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Heat shock protein 70 and glycoprotein 96 are differentially expressed on the surface of malignant and nonmalignant breast cells

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Abstract Heat shock proteins (HSPs), which are important for a number of different intracellular functions, are occasionally found on the surface of cells. The function of heat shock protein on the cell surface is not understood, although it has been shown to be greater in some tumor cells and some virally infected cells. Surface expression of both glycoprotein 96 (gp96) and Hsp70 occurs on tumor cells, and this expression correlates with natural killer cell killing of the cells. We examined the surface expression of gp96 and Hsp70 on human breast cell lines MCF7, MCF10A, AU565, and HS578, and in primary human mammary epithelial cells by immunofluorescence microscopy and flow cytometry. The nonmalignant cell lines HS578, MCF10A, and HMEC showed no surface expression of gp96, whereas malignant cell lines MCF7 and AU565 were positive for gp96 surface expression. All of the breast cell lines examined showed Hsp70 surface expression. These results also confirm previous studies, demonstrating that Hsp70 is on the plasma membrane of tumor cell lines. Given the involvement of heat shock proteins, gp96 and Hsp70, in innate and adaptive immunity, these observations may be important in the immune response to tumor cells.

INTRODUCTION

Heat shock proteins (HSPs) are ubiquitous in eukaryotes and accumulate in response to a variety of stresses including hyperthermia, viral infection, glucose deprivation, and oxidative stress (Morimoto 1991; Welch 1993; Moseley 2000). Hsp70 is the most highly heat inducible of the HSPs, and functions in at least 3 capacities: folding newly made proteins, prevention of stress-related protein aggregation, and translocation of proteins across membranes. The expression of stress proteins in a cell correlates with a state of increased tolerance to subsequent, otherwise lethal exposure to these and other stresses. Although the HSPs function primarily in the cytoplasm, several members of the HSP family, including glycoprotein 96 (gp96), Hsp70, Hsp40, and Hsp60, have been reported to be on the plasma membrane surface (Di Cesare et al 1992; Altmeyer et al 1996; Roigas et al 1998). Hsp70 surface expression has been observed on virally infected cells, on biopsy material of colorectal, lung, neuronal, pancreas, oral dysphasia, and squamous cell carcinoma, but not on normal cells (Ferrarini et al 1992; Botzler et al 1996a; Kaur et al 1998). Surface expression of Hsp70 can be increased in some tumor cells by passive hyperthermia, but the amount of cytosolic Hsp70 does not determine the amount of Hsp70 observed on the surface (Multhoff 1997; Roigas et al 1998).

The plasma membrane expression of stress proteins may have relevance to immune surveillance. Hsp70 correlates with MHC-independent natural killer (NK) cell

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cytotoxicity of tumor cells, which can be blocked using specific antibodies to Hsp70 (Botzler et al 1996a, 1996b; Multhoff 1997; Roigas et al 1998). In contrast to Hsp70, the role of surface gp96 in NK cytotoxicity has not been evaluated, but studies showing that secretion of gp96 mediates NK cell expansion suggest similar function (Strbo et al 2002). Neither Hsp40 nor Bag-1 surface expression correlated with NK cytotoxicity (Gehrmann et al 2005). Given the effect of surface expression of heat shock proteins as a potential target of immune surveillance, understanding the heat shock response and factors that control subsequent surface expression of stress proteins may lead to the development of novel strategies to target cells for destruction by the immune system.

In the present study, we used several immunofluorescent techniques to examine the surface expression of the stress proteins Hsp70 and gp96 on human breast cells. We examined both malignant and nonmalignant human breast cells lines and a primary breast cell culture and asked whether a correlation existed between malignancy and Hsp70 and gp96 surface expression. We also sought to determine how surface expression of Hsp70 and gp96 correlated with NK cytotoxicity. Finally, to address the issue of relying on detection by immunofluorescent techniques, we used mass spectrometry to identify the Hsp70 species on the plasma membranes of the human breast cell line AU565.

MATERIALS AND METHODS

Chemicals and reagents

The following reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA): dithiothreitol (DTT), Ready Prep Sequential Extraction Kit Reagent 3, tributylphosphine (TBP), urea, 11-cm immobilized pH gradient (IPG) strips, 10% criterion polyacrylimide gels, and Biosafe Coomassie blue. The following regents were purchased from Sigma (St Louis, MO, USA): RPMI media, fetal bovine serum (FBS), glucose, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), sodium pyruvate, KCl, NaCl, $MgCl_2$, acetic acid, propidium iodide, isotype controls mouse IgG2a, acetonitrile (ACN), trifluoroacetic acid (TFA), trichloroacetic acid (TCA), NH_4HCO_3, sodium dodecyl sulfate (SDS), Tris-HCl, glycerol, iodoacetimide, α-cyano-4-hydroxycinnamic acid, and acetone. Complete mini-protease inhibitor cocktail was purchased from Boehringer Mannheim (Mannheim, Germany). C18 ZipTip Pipette tips were purchased from Millipore (Bedford, MA, USA). Sequazyme Peptide Mass Standards kit was purchased from Applied Biosystems (Foster City, CA, USA). Modified sequencing grade trypsin was purchased from Promega (Madison, WI, USA). Antibodies were purchased from the following: StressGen Biotechnologies (Victoria, British Columbia, Canada) (Hsp70, SPA 822 and SPA 810; gp96 (grp94), SPA 850); Santa Cruz Biotechnologies (Santa Cruz, CA, USA) (epidermal growth factor receptor [EGFR] sc-120); Amersham Biosciences (Piscataway, NJ, USA) (Hsp70, RPN1197); Caltag Laboratories (Burlingame, CA, USA) (anti-rat FITC-conjugated no. R2a00); and Chappel (Aurora, OH, USA) (anti-mouse FITC-conjugated no. 55522).

Cell lines and culture

The human breast adenocarcinoma cell line MCF7 and nontransformed human mammary epithelial cell line MCF10A were gifts from Dr. Scott Burchiel (University of New Mexico, Albuquerque, NM, USA). The human mammary breast cell line HS578, of myoepithelial origin, and the human breast adenocarcinoma cell line AU565 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Human mammary epithelial cells primary culture was obtained from Clonetics (Walkersville, MD, USA). MCF7 cells were grown in modified Eagle's medium with Earls salts with nonessential amino acids, supplemented with 1.0 mM sodium pyruvate and 0.01 mg/mL insulin, and 10% FBS (v/v). AU565 cells were grown in RPMI 1640 medium supplemented with 4.5 g/L glucose, 10 mM HEPES, 1.0 mL of sodium pyruvate, and 10% FBS (v/v). MCF10A cells were grown in 1:1 DMEM:F-12 Ham's media supplemented with 1 mg/mL bovine serum albumin, 1 μg/mL hydrocortisone, 10 ng/mL epidermal growth factor, 5 μg/mL transferrin, 5 μg/mL gentamycin, 5 μg/mL transferrin, 50 μM sodium selenite, 10 μM 3,3′-5-triiodo-L-thionine, 5 μM ethanolamine, 10 mM HEPES, 5 μg/mL amphotericin B solution, and 5% horse serum (HS) (v/v). HS578 cells were cultured in Hybrid Care (ATCC) medium supplemented with 30 ng/mL epidermal growth factor and 10% FBS. Human mammary epithelial cells primary culture were cultured in Mammary Epithelial Cell Growth Medium Bullet kit (Clonetics).

Immunofluorescence microscopy

Cells were grown on coverslips in 6-well plates for 24 hours, and fixed with 2% paraformaldehyde for 15 minutes, and then rinsed 3 times for 10 minutes in phosphate-buffered saline (PBS). Selected cells were then permeabilized with 0.5% Triton X for 10 minutes. All coverslips were incubated with PBS and 1% HS to decrease nonspecific binding. Coverslips were incubated with the primary antibody for 1 hour at room temperature. To detect Hsp70, gp96, and EGFR surface expression, the following antibodies were used: anti-human inducible Hsp70 mouse monoclonal antibody (RPN1197), anti-human inducible and constitutive Hsp70 mouse monoclonal anti-

body (SPA-822), anti-human GRP94 rat monoclonal antibody, and anti-human EGFR mouse monoclonal antibody. Following 3 washes with PBS and 1% HS, the cells were incubated with the anti-mouse goat IgG fluorescein isothiocyanate (FITC) conjugate or anti-rat IgG FITC-conjugated secondary antibodies for 1 hour at room temperature. Coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescence microscopy was carried out on a Zeiss Axioplan 2 with a Hamamatsu ORCA 100 CCD camera interfaced to a Kontron 300 image acquisition and analysis system. Two hundred cells were counted from each experiment to assess surface staining.

NK isolation

The buffy coat was isolated from healthy human blood donors by differential layering over 4% Percoll at 250 × *g* for 30 minutes at room temperature. NK cells were isolated from the buffy layer using the MACS NK cell isolation Kit (Mitenyi Biotec, Auburn, CA, USA). NK cells were incubated in RPMI medium plus 10% FBS (v/v) with 100 U/mL interleukin 2 (IL-2) for 5 days.

NK cytotoxicity assays

NK cytotoxicity was determined by measurement of lactate dehydrogenase (LDH) release using the CytoTox96 Non-Radioacitve Cytotoxicity Assay (Promega). Target cells were pretreated with either anti-Hsp70 or anti-gp96 antibodies for 30 minutes prior to the addition of NK cells.

Hyperthermia

Cells were exposed to nonlethal hyperthermia to induce a heat shock response. Cells were heated at 42.5°C with 5% CO_2 for 3 hours. They were allowed to recover at 37°C with 5% CO_2 for 48 hours.

Membrane separation

Using liquid nitrogen, samples were subjected to a rapid freeze-thaw cycle in lysis buffer A, which consists of 20 mM HEPES-NaOH, 10 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 1 Complete mini-protease inhibitor cocktail tablet. Samples were centrifuged at 15 000 × *g* to separate unlysed cells. The supernatant was centrifuged at 100 000 × *g* for 30 minutes to generate membrane (pellet) and cytosolic (supernatant) fractions. The membrane fraction was resuspended in lysis buffer A.

Concentration of proteins

To clean and concentrate proteins, 100% TCA was added to get a final concentration of 20% TCA. The samples were incubated on ice for 20 minutes and centrifuged at 15 000 × *g* for 20 minutes at 4°C. The pellet was washed once in acetone, and then centrifuged at 15 000 × *g* for 20 minutes at 4°C. The samples were air dried.

Two-dimensional analysis

Samples were dissolved in Ready Prep Sequential Extraction Kit Reagent 3, supplemented with TBP, and then rehydrated overnight on an 11-cm IPG strip, pH 3–10. Proteins were focused on a Multiphor II (Amersham Biosciences) to at least 30 000 V hours. Focusing was 20 minutes at 250 V, and then 20 minutes from 250 to 500 V, and then 2.5 hours from 500 to 8000 V, and then 8000 V until at least 30 000 V hours was achieved. IPG strips were reduced in 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 130 mM DTT for 10 minutes, and then alkylated in 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and iodoacetimide for 10 minutes. The IPG strips were rinsed in running buffer and placed on a 10% acrylamide gel for the second dimension. The proteins were electrophoresed at 200 V for 1 hour. To identify proteins for matrix-assisted laser desorption/ionization (MALDI) analysis, gels were stained with Biosafe Coomassie blue. To recognize which spots on the two-dimensional (2-D) gels were Hsp70, the proteins from duplicate 2-D gels were transferred to a nitrocellulose membrane at 60 V for 2 hours at 4°C. The membrane was blocked for at least 2 hours. The membrane was then washed twice in Tris-buffered saline with Tween 20 (TBST) and once in Tris-buffered saline (TBS). The membrane was incubated with anti-Hsp70 (SPA 822 and 810) to detect both inducible and constitutive Hsp70 molecules. The membrane was washed twice in TBST and once in TBS, and incubated for 30 minutes with anti-mouse IgG alkaline phosphatase–conjugated antibody. The membrane was washed twice in TBST and once in alkaline phosphatase buffer, and then developed with nitroblue tetrazoliun chloride NIB/5-bromo-4-chloro-3-indolyl phosphate.

Trypsin digest

Excised gel spots were washed 3 times in 25 mM NH_4HCO_3/50% ACN for 20 minutes at 37°C. To digest the protein, gel pieces were incubated with 12.5 ng/μL trypsin in 25 mM NH_4HCO_3/10% ACN for 30 minutes at 37°C, and then 25 mM NH_4HCO_3 was added to cover all of the gel and the proteins were incubated overnight at 37°C.

MALDI–time of flight mass fingerprinting of tryptic peptides

Five microliters of peptide digest were bound to a C18 zip tipped and eluted in 2 spots onto a MALDI plate with 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA and allowed to air dry. Peptide spectra were acquired using a Voyager-DE Elite (Applied Biosystems) instrument housed at the Lovelace Respiratory Research Institute (Albuquerque, NM, USA). The instrument was in reflector mode and calibrated using close external standards. Monoisotopic peptides were identified and using MS-FIT program Protein Prospector from University of California, San Francisco (www.ucsf.edu), and the peptide mass-fingerprinting search data were searched against the Swiss-Prot/TrEMBL database.

Acid wash

Cells were collected by trypsinization and incubated on ice for 10 minutes in acid solution of 0.5 M NaCl and 0.2 M acetic acid (pH 2.7), followed by 1 wash in PBS (Haigler et al 1980).

Flow cytometry

Flow cytometric analysis was performed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA, USA). Ranges of 5×10^4 to 1×10^5 cells were placed in a 96-well plate and centrifuged at $400 \times g$ for 5 minutes. Excess medium was removed, and then 10 μg/μL primary antibody for Hsp70, gp96, EGFR, or isotype controls was added to each well. The plate was incubated on ice for at least 30 minutes, and then washed with 50 μL of cell medium plus 2% FBS. Cells were stained with FITC-labeled secondary antibodies and incubated on ice for 30 minutes. Cells were washed once with 1× PBS. The relative mean fluorescence intensity values are calculated as averages of at least 3 independent experiments. Flow cytometry analysis was performed on 10 000 cells for each experiment. Dead cells were detected by negative propidium iodide staining and excluded from the analysis.

Statistical analysis

A 2-way analysis of variance (ANOVA) followed by Student's *t*-test was performed for cell counts, and Student's t test was performed on mean fluorescence intensity using StatView Statistical software (SAS Institute, Cary, NC, USA). One-way ANOVA followed by a multiple comparison against the control group was performed on NK cytotoxicity using SigmaStat software (Janel Scientific, San Rafael, CA, USA).

RESULTS

Characterization of human breast cell Hsp70 and gp96 surface expression

Flow cytometric analysis detected the presence of Hsp70 on the plasma membrane on both malignant and nonmalignant breast cells (Fig 1). In contrast, only the plasma membranes of the malignant cell lines (AU565 and MCF7) were positive for gp96. All cells stained positive for EGFR, which served as the positive control. The data shown in Figures 1–3 were obtained using the Amersham RPN 1197-inducible Hsp70 mouse monoclonal antibody. Similar mean fluorescence values were obtained for each of the Hsp70 antibodies used.

The results of flow cytometry were confirmed by immunofluorescence microscopy studies (Fig 2). The malignant breast cell lines had positive surface staining for both Hsp70 and gp96. The nonmalignant human breast cell HS578 demonstrated Hsp70 plasma surface expression, and very slight surface staining for gp96. The nonmalignant breast cell line MCF10A and the primary HMEC, showed very little to no surface staining for either Hsp70 or gp96.

Cell counts were performed on 200 consecutive cells to determine the percentage of cells with surface staining (Fig 3). Hsp70 surface expression was detected in 70% of the cells for both malignant cell lines, MCF7 and AU565, which is comparable to gp96 surface expression, which was 77% and 66% in MCF7 and AU565 cell lines, respectively.

Fifty-five percent of the nonmalignant human breast cell line, HS578, cells stained positive for plasma membrane surface expression of Hsp70 (Fig 3). In contrast, only 9% stained positive for gp96. This degree of gp96 surface staining was significantly lower than MCF7 and AU565 ($P < 0.05$). Hsp70 was present in a significant percentage of both malignant and nonmalignant cell lines. Twenty percent of the nonmalignant human breast cell line, MCF10A, stained positive for plasma membrane surface Hsp70 (Fig 3). Surface expression of gp96 on MCF10A cells was not detected by fluorescence microscopy. There was also a significant difference between the surface expression of Hsp70 and gp96 within the normal breast cell line, MCF10A ($P < 0.05$). The primary HMEC showed 22% staining positive for plasma membrane surface expression of Hsp70 and 4% staining positive for gp96 (Fig 3) ($P < 0.05$).

Surface expression of Hsp70 and gp96 did not correlate with intracellular levels. Total intracellular levels of Hsp70 and gp96 were detected by Western analysis (Fig 4). The intracellular of Hsp70 varied among the different cell lines, but did not correlate with the amounts seen on the surface or with malignancy. The intracellular levels of gp96 were similar in all cell lines tested.

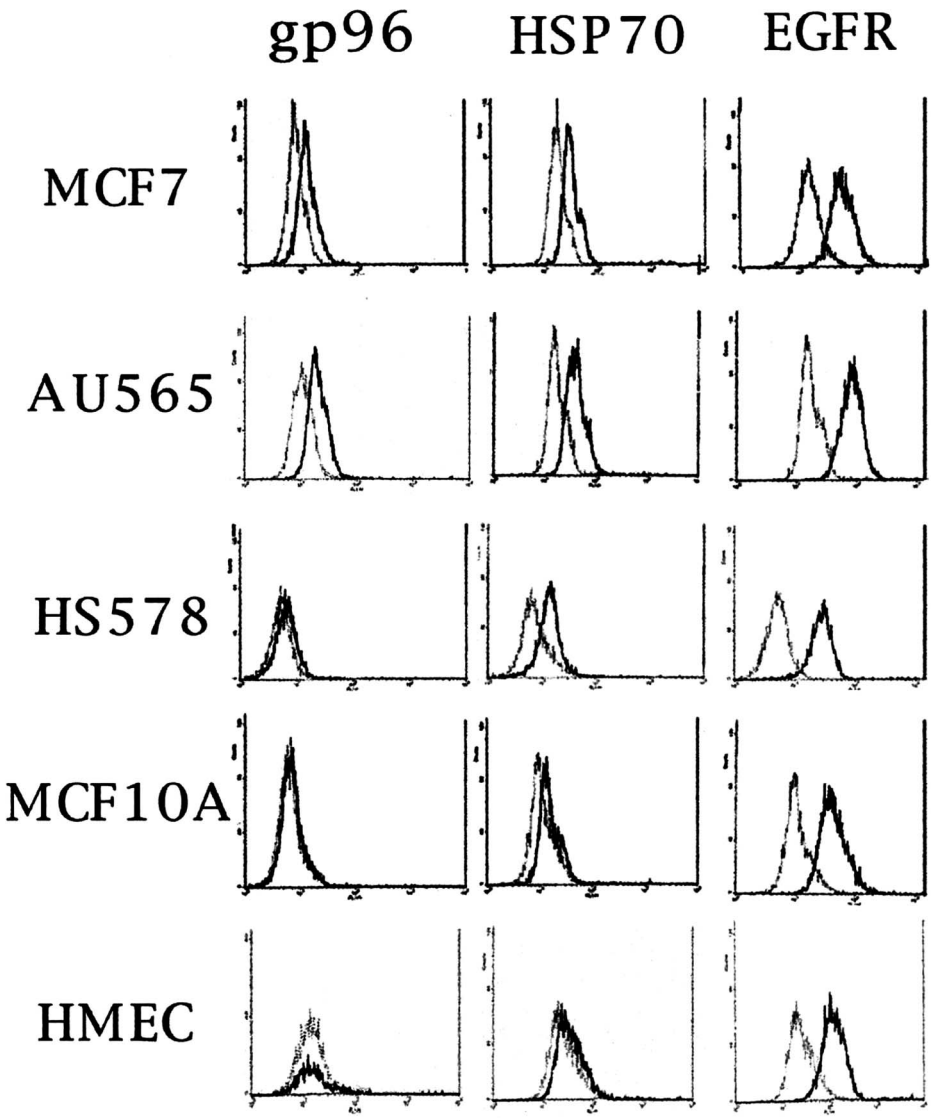

Fig 1. MCF7, AU565, HS578, MCF10A, and HMEC breast cell lines were labeled with Hsp70, gp96, or EGFR antibody and analyzed on a FACScan. The relative fluorescence intensity of FITC-labeled cells (*x*-axis, 4 decade log scale) is plotted vs the relative number of cells. The bold lines represent specific antibody, and the light lines represent cells incubated with secondary antibody only (control). Data shown are representative of 3 independent experiments. EGFR was used as a positive control. The mean fluorescence intensity values for expression of Hsp70 were MCF7 (13.96 ± 6.05), AU565 (22.31 ± 1.33), HS578 (10.81 ± 4.41), MCF10A (27.82 ± 24.95), and HMEC (19.05 ± 3.27). The mean fluorescence intensity values for expression of gp96 were MCF7 (6.96 ± 1.57), AU565 (15.27 ± 6.18), HS578 (1.74 ± 0.69), MCF10A (2.37 ± 1.38), and HMEC (4.09 ± 3.37). The mean fluorescence intensity values for EGFR surface expression were MCF7 (37.94 ± 5.54), AU565 (109.32 ± 18.23), HS578 (8.36 ± 1.31), MCF10A (65.03 ± 17.83), and HMEC (79.35 ± 20.76).

NK cell cytotoxicity correlates with both Hsp70 and gp96 surface expression

Surface levels of Hsp70 have been shown to be correlated with NK cell killing (Roigas et al 1998) in other cell line; this experiment was to determine whether NK activity correlated with surface expression in AU565 and the nonmalignant cell line, MCF10A, cells. The malignant cell line, AU565, showed lysis upon addition of NK cells (Fig 5A). When target cells were incubated with either anti-Hsp70 antibody or anti-gp96 antibody prior to the addition of NK cells, there was a significant decrease in NK cytotoxicity. The nonmalignant cell line also showed lysis upon addition of NK cells (Fig 5B). When target cells were incubated with anti-Hsp70 or anti-gp96, there was a slight decrease in lysis by NK cells.

MALDI–time of flight identification of Hsp70 in the plasma membrane

To verify detection of Hsp70 by immunofluorescence techniques, we used a proteomic approach to detect Hsp70 in the plasma membrane of AU565 cells because these cells showed the highest level of Hsp70 surface expression by flow cytometry. Cellular proteins were separated into cytoplasmic and membrane fractions, before and after exposure to nonlethal hyperthermia. Proteins from each fraction were separated using 2-D electrophoresis (Fig 6). To increase the amount of integral proteins in the membrane fraction, samples were rehydrated using rehydration buffer 3 from Bio-Rad, which contained thiourea, which has been shown to help recover membrane proteins and eliminate cytosolic proteins from sample preparations (Friso and Wikstrom 1999). To identify pro-

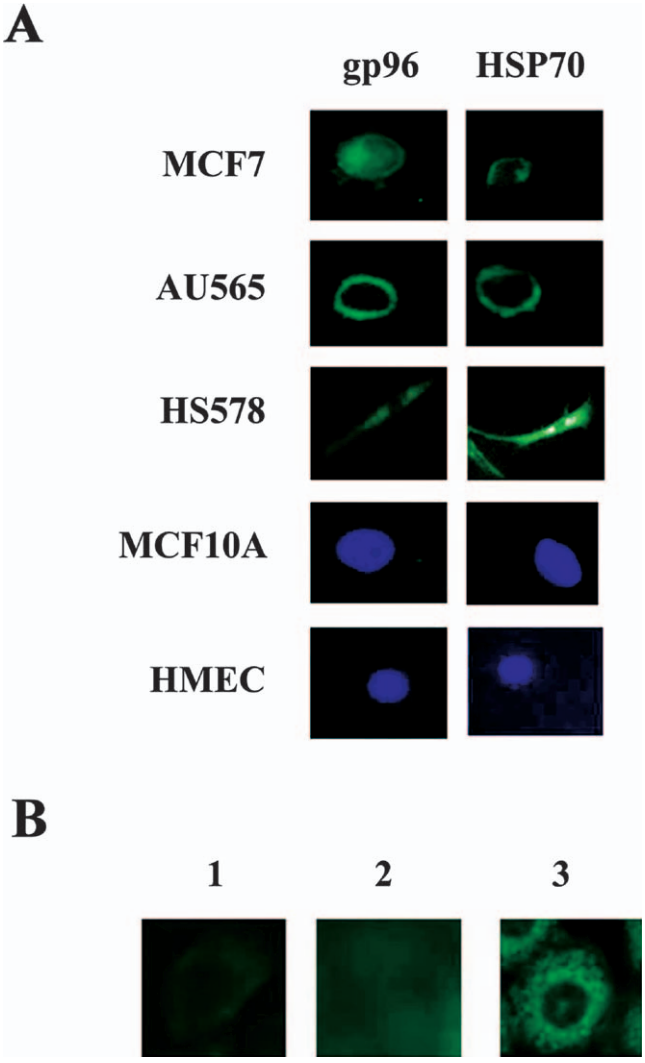

Fig 2. Microscopy analysis of surface expression of Hsp70 and gp96. Representative microscopy pictures of at least 3 independent experiments are depicted. (A) Nonpermeabilized cells were stained with either Hsp70 or gp96. (B) AU565 controls are (1) nonpermeabilized cells stained with actin to determine whether cells were permeabilized to antibodies, (2) nonpermeablized cells stained with secondary antibody for nonspecific labeling, and (3) permeabilized cells stained with Hsp70 to distinguish between surface staining and intracellular staining.

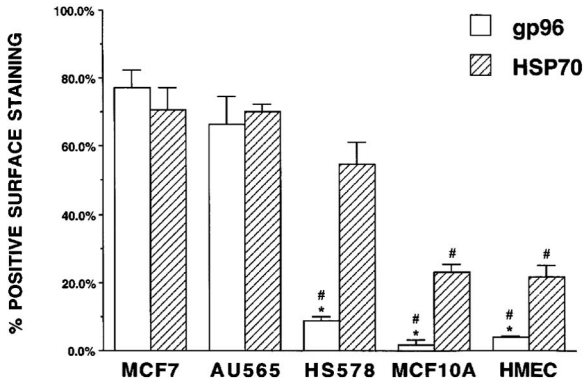

Fig 3. Percentage of positive surface staining of Hsp70 and gp96 in human breast cells MCF7, AU565, MCF10A, HS578, and HMEC. Percentage of MCF7, AU565, HS578, and MCF10A cell lines that showed positive surface plasma membrane staining for gp96 (open bars) and Hsp70 (hatched bars). Data was collected from 3 independent experiments in which 200 consecutive cells were counted. Error bars are the standard error obtained from the 3 independent experiments. The nonmalignant cells MCF10A were significantly different from both the AU565 and MCF7 malignant breast cell line in gp96 and Hsp70 staining. The asterisk (*) denotes significance between Hsp70 and gp96 within cells. The number sign (#) denotes significance from the malignant cells lines AU565 and MCF7.

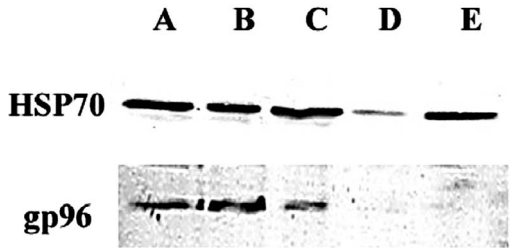

Fig 4. Western analysis of total cellular Hsp70 and gp96 in human breast cells. Whole-cell lysates of human breast cells probed with either anti-Hsp70 or anti-gp96 antibodies to determine total cellular quantity.

teins for analysis by MALDI–time of flight (TOF) mass spectrometry, the spots corresponding to an isoelectric point of 5.5–6.0 and a molecular weight of 70–72 kDa (Fig 6, arrows) were excised from Coomassie-stained gels and digested with trypsin. Mass spectra were acquired using MALDI-TOF for each sample of heated and control cells. Monoisotopic peptide fingerprints were searched against the Swiss protein database and Hsp70 was identified in both the membrane and cytosolic fractions of heated and control AU565, human breast cells (Table 1). To confirm that the proteins excised could be detected by antibody to Hsp70, 2-D gels were transferred to nitrocellulose membranes and exposed to anti-Hsp70 antibody.

Acid washing of Au565 cells

To determine whether Hsp70 was an integral membrane protein or noncovalently bound, AU565 cells were subjected to acid washing. Acid washing of cells strips off proteins that are noncovalently bound, leaving proteins that are tightly bound to the membrane. EGFR, a known integral membrane receptor, was used as the negative control and surface-bound antibodies were used as a noncovalently bound positive control. AU565 cells were subjected to acid washing, and then analyzed by flow cytometry to detect any Hsp70 surface molecules left. The Hsp70 molecule could not be removed from the surface of AU565 cells after treatment with acid (Fig 7). Cells exposed to anti-EGFR antibody were subjected to acid

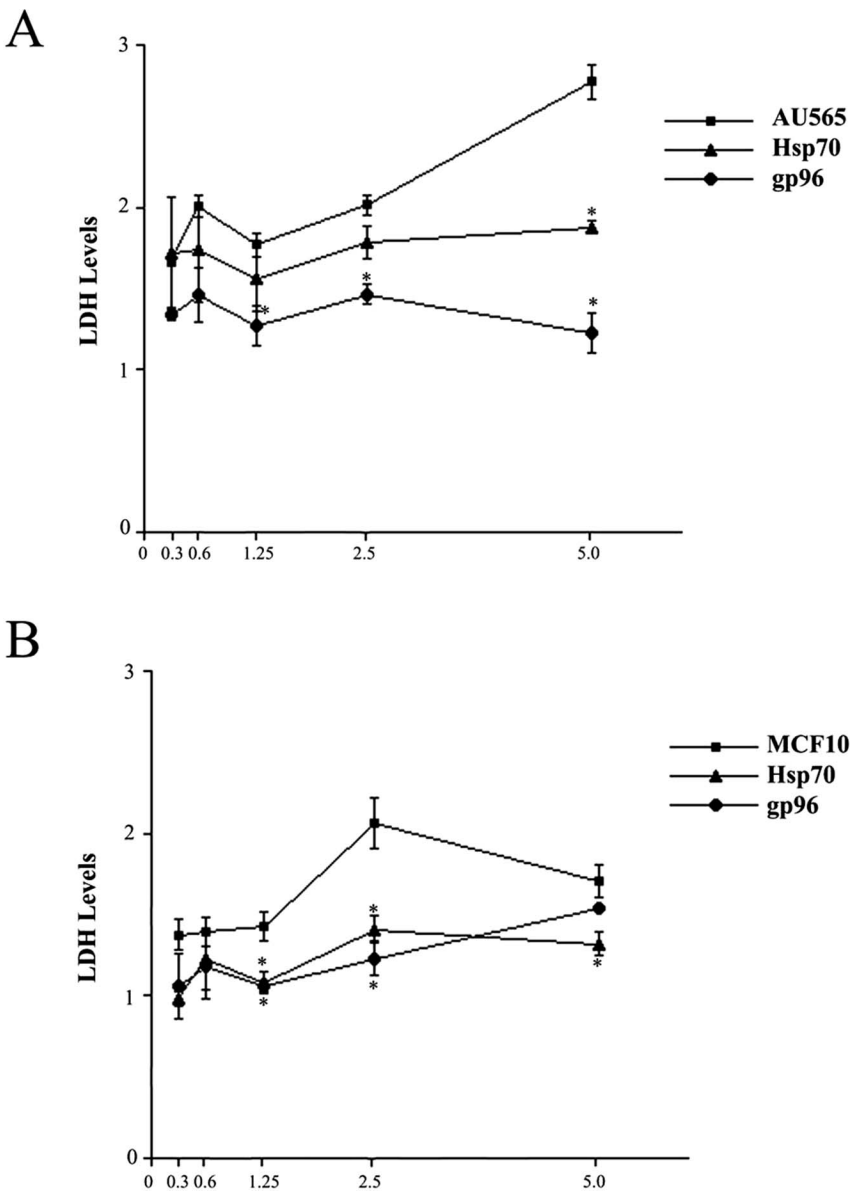

Fig 5. NK cytotoxicity of malignant AU565 cells. NK cytotoxicity was measured by LDH levels after 4-hour incubation of (A) AU565 (diamonds), AU565 cells preincubated with anti-Hsp70 antibody (square), or AU565 cells preincubated with anti-gp96 antibody (triangles) with IL-2-stimulated NK cells. (B) NK cytotoxicity was measured by LDH levels after 4-hour incubation of MCF10A (diamonds), MCF10A cells preincubated with anti-Hsp70 antibody (square), or MCF10A cells preincubated with anti-gp96 antibody (triangles) with IL-2-stimulated NK cells. The asterisk (*) denotes significance from control cells.

washing and anti-EGFR antibody was stripped off, and thus noncovalently bound proteins were stripped off (data not shown).

DISCUSSION

Our results demonstrate that the both malignant and nonmalignant human breast cell lines tested expressed Hsp70 on their surface, whereas only the malignant cell lines expressed gp96. Surface expression of both Hsp70 and gp96 correlated with specific NK-mediated cytotoxicity, which could be blocked with specific antibodies for either Hsp70 or gp96.

These findings both expand and support the observations that Hsp70 can be found on the surface of a variety of different tumor cells (Ferrarini et al 1992; Botzler et al 1996a; Kaur et al 1998) and that Hsp70 on the surface of cells can serve as a target for NK cells (Botzler et al 1998; Roigas et al 1998). This study extends these findings to include a number of commonly used breast cell lines (Bacus 1990). The ability of activated NK cells to target Hsp70 on the surface has been described in several different cancer models, and activation of NK cells by ex vivo Hsp70 is currently being used in clinical trials of colon and lung cancer patients (Krause et al 2004).

Hsp70 on the cell surface has been studied in a number of different cell lines but the cell surface characteristics and immune functions of gp96 have received much less attention despite the fact that both appear to be have important functions in immune signaling and peptide pre-

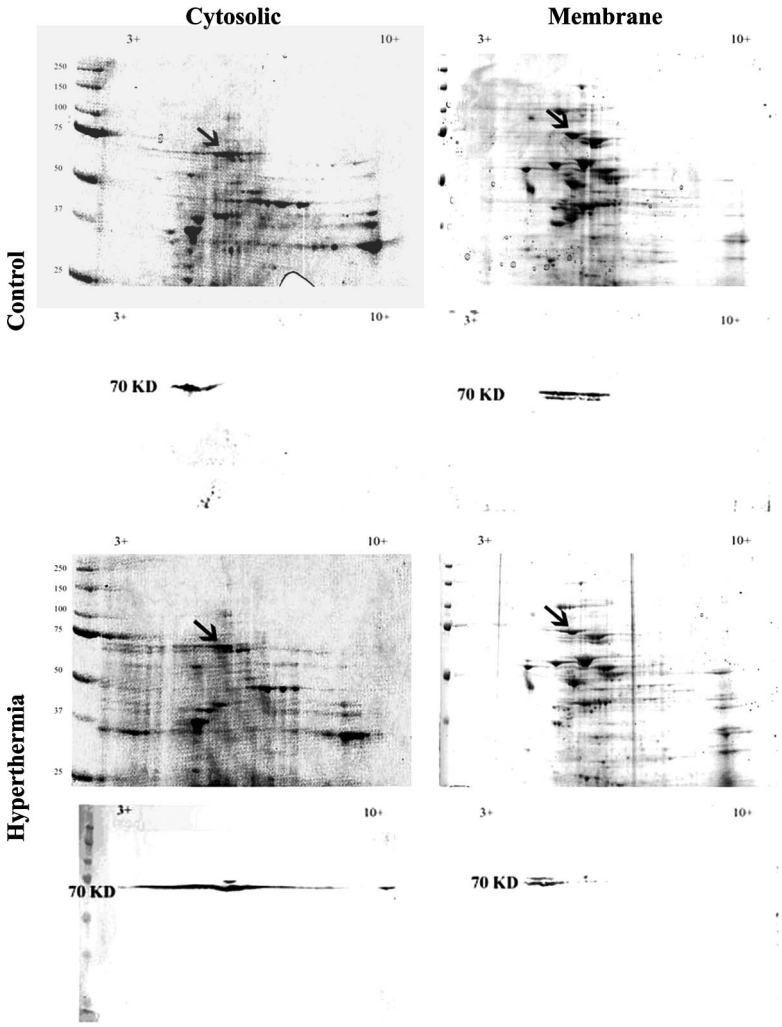

Fig 6. 2-D analysis of the membrane and cytosolic fractions of human breast cells, AU565. AU565 cells were subjected to hyperthermia to increase intracellular levels of Hsp70. The samples were fractionated to obtain cytosolic and membrane fractions. Each protein fraction was separated by 2-D gel electrophoresis. Protein spots were excised and identified by MALDI-TOF mass spectrometry. The arrows point to those spots that were identified as Hsp70; associated Western blots for each experiment are also shown.

Table 1 Identification of protein from AU565 cells using peptide maps against the MS-FIT program in the Swiss-Prot/TrEMBL database

Sample	Fraction	Protein identification
Control	Cytosolic	Heat shock cognate 71-kDa protein
Control	Membrane	Heat shock 70-kDa protein
Heated	Cytosolic	Heat shock cognate 70-kDa protein
Heated	Membrane	Heat shock 70-kDa protein

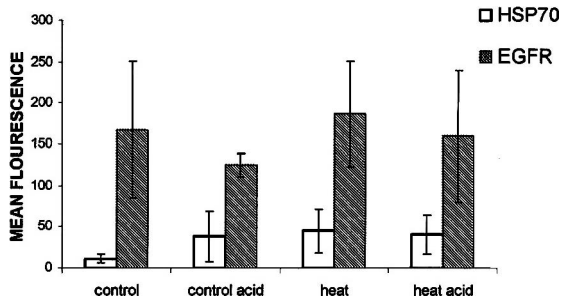

Fig 7. Flow cytometry analysis of AU565 human breast cancer cells treated with acetic acid. Mean fluorescence values of Hsp70 (open bars) and EGFR (hatched bars) plasma surface expression are presented before and after acid washing. Data were collected from 3 independent experiments. The error bars denote the standard error.

sentation to antigen-presenting cells (Srivastava et al 1990; Udono and Srivastava 1993; Srivastava 2002). Intracellular levels of Hsp70 are regulated differently in different kinds of tumors. In a study of breast cancer patients, intracellular Hsp70 levels were found to be lower in patients with metastatic breast cancer, and it was speculated that the metastatic cells may have down-regulated Hsp70 in order to elude immune surveillance (Torronteguy et al 2006). Surface levels of Hsp70 can be influenced by certain chemotherapeutics (Botzler et al 1999); the surface expression of gp96 under various chemical conditions has not been identified.

gp96 has been found on the surface of some tumor cells. Altmeyer et al (1996) found gp96 on the surface of mouse Meth-A sarcomas but not on the surface of normal fibroblasts. gp96 on the surface of cells is capable of sig-

naling the immune system. Zheng et al (2001) used genetic engineering to create cells that express gp96 on their surface. They found that these cells were capable of inducing maturation of dendritic cells and the subsequent release of proinflammatory cytokines IL-1β and IL-2.

Because gp96 is an important immune-stimulatory and signaling molecule, it may be important to determine whether it possesses the ability to signal and activate immune cells in a manner similar to, or complementary to, the function of Hsp70 on the cell surface. The finding that the malignant cell lines tested in this study have increased levels of gp96 on their surface suggests that methods to specifically target this molecule might be useful, especially because gp96 may have a different pattern of receptors than Hsp70 (Binder et al 2004). The results of this study show that, in this limited sample of breast cell lines tested, surface expression of gp96 is different for malignant and nonmalignant cells and that surface expression of gp96 on malignant cells may have therapeutic implications.

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