Changes in the Localization of Type I, III and IV Collagen mRNAs in the Kidneys of Hereditary Nephritic (ICGN) Mice with Renal Fibrosis

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ABSTRACT. Renal fibrotic change, extreme accumulation of extracellular matrix (ECM) components in glomeruli and tubulointerstitium, is one of the characteristic features of ICR-derived glomerulonephritis (ICGN) mice. Decreased degradation of ECMs by matrix metalloproteinases was demonstrated in kidneys of ICGN mice. To determine the balance between production and degradation of ECMs in kidneys of ICGN mice, we examined expression of mRNAs of ECMs in those. To demonstrate the localization of type I, III and IV collagen mRNAs in kidney sections of ICGN and control ICR mice, *in situ* hybridization using digoxigenin-labeled oligonucleotide antisense probes for procollagen- α_1 (I), - α_1 (III) and - α_1 (IV) mRNAs, respectively, was performed. Negative or trace expressions of type I and III collagen mRNAs were observed in the kidneys of control mice, but stronger expressions of those were seen in glomeruli and in jured renal tubules of the kidneys of ICGN mice. Moderate expression of type IV collagen mRNA was demonstrated in a part of glomeruli and renal tubules of both control and ICGN mice, and no remarkable difference was seen between them. Severe renal fibrosis, extreme accumulation of interstitial type I and III collagens is caused by increased production and decreased degradation in the kidneys of ICGN mice. Thus, the profiles of metabolism between interstitial and membranous collagens may be different in the kidneys of ICGN mice, and excessive production of interstitial collagens may be the dominant cause of renal disease in them.

KEY WORDS: extracellular matrix (ECM), ICR-derived glomerulonephritis (ICGN) mouse, *in situ* hybridization, type I, III and IV collagen mRNA.

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Institute of Cancer Research (ICR)-derived glomerulonephritis (ICGN) mice, a novel inbred strain of mice with a hereditary nephrotic syndrome of unknown etiology, were established in the National Institute of Infectious Diseases (NIID, Tokyo, Japan) and considered a good model of human idiopathic nephrotic syndrome [14–20]. The ICGN mice develop proteinuria, hypoproteinemia and hyperlipidemia, and approximately 40% of aged (more than 30-week-old) mice develop severe systemic edema. Conventional 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 accompanied by cellular proliferation [14, 15, 19, 20]. Ultrastructurally, multilaminar splitting of the lamina densa of the thickened glomerular basement membrane and fusion of the epithelial foot process were noted [14, 15]. The characteristic changes in the structure of cell-surface carbohydrates histochemically estimated with 24 different lectins were seen in the tubulointerstitial cells in ICGN mice [25]. Previously, we immunohistochemically showed that excessive accumulation of type I, III and IV collagens was demonstrated in glomeruli and tubulointerstitium of ICGN mice as compared with those of sex- and age-matched ICR mice [26, 28]. Marked deposition of type I collagen, which was not

detected in the glomeruli of ICR mice, was seen in the glomeruli of ICGN mice.

Extracellular matrices (ECMs) play important roles in maintenance of the homeostasis of renal functions, and excessive accumulation of them in kidneys leads to progression of nephropathy [3]. The maintenance of normal kidney structure and function depends on an appropriate regulation of synthesis and degradation of ECMs, and unbalance causes gradual development of the glomerular and/or tubulointerstitial fibrotic lesion [23, 24]. We compared the activities of matrix metalloproteinases (MMPs) in the kidneys of ICGN and control ICR mice [23]. The MMPs can be divided into three groups on the basis of substrate specificity [2, 7, 24, 30]: (1) the interstitial collagenases (MMP-1) primarily responsible for the degradation of type I collagen, (2) the gelatinases (MMP-2 and -9) that dominantly degrade type IV collagen, and (3) the stromelysins (MMP-3), which have a broader substrate specificity and degrade type IV and V collagens, proteoglycans and laminin. We reported that lower activities of MMP-1 and MMP-2 and -9 were demonstrated in the kidneys of ICGN mice as compared with those of ICR mice, but there was no significant difference in the MMP-3 activities between these strains [27], indicating decreased MMP activities cause abnormal accumulation of ECM in ICGN mouse kidneys. However, the mechanisms responsible for the excessive accumulation of ECMs in the kidneys of ICGN mice have not been fully understood. In the present study, to make clear the changes in synthesis levels of the ECMs, we compared the localiza-

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tion of type I, III and IV collagen mRNAs [procollagen- α_1 (I), - α_1 (III) and - α_1 (IV) mRNAs, respectively] in the kidney sections of ICGN and ICR mice demonstrated by *in situ* hybridization technique.

MATERIALS AND METHODS

Animals and tissue preparation: Nephrotic ICGN mice were prepared by mating between homozygous males (*nep/nep*) and heterozygous females (*nep/-*) at the laboratory of NIID [14–20]. Male homozygous ICGN mice (30-week-old) from a specific-pathogen-free colony in NIID and sex- and age-matched ICR mice purchased from Clea Japan (Tokyo, Japan) were used. All animals were housed in autoclaved metal cages and were given a standard diet (Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* in an air conditioned room ($23 \pm 1^\circ\text{C}$), under controlled lighting conditions (12 hr light/12 hr dark). They received humane care as outlined in the “Guide for the Care and Use of Laboratory Animals” (Kyoto University Animal Care Committee according to NIH #86–23; revised 2000). For biochemical analyses, urine samples during the 24 hr before sacrifice (24-hr-urine samples) were collected, and blood samples were obtained from the cervical vein under ether anesthesia. The animals were sacrificed under deep ether anesthesia, and then the kidneys were rapidly removed. One kidney was immediately fixed in 10% neutral-buffered formalin, pH 7.4, for conventional histopathological evaluation, while the other was put on filter paper, mounted in OCT compound (Ames, Elkhart, IN, U.S.A.), and then rapidly frozen in dry ice-isopentane mixture for histochemical measurement of total and type I collagens and for *in situ* hybridization.

Clinical biochemistry: To evaluate nephrotic state and loss of renal function, blood and 24-hr urine samples were examined on the basis of the following biochemical parameters. Serum and urinary albumin (sAlb and uAlb, respectively), serum creatinine (sCr) and blood urea nitrogen (BUN), serum total cholesterol (sTC) levels were enzymatically measured by an automatic analyzer (Dri-Chem 3500U, Fuji Film Co., Tokyo, Japan). All procedures were performed according to the manufacturers’ protocols.

Renal histopathology: Formalin fixed kidney samples were dehydrated through a graded ethanol series and embedded in Histosec (Merck, Darmstadt, Germany). Sections 3 μm thick were mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Silane; Sigma Aldrich Chemical, St. Louis, MO, U.S.A.), deparaffinized with xylene and rehydrated through a graded ethanol series. For conventional histopathological evaluation, some of the sections were stained with hematoxylin and eosin. As previously reported [6, 25, 26], the extent of glomerulosclerosis was expressed as the degree of ECM deposition, which was assessed on sections stained with Sirius red solution (saturated picric acid in distilled water containing 0.1% Sirius red F3B; BDH Chemicals, Poole, U.K.). Sirius red staining detects interstitial collagens. All slides were mounted with

Entellan (Merck), and examined by light microscopy (at least six sections/mouse). In each kidney specimen, approximately 100 glomeruli were selected at random and evaluated. Briefly, the mesangial expansion in the glomeruli scored according to the extent of the sclerotic lesion in the glomerulus, the morphological changes in the 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 evaluated [6, 25, 26].

Histochemical quantification of total and type I collagens: Type I collagen levels in each kidney cortex section were measured by microquantitative methods [6, 25, 26]. Briefly, frozen sections 5 μm thick were prepared on a cryostat (Jung CM1500; Leica, Heidelberg, Germany), mounted on Silane-precoated slides and fixed with cold acetone (-80°C) for 5 min. The renal medulla area of each fixed section was removed under a surgical dissecting microscope. The kidney cortex sections were incubated with rabbit anti-mouse tail type I collagen antibody (LSL, Tokyo, Japan) for 12 hr at 4°C , washed in phosphate buffers saline (PBS; pH 7.4), and then incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG antibody (American Qualex, La Mirada, CA, U.S.A.) for 1 hr at 25°C . After washing with 50 mM Tris-HCl-saline (pH 7.4), chromogenic substrate solution (30 mM phenol, 3 mM 4-aminopyridine and 2 mM H_2O_2 in 50 mM Tris-HCl, pH 7.4; Wako Pure Chemicals, Osaka, Japan) was applied to each section and incubated for 10 min, and the optical density of the solution at 450 nm was determined with a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Uppsala, Sweden). Type I collagen content in each section was calculated. After measurement of the type I collagen content, total collagen level in each section was measured colorimetrically [6].

In situ hybridization: As previously reported [13, 29], frozen sections 5 μm thick mounted on Silane-precoated slides were fixed with 4% paraformaldehyde (Wako) in PBS for 8 min at room temperature (RT; $22\text{--}25^\circ\text{C}$), treated with 0.2 N HCl for 10 min, and then digested with proteinase K (0.5 $\mu\text{g}/\text{ml}$; Sigma) in PBS for 10 min at 37°C . The sections were postfixed with 4% paraformaldehyde in PBS for 5 min, rinsed twice with 2 mg/ml glycine in PBS for 10 min, and then prehybridized in hybridization buffer (0.1 M Tris HCl, pH 7.4, $1 \times$ Denhart’s solution, 10% dextran sulfate, 50% deionized formamide containing 0.1 mg/ml salmon sperm DNA and 0.1 mg/ml yeast tRNA) for 1 hr at RT. Digoxigenin (DIG)-labeled oligonucleotide probes for type I, III and IV collagen mRNAs [antisense probes for procollagen- α_1 (I), - α_1 (III) and - α_1 (IV) mRNAs, respectively] were mixed with hybridization buffer (200 pM/ml), and then applied on each section (50 $\mu\text{l}/\text{section}$). Sequences of oligo-DNA probes for type I, III and IV collagens were TTTTA CCTTT GACCA ACTGA ACGTG ACCAA [5, 21], TATGA CATTG GGGGT CCTGA TCAAG AATTT [5, 21] and GTACC CCATC TGTGG ACCAT GGCTT

CCTTG [22], respectively. The sections were incubated for 18 hr at 37°C and washed as follows: 4 times 2 × standard saline citrate (SSC) and 50% formamide for 60 min at 37°C, 4 times 2 × SSC containing 0.075% Brij-35 (Sigma) for 30 min at 37°C, 4 times 0.5 × SSC containing 0.075% Brij-35 for 30 min at 37°C, 2 times 0.2 × SSC containing 0.075% Brij-35 for 30 min at 37°C, and then 3 times 0.1 M Tris-HCl, pH 7.4, containing 0.15 M NaCl for 5 min at RT. Immunohistochemical detection for each hybridized probe was performed using a Nucleic acid detection kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturers' protocols. Briefly, the sections were rinsed with 1% blocking reagent diluted in 0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl (THS) for 1 hr and incubated with alkaline phosphatase (ALP)-conjugated anti-DIG antibody (diluted 1/500 with THS) for 90 min at RT. After washing 3 times with THS for 5 min with 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl and 0.05 M MgCl₂ (alkaline buffer) for 5 min, tissue sections were incubated with 0.4 mM nitroblue tetrazolium (Sigma), 0.4 mM 5-bromo-4-chloro-3-indolylphosphate (Sigma) and 1 mM levamisole (Sigma) in alkaline buffer for 2 hr at 4°C, and then rinsed with alkaline buffer. They were counter-stained with methyl green (Merck), mounted with Histofine (Nichirei, Tokyo, Japan) and examined with a microscope (BX51, Olympus, Tokyo, Japan). The specificity of the obtained *in situ* hybridization signal was verified by incubation with sense probe or without antisense probe as negative controls. As further negative controls, serial sections were processed by omission of the anti-DIG antibody. Any controls yielded completely negative results.

Statistical methods: ANOVA analysis with Fisher's least significant differences test comparison for biochemical data, and Wilcoxon's signed rank test for histopathological estimation were carried out with the StatView IV program using a Macintosh computer. Differences at a probability of $P < 0.05$ were considered significant. All data are expressed as mean values $\pm$ SD ($n=6$ mice in each group).

RESULTS

Clinical parameters: Aged ICGN mice (30-week-old) manifested clinical abnormalities such as exercise intolerance, pale ears and weight loss. Table 1 shows biochemical parameters that were measured to assess the renal function. ICGN mice showed severe hypoalbuminemia (1.49-fold decreases as compared with age-matched ICR mice; $P < 0.001$) and albuminuria (no urinary albumin was detected in ICR mice). Marked increases were observed in sCr (3.00-fold increases as compared with ICR mice; $P < 0.001$) and BUN (2.22-fold increase as compared with ICR mice; $P < 0.001$) in ICGN mice, indicating the loss of renal function in these animals. Increased sTC (1.70-fold increases as compared with ICR mice; $P < 0.001$) suggested severe hypercholesterolemia in ICGN mice. Thus, aged ICGN mice used in the present study were in the nephrotic state.

Kidney histopathology and collagen levels: As shown in Table 2, histopathological examination revealed that aged

Table 1. Serum and urine biochemical features in control ICR and ICGN mice

	ICR mice	ICGN mice
sAlb ^{a)} (g/dl)	3.18 $\pm$ 0.13	2.13 $\pm$ 0.16***
uAlb ^{a)} (mg/ml)	not detected	13.15 $\pm$ 3.56
sCr ^{a)} (mg/dl)	0.30 $\pm$ 0.03	0.90 $\pm$ 0.10***
BUN ^{a)} (mg/dl)	31.8 $\pm$ 1.9	70.7 $\pm$ 5.4***
sTC ^{a)} (mg/dl)	120.2 $\pm$ 5.1	204.5 $\pm$ 15.1***

^{a)} For details, see Methods.

***: $P < 0.001$ versus each control ICR mice.

Table 2. Histopathological changes of glomeruli, and total and type I collagen levels in the renal cortex of control ICR and ICGN mice

	ICR mice	ICGN mice
Normal ^{a)} (%)	97.8 $\pm$ 1.2	32.7 $\pm$ 3.1***
Expansion of mesangial areas ^{a)} (%)	2.2 $\pm$ 1.2	54.8 $\pm$ 3.8***
Capillary aneurysm ^{a)} (%)	not observed	12.5 $\pm$ 2.3
Total collagen ^{b)} (mg/g protein)	4.35 $\pm$ 0.47	7.52 $\pm$ 0.43***
Type I collagen ^{b)} (mg/g protein)	2.00 $\pm$ 0.20	3.50 $\pm$ 0.20***

^{a)} Histopathological changes are described in detail in Methods.

^{b)} For details, see Methods.

***: $P < 0.001$ versus each control ICR mice.

ICGN mice had abnormal kidneys (54.8 $\pm$ 3.8 and 12.5 $\pm$ 2.3% of kidney sections with expanded mesangial area and capillary aneurysm, respectively; $P < 0.001$), but most age-matched ICR mice (97.8%) had normal kidneys. When compared with control ICR mice, enlargement of the mesangial region without cellular proliferation, tubulointerstitial expansion, appearance of cysts, expansion of renal tubules, and infiltration of inflammatory cells were observed in the kidney sections of ICGN mice, and such histopathological abnormalities progressed in an age-dependent manner in ICGN mice. Moreover, as compared with ICR mice, progressed fibrotic change in the glomeruli and tubulointerstitium was observed in the kidney cortex area of ICGN mice, but no progressed fibrotic change was seen in the kidney medulla area. Thus, glomerulonephritic, and glomerular- and tubulointerstitial-fibrotic change became severe only in ICGN mice.

Total and type I collagen levels in the renal cortex sections of ICR and ICGN mice assessed by a microquantification method are shown in Table 2. ICGN mice showed 1.73-fold increases in total collagen levels ($P < 0.001$) and 1.75-fold increases in type I collagen levels, as compared to controls ($P < 0.001$), indicating that fibrotic change in the renal cortex progressed in aged ICGN mice.

In situ hybridization: Moderate to strong expression of type I collagen mRNA was detected in glomeruli and proximal renal tubules with moderate and/or severe lesion in kidney sections of aged ICGN mice with manifested clinical abnormalities (Fig. 1B), but negative or trace expression of that was observed in those of ICR mice (Fig. 1A). In the kidneys of aged ICGN mice, type III collagen mRNA was detected in glomeruli and tubular epithelia (Fig. 1D). A part of interstitial myofibroblast-like cells were positive for type I and III collagen mRNAs (shown in insets in Fig. 1B and

Fig. 1. Representative renal sections of ICR (A, C and E) and ICGN mice (B, D and F) were subjected to *in situ* hybridization with specific probes for procollagen α_1 (I) (A and B), α_1 (III) (C and D) and α_1 (IV) (E and F) mRNAs. Insets in B and D are interstitial regions, and arrowheads show positive interstitial cells. Scale bars indicate 50 μ m.

D). Negative or trace reaction for Type III collagen mRNA was observed in the kidneys of ICR mice (Fig. 1C). No remarkable differences were seen in the localization of type I and III collagen mRNAs in the kidneys of aged ICGN mice. Slight expression of type IV collagen mRNA was demonstrated in every renal tubules in kidneys of both ICGN and ICR mice, and no remarkable difference in the expression of that was noted between these mice.

DISCUSSION

Chronic renal disease is characterized by clinical and morphological features caused by a progressive and irreversible loss of renal function. ICGN mice, a spontaneous nephrotic model, are considered a useful model for pathogenic analyses of progressive and chronic nephrotic syn-

drome and for investigating not only nephrotic conditions but also the cellular and molecular pathogenesis of diffuse mesangial sclerosis in steroid-resistant infantile nephrotic syndrome [9, 10]. Moreover, recent studies using the ICGN mice showed that an angiotensin converting enzyme inhibitor can attenuate the progress of renal dysfunction and fibrosis [8], and that recombinant hepatocyte growth factor (HGF) inhibits the onset of tubulointerstitial fibrosis and attenuates the progression of glomerulosclerosis [9, 10]. ICGN mice are believed to be a useful hereditary animal model for evaluation of therapeutic agents for nephrotic syndrome.

We demonstrated that both interstitial and membranous ECM components were abnormally accumulated in renal glomeruli and tubulointerstitium of ICGN mice, and such ECM accumulation developed in an age-dependent manner

[26]. As the mechanisms responsible for the excessive accumulation of ECM components in the kidneys of ICGN mice have not yet been revealed, we compared the activities of MMPs in the kidneys of aged ICGN mice and age-matched ICR mice, and revealed that lower activities of collagenase (MMP-1) and gelatinase (MMP-2 and -9) were demonstrated in the kidneys of ICGN mice as compared with those of ICR mice, but there were no significant differences in the stromelysin (MMP-3) activities between these mice [27]. These results indicate that decrease of MMP activity is one of the causes for abnormal accumulation of ECMs in ICGN mouse kidneys. Extremely low levels of ECM degradation together with reduced ECM production cause abnormal ECM accumulation, and extremely high levels of ECM production together with increased ECM degradation also cause abnormal ECM accumulation [1, 3, 4, 23, 24]. Clarification of the balance between the rates of ECM production and degradation is necessary to explain the abnormal ECM augmentation in the tissues with pathological conditions, but this balance has not been fully determined in the kidneys of ICGN mice. In the present study, to reveal the mechanisms of abnormal ECM accumulation in the kidneys of ICGN mice, we investigated the localization of mRNAs of interstitial type I and III collagens and membranous type IV collagen, because these collagens were excessively accumulated in the kidneys of aged ICGN mice [26]. *In situ* hybridization for these mRNAs showed that increased expression of mRNAs of type I and III collagens were detected in glomeruli and expanded proximal tubules of aged ICGN mice, but no notable difference in the localization of type IV collagen mRNA, which was demonstrated in all glomeruli and tubule regions, was seen between the kidneys of ICR and ICGN mice. Our findings of the localization of type I and III collagen proteins assessed by immunohistochemical stainings [26] were correspond with the present data of the localization of their mRNAs. Excessive accumulation of type IV collagen protein was immunohistochemically demonstrated in glomeruli and tubulointerstitium of ICGN mice [26], but no distinct changes in the localization of type IV collagen mRNA. We biochemically showed that lower activities of MMP-1 and MMP-2 and -9 were demonstrated in the kidneys of ICGN mice as compared with those of ICR mice [28], indicating that lower levels of MMPs may cause excessive accumulation type IV collagen. The present *in situ* hybridization studies demonstrated that increased production levels of interstitial collagens but not of membranous ones were concluded in the kidneys of aged ICGN mice. We immunohistochemically demonstrated the increased level of the active form of TGF- β_1 , which promotes ECM synthesis and suppresses its breakdown [1, 4], in the kidney sections of ICGN mice with progressed renal failure [26]. In the kidneys, the balance of TGF- β_1 , which acts as an accelerator, and HGF, which acts as an inhibitor, is considered crucial in the progression of renal fibrotic change [9, 10]. Not only an increase in TGF- β_1 levels but also a decrease in local HGF levels may be responsible for the manifestation of renal fibrosis, particu-

larly tubular destruction. High level of TGF- β_1 and low level of HGF cause renal fibrosis [26, 27], and recombinant human HGF treatment prevents the progression of renal fibrotic change in ICGN mice [10–12]. Moreover, we immunohistochemically demonstrated the abnormal localization of integrins, specific ECM receptors, corresponding for the fibrotic change in the kidneys of ICGN mice [28]. We immunohistochemically compared the cell kinetics (localization of proliferating cells and apoptotic cells) in the kidneys of ICGN mice with ICR mice [31]. Proliferating glomerular mesangial cells, epithelial cells of renal tubules and tubulointerstitial myofibroblast-like cells were observed in the kidneys of ICGN mice, but no proliferating cells were seen in those of ICR mice. Apoptotic cells were observed only in the tubulointerstitium in the kidneys of ICGN mice but not in those of controls. These proliferating mesangial cells and epithelial cells produce type I and III collagens, and a part of tubulointerstitial cells produce interstitial collagens. Because a part of tubulointerstitial cells may be a process of apoptotic cell death and a part of them may not be mature, such tubulointerstitial cells can not produce interstitial collagens. Increased population of these fibrogenic cells and activated metabolisms of ECM production may cause severe fibrotic change in the kidneys of ICGN mice. The proliferation of renal tubular epithelial cells may represent a compensatory response, and that of mesangial and myofibroblast-like cells may play a pathogenic role in nephrotic syndrome.

We concluded that excessive accumulation of interstitial type I and III collagens in kidneys of ICGN mice is caused by increased production of these collagens and decreased degradation of them, and that moderate accumulation of membranous type IV collagen is caused by decreased degradation but not increased production. Interstitial collagen accumulation may be caused by activation of TGF- β_1 . Interestingly, the mechanisms of ECM accumulation of interstitial collagens are different from those of membranous collagen. Our findings should contribute to elucidation of the mechanism of excessive accumulation of ECMs in patients with hereditary nephrotic syndrome. Further detailed studies to reveal the pathogenic causes of the renal failure in ICGN mice, especially how to upregulate the collagen mRNAs and to activate the TGF- β_1 , are in progress in our laboratory.

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