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Heat shock protein expression in the transplanted human kidney

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Abstract Heat shock proteins (HSPs) have been shown to represent potential target molecules for T-cell-mediated allograft rejection in heart and kidney transplants. In the present study, we therefore investigated the expression of HSP subtypes 60, 72, and 73 in normal kidneys and qualitative and/or quantitative changes in rejected renal allografts. Six normal kidney tissue specimens, three biopsies from patients with minimal change nephritis, as well as 37 biopsies and eight transplant nephrectomy specimens of patients with renal allograft rejection were studied. Type and severity of rejection were assessed according to the Banff classification. Immunohistochemical demonstration of HSP expression was performed using specific monoclonal antibodies after wet autoclave antigen retrieval on sections from either Carnoy-fixed (biopsies) or formalin-fixed (transplant nephrectomies) and paraffin-embedded tissue. The expression was scored in a semiquantitative manner. All three subtypes were found to be constitutively expressed in normal kidney tissue and in non-inflammatory minimal change nephritis, albeit with a characteristic compartmental and cellular distribution. Rejection resulted in a higher immunohistochemical scoring for all three HSP subtypes in compartments in which they were normally present; in addition, a de novo expression of HSP60 was found in the vascular compartment and, moreover, infiltrating mononuclear cells were strongly immunoreactive for HSP60 and HSP73. Only quantitative differences were observed for HSP72 immunoreactivity. These results indicate that rejection episodes are paralleled by an increased but differential expression of HSPs in the glomerular, tubular, and vascular compartments of the kidney. This enhancement as well as the de novo appearance of HSP60 on vascular endothelial cells might explain the presence of HSP-reactive T lymphocytes in rejected allografts.

Keywords Heat shock protein · Human kidney allograft · Immunohistochemistry · Rejection · Transplantation

Abbreviations *HSP* Heat shock protein

Introduction

The cellular and inflammatory immune response leading to rejection of an allograft is mainly mediated by T lymphocytes [13]. Although alloreactive T cells are of major importance for this reaction, limiting dilution experiments have shown that they comprise only a small fraction of all infiltrating lymphocytes [6]. Therefore, it seems likely that other subpopulations with different antigen specificities also participate in graft rejection [12].

Beside other molecules, heat shock proteins (HSPs) have been suggested as possible candidate antigens for such a T-cell-mediated immune reaction [10, 11, 15].

HSPs are evolutionary, highly conserved proteins involved in a variety of vital cellular functions by facilitating intracellular protein folding and transport. They are subdivided into several families according to their molecular weight and into constitutive and/or inducible forms. In all cells, HSP production is uniformly increased in response to exogenous or endogenous stress, essentially to protect themselves from harmful effects [5, 18]. Beside chaperoning functions and protection, HSPs have been shown to be target molecules of the immune system in autoimmune disease, in cancer immunity, and in antigen presentation [3, 7, 8, 16]. Additionally, recent studies have demonstrated the presence of HSP-specific T lymphocytes during rejection of allografts. Thus, T cells reactive to mycobacterial HSP65 were isolated from endomyocardial biopsies of transplanted human hearts [11], and T cells reactive to HSP65 and HSP70, from heterotopic rat cardiac allografts [10]. We have isolated HSP70-specific T lymphocytes from human allograft kidneys removed for irreversible rejection [15]. Furthermore, an increased expression of HSP45 and HSP60 mRNA was recently described in biopsies of rejected human kidneys as compared to nonrejected organs [1].

In view of these findings, the present immunohistochemical study has assessed the in vivo expression of HSP60 and HSP70 in rejected human renal allografts and their potential qualitative and quantitative changes in comparison to normal kidney tissue.

Materials and methods

Patients and biopsies

A total of 54 specimens from 42 patients were analyzed. This includes 37 fine-needle renal biopsies and eight nephrectomies from renal transplants, as well as normal kidney tissue from six patients (two biopsies, four nephrectomy specimens with renal oncocytoma) and three biopsies with minimal change nephritis. Four consecutive specimens were available from two patients; in nine cases, two specimens were investigated, and in 20 patients, only a single biopsy or nephrectomy was available for HSP immunostaining.

Immunohistochemistry

Biopsy specimens from transplanted kidneys were fixed in Carnoy and embedded in paraffin. Nephrectomy specimens and biopsies from patients with minimal change nephritis were formalin-fixed. Grading of rejection followed the Banff classification [14].

Immunohistochemistry was performed using specific monoclonal antibodies against HSP60 (clone SPA 804, dilution 1:500), HSP72 (the inducible member of the HSP70 family; clone SPA 810, dilution 1:200), and HSP73 (the constitutive member of the HSP70 family; clone SPA 815, dilution 1:300; StressGen Biotechnologies, Victoria, B.C., Canada) in appropriate dilutions; biotinylated secondary reagents and a streptavidin-biotin complex (DAKO, Copenhagen, Denmark) together with DAB-development (Sigma Biochemicals, Munich, Germany) were applied for visualization of the immune reaction. Wet autoclave pretreatment (10 min, 1.8 bar) of the sections in citrate buffer (0.5 mol/l, pH7.4) was used for antigen retrieval [2]. Endogenous peroxidase activity was blocked by incubation of the sections in 3% H_2O_2 in methanol after rehydration in graded alcohol. Omission of the primary antibodies and, in the case of HSPs, the application of antibodies after pre-incubation with the respective peptides (StressGen) were included as specificity controls.

The immunostained sections were evaluated semiquantitatively by light microscopy and scored either negative (–) or weakly (+) and moderately to strongly positive (++) in the various histological (i.e., glomerular, tubular, interstitial, and vascular) compartments as determined by two independent observers (H.F. and N.K.). In addition, the differential expression of HSP60, HSP72, and HSP73 in the nucleus and/or cytoplasm of cells was taken into consideration.

Table 1 Distribution of HSP60, HSP72, and HSP73 in normal kidney

Compartment	HSP60	HSP72	HSP73
Tubules			
Proximal convolute	+	+/–	+/–[b]
Distal convolute	++	+[b]	+[b]
Henle loop	++	+	+
Collecting ducts	+	+[b]	+
Glomeruli			
Epithelial cells	–[a]	++[c]	+[c]

[a] Single cells positive
[b] Nuclear and cytoplasmic
[c] Nuclear

Western blot analysis

Western blot analysis was performed on homogenized normal kidney specimens obtained from two nephrectomies with angiomyolipoma. Protein loading was assured by staining of gels with Coomassie Blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) and absorption at UV280. The blots were analyzed with a monoclonal antibody specifically recognizing HSP60 (dilution 1:5000, 1.5 h at room temperature; SPA 804, StressGen). Antibody binding was detected with a horseradish peroxidase-conjugated secondary antibody (dilution 1:30,000) and determined with a commercially available chemoluminescence kit (Pierce SuperSignal chemoluminescent system).

Results

HSP60

In normal kidney tissue, including minimal change glomerular disease, HSP60 was found predominantly in the tubular compartment with moderate immunoreactivity in the distal convolute and the Henle loop. A weak immunostaining was observed in the epithelial lining of the proximal tubules and in collecting ducts. The intracellu-

Fig. 1 Western blot analysis of HSP60 expression in normal kidney tissue (immunoblot). Samples derived from two different kidneys; two samples of each kidney were analyzed (the *left* two lanes derived from one kidney and the *right* two lanes from the other) and demonstrate expression of HSP60 in normal kidneys

lar distribution was purely cytoplasmic and exhibited a fine granular pattern. HSP60 was also detectable in single visceral and parietal epithelial cells within glomeruli. No immunoreactivity was found in the interstitial and vascular compartment (Table 1). The presence of HSP60 in normal kidney was also confirmed by Western blot analysis of homogenized normal renal tissue (Fig. 1).

In contrast, biopsies of kidney allografts with acute or chronic rejection and specimens of renal allografts removed because of irreversible rejection not only revealed markedly enhanced immunoreactivity for HSP60 in all parts of the tubular and glomerular compartment, but also showed a de novo expression in the smooth muscle layer and in the endothelium of blood vessels. Furthermore, HSP60 was prominently present in a significant number of infiltrating mononuclear cells in pure interstitial as well as in vascular rejection. (Fig. 2 a, b, c; Table 2).

HSP72

Since the immunolocalization of HSP72 was restricted to formalin-fixed tissue, only specimens from normal renal tissue, minimal change glomerular disease, and explanted allografts with severe rejection were testable. Normal kidneys showed a relatively strong expression of HSP72 in the epithelial and mesangial cells of the glomerular compartment in a predominantly nuclear distribution. The staining pattern within the tubular part of the nephron was more or less identical to that of HSP60 and showed a similar granular staining pattern in the cytoplasm, sometimes associated with weak nuclear reactivity. No immunoreactive cells were seen in the interstitium and in blood vessels (Table 1).

Severe rejection was associated with an increase in intensity of immunostaining in glomerular epithelial cells as well as in the tubular compartment, predominantly in the proximal and distal convolutes. Mononuclear infiltrating cells and blood vessels remained negative (Fig. 3 a, b; Table 2).

HSP73

In contrast to the more restricted expression of HSP60 and HSP72, HSP73 was widely found in normal kidney

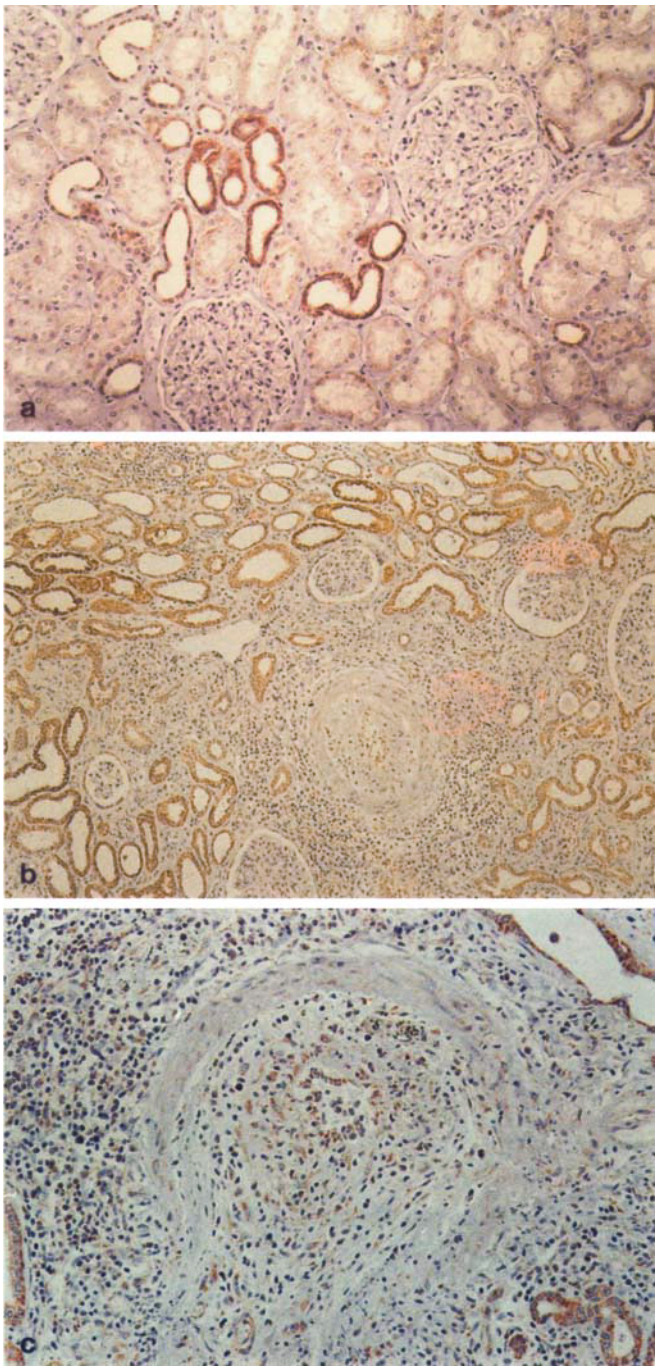

Fig. 2 Expression of HSP60. **a** Renal cortex of normal kidney predominantly in distal tubules (× 125), **b** rejected allograft showing immunostaining of the tubular epithelia as well as immunoreactive cells within the glomeruli and in an affected blood vessel (× 67), **c** high magnification of a blood vessel in a case of proliferative vascular rejection exhibiting immunoreactive endothelial cells and infiltrating mononuclear cells (× 270)

Table 2 Expression of HSP60, HSP72, and HSP73 in normal kidney and rejected allografts (– negative, + weak, ++ strong immunoreactivity; *ND* not done)

	HSP60			HSP72			HSP73		
	Glomerular[a]	Tubular[b]	Vascular[c]	Glomerular	Tubular	Vascular	Glomerular	Tubular	Vascular
Normal kidney	+ (9/9)	+ (9/9)	– (9/9)	+ (8/8)	+ (8/8)	– (8/8)	+ (9 /9)	+ (9/9)	+ (9/9)
Borderline	+ (3/3)	++ (3/3)	+ (3/3)	ND	ND	ND	+ (3/3)	+ (3/3)	+ (3/3)
Acute tubulointerstitial rejection	+ (9/9)	++ (9/9)	+ (9/9)	ND (9/9)	ND	ND	+	++ (9/9)	+ (5/9)
Acute vascular rejection	++ (13/15)	++ (15/15)	+ (11/15)	ND	ND	ND	++ (6/14)	++ (10/14)	++ (10/14)
Chronic rejection	+ (8/8)	++ (8/8)	++ (5/8)	++ (5/8)	++ (5/8)	– (8/8)	++ (8/8)	++ (8/8)	++ (8/8)

[a] Visceral and parietal epithelial cells
[b] Nuclear and cytoplasmic staining
[c] Smooth muscle and endothelial cells

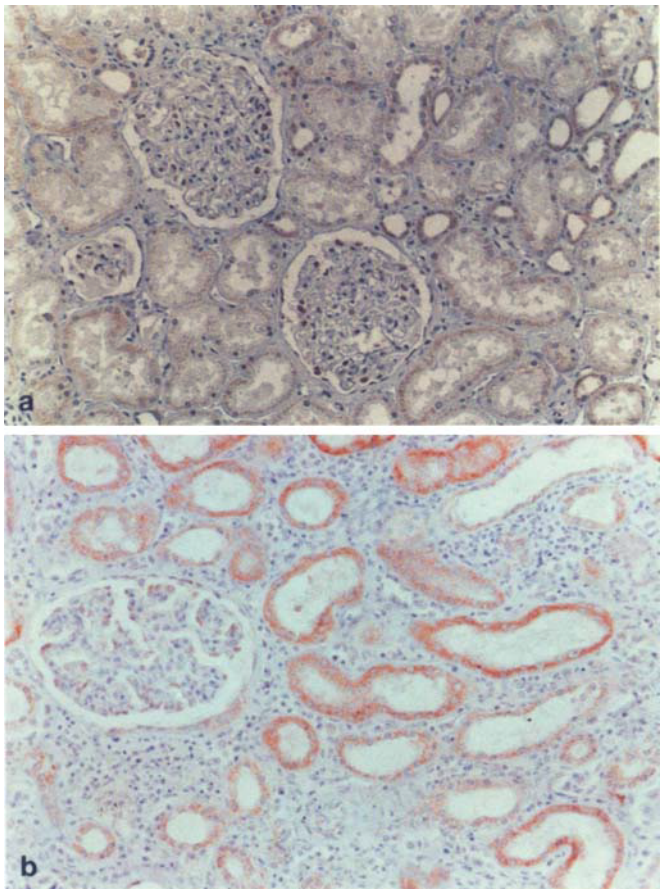

Fig. 3 Expression of HSP72. **a** Normal kidney mainly restricted to the distal tubular convolute and to glomerular epithelia/podocytes (× 130), **b** rejection with cytoplasmic immunoreactivity also in the proximal tubular epithelium; blood vessels are negative (× 130)

tissue and in biopsies from patients with minimal change nephritis; thus, HSP73 was observed in epithelial cells as well as in single endothelial cells of the capillary loops within glomeruli, in all parts of the tubular system, and in some smooth muscle cells and endothelial cells of blood vessels. Immunostaining was seen in both the cytoplasm and the nucleus; however, with nuclear predominance. Staining intensity was generally weak to moderate (Table 1).

Allograft rejection resulted in a marked increase of nuclear and cytoplasmic HSP73 immunoreactivity in all compartments in which it was normally present; in addition, the vast majority of infiltrating mononuclear cells showed a considerable expression of HSP73, irrespective of type and severity of rejection (Fig. 4 a, b, c; Table 2).

Discussion

The present study shows that HSP subtypes 60, 72, and 73 are constitutively expressed in normal kidney tissue in a distinct compartmental pattern and cellular distribution. HSP60 and HSP72 were predominantly present in the distal convoluted tubules, the loop of Henle, and in collecting ducts. They were weakly expressed in the epithelial lining of proximal tubules and in epithelial cells of the glomeruli. HSP73 was the only molecule demonstrable in all compartments, i.e., including also the smooth muscle layer and the endothelium of blood vessels. This is in accordance with studies in the rat [9, 10]. Observations in human kidneys are scanty and somewhat controversial. Thus, in the present study HSP60 could be detected in normal kidney by immunohistochemistry as well as by Western blot. This is somewhat in contrast to other findings [1] which, however, were based on mRNA detection by Northern blot. Whereas HSP72 was reported as negative in one investigation [4], in a recent study [17] HSP72/73 was demonstrated in the normal human kidney, predominantly in distal tu-

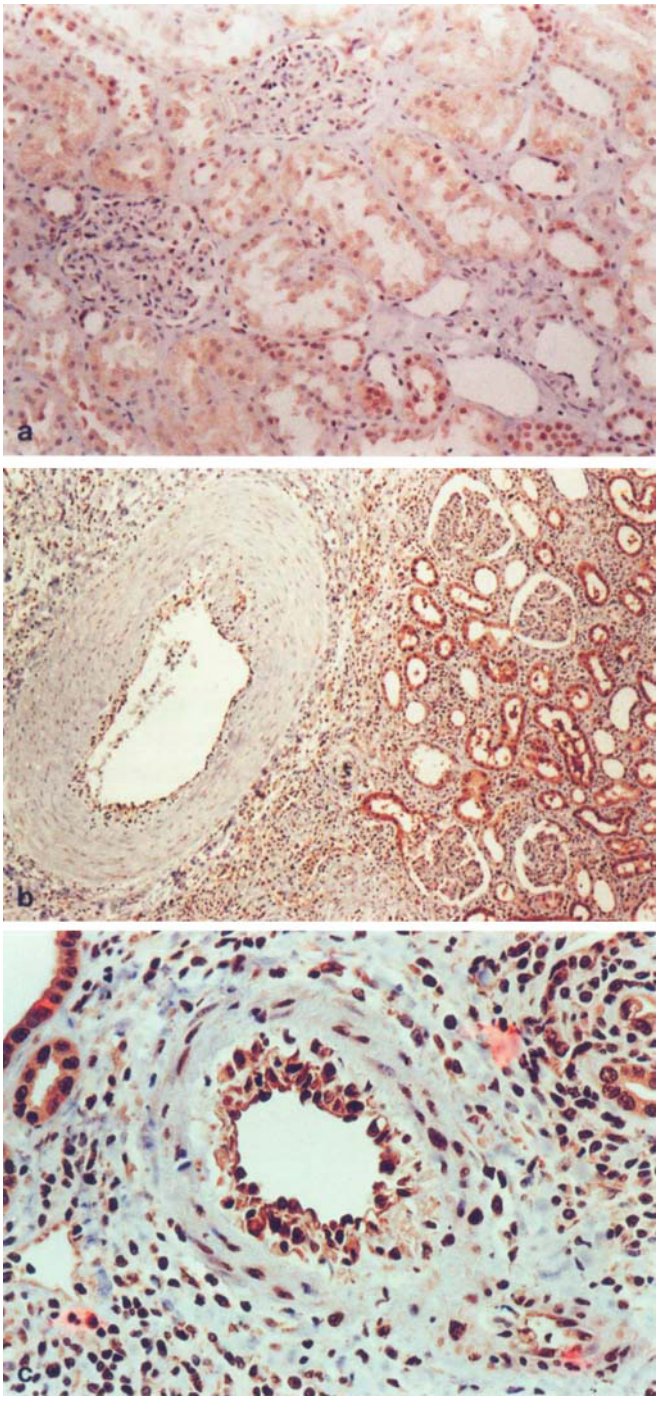

Fig. 4 Expression of HSP73. **a** Normal kidney in glomeruli, proximal and distal convolute, and in endothelial cells (× 140), **b** allograft rejection with strong nuclear and cytoplasmic immunoreactivity in all compartments including infiltrating mononuclear cells (× 70), **c** high magnification of HSP73 expression in blood vessels severely affected by proliferative vascular rejection (× 280)

bules and collecting ducts and similarly to our findings. Such differences concerning detectability as well as compatibility and cellular distribution of immunoreactive cells might be due to the different methodologies used, like Northern blotting, Western blotting, and immunohistochemistry. For the latter, antigen retrieval by wet autoclave pretreatment, which has been shown to be a very effective method for unmasking a variety of different antigens in fixed tissues, may be relevant. It proved most useful in the present study when compared to microwaving and immunostaining procedures without any pretreatment. The importance of fixation and antigen unmasking was also underlined by the fact that low-pH fixatives were more deleterious to HSP72, as demonstrated by the results observed in Carnoy-fixed biopsies versus formalin-fixed explants and normal kidney tissue. Immunohistochemically, HSP72 was localized exclusively in tubules and glomeruli without any expression in the vascular compartment. This distinct distribution might indicate that HSP72 is immunologically more relevant as an antigen released by tubular cell damage and is thus pathogenetically linked with the tubulo-interstitial type of acute rejection.

So far, the role of increased HSP expression during rejection episodes is not completely clear. For instance, HSPs can be increased during transplantation by surgery itself, reperfusion, and ischemia. In this stage, HSPs are assumed to protect cells from damage and to increase transplant survival. If this upregulation is not temporary, later on this protecting effect might change. As precursor T lymphocytes specific for HSPs can be isolated from normal individuals, the ongoing increased expression of HSPs might lead to a release of HSPs from dying cells. These released HSPs are in turn internalized and processed by mononuclear antigen-presenting cells already present in the graft. This leads to a specific presentation of HSPs via major histocompatibility complex molecules to specific T lymphocytes. The thus activated T lymphocytes recognize the still overexpressed HSPs in the transplanted kidney cells, which results in aggravation and longer duration of the rejection process. This theory is supported by the fact that T cells reactive to HSP were recently isolated from two of the transplant nephrectomies included in this study [15] and from rejected cardiac allografts [10, 11]. These T lymphocytes could be stimulated with recombinant human HSP72 in combination with antigen-presenting cells, suggesting a role of HSP72 either as transplantation antigen or as an unspecific trigger of T-cell activation associated with severe inflammation [15].

The distinct expression of HSPs in rejected kidneys, together with corresponding in vitro findings of HSP-specific T cells, suggest a participation as immunogens in graft rejection. Whether HSPs thereby act as primary inducers of rejection or as secondary stimulators of an immune response is subject of future investigations.

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