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Expression of the heat shock protein 47 in gentamicin-treated rat kidneys

M. CHENG, M. S. RAZZAQUE, A. NAZNEEN AND T. TAGUCHI
Second Department of Pathology, Nagasaki University School of Medicine, Nagasaki, Japan

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Summary. Heat-shock proteins (HSPs) are rapidly synthesized in cells in response to various cytotoxic agents. Although several stress proteins are actively involved in the gentamicin-induced renal damages, the possible role of HSP47 in this condition is not yet clear. In this study, the expression of HSP47 in the gentamicin nephrotoxicity was examined by immunohistochemistry. Twenty male Wistar rats were sacrificed at day 0, 3, 7, 14 and 28 after subcutaneous injection of gentamicin. Gentamicin treatment causes tubular necrosis at day 3, followed by tubular regenerative changes and interstitial fibrosis, which was most prominent at day 14. The renal structures returned to almost normal architectures at day 28. By immunohistochemistry, HSP47 was weakly expressed in most of the glomeruli and occasionally in interstitial cells in the control rat kidneys. In contrast, strong immunostaining for HSP47 was noted in the tubular epithelial cells and interstitial cells in gentamicin treated rat kidneys, and strongest staining was observed at day 7. The immunostaining for HSP47 then gradually decreased, and returned to the normal level at day 28. In the whole experimental period, staining pattern of HSP47 in the glomeruli was not changed. In addition, phenotypically altered tubulointerstitial cells including regenerative tubular epithelial cells (immuno-positive for vimentin) and interstitial cells (immuno-positive for α-smooth muscle actin) were found in gentamicin nephrotoxicity. Expression of type III collagen increased in the areas of interstitial fibrosis. By double immunostaining, the regenerated and phenotypically altered tubulointerstitial cells were found to express HSP47 in and around interstitial fibrosis.
It is concluded that overexpression of HSP47 by phenotypically altered renal cells might play a significant role in the development of gentamicin nephrotoxicity.

Keywords: gentamicin, nephrotoxicity, HSP47, immunohistochemistry

Heat-shock proteins (HSPs), referred to as stress proteins are highly conserved proteins throughout evaluation and are rapidly synthesized in cells in response to elevated temperature, cytotoxic agents and hypoxia (Wakui *et al*. 1995).

Aminoglucoside antibiotics including gentamicin are widely used in the treatment of gram-negative infections. A major complication of the use of these drugs is

Correspondence: T. Taguchi, Second Department of Pathology, Nagasaki University School of Medicine, 1–12–4, Sakamoto, Nagasaki 852, Japan. Fax: +81 95 8497056; E mail: taguchi@net.nagasaki-u.ac.jp

nephrotoxicity. The specificity of gentamicin for renal toxicity is apparently related to its preferential accumulation in the renal proximal convoluted tubules (Humes *et al.* 1986). Although several hypotheses have been suggested to elucidate the possible mechanism(s) of gentamicin nephrotoxicity (Ali 1995), the exact mechanism(s) of gentamicin nephrotoxicity is unknown.

Recent reports have shown the important role(s) of HSP73 and 90 in the gentamicin-nephrotoxicity (Komatsuda *et al.* 1993; Ohtani *et al.* 1995). HSP47, a 47 kD protein, differs in function from that of other HSPs of higher molecular weight, such as HSP70 and HSP90 (Nagata *et al.* 1986; Ferreira *et al.* 1994). HSP47 is thought to be mainly related to the sclerotic/fibrotic process (Masuda *et al.* 1994; Razzaque & Taguchi 1997; Razzaque *et al.* 1998) as demonstrated in various fibrotic models. Most of these studies were performed in the progressive models for fibrosis.

In this study, we examined the expression of HSP47 in a more acute reaction gentamicin-nephropathy, a model which is characterized by early tubulointerstitial changes with increased deposition of collagens followed by restoration of damaged structures.

To the best of our knowledge, the expression of HSP47 in gentamicin-nephrotoxicity has not yet been reported. To elucidate the role of HSP47 in gentamicin-nephrotoxicity, we studied the expression pattern of HSP47 in the gentamicin-treated kidney, especially focusing on the recovery stage of fibrosis.

Materials and methods

Animals

Male Wistar rats ($n = 20$) were given 250 mg/kg body weight/day of gentamicin by subcutaneous injection in the neck, at 8-h intervals for 2 days. Age-matched controls ($n = 15$) received an equal volume of physiological saline instead.

Tissue collection

Rats were sacrificed by exsanguination under ether anaesthesia at day 0, 3, 7, 14, 28 after gentamicin injection. The kidneys were rapidly removed and weighed, then fixed immediately in either 10% formalin for 24 h or in Carnoy's solution for 2 h and processed further for histological and immunohistochemical examination.

Biochemical determinations

Blood was collected from the inferior vena cava of each rat and level of blood urea nitrogen (BUN) and serum creatinine were measured by auto analyser (Hitachi 7170, Japan).

Histological studies

Tissues were routinely processed, embedded in paraffin, cut into 4 μm sections and stained with haematoxylin-eosin (HE), Periodic acid Schiff (PAS), Peroiadic acid methenamine silver (PAM) and Masson's trichrome. The extent of renal damages was determined by light microscopic examination of the sections stained by these methods.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections as described previously (Razzaque *et al.* 1994, 1997). Briefly, paraffin sections were deparaffinized with xylene, then pretreated with hydrogen peroxide to abolish endogenous peroxidase activity. After mild treatment with trypsin, the sections were incubated with either 10% goat serum or 10% rabbit serum, and then incubated overnight by antibodies against α -smooth muscle actin (Dako Co, Denmark), vimentin (Dako), type III collagen (Chemicon, USA), HSP 72/73 (Oncogene, USA) and HSP47 (Biotechnologies Corp, Canada). As immunohistochemical controls, the antibodies were replaced with 0.01 M PBS or with similar concentration of either mouse or rabbit IgG. After washing with PBS, the sections were processed further using Histofine SAB-PO kit, as directed by the manufacturer and the sections were developed with 0.01% hydrogen peroxide and 3,3'-diaminobenzidine.

The staining intensity of α -smooth muscle actin, vimentin, type III collagen, HSP 72/73 and HSP47 were graded semiquantitatively according to the following scales; 0: no staining, $+$: weak staining, $++$: moderate staining, $+++$: strong staining.

Double immunostaining

Double immunostaining was performed to localize HSP47 expressing cells as described previously (Razzaque & Taguchi 1997; Razzaque *et al.* 1998). Briefly, HSP47 was initially stained by alkaline phosphatase method and developed with BCIP/NBT, which produced dark purple staining. The sections were further stained for type III collagen, HSP 72/73, α-smooth muscle actin or vimentin by the peroxidase method and visualized with hydrogen peroxide/ACE, producing an intense red stain.

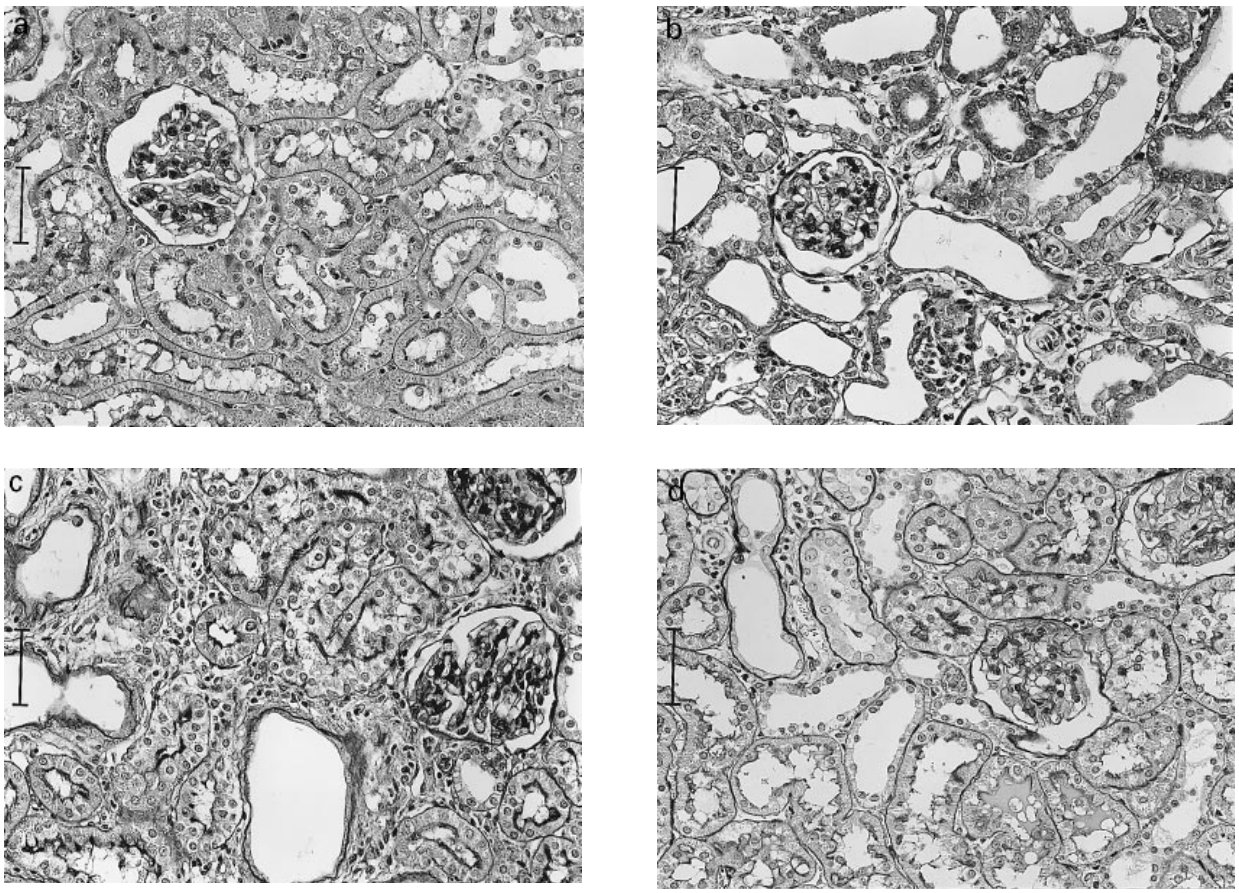

Figure 1. Histological features of gentamicin-treated kidneys. (a) control rat kidney, (b-d) gentamicin-treated kidney. (b) day 7, mononuclear cell infiltration and regenerative change of the proximal tubules. (c) day 14, interstitial inflammatory cell infiltration with fibrosis and regenerative changes. (d) day 28, almost normal renal structure. (PAS stain; Bars $= 50\,\mu$m).

Results

Morphology

In gentamicin induced rats, tubular necrosis appeared on day 3. Subsequently, tubulointerstitial injuries included regenerative changes of proximal tubular epithelium, focal infiltration of mononuclear cells and increased deposition of collagen on day 7 (Figure 1b). The tubulointerstitial changes became most prominent on day 14 (Figure 1c). The renal structures then gradually returned to almost normal architecture at day 28 (Figure 1d). The glomeruli showed no significant pathological alterations during the experimental periods. No significant histological changes were noted in the control kidneys in the whole experimental period (Figure 1a).

Blood chemistry

The level of blood urea nitrogen (BUN) and serum creatinine in gentamicin treated rats and control rats were shown in Figure 2(a,b). The level of BUN and serum creatinine began to increase on day 3, reached a peak level on day 7, then gradually decreased and returns to normal levels on day 28. The renal function was parallel to the histological behaviours.

Expression of HSP47

By immunohistochemistry, HSP47 was weakly detected in the glomerular cells and interstitial cells in control rat kidneys (Figure 3a). In contrast, increased immunostaining of HSP47 was noted in the interstitial cells and in the tubular epithelial cells in the gentamicin-treated rat kidney (Figure 3b, 3c). The expression was stronger and more widely distributed on day 7 (Figure 3b) than that on day 14 (Figure 3c) and its expression was decreased to almost normal level on day 28 (Figure 3d).

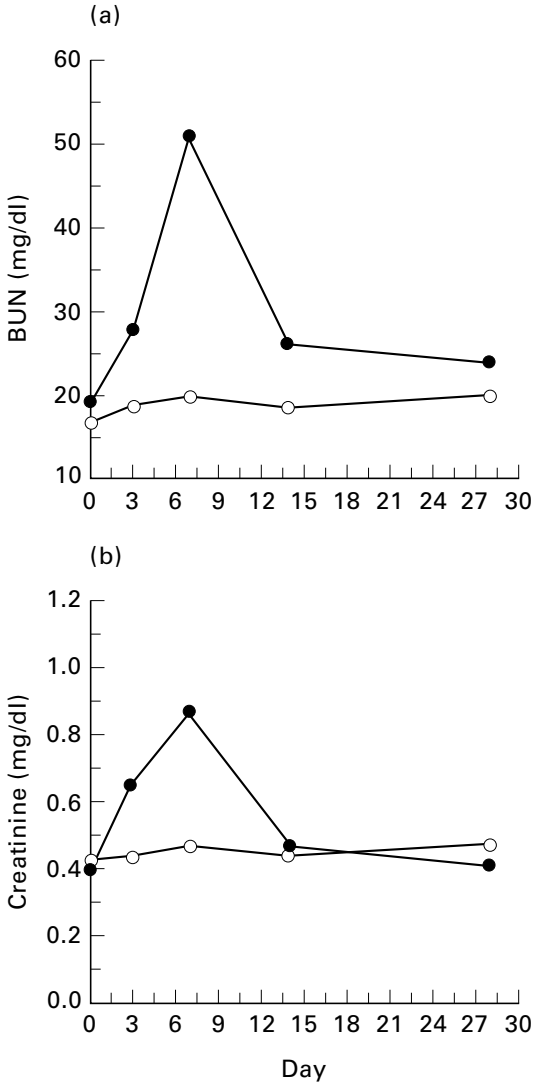

Figure 2. The level of (a) blood urea nitrogen and (b**)** serum creatinine in gentamicin treated rats in time course.
● representative of gentamicin treated rats; ○ representative of control rats.

Expression of α-smooth muscle actin and vimentin

α-smooth muscle actin was present mainly in the vascular wall in the control rat kidneys (Figure 4a). Increased number of interstitial cells expressed actin in gentamicin-treated rat kidneys from day 3 to day 14 (Figure 4b). On the other hand, vimentin was weakly positive in the glomeruli, but negative in the tubular epithelial cells in control rat kidneys (Figure 4c). In the gentamicin-treated rat kidneys, interstitial cells and tubular epithelial cells in and around the fibrous areas showed strong immunostaining for vimentin (Figure 4d). Interestingly, immunostaining for both α-smooth muscle actin and vimentin at day 28 was almost similar to the control kidney (data not shown).

Expression of type III collagen

Type III collagen was weakly stained in the control rat kidneys (FIgure 5a). Increased expression of type III collagen was noted in the fibrotic areas in gentamicin-treated kidneys, with a peak at day 14 (Figure 5b), and returned to normal pattern at day 28 (figure 5c).

Co-localization of HSP47 and type III collagen

In order to clarify whether HSP47 expression is related to collagen expression, dual staining for HSP47 and type III collagen was performed. HSP47 expressing cells and type III collagen (Figure 6a, b) were colocalized especially in the area of interstitial fibrosis in gentamicin-treated rats at day 14.

Co-expression of HSP 47 and α-smooth muscle actin or vimentin

To examine whether phenotypically altered cells, which were positive for α-smooth muscle actin or vimentin, are related to increased expression of HSP47 in gentamicin treated kidneys, double immunostaining of HSP47 and α-smooth muscle actin or vimentin was done. HSP47 positive cells were coexpressed with α-smooth muscle actin-positive cells (Figure 7b) and vimentin-positive cells in gentamicin treated kidneys (figure 7a). These results showed that α-smooth muscle actin-positive myofibroblasts and/or vimentin-positive tubular epithelial cells are responsible for increased expression of HSP47 in the gentamicin treated kidneys.

Expresssion of HSP72/73

Weak immunostaining for HSP72/73 was occasionally noted in the tubular epithelial cells in the control rat kidney. In contrast, increased numbers of tubular epithelial cells showed positive immunostaining for HSP72/73 in the gentamicin treated rat kidney on day 3 (figure 8a), day 7 (figure 8b) and day 14 (figure 8c). The peak expression of HSP72/73 was noted on day 3 and then gradually decreased with time and the expression returned to almost normal pattern on day 28 (data not shown). When HSP72/73 was double stained with HSP47, the disribution of the HSP72/73 expressing cells were found to be different from that of the HSP47 expressing cells, although a few tubular epithelial cells revealed their co-expression on day 14 (figure 8d).

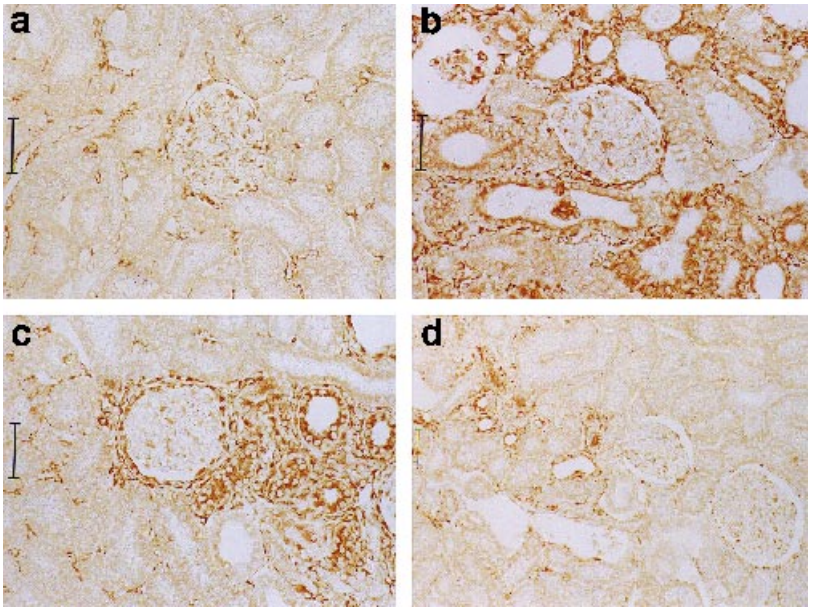

Figure 3. Immunostaining of HSP47. Weak staining for HSP47 in the glomerulus and interstitial cells is noted in the control rat kidney (a). In gentamicin treated rat kidney, markedly increased HSP47 immunostaining is noted in the tubular epithelial cells and interstitial cells on day 7 (b) and on day 14 (c). The staining was markedly decreased on day 28 (d). (Bars $= 50\,\mu$m).

Discussion

Aminoglycosides including gentamicin are still widely prescribed for the treatment of serious infection despite the well-documented acute nephrotoxicity occurring in approximately 10% of all clinical cases (Smoth *et al.* 1980). Although extensive studies have been performed to elucidate the pathophysiololgic mechanisms of gentamicin-induced nephrotoxicity, the exact mechanism has not been well defined. Recent studies suggested that HSPs, HSP73 (Komatsuda *et al.* 1993) and HSP90 (Ohtani *et al.* 1995) were actively related to the pathogenesis of gentamicin-nephrotoxicity. HSP73 was found to express rapidly in gentamicin-nephrotoxicity, and thought to act in a cell protective manner (Komatsuda *et al.* 1993). Similarly, increased HSP90 in gentamicin-nephrotoxicity was suggested to be responsible for disposition of degenerated proteins and in the synthesis of new proteins for the protection and repair of the gentamicin-injured cells (Ohtani *et al.* 1995). HSP47 is

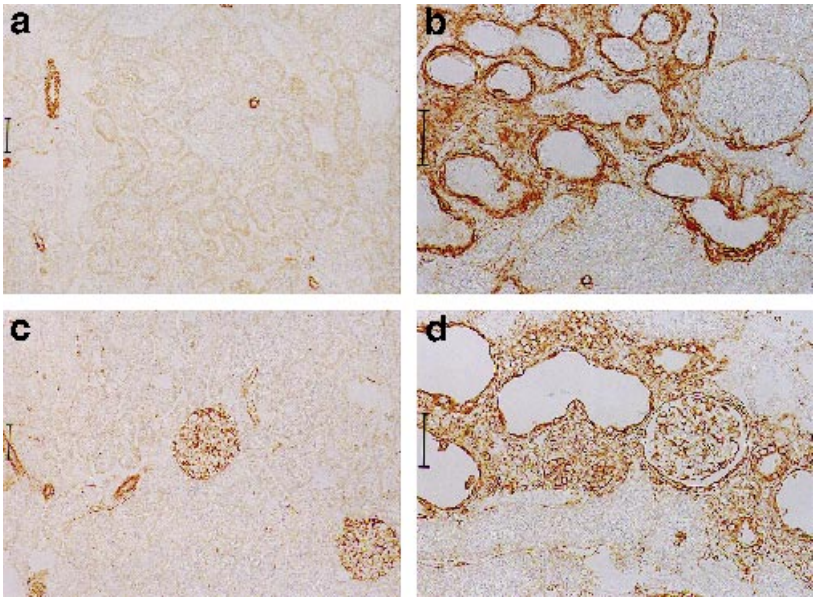

Figure 4. Immunostaining of α-smooth muscle actin and vimentin. Mainly blood vessels showing immnostaining for α-smooth muscle actin in the control kidney (a). Strong staining for α-smooth muscle actin is noted in the interstitium in gentamicin treated rat kidney on day 14 (b). Vimentin is positive in glomerulus, but mostly negative in tubular epithelial cells in control kidney (c). in gentamicin treated rat kidney, strongly positive immunostaining for vimentin is noted in the tubular epithelial cells on day 14 (d). (bars $= 50\,\mu$m).

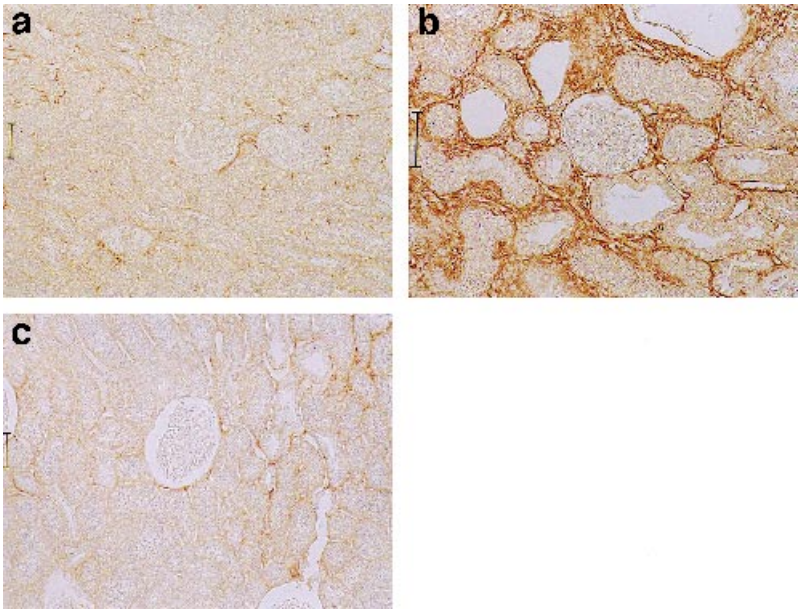

Figure 5. Immunostaining of type III collagen. Focal deposition of type III collagen in the interstitium in control kidney (a). Markedly increased deposition of type III collagen in the fibrotic areas in the gentamicin treated rat kidney on day 14 (b). Almost normal staining pattern on day 28 (c). (bars $= 50\,\mu$m).

a heat-inducible protein similar to other HSPs, also possesses collagen-binding activities and acts as a collagen-specific chaperone in the ER (Miyaishi *et al.* 1992; Shroff *et al.* 1993; Nagata 1996). the definitive role of HSP47 in gentamicin-nephrotoxicity is not yet known, nevertheless the potential exists for a pathological role of this HSP47 in gentamicin-nephrotoxicity.

Our study revealed regeneration of tubular epithelial cells and interstitial fibrosis following tubular necrosis in gentamicin-treated rat kidneys. These pathological changes resolved within 4 weeks and the kidney returned to an almost normal structure. Using immunohistochemical technique, the expression of HSP47 was markedly increased in the 1st week after gentamicin injection, which was followed by increased deposition of collagen in the fibrotic mass. Dual staining clearly showed that increased expression of HSP47 in the gentamicin-treated kidney was associated with increased deposition of type III collagen. Our results are similar to the earlier studies where increased expression of HSP47 was related to increase accumulation of collagens in experimental fibrotic diseases, including CCl_4-induced liver fibrosis (Masuda *et al.* 1994), ATS-induced glomerulosclerosis (Razzaque & Taguchi 1997), cisplatin-induced interstitial fibrosis (Razzaque *et al.* 1996) and Bleomycin-induced pulmonary fibrosis (Razzaque *et al.* 1998).

The major finding of the present study was that HSP47 was strongly expressed during fibrosis but that its expression returned to almost normal levels when fibrotic changes resolved within 4 weeks. Although factors regulating the expression of HSP47 in gentamicin-treated kidney are not clear from this study, the results raise the possibility of possible beneficial effects of therapeutic intervention of HSP47 in progressive fibrotic diseases.

Gentamicin treatment can cause phenotypic alteration of tubulointerstitial cells, such as α-smooth muscle actin positive interstitial cells and vimentin-positive tubular epithelial cells. Phenotypic alteration of renal cells has also been reported in other rat models of renal diseases

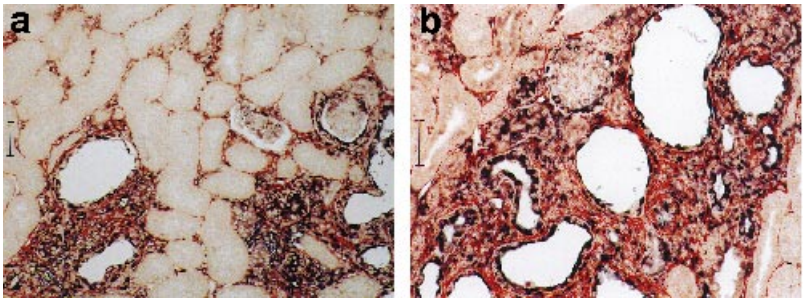

Figure 6. Double staining for HSP47 and type III collagen in gentamicin treated rat kidney. Increased expression of HSP47 (dark purple) is related to increased deposition of type III collagen (intense red) in and around the interstitial fibrosis in gentamicin treated rat kidney (a, b). (bars $= 50\,\mu$m).

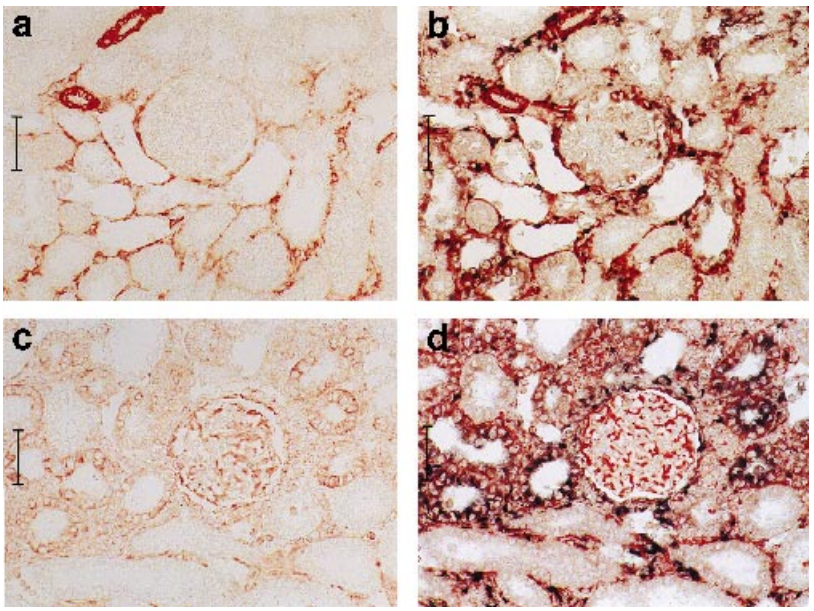

Figure 7. Double staining of HSP47 and α-smooth muscle actin (b) or vimentin (d) in gentamicin treated rat kidney. HSP47 positive cells (dark purple) are coexpressed with α -smooth muscle actin-positive interstitial cells (red) (b) and vimentin-positive tubular epithelial cells (red) (d). The expression pattern of α-smooth muscle actin (a) and vimentin (c) in the serial sections as those of double stained sections are shown in 7a and c respectively. (bars $= 50\,\mu$m).

(Johnson *et al*. 1991; Hamaguchi *et al*. 1995). Double staining procedure revealed that phenotypic alteration of tubulointerstitial cells were responsible for the expression of HSP47 in gentamicin-treated rat kidneys. Earlier reports showed increased expression of HSPs (HSP73 and HSP90) in gentamicin treated kidney, and mentioned that HSPs might act as a cell protective manner (Komatsuda *et al*. 1993; Ohtani *et al*. 1995). Strong expression of HSP47 in vimentin-positive tubular epithelial cells, which are regenerative cells (Nouwen *et al*. 1994; Ichimura *et al*. 1995) was also noted in gentamicin-treated kidneys. In our study, we found a diffuse peak expression of HSP47 in around day 7 after gentamicin treatment which gradually decreased and returned to almost normal levels on around day 28. Previous studies have shown the expression of HSP73 and HSP90, as early as 36 h after gentamicin treatment, gradually increased up to day 12 and then decreased from day 18 and no expression on day 27 (Komatsuda *et al*. 1993; Ohtani *et al*. 1995). Comparing

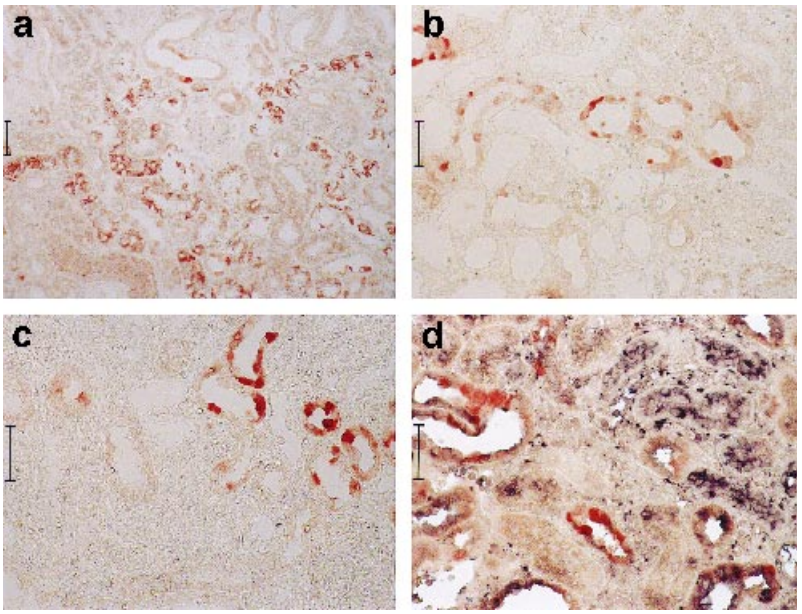

Figure 8. Immunostaining of HSP72/73. Markedly increased HSP72/73 immunostaining is noted mainly in the damaged tubular epithelial cells on day 3 (a) with relatively lower expression on day 7 (b) and on day 14 (c). Double immunostaining of HSP72/73 (red) with HSP47 (dark purple) on day 14 (d) revealed differential localization of these HSPs although a few tubular epithelial cells showed their co-expression (Bars $= 50\,\mu$m).

the expression pattern of HSP47 with other HSPs, it seems that HSP73 and HSP90 appears earlier than HSP47. This suggests that the induction of HSP73 and HSP90 may be a direct effect of gentamicin as a stressor to protect the cells from the damage, while the induction of HSP47 may be more related to fibrotic changes. It is of interest, that dual immunostaining of HSP47 and HSP72/73 was performed and revealed that most of the HSP47 expressing cells and HSP72/73 expressing cells in gentamicin-treated kidney located separately, although some proximal tubular epithelial cells co-expressed both HSP47 and HSP72/73.

In summary, HSP47 was expressed mainly by the phenotypically altered tubulointerstitial cells in gentamicin-treated kidney, and abnormal expression of HSP47 might contribute significantly to gentamicin nephrotoxicity.

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