Inducible heat shock protein 70 expression as a potential predictive marker of metastasis in breast tumors

Carolina Torronteguy,¹ Antonio Frasson,² Felipe Zerwes,² Erik Winnikov,²
Vinicius Duval da Silva,³ Antoine Ménoret,⁴ and Cristina Bonorino¹

¹Faculdade de Biociências e, Instituto de Pesquisas Biomédicas, PUCRS, Avenida Ipiranga, 6690 2o andar, 90010, Porto Alegre, RS, Brazil

²Centro de Mama, Hospital São Lucas, PUCRS, Porto Alegre, RS, Brazil

³Departamento de Patologia, Faculdade de Medicina, PUCRS, Porto Alegre, RS, Brazil

⁴University of Connecticut, Farmington, CT 06032, USA

Abstract Heat shock protein (Hsp)–peptide complexes purified from tumors can prime the immune system against tumor antigens, but how they contribute to the generation of immune responses against naturally occurring tumors is unknown. Murine tumors expressing high amounts of Hsp70 are preferentially rejected by the immune system, suggesting that low Hsp70 expression is advantageous for tumor growth in the host. To determine whether Hsp70 was differentially expressed in human tumors, inducible Hsp70 expression was quantitatively (by Western blot) and qualitatively (by immunohistology) analyzed in 53 biopsies of tumor and normal breast tissue. The mean expression of inducible Hsp70 was significantly higher in tumor compared with normal tissue ($U = 899.0$; $P = 0.0033$). However, a significant negative association of the amount of Hsp70 expressed by tumor tissue was found with metastasis ($r^2 = -0.309$; $P = 0.05$). After 3 years, follow-up analysis determined that 7 of the 53 patients relapsed, and 5 died. Hsp70 expression in tumor (but not normal) cells was significantly lower in relapse patients and patients with metastatic disease than in patients with no relapse or metastasis. Together, these observations support the hypothesis that Hsp70 plays a role in tumor expansion in vivo, and tumors that downregulate it may be able to evade immunosurveillance and grow.

INTRODUCTION

The stress-inducible heat shock protein 70 (Hsp70) is the main protein produced during cellular responses to varied stresses such as heat shock, ischemia/reperfusion, and oxidative changes (Lindquist 1986; Subjeck and Shyy 1986). A cognate form, Hsc70, is constitutively expressed in cells not experiencing stress. Hsp70, but not Hsc70, is overexpressed in human and animal tumors of varied origins (Clark and Menoret 2001). Because Hsp70 confers cell protection against different stresses, it has been hy-

pothesized that this protein plays a protective role in tumor growth in vivo (Barnes et al 2001; Ravagnan et al 2001). Indeed, CH1 lymphoma cells in culture methylate and silence their Hsp70 promoter, but upon injection into a mouse Hsp70 production in the CH1 cells is restored (Davidson et al 1995). These data suggest that Hsp70 is needed for in vivo tumor progression.

One of the major stresses faced by a tumor in vivo is the pressure of an immune response (Dunn et al 2004). Interestingly, it has been demonstrated that Hsp70 and other heat shock proteins can interact with the immune system. HSPs purified from tumors, particularly Hsp70, HSP90, calreticulin, and gp96, promote the generation of CD8⁺ T cell responses against tumor antigens, both in mice (Udono and Srivastava 1993; Janetzki et al 1998, 2000; Basu and Srivastava 1999) and humans (Parmiani et al 2004). HSPs carry tumor peptides to antigen-pre-

Received 23 August 2005; Revised 16 September 2005; Accepted 4 October 2005.

Correspondence to: Cristina Bonorino, Tel: 55 51 3320 3000 ext 2725; Fax: 55 51 3320 3312; E-mail: cbonorino@pucrs.br.

Current address: Cristina Bonorino, Center for Immunology, University of Minnesota, 312 Church Street, BSBE 6th Floor, Minneapolis, MN 55455.

senting cells (APCs), routing them for antigen presentation via MHC class I and II (Arnold-Schild et al 1999; Castellino et al 2000; Ueda et al 2004). Nevertheless, it is unknown whether Hsp70-peptide complexes are indeed released from tumor cells in concentrations capable of activating immune cells, contributing to the naturally occurring immune response against the tumor. In rodent carcinomas, Hsp70-producing clones were rejected by the immune system in a T cell-dependent fashion, whereas Hsp70-negative clones were not rejected and progressed in the host (Menoret et al 1995; Melcher et al 1998).

The tumor apparently has a dilemma: it needs to produce Hsp70 to grow *in vivo*; however, overexpression of this protein might activate a specific immune response against it (Menoret et al 2001). A prediction that follows is that Hsp70 production should be high in early-stage tumors but should decrease over time, enabling the tumor to evade the immune system and metastasize. Therefore, high Hsp70 expression in a recently developed tumor should offer a better prognosis for a patient, whereas an advanced tumor with low Hsp70 expression would correlate with poor prognosis.

To test this hypothesis, we analyzed expression of Hsp70 and Hsc70 quantitatively, by Western blot, as well as qualitatively, by immunohistology, in normal and tumor tissue of breast cancer patients. We also performed a 3-year follow-up on the patients. Our results show that Hsp70 is expressed by all tumors, in all stages, and usually at significantly higher concentrations than observed in normal, adjacent tissue. However, production of this protein in tumor, but not normal tissue, is lower in patients with advanced and metastatic tumors. Hsp70 expression was also significantly reduced in patients that experienced relapse, including death, in a period of 3 years. Our results support the hypothesis that expression of Hsp70 in breast cancer may indeed favor immune activation against a naturally occurring tumor.

MATERIALS AND METHODS

Patients

Samples of tumor and normal tissue were collected from 53 patients with primary breast cancer. All patients enrolled in the study signed an informed consent form, and the study protocol was previously approved by the Ethics Committee of the Sao Lucas Hospital. Exclusion criteria were previous radio- or chemotherapy, familial cancer, hormonal therapy, and use of antidepressants or corticosteroids. All patients were submitted to complete excision of tumor or total mastectomy.

Western blot

Fresh tissue samples from either the invasive margins of the tumors, as well as adjacent normal breast tissue, as

Table 1 Patient and tumor characteristics

Number of patients†	53
Age (mean $\pm$ SD)	54.84 $\pm$ 13.27
Menopausal status (mean $\pm$ SD)	49.33 $\pm$ 3.07
Size (mean $\pm$ SD)	2.48 $\pm$ 1.19 cm (range: 1.10–13.50)
Staging	
I	30.18%
IIA	24.52%
IIB	24.52%
IIIA	11.32%
IIIB	3.77%
IV	1.88%
Tumor histologic type	
Invasive ductal	64.15%
Invasive lobular	18.86%
Mucinous	3.7%
Metaplastic	1.88%
Intraductal	3.77%
Tumor differentiation	
Grade 1	3.77%
Grade 2	50.94%
Grade 3	18.86%
Grade not applicable	26.41%
Axillary lymph node status	
Node negative	39.62%
Node positive	63.38%
1–3	30.07%
4–10	13.20%
>10	5.66%
Lymphocyte infiltrate	
Absent (1)	9.43%
Slight (2)	45.28%
Moderate (3)	15.094%
High (4)	3.77%
Not determined	26.41
Estrogen receptor	65.95% positive and 8.5% negative
Progesterone receptor	44.68% positive and 29.79% negative

determined macroscopically, were retrieved from each patient and placed in RPMI media for immediate processing. Total protein was extracted by incubation of tissue fragment in ice with lysis buffer (10 mM Tris-HCl, pH 7.5; 1 mM MgCl₂; 1 mM ethylenediamine-tetraacetic acid [EDTA]; 0.1 mM phenylmethylsulfonyl fluoride [PMSF]; 5 mM 2-mercaptoethanol; 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS]; 10% glycerol), followed by centrifugation at 14 000 rpm for 1 hour at 4°C. Protein concentration was estimated by Bradford assay, using the Dye Reagent Concentrate (Bio-Rad, Hercules, CA; catalog no. 500-0006). Hsp70 expression by tumor and normal tissue was determined by Western blot. A total of 5 μ g of tissue lysate of each sample were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, as well as a purified Hsp70 (Stressgen, Victoria, British Columbia, Canada) standard curve of 2-fold serial dilu-

Fig 1. Western blot analysis of Hsp70 expression in normal and tumor tissue. Tissue samples were analyzed by Western blot for Hsp70 expression and compared with a standard curve of purified Hsp70. (A) T, Tumor; N, normal. Numbers next to these letters denote the patient. 1–4, Standard curve of Hsp70, in a serial 2-fold dilution, starting (on 4) at 0.2 μ g. (B) Tumor and normal sample of the same patients, blotted with both anti-Hsp70 and anti-actin antibodies. (C) Quantitation of Hsp70 expression in normal and tumor tissue of breast cancer patients. Hsp70 expression was determined in tumor and normal tissue by Western blot and quantitated by the Imagemaster software (Pharmacia). Tumor tissue expresses significantly more Hsp70 than normal tissue (Mann-Whitney $U = 924.0$; $P = 0.0024$).

tions, starting at 0.2 μ g. After electrophoresis, the gels were transferred to nitrocellulose membranes and blotted with anti-human Hsp70 antibody (SPA810; Stressgen) or anti-HSP/Hsc70 (SPA820; Stressgen). Actin expression was used as a control for basal constitutive protein synthesis, using an anti-human actin (A4700; Sigma, St Louis, MO) antibody. Samples in which actin was not detected were discarded. Secondary antibody was anti-mouse IgG conjugated with peroxidase (Invitrogen, San Diego, CA), and the blots were developed using the ECL system (Pharmacia, Peapack, NJ). The concentration of Hsp70 in tumor and normal tissue bands were determined according to the area of these bands in relation to a curve of areas plotted by the Imagemaster software

Fig 2. Expression of Hsp70, but not Hsc70, is significantly higher in tumor compared with normal tissue. Tumor and normal samples from 23 patients were screened for Hsp70 expression with either the C92 antibody (A), which recognizes exclusively the stress-induced protein, or the N27 (B) antibody, which recognizes both stress-induced and constitutive homologs. Hsp70 expression is expressed in nanograms per microgram of total protein.

from Pharmacia. Briefly, the Western blot images were captured using a scanner, and saved in a scale of gray with a resolution of 1200 dpi. Concentration values were assigned to Hsp70 standard curve bands according to the amount of protein applied to the respective gel lane. Quantitations were repeated for at least 3 times.

Histology

In tumor samples analyzed by immunohistochemistry, part of the tissue was fixed in paraffin and cut in sections of 4 μ m and applied to histology slides. Antigen recovery was performed in citrate buffer by microwave, in 3 cycles of maximum power for 5 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and 0.1% sodium azide. Additional blocking was performed with normal mouse serum (Novastain Universal Detection kit). Incubation with primary antibody (anti-Hsp70 from Stressgen or anti-IFN- γ from R&D Systems [Minneapolis, MN]) was performed overnight at room temperature, followed by incubation with biotinylated anti-mouse IgG and avidin-peroxidase (Novastain). Slides

Fig 3. Immunohistochemical analysis of Hsp70 expression in tumor tissue. Tissue samples were analyzed by immunohistochemistry for Hsp70 or control stain (interferon- γ) expression. (A) Invasive ductal tumor with hematoxylin-eosin stain. (B) Same tumor, stained for Hsp70 (arrow). (C) Tumor from a different patient, staining (arrows) for Hsp70. (D) Expression of Hsp70 quantified by stereoscopic analysis exclusively in tumors from 9 different patients. In a given tumor sample, Hsp70 is detected in the cytoplasm of tumor, but not normal, stromal cells permeating tumor masses ($t = 1.22$; $df = 18$; $P = 0.0001$).

were developed with diaminobenzidine (DAB) and hydrogen peroxide. Quantitation was performed by stereological microscopy, using the ImagePro Plus software. Images were captured at 200 $\times$ magnification, and 20 fields were picked per slide, 10 quadrants to the left and 10 to the right, at random. After capturing the images, a grid with 77 points randomly distributed was applied on each slide. Only cells that stained with Hsp70 and that were under a prelocalized spot were counted. Additionally, routine histologic assessment was performed for all samples by a pathologist specializing in cancer, and these established prognostic parameters, such as size, grade, volume, and staging (TNM classification) were attributed according to the criteria of the American Joint Committee on Cancer (AJCC). These parameters were analyzed for association with Hsp70 expression by Spearman correlation, and significance of difference of means was tested using the Mann-Whitney test. Statistical analysis was performed using the SPSS software, version 10 (SPSS, Chicago, IL).

RESULTS

Patient and tumor characteristics

Background data of patients with regard to age, menopausal status, histologic type of the tumor, tumor size, lymph node invasion by tumor, and estrogen receptor (ER) and progesterone receptor (PR) status are summa-

rized in Table 1. The majority of the patients presented tumors in early (I) and intermediate (IIA, IIB, and III) stages. Most of the tumors were of the invasive ductal type. Over 60% of the patients presented lymph nodes invaded by tumor cells.

Hsp70 expression by tumor and normal tissue

Previous studies on HSP expression by tumors focused on the percentage of tumor cells that expressed Hsp70 (Takeno et al 2001; Noguchi et al 2003). However, that approach cannot estimate the actual amount of protein being produced by the tissue. This is a critical issue if we hypothesize that a certain amount of Hsp70 must be produced or released by the tumor in order to activate APCs in a naturally occurring antitumor response. We therefore decided to quantitate the amount of Hsp70 in tumor tissue samples, as well as in normal, adjacent tissue. Quantitation was performed by Western blot, comparing samples from tumor and normal tissue of each patient (Fig 1 A,B) to a curve of serial 2-fold dilutions of recombinant Hsp70 (Fig 1A). Figure 1C shows the results of Hsp70 quantitation. Tumor samples exhibited a significantly higher mean expression of Hsp70 compared to normal tissue samples (Mann-Whitney $U = 824.5$; $P = 0.0006$). Whereas the distribution of Hsp70 values for tumor tissue varies greatly, in normal tissue Hsp70 values cluster together, showing less variation and seldom reaching amounts similar to those observed for tumors in the same patients.

In animals models, HSP, but not Hsc70, is associated with tumor progression/regression (Melcher et al 1998). To investigate whether this also occurs in breast cancer patients, samples were analyzed using either an antibody that recognizes both HSP and Hsc70 or an antibody that recognizes exclusively Hsp70. When samples of randomly selected 23 patients were probed with an antibody exclusively recognizing Hsp70, tumors showed a significantly ($P = 0.002$) higher expression of this protein over normal tissue (Fig 2A). However, when the antibody that recognizes both HSP/Hsc70 was used, normal tissue showed a higher mean expression ($P = 0.0036$) over tumor tissue (Fig 2B). This indicates that normal breast tissues express mainly Hsc70, whereas malignant breast tissues express mainly Hsp70.

Immunohistochemistry of Hsp70 in tumor samples

Higher Hsp70 expression in malignant vs normal breast tissues suggests that tumor, but not normal, cells were under stress. Naturally arising tumors are frequently permeated by stroma, untransformed connective tissue consisting of fibroblasts and nontransformed cells recruited by the tumor (Kiaris et al 2004). It was therefore possible

Table 2 Association between established prognostic markers by Spearman's correlation in the 53 studied patients

	Lymph node metastasis		Size		Metastasis		Relapse	
	r^2	P value	r^2	P value	r^2	P value	r^2	P value
LN metastasis	1.000	0.000	0.264	0.064	0.465*	0.003	0.356*	0.031
Limits	0.061	0.675	-0.038	0.798	-0.018	0.918	0.182	0.287
Grade	0.328	0.045*			0.290	0.127	0.477**	0.010
Infiltrate	-0.02	0.905	-0.04	0.794	-0.007	0.973	0.085	0.681
Staging	0.817***	0.000	0.561***	0.000	0.616***	0.000	0.333*	0.041
Metastasis	0.465**	0.003	0.203	0.222	1.000	0.000	0.545***	0.000
Age	-0.31*	0.029	-0.113	0.438	-0.209	0.194	-0.259	0.112
Skin invasion	0.204	0.232	-0.007	0.967	0.351	0.078	0.117	0.578
Vascular invasion	0.279	0.095	0.310	0.062	0.623**	0.001	0.345	0.091
Menopausal status	0.103	0.665	0.041	0.862	-0.343	0.138	-0.390	0.099
Estrogen receptor	-0.061	0.716	-0.139	0.405	0.212	0.195	0.221	0.182
Progesterone receptor	-0.098	0.560	-0.278	0.090	-0.067	0.687	0.038	0.822
Size								

LN, Lymph node.

that variation in tumor Hsp70 expression reflected variations in stromal, and not tumor tissue. We thus examined Hsp70 expression by immunohistochemistry in tumor sections. The typical pattern observed in tumor samples for Hsp70 staining (Fig 3) was that mainly tumor, rather than connective/stromal tissue, showed significant Hsp70 expression in the cytoplasm (Fig 3 B,C). Analysis of Hsp70 expression using stereotaxic method was performed in 10 tumor samples from patients with similar amounts of Hsp70 as estimated by Western blot (mean = 5.05 ± 1.89 ng/ μ g of total protein). Samples were from patients in different tumor stages, and a similar pattern was observed in all of them. By counting the number of Hsp70-positive cells in tumor tissue vs positive cells in tumor stroma, we confirmed that Hsp70 expression was indeed mostly from tumor, and not normal stromal cells (Fig 3D) ($P = 0.0012$). That suggested that the stromal content of tumor samples did not significantly influence the Hsp70 expression estimates, and that the amount of Hsp70 by tumors on a per-cell basis was actually higher than what we could estimate by Western blot. The normal stromal cells, although accounting for part of the tumor sample, did not show significant Hsp70 expression, behaving like cells in the adjacent, normal tissue samples.

Association between conventional prognostic markers

All tumor samples were analyzed by a clinical pathologist, as part of the routine clinical procedures. Association between conventional prognostic markers was analyzed using the Spearman test (Table 2). As expected, tumor staging was significantly associated with distant metastasis ($r^2 = 0.616$; $P = 0.000$), lymph node metastasis ($r^2 = 0.817$; $P = 0.000$), as well as with tumor size ($r^2 = 0.372$; $P = 0.02$), metastasis ($r^2 = 0.372$; $P = 0.02$), skin invasion ($r^2 = 0.372$; $P = 0.02$), and death ($r^2 = 0.577$; $P = 0.000$). In addition, lymph node invasion by tumor was signifi-

cantly and positively associated with distant metastasis ($r^2 = 0.465$; $P = 0.003$), relapse ($r^2 = 0.356$; $P = 0.031$), and death ($r^2 = 0.401$; $P = 0.013$). However, it correlated negatively with age ($r^2 = -0.31$; $P = 0.029$). Metastasis also showed a highly significant positive association with vascular invasion ($r^2 = 0.623$; $P = 0.001$), relapse ($r^2 = 0.545$; $P = 0.000$), and death ($r^2 = 0.804$; $P = 0.000$). Finally, the expression of progesterone receptors correlated positively with expression of estrogen receptors ($r^2 = 0.514$; $P = 0.001$).

Association between Hsp70 expression and prognostic markers

We hypothesized that, if tumor Hsp70 had a role in the generation of immune responses to the tumor, associations should be found between markers of tumor aggressiveness and Hsp70 expression in tumor samples. We performed a correlation analysis of tumor and normal tissue Hsp70 with the established prognostic tumor markers analyzed above. Table 3 summarizes these results. Spearman correlation analysis showed that Hsp70 expression in tumor and normal tissue were significantly and positively correlated ($r^2 = 0.292$; $P = 0.035$). Interestingly, Hsp70 expression in tumor, but not in normal tissue, was significantly and negatively associated with metastasis ($r^2 = -0.309$; $P = 0.05$).

These results led us to hypothesize that high Hsp70 expression is important for tumor growth, whereas low Hsp70 expression related to immune evasion and metastasis. Higher Hsp70 expression could trigger an immune response and lead to tumor regression, selecting for tumor clones that expressed less Hsp70, thus decreasing tumor detection by immune cells and increasing the chances of metastasis for the tumor. A prediction of this hypothesis is that Hsp70 would be higher in early neo-

Table 2 Continued

Death		Infiltrate		Skin invasion		Estrogen receptor		Vascular invasion	
r^2	<i>P</i> value	r^2	<i>P</i> value	r^2	<i>P</i> value	r^2	<i>P</i> value	r^2	<i>P</i> value
0.401	0.013*	-0.020	0.905	0.204	0.232	-0.061	0.716	0.279	0.095
-0.181	0.285	-0.161	0.340	0.070	0.688	0.092	0.587	0.266	0.117
0.084	0.665	0.060	0.764	0.134	0.514	0.325	0.085	0.107	0.594
-0.007	0.973	1.000	0.000	0.219	0.253	0.295	0.135	-0.091	0.620
0.577***	0.000	-0.155	0.352	0.249	0.143	0.000	1.000	0.372*	0.023
0.804***	0.000			0.351	0.078	0.212	0.195	0.623**	0.001
-0.024	0.882	-0.109	0.520	-0.180	0.301	-0.112	0.490	-0.085	0.621
0.351	0.078	0.219	0.253	1.000	0.000	0.389	0.055	0.215	0.238
0.246	0.225	-0.091	0.620	0.215	0.238	-0.161	0.442	1.000	0.0000
-0.252	0.284	0.253	0.363	0.089	0.752	-0.040	0.871	-0.077	0.794
0.212	0.195	0.295	0.135	-0.161	0.442	1.000	0.000	-0.161	0.442
-0.067	0.687	0.130	0.518	0.240	0.249	0.514**	0.001	-0.050	0.811
		-0.044	0.794	-0.007	0.967	-0.139	0.405	0.310	0.062

* = $p < 0.05$; ** = $p < 0.01$; *** = $p = 0.001$.

Table 3 Association between Hsp70 expression and established prognostic markers by Spearman's correlation

	Hsp70 in tumor tissue		Hsp 70 in normal tissue	
	r^2	<i>P</i> value	r^2	<i>P</i> value
LN metastasis	-0.050	0.729	0.091	0.530
Limits	-0.266	0.064	-0.186	0.202
Vascular invasion	-0.302	0.07	-0.318	0.055
Size	0.033	0.822	0.127	0.379
Grade	-0.091	0.585	0.086	0.610
Infiltrate	-0.332	0.042	-0.325	0.047
Staging	-0.064	0.657	0.186	0.190
Metastasis	-0.309*	0.050	0.067	0.683
Relapse	-0.201	0.219	0.052	0.755
Death	-0.255	0.113	0.164	0.313
Hsp70 in normal tissue	0.292*	0.035	1	0.000
Hsp70 in tumor tissue	1	0.000	0.292*	0.035

LN, Lymph node; r^2 , correlation coefficient.

Fig 4. Comparison of mean inducible Hsp70 expression values between tumors in different staging groups. Mean values of Hsp70 (nanograms per microgram of total protein) in various stages of tumor growth: early (stage I tumors), intermediary (stages IIA, IIB, and IIIA), and advanced (stages IIIB, IIIC, and IV) were compared using the Mann-Whitney test. *, ≤ 0.05 .

plasms and get lower with time under the selective pressure of the immune system.

To test this prediction, we investigated whether the mean Hsp70 expression was significantly different in various stages of tumor growth: early (stage I tumors), intermediary (stages IIA, IIB, and IIIA), and advanced (stages IIIB, IIIC, and IV). The results are displayed in Figure 4. Whereas early tumors did not differ significantly from intermediary tumors regarding their mean inducible Hsp70 expression, advanced tumors expressed significantly lower Hsp70 than both early- and intermediate-stage tumors.

Follow-up on breast cancer patients

After a 3-year period, the clinical status of the patients in this study was determined. Of the 53 patients, 7 (13.2 %) presented some sort of relapse (Table 4). The Hsp70 values displayed are the ones detected at diagnosis, because we did not have access to samples of the relapsing tu-

Table 4 Characteristics of patients with relapse (in a period of 2 to 3 years)

Patient no.	Diagnosis	Age at diagnosis	Staging	Tumor Hsp70 (ng/ μ g)	Normal Hsp70 (ng/ μ g)	Infiltrate	Follow-up
B2	August 2001	48	ND	3.36	0.10	Mild	Bilateral breast cancer
B8	January 2002	46	IIIA	6.93	3.56	Mild	Bone metastasis (August 2003), liver metastasis, and death (May 2004)
B12	May 2002	70	IV A	0.10	28.42	Not available	Submitted to palliative chemotherapy (May 2002) due to supraclavicular lymph node metastasis at diagnosis. Neoplasia responded, and subsequent mammography showed no tumor evidence (January 2003). Surgery was performed (May 2003), and there was no breast or axillary lymph node neoplasia. Symptomatic central nervous system metastasis occurred in June 2003, and patient died.
B13	July 2002	44	IIIB	0.10	11.28	Mild	Positive node at diagnosis. Relapse (June 2003), multiple bone metastasis (September 2003), and death by terminal neoplasia (December 2003)
P14	July 2001	73	IIB	6.92	4.46	Mild	Another neoplasia (gastric cancer with liver metastasis—September 2001), and death by upper gastric hemorrhage (July 2004)
P16 F4	August 2001 June 2001	41 81	IIIA I	4.37 ND	0.54 ND	Mild Absent	Relapse Liver metastasis (June 2003) and death (March 2004)

Fig 5. Comparison of mean inducible Hsp70 expression values between groups of tumor patients with different outcomes. Mean inducible Hsp70 values (nanograms per microgram of total protein) in either tumor (A) or normal (B) tissue were compared between patients positive (striped bars) or negative (white bars) for metastasis, relapse, and death, using the Mann-Whitney test. *, ≤ 0.05 .

mors. Remarkably, in all 7 patients, tumor Hsp70 expression at the time of diagnosis was low (mean = 3.63 ± 3.07 ng/ μ g of total protein). Among these relapsing patients, those who had lymph node metastasis at the time of diagnosis also had the lowest tumor (but not normal) tissue Hsp70 expression (Table 4, B12 and B13, mean 0.1 ng/ μ g of total protein). The relapsing patients with higher Hsp70 values in tumor tissue at the time of diagnosis showed metastasis only 1 year later (Table 4, B2, B8, P14, and P16, mean = 5.395 ± 1.8 ng/ μ g of total protein).

Because these findings suggested that low production of Hsp70 could indeed be a characteristic of metastatic tumors, we then compared mean inducible Hsp70 expression values between the following groups: patients with or without metastasis, with or without relapse, and patients who died vs patients who were still alive. Results are shown in Figure 5. All patients in groups with either metastasis, relapse, or death showed a low Hsp70 expression in tumor (Fig 5A), but not in normal (Fig 5B) tissue. A significant difference in tumor Hsp70 mean expression was found only between patients with vs with-

out metastasis (Fig 5A). No significant differences were found between groups of each outcome for mean Hsp70 expression in normal tissue.

DISCUSSION

Due to central immune tolerance, a lack of response against self-tumor-associated antigens is expected. Peripheral tolerance may explain the lack of response against tumor-specific antigens. The immune system, however, is capable of responding to breast cancer as evidenced by systemic, regional, and intratumoral leukocyte activation (Hamlin 1968; Kreider et al 1984). During tumor progression, tumors adopt immunosuppressive strategies, such as downregulation of HLA class I molecules (Lassam and Jay 1989) and secretion of anti-inflammatory cytokines such as TGF- β (Daughaday and Deuel 1991; Hazelbag et al 2002), leading to further tolerization of natural antitumor responses. Activation of dendritic cells, mainly via Toll-like receptor (TLR) ligands, seems to be crucial for the reversal of the tumor-induced state of tolerance of the tumor-specific T cells (Yang et al 2004). HSP-rich tumor lysates activate dendritic cells (Somersan et al 2001), suggesting that immune-mediated tumor regression by Hsp70 occurs via dendritic cell activation.

In this study, we asked whether Hsp70 expression in spontaneously arising tumors would be correlated with tumor progression and therefore would be significant for patient outcome. We observed that breast tumor tissue expresses significantly more Hsp70 than the adjacent normal, healthy tissue. Also, we showed that it is the neoplastic cells within the tumor mass, and not the recruited stroma, that express high amounts of this protein. Because Hsp70 is synthesized upon stress situations in cells of all living organisms, the fact that tumor, but not normal, cells express Hsp70 suggests tumor cells are under stress. Different hypotheses have been raised to explain Hsp70 expression by tumors. HSP expression may protect cells from apoptosis and toxic effects of tumor necrosis factor α (TNF- α) (Wang et al 1996; Van Molle et al 2002). However, TNF also inhibits the Hsp70 stress response by blocking TNF receptor 1 (TNF-R1) and activating protein phosphatases (Schett et al 2003). Cell surface-bound Hsp70 increases sensitivity to apoptosis induced by granzymes (Gross et al 2003). Nuclear Hsp70 expression led to the suggestion that Hsp70 would protect tumors from death by protecting transcription factors from degradation (Ravagnan et al 2001; Gurbuxani et al 2003). Together, these results suggest that high Hsp70 expression is critical for tumor growth, but most importantly is highly associated with, and modulated by, immune-induced apoptosis.

Tumors that arise from high Hsp70-expressing normal cells are likely to be high Hsp70 expressers. If the tumor

managed to progress even with low amounts of Hsp70, then immune activation by tumor Hsp70 would be lessened. This situation would select for clones with down-regulated Hsp70 expression and enhanced potential for growth and metastasis. This hypothesis predicts that Hsp70 expression should be higher in early-stage tumors, which mainly need Hsp70 to grow, and should be low under the selective pressure of the immune system. Indeed, we found Hsp70 expression was significantly lower in early- and intermediate-stage tumors compared with the advanced-stage ones, and tumors that showed an expressive cellular infiltrate were low in Hsp70. Support for this comes from the striking and statistically consistent finding that inducible Hsp70 expression in tumor, but not normal tissue, is negatively associated with metastasis. It suggests that tumor clones with low Hsp70 expression were able to evade an immune response and invade other tissues. The fact that all of the patients that experienced either relapse, metastasis, and/or died in 2 years had tumors that were low in Hsp70 expression agrees with an antitumor immune-activating role for this protein. Consistent with this is the finding that, among the relapsing patients, Hsp70 expression was very low in those with lymph node invasion at the time of diagnosis, compared with those with no signs of metastasis.

What is the mechanism that leads to tumor-specific activation by tumor Hsp70? Although a chaperone activity for tumor peptides has been consistently observed for Hsp70 purified from tumors, it is still not conclusive how and if this protein can bind to and activate APCs. Hsp70 has been shown to activate NK cells, leading to the development of antitumor responses (Multhoff et al 1999; Multhoff 2002). Different studies refer to different cell surface receptors, such as CD91 (Basu et al 2001), CD40 (Becker et al 2002), or TLR2 (Vabulas et al 2002). More recent reports suggest a role for scavenger receptors such as lox-1 (Delneste et al 2002) as a receptor for Hsp70. An additional mechanism has been reported (Faure et al 2004) for Hsp70 as a tumor antigen itself, due to its intense production in tumor cells. Our findings demonstrate that, in breast cancer patients, Hsp70 expression can be significantly associated with tumor progression. Together, they suggest that inducible Hsp70 expression in tumor tissue might be important as a prognostic factor for patients with breast cancer.

ACKNOWLEDGMENTS

We thank the patients who consented to participate in this study; Tiago Giuliane Lopes and Erlinda Furtado da Silva in the Pathology Department for the expert technical support with the histology studies; clinical pathologists Aluisio de Castro Antunes Maciel, Josenel Barcelos Marçal, and Sidnei Pacheco Júnior for excising samples of tu-

mor tissue; Carlos Luiz Reichel for sharing samples with us; and Pedro Pasetto for help in compiling patient chart information. This work was supported by FAPERGS and CNPq.

REFERENCES

- Arnold-Schild D, Hanau D, Spehner D, Schmid C, Rammensee HG, de la Salle H, Schild H. 1999. Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J Immunol* 162: 3757–3760.
- Barnes JA, Dix DJ, Collins BW, Luft C, Allen JW. 2001. Expression of inducible Hsp70 enhances the proliferation of MCF-7 breast cancer cells and protects against the cytotoxic effects of hyperthermia. *Cell Stress Chaperones* 6: 316–325.
- Basu S, Binder RJ, Ramalingam T, Srivastava PK. 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* 14: 303–313.
- Basu S, Srivastava PK. 1999. Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. *J Exp Med* 189: 797–802.
- Becker T, Hartl FU, Wieland F. 2002. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. *J Cell Biol* 158: 1277–1285.
- Castellino F, Boucher PE, Eichelberg K, Mayhew M, Rothman JE, Houghton AN, Germain RN. 2000. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. *J Exp Med* 191: 1957–1964.
- Clark PR, Menoret A. 2001. The inducible Hsp70 as a marker of tumor immunogenicity. *Cell Stress Chaperones* 6: 121–125.
- Daughaday WH, Deuel TF. 1991. Tumor secretion of growth factors. *Endocrinol Metab Clin North Am* 20: 539–563.
- Davidson S, Hoj P, Gabriele T, Anderson RL. 1995. In vivo growth of a murine lymphoma cell line alters regulation of expression of HSP72. *Mol Cell Biol* 15: 1071–1078.
- Delneste Y, Magistrelli G, Gauchat J, et al. 2002. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 17: 353–362.
- Dunn GP, Old LJ, Schreiber RD. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21: 137–148.
- Faure O, Graff-Dubois S, Bretaudeau L, et al. 2004. Inducible Hsp70 as target of anticancer immunotherapy: identification of HLA-A*0201-restricted epitopes. *Int J Cancer* 108: 863–870.
- Gross C, Koelch W, DeMaio A, Arispe N, Multhoff G. 2003. Cell surface-bound heat shock protein 70 (Hsp70) mediates perforin-independent apoptosis by specific binding and uptake of granzyme B. *J Biol Chem* 278: 41173–41181.
- Gurbuxani S, Schmitt E, Cande C, et al. 2003. Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. *Oncogene* 22: 6669–6678.
- Hamlin IM. 1968. Possible host resistance in carcinoma of the breast: a histological study. *Br J Cancer* 22: 383–401.
- Hazebag S, Gorter A, Kenter GG, van den Broek L, Fleuren G. 2002. Transforming growth factor-beta1 induces tumor stroma and reduces tumor infiltrate in cervical cancer. *Hum Pathol* 33: 1193–1199.
- Janetzki S, Blachere NE, Srivastava PK. 1998. Generation of tumor-specific cytotoxic T lymphocytes and memory T cells by immunization with tumor-derived heat shock protein gp96. *J Immunother* 21: 269–276.
- Janetzki S, Palla D, Rosenhauer V, Lochs H, Lewis JJ, Srivastava PK. 2000. Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study. *Int J Cancer* 88: 232–238.
- Kiaris H, Chatzistamou I, Kalofoutis C, Koutselini H, Piperi C, Kalofoutis A. 2004. Tumor-stroma interactions in carcinogenesis: basic aspects and perspectives. *Mol Cell Biochem* 261: 117–122.
- Kreider JW, Bartlett GL, Butkiewicz BL. 1984. Relationship of tumor leucocytic infiltration to host defense mechanisms and prognosis. *Cancer Metastasis Rev* 3: 53–74.
- Lassam N, Jay G. 1989. Suppression of MHC class I RNA in highly oncogenic cells occurs at the level of transcription initiation. *J Immunol* 143: 3792–3797.
- Lindquist S. 1986. The heat-shock response. *Annu Rev Biochem* 55: 1151–1191.
- Melcher A, Todryk S, Hardwick N, Ford M, Jacobson M, Vile RG. 1998. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. *Nat Med* 4: 581–587.
- Menoret A, Li Z, Niswonger ML, Altmeyer A, Srivastava PK. 2001. An endoplasmic reticulum protein implicated in chaperoning peptides to major histocompatibility of class I is an aminopeptidase. *J Biol Chem* 276: 33313–33318.
- Menoret A, Patry Y, Burg C, Le Pendu J. 1995. Co-segregation of tumor immunogenicity with expression of inducible but not constitutive hsp70 in rat colon carcinomas. *J Immunol* 155: 740–747.
- Multhoff G. 2002. Activation of natural killer cells by heat shock protein 70. *Int J Hyperthermia* 18: 576–585.
- Multhoff G, Mizzen L, Winchester CC, et al. 1999. Heat shock protein 70 (Hsp70) stimulates proliferation and cytolytic activity of natural killer cells. *Exp Hematol* 27: 1627–1636.
- Noguchi T, Wada S, Takeno S, Moriyama H, Kimura Y, Uchida Y. 2003. Lymph node metastasis could be predicted by evaluation of macrophage infiltration and hsp70 expression in superficial carcinoma of the esophagus. *Oncol Rep* 10: 1161–1164.
- Parmiani A, Testori A, Maio M, et al. 2004. Heat shock proteins and their use as anticancer vaccines. *Clin Cancer Res* 10: 8142–8146.
- Ravagnan L, Gurbuxani S, Susin SA, et al. 2001. Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* 3: 839–843.
- Schett G, Steiner CW, Xu Q, Smolen JS, Steiner G. 2003. TNFalpha mediates susceptibility to heat-induced apoptosis by protein phosphatase-mediated inhibition of the HSF1/hsp70 stress response. *Cell Death Differ* 10: 1126–1136.
- Somersan S, Larsson M, Fonteneau JE, Basu S, Srivastava P, Bhardwaj N. 2001. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. *J Immunol* 167: 4844–4852.
- Subjeck JR, Shyy TT. 1986. Stress protein systems of mammalian cells. *Am J Physiol* 250: C1–C17.
- Takeno S, Noguchi T, Kikuchi R, Wada S, Sato T, Uchida Y. 2001. Immunohistochemical study of leukocyte infiltration and expression of hsp70 in esophageal squamous cell carcinoma. *Oncol Rep* 8: 585–590.
- Udono H, Srivastava PK. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* 178: 1391–1396.
- Ueda G, Tamura Y, Hirai I, et al. 2004. Tumor-derived heat shock protein 70-pulsed dendritic cells elicit tumor-specific cytotoxic T lymphocytes (CTLs) and tumor immunity. *Cancer Sci* 95: 248–253.
- Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H. 2002. HSP70 as endogenous stimulus of the Toll/

- interleukin-1 receptor signal pathway. *J Biol Chem* 277: 15107–15112.
- Van Molle W, Wielockx B, Mahieu T, Takada M, Taniguchi T, Sekikawa K, Libert C. 2002. HSP70 protects against TNF-induced lethal inflammatory shock. *Immunity* 16: 685–695.
- Wang D, McMillin JB, Bick R, Buja LM. 1996. Response of the neonatal rat cardiomyocyte in culture to energy depletion: effects of cytokines, nitric oxide, and heat shock proteins. *Lab Invest* 75: 809–818.
- Yang Y, Huang CT, Huang X, Pardoll DM. 2004. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol* 5: 508–515.