Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases

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The major heat shock protein, Hsp70, is an effective inhibitor of apoptosis. To study its mechanism of action, we created tumor cell lines with altered Hsp70 levels. The expression levels of Hsp70 in the cells obtained correlated well with their survival following treatments with tumor necrosis factor, staurosporine and doxorubicin. Surprisingly, the surviving Hsp70-expressing cells responded to the apoptotic stimuli by activation of stress-activated protein kinases, generation of free radicals, early disruption of mitochondrial transmembrane potential, release of cytochrome *c* from mitochondria and activation of caspase-3-like proteases in a manner essentially similar to that of the dying cells with low Hsp70 levels. However, Hsp70 inhibited late caspase-dependent events such as activation of cytosolic phospholipase A₂ and changes in nuclear morphology. Furthermore, Hsp70 conferred significant protection against cell death induced by enforced expression of caspase-3. Thus, Hsp70 rescues cells from apoptosis later in the death signaling pathway than any known anti-apoptotic protein, making it a tempting target for therapeutic interventions.

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Introduction

Tissue homeostasis is dependent on the dynamic balance between cell proliferation and apoptosis (Kerr *et al.*, 1972; reviewed by White, 1996; Nagata, 1997). Until recently, cancer researchers have focused most of their attention on pathways within the cell that induce proliferation, but the realization that malignant tumors may grow because cells have lost the ability to die has provided a new direction for investigation. Apoptosis occurs in tumors responding to tumor necrosis factor (TNF) and related cytokines, cytotoxic lymphocytes, hormone ablation, starvation, irradiation and chemotherapy (reviewed by Kerr *et al.*, 1994; White, 1996; Nagata, 1997). In spite of the wide variety of stimuli that can activate apoptosis, the signaling pathways induced by them converge into a common death pathway either at a mitochondrial step culminating in the release of cytochrome *c* from mitochondria to the cytosol (X.Liu *et al.*, 1996; Kluck *et al.*, 1997; Li *et al.*, 1997; Yang *et al.*, 1997; Bossy-Wetzel *et al.*,

1998) or at the following step at which Asp-Glu-Val-Asp (DEVD)-specific caspase-3-like cysteine proteases, also called the executioners of apoptosis, are activated (X.Liu *et al.*, 1996; Li *et al.*, 1997). Active caspases cleave a limited set of cellular proteins. Caspase-mediated cleavage often inhibits the function of their substrates but, interestingly, some enzymes, such as protein kinase C δ (PKC δ) (Ghayur *et al.*, 1996), MEKK1 (Cardone *et al.*, 1997), DNA fragmentation factor (Liu *et al.*, 1997), PITSLRE kinases (Beyaer *et al.*, 1997), p21-activated kinase hPAK65 (Lee *et al.*, 1997), Mst1 kinase (Graves *et al.*, 1998) and cytosolic phospholipase A₂ (cPLA₂) (Wissing *et al.*, 1997), are actually activated by caspases either directly by the cleavage of the enzyme or indirectly by activation/inactivation of regulatory proteins. These and other as yet unknown caspase-activated enzymes could be the actual downstream effectors of apoptosis, whose activation might result in the typical apoptotic morphology including membrane blebbing, nuclear condensation and cell shrinkage.

Tumor cells express several proteins that suppress apoptosis. Of special interest in cancer research are those that function at the common part of the apoptotic signaling pathway and thus inhibit apoptosis induced by a wide range of stimuli. Increased activity of such protective proteins, such as anti-apoptotic members of the Bcl-2 protein family (Vaux *et al.*, 1988; Boise *et al.*, 1993), members of the inhibitor of apoptosis protein (IAP) family (Rothe *et al.*, 1995; Deveraux *et al.*, 1997) and Hsp70 (Jäättelä *et al.*, 1992; Mosser *et al.*, 1997), may result in aggressively growing and therapy-resistant tumors. Recent results have elucidated the mechanisms of action of some of the anti-apoptotic proteins. Bcl-2 inhibits the fall in the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the release of cytochrome *c* from the mitochondria (Shimizu *et al.*, 1996; Kluck *et al.*, 1997; Yang *et al.*, 1997), whereas IAPs function by inhibiting the caspase activity (Deveraux *et al.*, 1997).

The mechanism of action by which Hsp70 exerts its anti-apoptotic effect is obscure. Its role in cellular resistance against stress was first suggested based on the correlation between its synthesis and the development of resistance to elevated temperatures (Li and Werb, 1982). More direct evidence supporting the role of Hsp70 in thermotolerance came from studies showing that inhibition of its expression or neutralization of its function rendered cells extremely sensitive to heat (Jonston and Kucey, 1988; Riabowol *et al.*, 1988). Recent results have made it clear that Hsp70 is a general anti-apoptotic protein, which protects cells from cytotoxicity induced by TNF, monocytes, oxidative stress, chemotherapeutic agents, ceramide and radiation (Jäättelä *et al.*, 1992; Jäättelä and Wissing, 1993; Simon *et al.*, 1995; Karlseder *et al.*, 1996; Mosser *et al.*, 1997).

To study at which step of the apoptotic signaling

pathway Hsp70 functions, we used two model systems; inhibition of Hsp70 expression by antisense *hsp70* cDNA in ME-180 cervix carcinoma cells with high constitutive Hsp70 expression, and overexpression of Hsp70 in WEHI-S fibrosarcoma cells with low endogenous Hsp70 levels. Interestingly, the analysis of death signaling path-

ways in these cells revealed that while Hsp70 protected cells from apoptosis, it did not inhibit activation of stress-activated protein kinases (SAPK or c-Jun N-terminal kinase, JNK), the early mitochondrial changes or the activation of caspase-3-like proteases. Hsp70 did, however, inhibit the function of a caspase-activated cPLA₂, the ensuing late $\Delta\psi_m$ collapse and apoptotic changes in nuclear morphology. Remarkably, Hsp70-expressing cells in which cytochrome *c* was released from mitochondria and downstream caspases were activated acquired normal growth shortly after the otherwise lethal stimuli. These data indicate that 'the point of no return' in apoptosis signaling is further downstream than previously believed.

Results

Hsp70 protects cells against TNF- and drug-induced apoptosis

ME-180 human cervical carcinoma cells express high levels of Hsp70 constitutively (Figure 1A). To reduce the Hsp70 expression, we transfected them with three different antisense *hsp70* cDNA constructs. G418-resistant cell pools (ME-ashsp1-P, ME-ashsp2-P and ME-ashsp3-P) and single-cell clones (ME-ashsp1-1, ME-ashsp1-2, ME-ashsp2-1 and ME-ashsp3-1) together with their vector-transfected controls (ME-vector-P and ME-vector-1) were then tested for Hsp70 expression by immunoblot analysis (Figure 1A). All three antisense constructs reduced Hsp70 protein levels as compared with vector-transfected control cells. The expression of the closely related Hsc70 was not affected. The antisense Hsp70 transfectants were significantly more susceptible than vector-transfected control cells to the cytotoxicity induced by TNF as analyzed by a 48 h cell viability assay (Figure 1A). The single cell clone ME-ashsp2-1 and the pool ME-ashsp3-P with lowest Hsp70 levels were the most TNF sensitive. In addition to TNF sensitivity, low Hsp70 expression correlated with the sensitivity of ME-180 transfectants to death induced by staurosporine and doxorubicin (Figure 1B).

Unstressed WEHI-S murine fibrosarcoma cells express very low levels of Hsp70. To obtain another model system to study the mechanism of action of Hsp70, we transfected WEHI-S cells with human *hsp70* cDNA. Two single-cell clones with the highest Hsp70 expression levels (WEHI-hsp-2 and WEHI-hsp-18) and their vector-transfected

Fig. 1. Inhibition of Hsp70 synthesis sensitizes ME-180 cells to TNF, staurosporine and doxorubicin. (A) Single-cell clones (ME-ashsp1-1, ME-ashsp1-2, ME-ashsp2-1 and ME-ashsp3-1) and pools (ME-ashsp1-P, ME-ashsp2-P and ME-ashsp3-P) of ME-180 cells transfected with antisense Hsp70 constructs and their respective controls (ME-vector-1 and ME-vector-P) were plated on 96-well plates at a density of 8000 cells/0.1 ml of RPMI medium supplemented with 0.5% FCS and, 24 h later, treated with TNF for 48 h. The survival of TNF-treated cells is presented as a percentage of the survival of untreated cells as measured by an MTT assay. The experiment was repeated four times with essentially the same results. The amount of Hsp70 and Hsc70 in cell lysates from $\sim 3 \times 10^5$ cells was determined by immunoblot. (B) The sensitivity of cells to 100 nM staurosporine and 500 ng/ml of doxorubicin was analyzed by a 48 h MTT assay as above. The experiment was repeated twice with essentially the same results. (C) Approximately 1000 cells were plated in 96-well plates in RPMI + 0.5% FCS. After 24 h incubation, 10 ng/ml of TNF was added and the cell density was measured at the indicated times by an MTT assay. The experiment was repeated once with essentially the same results. The means of triplicate samples $\pm$ SD are shown (A–C).

Fig. 2. Overexpression of Hsp70 renders WEHI-S cells resistant to TNF, staurosporine and doxorubicin. (A) Immunoblot of Hsp70 protein expression in vector-transfected (WEHI-vector-2 and WEHI-vector-3) and Hsp70-transfected (WEHI-hsp-2 and WEHI-hsp-18) single-cell clones of WEHI-S cells. (B) Cells were plated on 96-well plates at a density of 15 000 cells/0.1 ml of complete medium. After 24 h, 100 pg/ml of TNF, 100 nM staurosporine or 1 µg/ml of doxorubicin were added and the survival of the cells was measured 18 h later by an MTT assay. The experiment was repeated three times with essentially the same results. (C) Approximately 2000 cells were plated in 96-well plates in RPMI medium containing 5% FCS. After 24 h incubation, 100 pg/ml of TNF was added and the cell density was measured at the indicated times by an MTT assay. The experiment was repeated once with essentially the same results. The means of triplicate samples $\pm$ SD are shown (B and C).

control clones (WEHI-vector-2 and WEHI-vector-3) were tested for their sensitivity to TNF, staurosporine and doxorubicin in an 18 h cell viability assay. As expected based on earlier results (Jäättelä *et al.*, 1992; Simon *et al.*, 1995), Hsp70 expression in the transfectants obtained correlated with the resistance against cell death induced by TNF and doxorubicin (Figure 2). Moreover, Hsp70 conferred resistance against staurosporine, further indicating that Hsp70 protects cells from a wide range of inducers

of apoptosis (Figure 2). The protective effect of Hsp70 against TNF, staurosporine and doxorubicin in both ME-180 and WEHI-S cells was confirmed by a chromium release assay and by counting of cells with apoptotic morphology (data not shown).

To test whether the Hsp70-associated protection from apoptosis described above was due to a delay in the cell death, we followed the growth of TNF-treated ME-180 and WEHI-S transfectants for 96 and 60 h, respectively. Remarkably, ~80% of the Hsp70-expressing ME-vector-P and WEHI-hsp-2 cells, which survived the TNF treatment, reached a normal growth rate after 48 and 35 h, respectively (Figures 1C and 2C).

Hsp70 does not affect apoptosis-associated activation of SAPK/JNK

ME-180 and WEHI-S cells with altered levels of Hsp70 were then used to study the mechanism of the anti-apoptotic action of Hsp70. The AP-1-like transcription factors, which are activated by SAPK/JNK-mediated phosphorylation of c-Jun, have been suggested to mediate TNF- and stress-induced apoptosis (Xia *et al.*, 1995; Verheij *et al.*, 1996). Therefore, we studied the ability of Hsp70 to interfere with the SAPK/JNK pathway by a kinase assay employing c-Jun as a substrate and by an AP-1-responsive luciferase reporter gene assay. TNF activated SAPK/JNK in both ME-180 and WEHI-S transfectants, whereas only WEHI-S transfectants responded to staurosporine. In all cases, the SAPK/JNK activation was rapid and transient, reaching a peak at 30–60 min. In repeated experiments, no significant difference could be seen in the TNF-induced SAPK/JNK activities of ME-vector-2 and ME-ashsp2-1 cells, the maximal inductions being 72- and 58-fold, respectively (Figure 3A). WEHI-vector-2 and WEHI-hsp-2 cells responded to 100 pg/ml of TNF with up to 4.7- and 4.9-fold induction in SAPK/JNK activity, and to 100 nM staurosporine with up to 41- and 32-fold induction, respectively (data not shown). In conclusion, Hsp70 had no significant effect on the activation of SAPK/JNK. Surprisingly, neither TNF- nor staurosporine-induced activation of SAPK/JNK led to the activation of AP-1-like transcription factors as measured by a luciferase assay (Figure 3B). Contrary to apoptotic stimuli, phorbol 12-myristate-13-acetate (PMA) activated AP-1-like transcription factors (Figure 3B).

Hsp70 does not affect apoptosis-associated early mitochondrial changes but blocks the late fall in $\Delta\psi_m$

Heat shock has been shown to protect mitochondria from hydrogen peroxide-induced damage (Polla *et al.*, 1996). Thus, we studied the ability of Hsp70 to protect cells from apoptosis-associated mitochondrial alterations, i.e. free radical generation, reduction in the $\Delta\psi_m$ and release of cytochrome *c* from mitochondria. As shown in Figure 4A, staurosporine induced a similar early free radical burst in vector-transfected and Hsp70-overexpressing WEHI-S cells. Treatment of WEHI-S transfectants with up to 1 ng/ml of TNF and ME-180 transfectants with up to 100 ng/ml of TNF or 100 nM staurosporine failed to induce measurable generation of free radicals.

In some apoptosis models, the reduction in the $\Delta\psi_m$ is

Fig. 3. Hsp70 has no effect on apoptosis-associated activation of SAPK/JNK. (A) Cells were treated with 10 ng/ml of TNF for the indicated times prior to harvesting and measuring the SAPK/JNK activity using the solid-phase kinase assay. (B) Luciferase activity was measured in cells transiently transfected with pAP(4)-LUC plasmid containing four AP-1-binding sites upstream of the *Ppyralis* luciferase-coding sequence. Two days after the transfection, cells were stimulated for 5 h with 100 ng/ml of PMA, 20 ng/ml (ME-180 transfectants) or 1 ng/ml (WEHI transfectants) of TNF, or 100 nM staurosporine. The values represent means of a duplicate experiment $\pm$ SD. The experiment was repeated once with essentially the same results.

an early event which precedes the release of caspase-activating factors from mitochondria, whereas in others the cytochrome *c* release occurs independently of the fall in the $\Delta\psi_m$ (Zamzami *et al.*, 1995; Susin *et al.*, 1997; Bossy-Wetzel *et al.*, 1998). In models used in this study, only ME-180 transfectants treated with staurosporine responded with a reduction in $\Delta\psi_m$ prior to the cell death. Hsp70 had, however, no effect on this early staurosporine-induced $\Delta\psi_m$ fall (Figure 4B). In staurosporine-treated WEHI-S cells, $\Delta\psi_m$ fell first at the time of cell death, ~10–12 h after the addition of the drug. TNF induced a similar late $\Delta\psi_m$ disruption, which occurred simultaneously with the cell death, ~2–3 and 12 h after the release of cytochrome *c* in WEHI-S and ME-180 cells, respectively. As shown for TNF-treated ME-180 transfectants, the late $\Delta\psi_m$ fall was inhibited by Hsp70 in a manner similar to cell death (Figure 4C).

The release of cytochrome *c* from mitochondria into the cytosol is a crucial step in apoptosis signaling as it results in the activation of downstream caspases (Li *et al.*, 1997; Bossy-Wetzel *et al.*, 1998). Treatment of ME-180 cells with TNF or staurosporine resulted in a clear reduction in the mitochondrial cytochrome *c* levels at ~8 and 5 h, respectively. The disappearance of cytochrome *c* from the mitochondrial fraction was not due to a complete destruction of the mitochondria as the level of another mitochondrial protein, Grp75, remained unchanged in this fraction. Surprisingly, Hsp70 had no effect on either TNF- (Figure 5A) or staurosporine-induced (Table I) cytochrome *c* efflux. WEHI-S cells also responded to TNF and staurosporine by a clear reduction in mitochondrial

cytochrome *c* levels at ~1.5 and 2.5 h, respectively. Hsp70 expression levels in WEHI-S cells had no effect on apoptosis-associated release of cytochrome *c* (Figure 5B, Table I).

Hsp70-mediated protection from apoptosis occurs downstream of caspase-3-like proteases

The next possible step at which Hsp70 could inhibit apoptosis signaling is the cytochrome *c*-mediated activation of downstream caspases. Thus, we tested the effect of Hsp70 on the activation of DEVD-specific caspases using a colorimetric probe Ac-DEVD-pNA. Surprisingly, treatment of ME-180 transfectants with TNF, staurosporine or doxorubicin induced as strong an activation of DEVD-specific caspases in apoptosis-resistant control cells with high Hsp70 expression as in apoptosis-sensitive antisense Hsp70 transfectants (Figure 6A–C). TNF- but not staurosporine- or doxorubicin-induced activation of caspases was delayed by ~4 h in control cells as compared with the antisense Hsp70 transfectants. WEHI-S cells also responded to apoptotic stimuli by activation of DEVD-specific caspases. In spite of the strong protective effect of Hsp70, it could not attenuate the activation of DEVD-specific caspases, but delayed their activation by TNF and staurosporine by ~1 and 4 h, respectively (Figure 6D–F). Contrary to Hsp70, both Bcl-2, which protects ME-180 cells (Jäättelä *et al.*, 1995), and CrmA, which protects WEHI-S cells from TNF-mediated apoptosis (Wissing *et al.*, 1997), inhibited the activation of caspases (Figure 6).

As the assay system used measures the ability of caspases to cleave an artificial peptide target, Hsp70 could bind to important caspase substrates *in vivo* and thereby inhibit their cleavage. Therefore, we studied the effect of Hsp70 on TNF-induced cleavage of known targets for DEVD-specific caspases. As shown in Figure 7, poly(ADP-ribose) polymerase (PARP), cPLA₂ and PKC δ are cleaved to the same extent in ME-180 cells with high and low Hsp70 expression. Similarly, PARP and cPLA₂ cleavage were unaffected by Hsp70 in WEHI-S transfectants (data not shown). These data indicate either that Hsp70 protects cells from the consequences of proteolysis by caspase-3-like proteases or that apoptosis is mediated by a caspase-independent mechanism. To test the latter hypothesis, we first studied the ability of a specific tetrapeptide inhibitor of caspase-3-like proteases, Ac-DEVD-aldehyde (DEVD), to interfere with death signaling. As DEVD conferred a clear protection from cell death in both ME-180 and WEHI-S transfectants with low Hsp70 expression levels, the activity of DEVD-specific caspases must be necessary for the cytotoxicity in the model systems used (Figure 8).

Hsp70 protects WEHI-S cells from death induced by enforced expression of caspase-3

The above data suggest that Hsp70 protects cells from caspase-3-like proteases. Thus, we established a model system to investigate directly the ability of Hsp70 to protect cells from enforced expression of caspase-3. Caspase-3 cDNA and/or an empty vector were co-transfected with β -galactosidase cDNA into WEHI-S cells, and cells were incubated for 24 h in the presence or absence of a caspase-3 inhibitor, DEVD. Thereafter, cells were fixed

Fig. 4. Hsp70 has no effect on the generation of free radicals or the early reduction in $\Delta\Psi_m$, but inhibits the late $\Delta\Psi_m$ fall. (A) Generation of free radicals in untreated cells and cells treated with 100 nM staurosporine was analyzed by flow cytometry analysis of DHR123-labeled cells. The data are presented as mean fluorescence intensity. (B and C) The $\Delta\Psi_m$ was analyzed by flow cytometry analysis of RH123-labeled cells. The data are presented as the percentage of the mean fluorescence intensity in staurosporine-treated cells as compared with untreated cells (B) or as the percentage of TNF-treated cells with reduced $\Delta\Psi_m$ (C).

Table I. A summary of the effects of Hsp70 on TNF- and staurosporine-induced cellular events

Event ^a	WEHI-hsp/WEHI-vector (%)		ME-vector/ME-ashsp (%)	
	TNF	Staurosporine	TNF	Staurosporine
JNK activation	138 ± 36 (2) ^b	103 ± 25 (2)	99 ± 3 (2)	N.A.
ROI release N.A. ^c	96 ± 11 (3)	N.A.	N.A.	N.A.
Early $\Delta\Psi_m$ fall N.A.	N.A.	N.A.	110 ± 2 (2)	
Cytochrome <i>c</i> release	100 ± 0 (3)	100 ± 0 (2)	97 ± 9 (3)	100 (1)
DEVDase activation	106 ± 4 (8)	115 ± 14 (4)	117 ± 9 (8)	114 ± 8 (4)
AA release	15 ± 4 (4)	45 ± 5 (4)	35 ± 8 (4)	51 ± 9 (4)
Nuclear changes	0 ± 0 (2)	27 ± 24 (2)	N.D. ^d	N.D.
Late $\Delta\Psi_m$ fall	15 ± 5 (2)	29 ± 1 (2)	33 ± 12 (2)	N.A.
Death	25 ± (10)	55 ± 11 (6)	30 ± 6 (10)	65 ± 5 (6)

^aTNF- and staurosporine-induced cellular events are presented in the order of their appearance.

^bThe effect of Hsp70 expression on the induction of the indicated events is expressed as a percentage ± SD of the TNF- or staurosporine-induced change in cells with high Hsp70 expression as compared with the change in cells with low Hsp70 expression. The calculations were made using values representing the maximal inductions of signaling events occurring before the onset of death, except for values for late $\Delta\Psi_m$ fall, which occurred at the time at which cells began to die, and death, which was monitored 18 (WEHI-S) or 48 h after the addition of TNF or staurosporine. The number of experiments performed is given in parentheses. For methods used, see the legends to Figures 1–6 and 10.

^cN.A., not applicable as the indicated event does not occur.

^dN.D., not detected.

and stained for β -galactosidase and the number of surviving transfectants was determined by counting β -galactosidase-positive blue cells. DEVD had no effect on the survival of vector-transfected cells but increased that of cells transfected with caspase-3 by >100% (Figure 9). These data indicate that enforced expression of caspase-3 leads to the activation of caspase-3 and to the death of WEHI-S cells. Remarkably, co-transfection of Hsp70 cDNA together with caspase-3 cDNA reduced the ability of DEVD to increase the survival of transfected cells to 16%, indicating that Hsp70 protects cells from caspase-3 almost as effectively as DEVD (Figure 9). Co-transfection of a cDNA encoding CrmA, a known caspase inhibitor, together with caspase-3 cDNA resulted in a protection from caspase-3-induced death similar to co-transfection with Hsp70, whereas a functionally defective mutant of CrmA could not protect WEHI-S cells from caspase-3. Thus, Hsp70 protects WEHI-S cells from caspase-3-induced cell death as effectively as CrmA.

Hsp70 inhibits caspase-3-mediated apoptotic events

The ability of Hsp70 to inhibit caspase-3-induced cell death without inhibiting its proteolytic activity suggests that Hsp70 protects cells from the consequences of caspase-3-mediated proteolysis. We have shown earlier that cPLA₂, which mediates the release of arachidonic acid (AA) from the sn-2 position of phospholipids, is one of the caspase-activated mediators of TNF-induced cell death (Wissing *et al.*, 1997). As shown in Figure 10A, staurosporine and doxorubicin also induced a caspase-dependent release of AA from ME-ashsp3-P and WEHI-vector-2 cells with low Hsp70 expression before the onset of cell death. Interestingly, the AA release induced by TNF, staurosporine and doxorubicin was significantly reduced in Hsp70-expressing ME-vector-P and WEHI-hsp-2 cells as compared with low Hsp70-expressing ME-ashsp3-P and WEHI-vector-2 cells, respectively (Figure 10A).

To investigate whether Hsp70 could inhibit other cas-

Fig. 5. Hsp70 does not inhibit TNF-induced release of cytochrome *c* from mitochondria. ME-180 transfectants were treated with 10 ng/ml of TNF (**A**) and WEHI-S transfectants with 100 pg/ml of TNF (**B**) for the indicated times before harvesting. Mitochondrial extracts were then prepared and analyzed for cytochrome *c* (cc) and mitochondrial heat shock protein (grp75) by immunoblot.

pase-mediated events, we tested its ability to inhibit apoptosis-associated nuclear changes, chromatin condensation and fragmentation, employing TdT-mediated dUTP-fluorescein nick end labeling (TUNEL). As shown in Figure 10B, treatment of WEHI-vector-2 cells with 100 nM staurosporine for 7 h increased the number of apoptotic nuclei >18-fold. Both DEVD and Hsp70 inhibited these changes almost completely (Figure 10B). Due to a rapid detachment of apoptotic cells from the surface, treatment of WEHI-vector-2 cells with 1 ng/ml of TNF for 4, 5 or 10 h resulted in a significantly smaller increase (2- to 2.5-fold) in cells with apoptotic nuclear changes as analyzed by the TUNEL assay at the indicated time points. The TNF-induced increase in TUNEL-positive cells was inhibited completely by both pre-treatment with DEVD (data not shown) and overexpression of Hsp70 (Table I). Due to the strong autofluorescence of doxorubicin, the TUNEL assay could not be used to detect apoptotic nuclei in doxorubicin-treated cells.

Discussion

The data presented above clearly show that Hsp70 is a general anti-apoptosis protein. The apoptotic signaling pathways culminating in the release of cytochrome *c* from mitochondria to the cytosol and the subsequent activation of caspase-3-like proteases were activated in a surprisingly similar manner in Hsp70-expressing apoptosis-resistant cells and in apoptosis-sensitive cells (Table I). Remarkably, the Hsp70-expressing cells were able to regain a normal growth rate shortly after the otherwise lethal stimuli in spite of the cytochrome *c* efflux and the cleavage of caspase substrates. These data suggest that Hsp70 exerts its protective effect downstream of caspase-3-like proteases or that the caspase-dependent death pathway is not essential for the cell death in these cells. The latter is, however, unlikely, as death can be inhibited effectively in these cells by a specific caspase-3 inhibitor, DEVD, a general

caspase inhibitor, CrmA (Wissing *et al.*, 1997), as well as by the well characterized anti-apoptotic protein, Bcl-2 (Jäättelä *et al.*, 1995), which acts by inhibiting the release of cytochrome *c* from mitochondria (Kluck *et al.*, 1997; Yang *et al.*, 1997). Furthermore, Hsp70 conferred a significant protection from cell death induced by enforced expression of caspase-3. Thus, Hsp70 is likely to protect cells from the consequences of caspase-mediated proteolysis. How this proteolysis leads to cell death is still unclear. Recent data suggest that caspase-activated enzymes including PKC δ , MEKK1, DNA fragmentation factor, PITSRE kinases, hPAK65, Mst1 kinase and cPLA₂ may act in concert to execute the cell (Ghayur *et al.*, 1996; Beyaer *et al.*, 1997; Cardone *et al.*, 1997; Lee *et al.*, 1997; Liu *et al.*, 1997; Wissing *et al.*, 1997; Graves *et al.*, 1998). Thus, variations in their expression levels in different cell types may explain differences observed in the final steps of apoptosis. Recent results showing that the specific inhibition of the enzymatic activity of a caspase-activated cPLA₂ represses TNF-induced apoptosis in WEHI-S cells strongly suggest that cPLA₂ is one of the crucial downstream effector molecules in these cells (Wissing *et al.*, 1997). Our earlier data showing that Hsp70 inhibits TNF-induced cPLA₂ activity in WEHI-S cells (Jäättelä, 1993) prompted us to study whether the inhibition of this enzyme activity by Hsp70 could be a more general phenomenon. First, we confirmed that in addition to TNF, staurosporine and doxorubicin also induced a caspase-dependent release of AA from both WEHI-S and ME-180 cells. Remarkably, the AA release induced by TNF, staurosporine and doxorubicin was inhibited effectively by Hsp70. It is important to note that, whether caspases activate cPLA₂ directly by cleavage or indirectly by activating/inactivating other proteins regulating its activity, is still unclear. Hsp70 had, however, no effect on the cleavage of cPLA₂ or other caspase substrates analyzed. Our data showing that DEVD effectively inhibits cPLA₂-like activity induced by TNF, staurosporine and doxorubicin suggest that Hsp70 acts by inhibiting the action of cPLA₂ or that of a putative caspase substrate which regulates the enzymatic activity of cPLA₂. It remains to be determined whether Hsp70 specifically inhibits the activation of cPLA₂ or whether activities of other caspase-activated enzymes are repressed as well. The latter hypothesis is more likely as WEHI-S cells are rendered more resistant to TNF-mediated cell death by enforced expression of Hsp70 than by a specific inhibitor of cPLA₂ (Wissing *et al.*, 1997). Moreover, Hsp70 effectively inhibited the appearance of apoptotic nuclear changes, which are likely to be mediated by a caspase-3-dependent, but cPLA₂-independent pathway (Lee *et al.*, 1997; Woo *et al.*, 1998).

Staurosporine induced an early Hsp70-insensitive $\Delta\psi_m$ reduction in ME-180 cells, whereas the TNF-induced reduction of the $\Delta\psi_m$ in both ME-180 and WEHI-S cells, as well as the staurosporine-induced $\Delta\psi_m$ fall in WEHI-S cells, occurred late and were inhibited by Hsp70 (Table I). These data suggest that the disruption of the $\Delta\psi_m$ can be triggered by different mechanisms. It has been suggested that the early $\Delta\psi_m$ collapse results in the free radical burst followed by the release of apoptotic factors from mitochondria (Zamzami *et al.*, 1995; Susin *et al.*, 1997). In both WEHI-S and ME-180 cells, TNF and staurosporine

Fig. 6. Hsp70 does not inhibit TNF-, staurosporine- or doxorubicin-induced activation of DEVD-specific caspases. ME-180 transfectants (A–C) and WEHI-S transfectants (D–F) were treated with the indicated concentrations of TNF (A and D), staurosporine (B and E) or doxorubicin (C and F). The DEVDase activity of the cell lysates was determined based on their ability to cleave Ac-DEVD-pNA. The mean values of triplicate samples $\pm$ SD are shown. The experiment was repeated three times (A and D) or once (B, C, E and F) with essentially the same results.

Fig. 7. Hsp70 has no effect on the TNF-induced cleavage of caspase substrates. Subconfluent ME-180 transfectants were treated with 10 ng/ml of TNF for the indicated times, whole cell extracts were prepared and an equal amount of protein from each extract was analyzed for PARP, PKC δ and cPLA $_2$ by immunoblot.

induced an early cytochrome *c* efflux, whereas an early $\Delta\psi_m$ was seen only in staurosporine-treated ME-180 cells and the free radical burst only in staurosporine-treated WEHI-S cells. These data indicate that $\Delta\psi_m$ disruption and free radical burst are not necessarily connected to each other or to the cytochrome *c* release.

Fig. 8. DEVD-specific caspases are mediators of cell death induced by TNF, staurosporine and doxorubicin. The effect of DEVD on the sensitivity of ME-ashsp2-1 cells to 10 ng/ml of TNF (24 h), 100 nM staurosporine (24 h) and 1 μ g/ml of doxorubicin (28 h) and that of WEHI-vector-2 cells to 1 ng/ml of TNF (12 h), 100 nM staurosporine (12 h) and 1 μ g/ml of doxorubicin (22 h) was analyzed by a chromium release assay. When indicated (+ DEVD), DEVD at the final concentration of 200 (ME-ashsp2-1) or 50 μ M (WEHI-vector-2) was added to the cells 1 h before the addition of the cytotoxic agent. The mean values of triplicate samples $\pm$ SD are shown. The experiment was repeated once with essentially the same results.

The role of SAPK/JNK in apoptosis signaling is controversial. Based on results obtained with kinase-inactive mutants of activators of SAPK/JNK as well as with a dominant-negative mutant of a SAPK/JNK substrate, c-Jun, the SAPK/JNK-mediated activation of transcription

Fig. 9. Hsp70, CrmA and DEVD protect WEHI-S cells from caspase-3-induced cell death. WEHI-S cells were transiently transfected with 3 μ g of pcDNA3 or pcDNA-caspase-3 (caspase-3) and 6 μ g of pSV2neo, pSV-Hsp70-tag (Hsp70), pcDNA-CrmA (CrmA) or pcDNA-CrmA-mut (CrmA-m) together with 2 μ g of pCMV- β Gal by electroporation. Transfected cells were incubated for 24 h in the absence (- DEVD) or presence (+ DEVD) of 75 μ M DEVD, fixed and stained for β -galactosidase. The number of blue cells was determined by counting 2000 cells in five blindly chosen fields. The means of five determinations $\pm$ SD are shown. The experiment was repeated once with essentially the same results.

factor AP-1 has been suggested to be essential for TNF- and stress-induced apoptosis (Xia *et al.*, 1995; Verheij *et al.*, 1996). Other results suggest, however, that TNF-induced activation of SAPK/JNK is not linked to the apoptotic pathway (Z.-G.Liu *et al.*, 1996; Natoli *et al.*, 1997). TNF induced a rapid activation of SAPK/JNK in both WEHI-S and ME-180 cells, and staurosporine activated it in WEHI-S cells. Hsp70 had no effect on TNF- or staurosporine-induced SAPK/JNK activity. This observation is in agreement with earlier data showing that the constitutive expression of Hsp70 does not inhibit heat- or ceramide-induced activation of SAPK/JNK (Mosser *et al.*, 1997). Contrary to the constitutive expression of Hsp70, its inducible expression blocks SAPK/JNK activation. This inhibition is, however, not likely to be crucial for the protective effect as induced and constitutive expression of Hsp70 are equally potent in conferring resistance against heat and ceramide (Mosser *et al.*, 1997).

The following data suggest that the ability of Hsp70 to prevent apoptosis could enhance tumorigenesis and limit the efficacy of cancer therapy: (i) Hsp70 is highly expressed in many tumor cells (Mivechi and Rossi, 1990; Ferrarini *et al.*, 1993; Kaur and Ralhan, 1995); (ii) transgenic mice expressing the human *hsp70* gene develop T-cell lymphomas (Seo *et al.*, 1996); (iii) levels of Hsp70 in fibrosarcoma cells correlate with their tumorigenic potential in mice (Jäättelä, 1995); and (iv) the expression of Hsp70 is an indicator of poor therapeutic outcome in breast cancer (Ciocca *et al.*, 1993). In this report, we show that Hsp70 rescues tumor cells from apoptosis late in the death signaling pathway. Thus, neutralization of the inhibitory activity of Hsp70 in tumor cells may prove useful in cancer therapy.

Fig. 10. DEVD and Hsp70 inhibit apoptosis-associated arachidonic acid release (A) and nuclear changes (B). (A) [3 H]Arachidonic acid-labeled cells were treated with 100 pg/ml of TNF for 4 h (WEHI transfectants), 10 ng/ml of TNF for 16 h (ME-180 transfectants), 100 nM staurosporine for 5 h or 1 μ g/ml of doxorubicin for 16 h. The release of radioactivity into the supernatant was measured and is presented as a percentage of the release from untreated cells. When indicated (+ DEVD), cells were pre-treated for 1 h with 50 (WEHI-vector-2) or 200 μ M (ME-ashsp3-P) DEVD. The mean values of triplicate samples $\pm$ SD are shown. (B) WEHI-S transfectants were left untreated (spont.) or treated with 100 μ M staurosporine for 7 h, fixed and analyzed for apoptotic nuclear changes by TUNEL assay (Boehringer-Mannheim). When indicated (+ DEVD), cells were pre-treated for 1 h with 200 μ M DEVD. The number of TUNEL-positive cells was determined by counting four independent fields and the means of four determinations $\pm$ SD are shown. The experiments were repeated once with essentially the same results.

Materials and methods

Plasmids

Four PCR primers corresponding to the indicated bases in the human *hsp70* sequence (Milarski and Morimoto, 1986), and containing *KpnI* (primers 2 and 4) and *XhoI* (primers 1 and 3) restriction sites, were synthesized: primer 1 (5'-ATATCTCGAGCAGGGAACCGCATG-3') contained bases 475-492 in the sense orientation; primer 2 (5'-TAATGGTACCTTGGGCTTGTCTCC-3') contained bases 780-796 in the antisense orientation; primer 3 (5'-ATATCTCGAGGAGATCTCGT-CCA-3') contained bases 836-852 in the sense orientation; and primer 4 (5'-ATATGGTACCGCATCCTTGGTGG-3') contained bases 958-974 in the antisense orientation. The antisense Hsp70 sequences were amplified from pSV-hsp70-tag plasmid (Jäättelä *et al.*, 1992) by PCR using primers 1 and 2 (ashsp1), 3 and 4 (ashsp2), and 1 and 4 (ashsp3) and Vent DNA polymerase (New England Biolabs, Beverly, MA). Following digestion with *KpnI* and *XhoI*, PCR fragments were subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) in the antisense orientation, creating three antisense Hsp70 expression plasmids, pcDNA-ashsp1, pcDNA-ashsp2 and pcDNA-ashsp3.

Plasmids pcDNA-caspase-3 (Tewari *et al.*, 1995), pcDNA-CrmA and pcDNA-CrmA-mut (Tewari and Dixit, 1995) were kindly provided by Dr Vishva Dixit (Genentech, CA), pCMV- β Gal (Saksela and Baltimore, 1993) by Dr Kalle Saksela (Institute for Medical Technology, Tampere, Finland) and pAP(4)-LUC by Dr Ami Aronheim (Technion, Haifa, Israel).

Cell lines, transfections and culture conditions

To create cells with reduced Hsp70 expression, pcDNA-ashsp1, pcDNA-ashsp2 and pcDNA-ashsp3 plasmids and pcDNA3 vector were introduced into ME-180 human cervix carcinoma cells by electroporation as described previously (Jäättelä *et al.*, 1995). G418-resistant cells were grown as pool populations originating from a minimum of 10 000 colonies (ME-vector1-P, ME-ashsp1-P, ME-ashsp2-P and ME-ashsp3-P) or cloned by limiting dilution to create single-cell clones (ME-ashsp1-1, ME-ashsp1-2, ME-ashsp2-1 and ME-ashsp3-1). ME-Bcl-2 cells are ME-180 cells successfully transfected with pEBS7-Bcl-2 plasmid (Jäättelä *et al.*, 1995).

To create Hsp70-overexpressing cells, WEHI-S cells were transfected by electroporation with pSV-hsp70-tag plasmid along with pSV2neo plasmid containing the neomycin transferase gene as described previously (Jäättelä *et al.*, 1992). G418-resistant cells were cloned by limiting dilution to create single-cell clones (WEHI-vector-2, WEHI-vector-3, WEHI-hsp-2 and WEHI-hsp-18). The WEHI-S cell line is a highly TNF-sensitive clone of WEHI 164 murine fibrosarcoma cells and WEHI-CrmA-4 is a single-cell clone of WEHI-S cells successfully transfected with pcDNA-CrmA plasmid (Wissing *et al.*, 1997).

Transient transfections of WEHI-S cells were performed by electroporation (125 μ F, 270 V and 1000 Ω) employing 2×10^6 cells and the indicated amounts of DNA in 50 μ l of phosphate-buffered saline (PBS). Transfected cells were plated on 24-well plates at a density of 3×10^5 cells/ml/well. At 24 h after transfection, cells were washed twice with PBS, fixed with methanol for 10 min at -20°C and washed again twice with PBS. Staining for β -galactosidase was performed by incubating the fixed cells for a minimum of 1 h at 37°C with 0.3 ml of staining solution (1 mg/ml Xgal from Amersham Pharmacia Biotech in PBS supplemented with 50 μ M MgCl_2 , 1.64 mg/ml potassium ferricyanide and 2.12 mg/ml potassium ferrocyanide).

Cells were cultured at 37°C in a humidified air atmosphere with 5% CO_2 . RPMI 1640 medium (Gibco Ltd, Paisley, UK) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco Ltd) and glutamax was used as a growth medium for all cell lines and is referred to as complete medium. Medium used for the culture of transfected cell lines was supplemented further with 0.2 mg/ml G418 (Gibco Ltd) or 150 U/ml of hygromycin (Calbiochem, La Jolla, CA). All cell lines were tested and found negative for mycoplasma.

TNF was kindly provided by Dr Anthony Cerami (Kenneth Warren Laboratories, Tarrytown, NY). Staurosporine, doxorubicin and PMA were from Sigma Chemical Co. (St Louis, MO) and Ac-DEVD-CHO from Biomol (Plymouth Meeting, PA). All experiments with ME-180 transfectants were performed in RPMI supplemented with 0.5% FCS following a pre-incubation of cells in this medium for 18–24 h. Experiments with WEHI-S transfectants were performed in complete medium.

Immunoblot analysis

Immunoblot analyses were performed as described previously (Wissing *et al.*, 1997). The primary antibodies used were mouse monoclonal antibody SPA810 recognizing both human Hsp70 and mouse Hsp68 (StressGen, Victoria, BC, Canada), mouse monoclonal antibody to cytochrome *c* (Pharmingen), mouse monoclonal antibody to mitochondrial Grp75 (StressGen), mouse monoclonal antibody C2-10 recognizing PARP (Lazebnik *et al.*, 1994), rabbit polyclonal antiserum specific for cPLA₂ (Wissing *et al.*, 1997) and rabbit polyclonal antibody to human PKC δ (Santa Cruz, CA). The appropriate peroxidase-conjugated secondary antibodies were from Dako-Immunoglobulins A/S, Denmark. Chemiluminescence reactions were performed according to the manufacturer's instructions employing ECL Western blotting reagents (Amersham International, UK).

Survival and apoptosis assays

The dimethylthiazol-diphenyltetrazolium bromide (MTT) assay was used to measure the viability, and the ^{51}Cr release assay to measure the death of cells as described previously (Jäättelä *et al.*, 1989, 1995). Apoptotic nuclear changes were detected by a TUNEL kit (Boehringer Mannheim) following the manufacturer's instructions. After the TUNEL reaction, slides were contrasted with 50 ng/ml of Hoechst 33258 (Sigma) in PBS for 5 min at 25°C . Slides were mounted with fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL) supplemented with 2.5 mg/ml propylgalate and examined by fluorescence microscopy (Olympus BX50, Japan). A minimum of three areas of each sample were photographed both for TUNEL labeling and Hoechst 33258 staining by an Olympus PMPB20 camera using Kodak TMax 400 pro

film. The apoptotic index was determined by counting the percentage of TUNEL-positive cells in the total number of cells.

Activation of the SAPK/JNK pathway

The cell lysates for the SAPK/JNK assay were prepared as described (Kallunki *et al.*, 1994). The solid-phase kinase assay was slightly modified from a previously described assay (Hibi *et al.*, 1993). Cell lysates containing 200 μ g of total protein were diluted to a 600 μ l volume in the lysis buffer. The diluted extracts were incubated with rotation for 2 h at 4°C with 10 μ g of GST-c-Jun 1–79 pre-bound to GST-Sepharose beads (Pharmacia). The beads were washed four times with the lysis buffer and once with the kinase buffer, after which the kinase reaction was carried out and the samples were processed as described previously (Kallunki *et al.*, 1994). To investigate the activation of AP-1-like transcription factors, pAP(4)-LUC plasmid containing four AP-1 sites upstream of the minimal rat prolactin promoter and the *Photinus pyralis* luciferase-coding sequence was introduced into cells by electroporation using 40 μ g of plasmid per 1.5×10^7 cells. Transfected cells were plated in 6- or 24-well plates in complete medium. After 48 h, cells were treated as indicated and the luciferase assay was performed using the Promega Luciferase Assay System.

Detection of mitochondrial events

Intracellular free radical formation and $\Delta\psi_m$ were measured as described previously employing specific probes, dihydrorhodamine 123 (DHR123, Molecular Probes, Leiden, The Netherlands) and rhodamine123 (RH123, Molecular Probes), respectively (Wissing *et al.*, 1998).

To investigate the release of cytochrome *c* from mitochondria, subconfluent cells growing on Petri dishes were treated as indicated. At the end of the treatment, Petri dishes were placed on ice, cells were scraped, washed in PBS, washed in a sucrose buffer [20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 40 μ g/ml leupeptin, 25 μ g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 mM dithiothreitol (DTT)] and resuspended in the same buffer at an approximate density of 5×10^7 cells/ml. After 1 h incubation on ice, cells were lysed by forcing them through a 27 gauge needle 20–30 times. The lysate was centrifuged at 750 g for 5 min and the supernatant was collected and centrifuged at 10 000 g for 15 min. The resulting mitochondrial pellet was washed once in the sucrose buffer, lysed in Laemmli sample buffer, boiled for 3 min, sonicated and stored at 80°C until analyzed by immunoblot.

In vitro measurement of caspase activity

The activity of caspase-3-like proteases in cell lysates was measured using a colorimetric probe Ac-DEVD-pNA (Biomol), which was dissolved in dimethylsulfoxide (DMSO) and stored as a 10 mM stock solution at -20°C . Subconfluent cells growing on Petri dishes were treated as indicated. At the end of the treatment, the Petri dishes were placed on ice and the cells were scraped, washed in PBS and resuspended in buffer A (80 mM KCl; 10 mM HEPES pH 7.4, 1 mM EDTA, 1 mM EGTA, 40 μ g/ml leupeptin, 25 μ g/ml pepstatin A, 1 mM PMSF, 4 mM DTT) at an approximate density of 10^7 /ml. Cells were lysed by sonication. Homogenates were centrifuged at 20 000 g for 30 min and the protein concentrations of the supernatants were determined by a BioRad protein assay (Bio-Rad Laboratories, Hercules, CA). Supernatants containing equal amounts of protein (corresponding to $\sim 5 \times 10^5$ cells) were mixed in a microtiter plate with 150 μ l of 100 μ M DEVD-pNA in buffer B (100 mM HEPES pH 7.5, 20% glycerol, 5 mM DTT, 0.5 mM Na-EDTA). After 0.5–4 h incubation at 37°C , the absorbance at 405 nm was measured by a microtiter plate reader (Molecular Devices). The absorbance of 50 μ l of buffer A + 150 μ l of 100 mM DEVD-pNA was subtracted from specific values.

cPLA₂ assay

Activation of cPLA₂ was analyzed as described previously by an assay based on labeling of cells with [^3H]arachidonic acid [5,6,8,9,11,12,14,15- ^3H (N)] (80 Ci/mmol, 0.1 mCi/ml; NEN, Boston, MA) and measurement of radioactivity released from the supernatant following the indicated treatment (Jäättelä, 1993). The spontaneous release of radioactivity was always <10% of the maximal counts.

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