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Original Paper

Expression of Heat Shock Protein 72 in Renal Cell Carcinoma: Possible Role and Prognostic Implications in Cancer Patients

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Renal cancer cells from 43 patients and normal renal cells from 10 of them were studied for the expression of highly stress-inducible heat shock protein 72 (HSP72) by means of immunoperoxidase analysis. It was found that HSP72 was expressed in a significantly higher percentage of renal cancer cells than normal renal cells ($P = 0.0001$), the mean percentage of positive cells being 33.1 ± 18% and 8 ± 5%, respectively. Moreover, a percentage of HSP72-positive cells that was less than the cut-off point (18%, mean value of normal cells + 2 S.D.) significantly correlated with shorter disease-free survival ($P = 0.002$). The renal cancer cell populations taken from the 21 patients who relapsed after a median time of 13 months (range 3–73 months) had a significantly lower percentage of HSP72-positive cells (mean value 25.1 ± 17%) than the cells taken from the patients who remained tumour-free (mean value 40.8 ± 15%) after a median period of 72 months (range 19–96 months, $P = 0.003$). It was also demonstrated that HSP72 expression can be significantly increased by 48-h *in vitro* incubation with rIFN-γ ($P = 0.007$). These data suggest that HSP72 may represent a favourable prognostic factor regardless of stage and histological grade and its expression may be increased by treatment with rIFN-γ. Further studies are needed in order to investigate the relationship between HSP72 and the immunoeffector cells. © **1997 Elsevier Science Ltd.**

Key words: renal cell carcinoma, heat shock protein 72

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INTRODUCTION

MANY STRESS stimuli, such as heat, virus transformation, oxidative injury and heavy metal and serum deprivation, lead to the increased expression of heat shock proteins (HSPs) by prokaryotic and eukaryotic cells [1, 2]. On the basis of their molecular weight, HSPs are classified into five main families: low molecular weight, HSP65, HSP70, HSP90 and HSP100. The families consist of several members, each with a different pattern of induction and expression. Some HSP proteins, such as constitutive HSP73, are expressed under normal conditions in a cell-cycle dependent manner [2], whereas the highly-inducible HSP72, which is hardly detectable at normal temperatures, becomes one of the most actively synthesised proteins after heat shock [3]. In contrast, HSP90 and HSP60 are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat [4, 5]. HSPs appear to have the physiological functions of assembling and disassembling the protein complex, and are involved in the translocation of certain proteins through intracellular membranes [1].

Over the last few years, HSPs have also been shown to play a role in the antigenicity of tumours. The capacity of HSPs to be expressed on the surface of tumour cells, instead of their normal intracellular location, suggests they may play a role in inducing an immune response against cancer [6–8]. In a chemically-induced mouse sarcoma, Ullrich and associates identified a tumour-specific transplantation antigen that seems to be an HSP, which is expressed on the cell surface and induces protective immunity [9]. Furthermore, a protein related to the HSP70 family has been shown to be immunogenic in oncogene-transformed rat fibroblasts [10], and the HSP70 derived from MethA sarcoma (but not from normal tissue) has been demonstrated to be immunogenic not *per se* but in association with tumour peptides [11].

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All the studies reported so far in the literature focus on *in vitro* and animal models or just a few human malignant diseases (breast, lung, pancreatic carcinoma). This is the case of HSP72, which is hardly detectable under normal conditions, but is very actively synthesised after heat shock.

The aim of the present study, therefore, was to investigate the expression of highly stress-inducible HSP72 in tumours and normal renal cells and its possible clinical implications. Furthermore, since HSP70 is believed to act as a carrier of peptides in a manner similar to that of MHC in the antigen presenting cells, we also studied a possible immunomodulation of HSP72 by rIFN-γ (interferon-gamma).

PATIENTS AND METHODS

Patients

The renal cancer cells were obtained from 43 patients (29 male and 14 female) who had undergone radical nephrectomy for non-metastatic renal cell carcinoma (RCC); their median age at the time of surgery was 59 years (range 28–75). The normal renal cells were obtained from 10 of these patients. All the patients were scheduled for monitoring at 3-month intervals for the first year, every 4 months until the third year, every 6 months for the next 2 years, and once a year after 5 years. None of the patients had received any chemotherapeutic or immunomodulatory agents, and none showed any evidence of infection before surgery. All the patients gave their oral consent to tumour dissociation and the biological evaluation of cell suspensions.

Tissue specimens and cell line

The cell line CAKI-2 (human renal carcinoma) was cultured in Eagle's minimal essential medium (EMEM) (Flow, Irvine, U.K.) plus 10% fetal calf serum (FCS; Sera Lab, Crawley Down, U.K.).

The primary tumours obtained at the time of surgery were examined by the surgical pathologist who excised the specimens for both histological diagnosis and dissection from macroscopical tumour lesions.

Non-necrotic tumour tissue was dissected, minced with a scalpel and dissociated into a single-cell suspension with collagenase and DNase as previously described [12]. Normal renal tissue from a site distant from the tumour, histologically determined to be tumour-free, was processed similarly. The cells were cryopreserved and maintained in the vapour phase of liquid nitrogen until needed.

The cryopreserved tumour and normal cells were thawed in Hank's balanced salt solution (HBSS) with 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, California, U.S.A.), and then assessed for viability by means of the Trypan-blue exclusion test. With our dissociation and freezing procedures, viability was always 80% or more. The viable cells were counted, cytocentrifuged on to slides at 50*g* for 5 min (2×10^4 cells/slide), air-dried and frozen at −70°C.

Cell culture

The dissociated cryopreserved tumour cells and cell line were rapidly thawed and slowly diluted in HBSS with 10% FBS or 10% FCS over a period of 10 min. The dissociated cells were centrifuged, resuspended in RPMI plus 10% FBS medium, and then controlled for viability. The viable cells were plated in 3 ml of medium (1×10^6 cells/well). When the cells reached confluence, they were removed from the dish by means of a rubber-tipped cell scraper and transferred to a new well. The CAKI-2 cell line was resuspended and plated in EMEM plus 10% FCS under the same conditions.

After sufficient growth had occurred, the cells were fed with fresh medium alone or 100 units/ml of rIFN-γ (specific activity $>2.7 \times 10^4$ units/mg protein; Boehringer Mannheim, Germany) for 48 h. The adherent cells were scraped and cytocentrifuged on to slides (2×10^4 cells/slide) for the immunoperoxidase study.

Immunoperoxidase

After being cytocentrifuged on to slides, the cells were stained for immunoperoxidase study as previously described [13]. The anti-72 kDa HSP antibody (Amersham, Aylesbury, U.K.) was used to recognise the stress-inducible 72 kDa protein. Anti-HLA-ABC antibody (Becton Dickinson, Mountain View, California, U.S.A.) was used as a positive control, the anti-cytokeratin-18 MAb (Boehringer, Mannheim) as a means of verifying the epithelial origin of the examined cells, and anti- CD45 antibodies (Becton Dickinson) were used to identified the tumour infiltrating leucocytes.

An appropriate class-matched control (Sigma, St. Louis, Missouri, U.S.A.) was used, and no background reactivity was observed.

At least 200 cells were counted blind for positive or negative reactivity using light microscopy. All the antibodies used in this study induced positive staining of remarkable intensity, clearly distinguishable from negative staining. Only non-leucocytic cells were evaluated for the expression of HSP72.

Statistical analysis

The statistical significance of the differences between the study groups was determined using one-way ANOVA. When two groups of data were considered, a *t*-test was used. Disease-free interval curves were defined according to the product-limit method [14]. Differences between subgroups were first assessed by means of the log-rank test [15] and then by the Cox model [16], including the variables that were significantly associated with disease-free survival in the univariate analysis.

RESULTS

Clinical and pathological features

The tumour cells were obtained from 43 patients who had undergone radical nephrectomy for RCC, and normal renal cells were obtained from 10 of them. All the patients were available for follow-up (median duration 63 months, range 9–96 months). Of the 43 patients, 22 were tumour-free after a median of 72 months (range 19–96 months) and 21 relapsed (6 in the lung, 2 in the bone, 2 in the lymph nodes, 2 in the skin and 9 in other sites) after a median period of 13 months (range 2–73 months).

The patient stage (according to TNM) and histological grading are given in Table 1. At the time of nephrectomy, all the patients were lymph node-negative and without metastases.

HSP72 expression in RCC

HSP72 was expressed in a very low percentage of cells in eight, and not expressed in two of the ten normal renal cell

Table 1. Patient characteristics

	No. of patients	Mean % ± S.D. of HSP72 positive cells	No. (%) of HSP72 positive tumours*
Stage (TNM)			
I	2	22.5 ± 18	1 (50)
II	13	24.6 ± 18	6 (46)
III	28	36.8 ± 16	23 (82)
		P = NS†	
Grading			
G1	4	28.0 ± 18	2 (50)
G2	18	35.6 ± 15	15 (83)
G3	4	32.5 ± 20	3 (75)
Missing	17	-	10 (59)
		P = NS	

* Number of tumours expressing HSP72 levels higher than the cut-off point (18%, equal to the mean value of normal cells ±2 S.D.).
† Analysis was performed using one-way ANOVA.
NS, not significant.

populations analysed. The mean percentage of positive cells was 8 ± 5%. On the basis of this finding, 18% of positive cells (mean + 2 S.D.) was considered the cut-off point of normal expression, and values higher than this were considered high.

HSP72 was expressed at various levels in the renal cancer cell populations, and the mean percentage of positive cells was 33.1 ± 18%. A significant difference between levels of HSP72 expression in normal and renal cancer cells was observed not only in all the available renal cancer cell populations ($P = 0.0001$) (Table 2), but also between the 10 normal and cancer cell populations from the same patients ($P = 0.009$) in a paired t-test.

Thirty of the 43 renal cancers had a high percentage of HSP72-positive cells (19–50% in 22 cases and more than 50% in eight); the levels in the remaining 13 being lower than the cut-off point.

HSP72 appeared to be correlated with the probability of remaining tumour-free: the 13 patents with a percentage of HSP72-positive cells lower than the cut-off point had a significantly shorter disease-free survival (DFS) than those with higher HSP72 levels ($P = 0.002$) (Figure 1 and Table 3). Furthermore, the DFS curves were adjusted, in a multivariate analysis, by stage and histological grading. Nevertheless, the difference in DFS between the patients with high levels of HSP72-positive cells and those with low levels remained statistically significant ($P < 0.01$, Figure 1). HSP72, therefore, appears to be an independent prognostic

Table 2. Levels of HSP72 expression

	Mean % ± S.D. of HSP72 positive cells	
Normal renal cell populations (n = 10)	8.0% ± 5	
		$P = 0.0001$*
Renal cancer cell populations (n = 43)	33.1% ± 18	
Patients remaining tumour-free (n = 22)	40.8% ± 15	
		$P = 0.003$
Relapsing patients (n = 21)	25.1% ± 17	

* Analysis was performed using t-test.

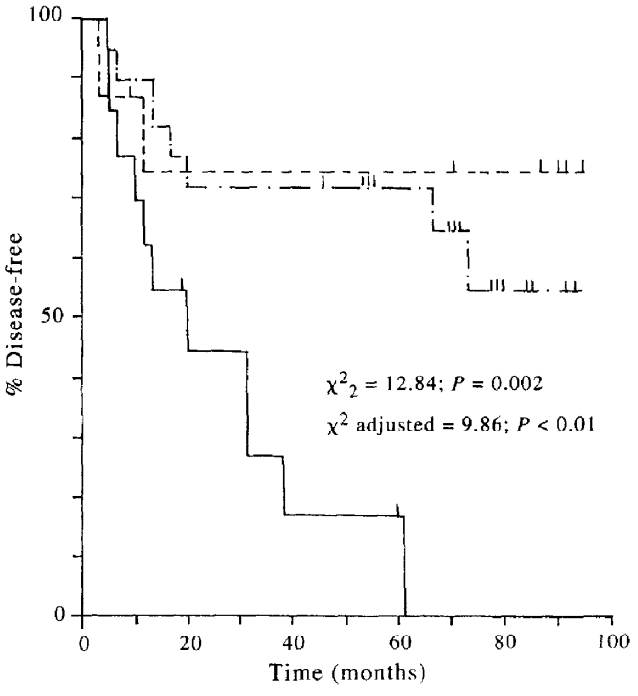

Figure 1. Disease-free probability of 43 patients with renal cell carcinoma according to HSP72 expression: ——, <18%: 13 patients; –·–·–, 19–50%: 22 patients; – – – –, <50%: 8 patients. χ^2adjusted = adjusted for stage and histological grade.

factor. Similarly, the tumour cells from the 22 patients who remained tumour-free expressed a percentage of HSP72-positive cells (40.8 ± 15%) significantly higher than the cells from the 21 patients who relapsed (25.1 ± 17%) ($P = 0.003$, Table 2).

11 patients have died; 2 from causes unrelated to the tumour and 9 of cancer; of these 5 had low HSP72 levels (38% of all the patients with low HSP72 levels) and 6 had more than 18% HSP72-positive tumour cells (20% of all the patients with high HSP72 levels). There were no statistical differences in terms of overall survival between the patients with high and low HSP72 levels.

Effect of rIFN-γ on HSP72 expression

In order to study the effect of rIFN-γ on renal cancer cells, six cell populations and one renal cell line (CAKI-2) were incubated with either rIFN-γ (100 U/ml) or control medium for 48 h. In all the cell populations and the CAKI-2 cell line, the percentage of HSP72-positive cells was higher than the cut-off point (mean value 50.5 ± 23%). After stimulation with rIFN-γ, HSP72 expression significantly increased in all the cell populations and the CAKI-2 cell line (overall mean value 71.3 ± 14%; $P = 0.007$, in a paired t-test) (Figure 2).

Table 3. Expression of HSP72 and other tumour characteristics in a univariate analysis for the prediction of disease-free survival

	χ^2_2	P
HSP72 expression	12.84	0.002
Stage	7.58	0.022
Grading	2.62	0.269

The analysis was performed as described in Materials and Methods.

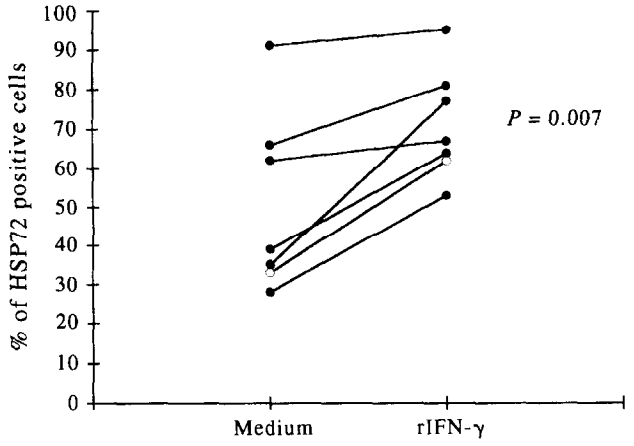

Figure 2. Effect of rIFN-γ on the expression of HSP72 in renal cancer cells in culture. CAKI-2 cell line –○– and six renal cancer cell populations –●– were exposed *in vitro* to rIFN-γ (100 U/ml) for 48 h and then tested by means of immunoperoxidase analysis for the expression of HSP72. The statistical analysis was performed using a paired *t*-test.

DISCUSSION

Although some evidence indicates that HSPs are involved in various aspects of cell transformation and the immune response against cancer [17], their biological role and its implications for the clinical course in cancer patients are not clear.

Inducible HSP72 has been shown to be a negative prognostic factor for DFS in lymph node-negative breast cancer patients [18], whereas other authors have shown that HSP72 positively correlates with oestrogen receptors and inversely with the expression of mutant p53 [19].

In our study, inducible HSP72 was expressed in a significantly higher percentage of renal cancer than normal cells. However, the cell populations from relapsed patients had a significantly lower percentage of HSP72-positive cells than those from the patients who remained tumour-free. Low percentages of HSP72-positive cells were found to be associated with a significantly shorter DFS in a multivariate analysis adjusted for stage and histological grade. These data were supported by the observation that three cell populations obtained from metastatic RCC lesions expressed very low levels of HSP72-positive cells (10%, 14% and 18%, respectively, data not shown). HSP72 expression may be one of the consequences of tumour transformation, although its role is not clear as it appears to be associated with a favourable clinical course. In fact, in our series HSP72 may be considered a favourable independent prognostic factor.

In the present study, HSP72 expression was not useful for identifying patients with shorter overall survival; the difference between the number of deceased patients with high and low levels of HSP72 did not have statistical significance. A longer follow-up period is probably required.

HSP72 appears to have a different role in different tumours. The same has been observed with HSP27, which seems to be associated with a shorter DFS in node-negative breast cancer patients [20], but a more favourable prognosis in malignant fibrous histiocytoma [21]. The reason for these conflicting aspects of HSPs could lie in the different functions in which they are involved.

For example, HSP70 has been demonstrated to be overexpressed and involved in the regulatory process in cultured cells after transformation with oncogenes [10, 22, 23], and high HSP70 expression (possibly of the constitutive form, HSP73) has been shown to provide protection from cell death induced by tumour necrosis factors-α and -β [24].

HSP70 proteins may also play an important role in tumour immunogenicity as it has been shown that the HSP72 expressed in tumour cells is recognised by T-lymphocytes [25]. Moreover, HSP70 was able to elicit immunity to the tumour from which it was isolated. HSP70 seems to be very important in the binding of tumour peptides [11, 26]. It has recently been proposed that HSP70 binding tumour peptides could be released by tumour cells and taken up by macrophages or other specialised antigen presenting cells, possibly by a receptor-mediated mechanism [27]. It appears, therefore, that stress-inducible HSP72 may either function as a tumour antigen or facilitate the presentation of tumour specific peptides by major histocompatibility complex molecules [7, 8, 17].

In an attempt to verify a possible immunomodulation of this protein, we studied the influence of rIFN-γ on HSP72 expression and we found that, in culture concentrations similar to those obtainable *in vivo*, rIFN-γ was able to increase significantly the percentage of HSP72-positive cells in all the tested tumour cell populations and the cell line.

In conclusion, the expression of inducible HSP72 is significantly greater in renal cancer than normal renal cells. Moreover, expression of HSP72 was greater in cell populations from patients who remained tumour-free than in those from relapsed patients. HSP72 may, therefore, represent a favourable prognostic factor, regardless of stage and histological grade. The expression of HSP72 can be significantly increased by *in vitro* incubation with rIFN-γ. However, further studies are needed to investigate the role of HSP72 and its interaction with immunoeffector cells.

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