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Binding of Caspase-3 Prodomain to Heat Shock Protein 27 Regulates Monocyte Apoptosis by Inhibiting Caspase-3 Proteolytic Activation*

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Caspase-3 is an essential executioner of apoptosis responsible for regulating many important cellular processes, among them the number of circulating monocytes, central players in the innate immune response. The activation of caspase-3 requires its processing from an inactive precursor. Here we show that the small heat shock protein 27 (Hsp27) associates with caspase-3 and protein-protein interaction experiments *in vivo* and with purified proteins demonstrate a direct interaction between Hsp27 and the amino-terminal prodomain of caspase-3. Using an *in vitro* caspase-3 activation assay, our results further establish that the interaction of Hsp27 with the caspase-3 prodomain inhibits the second proteolytic cleavage necessary for caspase-3 activation, revealing a novel mechanism for the regulation of this effector caspase. Hsp27 expression in monocytes is constitutive. Consistent with a central role of Hsp27 in blocking caspase-3 activation, Hsp27 down-regulation by double-stranded RNA interference induces apoptosis of macrophages, whereas Hsp27 overexpression increases the life span of monocytes by inhibiting apoptosis. Highlighting the importance of cell partitioning in the regulation of apoptosis, immunofluorescence, and subcellular fractionation studies revealed that whereas both caspase-3 and Hsp27 are cytoplasmic in fresh monocytes (*i.e.* not undergoing apoptosis), Hsp27 moves to the nucleus during apoptosis, a relocalization that can be blocked by promoting the differentiation of monocytes to macrophages or by inhibiting cell death. These results reveal a novel mechanism of caspase-3 regulation and underscore a novel and fundamental role of Hsp27 in the regulation of monocyte life span.

Apoptosis is a central homeostatic process that regulates cell number in metazoans. Caspases are highly conserved cysteine proteases essential for apoptosis (1). They are constitutively expressed as inactive precursors that become activated by proteolytic cleavage (2, 3). Caspase-3 is a central effector caspase involved in numerous apoptotic pathways. The first step for caspase-3 activation is mediated by initiator caspases such as caspase-8 and caspase-9 (4). The former binds to cytochrome *c* and, through a mitochondrial-dependent pathway, initiated the apoptotic cascade (5).

Monocytes, key components of the innate immune system, originate in the bone marrow daily and circulate in the bloodstream for 24–48 h (6). In the absence of survival stimuli, monocytes undergo spontaneous apoptosis (7). The monocyte apoptotic fate is halted by inflammatory stimuli, differentiation factors, or malignant transformation, all of which prolong monocyte survival by somehow inhibiting the activation of caspases (6–9). Macrophages are derived from monocytes, but unlike monocytes, they can live up to 3 months and are more resistant to apoptosis (10). Macrophages are distributed throughout every organ where they recognize a wide range of antigens (11). Monocytes and macrophages have distinct cell surface receptors like CD14 and mannose receptor, respectively, which allow these cells to respond to particular antigens and provide convenient markers to identifying both populations (12). Thus monocytes/macrophages constitute the main innate line of defense in the blood and organs. The activation of caspase-3 is central in the execution of spontaneous monocyte apoptosis, and prolonged monocyte survival is mediated by the inhibition of caspase-3 activation (7, 9, 13). Monocyte/macrophage accumulation participates in the pathogenesis and progression of a number of diseases, including atherosclerosis, pulmonary fibrosis, chronic inflammation, and cancer (14–16). Hence, understanding the mechanisms that regulate the activation of caspases is of great importance to manipulate cellular life span.

Recent attention has focused on heat shock proteins (Hsps)[4] as regulators of cell death and survival. Hsp represent a con-

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[4] The abbreviations used are: Hsp, heat shock protein; sHsp, small Hsp; M-CSF, macrophage colony stimulating factor; MDM, monocyte-derived macrophage; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; 7AAD, 7-amino-actinomycin *D*; DTT, dithiothreitol; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAPI, 4′6-diamidino-2-phenylindole; IP, immunoprecipitate; GFP, green fluorescent protein; siRNA, small interfering RNA; AFC, aminofluoromethylcoumarin; Casp-3, caspase-3.

served family of proteins induced by stress conditions that have been mainly studied for their participation in protein folding (17). Hsps are classified based on their molecular weight into large and small (sHsp). Hsp70 and Hsp90 are members of the large Hsp group, whereas Hsp27 and αB-crystallin belong to the sHsp group and have recently emerged as regulators of apoptosis (18–20). In human monocytes heat shock-induced expression of Hsp70 has been correlated with monocyte survival (21). High levels of Hsp27 are a marker for increased malignancy in breast cancer (22), and Hsp27 overexpression prevents apoptosis by associating with cytochrome *c*, resulting in the inhibition of caspase-9 (23). In addition, αB-crystallin has been suggested to act as a negative regulator of apoptosis during skeletal muscle development by inhibiting caspase-3 activation (24).

In this study we show a new checkpoint for the anti-apoptotic activity of Hsp27 in the regulation of apoptosis. We describe here that, unlike Hsp70, Hsp27 is higher and constitutively expressed in the monocyte lineage. In contrast, αB-crystallin is undetectable, even after heat shock stimulation. We found that Hsp27 relocalizes to the nucleus during spontaneous monocyte apoptosis. The relocalization of Hsp27 is blocked by caspase-3 inhibitors or by the differentiation factor macrophage colony stimulating factor (M-CSF). Hsp27 expression increases during monocyte-to-macrophage differentiation and is exclusively localized in the cytoplasm. We show that Hsp27 associates with caspase-3 in non-apoptotic cells. We determined that the prodomain of caspase-3 is necessary for the association and that Hsp27 acts by inhibiting caspase-3 proteolytic processing. We demonstrate that the overexpression of Hsp27 in monocytic cells reduced apoptosis, whereas silencing of Hsp27 expression in macrophages with double-stranded RNA interference increased macrophage apoptosis. Taken together, our findings suggest an additional role of Hsp27 as an anti-apoptotic modulator of caspase-3, providing a novel checkpoint in the regulation of monocyte/macrophage life span.

MATERIALS AND METHODS

Monocyte Purification and Cell Culture—Human monocytes were purified by clumping or CD14+ selection, as previously described (25). The population of monocytes was on average 85–90% pure as estimated by flow cytometry using an anti-CD14 marker (BD Biosciences). Monocytes were resuspended in RPMI 1640 (BioWhittaker, Walkersville, MD) to a final concentration of 3×10^6 cells/ml and cultured at 37 °C in 5% CO_2. THP-1 monocytic cells and RAW264.7 cells were obtained from ATCC and cultured in RPMI 1640, 5% fetal bovine serum. HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin. For monocyte/macrophage differentiation (monocyte-derived macrophages (MDMs), purified human monocytes were cultured on plates up to 7 days in RPMI 1640, 5% fetal bovine serum, 1% penicillin-streptomycin with 100 ng/ml M-CSF (R&D Systems, Minneapolis, MN). MDM differentiation was confirmed by changes in morphology and increased levels of macrophage mannose receptor, detected by flow cytometry (12). Heat shock was performed at 42 °C in 5% CO_2 incubator for 30 min in monocytes and 2 h in THP-1 cells followed by a recovery at 37 °C for different times.

Extract Preparation, Immunoprecipitation, and Immunoblotting—Extracts were collected by centrifugation and washed with KPM buffer (50 mM KCl, 50 mM PIPES, 10 mM EGTA, 1.92 mM $MgCl_2$, pH 7.0). Cells were lysed in ice-cold buffer B (50 mM Tris-HCl, pH 7.5, 0.1% Triton-X, 1 mM EGTA, 1 mM EDTA containing 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of protease inhibitors chymostatin, pepstatin, leupeptin, antipain, and phosphatase inhibitors) for 30 min on ice with intermittent vortexing, and extracts were then centrifuged for 20 min at 14,000 × *g*. Supernatants (soluble fractions in Fig. 1) were snap-frozen and stored at −70 °C for future use. The pellets (insoluble fractions in Fig. 1) were resuspended in buffer C (8 M urea, 4% CHAPS, 40 mM Tris-HCl containing DTT, phenylmethylsulfonyl fluoride, and protease inhibitors) for 30 min. Whole cell lysates (used in Fig. 1*A*) were prepared by sonication of extracts in the presence of buffer C. Lysates were boiled 5 min in Laemmli buffer containing 1% β-mercaptoethanol before loading onto gels. Gels were stained overnight with Coomassie Brilliant Blue R-250 to determine equal loading. For immunoprecipitations, HeLa cells expressing different domains of caspase-3 (26) were lysed in buffer A (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol containing DTT, phenylmethylsulfonyl fluoride, and protease inhibitors). Lysates were immunoprecipitated with anti-Hsp27, anti-caspase-9 (BD Pharmingen), and anti-Xpress (Invitrogen) antibodies and G-agarose beads (Invitrogen) for 5 h at 4 °C. Immunoprecipitates were washed four times with buffer A. Recombinant proteins were preincubated for 30 min on ice followed by immunoprecipitation with anti-Hsp27 for 5 h at 4 °C. Immunoblotting was carried out with anti-Hsp27 (SPA-803), Hsp70 (SPA-810), αB-crystallin (SPA-223), and Hsp25 (SPA-801) antibodies obtained from Stressgen. β-Tubulin antibody was obtained from Upstate (Charlottesville, VA). Anti-caspase-3 (clone C-31720) antibody was obtained from BD Transduction Laboratories and anti-acetylated-H4 from Cell Signaling (Danvers, MA). Densitometry analyses were done using Quantity-One (Bio-Rad). In experiments performed to detect Hsp27 and Hsp70, an equal amount of protein was loaded in each lane. For the detection of αB-crystallin, 100 μg of total protein were loaded per well. Secondary antibodies linked to horseradish peroxidase and ECL were purchased from Amersham Biosciences.

Subcellular Fractionation and Western Blots—To obtain subcellular fractions, 2×10^7 primary human monocytes were collected for each time point. Nuclear and cytoplasm fractions were obtained using the Qproteome Cell Compartment Reagent (Qiagen) following the manufacturer's recommendations. Equal amounts of protein for each fraction were subjected to SDS-PAGE. Western blot analysis was conducted with anti-glyceraldehyde-3-phosphate dehydrogenase and lamin B (Santa Cruz), markers of cytoplasm and nuclear fractions, respectively. The same membranes were immunoblotted with anti-Hsp27 and anti-caspase-3 active and inactive antibodies.

Flow Cytometry—For flow cytometry cells were washed with PBS and resuspended at a concentration of 2×10^7 cells/ml in blocking buffer (cold PBS containing 1% fetal bovine serum and 200 μg/ml human total IgG) and incubated for 30 min on ice. After gentle mixing, the cells were divided into individual tubes

containing 1×10^6 cells each, and 5 μl of allophycocyanin-conjugated anti-CD14 or isotype control was added and incubated for 30 min on ice. After 2 washes with blocking buffer, cells were resuspended in 250 μl of Cytofix/Cytoperm® and incubated for 20 min on ice with 20 μl of FITC-conjugated anti-active-caspase-3 (casp-3; BD Pharmingen). Cells were washed 2 times with Perm/Wash® buffer and finally resuspended with 200 μl of blocking buffer. Alternatively, cells were stained with annexin V-FITC and 7AAD using the annexin V-FITC apoptosis detection kit by following the manufacturer's specification (BD Pharmingen). Levels of mannose receptor were detected using anti-mannose-receptor-phosphatidylethanolamine (PE)-conjugated antibody or an isotype-PE-conjugated control (BD Pharmingen). Hsp27 expression levels were detected using an anti-Hsp27 and anti-Hsp25 (Stressgen, SPA-801) and Alexa-633 (Molecular Probes, Invitrogen) antibodies. Flow cytometry analysis was performed using BD Biosciences FACS ARIA using FCS Express V3 software.

Transient Transfection, RNA Interference, and Immunofluorescence—Full-length cDNA of Hsp27 (accession number BC012768) was obtained from OpenBiosytems and cloned in-frame into the green fluorescent vector pEGFP-C2 (GFP from Clontech, Mountain View, CA). THP-1 cells were washed in PBS and resuspended in the specified electroporation buffer provided by kit V Amaxa (catalog no. VCA-1003, Amaxa, Cologne, Germany) to a final concentration of 5×10^6 cells/ml. One μg of vector alone (vector-GFP) and vector containing Hsp27 (Hsp27-GFP) was mixed with 0.1 ml of cell suspension and nucleofected using the Amaxa Nucleofector™ (Program V-01 as recommended by the manufacturer). Twenty-four hours after transfection, cells were treated with 10 μM etoposide for 12 h to induce apoptosis. HeLa cells were transfected with different domains of caspase-3 as previously described (27) using Lipofectamine® 2000 (Invitrogen). For silencing experiments, pre-validated siRNA for human Hsp27 was obtained from Qiagen (Valencia, CA). siRNA for Hsp25 used in mouse Raw cells was from Ambion (Austin, TX). MDMs and RAW 264.7 cells were transfected with 150 nM siRNA or a scramble control (Qiagen) for 72 or 24 h, respectively. Apoptosis was induced for 12 h with 10 μM etoposide in MDMs or 100 μM for RAW cells. For immunofluorescence studies, the cells were fixed in PBS containing 2% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100 and 0.5% bovine serum albumin for 15 min at 4 °C. Cells were incubated in blocking solution (0.5% bovine serum albumin and 2 mg/ml human IgG) for 30 min at room temperature. Staining was conducted in blocking solution with anti-Hsp27 (SPA-803), anti-active caspase-3 (Asp175, Cell Signaling, Beverly, MA), or inactive caspase-3 (Transduction Laboratories) antibodies followed by secondary antibodies conjugated to FITC or phosphatidylethanolamine, respectively (Alexa 488 or Alexa 633, Molecular Probes, Eugene, OR). Nuclear staining with 50 ng/ml 4′6-diamidino-2-phenylindole (DAPI), Sigma) was conducted as previously described (27), and percentage of apoptosis was determined by nuclear fragmentation by counting at least 200 GFP-positive cells. Fluorescence was visualized on an epifluorescence microscope (Olympus, Melville, NY), and digital images were captured using Optronics Imaging System (Goleta, CA) and ImageProPlus software (excitation at 300 nm; emission at 461 nm).

Caspase Activity—Active caspases was determined by the AFC assay as previously described (27). Mixes were incubated with DEVD-AFC to determine the presence of active caspase-3 in a cyto-buffer (10% glycerol, 50 mM PIPES, pH 7.0, 1 mM EDTA, containing 1 mM DTT, and 20 μM tetrapeptide substrate DEVD-AFC). To determine the presence of active caspase-9, we used the tetrapeptide LEHD-AFC in a cyto-buffer (0.1 M MES, pH 6.5, 10% polyethylene glycol, 0.1% CHAPS, 10 mM DTT, and 500 μM LEHD-AFC). The tetrapeptides were obtained from Enzyme Systems Products (Livermore, CA). Release of free AFC was determined using a Cytofluor 4000 fluorometer (Perseptive Co., Framingham, MA.; excitation, 400 nm; emission, 508 nm). Recombinant Hsp27 and Hsp90 were obtained from Stressgen. Hsp27 was mixed at equal amounts (1:1) or double amounts with rcasp-3 (2:1) for 30 min on ice. Dilutions of Hsp27 were prepared in a buffer containing 20 mM Tris, pH 7.4, 10 mM NaCl, 1 mM EDTA, 1 mM DTT. Hsp90 was diluted in 50 mM Tris, pH 7.4. Caspase-9 obtained from Biomol (Plymouth Meeting, PA) was then added, and samples were incubated at 37 °C for 2 h in the presence of a buffer containing 50 mM Hepes, pH 7.4, 50 mM NaCl, 10% sucrose 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride.

Statistical Analysis—All data are expressed as the mean ± S.E. Student's *t* test comparisons or individual comparisons were made by using the contrast method. Statistical significance is stated in Figs. 4 and 5.

RESULTS

Hsp27 Is Expressed Constitutively in Human Monocytes—To evaluate the role of sHsps in the regulation of human monocytes life span, we investigated the level of expression of Hsp27 and αB-crystallin in human primary monocytes and THP-1 monocytic cells. THP-1 cells originate from a monocytic leukemia, and unlike primary human monocytes, do not undergo spontaneous apoptosis. Western blot analyses of whole cell lysates from freshly isolated human monocytes (Fig. 1*A*, *NT*) or THP-1 cells (Fig. 1*B*, *NT*) reveal high constitutively expression of Hsp27. Similar levels of Hsp27 are found in whole-cell lysates from monocytes undergoing apoptosis when compared with freshly isolated monocytes lysates (Fig. 1*A, A*). In contrast, no significant increase of Hsp27 expression was observed in lysates from heat-shocked-treated monocytes and THP-1 cells (Fig. 1, *A–C*, *HS*). Unlike Hsp27, αB-crystallin was undetectable by immunoblotting in the primary human monocytes under all of the conditions tested (Fig. 1*A*). A low constitutive level of αB-crystallin expression that remained unaltered during heat shock was found in THP-1 cells (Fig. 1*B*, *NT* and *HS*). In contrast to Hsp27, Hsp70 expression was low in fresh monocytes apoptotic monocytes, and untreated THP-1 cells but increased with heat shock at least 3-folds and in THP-1 cells by ~30-fold (Fig. 1, *A* and *B*, Hsp70), consistent with previous studies (21).

Together these results show a differential pattern of expression of Hsp70, αB-crystallin, and Hsp27 in monocytes. The constitutively high levels of Hsp27 expression in primary human monocytes and THP-1 cells in the absence of detectable

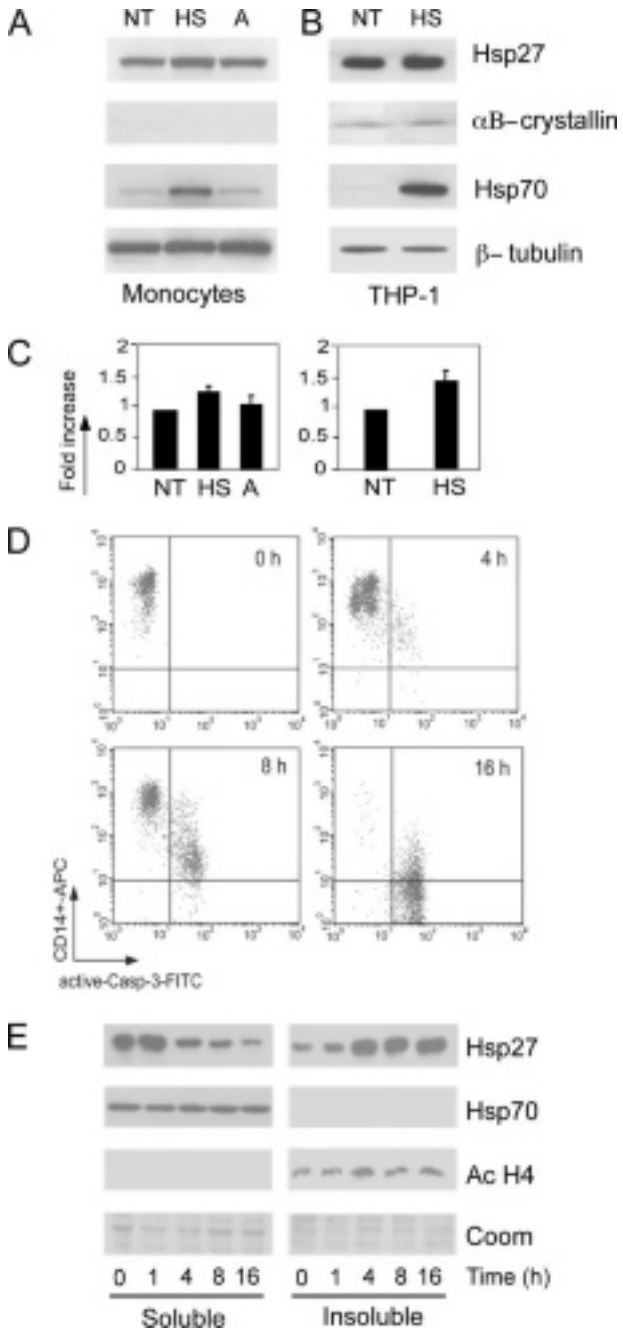

FIGURE 1. **Hsp27 expression and localization in human monocytes.** Whole cell lysates of fresh non-apoptotic (*NT*), monocytes cultured for 16 h (apoptotic (*A*)), and heat shock monocytes (*HS*) (*A*) or THP-1 whole cell lysates (*NT*) or heat shock (*HS*) (*B*) were resolved by SDS-PAGE and immunoblotted with anti-Hsp27, anti-αB-crystallin, anti-Hsp70, and anti-β-tubulin antibodies. *C*, relative amounts of Hsp27 protein found in monocytes or THP-1 cells (*right panel*) expressed as -fold increase relative to the amount found in NT monocytes and normalized by equal loading using β-tubulin ($p > 0.05$; $n = 3$). *D*, human monocytes CD14+ were cultured for different lengths of time and labeled with CD14-allophycocyanin and anti-active-caspase-3-FITC antibodies and analyzed by flow cytometry. *E*, lysates from soluble and insoluble cell fractions (see "Material and Methods") were resolved by SDS-PAGE and immunoblotted with anti-Hsp27, anti-Hsp70, and anti-acetylated H4 antibodies or gels were stained with Coomassie (*Coom*).

αB-crystallin are likely to suggest an important role of Hsp27 in the monocytic lineage.

Localization of Hsp27 Changes during Spontaneous Monocyte Apoptosis—We have previously shown that human monocytes undergo spontaneous apoptosis, a process that requires activation of caspase-3 (7). Consistently, we found that purified $CD14^+$ monocytes cultured for different lengths of time showed active caspase-3 as early as 4 h during monocyte life span and ~40% of the cells have active caspase-3 at 8 h (Fig. 1*D*). Because Hsp27 was previously shown to relocalize upon heat shock (28), we next determined the localization of Hsp27 during spontaneous monocyte apoptosis. Freshly isolated monocytes cultured for different lengths of time were separated into soluble (cytoplasm) and insoluble (nuclei and other organelles) fractions. Western blots performed with anti-Hsp27 antibodies showed that in fresh, non-apoptotic monocytes Hsp27 localized mostly to the soluble fraction, with significantly lower levels in the insoluble fraction (Fig. 1*E*, *0* and *1 h*). A significant increase of Hsp27 in the insoluble fraction was observed after 4 h of culturing (Fig. 1*E*, *4 h*). In apoptotic monocytes most of the Hsp27 protein was present in the insoluble fraction (Fig. 1*E*, *8–16 h*). In sharp contrast, Hsp70 was localized in the soluble fraction throughout the life span of monocytes (Fig. 1*E*). To rule out that the relocalization of Hsp27 was due to nonspecific localization during apoptosis, the same membranes were reblotted with acetylated histone 4 (*AcH4*). AcH4 was found in the insoluble fraction consisting of nuclei and other cellular organelles at all the time points tested (Fig. 1*E*).

Next, we determined the localization of endogenous Hsp27 by immunofluorescence during the life span of primary human monocytes. We found that Hsp27 localized to the cytoplasm of fresh monocytes, but at 8 h Hsp27 was found in the nucleus of monocytes undergoing spontaneous apoptosis (Fig. 2*A*). The localization of endogenous caspase-3 was determined using two different antibodies. The inactive caspase-3, shown in *red*, and the active caspase-3 (in *green*) were found routinely in the cytoplasm in both fresh and apoptotic primary human monocytes (Fig. 2*A*).

To determine whether the cytoplasm localization of Hsp27 was dependent on prolonged monocyte survival, we inhibited monocyte apoptosis by culturing monocytes for 8 h with the survival factor M-CSF, which blocks apoptosis by inhibiting caspase-3 activation (Ref. 9). We found that Hsp27 remained localized in the cytoplasm (Fig. 2*A*, see *M-CSF*). Next, we studied whether the relocalization of Hsp27 was dependent on caspase-3-mediated apoptosis. Monocytes were cultured for 8 h with 75 μM caspase-3 inhibitor DEVD-FMK (Fig. 2*A*, see *DEVD*). This inhibitor allows the first cleavage of caspase-3 but blocks the active site, preventing the autocatalytic processing of the prodomain of caspase-3 (4). We found that under these conditions, Hsp27 continued to be localized in the cytoplasm.

Next, we obtained nuclear and cytoplasmic fractions from freshly isolated primary human monocytes and monocytes cultured for 8 or 16 h, respectively. We found that Hsp27 localized in the cytoplasm in fresh monocytes. During monocyte spontaneous apoptosis at 8 h, Hsp27 was found mostly in nucleus, and by 16 h Hsp27 was found completely in the nucleus (Fig. 2*B*). In contrast, inactive full-length caspase-3 and the active cleaved caspase-3 were always found in the cytoplasm (Fig. 2*B*). Taken together, these results indicate that Hsp27 localizes with the inactive caspase-3 precursor in the cytoplasm of non-apoptotic cells. Although Hsp27 relocalized to the nucleus during apoptosis, active caspase-3 remained in the cytoplasm. Relocaliza-

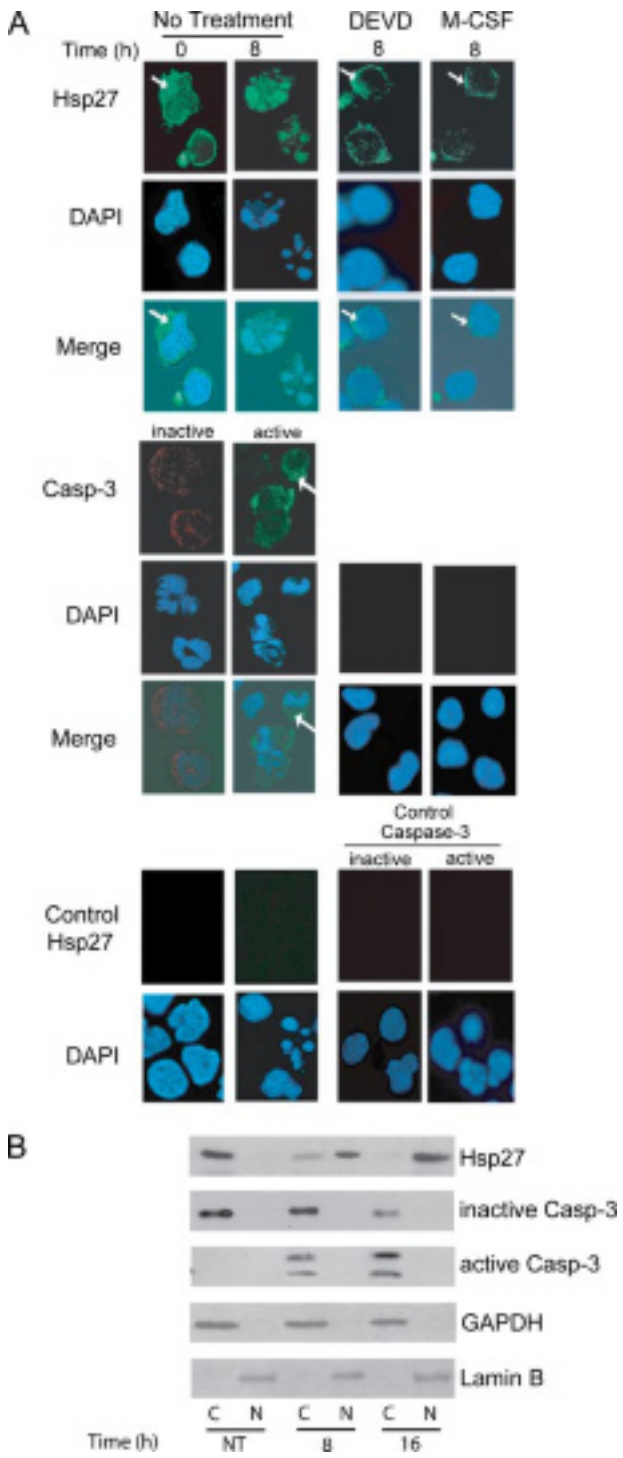

FIGURE 2. **Localization of Hsp27 and caspase-3 in monocytes.** *A*, nuclear and cytoplasmic fractions were obtained from freshly isolated untreated monocytes or undergoing spontaneous apoptosis cultured for 8 and 16 h. Fractions were resolved by SDS-PAGE and immunoblotted with anti-Hsp27, anti-inactive, and active caspase-3 antibodies. The same membranes were immunoblotted with anti-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and lamin B antibodies as cytoplasmic and nuclear markers, respectively. *B*, fresh and monocytes undergoing spontaneous apoptosis (cultured for 8 h) were stained with anti-Hsp27 (*green*). Fresh monocytes were stained with an anti-caspase-3 antibody (inactive in *red*). Monocytes cultured for 8 h were stained with an anti-active-caspase-3 (*green*) that only recognizes active caspase-3. Nuclei were stained with DAPI. Immunostaining using anti-Hsp27 or anti-caspase-3 antibodies was performed in monocytes cultured for 8 h with 75 μM DEVD-FMK or 20 μM M-CSF to promote survival. *C*, cytoplasm; *N*, nucleus.

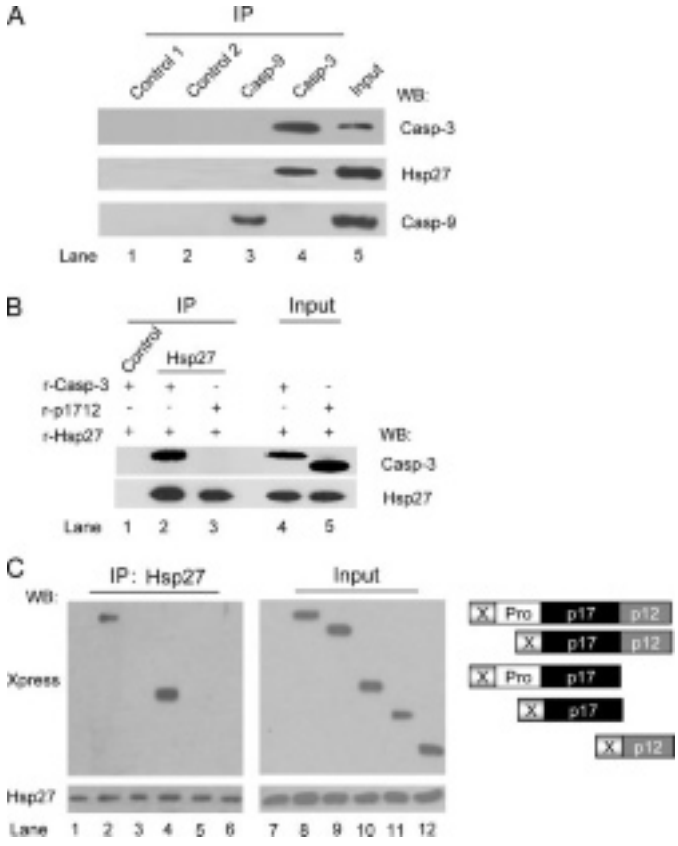

FIGURE 3. **Hsp27 associates with caspase-3 in human monocytes.** *A*, lysates of fresh human monocytes were immunoprecipitated using an anti-Casp-3, isotype controls (*Control*), or anti-caspase-9 antibody (*Casp-9*). IP and input were immunoblotted with anti-caspase-antibodies or anti-Hsp27 antibodies. *WB*, Western blot. *B*, purified Hsp27 and full-length casp-3 (*r-Casp-3*) or r-p1712 were mixed, and immunoprecipitations carried out with anti-Hsp27 or isogenic control (*lanes 1–33*). An aliquot of the mixes used in the immunoprecipitations was also analyzed by immunoblotting (*lanes 4* and *5*). Immunoblotting with anti-caspase-3 and anti-Hsp27. *C*, lysates of cells expressing various domains of caspase-3 (*Input*) were immunoprecipitated (*IP*) with anti-Hsp27, and IP and input were immunoblotted with anti-Xpress and anti-Hsp27 antibodies.

tion of Hsp27 appears to be dependent on the activation of caspase-3, since cells treated with the caspase-3 inhibitor DEVD-FMK or the differentiation factor M-CSF, both inhibitors of caspase-3-dependent-apoptosis, showed Hsp27 localization to the cytoplasm.

Hsp27 Associates with Caspase-3—Previous studies suggested roles for sHsp at multiple levels in the apoptotic cascade (29). To determine the role of Hsp27 in monocyte life span, we next investigated whether Hsp27 associated with caspase-3. Lysates from freshly isolated human monocytes were immunoprecipitated (IP) with an anti-caspase-3 antibody (*IP*, *anti-Casp-3*, Fig. 3*A*, *lane 4*) or with isotypes controls (IP, *control*, Fig. 3*A*, *lane 1* and *lane 2*, anti-rabbit and anti-mouse IgG controls, respectively) and analyzed by immunoblotting. We found that caspase-3 associated with Hsp27 (Fig. 3*A*, *lane 4*), whereas neither caspase-3 nor Hsp27 was detected in the control IP (Fig. 3*A*, *lanes 1* and *2*). To demonstrate the specificity of this interaction, caspase-9 was immunoprecipitated from monocyte lysates. Western analysis with anti-Hsp27 antibodies showed no association of caspase-9 with Hsp27 (Fig. 3*A*, *lane 3*).

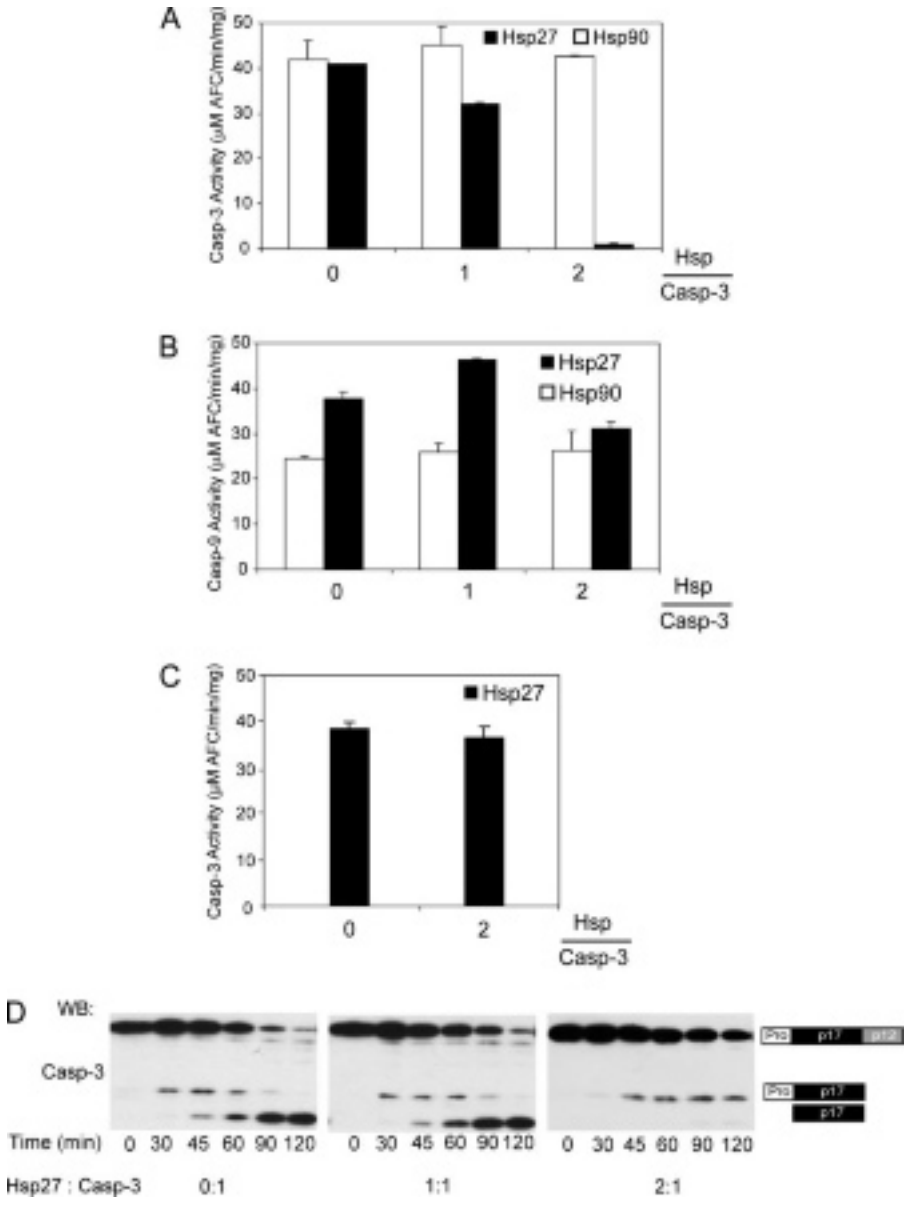

FIGURE 4. **Hsp27 inhibits the activation of caspase-3.** *A*, caspase-3 activity was determined using the fluorescent substrate DEVD-AFC activity assays. Purified full-length rCasp-3 was mixed for 30 min at 4 °C with equal amounts of Hsp27 (1) or with twice the amount of Hsp27 (2) or in the presence of Hsp27 buffer (0) to promote the formation of the complex. Then active caspase-9 was added to promote the first cleavage of casp-3, and the mix was incubated for 2 h at 37 °C before determining caspase-3 activity (*black bars*). Similar experiments using Hsp90 are shown in *white bars*. *B*, the activity of caspase-9 was determined in the samples used above using the fluorescent substrate LEHD-AFC. Results are expressed as average ± S.E. ($n = 3$). *C*, caspase-3 activity was determined as in *A* using purified p17p12 caspase-3 in the absence of Hsp27 (*0*) or with twice the amount of Hsp27 (2). *D*, purified full-length rCasp-3 was mixed for 30 min at 4 °C with equal amounts of Hsp27 (*1:1*) or with twice the amount of Hsp27 (*2:1*) or in the presence of Hsp27 buffer (*0:1*) to promote the formation of the complex. Then active caspase-9 was added to promote the first cleavage of caspase-3, and the mixes were incubated for different times at 37 °C. Processing of caspase-3 was determined by immunoblotting with anti-caspase-3 antibodies.

To determine whether Hsp27 associates directly with caspase-3, purified recombinant Hsp27 and wild type full-length caspase-3 or a caspase-3 version lacking the 28 amino acids of the amino-terminal prodomain were mixed and immunoprecipitated with anti-Hsp27 antibodies (Fig. 3*B*, *Input*, *lanes 4* and *5*). Western analysis with anti-caspase-3 antibodies demonstrated that full-length caspase-3 associates with Hsp27 (Fig. 3*B*, *lane 2*), whereas the caspase-3 p17p12 polypeptide, which lacks the prodomain, failed to associate with Hsp27 (Fig. 3*B*, *lane 3*).

To map the Hsp27/caspase-3 interaction, HeLa cells were transfected with pcDNA4HisMax plasmids encoding the caspase-3 polypeptide; that is, p12, p17, prop17, p17p12, or full-length prop17p12 tagged in the amino terminus with the Xpress epitope or a vector control as previously described (26). Twenty-four hours after transfection, lysates (Fig. 3*C*, *Input*, *lanes 7–12*) were immunoprecipitated with anti-Hsp27 antibodies (Fig. 3*C*, *lanes 1–6*). Immunoblots with the anti-Xpress antibodies showed that Hsp27 associates with full-length caspase-3 (Fig. 3*C*, *lane 2*) and with the prop17 polypeptide (Fig. 3, *lane 4*). All the other caspase-3 polypeptides failed to associate with Hsp27 (Fig. 3, *lanes 3*, *5*, and *6*). These results demonstrate the specific interaction of Hsp27 with caspase-3 and show that the prodomain of caspase-3 is necessary for this association.

Hsp27 Inhibits Caspase-3 Activation—To investigate the functional role of the caspase-3-Hsp27 association, we next determined the effect of Hsp27 on the activation of caspase-3. Recombinant full-length Casp-3 was mixed with recombinant Hsp27 at various molar ratios (equimolecular 1:1 or 1:2, the latter representing two molecules of Hsp27 per molecule of caspase-3) for 30 min on ice to promote the association of the protein complex. Active caspase-9 was then added to the mix to induce the first cleavage and activation of caspase-3 for 2 h, and caspase-3 activity was determined via the DEVD-AFC assay. In this *in vitro* enzymatic assay, the addition of Hsp27 resulted in a dramatic inhibition of the activity of caspase-3 (Fig. 4*A*, *black bars*). When similar experiments were carried out using the caspase-3 p17p12 polypeptide, which lacks the prodomain, we found no inhibitory effect of Hsp27 on the caspase-3 activity (Fig. 4*C*). These results together with the previous observations (Fig. 3, *B* and *C*) indicate that Hsp27 regulates the activation of caspase-3 by associating with its prodomain. The inhibitory effect on caspase-3 activity appears to be specific to Hsp27, as Hsp90 had no effect in caspase-3 activity (Fig. 4*A*, *white bars*).

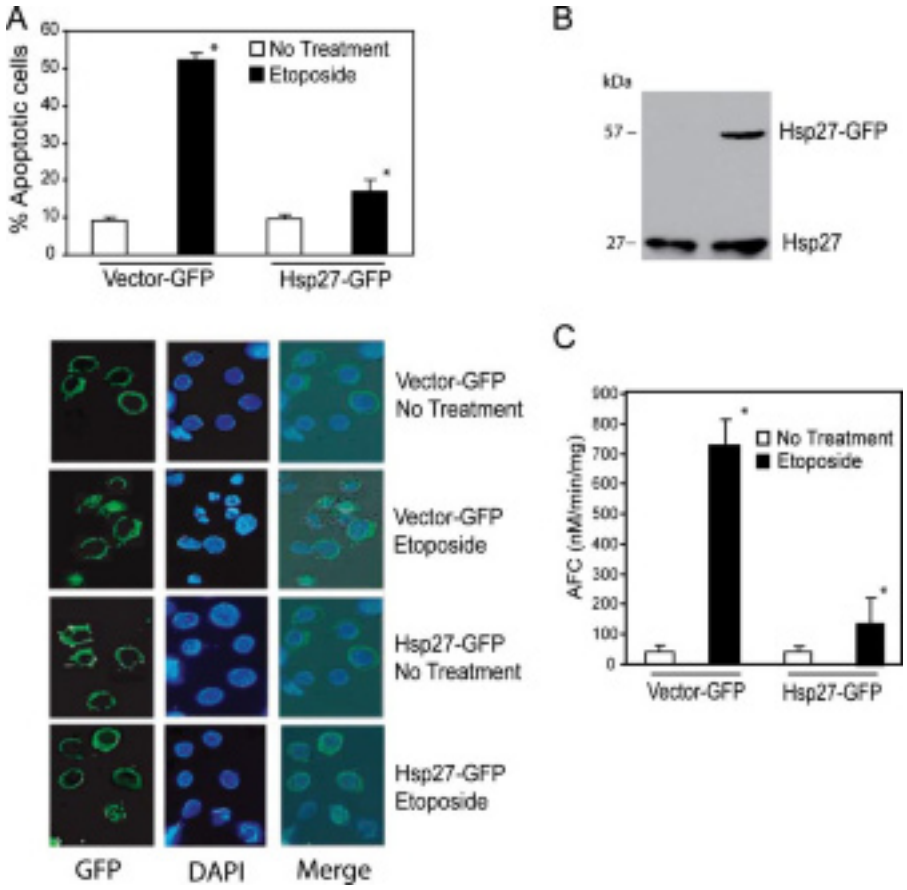

FIGURE 5. **Hsp27 overexpression inhibits caspase-3-dependent apoptosis.** THP-1 monocytic cells were transfected with Hsp27-GFP or an empty vector (*Vector-GFP*) and treated for 12 h with 10 μM etoposide to induce apoptosis or left untreated. *A*, the number of apoptotic cells was determined by nuclear fragmentation as seen by DAPI staining in cells Hsp27 determined by fluorescence. *B*, overexpression of Hsp27 determined by immunoblotting with anti-Hsp27 antibodies. *C*, caspase-3 activity determined by DEVD-AFC in lysates from the same cells treated for 12 h with etoposide to induce apoptosis or left untreated. Data are expressed as the average ± S.E. ($n = 3$; *, $p < 0.005$).

To rule out the possibility that the effect of Hsp27 on caspase-3 activity could be mediated by the Hsp27-dependent inhibition of caspase-9, we next determined the activity of caspase-9 in the same mixtures as shown in Fig. 4*A* using the caspase-9 substrate LEHD-AFC. Consistent with the specific interaction of Hsp27 with caspase-3 but not with caspase-9 (Fig. 3*A*), Hsp27 had no effect on caspase-9 activity (Fig. 4*B*, Hsp27 *black bars* or Hsp90 *white bars*).

Caspase-3 activation requires a two-step proteolytic processing. The first one, between p17 and p12, is mediated by an initiator caspase such as caspase-9, whereas the second processing involving the cleavage of the prodomain is caspase-3-dependent (4, 30). We next investigated the mechanisms by which Hsp27 affected caspase-3 activation by mixing recombinant full-length caspase-3 with recombinant Hsp27 at various molar ratios for 30 min on ice to promote the formation of the complex. Active caspase-9 was then added to the mix to induce the first cleavage, and the proteolytic processing of caspase-3 was verified at different times by Western blotting (Fig. 4*D*). In the absence of Hsp27 (represented by *Hsp27:Casp-3 0:1*, Fig. 4*D*), the pro-p17 fragment was first observed at 30 min, peaking at 45 min to then give to the appearance of the p17 fragment by the caspase-3-dependent second cleavage. By 120 min, most of the precursor caspase-3 had disappeared, and large quantities of p17 were observed (Fig. 4*D*, *0:1*). The addition of Hsp27 at a 1:1 molar ratio with caspase-3 slightly delayed the processing of the prop17 polypeptide (Fig. 4*D*, *1:1*). However, when the Hsp27:caspase-3 ratio was further increased to 2:1, the processing of prop17 was completely abolished (Fig. 4*D*, *2:1*). Interestingly, this concentration of Hsp27 also had a slight effect on the first cleavage, reflected by a delayed disappearance of the full-length caspase-3 band. Because Hsp27 was found to have no effect on the activity of caspase-9 (Fig. 4*B*), this result may suggest that the binding of Hsp27 to the prodomain caspase-3 somehow affects the conformation of full-length caspase-3, delaying the processing by caspase-9. Together, these experiments demonstrate that the binding of Hsp27 to the prodomain inhibits caspase-3 proteolytic activation.

Overexpression of Hsp27 Inhibits Apoptosis—A corollary of the experiments described earlier is that the ectopic expression of Hsp27 should affect the apoptotic potential of the cells. To test this hypothesis, THP-1 monocytic cells were transfected with Hsp27-GFP or empty vector control expressing GFP (Fig. 5*B*). Twenty-four hours after transfection, cells were either left untreated (Fig. 5, *No Treatment*) or induced to undergo apoptosis for 12 h with 10 μM etoposide, a chemotherapeutic drug that induces caspase-3-dependent apoptosis (27). The percentage of apoptotic cells was determined by the number of green transfected cells exhibiting nuclear fragmentation, a hallmark of caspase-3-dependent apoptosis. We found that the number of apoptotic cells was reduced approximately by 70% in cells overexpressing Hsp27 (Fig. 5*A*, *black bars*). A similar reduction of caspase-3 activity was observed in Hsp27-overexpressing cells treated with etoposide (Fig. 5*C*). These results demonstrate that Hsp27 functions as an anti-apoptotic regulator by inhibiting caspase-3-dependent apoptosis.

Hsp27 Localized in the Cytoplasm of Differentiated Monocytes—Based on our findings that the overexpression of Hsp27 can confer resistance to apoptosis, we hypothesized that the level of Hsp27 was likely to vary during the differentiation of monocytes to macrophages, cells from the same lineage but with a longer life span. To test this hypothesis, freshly isolated human monocytes obtained from circulating blood by a CD14+ selection were differentiated for 7 days to macrophages in the presence of M-CSF. Typical morphologic changes con-

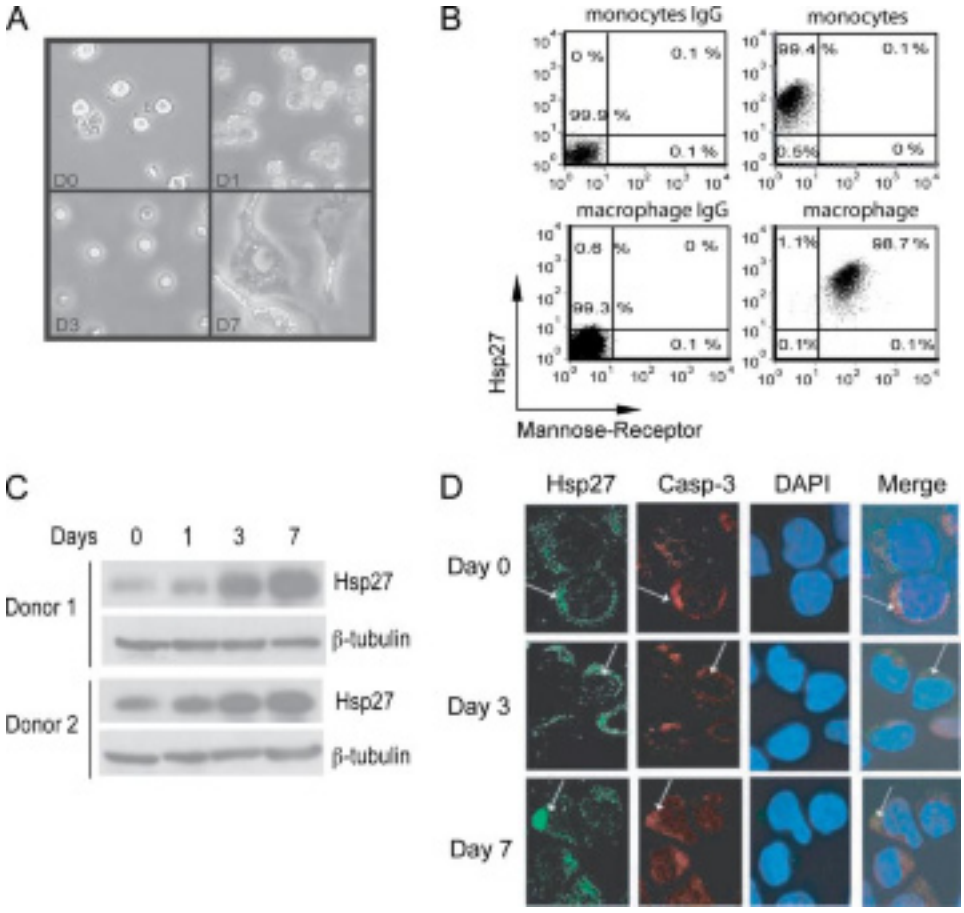

FIGURE 6. **Hsp27 expression is increased during monocyte differentiation and localized in the cytoplasm of macrophages.** Human monocytes were differentiated to macrophages (MDMs) *ex vivo* for different lengths of time. *A*, morphological changes were visualized during monocyte differentiation by microscopy. *B*, fresh monocytes or 7-day macrophages (MDMs) from the same donor were stained with anti-Hsp27 and anti-mannose receptor antibodies and analyzed by FACS. *C*, lysates from monocytes of two different donors at different days during differentiation were separated by SDS-PAGE and blotted with anti-Hsp27 and anti-β-tubulin antibodies. *D*, cells at different stages of differentiation were stained with anti-Hsp27 (*green*) and anti-caspase-3 (inactive, *red*) antibodies and DAPI.

sistent with increases in cell size and granularity and the increase of the macrophage mannose receptor marker (12, 31) were used to determine the differentiation of monocytes to macrophages (MDMs) (Fig. 6, *A* and *B*). We found that the total expression of Hsp27 remained constant during the first 24 h and increased at days 3 and 7 when most of the monocytes have differentiated into macrophages (Fig. 6*C*). Loading control was determined by immunoblotting the same membrane with anti-β-tubulin antibodies (Fig. 6*C*). A similar increase on Hsp27 expression was observed by flow cytometry (Fig. 6*B*). The localization of endogenous Hsp27 and caspase-3 was determined by immunofluorescence. We found that Hsp27 co-localized with caspase-3 in the cytoplasm of 7-day-old macrophages (Fig. 6*D*). These results demonstrate that in macrophages, cells of the monocytic lineage that have prolonged life span, Hsp27 localized in the cytoplasm with caspase-3.

Hsp27 Depletion Increases Apoptosis of Macrophages—It is well known that macrophages are very resistant to cell death induced by chemotherapeutic drugs (32, 33). To investigate whether the resistance to apoptosis was due to the higher level of expression of Hsp27, we inhibited the expression of Hsp27 by siRNA. First, monocytes were isolated from two normal donors. Half of the monocytes were differentiated to macrophages for 7 days (MDMs). The ability to undergo apoptosis was compared in monocytes and macrophages from the same donor. For this purpose, monocytes or macrophages were left untreated (Fig. 7*A*, *NT*) or treated with 10 μM etoposide for 12 h to induce cell death (Fig. 7*A*, *Etoposide*). We found no increase in the number of apoptotic cells in etoposide-treated macrophages compared with the macrophages left untreated in the two donors analyzed (Fig. 7*A*, shown in *red*). Consistent with these results, no increase in caspase-3 activity was found in macrophages treated with etoposide (Fig. 7*B*, shown in *red*). In contrast, treatment with etoposide for 12 h increased the number of apoptotic monocytes and caspase-3 activity when compared with the monocytes left untreated which were undergoing spontaneous apoptosis (Fig. 7, *A* and *B*, shown in *blue*). To determine whether a lower expression of Hsp27 affected apoptosis of macrophages, MDMs from two donors were transfected with siRNA duplexes that target Hsp27 (siRNA-Hsp27) or with a random duplex control (siRNA-Control) as previously reported (27). Immunoblotting analysis with anti-Hsp27 antibodies showed that Hsp27 expression was silenced by a 22–30% depending on the donor (Fig. 7*C*, *lane 2*). The same membrane was re-blotted with anti-Hsp70 antibodies to ensure the specificity of the silencing and with anti-β-tubulin for equal loading (Fig. 7*C*). After transfection with siRNA-Control or siRNA-Hsp27, macrophages were treated for 12 h with 10 μM etoposide to induce apoptosis or left untreated. We found that silencing of Hsp27 expression induced an increase to 15 and 20% in apoptosis for each donor, respectively, as determined by annexin V and 7AAD staining (Fig. 7*C*, *Donors 1* and *2*). In siRNA-Control etoposide-treated-macrophages, we found only 5% of apoptotic cells. Similarly, only 5% of cells undergoing apoptosis was also found in siRNA-Control and siRNA-Hsp27 no-treated macrophages.

Because of the difficulties associated with transfecting primary human macrophages, which make it difficult to obtain high transfection efficiency, we decided to investigate the effect of silencing the expression of Hsp27 using the mouse macrophage cell line RAW264.7. For this purpose mouse macrophages were transfected with siRNA duplexes that target Hsp25 (siRNA-Hsp25), the mouse Hsp27 homologue, or with a ran-

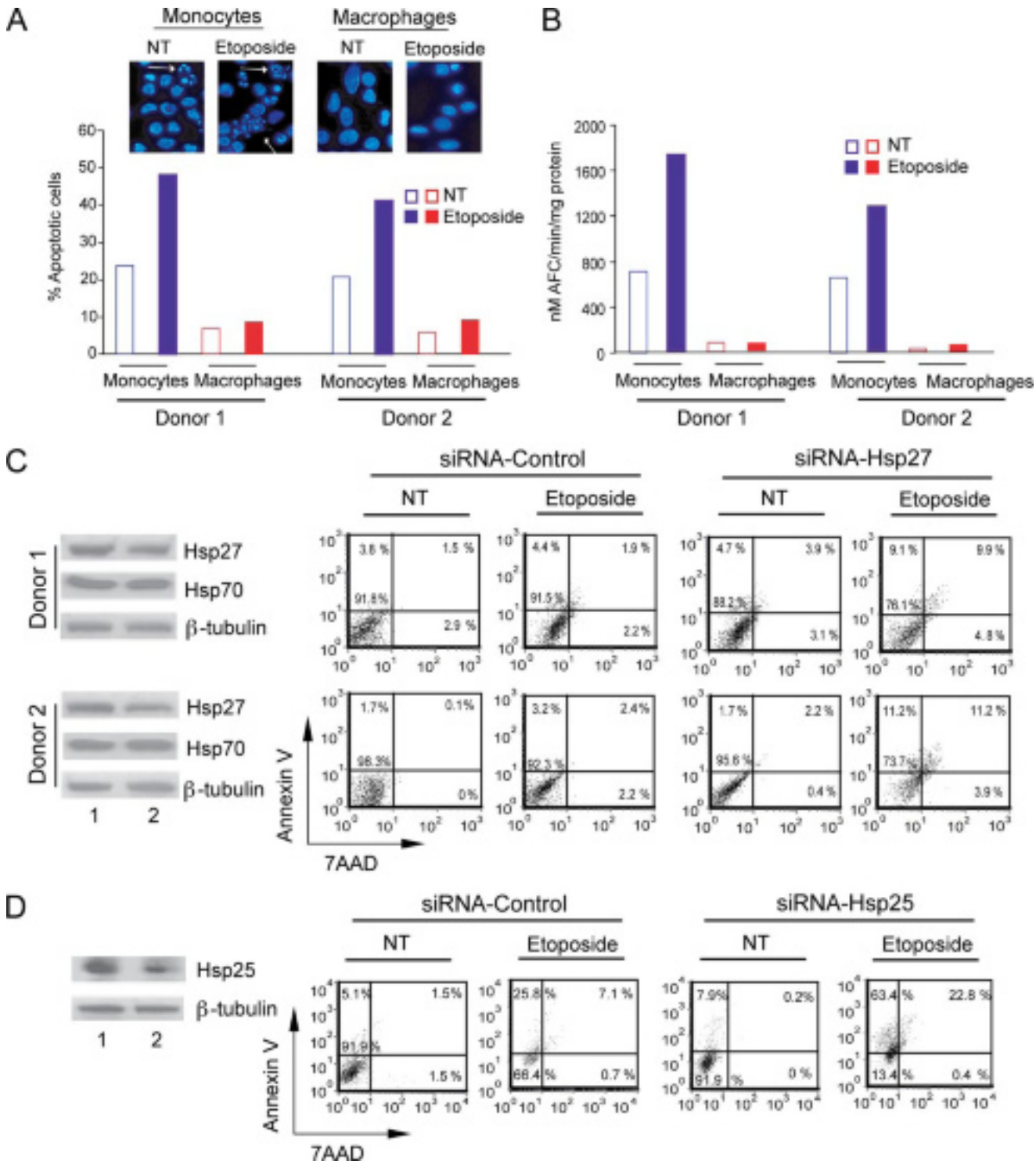

FIGURE 7. **Silencing of Hsp27 promotes macrophage apoptosis.** Human monocytes or macrophages (7-day MDMs) from two donors were left untreated (*NT*) or treated with 10 μM etoposide for 12 h to induce apoptosis. *A*, DAPI staining was used to score apoptosis. *B*, the same samples were used to determined caspase-3 activity by DEVD-AFC. *C*, macrophage lysates from two normal human donors transfected with scramble siRNA-Control (*lane 1*) or siRNA-Hsp27 (*lane 2*) were immunoblotted with anti-Hsp27, anti-Hsp70, and anti-β-tubulin antibodies. Same samples were left untreated or treated with etoposide as explained above, and the percentage of apoptotic cells was determined by staining with annexin V and 7AAD and analyzed by FACS. *D*, RAW macrophages were transfected with scramble siRNA-Control (*lane 1*) or siRNA-Hsp25 (*lane 2*) and treated with 10 μM etoposide for 12 h or left untreated (*NT*), and silencing was determined by immunoblotting with anti-Hsp25 and β-tubulin antibodies. The percentage of apoptotic cells was determined with annexin V and 7AAD in cells sorted by FACS based on their Hsp25 expression.

dom duplex control (siRNA-Control). The expression of Hsp25 was down-regulated by 70% 48 h after transfection (Fig. 7*D*, *lane 2*). The same membrane was immunoblotted with anti-β-tubulin antibodies showed equal loading (Fig. 7*D*). Next, macrophages transfected with siRNA-Hsp25 or siRNA-Control were treated for 12 h with 100 μM etoposide to induce apoptosis

or left untreated. The number of apoptotic cells was determined by annexin V, 7AAD staining after sorting Hsp25+ and Hsp25− cells. We found that 33% of the macrophages transfected with siRNA-Control undergo apoptosis after etoposide treatment (Fig. 7*D*). In sharp contrast, 85% of the macrophages transfected with siRNA-Hsp25 undergo apoptosis upon treatment with etoposide (Fig. 7*D*). These results taken together demonstrate that the reduction of Hsp27 expression increases the efficacy of the chemotherapeutic agent etoposide to induce apoptosis of macrophages, cells with reduced ability to undergo cell death.

DISCUSSION

The life span in cells of the monocytic lineage is determined by the activation of caspase-3, an essential component of the apoptotic machinery (7). The activation of caspases is regulated by pro- and anti-apoptotic factors (34). Although multiple regulators of the activator caspases have been identified, few direct modulators of caspase-3 have been found, and their mode of action remains elusive (34). We show here that Hsp27 regulates monocyte life span by associating with the prodomain of caspase-3 inhibiting its proteolytic activation.

Previous reports had already suggested an interaction of Hsp27 with components of the apoptotic cascade and an anti-apoptotic function. Indeed, the association of Hsp27 with DA*XX* blocks the formation of the DA*XX*-Ask complex, inhibiting the activation of caspase-8 (35). Hsp27 also associates with cytochrome *c* (a cofactor required for caspase-9 activation) halting the activation of caspase-9 (23). Pandey *et al.* (36) had previously reported that caspase-3 interacts with Hsp27, suggesting that this association blocks the caspase cascade at some point downstream of cytochrome *c*. However, these observations became controversial after the Bruey *et al.* (23) findings. First, these authors failed to reproduce the association of caspase-3 with Hsp27. Second, the activation of caspase-3 utilized by Pandey *et al.* (36) was dependent on the availability of cytochrome *c*, raising the possibility that the findings were just due to the ability of Hsp27 to block caspase-3 indirectly via sequestration of cytochrome *c*, as elegantly shown by Bruey *et al.* (23).

Using different strategies we unquestionably demonstrate here that Hsp27 directly associates with caspase-3 inhibiting apoptosis (Fig. 3). First, we used endogenous caspase-3 and Hsp27 to define the association between these two proteins (Fig. 3*A*). In agreement with Bruey *et al.* (23), we failed to see an association of Hsp27 with caspase-9 (Fig. 3*A*). Second we show using purified proteins that the Hsp27 association with caspase-3 is direct (Fig. 3*B*). Third, using transient transfection studies, we mapped the capase-3 domain responsible for the interaction *in vivo* (Fig. 3*C*). Finally, using purified proteins and transient transfection experiments we determined that the prodomain of caspase-3 is necessary for the association with Hsp27 (Fig. 3, *B* and *C*). The association of Hsp27 with caspase-3 inhibited specifically caspase-3 activity (Fig. 4). We found that Hsp90 did not affect caspase-3 activation (Fig. 4*A*), suggesting the specific role of Hsp27 in regulating caspase-3. Previous studies demonstrated that Hsp27 sequestered cytochrome *c*, subsequently blocking apoptosis by halting caspase-9 activation (23). It is of note, however, that in our results neither

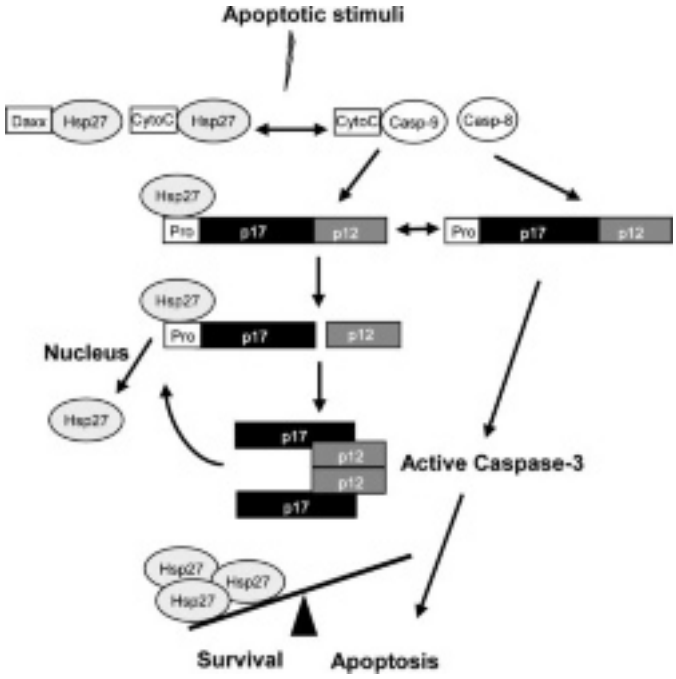

FIGURE 8. **Proposed anti-apoptotic checkpoints for Hsp27.** Hsp27 associates with several members of the apoptotic cascade. Cytochrome *c* and DA*XX* associate with Hsp27 inhibiting the apoptotic activation of upstream caspases (23, 35). Hsp27 associates directly with the prodomain of inactive caspase-3, halting the activation of this executioner caspase by inhibiting its proteolytic processing.

Hsp27 nor Hsp90 had any effect on the activity of caspase-9 (Fig. 4*B*). These findings are consistent with previous studies since cytochrome *c* affects the activation, but not the activity, of caspase-9 (4). We show that Hsp27 acts as an inhibitor of caspase-3 activation by blocking the proteolytic processing of caspase-3 (Fig. 4*D*). We found that increasing amounts of Hsp27 inhibit the formation of the p17 caspase-3 polypeptide (formed after the second cleavage of caspase-3). Interestingly, we also found a slight effect on the first cleavage, reflected by a delayed disappearance of the full-length caspase-3 band (Fig. 4*D*). These findings may suggest that the binding of Hsp27 to the prodomain of caspase-3 somehow affects the conformation of full-length caspase-3, delaying the processing by caspase-9. Together, these experiments demonstrate that the binding of Hsp27 to the prodomain inhibits caspase-3 activation. The prodomain of caspase-3 is well conserved throughout evolution. However, since its crystal structure is not yet available, its role *in vivo* has been mostly speculated (37). Our findings provide evidence for a biological role of the prodomain of caspase-3 by associating directly with the anti-apoptotic factor Hsp27 (Fig. 8).

Our studies show that the sHsp, Hsp27, is constitutively expressed in primary human monocytes and monocytic leukemia cells (Fig. 1). In contrast, the expression of αB-crystallin, an sHsp involved in myogenic differentiation, was undetectable in cells from the monocytic lineage even upon heat shock stimulation (Fig. 1) (24). The expression of Hsp27 did not increase upon heat shock and was unaltered during spontaneous apoptosis (Fig. 1). This is in sharp contrast with the observed induction of Hsp70 (21) and highlights the differential regulation of Hsp27 in monocytes. Notably, Hsp27 expression increased dra-

matically during monocyte-macrophage differentiation (Fig. 6). Unlike monocytes, macrophages have a long life span. High levels of Hsp27 have been also reported in several long-living cancer cells; however, the function of this increased expression has not yet been elucidated (22, 38–40). Our results show that overexpression of Hsp27 in monocytic leukemia cells blocked the caspase-3-dependent apoptosis induced by chemotherapeutic drugs (Fig. 5). In addition, our results demonstrate that silencing of Hsp27 in macrophage cell lines and in primary human macrophages facilitates the execution of cell death (Fig. 7). The increased expression of Hsp27 might contribute to tilting the balance toward cell survival, hence providing a molecular mechanism that may explain the resistance to cell death of macrophages. The constitutive expression of Hsp27 observed in fresh monocytes may ensure that Hsp27 acts by blocking the activation of the apoptotic cascade at several checkpoints, one of which involves the direct inhibition of at least a pool of caspase-3 (Fig. 8).

Changes in the subcellular localization of Hsp27 from the cytoplasm to the nucleus during heat shock have been reported (28, 41). Our results expand these studies by demonstrating that Hsp27, which is normally in the cytoplasm in non-apoptotic cells likely interacting with the inactive precursor of caspase-3, relocalizes to the nucleus during apoptosis (Figs. 1*E* and 2). However, in monocytes induced to differentiate through the addition of M-CSF, Hsp27 persisted in the cytoplasm, suggesting a mechanism by which Hsp27 can sense the "apoptotic potential" of the cell. Interestingly from a mechanistic perspective, blocking apoptosis by the addition of the caspase-3 inhibitor DEVD-FMK also prevents Hsp27 from going to the nucleus, indicating that the nuclear translocation of Hsp27 might be both necessary for caspase-3 activation and a consequence of caspase-3 activity, suggesting a positive regulatory mechanism which ensures that, once triggered, caspase-3 activation will result in apoptosis. Relocalization of Hsp27 to the nucleus may provide an additional mechanism to free the apoptotic caspases of its "anti-apoptotic-brake," allowing the execution of programmed cell death in primary cells that have a short life span.

Taken together these observations suggest an additional anti-apoptotic checkpoint of Hsp27 by regulating directly the caspase-3 proteolytic activation and, thus, controlling monocyte life span. Therapies involving depletion of Hsp27 might prove effective to help down-regulate the accumulation of monocytes/macrophages in cancer microenvironments or in chronic inflammatory diseases and, hence, be clinically useful for enhancing tumor cell death or controlling inflammation.

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REFERENCES

1. Steller, H. (1995) *Science* **267,** 1445–1462
2. Cohen, G. M. (1997) *Biochem. J.* **326,** 1–16
3. Nicholson, D. W., and Thornberry, N. A. (1997) *Trends Biochem. Sci.* **8,** 299–306
4. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312–1316
5. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489
6. Cline, M. J., Lehrer, R. I., Territo, C., and Golde, D. W. (1978) *Ann. Intern. Med.* **88,** 78–88
7. Fahy, R. J., Doseff, A. I., and Wewers, M. D. (1999) *J. Immunol.* **163,** 1755–1762
8. Mangan, D. F., Mergenhagen, S. E., and Wahl, S. M. (1993) *J. Periodontol.* **64,** 461–466
9. Kelley, T. W., Graham, M. M., Doseff, A. I., Pomerantz, R. W., Lau, S. M., Ostrowski, M. C., Franke, T. F., and Marsh, C. B. (1999) *J. Biol. Chem.* **274,** 26393–26398
10. Thomas, E. D., Ramberg, R. E., sale, G. E., Sparkes, R. S., and Golde, D. W. (1976) *Science* **192,** 1016–1018
11. Ross, J. A., and Auger, M. J. (2002) in *The Macrophage* (Burke, B., and Lewis, C. E., eds) 2nd, Oxford University Press, Oxford
12. Mahoney, J. A., Haworth, R., and Gordon, S. (2000) in *Haematopoietic and Lymphoid Cell Culture* (Dallman, M. J., and Lamb, J. R., eds) pp. 120–146, Cambridge University Press, Cambridge, UK
13. Goyal, A., Wang, Y., Graham, M. M., Doseff, A. I., Bhatt, N. Y., and Marsh, C. B. (2002) *Am. J. Respir. Cell Mol. Biol.* **26,** 224–230
14. Smith, J. D., Trogan, E., Ginsberg, M., Grigaux, C., Tian, J., and Miyata, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8264–8268
15. Rajavashisth, T., Qiao, J. H., Tripathi, S., Tripathi, J., Mishra, N., Hua, M., Wang, X. P., Loussararian, A., Clinton, S., Libby, P., and Lusis, A. (1998) *J. Clin. Investig.* **101,** 2702–2710
16. Hance, A. J., Douches, S., Winchester, R. J., Ferrans, V. J., and Crystal, R. G. (1985) *J. Immunol.* **134,** 284–292
17. Hartl, F. U., and Hayer-Hartl, M. (2002) *Science* **295,** 1852–1858
18. Jacquier-Sarlin, M. R., Jornot, L., and Polla, B. S. (1995) *J. Biol. Chem.* **270,** 14094–14099
19. Mehlen, P., Schulze-Osthoff, K., and Arrigo, A. P. (1996) *J. Biol. Chem.* **271,** 3395–3401
20. Concannon, C. G., Gorman, A. M., and Samali, A. (2003) *Apoptosis* **8,** 61–79
21. Lang, D., Hubrich, A., Dohle, F., Terstesse, M., Saleh, H., Schmidt, M., Pauels, H. G., and Heidenreich, S. (2000) *J. Leukocyte Biol.* **68,** 729–736
22. Hansen, R. K., Parra, I., Lemieux, P., Oesterreich, S., Hilsenbeck, S. G., and Fuqua, S. A. W. (1999) *Breast Cancer Res. Treat.* **56,** 187–196
23. Bruey, J., Ducasse, C., Bonniaud, P., Ravagnan, L., Susin, S., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A., Kroemer, G., Solary, E., and Garrido, C. (2000) *Nat. Cell Biol.* **2,** 645–652
24. Kamradt, M. C., Chen, F., Sam, S., and Cryns, V. L. (2002) *J. Biol. Chem.* **277,** 38731–38736
25. Doseff, A. I., Baker, J. H., Bourgeois, T. A., and Wewers, M. D. (2003) *Am. J. Respir. Cell Mol. Biol.* **29,** 367–374
26. Chen, L., Smith, L., Wang, Z., and Smith, J. B. (2003) *Mol. Pharmacol.* **64,** 334–345
27. Voss, O. H., Kim, S., Wewers, M. D., and Doseff, A. I. (2005) *J. Biol. Chem.* **280,** 17371–17379
28. Arrigo, A.-P., Suhan, J. P., and Welch, W. J. (1988) *Mol. Cell. Biol.* **8,** 5059–5071
29. Arrigo, A.-P., and Muller, W. E. G. (2002) *Small Heat Shock Proteins*, 1st Ed., pp. 185–200, Springer-Verlag, Berlin, Germany
30. Slee, E. A., Adrain, C., and Martin, S. J. (2001) *J. Biol. Chem.* **276,** 7320–7326
31. Ezekowitz, R. A. B., Williams, D. J., Koziel, H., Armstrong, M. Y. K., Warner, A., Richards, F. F., and Rose, R. M. (1991) *Nature* **351,** 155–158
32. Perlman, H., Pagliari, L. J., Georganas, C., Mano, T., Walsh, K., and Pope, R. M. (1999) *J. Exp. Med.* **190,** 1679–1688
33. Zhang, J., Li, Y., Yu, M., Chen, B., and Shen, B. (2003) *Hematol. J.* **4,** 277–284
34. Degterev, A., Boyce, M., and Yuan, J. (2003) *Oncogene* **22,** 8543–8567
35. Charette, S. J., Lavoie, J. N., Lambert, H., and Landry, J. (2000) *Mol. Cell. Biol.* **20,** 7602–7612
36. Pandey, P., Farber, R., Nakazawa, A., Kumar, S., Bharti, A., Nalin, C., Weichselbaum, R., Kufe, D., and Kharbanda, S. (2000) *Oncogene* **19,** 1975–1981

37. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kosturas, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casanol, F. J., Chin, J., Ding, G. J.-F., Egger, L. A., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992) *Nature* **356,** 768–774
38. Mese, H., Sasaki, A., Nakayama, S., Yoshioka, N., Yoshihama, Y., Kishimoto, K., and Matsumura, T. (2002) *Oncol. Rep.* **9,** 341–344
39. Arrigo, A.-P., and Paul, C. (2000) *Exp. Gerontol.* **35,** 757–766
40. Elpek, G. O., Karaveli, S., Simsek, T., Keles, N., and Aksoy, N. H. (2003) *APMIS* **111,** 523–530
41. McClaren, M., and Isseroff, R. R. (1994) *J. Investig. Dermatol.* **102,** 375–381