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Ectopic expression of Hsp70 confers resistance and silencing its expression sensitizes human colon cancer cells to curcumin-induced apoptosis

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We have shown earlier that heat shock renders human colon cancer cells resistant to curcumin-induced apoptosis, but the contribution of individual heat shock proteins (hsps) to this resistance has not been tested. High expression of hsp27 and hsp70 in breast, endometrial and gastric cancers has been associated with metastasis, poor prognosis and resistance to chemo- or radiotherapy. In this study, SW480 cells were transfected with *hsp70* cDNA in either the sense or antisense orientation and stable clones were selected and tested for their sensitivity to curcumin. The cells were protected from curcumin-induced cell death by hsp70 while cells harboring antisense hsp70 (Ashsp70) were highly sensitive to curcumin. Curcumin-induced nuclear condensation was less in hsp70 but more in Ashsp70 cells when compared with control vector-transfected cells. Loss of mitochondrial transmembrane potential induced by curcumin was further accelerated by antisense hsp70 expression and hsp70 restored it partly. Ashsp70 cells released more cytochrome c, AIF and Smac from mitochondria upon curcumin treatment than control cells. hsp70 partly prevented the release of AIF but not the other proteins. Activation of caspases 3 and 9 induced by curcumin was also inhibited by hsp70, whereas more activation could be seen in Ashsp70 cells, although caspase 8 activation was unaffected by changes in hsp70 expression. Curcumin-induced cleavage of PARP and DFF45 was inhibited by hsp70 but enhanced in Ashsp70 cells. The present study demonstrates the potential of hsp70 in protecting SW480 cells from curcumin-induced apoptosis and highlights that silencing the expression of hsp70 is an effective approach to augment curcumin-based therapy in cancers that are resistant due to hsp70 expression.

Introduction

In multicellular organisms, apoptosis or programmed cell death is achieved by at least two independent pathways that are initiated and executed by distinct caspases, a class of cysteine proteases that participates in the dismantling of critical cellular components. For instance, several members of the tumor necrosis factor receptor family, such as Fas and tumor necrosis factor receptor-1, recruit procaspase 8 to their cytosolic domains upon ligation to activate caspase 8, which subsequently activates distal caspases 3, 6 and 7 (1). Another pathway of caspase activation involves mitochondria, from which cytochrome c is released into the cytosol, which binds with apoptosis protease activating factor-1 (Apaf-1) and activates caspase 9 in an ATP/dATP-dependent manner (2,3). Another pro-apoptotic protein released from mitochondria along with cytochrome c is second mitochondria-derived activator of caspase (Smac), which functions by relieving the inhibitory function of inhibitor of apoptosis protein that associates with and inhibits caspases (4,5). Recently, caspase-independent apoptogenic proteins such as apoptosis-inducing factor (AIF) and endonuclease G have been characterized (6,7).

During the past decade, several proteins that promote tumorigenesis by inhibiting apoptosis have been identified in primary tumors, which include anti-apoptotic members of the Bcl-2 protein family and heat shock proteins, as well as members of the inhibitor of apoptosis protein family (8). Heat shock proteins (hsp) are highly conserved proteins known to protect cells from adverse environmental, physical and chemical stresses by their ability to prevent protein aggregation and promote the refolding of denatured proteins (9). The ability of HSPs to prevent apoptosis induced by several anticancer drugs also explains how these proteins could limit the efficacy of cancer therapy (10,11). Indirect experimental evidence and clinicopathological studies indicate that hsp70 is the major stress inducible, cancer-associated, anti-apoptotic protein (8). Increased expression of hsp70 has been reported in high grade malignant breast and endometrial tumors, osteosarcoma and renal cell tumors (10). Understanding the molecular mechanisms of action of these proteins is necessary to initiate novel modes of rationally and selectively manipulating the sensitivity of cancer cells to therapy (8).

Curcumin (diferuloylmethane), the major active ingredient of turmeric has been widely used in the treatment of inflammatory disorders for centuries and is also known to possess anti-proliferative, anti-mutagenic and anti-carcinogenic properties (12,13). Curcumin is known to induce apoptosis in several cancer cells, like colon (14), T lymphocytes (15), hepatocarcinoma (16) and breast carcinoma cells (17). Recently we have shown in human colon cancer cells that mild heat treatment renders them resistant to curcumin-induced apoptosis (18) and, in the present study, we have developed stable clones of human colon cancer cells (SW480) expressing hsp70 in the sense or antisense orientation and tested them for their sensitivity to curcumin treatment. We report that hsp70 expression makes colon cancer cells resistant to curcumin-induced apoptosis by inhibition of processing of caspases and inhibiting the release of AIF and when the

Abbreviations: AFC, 7-amino-4-(trifluoromethyl) coumarin; AIF, apoptosis-inducing factor; Apaf-1, apoptosis protease activating factor-1; DAPI, 4,6-diamidino-2-phenylindole; $\Delta\Psi_m$, mitochondrial transmembrane potential; DFF45, DNA fragmentation factor 45; FITC, fluorescein isothiocyanate; hsp, heat shock protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; Smac, second mitochondria-derived activator of caspase.

The first two authors contributed equally to this work.

expression of hsp70 is silenced, the cells are sensitized to curcumin, indicating the potential of such targets in cancer therapy.

Materials and methods

Cell culture

Human colon cancer cell line, SW480, was provided by Ajit Kumar, Indian Institute of Science, Bangalore, India and maintained on DMEM (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma) in an atmosphere of 95% air and 5% CO_2.

Reagents and antibodies

Curcumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were procured from Sigma. Rabbit polyclonal antibody to cytochrome c (sc-7159), AIF (sc-5586), poly(ADP-ribose) polymerase (PARP) (sc-7150) and DNA fragmentation factor 45 (DFF45) (sc-9066), goat polyclonal antibody to Smac (sc-12683) and mouse monoclonal antibody to hsp70 (sc-24) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody to β-actin (A-5441) and all the secondary antibodies were obtained from Sigma. Rabbit polyclonal antibodies to caspase 3 (no. 9662) and caspase 9 (no. 9502) and a mouse monoclonal antibody to caspase 8 (no. 9746) were obtained from Cell Signaling Technology (Beverly, MA).

Stable transfection

SW480 cells grown on 60 mm dishes were transfected with 8 μg of pSV-hsp70-tag, containing the cDNA for full-length human hsp70, or pcDNA3 hsp70AS, an expression construct for hsp70 in the antisense direction (both constructs from M. Jaattela, Danish Cancer Society), with lipofectamine 2000 (Life Technologies Inc.) as per the manufacturer's instructions. The transfected cells were selected with 800 μg/ml G418 (Life Technologies Inc.) and clones formed were picked up and maintained separately with 100 μg/ml G418.

MTT assay

MTT was used to measure cell viability (19). This assay is based on the conversion of MTT, a soluble tetrazolium salt, into insoluble formazan by mitochondrial dehydrogenase enzymes in viable cells. hsp70-transfected and vector-transfected cells seeded at a density of 5×10^3 cells/well in 96-well plates were allowed to grow for 24 h. Subsequently, the cells were incubated with curcumin (25 μM) for 16, 24 and 48 h and cell viability was determined by MTT assay. Cell viability was calculated as the amount of dye converted relative to that of an untreated control.

Isolation of cytosolic fraction by digitonin lysis method

Cells (untreated or after treatment) were harvested, washed twice with phosphate-buffered saline (PBS) and the cell pellet was resuspended in digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose and 190 μg/ml digitonin) containing protease inhibitors and incubated on ice for 5 min. The releasate was centrifuged at 15 000 r.p.m. at 4°C for 30 min and used for western blotting as described below using antibodies to AIF, Smac or cytochrome c and appropriate secondary antibodies.

Western blotting

Cells were harvested, washed three times in PBS and lysed in RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 40 mM NaF, 10 mM NaCl, 10 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol and 1 μg/ml each of leupeptin and aprotinin). The cell lysates (50 μg protein) were loaded onto SDS–PAGE gels and the separated proteins were transferred to nitrocellulose membrane by the wet transfer method using a Bio-Rad electrotransfer apparatus. After blocking with 10% non-fat milk in Tris-buffered saline containing 0.2% Tween 20, the membrane was incubated with a primary antibody followed by an alkaline phosphatase-conjugated secondary antibody and the protein bands were visualized with a 3,3′-diaminobenzidine/H_2O_2 mixture (Sigma).

Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

Briefly, cells grown in 96-well plates with or without treatment were washed with serum-free medium and stained with a cationic dye called Mitosensor (ApoAlert mitochondrial membrane sensor kit; Clontech) as per the manufacturer's protocol and analyzed by fluorescence microscopy using a bandpass filter. The dye is taken up in the mitochondria, where it forms aggregates that exhibit an intense red fluorescence. However, once $\Delta\Psi_m$ is lost, Mitosensor cannot accumulate in the mitochondria and stays in the cytosol as a monomer with green fluorescence. Cells showing loss of $\Delta\Psi_m$ (green) were expressed as a percentage by two different investigators counting the number of red and green cells in at least three different fields.

Assessment of chromatin condensation

The cells were grown on 12 mm coverslips and exposed to 25 μM curcumin in subconfluent stage for 24 h. The monolayer of cells was washed in PBS and fixed with 3% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and incubated with 0.5 μg/ml DAPI for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) were scored as a percentage from 200–300 cells/sample with at least two investigators using a fluorescent microscope (Nikon TE 300).

Immunofluorescent staining

Cells grown on glass coverslips, after appropriate treatments, were fixed and permeabilized as before and then incubated with the respective primary antibody overnight. After extensive washing with Tris-buffered saline containing 0.2% Tween 20, the cells were incubated with FITC (fluorescein isothiocyanate)-conjugated secondary antibody at 1:50 dilution for 45 min in the dark. The coverslips were mounted in 50% glycerol–PBS, viewed under a fluorescence microscope and photographed.

Determination of caspase activities

Subconfluent cells growing on 100 mm dishes treated with 25 μM curcumin for 8, 16 or 24 h or left untreated were assayed spectrofluorimetrically for the enzymatic activities of caspase 3, caspase 9 and caspase 8 (20). Briefly, whole cell lysate was incubated with 50 μM fluorimetric substrate of caspase 3 (Ac-DEVD-AFC), caspase 9 (Ac-LEHD-AFC) or caspase 8 (Z-IETD-AFC) in a total volume of 500 μl of reaction buffer [50 mM HEPES–KOH, pH 7.0, 10% glycerol, 0.1% 3-(cholamidopropyl)-dimethylammonio-1-propane sulfonate, 2 mM EDTA and 2 mM dithiothreitol] at 37°C for 1 h. The released AFC was quantitated using a spectrofluorimeter (LS-50 B; Perkin Elmer) with excitation and emission wavelengths of 405 and 500 nm, respectively. Values of relative fluorescence units released per milligram of protein were calculated. Further, the cleaved fragments of caspase 3, caspase 9 and caspase 8 were detected by western blotting using specific antibodies that detect the intact mother band as well as the corresponding cleaved fragments as described earlier.

Results

Ectopic expression of hsp70 renders SW480 cells resistant, whereas that of antisense hsp70 (Ashsp70) sensitizes them, to curcumin-induced cytotoxicity

To understand whether hsp70, known to possess anti-apoptotic properties, could regulate curcumin-induced apoptosis, SW480 human colon cancer cells were first stably transfected with pSV-hsp70-tag (with hsp70 cDNA in the sense orientation) or pcDNA3 hsp70AS (with hsp70 cDNA in the antisense orientation) constructs. The relative expression of hsp70 assessed by western blotting in different G418-resistant clones was quantitated using Quantity One software for image analysis (Bio-Rad) after normalizing for the value of the loading control (β-actin intensity). The vectors, pSV-tag and pcDNA3, contain neomycin resistance genes and we used one of them (pcDNA3) as a control. When compared with these pcDNA3 vector-transfected cells (neo), all the *hsp70*-transfected clones expressed a higher level of hsp70 (Figure 1A) and similarly all the three *Ashsp70*-transfected clones showed lower levels of hsp70 compared with neo cells (Figure 1B). A clone of hsp70 cells that expressed an 8-fold higher level of hsp70 (clone 1) (Figure 1A) and Ashsp70 clone 1, which showed very low expression of hsp70 (Figure 1B), were used for subsequent experiments. Then neo, hsp70 or Ashsp70 cells were incubated with 25 μM curcumin for 16, 24 or 48 h and cell viability was analyzed by MTT assay. Upon treatment with curcumin, the mean viability values for neo cells were 66, 53 and 35% over the untreated control for 16, 24 and 48 h, respectively, indicating an increase in the cytotoxicity of curcumin over this time period (Figure 1C). In hsp70 cells viabilities were much better, with mean values being 94 ($P < 0.038$), 82 ($P < 0.036$) and 68% ($P < 0.013$), while in contrast only 43 ($P < 0.004$),

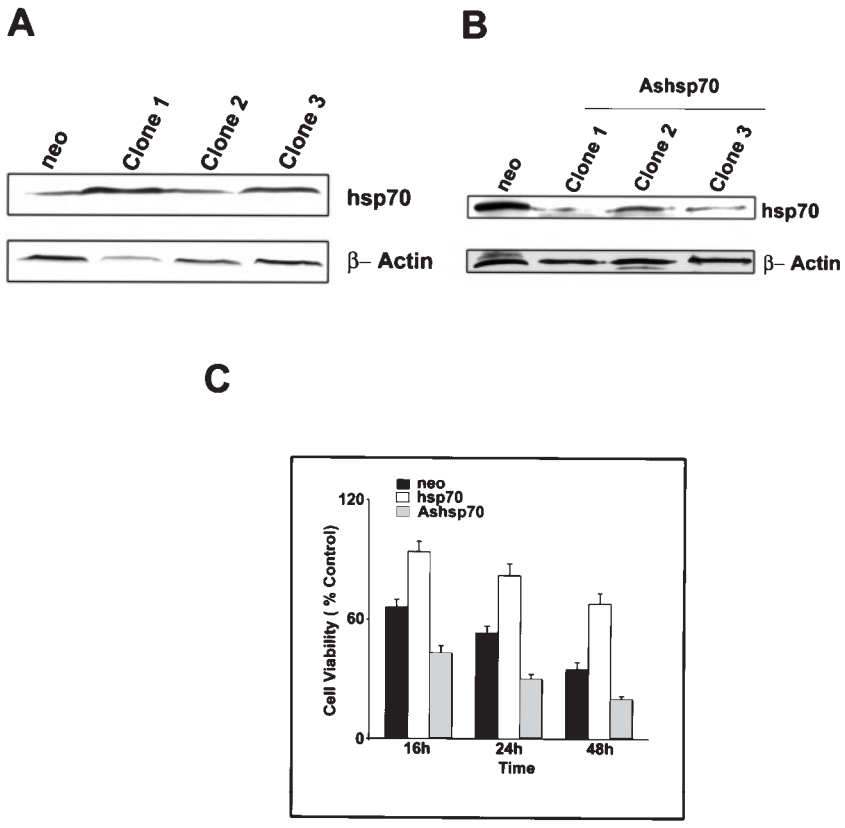

Fig. 1. Establishment of stable clones expressing hsp70 or its antisense and changes in cell viability induced by curcumin. (**A**) SW480 cells were stably transfected with pSV-hsp70-tag using lipofectamine 2000 as per the manufacturer's instructions (Life Technologies Inc.) and whole cell extracts prepared from three different G418-resistant clones were analyzed for hsp70 expression by western blot (10% gel) using a mouse monoclonal antibody as described in Materials and methods. (**B**) SW480 cells were transfected with pcDNA3 hsp70 AS and G418-resistant clones were analyzed for hsp70 expression by western blot as described above. For the above experiments, cells transfected with the empty vector pcDNA3 served as a control (neo) and β-actin as a loading control and they were repeated at least twice with similar results. (**C**) SW480 cells (neo, hsp70 or Ashsp70) grown on 96-well plates were exposed to curcumin (25 μM) for 16, 24 or 48 h and the cell viability (expressed as a percentage of the untreated control) was determined by MTT assay as described in Materials and methods. The mean values of triplicate samples are shown and error bars indicate standard deviations and the experiments were repeated three times with similar results.

30 ($P < 0.008$) and 20% ($P < 0.005$) of Ashsp70 cells survived upon curcumin treatment for the same time intervals (Figure 1C). These data suggest that hsp70 cells resist curcumin treatment, whereas neo and, more effectively, Ashsp70 cells are sensitive to curcumin-induced cell death.

Curcumin induces nuclear condensation effectively in neo and Ashsp70 cells but not in hsp70 cells

To determine whether the survival of hsp70 cells was associated with inhibition of apoptosis, neo, hsp70 or Ashsp70 cells were exposed to 25 μM curcumin for 24 h and chromatin condensation was analyzed by DAPI staining. All the untreated samples from neo, hsp70 or Ashsp70 cells showed uniform diffuse staining with DAPI, but upon treatment with curcumin, 65% of neo, 35% of hsp70 ($P < 0.022$) and 93% of Ashsp70 ($P < 0.011$) cells showed a condensed nuclear morphology (Figure 2A and B). These results indicate that curcumin-induced cytotoxicity involves nuclear condensation characteristic of apoptosis and ectopic expression of hsp70 promotes survival of SW480 cells, whereas down-regulating hsp70 sensitizes them to curcumin-induced apoptosis.

Ashsp70 expression augments curcumin-induced loss of $\Delta\Psi_m$ and hsp70 expression partly restores it

Loss of $\Delta\Psi_m$ is one of the earliest events of curcumin-induced apoptosis observed by us in SW480 cells (18). Hence, we analyzed whether hsp70 expression or its down-regulation could play a protective role by altering curcumin-induced loss of $\Delta\Psi_m$. Untreated neo, hsp70 and Ashsp70 cells showed a granular red fluorescence indicating integrity of $\Delta\Psi_m$ (Figure 3A). However, upon exposure to 25 μM curcumin for 24 h, only 23% of neo, 32% of hsp70 ($P < 0.075$) and 10% of Ashsp70 ($P < 0.012$) cells retained $\Delta\Psi_m$ (Figure 3B). These data indicate that the expression of hsp70 partly restores $\Delta\Psi_m$ loss induced by curcumin, but curcumin-induced $\Delta\Psi_m$ loss is more in Ashsp70 cells.

hsp70 partially prevents curcumin-induced AIF release, but not that of cytochrome c and Smac from mitochondria while AsHsp70 enhances their release

Cytochrome c, Smac and AIF are mitochondrial proteins that are safely stored within the intermembrane space of mitochondria in intact cells (21). We have tracked these molecules with corresponding primary antibodies and FITC-conjugated secondary antibodies after 24 h of curcumin (25 μM) treatment. neo control, hsp70 control and Ashsp70 control cells showed granular mitochondrial staining for cytochrome c, Smac and AIF (Figure 4A). Upon curcumin treatment, neo and Ashsp70 cells showed a diffuse pattern of staining for cytochrome c, Smac and AIF, indicating their release from mitochondria, with curcumin-induced release of all three molecules being relatively more in Ashsp70 cells (Figure 4A). Curcumin-induced

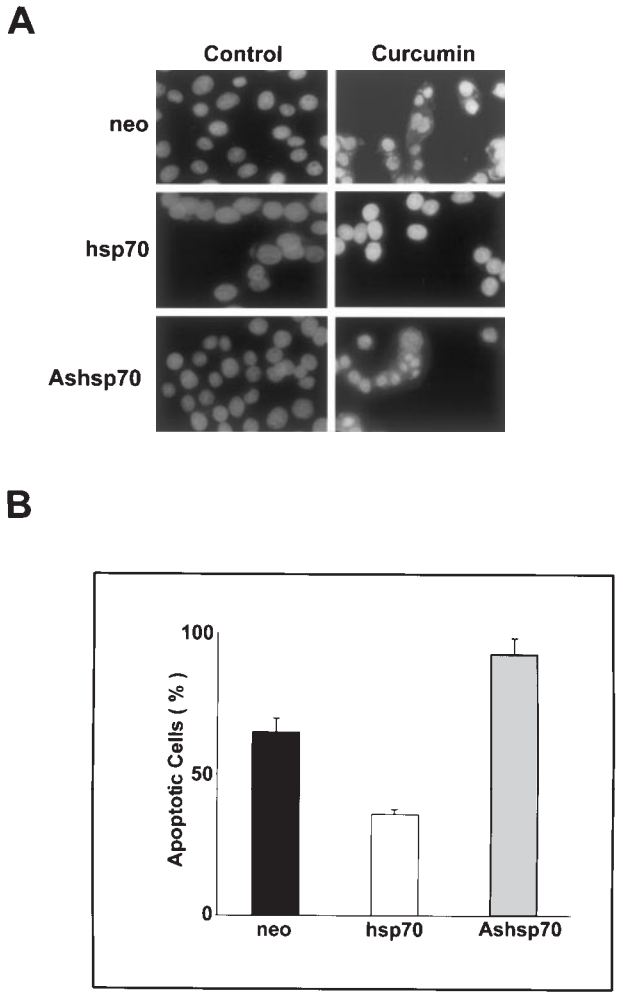

Fig. 2. Relative changes in nuclear condensation induced by curcumin in neo, hsp70 and Ashsp70 cells. (**A**) neo, hsp70 or Ashsp70 cells were seeded onto coverslips, treated with or without curcumin (25 μM) for 24 h, fixed using 3% paraformaldehyde and stained with DAPI as described in Materials and methods and representative fluorescent micrographs from untreated (control) and curcumin-treated cells are shown. (**B**) Cells with condensed and fragmented chromatin from the experiment described above were counted in five different fields and the mean values of triplicate samples expressed as percentages are shown. These results were confirmed by another independent experiment.

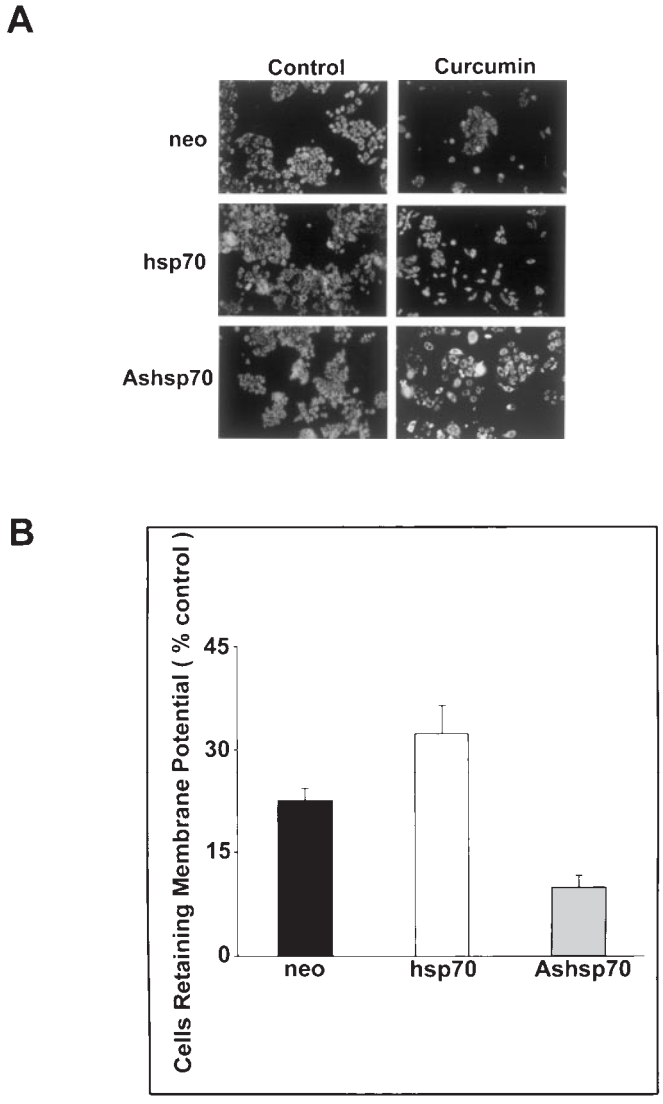

Fig. 3. Effects of curcumin on the $\Delta\Psi_m$ of neo, hsp70 or Ashsp70 cells. (**A**) neo, hsp70 or Ashsp70 cells grown on 96-well plates in sub-confluent stage were incubated with or without curcumin (25 μM) for 24 h, washed with serum-free medium and stained with a mitosensor dye for 15 min as described in Materials and methods. Representative fluorescent micrographs are shown and an intense red granular appearance indicates the integrity of $\Delta\Psi_m$ and green cytoplasmic fluorescence indicates the loss of $\Delta\Psi_m$. (**B**) Cells retaining $\Delta\Psi_m$ from the above experiment were counted in three different fields using triplicate samples and expressed as percentages of the control and the error bars indicate standard deviation. The experiment was repeated three times with similar results.

release of cytochrome c and Smac was also noticed in hsp70 cells, but the AIF release was incomplete (Figure 4A). The cytoplasmic release of cytochrome c, Smac and AIF was analyzed further by western blotting of digitonin-lysed samples treated with or without curcumin. Figure 4B shows that upon curcumin treatment, neo cells released all three molecules whereas hsp70 cells released cytochrome c and Smac with partial release of AIF, while the β-actin levels were unchanged with or without curcumin. Upon curcumin treatment, the release of cytochrome c, Smac and AIF was more in Ashsp70 than neo cells (Figure 4C). These data show that hsp70 expression partially prevents curcumin-induced release of AIF without inhibiting the release of cytochrome c and Smac from mitochondria and the expression of Ashsp70 sensitizes SW480 cells to release greater amounts of these molecules upon curcumin treatment.

hsp70 inhibits curcumin-induced activation of caspases 3 and 9 but not 8 whereas Ashsp70 potentiates the effects of curcumin

Cytochrome c released into the cytosol is known to bind Apaf-1 and procaspase 9, leading to activation of caspases 9 and 3 (22). In order to understand whether changes in hsp70 expression interfere with the activation of caspases during curcumin-induced apoptosis, the processing of procaspases 9, 3 and 8 was assessed using specific spectrofluorimetric substrates as well as immunodetection of cleaved fragments of these caspases. As can be seen from Figure 5A, caspase 9 was increasingly activated with increasing time of incubation with curcumin in neo and, more effectively, Ashsp70 cells, whereas hsp70 cells exhibited a lower level of activity that did

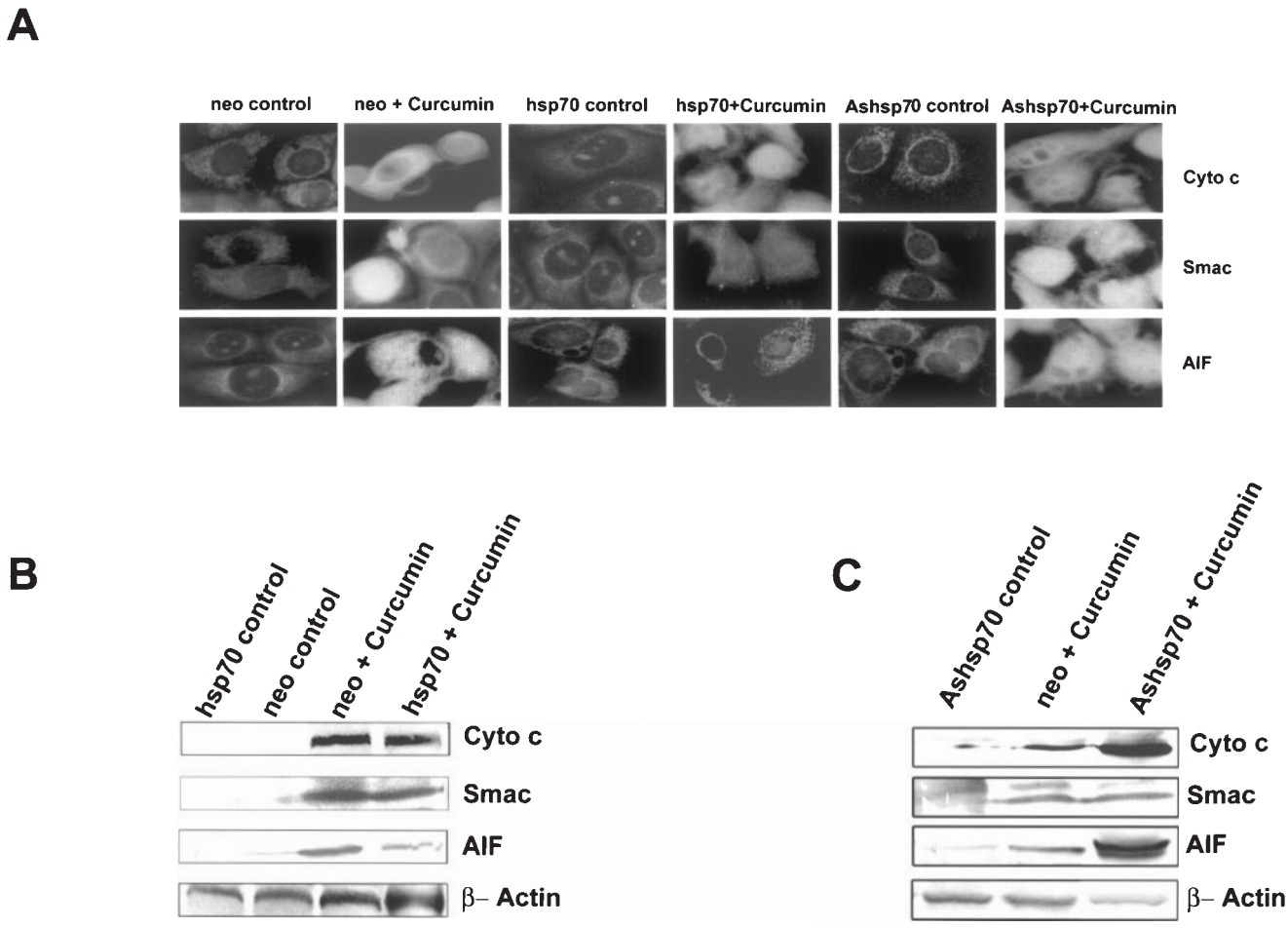

Fig. 4. Induction of release of cytochrome c, Smac and AIF from mitochondria induced by curcumin. (**A**) Cells grown on coverslips were fixed, permeabilized and incubated overnight with a primary antibody to Smac, cytochrome c or AIF and then incubated again with FITC-conjugated secondary antibody and visualized under a fluorescent microscope as described in Materials and methods. (**B**) neo or hsp70 cells with or without curcumin treatment were suspended in digitonin lysis buffer and the lysate was used for western blotting of AIF, Smac, cytochrome c or β-actin (control) as described in Materials and methods. (**C**) Ashsp70 cells treated with or without curcumin or neo cells treated with curcumin were analyzed for the release of cytochrome c, Smac, AIF or β-actin by western blotting as described above. All the above experiments were repeated at least twice with similar results.

not change throughout the experimental period. In western blot analyses cleaved fragments (37 kDa) indicating activation of caspase 9 by curcumin were visible in neo and Ashsp70 cells but not in hsp70 cells (Figure 5B). Similarly, caspase 3 activation by curcumin increased with time in neo and again more efficiently in Ashsp70 cells, but hsp70 cells exhibited lower activation that remained unchanged when analyzed by a spectrofluorimetric method (Figure 5C). These results were further confirmed by western blotting, which showed the cleaved fragment of procaspase 3 only in neo and Ashsp70 cells and not in hsp70 cells when induced by curcumin for the time periods shown (Figure 5D). When curcumin-induced activation of caspase 8 was assessed in neo, hsp70 and Ashsp70 cells using specific fluorimetric substrates, all of them showed increased activity with increasing time (Figure 5E). Similarly, the cleaved fragment could be seen in all three cells treated with curcumin throughout the experimental period (Figure 5F), suggesting that activation of caspase 8 by curcumin is unaffected by changes in hsp70 expression. Both fluorimetric as well as western blotting methods supported the finding that hsp70 remarkably reduced the activation of procaspases 9 and 3 but not 8. The experimental results substantiate the hypothesis that hsp70 predominantly blocks apoptosis downstream of mitochondrial release of cytochrome c, especially reducing the processing of caspase 9 and subsequent caspase 3 processing. Activation of these caspases was potentiated in Ashsp70 compared with neo cells.

hsp70 blocks curcumin-induced cleavage of PARP and DFF45 but Ashsp70 enhances the effects of curcumin

PARP and DFF45 are two important cellular proteins necessary for maintaining cellular integrity that are cleaved and rendered inactive during apoptosis. As shown in Figure 6A, curcumin cleaved 116 kDa PARP (producing an 85 kDa fragment) after 16 h and by 24 h the intensity of the cleaved fragment increased in neo cells, whereas in hsp70 cells the cleaved fragment was not visible up to 16 h, although a very faint 85 kDa band could be seen at 24 h. In contrast, cleaved fragment could be observed along with disappearance of the 116 kDa PARP band in Ashsp70 cells treated with curcumin for 16 or 24 h (Figure 6A). Similarly, DFF45 cleavage was observed in neo cells as early as 16 h and continued up to 24 h upon treatment with curcumin, whereas in hsp70 cells only a faint band was visible at 24 h. In contrast, Ashsp70 cells

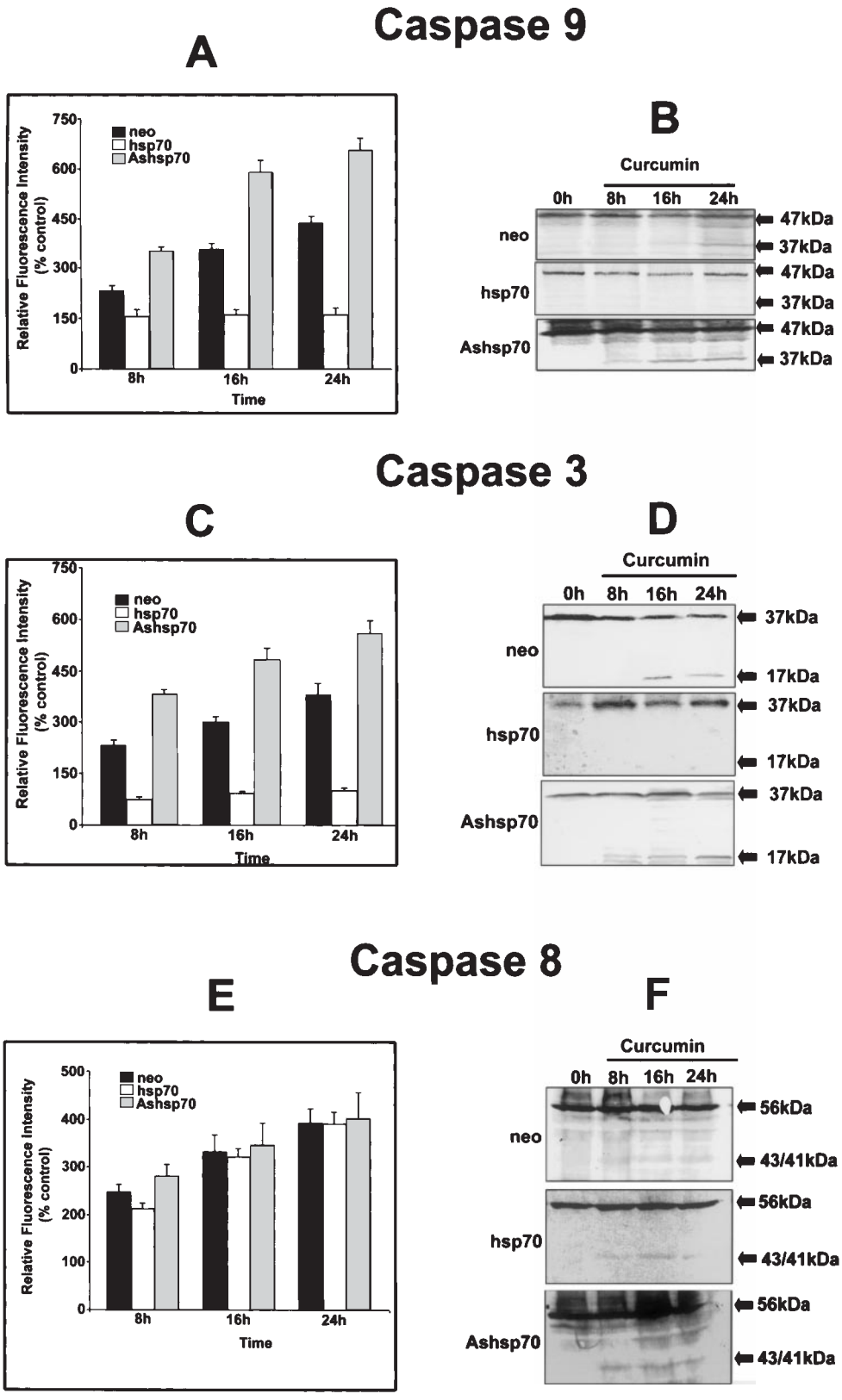

Fig. 5. Changes in the activities of caspases induced by curcumin. (**A**) Whole cell extracts (50 μg) prepared from neo, hsp70 or Ashsp70 cells treated with or without curcumin (25 μM) for 0, 8, 16 or 24 h were assessed for the activation of caspase 9 using a fluorimetric substrate (Ac-LEHD-AFC) in a reaction buffer at 37°C for 1 h as described in Materials and methods. (**B**) Caspase 9 activation was also assessed by western blotting as described in Materials and methods. (**C**) Activation of caspase 3 by curcumin at the indicated periods of time was determined by using a fluorimetric substrate of caspase 3 (Ac-DEVD-AFC) as described in Materials and methods. (**D**) Caspase 3 activation was confirmed by western blot analysis as described above. (**E**) Caspase 8 activation assessed by a spectrofluorimetric method with substrate Z-IETD-AFC was essentially the same as described above. (**F**) Western blot was used to assess caspase 8 activation. All these experiments were repeated at least twice with similar results and the error bars denote standard deviation.

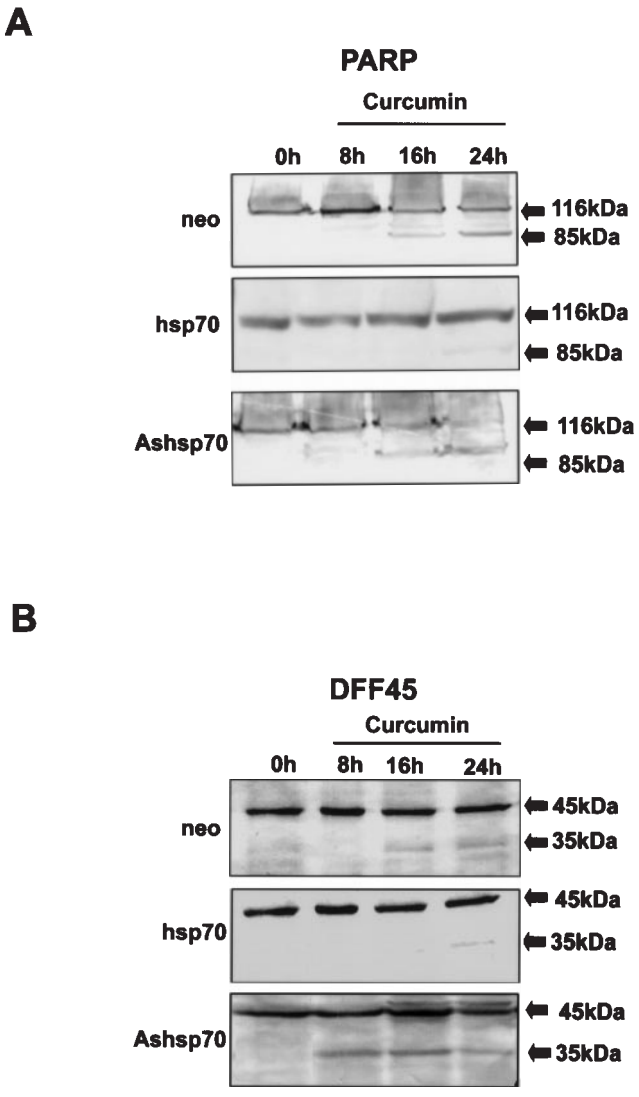

Fig. 6. Curcumin-induced cleavage of PARP and DFF45. (**A**) Lysates (60 μg protein) prepared after treating the cells with or without curcumin (25 μM) for the indicated time periods were analyzed for PARP with a specific polyclonal antibody as described in Materials and methods. (**B**) Western blotting was carried out for the detection of DFF45 and its cleaved fragment using a specific polyclonal antibody as described above and these experiments were repeated twice with similar results.

showed DFF45 cleavage for all the time periods of this experiment (Figure 6B). These results show that hsp70 expression suppresses curcumin-induced apoptosis predominantly by preventing the processing of caspases and by preventing the cleavage of caspase substrates like PARP and DFF45. A model showing the effects of hsp70 on curcumin-induced apoptosis is shown in Figure 7.

Discussion

Triggering apoptosis in cancer cells is the predominant mechanism by which most anticancer agents act and failure to elicit it not only leads to the development of tumors but also increases their resistance to therapy (23). High expression of hsps, especially hsp27 and hsp70, in breast, endometrial and gastric cancer has been associated with metastasis, poor prognosis and resistance to chemo- and radiotherapy (24). We have shown earlier that heat shock renders human colon cancer cells (SW480 and SW620) resistant to curcumin-induced apoptosis, but the potential contribution of any of the individual hsps to this resistance has not been tested (18). The present study demonstrates the potential of hsp70 in protecting human colon cancer cells from curcumin-induced apoptosis and highlights that silencing expression of hsp70 using is an effective approach to augment curcumin-based therapy. Overexpression of hsp70 was shown to confer tumorigenicity and resistance to the cytotoxic effects of tumor necrosis factor, staurosporine and doxorubicin (10,25). Selective depletion of hsp70 by its antisense oligo alone was sufficient to kill human breast cancer cells independent of caspases and apoptotic stimuli without affecting the survival of non-tumorigenic breast epithelial cells (11). Inhibition of curcumin-induced activation of caspases and partly blocking the release of AIF by hsp70 suggests that it interferes with caspase-dependent and -independent pathways induced by curcumin in human colon cancer cells.

Consistent with our results showing curcumin-induced release of AIF, others have shown the involvement of AIF-mediated large scale DNA fragmentation in response to curcumin (26,27). Since the release of cytochrome c or Smac is unaffected by hsp70 expression in human colon cancer cells, this suggests that hsp70 is acting downstream of cytochrome c release, whereas Bcl-2 prevents the release of AIF and cytochrome c from the mitochondrion (6,21,28). Our results support the notion that the loss of $\Delta\Psi_m$ and the release of cytochrome c, AIF and Smac from mitochondria are separate events (29,30). Accelerated loss of $\Delta\Psi_m$ and enhanced release of AIF, cytochrome c and Smac upon curcumin treatment in Ashsp70 cells may be secondary to increased caspase activation (31). Translocation of cytochrome c from mitochondria to cytosol is also said to occur very early (before $\Delta\Psi_m$ loss) during the apoptotic process (32).

Our results showing inhibition of curcumin-induced activation of both caspases 9 and 3 in hsp70 cells and the enhanced activation of these caspases in Ashsp70 cells are supported by the report that, unlike Bcl-2, hsp70 interacts directly with Apaf-1 with subsequent inhibition of cytochrome c-mediated oligomerization of Apaf-1 and processing of procaspase 9 (21,33). The reduced cleavage of caspase 3 substrates like PARP and DFF45 in hsp70 cells together with their increased cleavage by the selective depletion of hsp70 indicates a role for hsp70 in inhibiting apoptosis downstream of caspase 3 activation (25). However, hsp70 can also prevent cell death under conditions in which caspase activation doesn't occur, i.e. after inactivation of the *Apaf-1* gene, suggesting that caspase inhibition may not be the sole mechanism behind the survival effect of hsp70 (34).

Since hsp70 prevented the processing of caspase 9 and caspase 3 in human colon cancer cells in the present study, subsequent release of AIF probably required active caspases and, in agreement with this, it has been reported that release of AIF is downstream of cytochrome c release and requires active caspase 3 (35). It appears that there will be an initial loss of AIF from mitochondria along with the release of cytochrome c or Smac independent of caspases. However, subsequent mitochondrial release of AIF may require active caspases, an event likely to be blocked in hsp70 overexpressing cells. This notion is further substantiated by the accelerated release of AIF in Ashsp70 cells. Both immunoblot and immunofluorecence microscopic experiments substantiated the findings. Failure

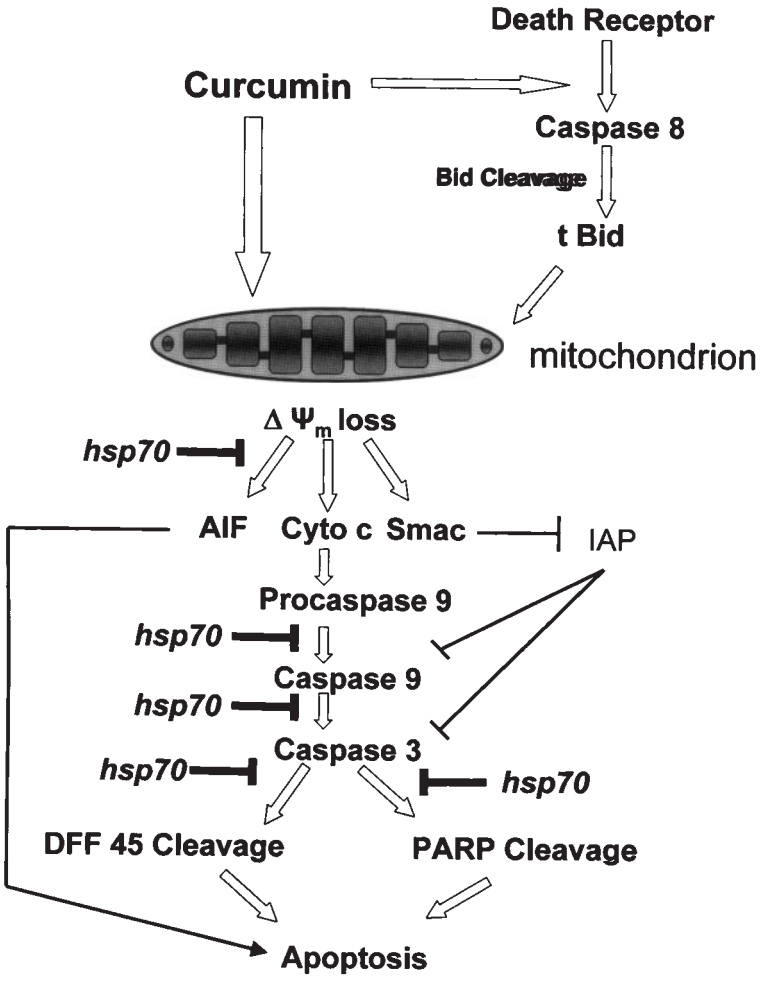

Fig. 7. A model depicting the role of hsp70 in curcumin-induced apoptosis. Curcumin induces apoptosis by both receptor- and mitochondria-mediated pathways. hsp70 blocks apoptosis by inhibiting curcumin-induced activation of caspases 9 and 3 and also the caspase 3-mediated cleavage of DFF45 and PARP. The release of AIF is also partly prevented by hsp70.

of hsp70 to completely prevent the release of AIF may be the reason for not getting complete protection in hsp70 over-expressing cells, using the MTT assay. In isolated mitochondria Bax (a pro-apoptotic member of the Bcl-2 family) causes the release of cytochrome c, but not of AIF, and it has been hypothesized that the association of AIF with the mitochondrial inner membrane could account for its lack of release upon Bax-mediated outer membrane permeabilization (35). However, the actual mechanism behind the selective retention of AIF within the mitochondria in hsp70 cells is not known and contradictory results from different groups make it difficult to discern whether AIF release depends upon different cell types or stimuli and if it occurs in a caspase-dependent or -independent fashion (36).

Although curcumin induces both the receptor- and mitochondria-mediated pathways of apoptosis (18,37,38), hsp70-mediated survival does not appear to inhibit the receptor-mediated pathway, as curcumin-induced cleavage of procaspase 8 remains unaffected by changes in hsp70 expression. Curcumin (facilitated by its hydrophobic nature) is said to induce oligomerization and aggregation of Fas receptor in the cell membrane to activate caspase 8, but to induce cell death further events such as the cleavage of Bid, release of cytochrome c and activation of other caspases (inhibited by hsp70) are necessary (39,40).

Our results suggest that potential drugs like curcumin may not be effective in eliminating chemoresistant cancers that overexpress hsp70, but down-regulation of hsp70 remarkably sensitizes human colon cancer cells to apoptosis induced by curcumin and interventional approaches to reduce the expression of hsp70 offer novel ways to eliminate such cancers.

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