

## SHORT COMMUNICATION

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**Localization of HSP47 in cisplatin-treated rat kidney: a possible role in tubulointerstitial damage**

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**Abstract**

**Background.** A 47-kDa heat-shock protein (HSP47) is a major collagen-binding stress protein and is assumed to play an important role in the fibrotic process, but its role in cisplatin-induced tubulointerstitial fibrosis is not yet clear. To explore the possible role(s) of collagen-binding stress protein HSP47 in cisplatin-induced tubulointerstitial fibrosis, the expression of HSP47 was examined in cisplatin-treated rat kidneys.

**Methods.** Eighteen male Wistar rats were divided into two groups; group I were age-matched controls and group II, animals were injected intraperitoneally with a single dose of cisplatin (6 mg/kg body weight). One hour after cisplatin injection, three rats from group II were killed along with control rats. The remaining rats in both groups were killed on the 7th, 14th, and 28th days of the experiment and the kidneys were collected for morphological and immunohistochemical study. The expression of collagen-binding HSP47 with various proteins implicated in phenotypic modulation ( $\alpha$ -smooth muscle actin and vimentin) and fibrosis (type I and type III collagens) was examined in control and cisplatin-treated kidneys.

**Results.** Cisplatin induced marked tubulointerstitial damage, including interstitial fibrosis, which was characterized by increased deposition of type I and type III collagens. Increased expression of HSP47 was also noted in and around the expanded interstitium in cisplatin-treated rats; by double-staining, HSP47-expressing cells were found to be  $\alpha$ -smooth muscle actin-positive myofibroblasts and vimentin-positive tubular epithelial cells. In addition,

colocalization of HSP47 and collagens was seen in and around the interstitial fibrosis in cisplatin-treated kidneys.

**Conclusion.** From the results, we concluded that overexpression of HSP47 by phenotypically altered renal cells may play an important role in the excessive assembly of collagens and could thereby contribute significantly to the development of the tubulointerstitial fibrosis found in cisplatin-treated rat kidneys.

**Key words** HSP47 · Collagen · Cisplatin · Interstitial fibrosis

**Introduction**

Cisplatin is widely used in the treatment of various tumors, but its use is restricted due to its nephrotoxicity. Cisplatin-induced tubulointerstitial nephritis, one of the major chronic problems encountered in clinical practice, is characterized by early inflammatory changes with damage to the tubular epithelial cells (apoptosis and necrosis), followed by tubulointerstitial fibrosis; all these structural alterations leading to gradual deterioration of renal function.<sup>1,2</sup> Despite extensive studies, the exact mechanism of cisplatin-induced tubulointerstitial damage in the kidney is not yet clear.

HSP47 is a collagen-binding stress protein, localized exclusively in the endoplasmic reticulum<sup>3</sup> and suggested to play a role in the intracellular processing, assembly, and/or secretion of procollagen molecules as a collagen-specific molecular chaperone.<sup>4,5</sup> In-vitro studies have shown that phosphorothioate antisense oligodeoxynucleotides to HSP47 inhibited HSP47 production and consequently diminished the production of type I procollagen  $\alpha$  (1) chains.<sup>6</sup> Recent in-vivo experimental studies disclosed an association between HSP47 and the fibrotic process in liver, lung, and kidney.<sup>7–10</sup> Because HSP47 is closely related to the sclerotic/fibrotic process in various experimental renal diseases,<sup>7,8,10</sup> it is tempting to speculate that the newly identified

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stress protein-HSP47 may also play a role in cisplatin-induced tubulointerstitial fibrosis, although this remains to be proven. This study was undertaken to investigate the expression of type I and type III collagens with their molecular chaperone HSP47 in cisplatin-treated rat kidneys. In addition, the cells responsible for the expression of HSP47 were also studied, using a double-staining technique.

## Materials and methods

### Animals

Male Wistar rats ( $n = 12$ ) were treated intraperitoneally with cisplatin (6 mg/kg body weight). Age-matched controls ( $n = 6$ ) received an equal volume of physiologic saline instead.

### Tissue collection

Rats were killed by exsanguination under ether anesthesia at 1 h and on days 7, 14, and 28 after cisplatin injection. The kidneys were removed, 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.

### Histological studies

Tissues were routinely processed to be embedded in paraffin and stained with hematoxylin-eosin (H&E), periodic acid Schiff (PAS), periodic 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.<sup>7,8,10-12</sup> Briefly, paraffin sections were deparaffinized with xylene, then pretreated with hydrogen peroxide to abolish the 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 with antibodies against  $\alpha$ -smooth muscle actin (Dako, Glostrup, Denmark), vimentin (Dako), ED-1 (Serotec, Oxford, UK), a marker for monocyte/macrophage, type I collagen (Chemicon, Temecula, CA, USA), type III collagen (Chemicon), and HSP47 (StressGen Biotechnologies Victoria Canada). After being washed with phosphate-buffered saline (PBS), the sections were processed further, using the Histofine SAB-PO kit (NICHIREI, Tokyo, Japan), as directed by the manufacturer; the sections were then developed with 0.01% hydrogen peroxide/3,3'-diaminobenzidine. The staining intensities of  $\alpha$ -smooth muscle actin, vimentin, type I collagen, type III collagen and HSP47 were graded semiquantitatively according to the following scales; 0, no

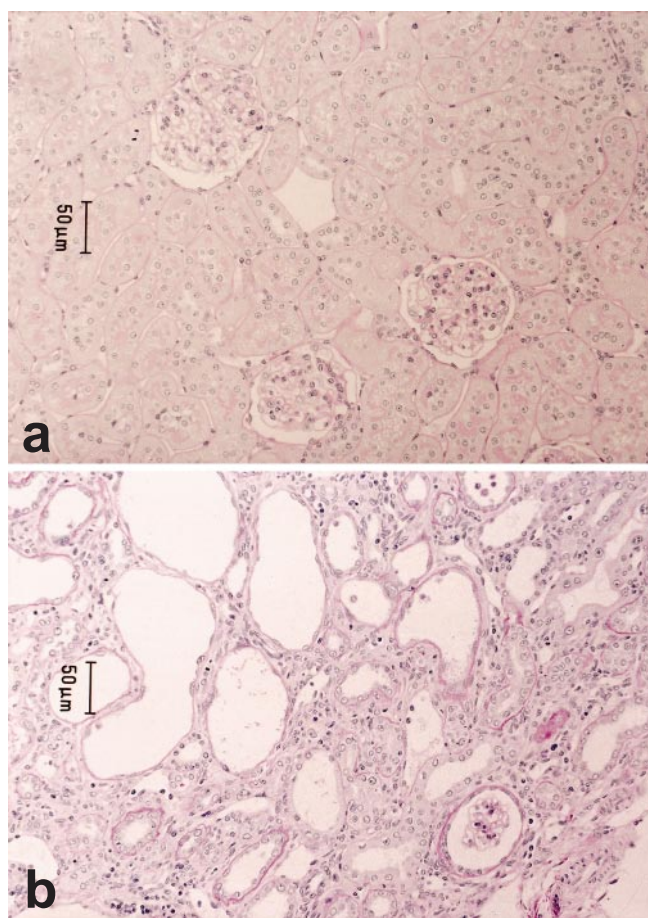
staining; +, weak staining; ++, moderate staining; +++, strong staining.

### Double immunostaining

Double immunostaining was performed to localize the expression of HSP47 and  $\alpha$ -smooth muscle actin, vimentin, and type I or type III collagen, as described previously.<sup>13,14</sup> Briefly, HSP47 was initially stained by the alkaline phosphatase method and developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitroblue tetrazolium (NBT), which produced dark-purple staining, then the sections were counterstained with  $\alpha$ -smooth muscle actin, vimentin, ED-1, type I collagen, or type III collagen by the peroxidase method, and visualized with hydrogen peroxide/aminoethyl carbazole (AEC), producing an intense red stain.

### Control experiment

The following control experiments were performed to verify the specificity of the immunostaining. (1) In some sections, primary antibodies were replaced with either



**Fig. 1a,b.** Histological features of kidney from cisplatin-treated rat killed 4 weeks after cisplatin injection (**b**) showing inflammatory cell infiltration, detachment of tubular epithelial cells, occasional dilatation of tubules and interstitial fibrosis. Kidney from age-matched control rat (**a**) disclosing no significant histological changes

0.01 M PBS or mouse IgG/rabbit IgG diluted with PBS (similar concentration to that of the primary antibody). (2) In some adjacent sections primary antibody was replaced with a solution containing a ten fold excess of recombinant HSP47 (StressGen Biotechnologies) in addition to anti-HSP47 antibody.

## Results

### Morphological analysis

Macroscopically, the cisplatin-treated kidney showed a granular surface, while the control kidney surface was smooth. Histological examination of slides stained with H&E, PAS, PAM, and Masson's trichome revealed no significant abnormalities in kidneys obtained from age-matched control rat kidneys (Fig. 1a), but marked tubulointerstitial damage, including detachment of tubular epithelial cells, tubular atrophy, inflammatory cell infiltration, and interstitial fibrosis, was always noted in all

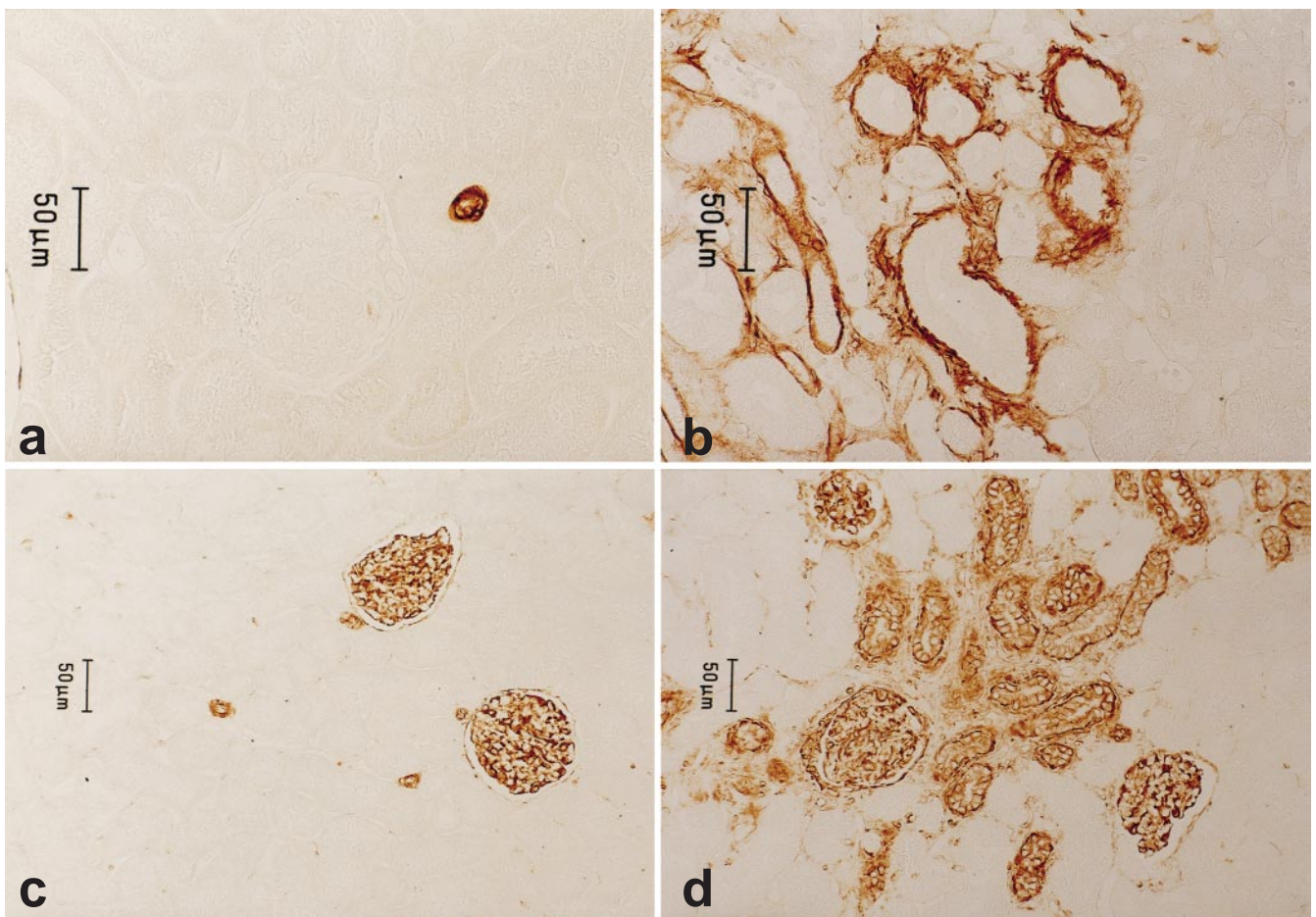
cisplatin-treated rat kidneys (Fig. 1b); however, interstitial fibrotic changes gradually increased with the passing of time.

### Immunohistochemical localization of $\alpha$ -smooth muscle actin and vimentin

$\alpha$ -Smooth muscle actin (Fig. 2a) and vimentin (Fig. 2c) were mostly undetectable in the tubulointerstitium of control kidneys. In contrast to the control, increased numbers of interstitial cells and tubular epithelial cells in cisplatin-treated kidneys showed strong immunostaining (+++) for  $\alpha$ -smooth muscle actin (Fig. 2b) and vimentin (Fig. 2d), respectively.

### Immunohistochemical localization of type I and type III collagens

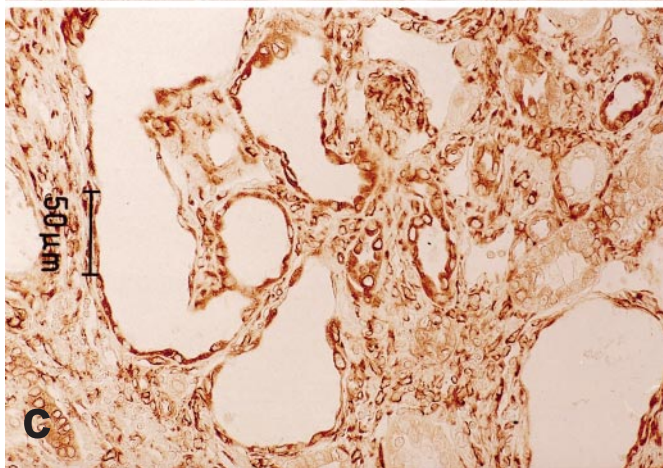
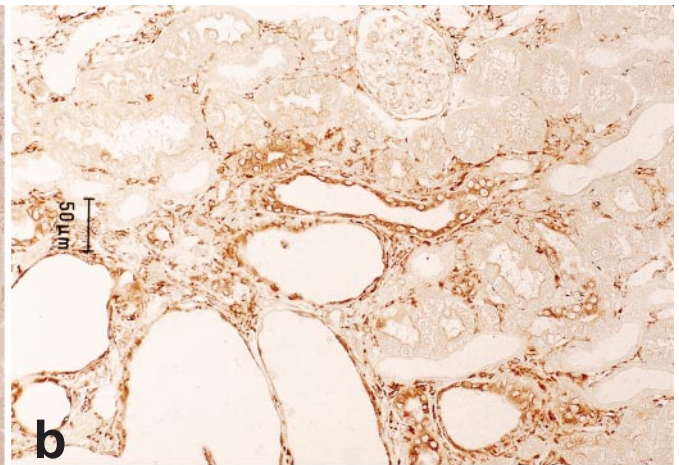
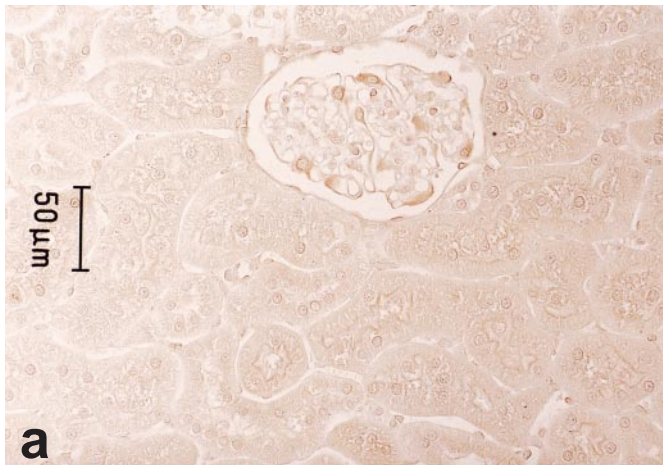
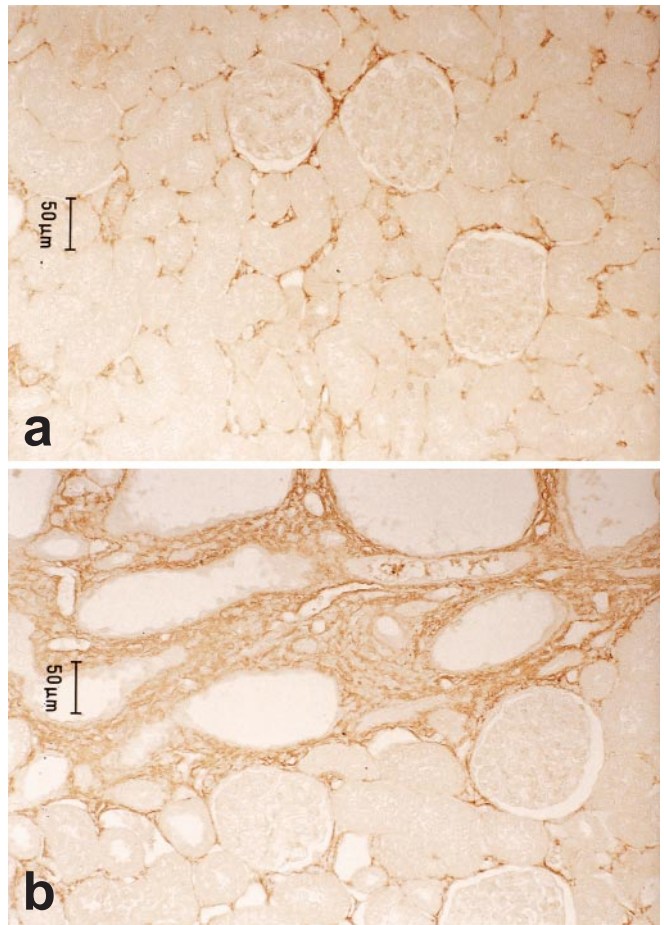
Mainly weak (+) interstitial immunostaining for type I (data not shown) and type III (Fig. 3a) collagens was noted



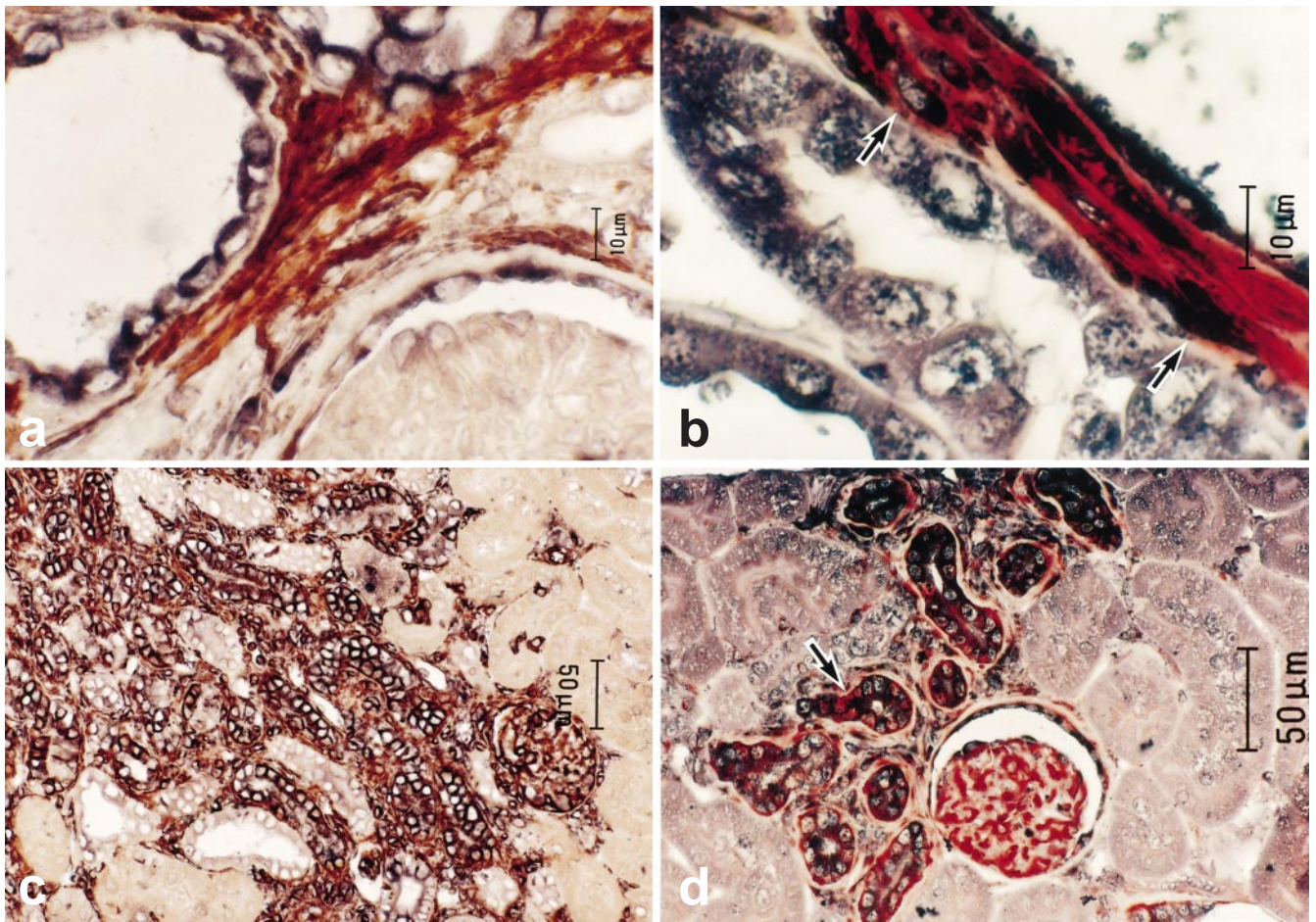
**Fig. 2a–d.** Distribution of  $\alpha$ -smooth muscle actin immunoreactivity in **a** control kidney, showing positive staining in the small vessels, while increased immunostaining is seen in the tubulointerstitium of **b** kidney from cisplatin-treated rat killed 4 weeks after cisplatin injection. **c** Immunolocalization of vimentin in control kidney showing

intraglomerular staining, but lack of staining in tubular epithelial cells. **d** In the kidney of the cisplatin-treated rat killed 4 weeks after cisplatin injection, strong immunostaining for vimentin is noted mostly in the tubular epithelial cells and in the interstitium

**Fig. 3a,b.** Immunostaining of type III collagen **a** is weak, noted mainly in the interstitium, in the control kidney. **b** In contrast to the control kidney, the immunostaining of type III collagen is strong in cisplatin-treated kidney with predominant deposition in the widened interstitium



**Fig. 4a–c.** Immunohistochemistry for HSP47 in a control kidney and **b,c** kidney of cisplatin-treated rat killed 4 weeks after cisplatin injection. Weak immunostaining for HSP47 is seen in the intraglomerular cells and interstitium in a the control kidney (**a**), while markedly increased HSP47 immunostaining is present in tubular epithelial cells and interstitial cells in and around the interstitial fibrosis (**b,c**)



**Fig. 5a–d.** Double staining on a paraffin section of kidney of cisplatin-treated rat killed 4 weeks after cisplatin injection; **a,b** with antibodies against HSP47/ $\alpha$  smooth muscle actin and **c,d** with antibodies against HSP47/vimentin. HSP47 is stained dark purple and  $\alpha$ -smooth muscle

actin and vimentin are stained intense red. Note HSP47-expressing cells are co-expressed with  $\alpha$ -smooth muscle actin-immunopositive peritubular cells (arrows) (**a,b**) and vimentin-immunopositive tubular epithelial cells (arrows) (**c,d**)

in the control kidney. Compared to the control (Fig. 3a), immunoreactive type I collagen (data not shown) and type III collagen (Fig. 3b) was increased in cisplatin-treated kidneys, and predominantly deposited in the widened interstitium.

#### Immunohistochemical localization of HSP47

In the age-matched control rat kidneys, immunoreactive HSP47 expression was weakly (+) detected in the intraglomerular cells and interstitial cells (Fig. 4a). In the kidneys of cisplatin-treated rats, HSP47 immunostaining was markedly increased and strongly (+++) present in and around the interstitial fibrosis (Fig. 4b,c).

#### Double staining of HSP47 and $\alpha$ -smooth muscle actin and vimentin

To determine the cells expressing HSP47 in the cisplatin-treated kidneys, double immunostaining for HSP47, with  $\alpha$ -smooth muscle actin and vimentin, was performed. Co-

expression of HSP47/ $\alpha$ -smooth muscle actin (Fig. 5a,b), and HSP47/vimentin (Fig. 5c,d) was noted in the cisplatin-treated kidneys, suggesting that HSP47-expressing cells are mostly phenotypically altered tubular epithelial cells and interstitial myofibroblasts. To clarify the relationship between the HSP47-expressing cells and infiltrating macrophages in the cisplatin-treated kidney, double immunostaining for HSP47 and ED-1 was performed in the same renal section and most of the ED-1-positive macrophages were negative for HSP47 staining (data not shown), suggesting that HSP47-expressing cells are mainly intrinsic renal cells rather than infiltrating inflammatory cells.

#### Double staining of HSP47 and collagens

To determine the relationship between the expression of HSP47 and collagens, double immunostaining for HSP47 with type I or type III collagens was performed. By double staining, co-expression of HSP47 and collagens was seen in the cisplatin-treated kidneys. Co-expression of HSP47 and type I collagen (data not shown) or type III collagen (Fig.

**Fig. 6a,b.** Double staining on paraffin section of kidney from cisplatin-treated rat killed 4 weeks after cisplatin injection; with antibodies against **a,b** HSP47/type III collagen. HSP47 is stained dark purple and collagen is stained intense red. Increased expression of HSP47 is associated with increased deposition of collagen in the region of interstitial fibrosis

6a,b) was mainly seen in and around the interstitial fibrosis in the cisplatin-treated kidneys.

## Discussion

It is well known that the pathological changes after cisplatin treatment include early inflammatory changes followed by tubulointerstitial fibrosis. Although numerous studies have been done to elucidate the early events after cisplatin treatment, and have convincingly showed that necrosis and apoptosis in the proximal tubular epithelial cells are mainly responsible for the nephrotoxicity, less attention has been given to the subsequent events of interstitial fibrosis. The mechanisms of tubulointerstitial damage in cisplatin-treated animals are not yet clear, but it has been shown that the extent of tubulointerstitial damage is partly related to the dose of cisplatin; with a very high dose of cisplatin (20mg/kg body weight), Marcussen<sup>15</sup> showed that 35% of glomeruli were atubular after 10 or more weeks, and found

a correlation with the severity of tubulointerstitial fibrosis. In contrast, we used a relatively low dose of cisplatin in our study (6mg/kg body weight) and observed fibrotic changes even 1 and 2 weeks after cisplatin treatment, along with necrotic/apoptotic and regenerative changes in different parts of the same kidney. Thus, we consider that cisplatin-induced tubulointerstitial fibrosis is not necessarily the secondary event following the initial injury, but rather, that it is a relatively late complication of cisplatin treatment.

HSP47 is one of the heat shock proteins that exert important biological effects on collagen synthesis, and thus could be actively involved in the fibrotic changes that are found in cisplatin-treated rat kidneys. By immunohistochemistry, we were able to demonstrate the precise localization of HSP47 in control and cisplatin-treated rat kidneys; increased expression of HSP47 was noted in the tubular epithelial cells and interstitial cells (mostly myofibroblasts) in and around the fibrotic areas. However, no significant difference in HSP47 expression was found in control rats and cisplatin-treated rats killed 1 h after cisplatin injection, indicating that the elevated level of HSP47 was not due to the direct effect of cisplatin as a stressor, but could be related to the progression of interstitial fibrosis. From the results, it is reasonable to speculate that HSP47 is more involved in the late complication of cisplatin-induced interstitial fibrosis rather than in the early events of necrosis and apoptosis of the proximal tubular epithelial cells.

Our results are in accord with earlier experimental fibrotic models, in which HSP47 expression was markedly induced in carbon tetrachloride-induced liver fibrosis,<sup>9</sup> antithymocyte serum-induced glomerulosclerosis,<sup>7</sup> bleomycin-induced pulmonary fibrosis,<sup>8</sup> and gentamicin-induced interstitial fibrosis.<sup>10</sup> Consistent with these earlier reports, we now showed that the expression of HSP47 is substantially increased in the cisplatin-treated kidney, in association with interstitial fibrosis. Double immunostaining has clearly shown that phenotypically altered interstitial cells and tubular epithelial cells were the main source of HSP47 and that increased expression of HSP47 in cisplatin-treated kidney co-localized with collagens in the expanded interstitium.

Recent studies have suggested that tubular epithelial cells are capable of tubular epithelial-myofibroblast transdifferentiation<sup>16</sup> and these pro-fibrogenitor cells are able to produce interstitial collagens.<sup>17</sup> In addition, both *in vivo* and *in vitro* studies have convincingly shown that renal tubular epithelial cells have the ability to synthesize various types of collagen, including types I, III, and IV.<sup>18-23</sup>

From the collagen-synthesizing ability of HSP47,<sup>3-6</sup> it is assumed that increased expression of HSP47 by phenotypically altered cells in the cisplatin-treated kidney may play a significant role in the subsequent manifestation of interstitial fibrosis, possibly by regulating the increased assembly/synthesis of collagens.

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