Downstream caspases are novel targets for the antiapoptotic activity of the molecular chaperone Hsp70

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Abstract The response of cancer cells to apoptosis-inducing agents can be characterized by 2 opposing factors, the proapoptotic caspase cascade and the antiapoptotic stress protein Hsp70. We show here that these factors interact in U-937 leukemia cells induced to apoptosis with anticancer drugs, etoposide and adriamycin (ADR). The protective effect of Hsp70 was verified using 2 approaches: mild heat stress and transfection-mediated overexpression of the Hsp70 gene. The increase in Hsp70 levels attained by these 2 methods was found to postpone caspase activation for 12–18 hours. An *in vitro* assay was developed using mouse myeloma NS0/1 cells, which lack the expression of Hsp70. Measurement of DEVD-ase activity in extracts of apoptotic NS0/1 cells incubated with purified Hsp70 showed that Hsp70 reduced caspase activity by up to 50% of its control value in a dose-dependent manner. The hypothesis that the inhibitory effect of Hsp70 on caspase-3/7 activity related to a direct interaction between Hsp70 and the caspases was tested by reciprocal immunoprecipitations and Far-western analyses. These tests were performed with extracts of Hsp70-overexpressing, control, and ADR-treated U-937 cells and using anti-caspase-3, caspase-7, and anti-Hsp70 antibodies, and the data clearly showed that Hsp70 was able to interact with the proforms of these caspases in cell lysates and with reconstituted purified proteins but did not bind the activated forms of either caspase-3 or -7. This association was also corroborated by a novel, enzyme-linked immunosorbent assay–like assay, protein interaction assay, that combined the advantages of immunoprecipitation and immunoblotting in a 96-well microplate–based assay. Thus, Hsp70 may act to suppress caspase-dependent apoptotic signaling through binding the precursor forms of both caspase-3 and caspase-7 and preventing their maturation.

INTRODUCTION

A perfect antitumor drug should kill cancer cells without affecting normal tissue, and the best way to achieve this is to stop the proliferation of tumor cells. As a result of growth arrest the cells undergo irreversible changes leading to redifferentiation or apoptosis (or both) (Vaux and Strasser 1996). A number of anticancer drugs include 2 chemicals, etoposide (ETO) and adriamycin (ADR), that stop cell growth through their effect on deoxyribonucleic acid (DNA) structure and inhibition of topoisomerase II activity. Apoptosis induced by these drugs shows all the hallmarks of the process, including the appearance of cy-

tochrome *c* in the cytoplasm, activation of caspase cascades leading to the fragmentation of chromatin, and the formation of apoptotic bodies (Mashima et al 1995; Perez et al 1997). The proapoptotic activity of caspases is established as a cascade or a chain of consequential proteolytic cleavages, so that the individual enzyme may cleave itself or other proteins like actin, poly(adenosine diphosphate)-polymerase (PARP); among these targets is a caspase-activated deoxyribonuclease (DNase) whose activation leads to the oligonucleosomal dissociation of chromatin (Kumar and Colussi 1999). Depending on their position in the cascade, caspases can be divided in 2 groups, upstream (caspase-2, -8, -9, and -10) and downstream, effector caspases (3, 6, 7, and 14). Although the function of these “apoptotic proteases” is not well established, caspase-3 and -7 appear to be ubiquitous and the most important effector

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caspases for the execution of apoptosis. In normal cells, caspases are expressed in a nonactive form with a mass of 30–50 kDa, which are named procaspases. All these possess an N-terminal prodomain of varying length, a large-subunit domain, a linker fringed by aspartic residues, and small-subunit domain. Maturation of a caspase starts with the dissociation of its own prodomain and linker and proceeds autocatalytically or under the action of another caspase (Creagh and Martin 2001). It has been suggested that activated caspases specifically cleave proteins essential for cell survival and for the modification of DNases responsible for oligonucleosomal dissociation of chromatin.

Among the array of known antiapoptotic molecules some of the most potent ones appear to be Bcl-2, nuclear factor- κ B (NF- κ B), and Hsp70. The last is known to possess 2 major properties, chaperone activity and cellular protective function. The chaperone activity is the ability to bind damaged or newly synthesized polypeptides and transport them across intracellular membranes, expose them to protein-modifying systems, or both (Morimoto et al 1997). In this activity, Hsp70 is accompanied by other proteins, the so-called cochaperones. Hsp70 was shown to form a potent and well-conserved cellular protective system, which has been verified in numerous experiments *in vitro* and *in vivo* (Jäättelä 1999). This protection appears to be safe for normal cells, and a research goal was to find novel inducers of Hsp70 in a whole organism. On the other hand, the high level of Hsp70 expression in cancer cells is an obstacle for many types of antitumor therapy, and high levels of Hsp70 appear to serve as a poor prognosis for a few types of cancers (Ciocca et al 1993; Ricaniadis et al 2001). Hsp70 was shown to rescue cells from several factors inducing apoptosis, such as tumor necrosis factor- α (TNF- α), staurosporine, heat stress, and to restrain the process of apoptosis at several different steps (Samali and Orrenius 1998). First, Hsp70 was shown to inhibit stress kinases, and this function was attributed to its chaperone activity (Mosser et al 2000). Second, 2 groups have demonstrated that the protein was able to bind APAF-1, and this binding prevented the formation of apoptosomes (Beere et al 2000; Saleh et al 2000). The most convincing evidence for the antiapoptotic function of Hsp70 came from experiments in which MCF-7 breast carcinoma cells were depleted of the protein by the use of antisense messenger ribonucleic acid (mRNA), and transfected cells were shown to enter apoptosis spontaneously (Nylandsted et al 2000).

The present study was performed to answer the following questions: first, does overexpression of Hsp70 inhibit the apoptosis-inducing activity of anticancer drugs, and second, can this inhibition be attributed to a direct binding of effector, “downstream” caspases by Hsp70.

MATERIALS AND METHODS

Materials

Culture medium and fetal calf serum were purchased from Gibco (BRL, Carlsbad, CA, USA) and Paneco (Moscow, Russia); reagents for electrophoresis and secondary antibodies conjugated with peroxidase or with Cy3 dye were from Sigma (St. Louis, MO, USA), and Jackson Immunochemicals (West Grove, PA, USA), respectively. DEVD-ase (caspase-3/7) chromogenic substrate was purchased from Calbiochem (Nottingham, UK). Reagents for electrophoresis, immunoblotting, and immunoprecipitation were from Amersham/Pharmacia (Chalfont St. Giles, UK).

Cells and treatments

Human myeloid leukemia U-937 cells were grown in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal calf serum. To induce accumulation of Hsp70, the cells were subjected to heat stress at 43°C for 30 minutes and were incubated at normal temperature for certain time intervals. Collected cell lysates were further analyzed with the aid of immunoblotting. To delineate the role of Hsp70, U-937 cells were transfected with the pcDNA3HSP plasmid. The plasmid was manufactured using pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) and the Hsp70 gene coding sequence excised from pH2.3 plasmid (generous gift from Dr R. Morimoto, Northwestern University); the final construct contained the whole Hsp70 gene under the control of cytomegalovirus gene promoter. The transfection was performed with the help of DMRIE liposomal reagent (Gibco BRL) in accordance with the protocol of the manufacturer; in typical experiment, 8 μ L of DMRIE and cholesterol was mixed with 1 mL of cell culture containing 3×10^6 cells, followed by 3 μ g of plasmid DNA. The efficacy of transfection was established using immunoblotting and immunofluorescence techniques. Stably transfected cells containing plasmid pcDNA3 or pcDNA3HSP were selected by growing the culture in medium supplemented with G418 (Sigma) at a concentration of 500 μ g/mL; 14 days later, cells were collected and routinely maintained in medium containing 200 μ g/mL G418.

Assessment of apoptosis

Apoptosis was induced by ETO and ADR (both donated by Dr P. Aller, Centro de Investigaciones Biológicas CSIC, Spain) introduced to cell cultures at concentrations of 20 μ M and 10 μ M, respectively. These concentrations were found earlier to induce apoptosis in 30–40% of the cell population.

To explore the dynamics of apoptosis, the cells were

placed in a 96-well microtiter plate, and ethidium bromide and acridine orange in phosphate-buffered saline (PBS) were added to give concentration of 5 µg/mL for each dye. Stained cells were analyzed with the aid of Zeiss Axioscope (Zeiss, Jena, Germany). Caspase activity was determined with the aid of immunoblotting using monoclonal antibodies to PARP (Novus Biologicals, Cambridge, UK) and to caspase-7 (Pharmingen, San Diego, CA, USA) and polyclonal antibody to caspase-3 (generous gift of Dr Yuri Lazebnik, Cold Spring Harbor Laboratory).

In NS0/1 mouse myeloma cells, caspase activity was determined using an *in vitro* test. In a typical experiment, cells were maintained in culture for 3 days without changing the medium; the depletion of nutrients induced the process of apoptosis in approximately half the cell population, as was confirmed by acridine orange and ethidium bromide staining. At certain periods of time, cells were taken to obtain lysates in following solution: 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES), 1 mM ethylenediaminetetracetic acid, 5 mM ethyleneglycoltetracetic acid, 1 mM phenylmethylsulfonic acid, 2 µg/mL leupeptin, and 2 µg/mL pepstatin. After clarification by centrifugation at $10\,000 \times g$ and the measurement of protein concentration (Bradford 1976), the extracts (total protein 1 mg/mL) were placed in a 96-well plate and mixed with the reaction buffer (50 mM HEPES, 20% glycerol, 5 mM dithiothreitol) to which the caspase-3/7 substrate *N*-acetyl-Asp-Glu-Val-Asp-p-nitroanilidine (Sigma) was added to give a final concentration of 100 µM. Dye release was measured using STAT FAX2100 multiplate reader (Awareness Technology, Palm City, FL, USA) at 405 nm. To test the inhibitory effect of Hsp70, a preparation of Hsp70/Hsc70 was isolated from bovine muscle and characterized as described elsewhere (Guzhova et al 1998); the preparation contained inducible (p) and constitutive (c) forms in the ratio of 65:35 (Guzhova et al 1998). Hsp70/Hsc70 was added to cell extracts at 0.2% of total cell protein. Ovalbumin was used as a control protein.

Hsp70 antibody blockade of DEVD-ase assay was used to prove that the removal of Hsp70 by the specific antibody 2H9 was able to restore caspase-3 and -7 activity. At the first stage, U-937 control extracts and extracts taken 6 hours after the induction of apoptosis with ETO were lysed in the same manner as above mentioned for NS0/1 cells. The protein concentrations of lysates were adjusted, and a stock of 1 mg/mL Hsp70/Hsc70 from bovine muscle was added to give final concentrations of 10, 25, and 50 µg/mL. An excess of Hsp70-specific antibody 2H9 was added to the samples. The latter were applied in duplicates to the wells of 96-well microtiter plate, followed by DEVD-p-anilidine colorimetric substrate, as

above described for NS0/1 cells for 4 hours. After that the plate was read at 405 nm.

Immunochemical studies

Electrophoresis was performed with 10% or 13% polyacrylamide gels, depending on molecular mass of protein species to be resolved. After the electrophoretic transfer of protein bands onto nitrocellulose membranes (Towbin et al 1979), the membranes were incubated with the antibodies to PARP and caspase-3 and -7 (see above) or with 2H9 antibody known to specifically recognize the inducible form of Hsp70 (Lasunskaja et al 1997), followed by appropriate secondary antibodies conjugated with horseradish peroxidase. ECL-plus chemiluminescent cocktail (Amersham/Pharmacia) was used to develop the immunoblots. For the immunocytochemical visualization of Hsp70 in transfected cells, the latter were treated as described elsewhere (Guzhova et al 2001); monoclonal antibody 2H9 and anti-mouse goat IgG-Cy3 conjugate (Jackson Immunochemicals) were used in these experiments. To reveal the possible reaction between Hsp70 and caspases, we used an immunoprecipitation assay with the use of antibodies against caspase-3 and -7 and 2H9 recognizing Hsp70. In brief, U-937 cells transfected with the Hsp70 gene, control, and ADR-treated cells for 6 hours, were collected and lysed in RIPA buffer (140 mM NaCl; 20 mM Tris-HCl, pH 7.5; 40 µg/mL leupeptin, and 25 µg/mL pepstatin). An aliquot containing 0.7 mg of total cell protein was incubated for 30 minutes either with 10 µL of 2H9 or preimmune IgG-Sepharose gel. The latter 2 carriers were prepared by coupling affinity-purified IgGs to CNBr-Sepharose (2 mg IgG/mL wet gel) as recommended by the manufacturer. After washing in RIPA buffer, Sepharose gel slurries were boiled in sodium dodecyl sulfate sample buffer (Laemmli 1970) and subjected to electrophoresis and immunoblotting with the use of antibodies to caspase-3 and -7 as described above. Reciprocal immunoprecipitations with anti-caspase-3 and -7 antibodies were performed using Protein-G-Sepharose and anti-caspase-3 and -7 antibodies, and Sepharose pellets were probed with 2H9 antibody. The immunoprecipitation assay with extracts of control and NS0/1 myeloma cells, induced to apoptosis by 2 day starvation, was performed using the same protocol.

Far-Western blotting

To reveal the interaction of Hsp70 with caspase-3 and -7, control and ADR-treated U-937HSP cells (6-hour treatment) were lysed in RIPA buffer. Cell lysates were used for immunoprecipitation with antibodies to caspase-3 or -7 using Protein-G-Sepharose. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

transfer, the membrane strip with immunoprecipitated caspase-3 and -7 was incubated with a solution of 5 µg/mL Hsp70/Hsc70 isolated from bovine muscle (see above) in Tris-buffered saline (TBS). After the incubation was completed, this nitrocellulose strip was washed in TBS, TBS with 0.05% Tween-20, and placed in solution containing 2H9 anti-Hsp70 antibody. To identify the position of both caspases on the blot, both cell lysates (50 µg of total protein per lane) were subjected to the same SDS-PAGE and transferred as described above; this part of membrane was probed with the anti-caspase-7 or anti-caspase-3 antibodies. After washings, all strips were incubated with the anti-mouse or anti-rabbit antibody-peroxidase conjugate and subsequently processed as before.

Protein-interaction assay was established based on the principle of the enzyme immunoassay. Hsp70 isolated from bovine muscle was immobilized in the wells of 96-well microtiter plate in concentration of 1.5 µg/mL for 2 hours at 37°C. After blocking with 3 mg/mL ovalbumin (1 hour, 37°C), cell lysates in RIPA buffer from U-937HSP cells, control ones, and after treatment with ADR were applied to wells in concentrations of 0.2, 0.5, and 1.0 mg/mL for 2 hours at 37°C. After washing with PBS containing 0.05% Tween-20, antibodies to caspase-3 and -7 were used in quadruplicate for each type of lysate, followed by biotinylated anti-mouse (for caspase-7) or anti-rabbit (for caspase-3) antibodies; peroxidase-conjugated streptavidin was finally applied to all wells. After developing with orthophenylenediamine and H₂O₂, optical density (OD) was measured at 450 nm.

RESULTS

Elevation of Hsp70 content delays ADR- and ETO-mediated apoptosis

Myeloid leukemia U-937 cells were used as a cell model because they have been shown to be an efficient target for such cytostatics as ETO and ADR (Samali and Cotter 1996; Troyano et al 2001). Both these drugs were used in our experiments, and both were shown to induce similar patterns of nuclear fragmentation in cells stained with acridine orange.

To induce Hsp70 accumulation, we used a mild heat shock of 43°C for 30 minutes. After the treatment, cells were allowed to recover for different periods of time to determine at which time the amount of Hsp70 was optimal for treatment with the anticancer drugs. Data from immunoblotting and immunofluorescence showed that a considerable increase in Hsp70 was attained 3 hours after the heat shock, and this time point was chosen to start treatments with ADR and ETO (Fig 1 A,B).

Hyperthermia is generally known to induce several other heat shock proteins, in addition to Hsp70, that may

also protect cells from various stressors. To distinguish the effect of Hsp70 from those of other proteins, we transfected U-937 cells with a plasmid expressing human Hsp70 under the control of a cytomegalovirus constitutive promoter, pcDNA3HSP. The resulting U-937HSP stable cell lines were shown to express a considerable amount of Hsp70 when analyzed by immunocytochemistry and immunoblotting with the use of the Hsp70-specific antibody 2H9 (see Fig 1 B,C).

Heat-stressed cells with enhanced levels of Hsp70, cells transfected with *hsp70* gene, and control cells were incubated with ETO and ADR for 6 and 18 hours. It was found that previous heat stress or transfection protected U-937 cells against ETO and ADR as shown by acridine orange staining. In each case, the heat stress-mediated inhibition of the level of apoptosis was almost 50% of the level without heat shock. Cells transfected with the Hsp70 expression plasmid were also shown to be less susceptible to the action of the 2 anticancer drugs. For instance, an 18-hour incubation with ADR led to apoptosis in 50% of the initial population, whereas this value reduced to 28% in the population of cells overexpressing the *hsp70* gene (Fig 1D). In U-937 cells, apoptosis signaling induces caspase activation (Widmann et al 1998), therefore, it was checked whether this process was suppressed in Hsp70-transfected cells. We studied the dynamics of caspase-3 and -7 cleavage within 24 hours after the addition of ADR to cell culture. Immunoblotting data showed that the cleavage of caspase-3 and -7 in control cells started 6 hours after ADR administration, whereas in cells overexpressing Hsp70, this event was observed 12–18 hours later (Fig 2). Cleavage of PARP by caspases was also postponed in U-937HSP cells for 18 hours, indicating that both these events have been inhibited by Hsp70.

Hsp70 inhibits effector caspase activation

To confirm the Hsp70-mediated inhibition of caspase activity, we designed an in vitro system that was based on extracts from mouse myeloma NS0/1 cells. Mouse plasmacytoma cells are known to lack the expression of Hsp70 mRNA even after heat shock (Aujame and Firko 1988). Moreover, it was found that both mono- and polyclonal anti-Hsp70 antibodies did not recognize Hsp70 irrespective of whether NS0/1 cells were heat stressed (Lanskaia et al 1997). Recently, it was found that if the culture medium was not replaced by fresh media after 2–3 days, NS0/1 cells entered into apoptosis characterized by fragmented nuclei and ladder-like pattern of DNA dissociation (Fridlianskaia et al 2000). The profile of caspase-3 and -7 activation in starving cells was established with the aid of Western blotting. It was found that the cleavage of both caspases started on the second day of starvation, and by day 3, the active fragments were

Fig 1. Elevation of Hsp70 levels attained by mild heat stress or by transfection with the *hsp70* gene reduces the effect of anticancer drugs on U-937 cells. (A) Accumulation of Hsp70 in U-937 heat shocked at 43°C for 30 minutes; after stress, cells were incubated at 37°C for times as indicated. Cell extracts taken at these time points were subjected to immunoblotting with anti-Hsp70 antibody 2H9. (B) Immunofluorescence staining of U-937 cells with anti-Hsp70 antibody 2H9: control (a); heat-stressed (b); *hsp70* gene transfected (c). (C) Increase of Hsp70 content in U-937 cells control (contr), heat stressed (HS), or transfected with the *hsp70* gene (*hsp70*). (D) Reduction of the percentage of apoptotic cells in heat-shocked and *hsp70*-transfected U-937 cells. Heat-stressed (HS), transfected with empty pcDNA3 (DNA3), or with pcDNA3HSP70 (Hsp70) plasmids as well as control (C) U-937 cells were treated with adriamycin or etoposide and at the indicated time were stained with acridine orange to measure the level of apoptosis.

observed to be dominant species (Fig 3A). In addition, the activity of caspase-3 and -7, also known as "DEVD-ase," was measured with the aid of a colorimetric assay and showed an increase within 3 days without changing the culture medium (Fig 3B). To prove the inhibition of DEVD-ase activity by Hsp70, we purified the protein from calf muscle and added it to NS0/1 cell extracts prepared at different time points of maintenance in culture without changing the medium. The measurement of DEVD-ase showed that after 48 and 60 hours of culture, Hsp70 was able to inhibit this activity down to 40% of the untreated value (Fig 3C). Only a small reduction in DEVD-ase activity was observed after 3 days, however, suggesting that the apoptotic cascade may have proceed-

ed beyond the point where Hsp70 can inhibit the cascade. The addition of a control protein, ovalbumin, to the cell extracts did not change the DEVD-ase activity (Fig 3C).

To prove that the inhibition of caspase activity was specific to Hsp70, we used an assay in which the action of Hsp70 might be blocked with the aid of the Hsp70-specific antibody 2H9. First, control and ETO-stimulated U-937 cells were lysed and the Hsp70/Hsc70 preparation was added, and as in case of NS0/1 cells, the addition of Hsp70 was found to inhibit the DEVD-ase activity (Fig 3D). The reduction of DEVD-ase activity was proportional to the amount of Hsp70 added. The addition of 2H9 anti-Hsp70 antibody to the extracts incubated with Hsp70 was shown to partially restore caspase activity,

Fig 2. Transfection with *hsp70* gene causes temporary inhibition of caspase-3 and -7 activation. U-937 and U-937HSP (*hsp70*) cells were treated with adriamycin and cell lysates collected at the indicated time points. Fifty micrograms of total protein from each cell lysate was loaded for electrophoresis-immunoblotting and the time course of caspase activation determined using antibodies to appropriate caspases and to poly(adenosine diphosphate)-polymerase (as indicated).

though lower than in apoptotic cells, but 30–40% higher than in Hsp70-treated cell lysates. Finally, addition of an irrelevant antibody to the incubation medium did not change the effect of Hsp70 on DEVD-ase activity (Fig 3D). Thus, the inhibition of caspase-3 and -7 activity found in extracts of NS0/1 and U-937 cells induced to apoptosis with 2 different stimuli was firmly demonstrated to be due specifically to exogenous Hsp70 introduced to the lysates.

Hsp70 binds caspase-3 and -7

We hypothesized that the inhibitory effect of Hsp70 could be due to Hsp70 forming a complex with immature caspases, as has been reported earlier for NF- κ B proteins in Molt-4 lymphoma (Guzhova et al 1997) and in U-937 cells affected by various stimuli (Guzhova et al 2000). To check

this, we used an immunoprecipitation assay using 2H9 anti-Hsp70 antibody purified and coupled to CNBr-Sepharose and a reciprocal system with anti-caspase-3 and -7 antibodies, followed by pull down with Protein-G-Sepharose gel (Fig 4A). The lysates of control and apoptosis-induced U-937HSP cells were passed through the 2H9 IgG column, and the proteins bound to the gel were analyzed with the aid of blotting using antibodies to caspase-3 and -7. In the reciprocal immunoprecipitation, caspases and bound proteins were probed by immunoblotting with anti-Hsp70 antibody. Both proforms of caspase-3 and -7 were found to interact with the chaperone in control and apoptosis-induced cells (Fig 4A). When non-immune IgG of the same isotype was used instead of 2H9 antibody, neither Hsp70 nor caspase bands were observed in the precipitates (Fig 4A). In cells treated with ADR, Hsp70 was associated with procaspase-7 (34 kDa) and partially with its 32-kDa component, the enzyme form that had lost its prodomain. No binding of Hsp70 was observed with the active 19-kDa form of caspase-7. Hsp70 was shown also to bind the procaspase-3 (32 kDa) and was not associated with the 17-kDa polypeptide corresponding to the final caspase-3 cleavage product.

To show that Hsp70 and caspases could form direct intramolecular complexes, we used a Far-western assay. The lysates of control U-937HSP and ADR-treated cells were incubated with antibodies to caspase-3 and -7 and immunoprecipitated as above (Fig 4A) and then subjected to SDS-PAGE and transferred onto nitrocellulose membrane. Blots with procaspase bands were incubated with a pure Hsp70/Hsc70 preparation, followed by probing with the 2H9 anti-Hsp70 antibody (Fig 4B). Other strips with immunoprecipitated caspase-3 or -7 were probed directly with 2H9 antibody to prove that there was no non-specific binding of the antibody to caspases. The position of the procaspase bands on the blots was confirmed by staining membranes of whole lysates with the appropriate anticaspase antibodies (Fig 4B). The results of these assays showed that Hsp70 from the lysates and the pure protein preparation recognized both procaspases purified by immunoprecipitation or contained in lysates of control or apoptosis-induced cells (Fig 4 A,B).

To prove that the binding of caspase-3 or -7 to 2H9 antibody-Sepharose was direct and due to a specific interaction with Hsp70 and not to a precipitation of the protease near its isoelectric point or some other nonspecific event, we probed extracts obtained from control and apoptotic NS0/1 mouse myeloma cells that do not express Hsp70. The cells were kept for 2 days without changing the medium to induce apoptosis (see previous section of Results). No bands corresponding to procaspase-3 or -7 were present in 2H9 immunoprecipitates, proving that the binding of caspase-3 and -7 to the 2H9

Fig 3. Hsp70 can inhibit caspase-3/7 (DEVD-ase) activity in vitro. NS0/1 mouse cells were used for the preparation of a cell-free system; the cells were kept without changes of medium for the indicated periods of time, and caspase-3/7 (DEVD-ase) activity was measured in cell extracts using DEVD-p-anilidine as colorimetric substrate. (A) Caspase activation in NS0/1 cells: upper panel, caspase 7; lower panel, caspase 3; time in hours indicated at top. (B) Time course of caspase activity in NS0/1 cells deprived of serum (in hours). (C) DEVD-ase activity in cell extracts after addition of Hsp70/Hsc70 isolated from bovine muscle or ovalbumin. See Materials and Methods and Results for details. (D) DEVD-ase activity in control and etoposide-treated U-937 cell extracts. Hsp70/Hsc70 was added as indicated and Hsp70 activity blocked by incubation with antibody 2H9 or nonspecific antibody as indicated.

antibody column occurred in specific manner and was dependent on the presence of Hsp70 (Fig 4C).

The association between Hsp70 and both downstream caspases was confirmed with the aid of protein interaction assay (PIA). This test was assembled in a manner analogous to a combined immunoprecipitation-immunoblotting system and formatted as an immunoenzyme assay. Hsp70 preparations from bovine muscle were applied to the wells of microtiter plate, and after blocking, extracts of U-937HSP cells containing caspases were applied, followed by specific anticaspase antibodies. PIA data revealed a dose-dependent interaction between both caspases and pure Hsp70 (Hsp/Hsc70) (Fig 5). Successful attachment of Hsp/Hsc70 to the microtiter plate was confirmed with 2H9 antibody (OD = 2.805).

DISCUSSION

Major stress protein Hsp70 is known to interact with different chains of the apoptotic signaling machinery upstream and downstream the effector caspase activation; these interactions in most of the cases lead to a suppression of apoptosis. To check whether the protein can inhibit apoptosis through the binding to these proteases, we chose human leukemia U-937 cells and treated them with well-established anticancer drugs. These cells were shown to exhibit all the attributes of apoptosis, eg, fragmented nuclei, DNA ladder, and activation of effector caspase-3 and -7, when treated with ADR or ETO (Samali and Cotter 1996). We used 2 methods to increase the in-

tracellular content of Hsp70, heat treatment and transfection with the *Hsp70* gene. In both cases the elevation of Hsp70 intracellular content was concomitant with a significant reduction of a number of apoptotic cells (Fig 1). Our data on ADR and ETO effects and earlier results obtained with the same cells treated with TNF- α (Jäättelä 1993; Guzhova et al 2000) show that a previous heat stress is able to postpone the onset of apoptosis rather than fully stop the process. These findings and the data of other groups working on the same cells (Samali and Cotter 1996; Perez et al 1997) prove that increased levels of Hsp70 are able to efficiently protect leukemic cells against lethal concentrations of antitumor drugs. Because the reduction of apoptosis might be partially explained by the inhibition of the activation of effector caspases, we determined caspase activity in control and transfected cells. Data from immunoblotting with antibodies to caspases and to PARP showed that the transfection caused significant delay in the onset of caspase activity (see Fig 2). We believed that this inhibition may have been due to a direct inhibition of caspase activation by Hsp70, in the addition to the effect of the protein on other initiating molecules in the apoptotic cascade, such as APAF-1 (Beere et al 2000; Saleh et al 2000). To test this, we chose NS0/1 myeloma cells that are known not to express Hsp70 and, therefore, to represent a unique model for these experiments (Aujame and Firco 1988; Lasunskia et al 1997; Lasunskia et al 2003). These cells were found earlier to undergo apoptosis with all the known attributes, including DEVD-ase activation, in conditions of serum deprivation (Frid-

A

B

C

Fig 4. Hsp70 binds caspase-3 and -7 in U-937 cells. (A) Immunoprecipitation assay. Cell extracts (500 μ g) obtained from control U-937HSP (C) cells and from cells treated with adriamycin (ADR) for 6 hours (A) were incubated with 15 μ L of 2H9 antibodies coupled to CNBr-Sepharose gel (lower panels) or with 5 μ L of anti-caspase-3 or anti-caspase-7 antibodies (upper panels, as indicated) or with 5 μ L of nonspecific antibody of the same class (IgG). Immunoprecipitates (immunoprecipitate) were analyzed by electrophoresis on a 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and after transfer, the membranes with caspase-3 or caspase-7 immunoprecipitates were probed with 2H9 antibody against Hsp70, whereas the membranes that contained the anti-Hsp70 immunoprecipitates were probed with antibodies to caspase-3 or caspase-7. Unbound proteins (supernatant) were also analyzed by immunoblotting with anti-Hsp70 (upper panels) or anti-caspase-3 or -7 antibodies (lower panels, as indicated). (B) Far-western assay; anti-caspase-3 and -7 immunoprecipitates of control or ADR-treated cells were electrophoresed and transferred onto a membrane that was then incubated with pure Hsp70/Hsc70, followed by 2H9 antibodies; caspase-3 and -7 membranes without exogenous Hsp70 were probed with 2H9 antibody to prove no reaction of 2H9 antibody with the caspases. Total cell lysates were probed with anti-caspase-3 or caspase-7 antibodies to confirm the position of caspase-3 and -7 bands. (C) Immunoprecipitation of NS0/1 myeloma cell proteins with anti-Hsp70 antibody. Extracts from control (C) and from NS0/1 cells starved for 60 hours (ST) were incubated with 15 μ L of Sepharose gel coupled with 2H9 antibodies. Immunoprecipitates and 50 μ g of each fraction of unbound protein were loaded onto 13% SDS-PAGE gel and immunoblotted with anti-caspase-7 (left) or anti-caspase-3 (right) antibodies.

lianskaia et al 2000). The addition of purified Hsp70 isolated from bovine muscle was found to inhibit the DEVDase activity, and this inhibition was particularly strong after 2.5 days of cultivation without serum changes (Fig

3). We observed only a weak effect of Hsp70 on day 3, probably indicating that the process of cleavage was near to its end. To further prove that the inhibitory effect is particularly attributed to Hsp70, we used the same test

Fig 5. Protein interaction assay demonstrates the interaction between Hsp70 and downstream caspases. Pure Hsp70/Hsc70 was plated to wells of 96-well plate, and after blocking, lysates of U-937HSP cells at concentrations of 0.2, 0.5, and 1.0 mg/mL were added. Bound caspases were revealed with a help of corresponding antibodies and secondary antibody conjugated with horseradish peroxidase. Data presented as optical density at 450 nm $\pm$ SE.

system with U-937 cells induced to apoptosis by anticancer drugs. It was shown that the addition of the specific antibody recognizing Hsp70 was able to restore caspase activity earlier suppressed by pure Hsp70. The results obtained with the similar *in vitro* system for the assessment of Hsp70 activity were reported by Li and coauthors (2000); they found that the addition of Hsp70 or of its C-terminal fragment to apoptotic cell extracts reduced the level of caspase-3 cleavage (Li et al 2000).

These observations led us to suggest that Hsp70 chaperone could influence caspase-3 and -7 processing by a direct association with these enzymes, and because the maximal inhibitory effect was attained on the third day of serum deprivation of NS0/1 cells, we suggested that the binding occurred earlier. To check whether the association of Hsp70 with caspases took place, we used a reciprocal immunoprecipitation, Far-western assay, and PIA. Two major conclusions from the results of these assays are that (1) pure caspase and Hsp70 can interact with each other in differently treated cells and (2) native molecules of both proteins are involved in this interaction. Thus one might suggest that this interaction may not be just a conventional binding of chaperone to unfolded substrate but may be mediated by specific domains within the caspase molecules. The results of immunoprecipitation assay clearly showed that Hsp70 binds predominantly procaspases and their "first-step" degradation products; this could mean that overexpressed Hsp70 inhibits caspase maturation at the early stage of the process.

To avoid artifacts, we have checked whether the capture of caspase-7 by the antibody-Sepharose gel might be due to precipitation of the protein near its isoelectric point or to nonspecific interaction with immunoglobulins, and passed NS0/1 cell extract known to contain no

Hsp70 through the same 2H9 antibody-Sepharose gel (Aujame and Firco 1988; Lasunskaja et al 2003).

To prove the association between Hsp70 and 2 caspases, we have used a novel test system, PIA that allowed us not only to confirm the data of immunoprecipitation and Far-western analyses but significantly reduce the duration of the experiment. Usually, combined immunoprecipitation-immunoblotting takes almost 2 working days, whereas PIA takes 4 hours. Another advantage of PIA is that it uses native-state proteins as opposed to Far-western assays. Finally, PIA can be used to provide quantitative results of binding capacity between 2 proteins.

There is a slight discordance between the results of the *in vitro* assay of Hsp70 inhibitory activity and the immunoprecipitation data showing the existence of binding between the above proteins. This apparent contradiction may stem from the difference between various pools of Hsp70 recognized by the antibody; earlier, we reported that in cells with the elevated content of Hsp70, approximately 20–45% cannot be recognized by various monospecific antibodies (Guzhova and Margulis 2000); if this happens in our experiments, there are at least 2 different subpopulations of Hsp70 molecules behaving in relation to caspases in distinct modes. This may be approved by the fact that the addition of 2H9 antibody was not able to fully restore caspase activity (Fig 3D). On the other hand, Hsp70 was shown to bind procaspase and partially its first-step product of degradation, and therefore the chaperone might have several sites on caspase molecules for binding. We suggest that Hsp70 forms multiple complexes with caspases and as result a certain part of the latter activity is reduced.

The process of apoptosis induced in tumor cells by anticancer drugs is enormously complicated, and a cell possesses many modes on how to "die decently" (Jäätelä 2002). The activation of a single receptor may cause the simultaneous switching of various signaling mechanisms (Stennicke et al 2002). To date, Hsp70 has been found to suppress apoptotic signaling at almost all its critical points, binding APAF-1, interacting with AIF (Ravagnan et al 2001), p65, p50 (Guzhova et al 1997, 2000), and I- κ B (Kohn et al 2002), parts of the NF- κ B transcription activator complex, inhibiting stress kinase activity (Mosser et al 2000) and caspase-activated DNase (Liu et al 2003), and now a novel target of the chaperone is revealed as the proforms of effector caspase-3 and -7. Hsp70 may act as a chaperone to bind these molecules during their conversion to active forms. It is also remarkable that Hsp70 can act at many different points of apoptotic signaling simultaneously. For instance, if the mitochondrial chain of signal transduction is broken because Hsp70 binds the CARD-domain of APAF-1 and inhibits its oligomerization (Beere et al 2000; Saleh et al 2000), the process of apoptosis can proceed by a mechanism involving caspase-8–

provoked activation of downstream, effector caspases. However, in this case, Hsp70 intervenes in this process by directly inhibiting the activation of these caspases. Even if the caspase cascade is fully switched off, Hsp70 can inhibit a novel caspase-independent death effector released from mitochondria, AIF (Cande et al 2002). Very recently, McLaughlin et al (2003) presented data indicating that Hsp70 colocalized with the activated caspase-3 in neural cells induced to apoptosis with KCN. Certainly, further experiments are needed to understand the nature of effector caspase binding to Hsp70 and to what extent this interaction influences the process of apoptosis induced by anticancer drugs.

In conclusion, the results of this study show that Hsp70 is an effective means of cancer cell defense against anti-tumor therapy because the selective increase of Hsp70 levels led to an elevation of drug resistance. Conversely, numerous proapoptotic and potential anticancer drugs, eg, bleomycin, quercetin, and staurosporine, are able to downregulate the expression of Hsp70. The increase of Hsp70 in tumor cells may damage several aspects of the apoptotic system, protecting them from cytostatic drugs and promoting tumorigenesis. In addition to the previously identified targets of Hsp70 in the apoptotic cascade, the data in this report show that Hsp70 can also inhibit proteolysis based on effector caspases. New effective cancer therapies may have to consider ways to prevent Hsp70 blocking apoptosis at several different levels from stress kinase and APAF-1 induction through to the execution of apoptosis by effector caspases.

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