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Elevated concentrations of the small stress protein HSP27 in rat renal tumors

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Abstract The expression of two small stress proteins, αB crystallin and the 27-kDa heat shock protein (HSP27), was studied quantitatively and immunohistochemically in normal kidney and renal tumors in rats. Levels of αB crystallin in renal cell tumors tended to be higher than in normal kidney ($P = 0.07$), but with a wide range of values, whereas they were significantly lower in mesenchymal tumors ($P < 0.0001$). In contrast, HSP27 concentrations in both renal cell (mean ± SD: 1790 ± 940 ng/mg protein, $n = 15$) and mesenchymal (1260 ± 1080 ng/mg protein, $n = 10$) tumors were significantly higher than the normal kidney value (142 ± 30 ng/mg protein, $n = 10$, $P < 0.0001$). A positive correlation was found between αB crystallin and HSP27 levels limited to the renal cell tumor case (Pearson's correlation coefficient, $r = 0.68$, $P < 0.01$). Immunohistochemistry revealed the loops of Henle to be positive for αB crystallin, whereas HSP27 staining was positive in glomerular and interstitial vascular walls and epithelial cells of proximal and distal tubules. Positive immunostaining for αB crystallin was demonstrated in six of nine renal cell tumors (67%) studied and for HSP27 in all of the nine cases (100%).

Key words αB crystallin · Heat shock protein · Rat · Kidney neoplasms

Introduction

Heat shock proteins (HSPs), a group of molecules induced in mammalian cells by heat shock and other stresses, are involved in the acquisition of thermotolerance and in protein folding and degradation [2, 9, 25]. Although low-molecular-weight (molecular masses of 20–30 kDa) and high-molecular-weight (60, 70, 90 or 100 kDa) HSPs have been identified, it remains unclear whether they play different biological roles in cells.

αB Crystallin, a major constituent of vertebrate lens, is a 23-kDa protein which is generally expressed as a polymeric form with a molecular mass of 500–800 kDa [5, 21]. Recent studies have revealed that it is present in various tissues other than the lens, including skeletal muscle, brain, and kidney [8, 18, 20]. The amino acid sequence of αB crystallin has common elements with those of a number of small HSPs [11, 16, 24] and it is in fact produced in response to heat shock [14, 23, 24]. Thus, the available data suggest that αB crystallin is a member of the small HSP family.

Another small HSP with a 27-kDa molecular mass (HSP27) is also detected in various normal tissues, including the kidney, in rats [21]. Several lines of evidence suggest that αB crystallin and HSP27 may be associated in cells [21, 22, 41]. However, while it is known that small HSPs, including αB crystallin, effectively prevent heat-induced aggregation of other proteins [12, 19, 28], their precise biological significance, alone or in combination, remains unclear.

To our knowledge there have been no studies of αB crystallin and HSP27 in chemical-induced renal tumors in rats. To clarify the biological significance of these HSPs for rat renal carcinogenesis, a quantitative determination of their concentrations in normal kidney and renal tumor tissues was therefore performed in addition to immunohistochemical localization.

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Materials and methods

Animals and treatment

N-Ethyl-*N*-hydroxyethylnitrosamine (EHEN, Sakai Laboratories, Fukui, Japan) was used for induction of kidney tumors as previ-

ously described [36]. In brief, a total of 20 male Wistar rats (Charles River Japan, Atsugi, Japan), aged 6 weeks at the beginning of the experiment, were maintained in a room at 25 ± 2°C, with a relative humidity of 55 ± 5% and a 12-h light-dark cycle. The animals were given basal diet (Oriental M, Oriental Yeast, Tokyo) and tap water ad libitum. They initially received 0.05% EHEN dissolved in their drinking water for 3 weeks, returned to normal tap water for the subsequent 49 weeks and were killed at the end of week 52. Histological assessment demonstrated renal cell tumors in 19 of the 20 animals (95%). Of these, 15 tumors could be used for the present study.

N-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN, Nakarai Chemicals, Kyoto, Japan) and uracil were used for the induction of mesenchymal tumors of the kidney as described by Nakano [29] and Takashi et al. [37]. A total of 27 female Sprague-Dawley rats (Charles River Japan, Atsugi, Japan), aged 5 weeks at the beginning of the experiment, were initially given 0.05% BBN dissolved in drinking water, together with a 3% uracil supplement in the diet, for 8 weeks, and then returned to a normal water and basal diet. At the 52-week time point mesenchymal tumors were found in 10 of 27 rats (37%). All of the ten tumors were used for the present study. Although transitional cell and squamous cell carcinomas were also found, their small sizes prevented their use for quantitative evaluation.

The kidneys were immediately excised for quantitative assays of tissue fractions and immunohistochemical examination. A group of control Wistar male rats ($n = 10$) not receiving any chemical treatment were also included.

Tissue samples

Tumor and control tissues were immediately resected at sacrifice and for immunoassays were promptly frozen and kept at −80°C until the analyses, when they were homogenized at 0°C with 10 volumes (V/W) of 50 mM TRIS-HCl (pH 7.4) containing 5 mM $MgSO_4$. Homogenates were centrifuged at 4°C at 20000 *g* for 20 min, and the soluble fractions were used for the analysis. For histological examination and immunohistochemistry, tissues from five cases of normal kidney and nine cases of renal cell tumors were fixed in periodate-lysine-4% paraformaldehyde for 6 h, washed in phosphate-buffered saline (PBS, pH 7.2) containing increasing concentrations of sucrose, and embedded in OCT compound (Tissue-Tek, Naperville, Illinois, USA).

Antibodies

Antibodies to αB crystallin and HSP27 were raised in New Zealand rabbits by injecting the respective antigens, purified from bovine lenses and rat skeletal muscles, respectively, with Freund's complete adjuvant, as described elsewhere [15, 20]. Antibodies monospecific for the two antigens were purified by immunoaffinity column chromatography using antigen-coupled Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The specificities of the purified antibodies to αB crystallin and HSP27 thus obtained were confirmed previously [15, 20].

As secondary antibodies for immunohistochemistry, horseradish peroxidase (HRP)-labeled rabbit IgG Fab' fragments against rabbit IgG were prepared [34].

Immunoassay methods

Concentrations of αB crystallin and HSP27 in the soluble fractions of tissues were determined by the sandwich-type enzyme immunoassay system developed by Kato et al. [20] and Inaguma et al. [15]. In brief, extracts were incubated with polystyrene balls bearing immobilized monospecific rabbit antibodies to the respective antigens, and then the balls were incubated with the same antibodies labeled with β-D-galactosidase from *Escherichia coli*. The bound galactosidase activity was assayed with 4-methylumbelliferyl-β-D-galactoside as a substrate. Purified rat αB crystallin and HSP27 were used as standards and the results were expressed as antigens equivalent to nanograms per milligram soluble protein. The assay systems were all highly sensitive, the limit of detection for each antigen being 10 pg/test tube.

Immunohistochemistry

The indirect HRP-labeled antibody method was employed for the immunostaining as described previously [34, 35]. In brief, 5-μm-thick cryostat sections were placed on albumin-coated slides and dried at room temperature. They were treated with 100% methanol and 0.3% hydrogen peroxide solution for 30 min to inactivate endogenous peroxidase, washed in PBS, and then incubated with purified anti-αB crystallin IgG or anti-HSP27 IgG (4 μg/ml) for 12 h at 4°C. For control sections, antibodies absorbed with the purified respective antigen were substituted for the primary antibodies. After being washed in PBS, all sections were incubated with HRP-labeled secondary antibodies for 60 min at room temperature. After further washing in PBS, they were reacted with 0.025% 3,3'-diaminobenzidine solution containing 10 mM hydrogen peroxide, and counterstained with methyl green.

Other methods

Protein concentrations of the tissue extracts were determined with the aid of a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, California, USA), utilizing the principle of protein-dye binding [4]. Quantitative data were expressed as mean ± standard deviation (SD) values and the results compared using the Wilcoxon's rank-sum test.

Results

Concentrations of αB crystallin and HSP27 in normal rat kidney tissues and the two types of renal tumors

Table 1 summarizes data for concentrations of αB crystallin and HSP27 in normal kidneys, renal cell tumors, and mesenchymal tumors. Values for αB crystallin in renal cell tumors tended to be higher than in the

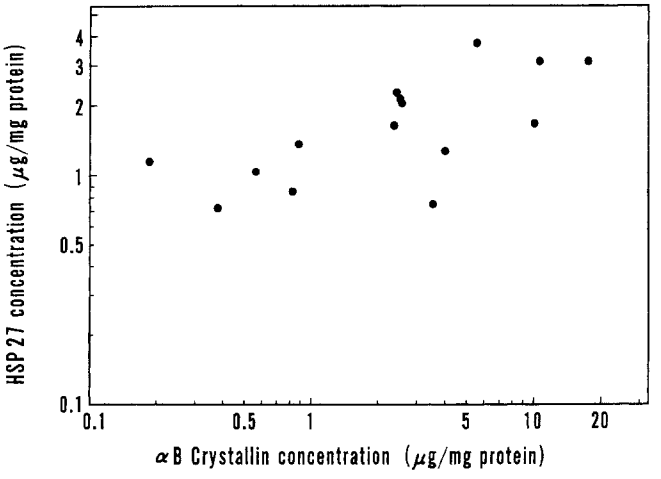

Fig. 1 Relation of αB crystallin to HSP27 levels in renal cell tumors (Pearson's correlation coefficient, $r = 0.68$, $n = 15$, $P = 0.0056$)

Table 1 Concentrations of αB crystallin and HSP27 in normal kidney tissues and two types of renal tumors in rats. Data are mean ± SD values (range limit).

* Significantly different from the normal kidney value ($P < 0.0001$)

Tissues	No. of samples	Tissue concentration (ng/mg protein)			
		αB Crystallin		HSP27	
Normal kidney	10	969 ± 251	(581–1380)	142 ± 30	(104–200)
Renal cell tumor	15	4240 ± 4890	(186–17600)	1790 ± 940*	(718–3720)
Mesenchymal tumor	10	73.7 ± 69.4*	(0.9–219)	1260 ± 1080*	(393–4190)

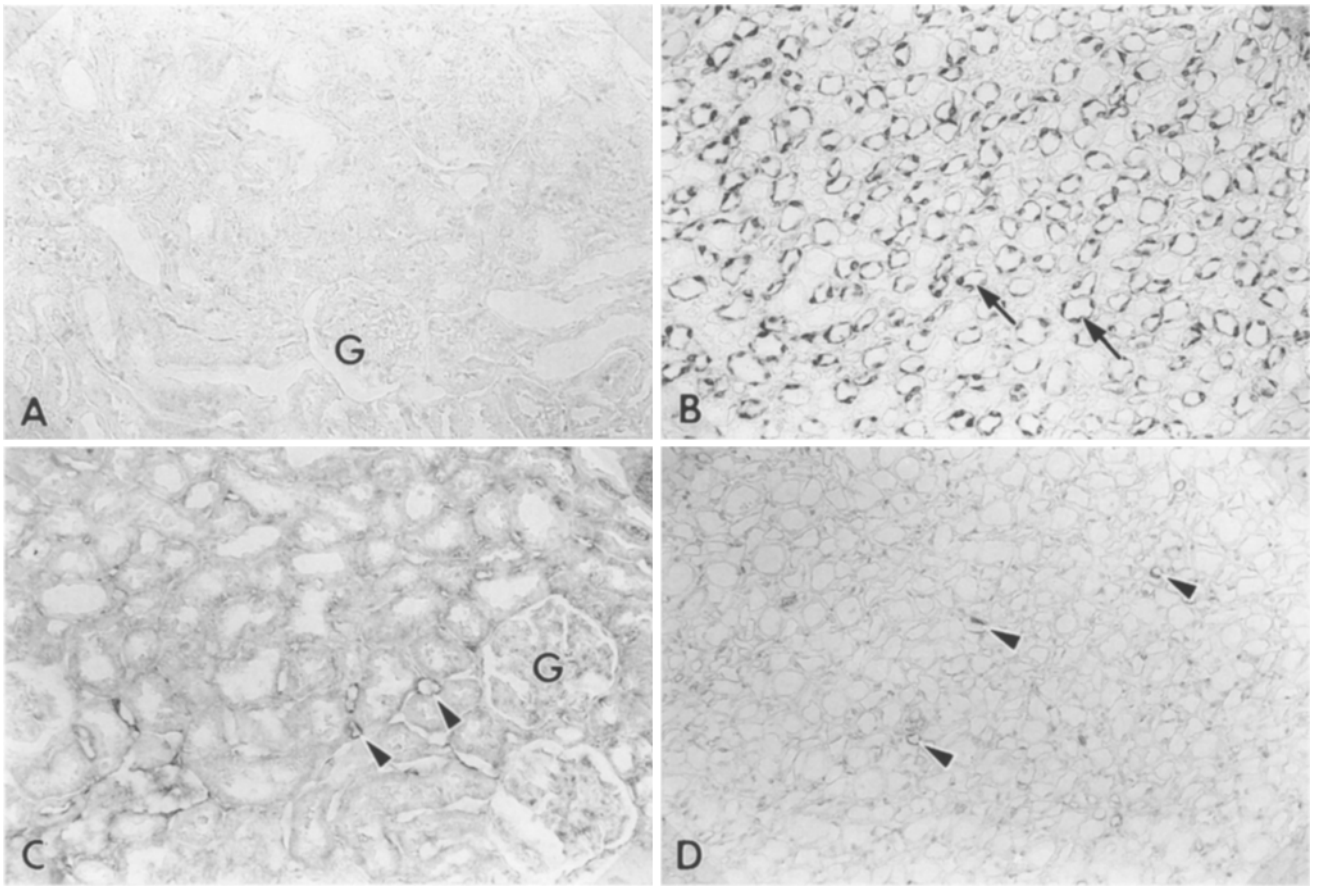

Fig. 2A–D Immunohistochemical localization of αB crystallin (**A, B**) and HSP27 (**C, D**) in the renal cortex and medulla. αB crystallin is negative in all of the nephron segments in the cortex (**A**) and positive in epithelial cells of the loops of Henle (*arrows*) in the medulla (**B**). In contrast, epithelial cells of proximal and distal tubules and glomerular and interstitial vascular walls (*arrowheads*) in the cortex (**C**) and vascular walls (*arrowheads*) in the medulla (**D**) demonstrate varying intensities of positive staining for HSP27. *G*, glomerulus. Indirect immunoperoxidase method, ×130

normal kidney, and in some cases massively, but without statistical significance ($P = 0.07$), whereas those in mesenchymal tumors were significantly depressed ($P < 0.0001$). In the HSP27 case, both renal cell and mesenchymal tumors demonstrated significantly higher values than found for the normal kidney samples ($P < 0.0001$).

Figure 1 illustrates the positive correlation between αB crystallin and HSP27 levels in renal cell tumors (Pearson's correlation coefficient, $r = 0.68$, $n = 15$, $P = 0.0056$). No such correlation was found between the two HSPs for the normal kidney or mesenchymal cases.

Immunohistochemical localization of αB crystallin and HSP27 in normal rat kidney and renal cell tumors

Figure 2 illustrates immunohistochemical localization of αB crystallin and HSP27 in normal rat kidney tissue. The former was found to be lacking in the proximal and distal tubules in the cortex (Fig. 2A) but present in the epithelial cells of loops of Henle (Fig. 2B). HSP27 staining was positive in the glomerular and interstitial vascular walls in the cortex and medulla (Fig. 2C,D), and also in the epithelial cells of the proximal and distal tubules, with considerable cell-to-cell variation in staining intensity (Fig. 2D).

Of nine cases of renal cell tumor tissues studied, six (67%) stained positively for αB crystallin and all cases

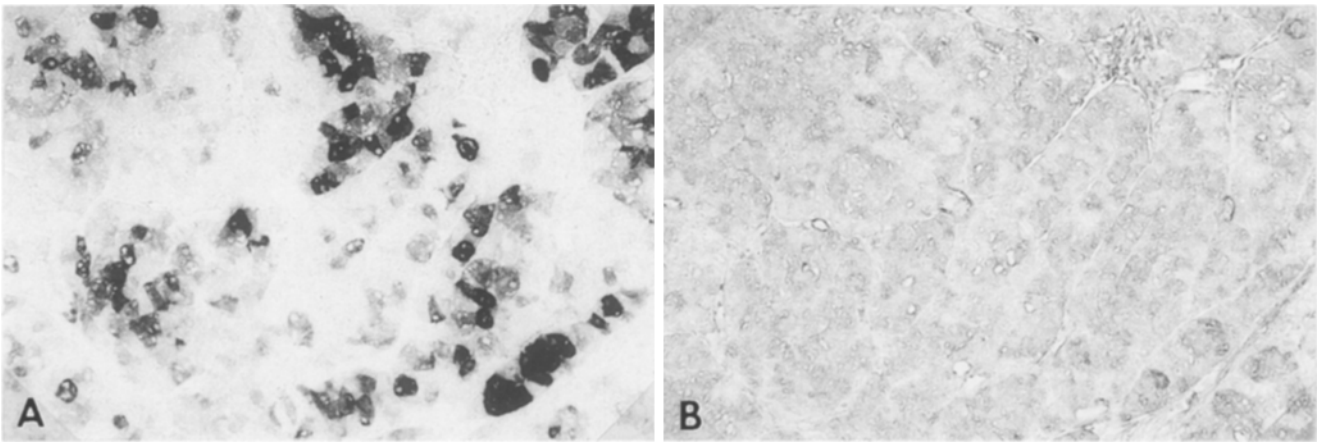

Fig. 3A,B Immunohistochemical localization of αB crystallin (**A**) and HSP27 (**B**) in a renal cell tumor in a rat. αB crystallin is positive in the cytoplasm of some of the tumor cells. In contrast, HSP27 is immunostained diffusely in the cytoplasm of all the tumor cells. Indirect immunoperoxidase method, ×130

(100%) for HSP27. Figure 3 illustrates the immunohistochemical localization of αB crystallin and HSP27 in a typical renal cell tumor. Both HSPs were found to be localized primarily in the cytoplasm but also occasionally in the nuclei of the tumor cells. The intensity of staining varied from case to case, and from site to site within individual tumors. Control sections of tissues treated with antibodies pre-absorbed with the respective antigens were uniformly negative.

Discussion

The present study of αB crystallin and HSP27 concentrations demonstrated clear differences from the normal kidney in both renal cell and mesenchymal tumors, with apparent separation of regulation of expression between the two types of neoplasm. Our results for immunohistochemical localization of αB crystallin in the normal kidney confirmed the findings of Iwaki et al. [18]. Regarding localization of HSP27 in the normal rat kidney, immunofluorescence staining recently showed the glomerular capillary loops to be positive [32]. Here we found HSP27 in the interstitial vascular walls and epithelial cells of proximal and distal tubules of the normal kidney, in clear contrast to the αB crystallin case. Expression of αB crystallin limited to only the loops of Henle in the medulla might be associated with the hypertonic environment, which is a stress factor for cells in the medulla [6, 10]. At present it remains unknown what are the exact biological functions of HSP27 and αB crystallin in cells. Clearly their regulation is cell specific, which would imply differences in action.

Only a few studies have been conducted to ascertain the relevance of small HSP to carcinogenesis. This is the first demonstration that HSP27 concentrations are elevated in chemical-induced renal cell tumors and mesenchymal tumors. In the rat liver, dimethylnitrosamine induces neoplastic nodules and hepatocellular carcinomas, and Mairesse et al. [27] found that a HSP27-like protein was enhanced in such lesions. Toxicity of chemical carcinogens and various adverse conditions (such as oxidative injury resulting from ischemia) that prevail in neoplastic tissues may bring about induction of HSP27 [7], but further studies are needed to clarify at what stage in tumorigenesis this might occur and also whether other tissues demonstrate similar alteration during neoplasia.

In recent years it has become clear that HSP27 is involved in some forms of chemoresistance and could participate in the loss of sensitivity to anticancer drugs [13]. For example, Oesterreich et al. [30] reported that HSP27 is correlated with growth and drug resistance in human breast cancer cell lines. A recent study demonstrated that human testicular cells overexpressing HSP27 are more resistant to heat shock, cisplatin, and doxorubicin, and suggested that low constitutive levels in others may contribute to their sensitivity to chemotherapy [31]. The authors concluded that targeting HSP27 may improve response rates in other types of cancer. The present study demonstrated the mean level of HSP27 in rat renal cell tumors to be 12.6 times higher than that of normal kidney. This finding might imply a role in the resistance of human renal cell carcinomas against various kinds of chemotherapeutic agents because the rat lesions have similar characteristics in terms of origin from proximal renal tubules and histological and immunohistochemical findings [1, 3, 17, 38].

Several studies have revealed that expression of HSP27 is associated with aggressive behavior and patient survival in several types of malignancy, including breast cancer and the malignant fibrous histiocytoma [26, 39, 40]. In contrast, no diagnostic or prognostic significance has been established for cases of prostate or bladder cancers [33]. We are now studying whether concentrations of HSP27 in renal cell carcinomas in man are related to histological grade, cell type, invasion, or patient survival.

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