Expression of heat shock protein 90 at the cell surface in human neuroblastoma cells

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Abstract In addition to the activity of heat shock protein 90 (Hsp90/HSPC) as a chaperone, some recent studies have reported expression of Hsp90 at the cell surface in certain types of cancer and nervous system cells. We study the expression of Hsp90 at the cell surface in human neuroblastoma (NB69) cells. Immunofluorescence experiments labeling with anti-Hsp90 antibodies on both nonpermeabilized cells and live cells detected Hsp90 at the cell surface. Hsp90 was also identified in a membrane fraction from subcellular fractionation. Cell-surface Hsp90 was significantly more expressed in undifferentiated proliferative spherical neuroblastoma cells than in differentiated flattened cells. In addition, spherical cells were significantly more sensitive to Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin compared to flattened cells. This paper describes the first evidence of cell-surface Hsp90 expression in a cancer cell line from nervous tissue and may indicate a novel target for anti-tumoral agents.

Keywords Hsp90 expression · Cell surface · Neuroblastoma · Hsp90 inhibitors · 17-allylamino-17-demethoxygeldanamycin

Abbreviations

Hsp90 heat shock protein 90
17-AAG 17-allylamino-17-demethoxygeldanamycin
OPCs oligodendrocyte precursor cells

Introduction

Heat shock protein 90 (Hsp90) is a conserved molecular chaperone that is essential for posttranslational control, as part of a multichaperone complex whose association is required for the stability and function of multiple mutated, chimeric, and overexpressed signaling proteins that promote cancer cell growth, differentiation, and apoptosis. Hsp90 client proteins comprise several key client proteins, including some that are oncogenic, such as mutated p53, ErbB2/Her2, Raf-1, cyclin-dependent kinase 1 and 4, Akt/PKB, Bcr-abl, or Hif-1 α (Zhang et al. 2007; Neckers 2007). In this paper, Hsp90 is used to designate human HSPC (Kampinga et al. 2008).

In addition to the activity of Hsp90 as a chaperone, some studies have reported expression of Hsp90 at the cell surface in certain types of cancer cells (reviewed in Tsutsumi and Neckers 2007) and in different cells from the nervous system (Kakimura et al. 2002; Cid et al. 2004; Sidera et al. 2004), which suggests an additional participation of Hsp90 in important processes, such as cell migration and maturation. The role of cell-surface Hsp90 in tumor cells is unknown, although it has been correlated with cancer metastasis and

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migration of malignant cells (Tsutsumi and Neckers 2007). We have recently demonstrated that Hsp90 β is expressed at the cell surface in oligodendrocyte precursor cells (OPCs), but not when these OPCs have differentiated into preoligodendrocytes (Cid et al. 2004, 2005). These results, in addition to the expression of Hsp90 at the cell surface in microglial, cerebellar, and Schwann cells (Kakimura et al. 2002; Sidera et al. 2004), suggest that cell-surface Hsp90 may have a role in the differentiation and development of certain types of cells in the nervous system.

One type of malignant cell in the nervous tissue is neuroblastoma cells. To date, no information about the expression of Hsp90 at the cell surface is available (Tsutsumi and Neckers 2007). We considered it of interest to study the possible expression of Hsp90 at the cell surface in live human neuroblastoma cells. In addition, Hsp90 inhibitors have emerged as potent anti-tumoral agents (Kamal et al. 2003). Among these, the geldanamycin-derived compound 17-allylamino-17-demethoxygeldanamycin (17-AAG) is currently in phase I and II clinical trials (Zhang et al. 2007; Bagatell et al. 2007). We therefore also investigated the potential cytotoxicity of this inhibitor in cultured human neuroblastoma cells. This paper describes for the first time the expression of Hsp90 at the cell surface in neuroblastoma cells. The expression of cell-surface Hsp90 has been described in some cancer cell lines (Tsutsumi and Neckers 2007) but not in neuroblastoma. Because the expression of cell-surface Hsp90 has previously been related to cell motility and cancer metastasis (see above), we consider this first evidence of cell-surface Hsp90 expression in a cancer cell line from nervous tissue to be of great interest.

Materials and methods

Reagents

Anti-Hsp90 antibodies: mouse monoclonal anti-Hsp90(α/β) (clone AC88, Calbiochem) and rabbit polyclonal anti-Hsp90 (β) (Hsp84, Chemicon) were used. The secondary antibodies used were fluorescein-conjugated goat anti-mouse IgG antibody and lissamine rhodamine-conjugated goat anti-rabbit IgG antibody (both from Chemicon). Monoclonal anti-S6 ribosomal protein antibody was supplied by Cell Signaling, Danvers, MA, USA. 17-AAG was supplied by Sigma, St. Louis, MO, USA.

Human neuroblastoma cell culture

Human neuroblastoma cell line NB69 (European Collection of Cell Cultures, ECACC, no. 99072802) was cultured at 37°C in a 6.5% CO₂ atmosphere in high-glucose Dulbecco's

medium supplemented with 15% heat-inactivated (56°C for 30 min) fetal calf serum. Human neuroblastoma cells were seeded on glass coverslips precoated with 0.05 mg/ml poly-D-lysine or in 35-mm-diameter plastic wells at 3×10^5 cells/ml and cultured for 3 days before use.

Immunofluorescence assay in fixed and live cells and laser confocal microscopy

Neuroblastoma cells cultured on glass coverslips for 3 days were fixed with 3.7% paraformaldehyde for 10 min and incubated for 3.5 h in a blocking solution (10% heat-inactivated fetal calf serum in phosphate-buffered saline). Fixed cells were incubated for 1 h at room temperature with the primary antibody (monoclonal anti-Hsp90). They were then exposed to the corresponding fluorochrome-conjugated secondary antibodies for 1 h at room temperature. The coverslips were mounted with anti-fade solution containing 30 μ M Hoechst 33342 to stain the cell nuclei. Immunofluorescence was visualized by laser scanning confocal microscopy (MRC-1024, Bio-Rad, Hercules, CA, USA) and a transmission light detector was used to phase-contrast images, as previously described (Alcázar et al. 2000; Cid et al. 2004).

Neuroblastoma cells were also labeled for Hsp90, labeling live cells with anti-Hsp90 antibodies. Living cells cultured on coverslips for 3 or 8 days were incubated with polyclonal anti-Hsp90 β antibodies for 30 min at 25°C and labeled with the fluorescent-conjugated secondary antibody for 1 h at 4°C, as previously described (Cid et al. 2004, 2005). The cells were then fixed and mounted as described above. Laser scanning confocal microscopy (MRC-1024; Bio-Rad) was used to visualize neuroblastoma cells labeling with anti-Hsp90 antibodies. Experiments omitting the primary antibodies were performed to test the background. The confocal fluorescence images presented in this paper are 11 optical sections merged to form one image. Sections (1 μ m each) were collected in each laser line channel. Fluorescence intensity was quantified in each channel separately using LaserSharp (Bio-Rad) software.

Subcellular fractionation

Subcellular fractionation was achieved as previously described (Cid et al. 2004), using a procedure similar to that described by Hovanessian et al. (Hovanessian et al. 1987). Briefly, cultured cells were lysed in a hypotonic buffer and the lysate centrifuged at 500 $\times$ g for 10 min. The supernatant was further centrifuged at 12,000 $\times$ g for 20 min to obtain a 12,000 $\times$ g cytosolic fraction (S12) and a mitochondrial pellet (P12). The 500 $\times$ g pellet was resuspended in a buffer containing 0.5% vol/vol Triton X-100 and centrifuged to

separate the detergent-extracted membrane fraction (M) and the nucleus pellet (N). The level of cytosolic contamination in the membrane fraction preparations was analyzed by Western blot using monoclonal anti-S6 ribosomal protein antibody.

Western blot

Subcellular fractions samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis with 5–12% acrylamide (2.6% C) discontinuous gels. The gels were blotted and the membranes incubated with polyclonal anti-Hsp90 β antibodies (1.0 μ g/ml) or with monoclonal anti-S6 antibody (1:1,000) for 1 h at room temperature. The membranes were then incubated at room temperature for 1 h with peroxidase-conjugated goat anti-species IgG antibody (GE Healthcare, Chalfont St. Giles) and developed with ECL reagent (GE Healthcare).

Cytotoxicity assay

Cells were cultured in 35-mm-well plates for 3 days before the addition of increasing concentrations of 17-AAG, and they were then incubated for 5 days. Cell death was calculated by comparing the number of cells after treatment to that in control experiments. The cells counted by phase-contrast microscopy were all intact, and showed a full cell body. At least 100–200 cells from nine (three by three) or more fields per well were counted in three or more separate experiments run in duplicate. Each experiment was performed with a different culture. Two independent observers counted the cells in each experiment. Data for induced cell death were expressed as the number of cells or as a percentage with respect to untreated control cells, which represent the 100% value.

Results

Expression of Hsp90 at the cell surface of human neuroblastoma cells

To investigate if Hsp90 is expressed on the cell membrane, human neuroblastoma cells were not permeabilized and incubated with monoclonal anti-Hsp90 antibody in an immunofluorescence assay. Because Hsp90 is mainly expressed in the cytosol, neuroblastoma cells were fixed but not permeabilized to detect Hsp90 at an extracellular membrane location. The paraformaldehyde fixation method used without a permeabilization procedure keeps the cells intact and allows immunostaining only on the surface (Harlow and Lane 1999). Thus, cells fixed in 3.7%

paraformaldehyde did not allow the entry of a nonpermeable fluorescent dye (tetramethylrhodamine-conjugated dextran 10 kDa, Molecular Probes4, Eugene, OR, USA), probing the integrity of extracellular membrane in these fixed cells. We found that human neuroblastoma cells were labeled with anti-Hsp90 antibody in the cell membrane (Fig. 1). The Hsp90 detected in confocal immunofluorescence sections was shown to have an extracellular membrane location without label in the cell body (Fig. 1A–B). No colocalization between nucleus and the label for Hsp90 was observed (Fig. 1C–D). The cells positive for anti-Hsp90 antibodies were $57 \pm 13\%$ of the total cell population, leaving a cell subpopulation that was not labeled. Because Hsp90 is mainly expressed in the cytosol, this fact demonstrated that cytosolic Hsp90 was not labeled.

To confirm the expression of Hsp90 on the cell surface, Hsp90 was labeled on live neuroblastoma cells. Living cultured cells were incubated with polyclonal anti-Hsp90 β antibodies and labeled with the fluorescent secondary antibody. Subsequently, cells were fixed and visualized under fluorescence confocal microscopy. Human neuroblastoma cells were labeled on live cells for Hsp90 (Fig. 2). In accordance with the results described above, the label for Hsp90 was observed in top sections of confocal images, corresponding to a cell surface localization (Fig. 2B). This is the first evidence of cell surface expression of Hsp90 in human neuroblastoma cells.

In addition, subcellular fractionation was carried out to corroborate the Hsp90 expression in cell membrane. Detergent-extracted membrane fraction and cytosolic, mitochondrial, and nuclear fractions were obtained and Hsp90 was identified by Western blotting. Hsp90 was detected mainly in the cytosolic fraction, as expected, and was also detected in the membrane fraction (Fig. 3), confirming the expression of Hsp90 in the cell membrane. The cytosolic S6 ribosomal protein was only detected as a trace in the membrane fraction, demonstrating the specificity of this fraction (Fig. 3).

Cell-surface Hsp90 was significantly more expressed in spheroids cells

A relationship was found between cell morphology and cell-surface Hsp90 label. Neuroblastoma cells can grow in aggregates, as well as in individual attached cells. These two cell types have different morphology and can be distinctly observed in the confluent phase of growth. Cells in aggregates appear as spheres and occur often in the cell division phase, whereas when the cells are attached to the dish, they differentiate into flattened cells (Fig. 2A). Interestingly, Hsp90 expression on the cell surface was detected in $84.7 \pm 1\%$ of the cells with spherical morphology

Fig. 1 Immunofluorescence of human neuroblastoma cells labeled for Hsp90. Cells were cultured for 3 days and then fixed. Nonpermeabilized cells were labeled in the cell membrane with monoclonal anti-Hsp90 antibody and fluorescein-conjugated secondary antibody. Cell nuclei were stained with Hoechst. Cells were simultaneously visualized by confocal microscopy using transmission light, green laser line, and blue channels for phase contrast (A), fluorescein-labeled Hsp90 (B), and nuclei detection (C) images, respectively. D Green and blue fluorescence images are overlapped. Scale bar in micrometers

(Fig. 2B, red arrows), whereas cell-surface Hsp90 was only detected in $20.3 \pm 6\%$ of the flattened cells (Fig. 2B, white arrows) ($p < 0.001$), as shown in experiments on live cells. This significant difference was also found when the fluorescence intensity of anti-Hsp90 antibodies in labeled cells was quantified in each type of cell. Thus, the mean of fluorescence intensity of the label for Hsp90 was 77 ± 6 compared to 136 ± 12 per labeled cell ($p = 0.0021$) for flattened and spherical cells, respectively (Fig. 2C).

Hsp90 inhibitor 17-AAG induced higher cytotoxicity in spheroids neuroblastoma cells

We studied the effect of the specific Hsp90 inhibitor 17-AAG on our cell cultures. Neuroblastoma cells from 3-day-old cultures were treated with increasing concentrations of 17-AAG, from 0 (vehicle, as control) to 100 nM, and were incubated for 5 days. 17-AAG was a potent cytotoxic agent for human neuroblastoma cells, inducing significant cell death at concentrations higher than 30 nM (Fig. 4A–C). Treatment with 60 nM induced cell death in 92% ($p = 0.0005$) of the total cell population, with an IC_{50} of $39 \pm$

2 nM (Fig. 4C and D, black line). Interestingly, 10 nM 17-AAG induced significant cell death only in spherical cells (Fig. 4D, red line), and at 30 nM of the inhibitor, both cell aggregates and intact spherical cells could not be observed (Fig. 4B), extinguishing this cell population. The IC_{50} values of 17-AAG were 15 ± 2 and 42 ± 3 nM for spherical and flattened cells, respectively (Fig. 4D, red and blue lines), indicating a higher sensitivity of spherical cells to 17-AAG.

Discussion

This study demonstrated the expression of Hsp90 at the surface of human neuroblastoma NB69 cells by three different experimental results: the label for Hsp90 on nonpermeabilized cells, labeling living cells with anti-Hsp90 antibodies, and the detection of Hsp90 in membrane fraction from a subcellular fractionation. Analyzing the morphology of neuroblastoma cells in culture, Hsp90 was significantly more expressed in cells with spherical morphology than in flattened cells. Neuroblastoma cells

Fig. 2 Hsp90 detection at the cell surface on live human neuroblastoma cells. Living neuroblastoma cells from 8-day cultures were labeled with polyclonal anti-Hsp90 β antibodies and rhodamine-conjugated secondary antibody. Cells were subsequently fixed and labeled cells were detected. Cells were simultaneously visualized by confocal microscopy using transmission light and red laser line channels for phase contrast (**A**) and rhodamine-labeled Hsp90 (**B**) images, respectively. The figure is a representative result. Scale bar in micrometers. The fluorescence intensity of anti-Hsp90 antibodies in labeled cells was quantified in flattened (white arrows) and spherical (red arrows) cells, and data (**C**) were expressed as mean pixel intensity in gray scale (0–255 values) corrected for the background. Fifty spherical cells and 90 flattened cells per sample were averaged in three independent experiments. Double asterisks, $p < 0.01$, compared to flattened cells by Student's t test

possessing spherical morphology have been described as characteristic of immature proliferative cells (also called neuroblasts), whereas flattened attached cells have been described as differentiated neuroblastoma cells (Morgan and Seeds 1975; Zucco et al. 1975). In addition, our results show a link between the expression of Hsp90 at the cell surface and the proliferation of neuroblastoma cells.

We have previously demonstrated that Hsp90 β is expressed at the cell surface in OPCs in both perinatal and adult cell cultures, but not when OPCs differentiate into oligodendrocytes (Cid et al. 2004, 2005). It is of interest that OPCs are proliferative cells and that most of them differentiate into postmitotic oligodendrocytes that do not proliferate (Temple and Raff 1986; Roy et al. 1999). Therefore, Hsp90 is present at the cell surface at the proliferative stage in both OPCs and neuroblastoma cells. This finding supports the suggestion that cell-surface Hsp90 has an important role in differentiation in certain types of nervous tissue cells.

Neuroblastoma is one of most common extracranial solid tumors of infancy and childhood. We studied the expression of Hsp90 at the surface of human neuroblastoma cells in an

effort to advance our knowledge of nervous tissue tumor cells. In addition, we also studied the effect of the Hsp90 inhibitor 17-AAG on these cells because geldanamycin and its derivatives are specific inhibitors of Hsp90 and have been

Fig. 3 Subcellular fractionation detected Hsp90 in membrane fraction. Subcellular fractions: detergent-extracted membrane fraction (M), 12,000 $\times$ g cytosolic fraction (S12), mitochondrial fraction (P12), and nuclear fraction (N). Samples (60 μ g each) were subjected to Western blot and the blotted membrane was detected using polyclonal anti-Hsp90 β antibodies (top panel), and the membrane was then reprobbed with monoclonal anti-S6 ribosomal protein antibody (bottom panel). The figure is a representative result. The positions of relative molecular weight standards (in thousands) are indicated

Fig. 4 Hsp90 inhibitor 17-AAG induced human neuroblastoma cell death. Cells cultured for 3 days were untreated (control) or treated with Hsp90 inhibitor 17-AAG for 5 days. Phase contrast microscopy of control (A) and treated cells with 30 nM (B) or 60 nM (C) 17-AAG. Scale bar in micrometers. **D** Dose-response curve of cytotoxicity induced by 17-AAG. Total cells (squares, black line), spherical cells (triangles, red line), and flattened cells (inverted triangles, blue line) were counted in untreated control and treated cells. The number in control cells, 98 ± 17 , 11 ± 3 , and 86 ± 19 per field, was considered as 100% for total, spherical, and flattened cells, respectively. The results were obtained from four independent experiments; error bars indicate standard error. Single asterisks, $p < 0.05$; double asterisks, $p < 0.01$; triple asterisks, $p < 0.001$, compared to respective 100% control by one sample Student's *t* test

described as potent anti-tumor agents in certain tumor cells (Kamal et al. 2003; Zhang et al. 2007), including some human neuroblastoma cell lines (Kim et al. 2003; Kang et al. 2006), but the neuroblastoma NB69 cell line has not yet been studied. Hsp90 inhibitor 17-AAG was a potent cytotoxic agent for human neuroblastoma NB69 cells, inducing cell death at concentrations ranging from 10 to 60 nM. These inhibitor concentrations were in the described range for SH-SY5Y cells (Kim et al. 2003) and other tumor cell types with higher binding affinity to 17-AAG (Kamal et al. 2003). Interestingly, proliferative spherical cells were significantly more sensitive to 17-AAG compared to differentiated flattened cells. The IC₅₀ value of 17-AAG for spherical cells, 15 ± 2 nM, was significantly lower compared to 42 ± 3 nM for flattened cells ($p = 0.0017$). This result was coincident with the increased expression of cell-surface Hsp90 in these spherical neuroblastoma cells. It has been reported that geldanamycin induces apoptosis in human neuroblastoma cells SH-SY5Y, LAN-1, and SK-N-SH (Kim et al. 2003; Shen et al. 2007). Hsp90 inhibitor 17-AAG, at the

described nanomolar concentrations, also induced cell death by apoptosis in cultured neuroblastoma NB69 cells (not shown).

The potential role of cell-surface Hsp90 in cancer-cell proliferation has recently been highlighted (Tsutsumi and Neckers 2007; Sidera and Patsavoudi 2008). The results of the present study demonstrate the cell-surface expression of Hsp90 in neuroblastoma NB69 cells, particularly in proliferative cells. This study has also confirmed the effectiveness of 17-AAG in inducing cell death in human neuroblastoma cells since those proliferative cells are more sensitive to the inhibitor. These results indicate a target for developing new inhibitors or agents with potential cytotoxicity for tumor cells and suggest new therapeutic strategies that target cell-surface Hsp90 to control tumor-cell proliferation, such as antibodies or nonpermeable Hsp90 inhibitors.

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