Small stress protein expression enhances the survival of mammalian cells exposed to numerous injuries that induce necrotic cell death. The cell surface receptor Fas/APO-1 and its ligand have been recently identified as important mediators of apoptosis. Here, we show that constitutive expression of human heat shock protein (hsp)27 in murine L929 cells blocks Fas/APO-1-mediated cell death. Expression of human hsp27 prevented anti-APO-1-induced DNA fragmentation and morphological changes. These results strongly suggest that human hsp27 acts as a cellular inhibitor of Fas/APO-1-induced apoptosis. We also report that the expression of small stress proteins from different species, such as human hsp27, Drosophila Dhsp27, or human aB-crystallin, confers resistance to apoptotic cell death induced by staurosporine, a protein kinase C inhibitor. Hence, small stress proteins are novel regulators that are able to block apoptosis induced by different pathways.

There are two major morphologically and biochemically distinct modes of death in nucleated eukaryotic cells, necrosis and apoptosis (1). The first to be described is necrotic cell death characterized by swelling of the cytoplasm and of the organelles, followed by lysis of the plasma membrane and random DNA degradation. In contrast, apoptosis or programmed cell death involves plasma membrane blebbing, volume loss, nuclear condensation, and endonucleolytic cleavage of DNA into fragments of oligonucleosomal length (2). Depending on cells, doses, and/or stimuli one can observe either apoptosis, necrosis, or both phenomena (3). The stimulation of the Fas/APO-1 receptor has recently been shown to induce death by apoptosis (4, 5). This receptor and its ligand represents a physiological system that allows the specific elimination of unwanted cells, particularly during the maturation and activation of the immune system (6). Fas/APO-1 is a member of the TNF1/nerve growth factor receptor superfamily (7, 8). The mechanism by which Fas/APO-1 induces cell death is unknown but occurs probably through the activation of interleukin converting enzyme-like proteases (9–11). Another way to induce apoptosis is the inhibition of the protein kinase C-dependent pathways. Staurosporine, an inhibitor of kinase C, has recently been shown to kill cells by apoptosis (12, 13). Because of the considerable importance of the in vivo regulation of apoptosis, the discovery of proteins that can rescue cells from undergoing apoptosis is of considerable interest. Some virally encoded proteins were found to possess this ability (14). To date, the only endogenous cellular proteins described as inhibitors of apoptosis induced by a wide range of different stimuli are Bcl-2 and related members of the Bcl-2/Ced-9 family (15).

Small stress proteins (shsp) belong to the family of heat shock proteins, and their expression enhances the survival of mammalian cells exposed to heat (16–18) or oxidative (17–19) stress. Recently, we described that different small heat stress proteins such as mammalian hsp27, Dhsp27 from Drosophila, and human aB-crystallin enhanced the resistance against TNFα cytotoxicity in the highly sensitive L929 fibrosarcoma cells (19–20). L929 cells are killed by TNFα through a necrotic process (21), although some parameters of apoptosis are also detected (22) suggesting that shsp may also interfere with apoptosis.

We show here that the constitutive expression of human hsp27 in L929 cells that express the cell surface Fas/APO-1 receptor suffices to block the apoptotic process generated by the agonistic anti-APO-1 antibody. Similarly, we show that staurosporine is unable to generate cell death in L929 cells that express either human hsp27, Drosophila hsp27, or human aB- crystallin. Thus, small stress proteins can be considered as novel negative regulators of programmed cell death.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The murine fibrosarcoma cell line L929-APO-1–6 as well as L929 cell lines that constitutively express either human hsp27 (L929–27–3), Drosophila Dhsp27 (L929–D27–3), or human aB-crystallin (L929–cry-3) were previously described (19, 20, 21). L929–27–3 express 0.88 ng of human hsp27/mg of cellular proteins and L929-cry-3 cells 0.70 ng/ml aB-crystallin. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum. The apoptosis-inducing monoclonal antibody anti-APO-1 (mouse IgG3) was already described (4, 21) as well as anti-human hsp27 (19, 20, 23). Actinomycin D, crystal violet, and staurosporine were from Sigma. G418 was from Clonetech (Palo Alto, CA). Purified human hsp27 was from Stressgen (Victoria, BC, Canada).

Transfection Procedures—Exponentially growing L929-APO-1–6 cells, plated at a density of 5303 cells/78 cm2 1 day before transfection, were co-transfected by the calcium phosphate procedure (19) with 20 μg of DNA containing 18 μg of either psvhsp27 (19) or psvk3 (19) vectors and 2 μg of pSV2neo plasmid (Clonetech, Palo Alto, CA) bearing the neomycin resistance gene. After 48 h, G418 at a concentration of 500 

heat shock protein; shsp, small stress proteins.
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μg/ml was added to the cells for selection. G418-resistant clones were isolated, grown in the presence of the antibiotic, and screened for the expression of hsp27.

Gel Electrophoresis and Immunoblotting—Gel electrophoresis and immunoblots probed with hsp27 antisera were performed as already described (19, 20, 23). The detection of the immunoblots was performed with the enhanced chemiluminescence kit from Amersham Corp. (U. K.). The duration of the exposure was calculated to be in the linear response of the film. Autoradiographs were scanned with the Bioprofil system (Vilber Lourmat, France). The level of human hsp27 expressed in L929-APO-1–6 cells was compared with serial dilutions of the purified protein.

Cytotoxicity Assays—Transfectants of L929-APO-1–6 or L929 cells (10^4 cells/well) were grown in 96-well plates for 24 h before being analyzed for their resistance to anti-APO-1 or staurosporine. Serial dilutions of anti-APO-1 in actinomycin D-containing medium (1 μg/ml) or staurosporine were added to cells. Subsequently, supernatants were discarded and the remaining viable cells were stained with 0.5% crystal violet in 50% methanol for 15 min. Microtiter plates were rinsed and dried. A solution containing 0.1 N sodium citrate, pH 5.4, and 20% methanol was then added to solubilize the stained cells. The absorbance of each well was read at 570 nm with an MR5000 microelisa reader (Dynatech Laboratories, Chantilly, VA). Percent of viability was defined as the relative absorbance of treated versus untreated control cells. Cytotoxicity was also monitored by the classical 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (24).

Analysis of APO-1-induced DNA Fragmentation—After anti-APO-1 treatments, DNA fragmentation was analyzed essentially as described by Hockenbery et al. (25). Briefly, cells were gently lysed for 20 min at 4°C in a buffer containing 5 mM Tris buffer, pH 7.4, 20 mM EDTA, and 0.5% Triton X-100. After centrifugation at 20,000 × g for 15 min, supernatants were collected and extracted with phenol/chloroform, and nucleic acids were precipitated in ethanol. Each sample was then electrophoresed on a 1.5% agarose gel (Nusieve, Tebu, France). Thereafter, the agarose gel was incubated for at least 3 h at 37°C with 20 μg/ml RNase A (Sigma) before being stained with ethidium bromide. Further incubations with RNase A were performed until the gel was devoid of contaminating RNA.

Analysis of APO-induced Morphological Changes—Analysis of morphological changes was performed as described by Tomasovic et al. (22). Cells were grown on chamber slides (Nunc, Naperville, IL) and treated with anti-APO-1. The medium was carefully poured off from the plates and replaced with 95% ethanol. After 30 min fixation, the alcohol was decanted and the coverslips air-dried overnight. The cells were then rehydrated in distilled water prior to transfer to Mayer's hematoxylin for 20 min. The color was intensified by a 30-min running tap water wash, and the cells were then counterstained with 1 min in eosin-phloxine and dehydrated through three changes each of 95% ethanol followed by a 100% ethanol wash. The stained coverslips were examined by light microscopy under low magnification and with an oil-immersion lens.

RESULTS

Previous studies have shown that the morphological changes and the mechanism driving cells to death following Fas/APO-1 ligation are characteristics of apoptosis (4, 9, 21). In order to determine if small stress proteins interfere with apoptosis, we constitutively expressed human hsp27 in Fas/APO-1-sensitive L929 cells (L929-APO-1–6 cells) (9, 21). These cells are rapidly destroyed by apoptosis when treated with anti-APO-1 antibody (21). L929-APO-1–6 cells, as well as parental L929 cells, are devoid of endogenous murine hsp27, whose expression is only observed after heat shock (not shown). Stable cell clones expressing human hsp27 and mock transfectants were obtained from transfections performed with either the psvhsp27 or psvK3 control plasmid (see "Experimental Procedures"). The immunoblot presented in Fig. 1A shows the constitutive presence of human hsp27 in three clones, denoted L929APO-27/2, L929APO-27/3, and L929APO-27/8 which contained 0.18, 0.25, and 0.07 ng of hsp27 per μg of cellular proteins, respectively. Similar immunoblots probed with anti-APO-1 antibody revealed that human hsp27 expression did not alter the level of Fas receptor polypeptide in L929-APO-1–6 cells (not shown).

We compared cell death induced by anti-APO-1 in L929-APO-1–6 transfectants to that of control L929APO-K3 or L929APO-27/2 cells treated with a 100 ng/ml dilution of anti-APO-1. Cells were harvested at 15 min or 2 h after treatment with anti-APO-1. As shown in Fig. 2, constitutive expression of human hsp27 resulted in a strong protection against cell death mediated by the expression of human hsp27.

![Figure 1](image-url)

**FIG. 1.** Constitutive expression of hsp27 in L929-APO-1–6 cells blocks anti-APO-1-induced cell death. A, constitutive expression of human hsp27 in L929-APO-1–6. Total cellular extracts of L929-APO-1–6 transfected L929APO-27/2, -27/3, and -27/8 cells were analyzed. Autoradiographs of enhanced chemiluminescence-developed immunoblots are presented. B and C, crystal violet assay, performed as described under "Experimental Procedures," on control L929APO-K3 or human hsp27-expressing L929APO-27/2 cells treated for different times with 100 ng/ml anti-APO-1 (B) or for 18 h with serial dilutions of anti-APO-1 (C). Standard deviations are indicated (n = 3). Note the strong protection against cell death mediated by the expression of human hsp27.
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Fig. 2. Constitutive expression of human hsp27 in L929-APO-1-6 cells blocks anti-APO-1-induced DNA fragmentation. Anti-APO-1-induced apoptosis was analyzed by agarose gel electrophoresis of oligonucleosomal-length DNA fragments from control L929APO-K3- or human hsp27-expressing L929APO-27/2 cells. 0, untreated cells; 100, 500, cells treated for 6 h with either 100 or 500 ng/ml anti-APO-1. Note the absence of oligonucleosomal-length DNA fragments in APO-1-treated L929-APO-1-27/2 cells that express human hsp27.

APO-1-6 cells expressing or not expressing human hsp27. An illustration of the killing induced by 100 ng/ml anti-APO-1 in control L929APO-K3 is presented in Fig. 1B. Five hours of treatment were necessary to decrease the viability of these cells by half. After 18 h, only about 10% of the cells were still viable. Remarkably, in L929APO-27/2 cells, anti-APO-1 treatment only very weakly decreased cell viability (Fig. 1B). Moreover, this inhibition of cell death was still observed when the concentration of anti-APO-1 antibody was increased (Fig. 1C). Similar observations were made when other control (L929APO-K2) or hsp27-expressing (L929APO-27/3, L929APO-27/8) cells were analyzed (not shown). Thus, expression of human hsp27 blocks the cell death induced by APO-1.

We then investigated the effect of human hsp27 expression on apoptotic features, such as DNA fragmentation and morphological changes. It is seen in Fig. 2 that stimulation of the Fas/APO-1 receptor by a 6-h treatment with 100 or 500 ng/ml anti-APO-1 induced an intense fragmentation of DNA in L929APO-K3 cells. Human hsp27 expression drastically inhibited this phenomenon. At the morphological level, it is seen in Fig. 3 that control L929APO-K3 cells, treated with 100 ng/ml anti-APO-1, displayed, in less than 1 h, membrane blebblings (Fig. 3C). These were rapidly followed by the condensation of nuclei (Fig. 3E). No such morphological changes were observed in L929APO cells that express human hsp27 (Fig. 3, D and F). Hence, it can be concluded that the presence of human hsp27 in L929-APO-1-6 cells blocks the apoptotic process generated by Fas/APO-1 ligation.

It was of interest to test whether the inhibitory action of human hsp27 was restricted to Fas/APO-1 or was also efficient in other apoptotic systems. Another classical inducer of apoptotic cell death is staurosporine, an inhibitor of protein kinase C (12, 13). This was assessed in plain L929 cell lines, devoid of human Fas/APO-1 receptors, that constitutively expressed (L929-27-3) or not expressed (L929-C3) human hsp27 (19, 20). In control L929-C3 cells, staurosporine, at the concentration of 0.5–5 μM in the growth medium, induced a rapid death (Fig. 4) that was accompanied by an intense fragmentation of DNA and morphological modifications characteristic of apoptosis (not shown). A similar result was observed when two other control cell lines L929-C1 and L929-C2 were analyzed. As seen in Fig. 4, the constitutive expression of human hsp27 inhibited the staurosporine-induced cellular death. Human hsp27 expression also blocked the staurosporine-mediated DNA fragmentation and morphological modifications (not shown). To test whether the anti-apoptotic activity described above was a conserved property of small stress proteins or restricted to human hsp27, we took advantage of previously described L929 cells that constitutively express Drosophila Dhsp27 or human αB-crystallin (19). It is seen in Fig. 4 that Drosophila Dhsp27 and human αB-crystallin also efficiently protected against staurosporine-induced cell death. Hence, small stress proteins from different species share the ability to block apoptosis, suggesting that these proteins are novel negative regulators of apoptosis and that this activity resides at the level of their conserved sequence homology (the α-crystallin box) (34).

**DISCUSSION**

We show here that the expression of human hsp27 blocks the apoptotic process generated by Fas/APO-1. Remarkably, hsp27 expression also blocks the cellular death induced by TNFα (19, 20). TNFα receptors are sequence-related to Fas/APO-1, but the pathways and the type of death induced by their stimulation are at least partially different (21, 26, 27). If the necrotic action of TNFα is very efficient in the eradication of tumor cells, Fas/APO-1-mediated apoptosis is more oriented in the physiological elimination of specific and nonpathological cells leading, for example, in the maturation and amplification of the immune system. The Fas/APO-1 system plays an important role during the maturation of lymphocytes by selecting cells that have to be eliminated (for a review see Ref. 6). Of interest, a specific and transient accumulation of hsp27 during the maturation of B or T cells has been described (28, 29). Hence, the transient accumulation of hsp27 during lymphocyte differentiation may determine the selectivity of Fas/APO-1 action. The high level of expression of transcripts encoding Fas/APO-1 ligand in gonads (30) and smooth and skeletal muscles (6) suggests that, in addition of being a regulator of the immune response, Fas/APO-1 is involved in tissue turnover (31). Interestingly, high levels of hsp27 were also found in these tissues (32, 33). Thus, hsp27 may be a key element that regulates Fas/APO-1 activity in vivo.

Another important result shown in this report is that the expression of three different small stress proteins, such as human hsp27, Drosophila hsp27, and human αB-crystallin, interferes with staurosporine-induced apoptosis. Hence, it can be concluded that shsp act as inhibitors of apoptosis even if this process is generated through different signal transduction pathways. Taking into account their transient expression during differentiation, shsp appear as physiological regulators of apoptosis with a broader spectrum of action than Bcl-2. Indeed, Bcl-2 only weakly inhibits the Fas/APO-1 pathway (35). Bcl-2 and shsp molecular functions are unknown (18, 36). Reports have described shsp as molecular chaperones (37, 38) or actin capping/decapping enzymes (39, 40). In a recent study, we have observed that shsp expression decreased intracellular-reactive
oxygen species levels and increased the cellular content of the antioxidant glutathione. This phenomenon appears to be essential for shps protective activity against TNFα- and oxidative stress-induced cell death (41). Bcl-2 was also reported to increase glutathione, but this phenomenon was not essential for the protective activity of this protein (42). It will be of interest to test whether shsp, similar to Bcl-2 (43, 44), will protect against apoptosis independently of their action on intracellular reactive oxygen species and glutathione levels. The rapidity of the death induced by Fas/APO-1 and the total freezing of this death program, as well as that induced by staurosporine, by hsp27 would suggest an action at the level of pathways or targets that are common to several apoptotic inducers. In shsp-expressing cells, the analysis of interleukin converting enzyme proteases that have been recently implicated as crucial mediators of apoptosis in both invertebrates and mammalian cells would be very informative in this respect.

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FIG. 3. Constitutive expression of hsp27 in L929-APO-1-6 cells blocks anti-APO-1-induced morphological changes. Control L929APO-K3 and human hsp27 expressing L929APO-27/2 cells were treated with anti-APO-1 (100 ng/ml) in actinomycin D-containing medium. At various time points, cells were fixed and stained as described under "Experimental Procedures." A, nontreated L929APO-K3 cells; B, nontreated L929-APO-27/2 cells; C, L929APO-K3 cells treated for 1 h with anti-APO-1; D same as C but L929APO-27/2 cells; E, L929-APO-K3 cells treated for 6 h with anti-APO-1; F, same as E but L929APO-27/2 cells. Note the absence of morphological changes in APO-1-treated cells that express human hsp27. Bar, 10 μm.

FIG. 4. Constitutive expression of human hsp27, Drosophila Dhsp27, or human αB-crystallin in L929 cells blocks staurosporine-induced apoptosis. Survival of L929 cells, treated for 24 h with increasing concentrations of staurosporine (0.5–5 μM), was analyzed by crystal violet assay as described under "Experimental Procedures." The values were normalized to 100% using the respective cells not treated with staurosporine. The cell lines shown are control L929-C3 (black bars), and the expressing clones L929–27–3 (L929-hsp27), L929–D27–3 (L929-Dhsp27), and L929-αBcry-3 (L929-αB-cry). The standard deviations are indicated (n = 6). Note the strong protection mediated by the expression of the exogenous small hsps. Similar results were obtained when survival was analyzed by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay.
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