Heat shock protein 90 suppresses tumor necrosis factor alpha induced apoptosis by preventing the cleavage of Bid in NIH3T3 fibroblasts

Chen Zhao\textsuperscript{a,b,}*\textsuperscript{,} Enhua Wang\textsuperscript{b}

\textsuperscript{a}Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinano-machi, Shinjuku, Tokyo 160-8582, Japan
\textsuperscript{b}Department of Pathology, China Medical University, 92 North Second Road, Shenyang, Liaoning 110001, PR China

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Abstract

Two highly conserved mechanisms for maintaining cellular homeostasis are apoptosis and the cellular stress response. Hsp90 is one of the most abundant, highly conserved, and inducible Hsps in eukaryotes. Recently, Hsp90 has been shown to play important antiapoptotic roles through binding with Apaf-1, RIP and kinase domain of IKK\textsubscript{a/\beta}. Our present studies demonstrate that Hsp90 can suppress tumor necrosis factor alpha (TNF\textalpha)‑induced apoptosis in stable Hsp90-overexpressing NIH3T3 cells by preventing the cleavage of Bid. The prevention of the cleavage of Bid can be partially explained by the direct interaction between Hsp90 and Bid. Furthermore, disrupting the function of Hsp90 by the addition of its specific inhibitor, geldanamycin, blocked Hsp90’s protection of Bid cleavage. These results show that Hsp90 can function at different levels within apoptotic signal transduction pathways.

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1. Introduction

Apoptosis and the cellular stress response are evolutionarily conserved mechanisms through which normal cellular development and homeostasis are maintained. Apoptosis, a cellular process of self-destruction with distinctive morphological features, is important in embryonic development, the maintenance of homeostasis, and the pathogenesis of a number of diseases [1–3]. Apoptosis can be induced by numerous stimuli, including the activation of members of the tumor necrosis factor receptor (TNFR)-1 superfamily, deprivation of growth factors, ionizing radiation, and genotoxic agents [4–6]. The signaling pathways involved in apoptosis have been extensively studied biochemically and genetically. It is now known that caspas, a family of cysteine proteases with aspartate specificity, are the major effectors of the process [7]. Caspas exist as inactive precursors in proliferating cells. When activated, they proteolytically cleave a wide variety of cellular proteins, including certain key substrates, which lead to morphological changes and the degradation of chromosomal DNA [8–13].

Two key pathways for transmitting a death signal to the apoptotic machinery are the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway [14,15]. When combined with actinomycin D or cycloheximide, tumor necrosis factor alpha (TNF\textalpha) induces cell apoptosis through the death receptor pathway [16]. TNF\textalpha is an inflammatory cytokine that can induce a diverse range of biological responses [17]. Due to its importance, it is now being targeted for therapies against widespread human diseases such as atherosclerosis, osteoporosis, autoimmune disorders, allograft rejection, and cancer [18]. TNF\textalpha signaling is initiated by binding to its receptor, TNFR-1. This causes the association of an adapter protein, TNFR1-associated death domain protein (TRADD) with the intracellular death domain of the TNFR-1 molecule [19]. TRADD mediates the subsequent recruitment of another adapter protein, Fas-associated death domain pro-
tein (FADD), thereby forming a death-inducing signaling complex. This complex initiates apoptosis through the activation of caspase-8, a proximally acting enzyme in the cascade of cysteine proteases [20–25]. Caspase-8 can induce apoptosis directly through the activation of procaspase-3 or indirectly by the cleavage of Bid, a proapoptotic member of the Bcl-2 family [26,27]. The cleaved C-terminal of Bid (tBid) translocates to the mitochondria and potently induces cytochrome c release [26–28]. Thus, Bid connects the death receptor pathway to the mitochondrial pathway and is responsible for cytochrome c release. 

Cytochrome c, together with a proapoptotic intermediate, apoptosis activating factor-1 (ApaF-1), activates procaspase-9, which leads to the downstream processing of procaspase-3 [29].

The cellular stress response, mediated through the expression of heat shock proteins (Hsp), is another evolutionarily conserved cellular protection mechanism. An increasing amount of evidence has shown that the cellular stress response and apoptosis are interconnected to form a regulatory network that mediates cell survival [30–32]. Induction of Hsps in response to stress serves to protect against the initial insult, augment recovery, and produce a state of resistance to subsequent stress in the cell [33]. This protective role of Hsps is attributed to several properties, most notably, the prevention of protein aggregation and the promotion of protein disaggregation by catalyzing the refolding of damaged or denatured proteins [34,35]. If the degree of stress exceeds the Hsps protective capacity, cells will die via apoptosis [36]. The mechanisms by which various Hsps protect against different apoptotic stimuli remain to be fully defined. Recently, the antiapoptotic mechanisms of Hsp70 and Hsp27 have been shown. Hsp70 prevents the oligomerization of Apaf-1 and thus, its association with procaspase-9. Hsp27 binds cytochrome-c, thereby preventing cytochrome-c-mediated interaction of Apaf-1 with procaspase-9 [37–39]. However, it should be mentioned that the role of the Hsps is complex and that they do not always function as antiapoptotic factors. In some cases, Hsp70 and Hsp90 may accelerate apoptosis [40,41]. Hsp90, which constitutively comprises 1–2% of cytosolic proteins, is highly conserved and essential for viability of eukaryotic cells [42–44]. In eukaryotes, Hsp90 has dual chaperone functions. They are involved in the conformational maturation of signal transduction molecules (for example, nuclear hormone receptors and kinases) and in the cellular stress response [42]. The finding that Hsp90 is induced in response to diverse apoptotic stimuli, such as UV, doxorubicin, and sodium arsenite, has supported its involvement in cell survival [35,45–47]. Recently, several mechanisms in which Hsp90 acts as an antiapoptotic factor have been reported. (1) Hsp90 forms a cytosolic complex with Apaf-1 and inhibits cytochrome c-mediated oligomerization of Apaf-1 and the activation of procaspase-9 [48]. (2) Hsp90 stabilizes receptor-interacting protein (RIP), a major antiapoptotic adaptor, to activate antiapoptotic pathways through NF-κB and MAPK [49]. (3) Hsp90 interacts with the kinase domain of IKKα/IKKβ, and disrupts the formation of this heterocomplex by geldanamycin, prevents TNF-induced activation of IKK and NF-κB [50,51].

In this report, we used Hsps90 overexpression systems to investigate whether Hsp90 can suppress TNFα-induced apoptosis and to examine the underlying mechanism. Since TNFα treatment alone does not lead to apoptosis due to the proliferation and survival effects of TNFR-1 signaling, we used TNFα in combination with the protein synthesis inhibitor cycloheximide (CHX) to induce apoptosis. CHX inhibits the induction of Hsps and other proliferation promoting factors allowing the function of Hsp90 in apoptosis to be clearly demonstrated. Our results show that Hsp90 can suppress TNFα-induced apoptosis by a mechanism involving the prevention of the cleavage of Bid through the direct interaction of Hsp90 with Bid. Inhibition of Hsp90 by geldanamycin increases Bid cleavage. Our studies, combined with previous reports, imply (1) that Hsp90 can function at different levels within apoptotic signal transduction pathways; and (2) that the abundant existence of Hsp90, even in physiological conditions, plays an important role in maintaining cellular homeostasis, not only by promoting protein folding, but also by keeping proapoptotic factors in an inert state.

2. Materials and methods

2.1. Cell culture and treatments

NIH3T3 mouse fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, MO, USA), supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 units/ml each of penicillin and streptomycin at 37 °C, in a humidified chamber supplemented with 5% CO2. Cells were split every 3 days to ensure logarithmic growth. The expression vector was constructed by subcloning in-frame the human hsp90β cDNA into pCruz Myc Mammalian Expression Vector (Santa Cruz Biotechnology, CA, USA). The expression vector was confirmed by DNA sequencing. The stable transfectants were generated by electroporation of 10 μg of expression vector into 5 × 105 NIH3T3 cells following selection with geneticin (G418, 500 μg/ml). Cells were incubated for the time periods indicated with 50 ng/ml TNFα (R&D Systems, Minneapolis, MN, USA) and 10 μg/ml cycloheximide (CHX, Sigma).

2.2. Immunofluorescence

The cells were attached to poly-L-lysine-coated slide glasses by cytopsin centrifugation. The apoptosis induced by TNFα/CHX was detected using Apoptosis Detection
System, Fluorescein (Promega, Wisconsin, USA) according to the manufacturer’s instruction. Briefly, cells were fixed with 4% methanol-free formaldehyde at 4°C for 25 min and permeabilized with 0.2% Triton X-100 for 5 min. After equilibration, TdT incubation buffer (45 μl equilibration buffer + 5 μl nucleotide mix + 1 μl TdT enzyme/sample) was added and cells were incubated at 37°C for 60 min. At the same time, negative controls (without TdT enzyme) and positive controls (treated with DNase I) were prepared and processed identical to the samples. The reactions were terminated by immersing the slides in 2 × saline sodium citrate (SSC) solution for 15 min at room temperature. Nuclei were stained with propidium iodide (1 μg/ml) for 15 min at room temperature. Cells were mounted using anti-Fade solution (Dako, Glostrup, Denmark) and analyzed using the ZEISS Axioskop microscope (Carl Zeiss, Heidelberg, Germany). A cell was scored as apoptotic if it positively displayed green fluorescence (FITC). More than 200 consecutive cells from different samples were counted to determine the percentage of apoptotic cells. Data shown are the means and standard deviation of at least three independent experiments.

2.3. Cell cycle analysis

Cells were seeded in 6-well plates at 2 × 10^5 cells/well and treated 24 h later with TNFα/CHX. Cells were detached using trypsin, washed twice with cold PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA), and resuspended in 1 ml of PBS containing 5 mM EDTA. Cells were fixed by adding 1 ml of ethanol and incubating for 1 h at room temperature or overnight at 4°C. Cells were harvested and resuspended in 0.5 ml of PBS containing 5 mM EDTA. RNA was removed by digestion with 20 μl of RNaseA (10 mg/ml) for 30 min at room temperature. One half a milliliter of staining solution (250–500 μg/ml propidium iodide in PBS, 5 mM EDTA) was added 30 min before cell cycle analysis was performed by flow cytometry (Beckman Coulter, Hialeah, USA).

2.4. Caspase-8 and caspase-3 colorimetric assay

Caspase-8 and caspase-3 activities were measured by the cleavage of Ile-Glu-Thr-Asp-p-Nitroaniline (IETD-pNA) and Asp-Glu-Val-Asp-p-Nitroaniline (DEVD-pNA), respectively, using the Apoalert Caspase-8 and Caspase-3 Colorimetric Assay kits (Clontech, Palo Alto, CA, USA). For the assay, NIH3T3 cells were seeded at a density of 1 × 10^6 cells/10 cm dish, left untreated, or treated with TNFα/CHX or TNFα/CHX plus DEVD-fmk (for caspase-3) for 6 h. Cells were then harvested, washed with PBS, and suspended in 50 μl of lysis buffer provided by the manufacturer. The rest of the assay was done according to the manufacturer’s instruction. Samples were measured at 405 nm using a microplate reader (Bio-Rad, model 550, Hercules, CA, USA).

2.5. SDS-page and Western blotting

Cells were lysed in buffer (pH 7.4) containing 10 mM N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES), 142 mM KCl, 5 mM MgCl2, 1 mM EDTA, 0.2% Nonidet P-40, and freshly added proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). The protein concentrations were determined by the Bradford method. Thirty micrograms of protein were resolved by electrophoresis on 4–12% SDS-polyacrylamide gels (Novex, Carlsbad, CA, USA) and transferred onto Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Bunkinghamshire, UK). After blocking with 5% milk in Tris buffer (10 mM Tris (hydroxymethyl) aminomethane Hydrochloride (Tris–HCl), pH 7.5, 150 mM NaCl with 0.1% Tween 20), membranes were probed with appropriate antibodies. The following primary antibodies were used: polyclonal antibodies (rabbit) against caspase-3 (StressGen, Victoria, Canada) and cytochrome c (Santa Cruz); monoclonal antibodies against c-myc (9E10) (Santa Cruz), Hsp90 (Affinity Bioreagents, Golden, CO, USA), Hsp70 (StressGen), bovine cytochrome oxidase subunit IV (Molecular Probes, Eugene, USA); and two rat monoclonal antibodies against mouse Bid (ZYMED, San Francisco, USA and R&D System). Recombinant human caspase-8 was purchased from Calbiochem (California, USA). To produce caspase-8 cleavage of Bid in vitro, recombinant caspase-8 (at 1000 U/ml) was mixed with an equal amount of lysate in the caspase assay buffer (20 mM HEPES, pH 7.4, 5% sucrose, 50 mM NaCl, 5 mM DTT and 1 mM EDTA) supplemented with protease inhibitors for 2 h. Where indicated, blots were stripped and reprobed with a monoclonal antibody against mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Advanced ImmunoChemical, Long Beach, CA, USA) to confirm equal protein loading. Proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

2.6. Cytochrome c release assay [16,28]

Cells were treated as indicated in figure legends, then pelleted, washed once with PBS, resuspended in 1 ml of ice-cold Buffer A (250 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM ethylene glycol-bis (β-aminoethylether) -N,N,N’,N’-tetraacetic acid (EGTA), 1 mM dithiothreitol, 170 μg/ml phenylmethylsulfonyl fluoride, 16 μg/ml aprotinin and 4 μg/ml leupeptin), and incubated for 10 min on ice. Cells were homogenized by 25 strokes of a dounce homogenizer and centrifuged at 750 × g for 15 min at 4°C to remove nuclei and unbroken cells. The supernatant was spun at 10,000 × g for 25 min at 4°C. The pellet from this spin, containing the mitochondrial fraction, was resuspended in 1 ml of Buffer A and spun at 100,000 × g for 1 h at 4°C. The supernatant from this spin contained the cytosolic S100 fraction. The protein concen-
trations were determined using a protein assay kit (BioRad). Thirty micrograms of the mitochondrial fraction or an equal volume of the S100 fraction were loaded on a 4–12% SDS-polyacrylamide gel and subjected to Western blotting for cytochrome c as described above. Where indicated, the membrane was reprobed with cytochrome oxidase subunit IV to confirm equal protein loading.

2.7. Immunoprecipitation

Immunoprecipitation was performed to investigate the interaction between Hsp90 and Bid. Cells were incubated with low salt lysis buffer [50 mM MOPS (pH 7.3), 25 mM β-glycerophosphate, 2 mM EDTA, 20 mM molybdate and a complete protease inhibitor cocktail set (Roche)] for 1 h at 4 °C, then gently passed 20 times through a 25 G needle. The lysates were microcentrifuged and 1 g of protein from each sample was precleared by incubation with rec-protein G Sepharose 4B (ZYMED) for 30 min at 4 °C. Rabbit anti-Bid polyclonal antibody (1:150 dilution, Cell Signaling Technology, Medellin, Colombia) or 5 µg polyclonal anti-myc-tag antibodies were added and the mixtures were incubated with rotation for 2 h at 4 °C. Protein G-Sepharose 4B beads (60 µl) were added and the mixture were incubated with rotation for another 2 h at 4 °C. Immunoprecipitates were then pelleted and washed five times with lysis buffer.

Fig. 1. Overexpression of Hsp90 inhibits TNFα/CHX-induced apoptosis in NIH3T3 cells. (A) The resistance to TNFα/CHX-induced apoptosis depends on Hsp90 expression levels. The upper figure shows the overall Hsp90 expressions in control and Hsp90 clones. The magnitude of Hsp90 expression levels calculated by NIH Image1.63 in Hsp90 clones is compared to control clone as indicated below the blot. The lower figure shows the quantification of apoptotic cells induced by TNFα/CHX in different Hsp90 clones compared to the control clone using flowcytometric analysis. The data represent mean values (mean ± SD). (B) The exogenous and overall Hsp90 expressions in control and Hsp90 cells. Thirty micrograms of total cell lysates were loaded in each lane of the SDS-PAGE gel. Western blotting was performed using anti-c-myc (9E10) (upper bands), anti-Hsp90 (middle bands) and anti-GAPDH (lower bands) antibodies. Exogenous Hsp90 was clearly expressed in Hsp90 cells. Total Hsp90 expression in Hsp90 cells was significantly greater than the expression in control cells. GAPDH expression was similar in control and Hsp90 cells. (C) Phase contrast photomicrographs. Cellular morphology of control and Hsp90 cells treated with TNFα/CHX for 6 h (upper panels). The residual adherent cells after PBS washing are shown in the lower micrographs. (D) Confocal microscopic analysis of TNFα/CHX-induced apoptosis in NIH3T3 cells. Control and Hsp90 cells were cultured untreated or treated with TNFα/CHX for 6 hours. Cells were collected, cytospun on poly-L-lysine coated glass slides and stained with FITC-conjugated dUTP (green) and propidium iodide (red) as described in Materials and methods. (E) Quantification of the protective effect of Hsp90 on TNFα/CHX-induced apoptosis in (D). The data represent mean values (mean ± SD) from three separate fields. (F) Quantification of apoptotic cells induced by TNFα/CHX using flowcytometric analysis.
buffer before the antibody–antigen complex was applied to SDS-PAGE. The following antibodies were used for immunoblotting: rat anti-Bid (ZYMED), mouse anti-Hsp90 (clone: 3B6, Affinity Bioreagents), mouse anti-myc-tag (clone: 9B11, Cell Signaling Technology), and rabbit anti-Hsp90 polyclonal antibodies (Santa Cruz). In mock immunoprecipitation experiments, rabbit IgG was used following the same procedures.

3. Results

3.1. Overexpression of Hsp90 inhibits TNFα/CHX-induced apoptosis

To investigate the functions of Hsp90 in the process of TNFα/CHX-induced apoptosis, myc-tagged human Hsp90-transfected (Hsp90) and vector only transfected (Con)

Fig. 2. Inhibition of caspase-3, but not caspase-8, activation by overexpression of Hsp90. Control and Hsp90 cells were cultured untreated or treated with TNFα/CHX at 37 °C for the indicated time periods. Caspase-8 (A) and caspase-3 (B) protease activities were determined using a colorimetric assay with Ac-IETD-pNA or Ac-DEVD-pNA, respectively, as substrates. Data are mean ± SD of three independent experiments, *p < 0.001(Student’s t-test-based statistics). While measuring caspase-3 activities, the inhibitor of caspase-3 (DEVD-fmk) was used to show specificity. (C) Western blot analysis caspase-3 activities. Equal amounts of protein were separated by SDS-PAGE and subjected to immunoblotting using rabbit antibodies against caspase-3 and monoclonal antibody against GAPDH. Arrows indicate pro- and activated caspases. Results are representative of at least three independent experiments with similar results.

Fig. 3. Hsp90 overexpression prevented the release of cytochrome c induced by TNFα/CHX treatment. Control and Hsp90 cells were either cultured untreated or treated with TNFα/CHX for the indicated times. The cells were harvested and the mitochondrial and S-100 fractions were isolated. Equivalent mitochondrial (A) and S-100 (B) fractions were analyzed by immunoblotting with anti-cytochrome c antibody. One representative immunoblot of three is shown. Anti-cytochrome oxidase IV and anti-GAPDH antibodies were used to demonstrate similar protein loading for the mitochondrial and S-100 fractions, respectively. The mitochondrial and cytosolic fractions of cytochrome c in control and Hsp90 cells are represented in the right portions of (A) and (B), respectively. Quantifications of the decrease in mitochondrial and increase in cytosolic cytochrome c were performed in the following manners: first, the 0 h cytochrome c band densities were divided by the 0 h Cox IV or GAPDH densities, respectively, to yield the 100% value; next, the 6- and 12-h cytochrome c bands densities were divided by corresponding Cox IV or GAPDH densities, and the results were divided by the corresponding 100% value to yield the percentage of cytochrome c at the different time points. Data are mean ± SD of three independent experiments.
 NIH3T3 cells were used. As shown in Fig. 1A (upper), the overall Hsp90 expression levels in these cells were greatly increased when compared to the control clone. The percentage increase in total Hsp90 was between 54% and 130%. Using confocal microscopy to examine these Hsp90 transfected cells after staining with anti-myc tag antibody, we found that more than 95% of cells expressed exogenous Hsp90 (the efficiency of transfection is more than 95%), therefore the total Hsp90 increase is due to a smaller increase in a larger number of cells showing no morphological differences to control and parental cells (data not shown). Hsp90 clone 1 was chosen to analyze the function of Hsp90 in apoptosis in detail. As shown in Fig. 1B, exogenous myc-Hsp90 clone 1 was clearly expressed in Hsp90 cells, while no signals could be detected in control cells (upper). The overall levels of Hsp90 were greatly increased in Hsp90 cells compared to the control cells (middle). The expression levels of Hsp70 and Hsp27 were not changed by Hsp90 overexpression (data not shown). In the first experiment, we exposed the cells to TNFα/CHX for a time course of 0, 3, 6, 9, 12 h and monitored the apoptosis induced. We found a significant difference in the levels of apoptosis between Hsp90 and control cells at 6 h of treatment with TNFα/CHX when analyzed with flow cytometry, while the level of Hsp90 and Hsp70 were not changed in this period (data not shown). Therefore, the 6-h time point was chosen for subsequent studies elucidating the functions of Hsp90 in apoptosis. At 6 h of treatment with TNFα/CHX, fewer floating cells could be detected in Hsp90-overexpressing cells (18%) compared with control cells (41%) under phase contrast microscopic examination (Fig. 1C, upper panel). The lower panels of Fig. 1C show the residual adherent cells after washing with PBS. No apoptosis was induced by TNFα or CHX treatment alone even after 8 h of culture (data not shown). To confirm the apoptotic characteristics of these cells and to quantify the difference, we performed TUNEL assay using confocal microscopy. As shown in Fig. 1D, a large proportion of control cells were undergoing apoptosis after 6 h of treatment with TNFα/CHX, while under the same conditions, considerably fewer Hsp90 cells were apoptotic. No difference existed between control and Hsp90 cells in non-treated conditions. Scoring of more than 200 cells from three different fields showed that Hsp90 significantly inhibits TNFα/CHX-induced apoptosis (Fig. 1E). We also used flow cytometry for analysis and quantification of the apoptosis induced by TNFα/CHX in control and Hsp90 cells. As shown in Fig. 1F, Hsp90 cells were significantly resistant to TNFα/CHX induced apoptosis, and this resistance depended on Hsp90 levels (Fig. 1A, lower). As shown in Fig. 1A, the clone that expressed less Hsp90 (clone 3) was less resistant to apoptosis than those (clones 1, 2 and 4) with higher Hsp90 levels. From our results, we believe that at least more than 50% increase in total Hsp90 expression was required to elicit its antiapoptotic roles. These Hsp90 clones were also resistant to its specific inhibitor, geldanamycin, induced apoptosis (data not shown). These results clearly demonstrate that overexpression of Hsp90 can significantly inhibit apoptosis and these results do not come from clonal variation.

3.2. Hsp90 functions downstream of caspase-8 but upstream of caspase-3

In order to investigate the molecular mechanism of Hsp90 inhibition of apoptosis, we first examined the activities of caspase-8 and caspase-3, the initiator and executioner caspases mediating the TNFα/CHX signal transduction pathway. For this analysis, we used colorimetric assays with Ac-DEVD-pNA and Ac-IETD-pNA as substrates. There were no differences in caspase-8 activities between Hsp90 cells and control cells before and after TNFα/CHX treatment (Fig. 2A), but there were significant differences in caspase-3 activities after TNFα/CHX induction (Fig. 2B). We also observed the proteolytic activation of caspase-3 in Hsp90 and control cells using immunoblotting analysis. As shown in Fig. 2C, the cleavage of procaspase-3 was delayed by Hsp90 overexpression; no cleaved caspase-3 was observed in Hsp90 cells after 6 h of TNFα/CHX treatment. In control cells, cleavage of procaspase-3 was evident after 6 h treatment. The difference in the levels of activated caspase-3 between Hsp90 and control cells was still apparent after 9 h of treatment. Levels of GAPDH immunoreac-

![Fig. 4. Cleavage of Bid was suppressed by Hsp90 overexpression.](image)
tivity were used as controls to demonstrate similar sample loading. The above results suggested that the apoptotic signals must pass through the mitochondria. To investigate this hypothesis, we examined the release of cytochrome c from the mitochondria of control and Hsp90 cells. The mitochondrial and S-100 fractions extracted from control and Hsp90 cells at the time points indicated in Fig. 3 were probed for cytochrome c by immunoblotting. The release of cytochrome c from mitochondria was suppressed in Hsp90 cells compared to the control cells at the 6-h time point. Corresponding with the inhibition of the release of cytochrome c from the mitochondria, the cytochrome c levels in S-100 fractions from Hsp90 cells were less than in control cells (Fig. 3B). However after 12 h of treatment with TNFα/CHX no differences were seen. The quantification of reduced mitochondrial and increased cytosolic cytochrome c is shown in the right panel, respectively. The levels of cytochrome oxidase four (COX IV) and GAPDH were used to assure similar sample loading of mitochondrial and S-100 fractions, respectively. Our results show that cytochrome c is released when apoptosis is induced by TNFα/CHX, and this event is blocked at early time point by overexpression of Hsp90.

3.3. Hsp90 inhibits cleavage of Bid

To clarify whether Hsp90 acts on mitochondria or on an upstream molecule, we investigated whether the cleavage
of Bid, which mediates signals from caspase-8 to the mitochondria, was affected by overexpression of Hsp90. We monitored the proteolytic cleavage of Bid by the loss of intact Bid and the increase of truncated Bid. As shown in Fig. 4A, the cleavage of Bid was inhibited in Hsp90 cells after 6 h of treatment as compared with control cells. Consistent with the loss of full length Bid, the truncated form of Bid (tBid) was increased in control cells. To test whether Hsp90 could directly prevent caspase-8 cleavage of Bid and the effect of geldanamycin in this process, human recombinant caspase-8 (1000 U/ml) was incubated with equal amount of lysates from control and Hsp90 cells in the presence or absence of geldanamycin (1 μM) for 2 h in caspase assay buffer. As shown in Fig. 4B, over-expression of Hsp90 impaired the cleavage of Bid and that this effect was suppressed by addition of geldanamycin.

We therefore tested whether Hsp90 could physically interact with Bid by performing coimmunoprecipitation experiments. Hsp90 coimmunoprecipitated with Bid in both control and Hsp90 cells (Fig. 5A). The same result was obtained by using another anti-Hsp90 antibody (SantaCruz) with another clone (data not shown). Fig. 5B showed that Bid was successfully immunoprecipitated from control and Hsp90 cells. To investigate whether exogenous myc-Hsp90 was included in the Bid coimmunoprecipitates, the membrane of Fig. 5B (left) was reprobed with anti myc-tag antibody (9B11). As shown in Fig. 5C, the exogenous Hsp90 simultaneously coimmunoprecipitated with Bid. We also investigated whether Bid coimmunoprecipitates with exogenous myc-Hsp90 by using anti-myc-tag antibody. As shown in Fig. 5D, under conditions where exogenous Hsp90 was successfully immunoprecipitated (upper), Bid also coimmunoprecipitated with exogenous myc-Hsp90.

4. Discussion

Our experiments suggest a role for Hsp90 within the pathways of TNFα-induced apoptosis in murine embryonic fibroblasts. This is consistent with the role of Hsp90 as a contributive, regulatory molecular chaperone that maintains its target molecules in an inactive, but competent condition [42,43]. In this sense then, our data are consistent with previous studies which showed that Hsp90 depletion by antisense techniques protects against apoptosis [41]. Hsp90 depletion might prevent apoptosis by the loss of a key proapoptotic mediator; whereas Hsp90 overexpression might block apoptosis by functionally repressing such an intermediate.

Our experiments show that one such intermediate might be Bid, and that Hsp90 functions as a negative regulator in the pathways downstream of TNFR-1. Our results clearly indicate that Hsp90 inhibits cell apoptosis through preventing the cleavage of the proapoptotic intermediate Bid by interacting directly with Bid. Bid is a BH-3 only domain molecule that links the death receptor pathway to the mitochondrial pathway of apoptosis. It has been suggested that caspase-8, following its own activation by TNFR/Fas engagement, directly cleaves Bid. Removal of the NH2 terminus of Bid would retain and potentially expose the predicted amphipathic α helix, BH3, on the active COOH-terminus p15 fragment. This proteolytic cleavage may alter an inert, intramolecular folded Bid or alternatively release Bid from a tethering chaperone-like molecule [28]. Our data verified this hypothesis and demonstrated the chaperone-like molecule is Hsp90. Our results also show that Hsp90 itself is stably maintained TNFα/CHX-induced apoptosis for at least 12 h (data not shown), reflecting the importance of Hsp90 for protection in cell survival. Our data showed that Bid interacts directly with Hsp90 and that the suppression of Hsp90 function by geldanamycin promotes the cleavage of Bid. This implies that one mechanism by which Hsp90’s inhibitor acts as an anti-cancer drug could be to promote the release of Bid from the Hsp90 complex. The subsequent increase in the cleavage of Bid, would in turn induce apoptosis in cancer cells. Recently, Vanden Berghe et al. [50] concluded that inhibition of HSP90 may alter the composition of the TNFR1 complex, favoring the caspase-8-dependent apoptotic pathway. Our findings support their conclusion. However, contrary to their results, our results showed apoptosis was caused by the activation of caspase-3 in NIH3T3 cells. One explanation for the differences in results could be the use of different cells. TNFα alone can induced cell death in their L929sA cells, but not in our NIH3T3 cells. So, what mechanism is responsible for the inhibition of Bid cleavage by Hsp90 binding? Recently, it has been reported that casein kinase I and II regulate the cleavage of Bid by affecting its phosphorylation [52]. The study showed that casein kinase I and II may cooperate to achieve complete phosphorylation of Bid. Interestingly, it has also been shown that Hsp90 binds and protects casein kinase II from self-aggregation and enhances its kinase activity [53]. Whether the prevention of cleavage of Bid is through the phosphorylation of Bid should be elucidated in the future. Though the phosphatase involved in dephosphorylation of Bid has not been identified [52], it is reasonable to postulate that Hsp90 might be involved in maintaining the balance of Bid phosphorylation and dephosphorylation. In addition, the region of Hsp90 responsible for binding Bid should also be determined by using deletion mutants of Hsp90.

In conclusion, we have shown that Hsp90 can suppress TNFα-induced apoptosis by preventing the cleavage of Bid. Our results, in conjunction with previously published data [48–51] suggest that Hsp90 can affect apoptosis at different
levels of regulation, depending on the stimuli. For cancer cells with high levels of Hsps, that are often resistant to chemical and/or radiation therapy, full unveiling of the functions of Hsp90 and its targets in apoptotic pathways would reveal novel targets for manipulating the sensitivity of cancer cells to therapies.

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