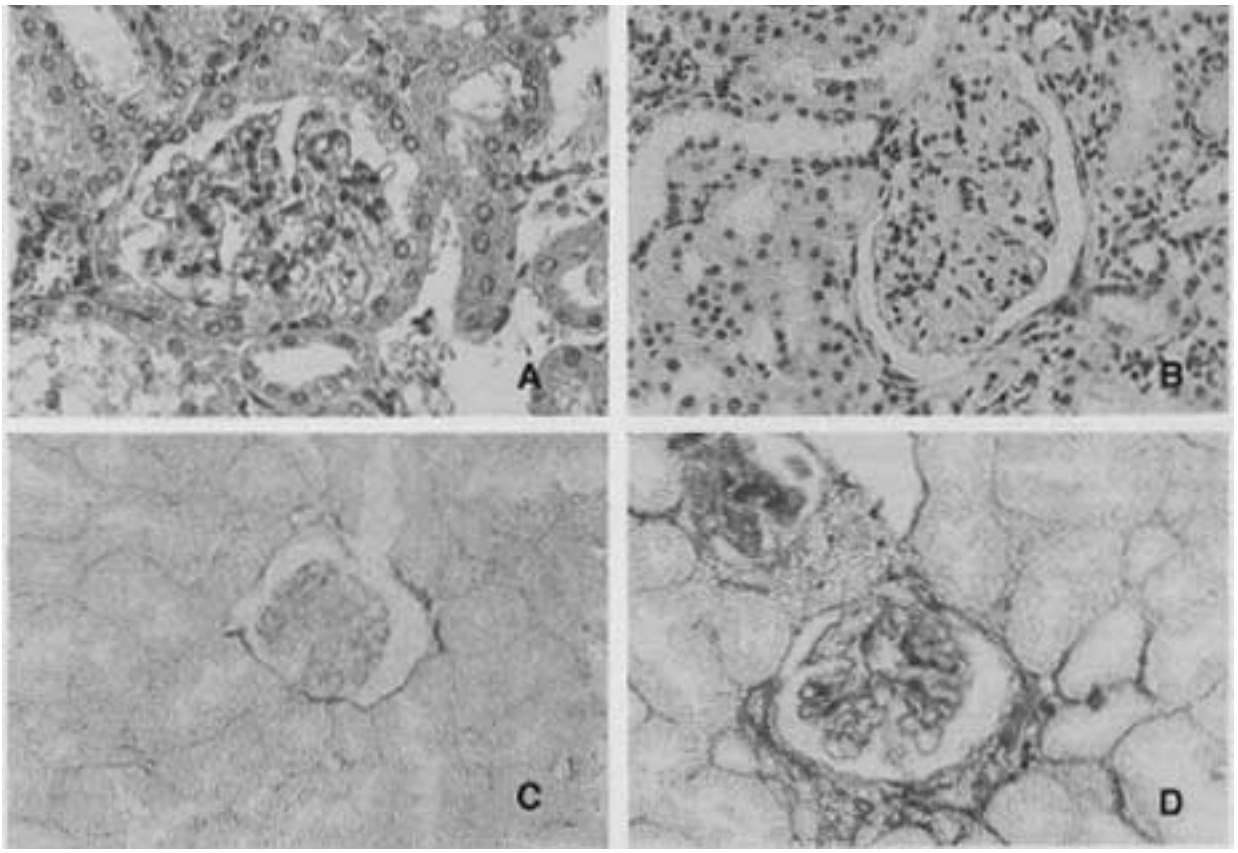

Fig. 1. Kidney sections prepared from 50-week-old ICR (A and C) and ICGN (B and D) mice were stained with hematoxylin-eosin (A and B) and with Sirius red (C and D). In the glomeruli of ICGN mouse kidney, mesangial expansion, expanded mesangial matrix, appearance of cysts, infiltration of inflammatory cells into glomeruli and no proliferation of mesangial cells were observed. Tubular lesions, i.e., cystic tubular dilation, epithelial cellular atrophy, intraluminal cast formation, and tubulointerstitial lesions, i.e., tubulointerstitial expansion and mononuclear cell filtration around arterioles, were also noted in sections of the ICGN mouse kidney (× 200).

Clinical blood biochemistry: To assess the loss of renal function and to evaluate nephrotic states, the blood samples were examined on the basis of the following biochemical parameters: blood creatinine (Cr) and blood urea nitrogen (BUN) levels were measured by Jaffé's method with a creatinine test kit (Wako Pure Chemical Co., Osaka, Japan) and by the urease indophenol method with a nitrogen-B test kit (Wako), respectively. All procedures were performed according to the manufacturers' protocols.

Statistical analysis: ANOVA with Fisher's least significant differences test for comparison of biochemical data was carried out with the StatView IV program using a Macintosh computer. Differences at a probability of $P<0.05$ were considered significant.

RESULTS

Renal histopathology and clinical biochemistry: In comparison with normal healthy controls, i.e., ICR mice (Fig. 1A and C), the following remarkable renal lesions were noted in ICGN mice (Fig. 1B and D). Mesangial expansion, expanded mesangial matrix, and appearance of cysts were observed only in the kidney of ICGN mice. The most typical renal lesion in the ICGN mice was mesangial expansion; the score of 5-week-old mice was 1 or 2, that of 10- and 15-week-old mice was 2 or 3, and that of 30- and 50-week-old mice was 3 or 4. In addition to such pathological findings, infiltration of inflammatory cells into glomeruli (hypercellularity) and no proliferation of mesangial cells were observed in the glomeruli of ICGN mouse kidneys. Cystic tubular dilation, epithelial cellular atrophy, intraluminal cast formation (tubular lesions), tubulointerstitial expansion and mononuclear cell filtration around arterioles (tubulointerstitial lesions) were also noted only in aged (30- and 50-week-old) ICGN mice. Then Sirius red staining was performed in ICR and ICGN mouse kidneys (Fig. 1C and D, respectively). Glomerulosclerosis were demonstrated only in the glomeruli and tubulointerstitum in the ICGN mouse kidneys. These findings implied that ECM accumulate in the glomerulus and tubulointerstitum in the

kidneys of ICGN mice. Moreover, renal clinical biochemistry was performed to assess the nephrotic state, and ICGN mice were found to have severe nephrotic disorder in comparison with control healthy ICR mice (Table 2).

Immunohistochemical detection of ECM components: Type I collagen was not detected immunohistochemically in normal glomeruli or the tubulointerstitum of ICR mice (Fig. 2A). In the kidneys of ICGN mice, however, marked deposition of type I collagen was seen in expanded glomeruli and tubulointerstitial lesions (Fig. 2B). In normal kidneys of ICR mice, trace deposits of fibronectin and type III collagen were observed in glomeruli and the interstitium (Fig. 2C and E, respectively). In ICGN mice, increased deposition of both fibronectin and type III collagen were noted in glomeruli and tubulointerstitial lesions (Fig. 2D and F, respectively). Trace staining for tenascin was seen in normal ICR kidneys (Fig. 3A), but strong positive staining for tenascin was detected in the expanded ICGN glomeruli but not in tubulointerstitial regions (Fig. 3B). In specimens stained for type IV collagen, a major component of the basement membrane, both the glomerular and tubular basement membranes of ICGN mouse kidneys showed a stronger positive staining than those of normal ICR mouse kidneys (Fig. 3C and D, respectively). Similar differences in immunofluorescence staining pattern were also observed for laminin, a major basement membrane component, between ICR and ICGN mice (Fig. 3E and F, respectively). In summary, basal membrane components (type IV collagen and laminin) and interstitial components (type I and III collagens, fibronectin and tenascin) accumulated in expanded glomeruli and tubulointerstitial lesions of the kidneys of ICGN mice.

Immunohistochemical localization of TGF-β1 and α-smooth muscle actin: In ICR and ICGN mice, positive staining for latent TGF-β1 was observed in glomeruli and tubulointerstitial regions (Fig. 4A and B, respectively), and no differences in intensity of staining or localization of latent TGF-β1 were seen between ICR and ICGN mouse kidneys. In normal ICR kidneys, weak immunoreactivity for active TGF-β1 was observed only in glomeruli (Fig. 4C). In disorders with abnormal glomerular and tubulounterstitial ECM accumulation, significant increases in active TGF-β1 reaction were demonstrated in glomeruli (in predominantly mesangial locations) of the kidneys in ICGN mice (Fig. 4D).

As α-smooth muscle actin (αSMA) is considered to be a good marker for activated mesangial cells, immunohistochemical localization of αSMA in the kidney sections was compared between normal ICR and hereditary nephrotic ICGN mice. In normal ICR kidneys, trace positive staining for αSMA was observed in glomeruli (Fig. 4E). In the kidneys of nephrotic ICGN mice, a marked increase in αSMA reaction was noted in expanded glomeruli (Fig. 4F), and these positive cells in ICGN glomeruli were mesangial cells.

Table 2. Blood biochemical characteristics in ICR and ICGN mice

	ICR mice	ICGN mice
Blood creatinine (mg/d*l*)	0.49 ± 0.07	0.68 ± 0.23**
Blood urea nitrogen (mg/d*l*)	17.9 ± 3.2	35.6 ± 10.1*

* and **: $P<0.05$ and 0.01, respectively, vs ICR mice as normal controls.

DISCUSSION

Earlier studies revealed that the important histopathological feature of kidney diseases is fibrotic degeneration, that is ECM accumulation in glomeruli and/or tubulointerstitum [5, 7]. In the present study, we confirmed that such fibrotic changes occur in the kidneys of ICGN mice, considered to be a new model for idiopathic nephrotic syndrome. We examined the type of ECM components are accumulated in the kidneys in this model to determine the clinical characteristic of the renal lesions of ICGN mice. Major ECM components, both interstitial (type I and III collagens, fibronectin, and tenascin) and basement membrane components (type IV collagen and laminin) were found to be markedly accumulated in glomeruli and the tubulointerstitum of ICGN mouse kidneys.

The mechanism of such deposition of ECMs in the ICGN kidneys, however, remains unclear. There are two possible mechanisms which may account for such ECM accumulation [21, 25]. One is ECMs in the kidney tissues may be overproduced in ICGN mice and another is ECM breakdown may be inhibited. Matrix metalloproteinases (MMPs) and plasmin play key roles in ECM degradation, and an inhibitor of MMPs (TIMP) arrests the ECM breakdown. When fibrotic degeneration occurs, levels of MMPs and activated plasmin are decreased, and the levels of TIMP and plasminogen activator inhibitor (PAI) are increased [21, 25]. In the present study, we demonstrated only ECM deposition, but had no detailed data concerning the mechanisms of ECM production and degradation. Interestingly, both of these possible mechanisms, i.e., acceleration of ECM synthesis and inhibition of ECM breakdown, are strongly related to TGFβ1, which is a potent accelerator of ECM production and is secreted in a latent nonactive form into the intercellular space [9, 22]. This latent form of TGF-β1 is partially digested by specific protease (activation of latent TGF-β1), and then the digested TGF-β1 (active form TGF-β1) shows strong bioactivity. Earlier *in vitro* and *in vivo* studies confirmed that TGF-β1 promotes the pathological progression of renal fibrotic degeneration characterized by ECM accumulation [3, 4]. When fibroblasts are treated with the active form of TGF-β1, they express αSMA and transform into myofibroblasts [6]. We showed that active TGF-β1 level was markedly increased in glomeruli of ICGN mice but not of ICR mice. In ICGN kidneys, most of glomerular mesangial cells in damaged glomeruli showed

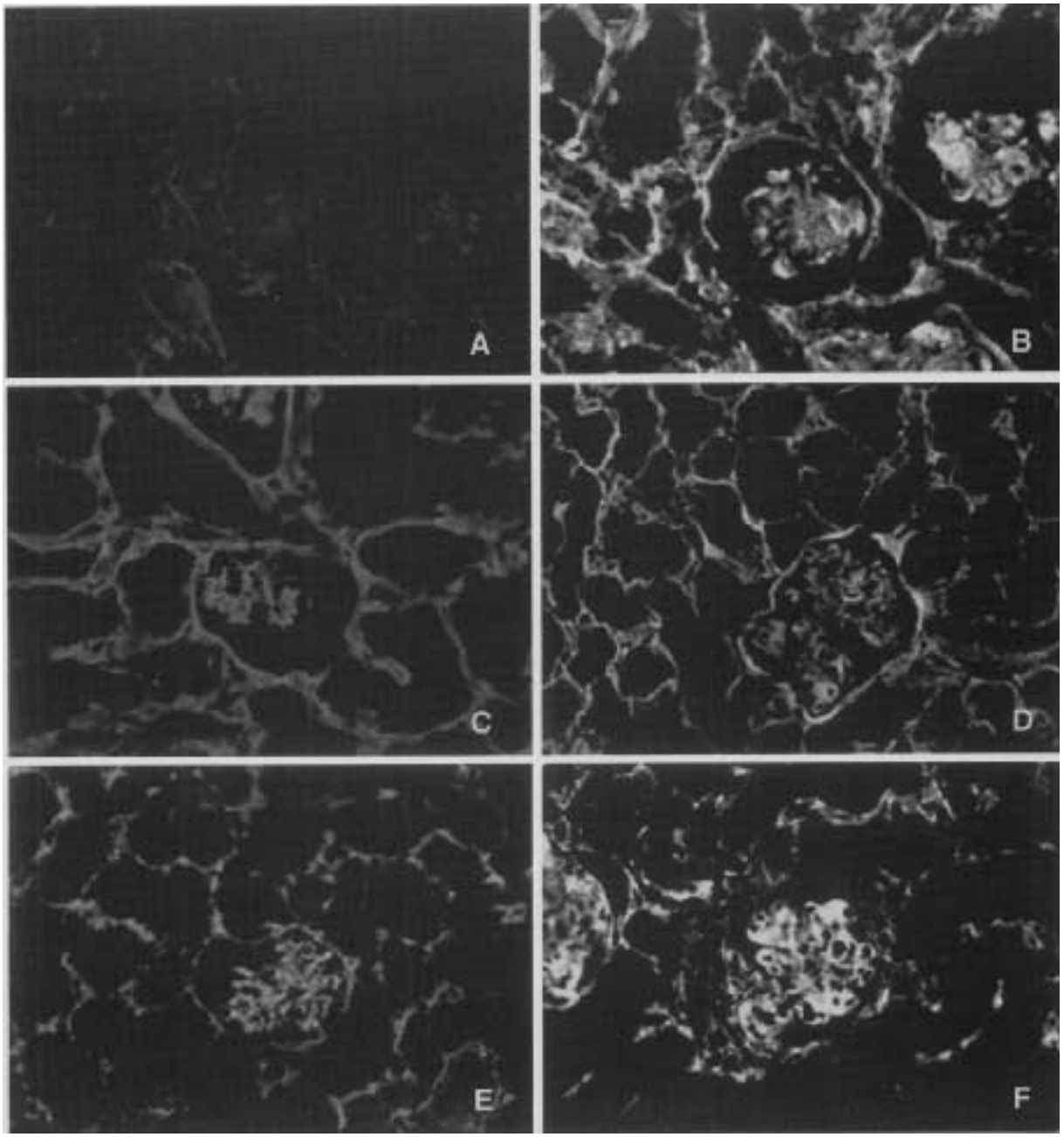

Fig. 2. Kidney sections prepared from 50-week-old ICR (A, C, and E) and ICGN (B, D, and F) mice were immunohistochemically stained for type I (A and B) and type III collagens (C and D) and fibronectin (E and F). In ICR mouse sections, no positive reactions for type I collagen was demonstrated. However, in the kidney sections of ICGN mice, strong positive reactions for type I collagen were seen in expanded glomeruli, and tubulointerstitial lesions. Weak reactions for fibronectin and type III collagen were observed in glomeruli and the interstitium of ICR mouse kidneys, but strong reactions were noted in glomeruli and tubulointerstitial lesions of ICGN mouse kidneys (× 200).

high αSMA expression, indicating that mesangial cells differentiated into myofibroblast-like cells induced by active TGF-β1. Thus, the pathogenetic process in the glomeruli of ICGN mouse kidneys is presumed to be mediated by the increased levels of active form of TGF-β1 which would induce mesangial cell transformation, and these activated mesangial cells would then overproduce ECMs. Tubulointerstitial fibrotic degeneration was also found in ICGN mice, but no increases in the levels of active form of TGF-β1 or αSMA were observed. The mechanism of this

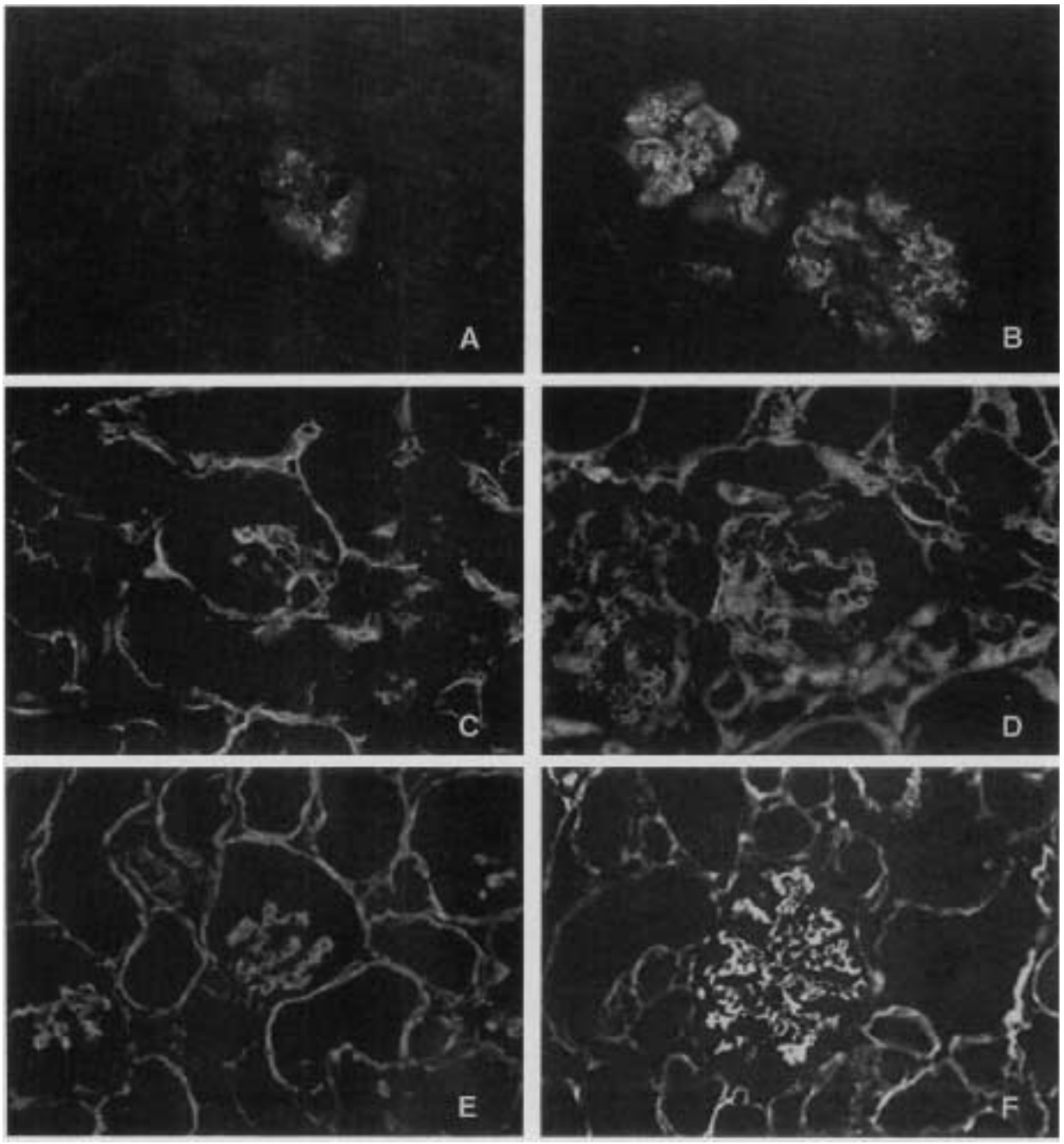

Fig. 3. Kidney sections prepared from 50-week-old ICR (A, C, and E) and ICGN (B, D, and F) mice were immunohistochemically stained for tenascin (G and H), type IV collagen (I and J) and laminin (K and L). In ICR mouse sections, trace reactions for tenascin were demonstrated. However, in the kidney sections of ICGN mice, positive staining for tenascin was detected in the expanded glomeruli. Both glomerular and tubular basement membranes in ICGN mouse kidneys showed stronger positive staining for type IV collagen and laminin than those in ICR mouse kidneys (× 200).

tubulointerstitial fibrotic degeneration is not yet clear. We presume that the tubulointerstitial cells were induced to differentiate into fibroblast-like cells by unknown stimulatory factor(s) in the ICGN mice, and then the differentiated tubulointerstitial cells would synthesize excessive ECMs into the intercellular spaces between the renal tubules. Further investigations to clarify the mechanism of tubulointerstitial fibrosis in ICGN mice are currently in progress in our laboratory.

In conclusion, a defect of TGF-β1 regulation may lead to abnormal accumulation of ECMs in the glomeruli of ICGN mice, and TGF-β1 plays a prominent role in the progression

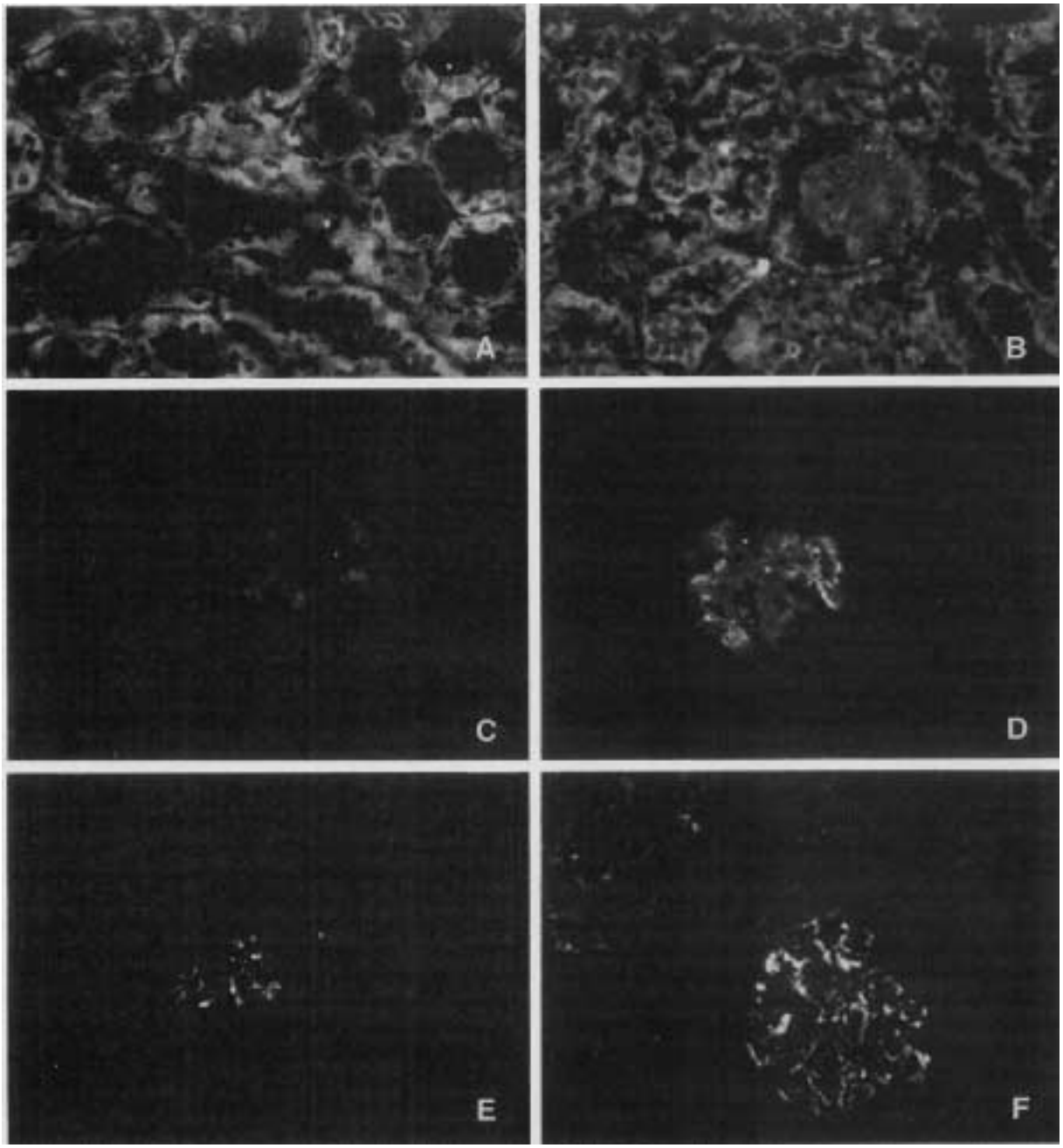

Fig. 4. Frozen kidney sections prepared from 50-week-old ICR (A, C and E) and ICGN (B, D and F) mice were immunohistochemically stained for latent (A and B) and active (C and D) TGF-β1 and αSMA (E and F). No differences in staining intensity or localization of latent TGF-β1 were seen between ICR and ICGN mouse kidneys (A and B, respectively). Weak reactivity for active TGF-β1 was observed only in glomeruli of ICR kidneys (C), but strong activity for active TGF-β1 was demonstrated in glomeruli, mainly in mesangial locations, of ICGN kidneys (D). Trace positive staining for αSMA was observed in glomeruli of ICR kidneys (E), but marked positive αSMA reaction was noted in expanded glomeruli of ICGN kidneys (F). The αSMA-positive cells in the ICGN glomeruli were glomerular mesangial cells (× 200).

of renal disease in these mice. The present findings help to explain the pathogenic mechanisms of not only nephrotic lesions of ICGN mice but also of human idiopathic nephrotic syndrome.

ACKNOWLEDGEMENTS. Part of this work was supported by a Grant-in-Aid to N. M. and H. M. from the Ministry of Education, Science, Sports and Culture of Japan.

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