Acta Med. Nagasaki 45 : 17－24

Increased Expression of Collagen-binding Heat Shock Protein 47 in Human and Experimentally-induced Rat Crescentic Glomerulonephritis: its Possible Role in Fibrotic Process

Arifa NAZNEEN, Takashi NAITO, Mohammed Shawkat RAZZAQUE, Diange LIU, Takashi TAGUCHI

Second Department of Pathology, Nagasaki University School of Medicine

The 47-kD heat shock protein 47 (HSP47), a major collagen-binding stress protein, is thought to play an important role in fibrotic diseases. To investigate the role of HSP47 in the fibrotic process of crescent formation in renal glomeruli, involvement of HSP47 was examined in both human and experimental crescentic glomerulonephritis (GN). Renal biopsy sections of 15 cases of crescentic GN and 5 cases of minimal change nephrotic syndrome were examined for the expression of HSP47. Experimental crescentic GN was induced by anti-glomerular basement membrane antibody in rats. HSP47 expression was examined in crescents at various stages of formation (days 7, 14, 21, 28 and 42). In human renal biopsy sections, increased expression of HSP47 was noted in the cellular stage of crescent formation. Double immunostaining demonstrated that the majority of α-smooth muscle actin-positive cells in the crescent expressed HSP47. However, its expression was less in fibrous crescents, which were predominantly composed of type III collagen. In the rat model of crescent GN, increased expression of HSP47, both at mRNA and protein levels, was noted in the early stages of cellular crescents. However, its expression was decreased in the late stages of fibrous crescents. Based on the collagen synthesis ability of HSP47, we speculate that overexpression of HSP47 in cellular crescents may contribute to the excessive synthesis/assembly of collagens and subsequently lead to irreversible fibrous crescent formation.

Key words: crescentic glomerulonephritis, HSP47, immunohistochemistry, RT-PCR

Address Correspondence : Arifa Nazneen, MBBS
Second Department of Pathology, Nagasaki University School of Medicine
1-12-4, Sakamoto, Nagasaki 852-8523, Japan
TEL:+81-95-849-7055 FAX:+81-95-849-7056

Introduction

Crescentic glomerulonephritis (GN) is a rapidly progressive renal disease, characterized histologically by the early appearance of cellular circumferential crescents in the glomeruli, which subsequently lead to fibrous crescent formation. While early treatment can resolve certain forms of cellular crescents, their transformation into fibrous crescents is an irreversible pathological process. Crescentic GN can be induced experimentally in laboratory animals by injection of anti-glomerular basement membrane (GBM) antibody. The renal tissues of these animals exhibit various stages of crescent formation with disease progression, resulting in prominent glomerular and interstitial fibrosis, similar to that seen in human crescentic GN. It has been widely noted that increased deposition of extracellular matrix (ECM), especially collagens, may contribute to fibrous crescent formation, but the factors that regulate increased synthesis of collagens during this process have not yet been identified (1, 2).

HSP47, a collagen-specific stress protein, is involved in the processing and/or assembly of procollagens (3, 4). Both human and experimental studies have shown that the expression of HSP47 is upregulated in the fibrotic process and correlates with excessive accumulation of collagens (5-10). Whether HSP47 plays any role in crescent formation is not yet known, but investigation of the involvement of HSP47 in the development of fibrous crescents is potentially important since such involvement implies the possible manipulation of fibrotic crescent formation (11, 12). The present study was undertaken to evaluate the role of HSP47 in the development of crescent formation in both human and experimentally-induced rat crescentic GN.

Materials and Methods

Tissue specimens

Human renal biopsy sections from 15 cases with histologically diagnosed crescentic GN were selected for this study. As control, we selected tissue sections from 5 cases of minimal change nephrotic syndrome (Table 1). The study protocol was approved by the Human Ethics Review Committee of Nagasaki University School of Medicine.

Table 1: Clinical and histopathological findings in human crescentic glomerulonephritis.

Case no	Age/sex	Clinical diagnosis	Serum creatinine (mg/dl)	Histopathological diagnosis
A) Crescentic glomerulonephritis				
1.	63 M	IgAN	3.8	Crescentic GN
2.	76 F	RPGN	3.4	Crescentic GN
3.	48 M	RPGN	3.1	Crescentic GN
4.	62 M	CGN, Hypertension	1.1	Crescentic GN
5.	70 M	NS, Lung ca, Liver dysf.	1.0	Crescentic GN
6.	53 F	NS	1.0	Crescentic GN
7.	64 M	CGN	1.4	Crescentic GN
8.	84 M	PN	1.5	Crescentic, necrotizing GN
9.	62 F	RPGN	4.6	Advanced crescentic GN
10.	57 M	ARF, ANCA related GN	7.9	Advanced crescentic GN
11.	76 M	Goodpasture's syndrome	11.2	Crescentic GN
12.	62 F	RPGN	11.7	Crescentic, necrotizing GN
13.	36 F	AGN, suspected	1.5	Crescentic GN
14.	76 F	CGN, ANCA related GN	1.3	Crescentic GN
15.	71 F	RPGN	8.1	Crescentic GN
B) Control : cases of MCNS				
1.	20 F	NS	0.5	MCNS
2.	31 M	NS	0.6	MCNS
3.	16 F	NS	0.8	MCNS
4.	21 M	NS	0.9	MCNS
5.	49 F	NS, DM	0.6	MCNS

IgAN : IgA nephritis, GN : glomerulonephritis, RPGN : Rapidly progressive glomerulonephritis, CGN : Chronic glomerulonephritis, NS : Nephrotic syndrome, ca : carcinoma, dysf. : dysfunction, PN : Polyarteritis nodosa, ARF : Acute renal failure, AGN : Acute glomerulonephritis, DM : Diabetes mellitus, MCNS : Minimal change nephrotic syndrome

Preparation of rat model of crescentic glomerulonephritis

Five-week old male Sprague Dawly rats (n=20) were primed with subcutaneous injection of rabbit IgG in Complete Freund's Adjuvant (5 mg/kg, Sigma Chemical Co., St. Louis, MO), five days prior to the induction of accelerated anti-GBM nephritis (13). Induction of crescentic GN was achieved by intravenous administration of 12.5 mg IgG/kg rabbit anti-rat GBM antibody (Otsuka, Japan). Rats were sacrificed on day 7, 14, 21, 28 or 42 after induction of nephritis. Control rats (n=5) were preimmunized, but GN was not induced. Both kidneys were dissected out, fixed, paraffin embedded, sectioned and stained with hematoxylin eosin (HE), periodic acid-Schiff (PAS), periodic acid-methenamine silver (PAM) and Mallory azan. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University School of Medicine.

Immunohistochemistry

Immunohistochemistry was performed as described earlier (14, 15). Briefly, paraffin embedded tissue sections were deparaffinized, rehydrated in graded alcohol, treated with 0.3% hydrogen peroxide/methanol to block endogenous peroxidase activity. After mild treatment with trypsin (T-8253, Sigma, 10 mg/100 ml of 0.01 M PBS, 3 min), tissue sections were incubated overnight with an antibody against HSP47 (StressGen Biotechnologies Corp., Canada). Sections were then processed with Histofine SAB kit (Nichirei, Japan) for staining by the immunoperoxidase method and the Histostain-SAP Kit (Zymed Laboratories Inc., San Francisco, CA) for staining by the alkaline phosphatase method. In addition to HSP47, both human and rat kidney sections were stained for collagens (type III and IV) and α-smooth muscle actin (α-SMA). Normal mouse IgG and rabbit IgG were used as negative control. The source of antibodies used in the present experiments has been reported previously (5, 6, 14, 15).

Table 2: Immunohistochemical expression of HSP47 in cases of human crescentic GN

case no.	Glomeruli				Interstitium	Tubules
	Tufts	crescent				
		Cellular	Fibrocellular	Fibrous		
(Crescentic glomerulonephritis)						
1	+	+++	±	-	+	++~+++
2	±	+++	±	-	+	++
3	+	+++	++	-	++	++
4	+~++	+++	NC	NC	++	±
5	+	+++	NC	-	+	++
6	+~++	+++	++	-	++	++
7	+	+++	++	-	+	++
8	+~++	+++	+	NC	+~++	±
9	+	+++	++	-	++	+
10	±~+	+++	+	-	++	++
11	±	+++	++	-	±	++~+++
12	±	++	++	-	+	++
13	++	+++	++	NC	+++	+
14	+	+++	±	-	+	++~+++
15	±	NC	+	-	+	+
(Control : cases of MCNS)						
1	±	NC			±	±
2	±	NC			±	±
3	±	NC			±	±
4	±	NC			±	±
5	±	NC			±	±

No crescent : NC No expression : - Faintly positive : ±
Mild expression : + Moderate expression : ++ Severe expression : +++

Double immunostaining

Double immunostaining was carried out to detect HSP47/collagen type III, HSP47/collagen type IV, HS

P47/ α-SMA in the same renal section as described previously (14, 15). Briefly, HSP47 was stained first by streptavidin-alkaline phosphatase method and developed by 5-bromo-4-chlor-3-indolyl-phosphate (BCIP) /nitroblue tetrazolium(NBT)which produced black purple colored signal. Then the slides were counterstained with either collagens (type III and IV) or α-SMA by streptavidin-biotin peroxidase method and developed by aminoethylcarbazole (AEC)/H_2O_2, which produced intense red colored stain.

RT-PCR

RT-PCR was performed using homogenized renal cortical tissue (homogenizer: Polytron, PT1200, Switzerland). Total RNA was extracted by phenol and guanidine isothiocyanate method using TRIzoL LS reagent (Gibco BRL, Long Island, NY). cDNA was prepared using the ssDNA synthesis kit (Superscript Preamplification kit, Gibco BRL) as instructed by the manufacturer. Briefly, 4 μm of total RNA was incubated with 50 ng of random hexamer at 70℃ for 10 minutes then rapidly chilled at 4℃ and kept at least for 1 minute. In the next step, deoxynucleoside triphosphate (dNTP) mix, 10 mM dithiothreitol (dTT), and 10 mM MgCl2 were added and further incubated for 5 minutes at 25℃. Then, Superscript II reverse transcriptase (RT) was added from the kit, and incubated at 42℃ for 50 minutes. The reaction was terminated by heating the mixture at 70℃ for 15 minutes. To remove residual RNA, Escherichia coli RNaseH was mixed gently with the product and kept at 37℃ for 20 minutes. Thus, the prepared cDNA was stored in -20 ℃ until PCR amplification. Amplification of target DNA of HSP47 was carried out with forward primer (5'-ACCACAGGATGGTGGACAACCGT-3') and reverse primer (5'-ATCTCGCATCTTGTCTCCCTTGGG-3') incorporation using the Gene Amp, PCR Reagent Kit with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT, Roche). Thirty cycles of denaturation at 94℃ for 1 minute, annealing at 55℃ for 1 minute and extension at 72℃ for 2 minutes were arranged in a thermocycler (PC701, ASTEC Co., Japan) and then PCR products were kept at 4℃. DNA electrophoresis of PCR products was performed through 2% agarose gel with ethidium bromide. 100 bp DNA ladder was used as a molecular marker to interpret the location of amplified DNA. PCR products were visualized by ultraviolet exposure and analyzed by densitometry with positive control of β-actin.

Results

Human renal biopsies

The clinical profiles of patients from whom renal biopsies were obtained are summarized in Table 1. All renal biopsies of crescentic GN showed various stages of circumferential crescents (cellular, fibrocellular and fibrous). Immunohistochemically, overexpression of HSP47 was identified in cellular (Fig. 1A) and fibrocellular crescents, but low expression was noted in fibrous crescents (Fig. 1B). Using double immunostaining, the major source of HSP47 was found to be α-SMA-immunopositive cells in the crescents (Fig. 1C). In addition to the crescents, intraglomerular cells and tubulointerstitial cells were also found to express HSP47 in the sclerotic glomeruli and interstitial fibrotic areas, respectively. The expression of HSP47 in different components of the studied cases is shown in Table 2. Increased deposition of type III collagen was also seen in fibrous crescents (Fig. 1D).

Morphological changes in experimental renal tissues

The renal tissue at day 7 after injection of rabbit anti-rat GBM antibody showed no apparent histopathological lesions, except for focal inflammatory cell infiltration in a few glomerular tufts. However, marked histopathological changes occurred at day 14, characterized by increased glomerular size, mild hypercellularity of the glomerular tufts, and exudative materials in Bowman's space. The glomerular lesions were subsequently followed by the appearance on day 21 of focal cellular and then circumferential crescents of cellular and fibrocellular variants (Fig. 2A and B). Progressive development of glomerular sclerosis with interstitial fibrosis was observed during the experimental period after day 21. The majority of crescents became fibrotic at day 42 (Fig. 2C). On the other hand, no morphological changes were noted in the control rat kidneys throughout the entire experimental period (Fig. 2D).

Immunohistochemical analysis of HSP47 in rat kidney sections

A few intraglomerular cells and interstitial cells showed weak expression of HSP47 in control renal tissues (Fig. 3A). In experimental rats, increased expression of HSP47 was noted in early cellular and fibrocellular crescents (Fig. 3B). In addition, cells of the contracted glomerular tufts showed strong expression of HSP47. A gradual increase in the expression of HSP47 was noted in the early stages while its

expression decreased in the late stages of fibrotic lesions (Fig. 3C).

Immunohistochemical analysis of collagens in rat kidney sections

Mild interstitial deposition of type III collagen was noted in control renal sections (Fig. 4A), while markedly increased expression was noted in and around the crescents, sclerotic glomeruli and widened interstitium (Fig. 4B). As for type IV collagen, a weak expression was noted in the GBM, tubular basement membrane and mesangial matrix of control renal sections (Fig. 4C). Increased expression was noted in the widened interstitium, thickened tubular basement membrane and sclerotic glomeruli (Fig. 4D). Increased deposition of collagens was noted in all stages of experimental nephritis. Double staining showed that overexpression of HSP47 correlated with increased deposition of collagens (Fig. 4E and F). However, the expression of HSP47 in crescents was noted only in cellular and fibrocellular components.

Identification of HSP47 expressing cells in rat kidney sections

Compared to control kidney sections, numerous α-SMA-positive cells were noted in cellular and fibrocellular crescents, glomerular tufts and in the interstitium in GN kidneys (Fig. 5B). Double immunostaining revealed that α-SMA-positive cells frequently coexpressed HSP47 in glomeruli (Fig. 5C) and interstitium (Fig. 5D).

RT-PCR analysis of mRNA for HSP47 in rat kidney sections

Compared to the control kidney, relatively stronger bands were seen in the kidneys in early crescentic GN in and around 600 bp (arrow), indicating increased expression of HSP47 mRNA in GN kidney until 28 days (Fig. 6). The HSP47 band decreased in intensity at day 42. RT-PCR products of β-actin of the corresponding lanes are shown at the bottom (Fig. 6).

Discussion

Crescentic GN is characterized by segmental or circumferential crescents associated with a variable degree of destruction of capillary tufts. These pathological structures either subsequently show a complete resolution, though rare, or persist and undergo transformation from a cellular to fibrocellular form and ultimately change to complete fibrous crescents (1, 2). In general, the presence of fibrous crescents represents irreversible damage, which leads to a permanent impairment of renal functions. When the crescents are circumferential, the glomerular tufts usually show collapse and gradually change to global sclerosis. Since advanced stage of fibrous crescent formation is closely related to subsequent glomerular damage, manipulation of crescents in the early stages might modulate glomerular damage. Although increased deposition of ECM including collagens is mainly responsible for the advanced stage of fibrous crescent formation, factors that regulate increased synthesis of collagens are not yet clear. In this study, the possible role(s) of HSP47 in fibrous crescent formation was investigated in human and experimental crescentic GN. The major finding of our study was the enhanced expression of HSP47 in early cellular crescents, which was followed by collagen-rich fibrous crescent formation.

Although the exact relationship between increased expression of HSP47 in the cellular crescent and increased deposition of collagens in the late stage of fibrous crescent is difficult to verify based on our results, previous studies have demonstrated that HSP47 is closely related to collagen synthesis in other sclero tic/fibrotic processes in various organs in both human and experimental animals (5, 6, 14-17). Furthermore, in vitro experiments have clearly shown the importance of HSP47 during collagen assembly/synthesis (3, 4). Thus, based on the results of these early studies and the present findings, we speculate that increased expression of HSP47 in early crescents might be associated with increased synthesis of collagens with subsequent fibrous crescent formation in the late stage. Consistent with earlier observations (5, 7, 8, 10-12, 14, 16, 17), apart from crescents, increased expression of HSP47 was also observed in sclerotic glomeruli and around the fibrotic interstitium in both human and experimental crescentic GN, suggesting the possible role of HSP47 in sclerotic/fibrotic process. These results imply that suppression of HSP47 expression might be of therapeutic value as it might potentially modulate the process of sclerosis/fibrosis in crescentic GN (11, 12).

In conclusion, we demonstrated in the present study increased expression of HSP47 in the early cellular crescents, which subsequently exhibited increased deposition of collagens in fibrous crescents. Based on the collagen synthesizing ability of HSP47, we speculate that increased synthesis of collagen might subsequently lead to irreversible fibrous crescent formation. In order to define that exact relationship between HSP47 and fibrous crescent formation, additional

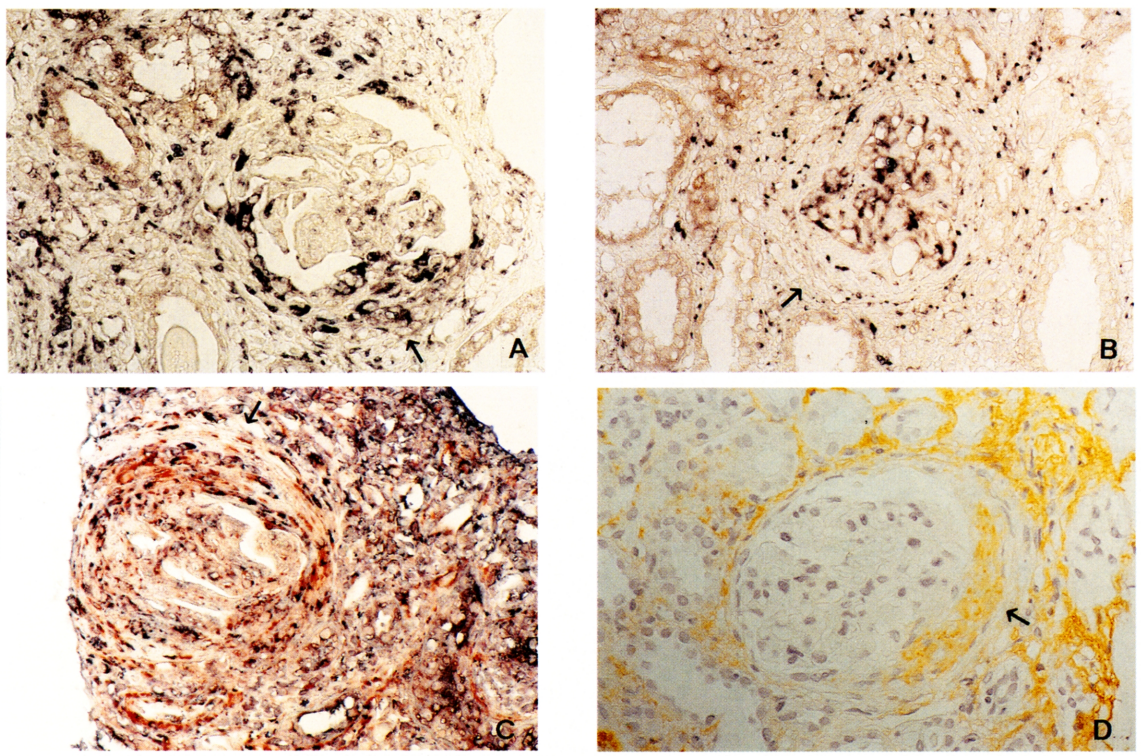

Fig. 1: Immunohistochemical expression of HSP47 in cases of human crescentic GN with cellular crescent (A) and fibrous crescent (B). Double immunostaining of HSP47 (black purple) and α-smooth muscle actin (red) showing colocalization in cells of cellular/fibrocellular crescents and interstitial cells (C). Expression of type III collagen in fibrous crescent (D). Arrows indicate crescents. Original magnification, x100.

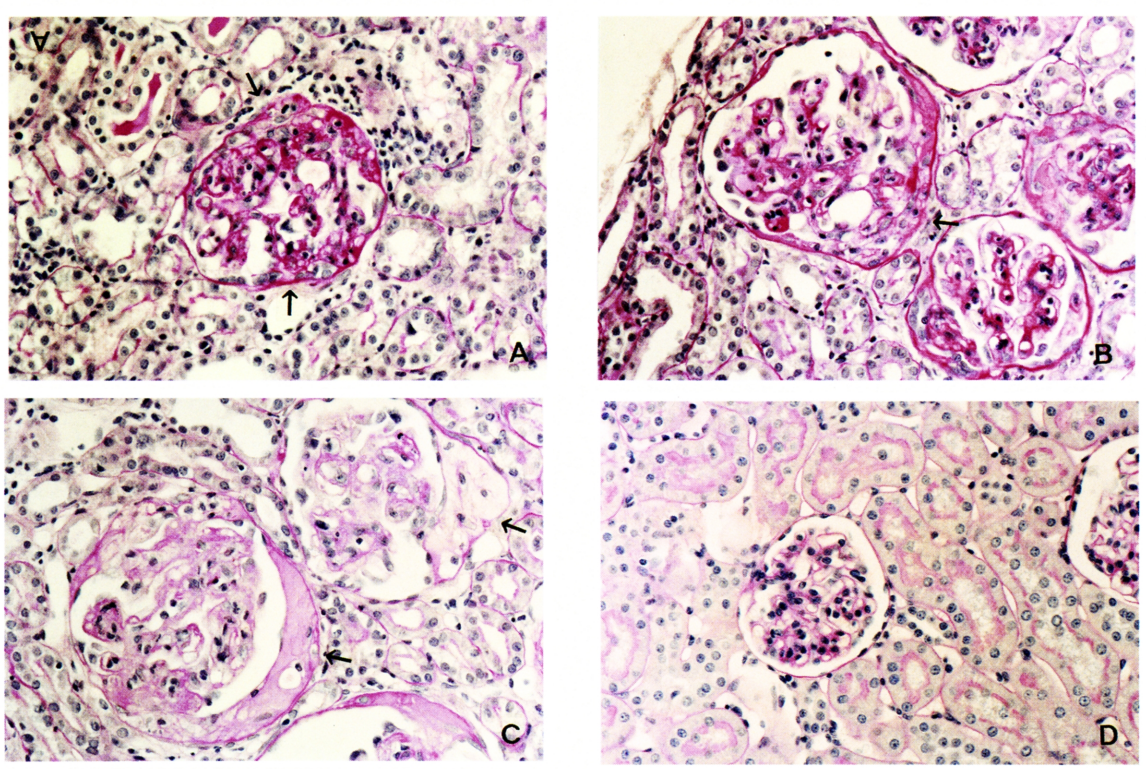

Fig. 2: Morphological changes in experimental GN. Cellular crescent at day 21 (A), fibrocellular crescent at day 28 (B), fibrous crescent at day 42 (C) and control (D). Arrows indicate crescents. (PAS stain, original magnification, x100).

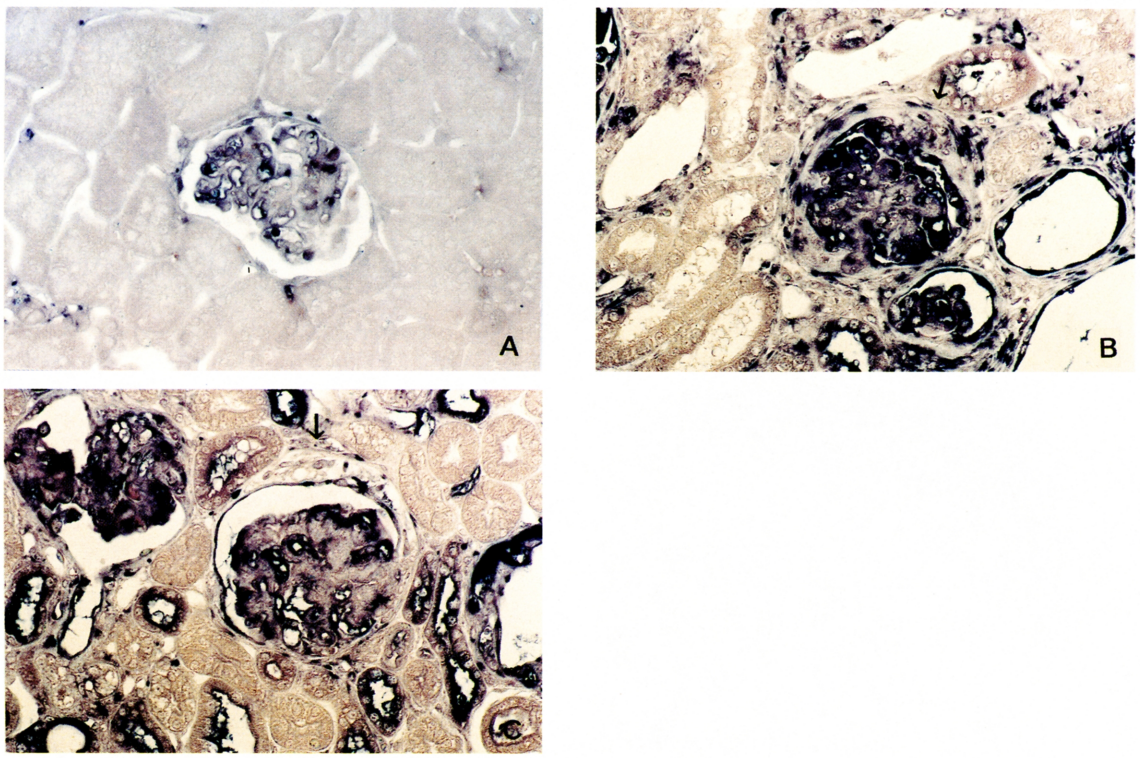

Fig. 3: Immunohistochemical expression of HSP47 in experimental GN. Control (A), fibrocellular crescent (B) and fibrous crescent (C). Arrows indicate crescents. Original magnification, x100.

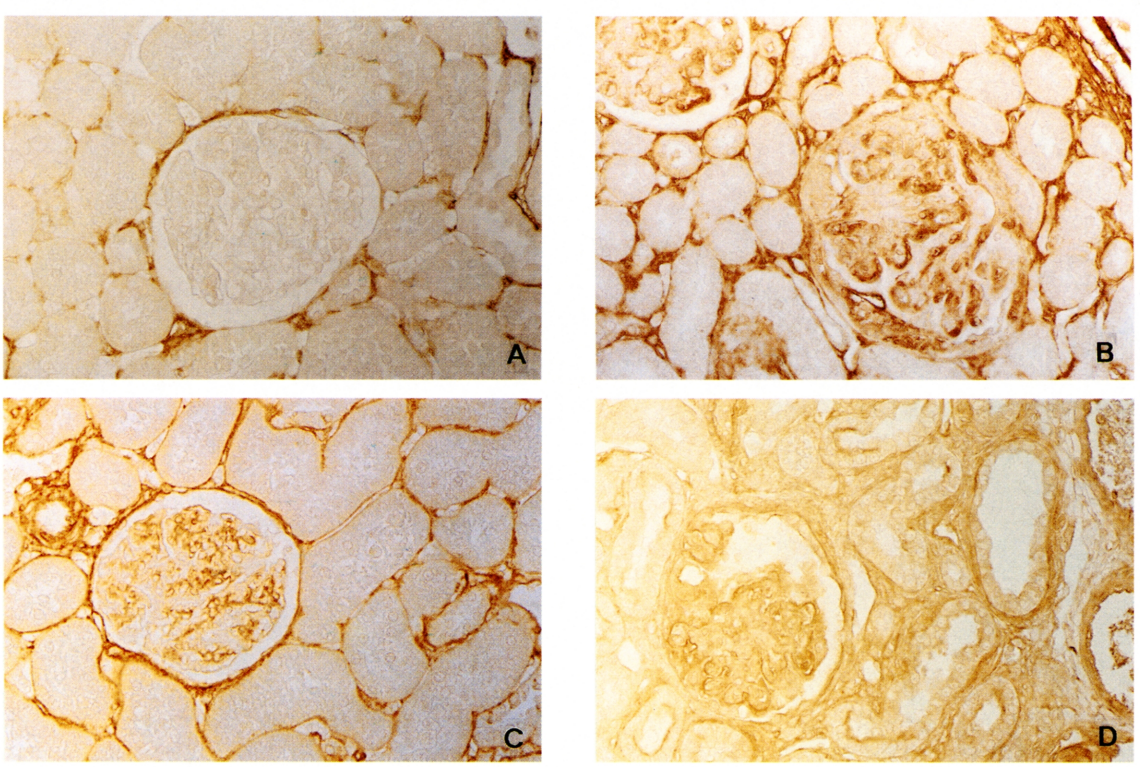

Fig. 4: Expression of collagens and HSP47 in experimental GN. Type III collagen in control (A) and experimental GN (B), Type IV collagen in control (C) and experimental GN (D). .

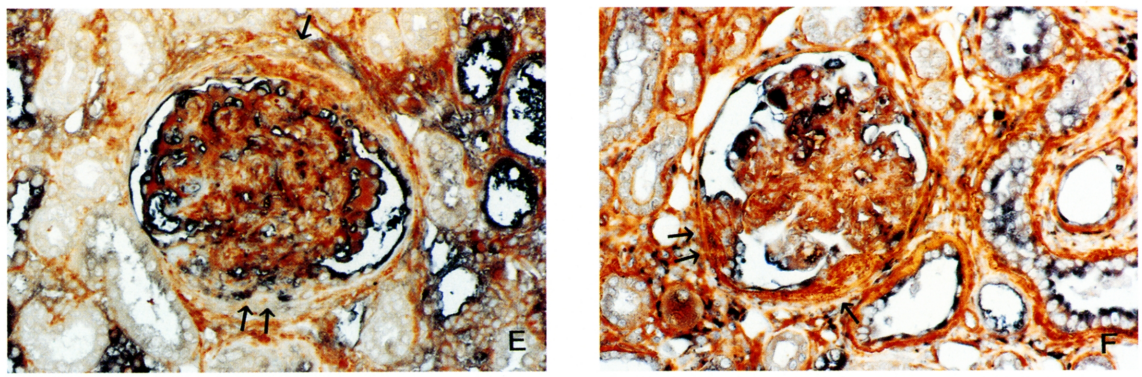

Fig. 4: Double immunostaining of HSP47 (black purple) and type III collagen (red) (E) and HSP47 and type IV collagen (red) (F). Collagens III and IV are abundant in fibrous (single arrow) and fibrocellular (double arrow) crescents, but the expression of HSP47 is only noted in fibrocellular crescents. Original magnification, x100.

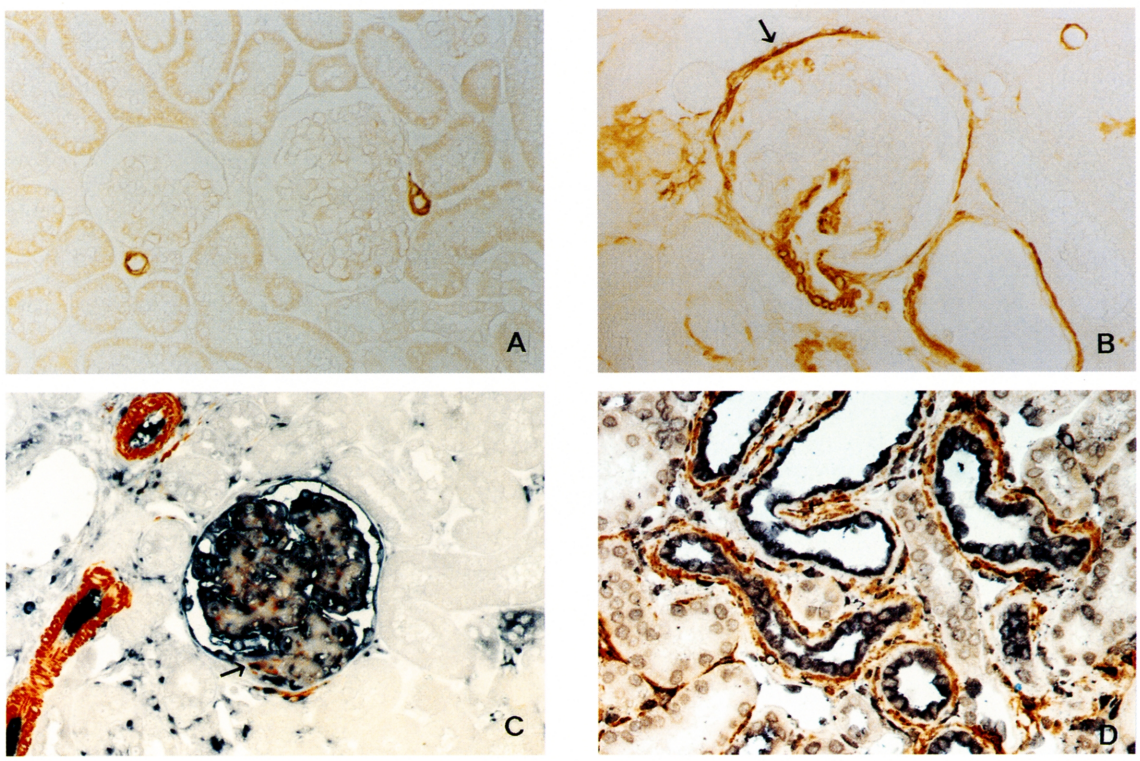

Fig. 5: Immunohistochemical expression of a-smooth muscle actin in control (A) and crescentic GN (B). Double immunostaining of HSP47 (black purple) and α-smooth muscle actin (red) in a glomerulus with cellular crescent (C) and interstitium (D) of GN kidney. Original magnification, x100.

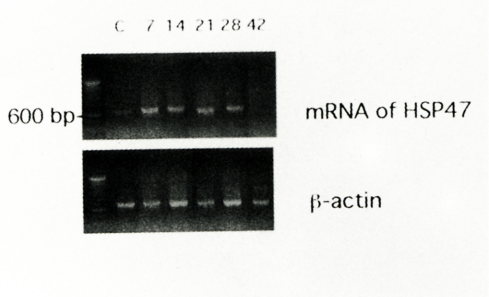

Fig. 6: Amplification of mRNA of HSP47 by RT-PCR shows prominent bands during 7th to 28th day of induction of glomerulonephritis. Lanes indicate control, 7th, 14th, 21st, 28th and 42nd days. The corresponding β-actin expression is shown at the bottom.

studies are needed in which the expression of HSP47 is modulated in experimental crescentic GN. Such studies would enhance our understanding of the role of HSP47 in humans particularly in the regulation of the fibrotic process in crescentic GN.

Acknowledgment

The authors thank the staff of the departments of the following hospitals for their help and permission to examine available renal biopsy specimens: Nagasaki Municipal Medical Center; Internal Medicine, Sasebo General Hospital; Second Department of Internal Medicine, Nagasaki University School of Medicine; Second Department of Internal Medicine, Oita Medical University and Second Department of Internal Medicine, Hiroshima University School of Medicine. We also thank all technical staff of the Second Department of Pathology, Nagasaki University School of Medicine.

References

1. Rosen S: Crescentic glomerulonephritis: occurrence, mechanisms and prognosis. Pathol Annu 10: 37-64, 1975.
2. Lewis EJ and Schwartz MM: Idiopathic crescentic glomerulonephritis. Semin Nephrol 2: 193-213, 1982.
3. Nagata K: HSP47: a collagen-specific molecular chaperone. Trends Biochem Sci 21: 22-26, 1996.
4. Ball EH, Jain N and Sanwal BD: Colligin, a collagen binding serpin. Adv Exp Med Biol 425: 239-45, 1997.
5. Razzaque MS, Kumatori A, Harada T and Taguchi T: Coexpression of collagens and collagen-binding heat shock protein 47 in human diabetic nephropathy and IgA nephropathy. Nephron 80; 434-443, 1998.
6. Razzaque MS, Nazneen A and Taguchi T: Immunolocalization of collagens and collagen-binding heat shock protein 47 in fibrotic lung diseases. Mod Pathol 11:1183-1188, 1998.
7. Cheng M, Razzaque MS, Nazneen A and Taguchi T: Expression of the heat shock protein 47 in gentamicin-treated rat kidneys. Int J Exp Path 79: 125-132, 1998.
8. Razzaque MS, Shimokawa I, Nazneen A, Higami Y and Taguchi T: Age-related nephropathy in the Fischer 344 rat is associated with overexpression of collagens and collagen-binding heat shock protein 47. Cell Tissue Res 293: 471-478, 1998.
9 Masuda H, Fukumoto M, Hirayoshi K and, Nagata K: Coexpression of the collagen-binding stress protein HSP47 gene and the a1(I) and a1(III) collagen genes in carbon tetrachloride-induced rat liver fibrosis. J Clin Invest 94: 2481-2488, 1994.
10. Razzaque MS and Taguchi T: Localization of HSP47 in cisplatin-treated rat kidney: a possible role in tubulointerstitial damage. Clin Exp Nephrol 3: 222-228, 1999.
11. Sunamoto M, Kuze K, Tsuji H, Ohishi N, Yagi K, Nagata K, Kita T and Doi T: Antisense oligonucleotides against collagen-binding stress protein HSP47 suppress collagen accumulation in experimental glomerulonephritis. Lab Invest 78: 967-972, 1998.
12. Razzaque MS, Shimokawa I, Nazneen A, Liu D, Naito T, Higami Y and Taguchi T: Life-long dietary restriction modulates the expression of collagen and collagen-binding heat shock protein 47 in aged Fischer 344 rat kidney. Histochem J 31: 123-132, 1999.
13. Lan HY, Bacher M, Yang N, Mu W, Nikolic-Paterson DJ, Metz C, Meinhardt A, Bucala R and Atkins RC: The pathogenic role of macrophage migration inhibitory factor in immunologically induced kidney disease in the rat. J Exp Med 185: 1455-1465, 1997.
14. Razzaque MS and Taguchi T: Collagen-binding heat shock protein (HSP) 47 expression in anti-thymocyte serum (ATS)-induced glomerulonephritis. J Pathol 181: 24-29, 1997.
15. Razzaque MS, Hossain MA, Kohno S and Taguchi T: Bleomycin-induced pulmonary fibrosis in rat is associated with increased expression of collagen-binding heat shock protein (HSP) 47: Virchows Arch 432: 455-460, 1998.
16. Razzaque MS and Taguchi T: Possible role of colligin/HSP47, a collagen-binding protein in the pathogenesis of human and experimental fibrotic diseases. Histol Histopathol; 14: 1199-1212, 1999.
17. Razzaque MS and Taguchi T: Role of glomerular epithelial cell-derived HSP47 in experimental lipid nephropathy. Kidney Int 71 (Suppl):S256-S259, 1999.