Human colon cancer cells lacking Bax resist curcumin-induced apoptosis and Bax requirement is dispensable with ectopic expression of Smac or downregulation of Bcl-XL

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Multiple apoptotic stimuli induce conformational changes in Bax, a proapoptotic protein from the Bcl-2 family and its deficiency is a frequent cause of chemoresistance in colon adenocarcinomas. Curcumin, a dietary compound from turmeric, is known to induce apoptosis in a variety of cancer cells. To understand the role of Bax in curcumin-induced apoptosis we used HCT116 human colon cancer cells with one allele of *Bax* gene (*Bax*$^{+/-}$) and Bax knockout HCT116 (*Bax*$^{-/-}$) cells in which *Bax* gene is inactivated by homologous recombination. Cell viability decreased in a concentration-dependent manner in Bax$^{+/-}$ cells treated with curcumin (0–50 μM) whereas only minimal changes in viability were observed in Bax$^{-/-}$ cells upon curcumin treatment. In Bax$^{-/-}$ cells curcumin-induced activation of caspases 9 and 3 was blocked and that of caspase 8 remained unaltered. Curcumin-induced release of cytochrome c, Second mitochondria derived activator of caspase (Smac) and apoptosis inducing factor (AIF) was also blocked in Bax$^{-/-}$ cells and reintroduction of Bax, downregulation of the antiapoptotic protein Bcl-XL by antisense DNA as well as the overexpression of Smac, highly sensitized the Bax$^{-/-}$ cells toward curcumin-induced apoptosis. There was no considerable difference in the percentage of apoptotic cells in Bak RNAi transfected Bax$^{+/-}$ or Bax$^{-/-}$ cells treated with curcumin when compared with their corresponding vector transfected cells treated with curcumin. The present study demonstrates the role of Bax but not Bak as a critical regulator of curcumin-induced apoptosis and implies the potential of targeting antiapoptotic proteins like Bcl-XL or overexpression of proapoptotic proteins like Smac as interventional approaches to deal with Bax-deficient chemoresistant cancers for curcumin-based therapy.

Introduction

Bcl-2 family members are important regulators of apoptosis that include antiapoptotic (Bcl-2, Bcl-XL and Mcl-1),

proapoptotic (Bax and Bak) and the BH3-domain-only (Bim, Bid and Bik) proteins (1). Anticancer drugs or radiation induce the release of cytochrome c from mitochondria which together with apoptosis protease activating factor-1 (Apaf-1) and pro-caspase 9 forms the apoptosome complex (2). Caspase 9 subsequently activates caspase 3, the cysteine protease that can cleave the majority of caspase substrates and other caspases ensuring peaceful elimination of the cell (3–5). Apart from cytochrome c, the release of second mitochondria derived activator of caspase (Smac) from mitochondria also ensures continued caspase activation needed for ultimate cell death by suppressing the caspase inhibitory function of inhibitors of apoptosis protein (IAPs) (6–11). Independent of caspases, apoptosis inducing factor (AIF) and endonuclease G can induce DNA fragmentation once they are released from the mitochondria (12,13). Caspase 8 cleaves Bid to form truncated Bid that translocates to mitochondria and oligomerizes Bak or Bax into pores so as to allow the release of cytochrome c and amplify the signals downstream of caspase activation (14,15). Bcl-2 and Bcl-XL perform their antiapoptotic function by inhibiting the release of cytochrome c from the mitochondria either by preventing the translocation or activation the Bax or Bak proteins (15,16).

Abrogation of the release of proapoptotic proteins from the mitochondria often leads to impaired apoptosis with subsequent chemoresistance. Experimental evidence involving various human tumors, animal models and *in vitro* reconstitution assays suggest the essential role of Bax, Bak or both for triggering apoptotic cell death (17–20). In non-apoptotic cells, Bax is a soluble monomeric protein, diffusely distributed in the cytoplasm (21). Diverse apoptotic stimuli induce a conformational change in Bax unmasking specific epitopes needed for membrane insertion, thereby facilitating its translocation to the outer mitochondrial membrane and oligomerization (22,23). Studies using mouse embryonic fibroblasts deficient in both Bax and Bak substantiated the essential role of these proteins in apoptosis (20). Recently, it has been shown that Bax deficiency renders cancer cells resistant to several anticancer drugs acting through the mitochondria or endoplasmic reticulum stress (18,24). A subset of human colon cancers with microsatellite mutator phenotype often shows chemoresistance to conventional therapy subsequent to loss in the function of Bax or Bak (25,26).

A proper understanding of how Bax deficiency results in resistance against conventional drugs could offer promising opportunities for circumventing the chemoresistance with conventional chemotherapeutics. Several studies have shown that curcumin, the yellow pigment isolated from *Curcuma longa*, is a potent inhibitor of the proliferation of several cancer cells (27–29). Recently, we have shown that heat shock provides resistance against curcumin-induced apoptosis of human colon cancer cells suggesting that interventional approaches to modify the expression of antiapoptotic proteins such as the heat shock proteins have the potential to make curcumin-based

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therapy more effective (30). In a subsequent study we have shown that antisense inhibition of hsp 70 restores the sensitivity of human colon cancer cells to curcumin-induced apoptosis (31).

In the present study we have attempted to understand the role of Bax in curcumin-induced apoptosis using isogenic human colon cancer cells that differ only in the presence or absence of Bax gene. Further, we have explored ways for using curcumin as a potential apoptosis-inducing compound for Bax-deficient colon cancers. The results from this study suggest the essential role of Bax in curcumin-induced apoptosis since Bax deficiency almost completely prevented the release of cytochrome c, AIF and Smac with the subsequent inhibition of caspases 3, 9 and 8. The reintroduction of Bax, the overexpression of Smac or AIF, or the downregulation of Bcl-XL are shown as some of the novel strategies to sensitize the human colon cancer cells to curcumin-induced cell death.

Materials and methods

Cell culture and vectors

The HCT116 human colon adenocarcinoma cell line having one intact Bax allele ($Bax^{+/-}$) and its Bax-deficient derivative ($Bax^{-/-}$) generated by gene targeting have been described previously (26) and provided by Dr Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). The cells were maintained on Dulbecco's Modified Eagle Medium (Life Technologies, Inc.) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (Sigma) in an atmosphere of 95% air and 5% CO_2. Bax–GFP expression vector was a gift from Dr Clark W.Distelhorst (Case Western Reserve University Medical School, Cleveland, Ohio). The full-length Smac cloned in pcDNA3 vector was obtained from Dr X.Wang (University of Texas South Western Medical Centre, Howard Hughes Medical Institute, Dallas, TX) and the antisense construct for full-length Bcl-XL cloned in pcDNA3 was obtained from Dr G.Filmus (University of Toronto, Canada). pBS/U6 Bak RNAi was a gift from Dr G.Chinnadurai (St Louis University, Institute for Molecular Virology, MO).

Reagents and antibodies

Curcumin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and DAPI (4,6-diamidino-2-phenylindole) were procured from Sigma (St Louis, MO). Rabbit polyclonal antibodies AIF (sc-5586), poly (ADP) ribose polymerase PARP (sc-7150), Bcl-XL (sc-7195), β-actin (sc-7120), goat polyclonal antibody to Smac (sc-12683) and mouse monoclonal antibody to Bax (sc-7480) were purchased from Santa Cruz Biotechnology and all the secondary antibodies were obtained from Sigma (St Louis, MO). Mouse monoclonal antibody to cytochrome c (clone 6H2.B4) was obtained from Imgenex. Rabbit polyclonal antibodies to caspase 3 (#9662), cleaved caspase 3 (#9661) and cleaved PARP (#9541) and caspase 9 (#9502) and a mouse monoclonal antibody to caspase 8 (#9746) were obtained from Cell Signaling Technology (Beverly, MA). Mouse monoclonal Bak antibody specific for conformationally active Bak was purchased from Oncogene (# AM03).

Transient and stable transfections

All transient transfections were performed with Lipofectamine 2000 as per the manufacturer's instructions (Life Technologies, Inc.) to achieve high transfection efficiency. The transfection reagent was diluted in OptiMeM medium, mixed with DNA and diluted again using the serum-free medium. The complex was incubated with the cell at 60–80% confluency for 12 h after which a completely fresh medium was added. For stable transfection of Bax–GFP Lipofectamine-mediated gene transfer was used as per manufacturer's protocol (Life Technologies, Inc.). After 24 h of transfection the cells were selected with 800 μg/ml of G418 for 45 days and stable clones were maintained in 400 μg/ml of G418 and analyzed for protein expression.

MTT assay

MTT was used to measure the viability (32). $Bax^{+/-}$ and $Bax^{-/-}$ cells seeded at a density of 5×10^3 cells per well in 96-well plates were allowed to grow for 24 h and incubated with or without curcumin (25 μM) for 24, 48 or 72 h. Aliquot of 2 mg/ml MTT in serum-free medium was then added to each well and incubated for 2 h. The formazan crystals formed were dissolved in isopropanol and spectrophotometric absorbance was measured using a 96-well

plate reader (Bio-Rad) at 540 nm and the results were expressed as a percentage over the untreated control.

Western blotting

The cells were scraped, washed three times in PBS, lysed in a buffer [50 mM Tris–Cl (pH 7.4), 1% NP-40, 40 mM NaF, 10 mM NaCl, 10 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mM DTT and 1 μg/ml each of leupeptin and aprotinin], centrifuged and protein concentration was determined by Bradford's method as per standard protocol. The samples were boiled in SDS sample buffer for 7 min and loaded onto SDS–PAGE; the separated proteins were transferred onto nitrocellulose membrane by wet transfer method using Bio-Rad electrotransfer apparatus. After blocking with 10% non-fat milk in TBS containing 0.2% Tween-20, the membrane was incubated with the primary antibody followed by an HRP conjugated secondary antibody and the protein bands were visualized by 3,3′-diaminobenzidine/H_2O_2 substrate mixture (Sigma).

Isolation of cytosolic fraction by digitonin lysis method

The cells (untreated or treated with curcumin) were harvested, washed two times with PBS and the 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 of 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 above using antibodies to AIF, Smac or cytochrome c and appropriate secondary antibodies.

Assessment of chromatin condensation

The cells were grown on 12 mm cover slips and exposed to 25 μM of curcumin in a subconfluent stage for 24 h. The monolayer of cells were 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 of DAPI for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) were scored in percentage from 200–300 cells/sample with at least two investigators using a fluorescent microscope (Nikon TE 300).

Immunofluorescent staining

The cells grown on glass cover slips, after appropriate treatments, were fixed, permeabilized as before and incubated with the respective primary antibody for 2 h at 37°C. After extensive washing with TBS containing 0.2% Tween-20, the cells were incubated with rhodamine conjugated secondary antibody at a dilution of 1:50 for 45 min in the dark. For immunofluorescent staining of Bax protein, the cells were permeabilized with 0.0125% zwitterionic detergent, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, in PBS to avoid artifactual activation of Bax (21). The cover slips were mounted with 50% glycerol–PBS, viewed under Nikon epifluorescent microscope and photographed.

Determination of caspase activities

The subconfluent cells growing on 100-mm dishes treated with or without curcumin (25 μM) for 8, 16 or 24 h were assayed spectrofluorimetrically for the enzymatic activities of caspases 3, 9 and 8. Briefly, the whole cell lysate was incubated with 50 μM of synthetic tetrapeptide substrates linked to a fluophor, 7-amino-4-trifluoromethyl coumarin (AFC), specific for 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 DTT] at 37°C for 1 h. The released AFC was quantitated using a spectrofluorimeter (Perkin Elmer, LS-50B) with the 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 caspases 3, 9 and 8 were detected by western blotting using specific antibodies that detect the intact mother band as well as the corresponding cleaved fragments described earlier.

Results

Expression of Bax in HCT116 $Bax^{+/-}$ and HCT116 $Bax^{-/-}$ cells and changes in cell viability and immunolocalization of Bax induced by curcumin

Multidomain proapoptotic proteins like Bax and Bak play pivotal roles in the release of apoptogenic proteins from the mitochondria into the cytosol in response to apoptotic stimuli. To study the involvement of Bax in curcumin-induced apoptosis, we used HCT116 human colon cancer cells with one

allele of *Bax* gene (Bax$^{+/-}$) and Bax knockout HCT116 (Bax$^{-/-}$) cells in which *Bax* gene is inactivated by homologous recombination (26). Both the cell lines were analyzed for the presence of *Bax* by western blotting of the total cell extracts and the results confirm the presence of *Bax* in Bax$^{+/-}$ cells and its absence in Bax$^{-/-}$ cells whereas β-actin was present in both the cell lines (Figure 1A). To study the relative cytotoxic effects of curcumin, Bax$^{+/-}$ or Bax$^{-/-}$ cells were incubated with or without different concentrations of curcumin for different time intervals and the cytotoxicity assay was done using MTT. In Bax$^{+/-}$ cells treated with 10, 25 or 50 μM curcumin, cell viability over the untreated control was 84, 40 and 18% at 24 h; 74, 24 and 10% at 48 h and 70, 16 and 4% at 72 h, respectively (Figure 1B). However, Bax$^{-/-}$ cells showed marked resistance to curcumin treatment at all the tested concentrations even after 72 h (Figure 1B) and thus the cell viability decreased in a concentration-dependent manner in Bax$^{+/-}$ cells treated with curcumin, whereas only minimal changes in viability were observed in Bax$^{-/-}$ cells upon curcumin treatment. We looked at the migration of native Bax protein using a monoclonal antibody and tracking it with rhodamine conjugated secondary antibody in the presence or absence of curcumin (25 μM) for 24 h. Bax$^{+/-}$ cells showed a uniform diffuse staining throughout the cytoplasm (control) and migrated to the mitochondrial outer membrane (intense granular staining pattern) upon treatment with curcumin whereas the Bax$^{-/-}$ cells showed no specific staining in the presence or absence of curcumin (Figure 1C). These results suggest that Bax is required for curcumin-induced cell death of human colon cancer cells, and curcumin stimulates the migration of Bax from cytoplasm to the outer membrane of mitochondria.

Release of cytochrome c, Smac or AIF from mitochondria assessed by immunofluorescence or western blotting in the presence or absence of curcumin

Early during apoptosis, cytochrome c, Smac and AIF, retained within mitochondria, are released from the intermembrane space into the cytosol. We performed immunofluorescence analysis with specific antibodies for cytochrome c, Smac and AIF in Bax$^{+/-}$ or Bax$^{-/-}$ cells treated with or without curcumin (25 μM) for 24 h. As shown in Figure 2A, cytochrome c, Smac and AIF showed a granular pattern of staining (mitochondrial localization) in untreated Bax$^{+/-}$ or Bax$^{-/-}$ cells and upon curcumin treatment a diffuse cytosolic distribution of cytochrome c, Smac and AIF was observed in Bax$^{+/-}$ but not Bax$^{-/-}$ cells. The patterns of release of cytochrome c, Smac and AIF into the cytosol were characterized further by western blotting of digitonin-permeabilized samples. Figure 2B shows that upon curcumin treatment, all three molecules were released from Bax$^{+/-}$ cells whereas their release was almost completely blocked in Bax$^{-/-}$ cells and β-actin levels of all the fractions were similar. These data indicate that curcumin-induced release of the mitochondrial apoptogenic molecules such as cytochrome c, Smac and AIF requires the presence of Bax.

Effect of curcumin on the activities of caspases 9, 3 and 8 assessed by spectrofluorometry and western blotting and PARP cleavage in Bax$^{+/-}$ or Bax$^{-/-}$ cells

In many systems, caspases 8 and 9 act as initiators and caspase 3, the effector, signals for the final execution of the cells. The processing of procaspases 9, 3 and 8 was assessed using
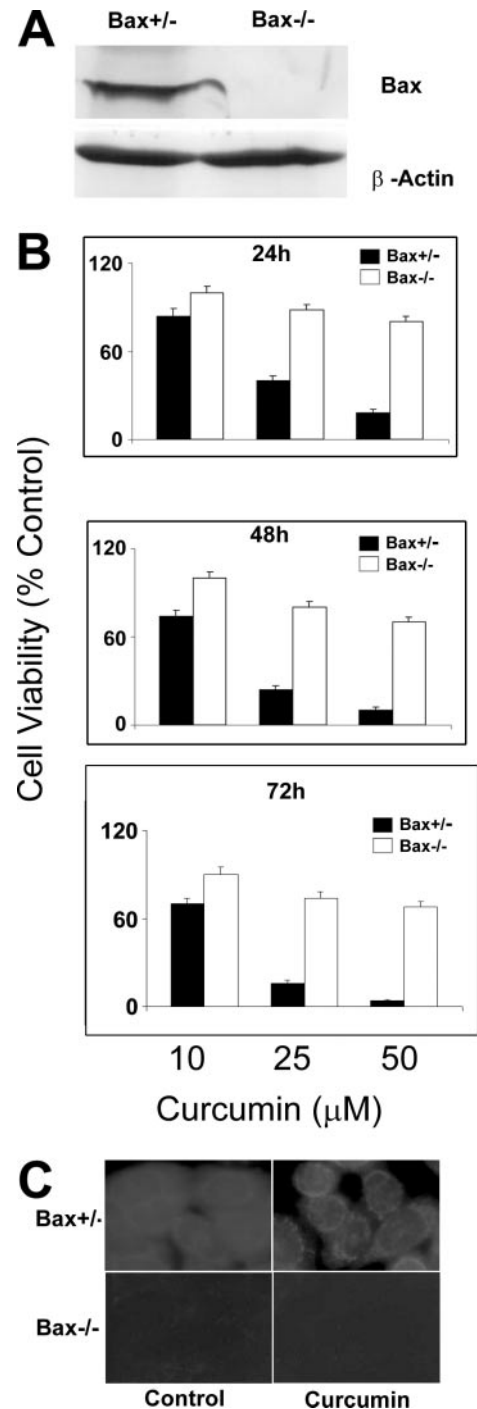
Fig. 1. Bax is required for curcumin-induced cell death. (**A**) Whole cell extracts (50 μg) prepared from HCT116 Bax$^{+/-}$ or HCT116 Bax$^{-/-}$ cells were separated on 12% SDS–PAGE and analyzed for Bax or β-actin (loading control) expression by western blotting. The experiment was repeated many times with similar results. (**B**) Bax$^{+/-}$ or Bax$^{-/-}$ cells grown on 96-well plates were exposed to different concentrations (0, 10, 25 or 50 μM) of curcumin for 24, 48 or 72 h and cell viability (expressed as a percentage over untreated control) was determined by MTT assay. The mean values of triplicate samples are shown and error bars indicate SDs and the experiments were repeated three times with similar results. (**C**) Bax$^{+/-}$ or Bax$^{-/-}$ cells grown on cover slips treated with or without curcumin for 24 h, were fixed with 3.7% paraformaldehyde, permeabilized with 0.0125% of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid in PBS and incubated overnight with a primary antibody to Bax and then incubated again with rhodamine-conjugated secondary antibody and visualized under a fluorescent microscope. The experimental results were confirmed in another independent experiment.
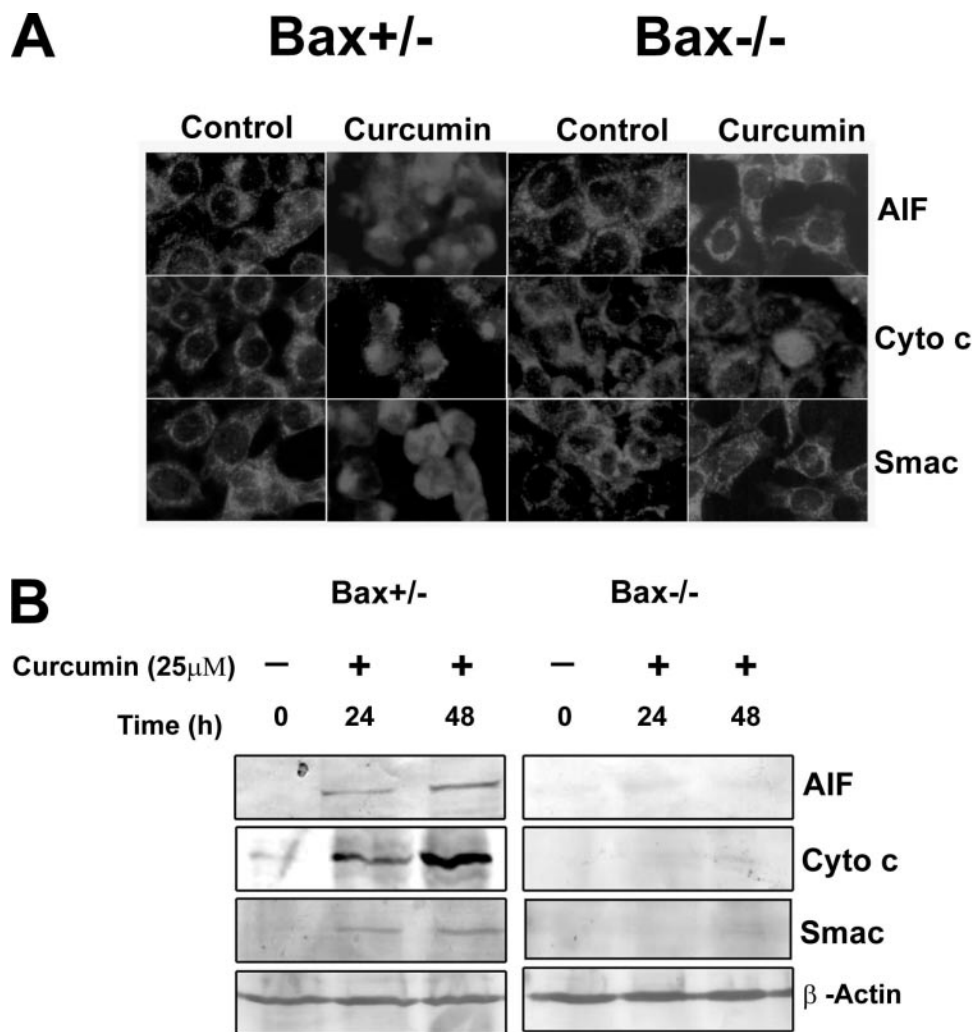
Fig. 2. Curcumin-induced release of AIF, cytochrome c and Smac requires Bax. (**A**) Bax$^{+/-}$ or Bax$^{-/-}$ cells grown on cover slips treated with or without curcumin for 24 h, were fixed with 3.7% paraformaldehyde, permeabilized with 0.2% Triton-X-100 and incubated overnight with a primary antibody to cytochrome c, Smac or AIF followed by rhodamine-conjugated secondary antibody and visualized under a fluorescent microscope. (**B**) For western blot-analysis, untreated or curcumin-treated cells (24 or 48 h) were suspended in digitonin lysis buffer containing protease inhibitors and incubated on ice for 5 min. The releasate was centrifuged and used for western blotting. These results were confirmed in another independent experiment.

specific spectrofluorimetric substrates as well as immunodetection of cleaved fragments of these caspases. It can be seen from Figure 3 that caspase 9 was activated by curcumin in Bax$^{+/-}$ cells in a time-dependent manner, whereas Bax$^{-/-}$ cells exhibited lower level of activity that did not change with increase in time. In western blot analysis, the intensity of the cleaved fragment increased with increase in time of curcumin treatment in Bax$^{+/-}$ cells but no cleavage fragment is visible in the Bax$^{-/-}$ treated cells (Figure 3). The extent of caspase 8 activation by curcumin in Bax$^{-/-}$ cells was low and did not differ from that of Bax$^{+/-}$ cells for all the time durations tested by fluorimetry (Figure 3). The cleaved fragment of procaspase 8 on western blot was also faint in both Bax$^{+/-}$ and Bax$^{-/-}$ cells treated with curcumin for the time periods studied (Figure 3). Curcumin-induced caspase 3 activation increased with time in Bax$^{+/-}$ cells whereas the extent of activation was relatively less in Bax$^{-/-}$ cells for the same time period as analyzed by spectrofluorimetric method (Figure 3). Western blot analysis clearly showed the cleaved fragment of procaspase 3 in Bax$^{+/-}$ but not in Bax$^{-/-}$ cells

treated with curcumin for the same experimental period (Figure 3). Further confirmation of caspase 3 activity was done by the western blot-analysis of PARP, a caspase 3 substrate (116-kDa modular protein) (33). In Bax$^{+/-}$ cells treated with curcumin, PARP cleavage band could be seen clearly whereas the cleavage product was completely absent in Bax$^{-/-}$ cells (Figure 3). The above results show that Bax deficiency blocks curcumin-induced caspase 9 activity and those of caspases 8 and 3 at least in part.

Curcumin-induced changes in chromatin condensation in Bax$^{+/-}$ and Bax$^{-/-}$ cells and the effects of reintroduction of Bax on curcumin-induced cell death

The disruption of nuclear integrity by the cleavage of its structural and functional components with the activation of caspases leads to fragmentation and condensation of the nuclear material, a characteristic feature of apoptosis. This was studied using DAPI staining before and after treating the cells with different concentrations of curcumin for different time periods. The untreated Bax$^{+/-}$ and Bax$^{-/-}$ cells showed
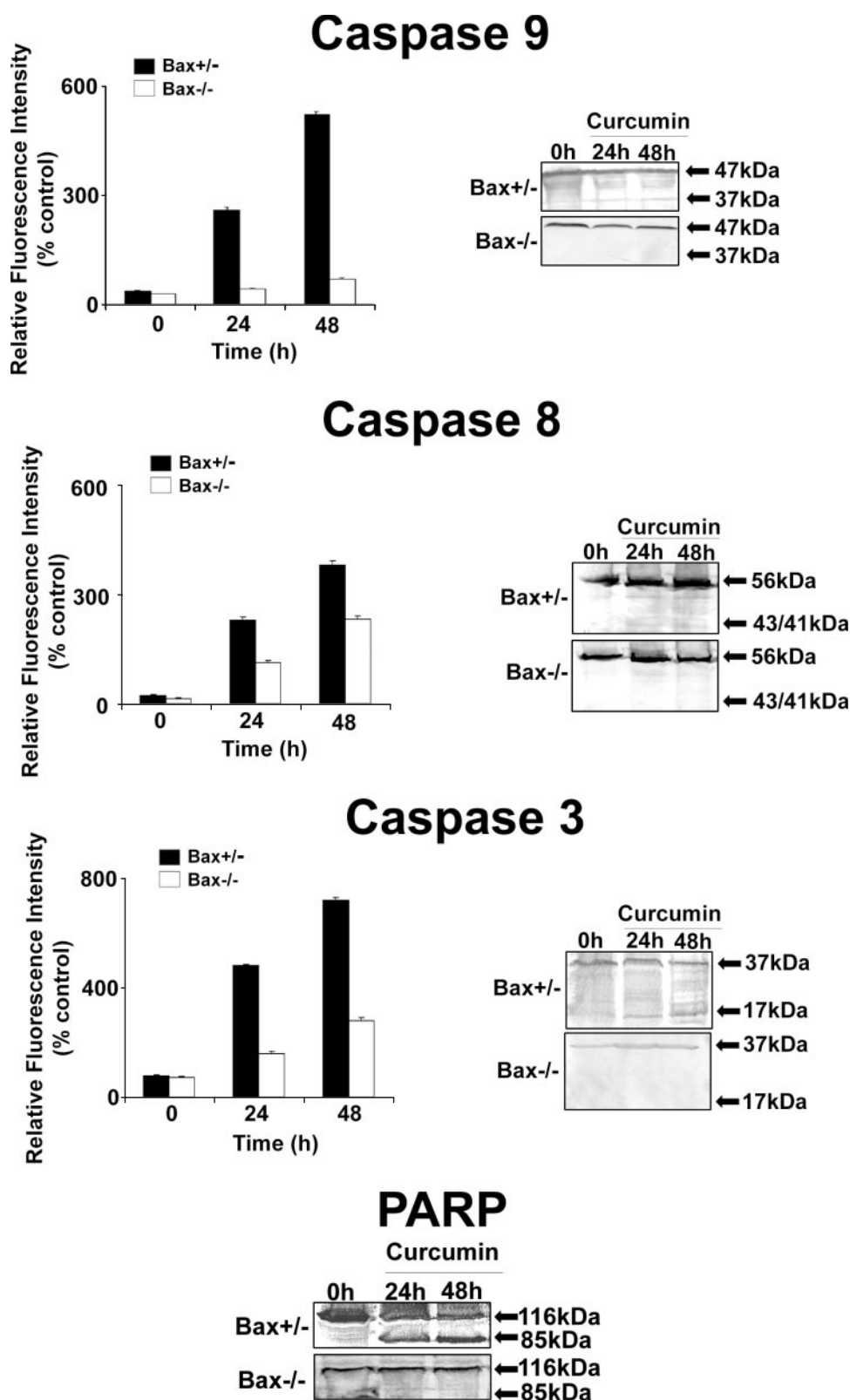
Fig. 3. Bax deficiency blocks curcumin-induced activation of caspases (9, 8 and 3) and PARP cleavage. Whole cell extracts (50 μg) prepared from cells treated with or without curcumin (25 μM) for 0, 24 or 48 h were assessed for the activation of caspase 9 using a fluorimetric substrate (Ac-LEHD-AFC) (**left panel**) in a reaction buffer at 37°C for 1 h. Caspase 9 activation was also assessed by western blotting (**right panel**). 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) (left panel) and by western blot-analysis (right panel). Caspase 8 activation was assessed using a spectrofluorimetric substrate (Z-IETD-AFC) (left panel) and western blot (right panel). All these experiments were repeated at least two times with similar results and the error bars denote standard deviation. Lysates (60 μg of protein) prepared after treating the Bax^{+/−} or Bax^{−/−} cells with or without curcumin (25 μM) for the indicated time periods were analyzed for PARP with a specific polyclonal antibody. The experiment was repeated twice with similar results.

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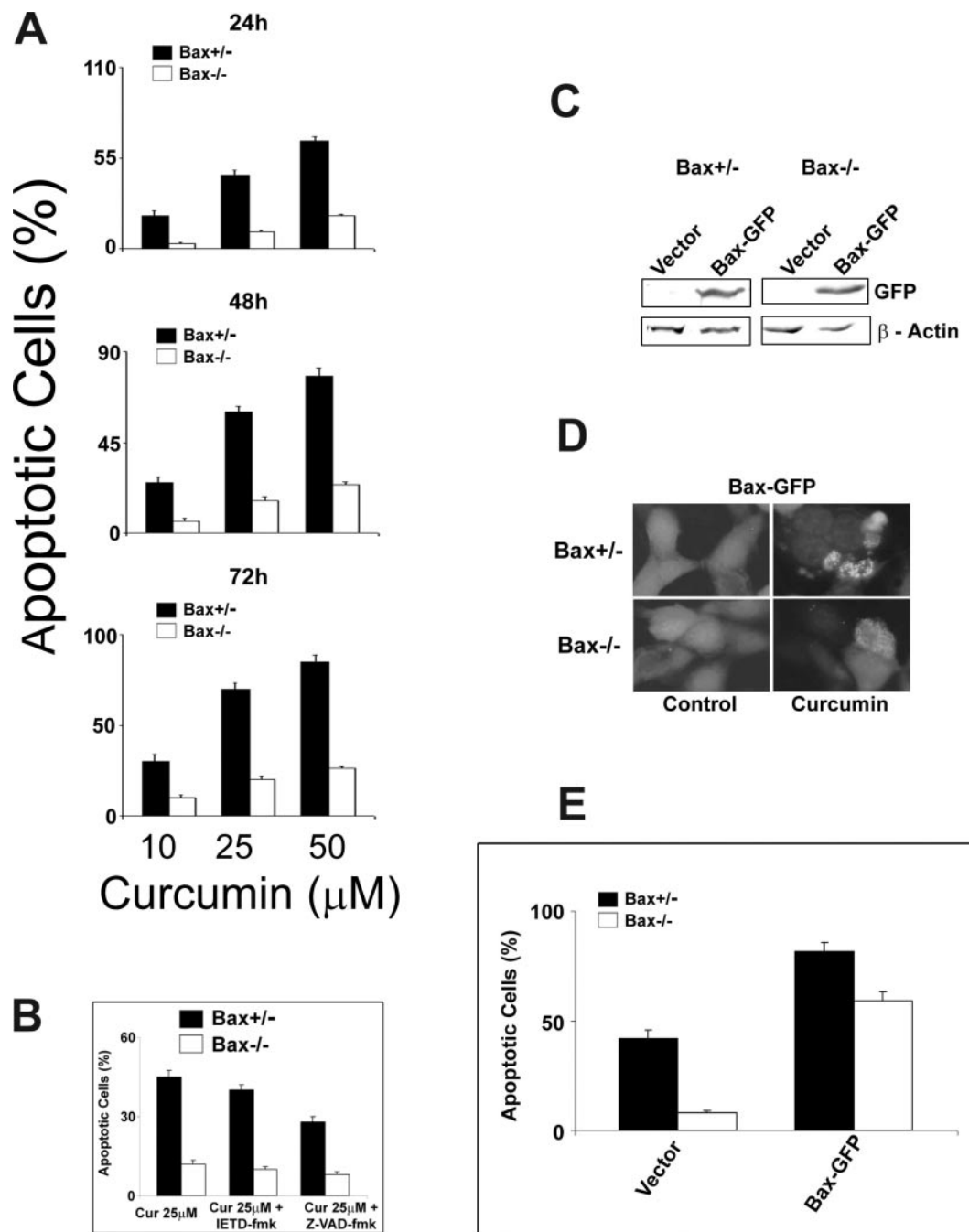
Fig. 4. Curcumin-induced condensation of chromatin and nuclei is prevented in Bax knockout cells and Bax transfection sensitizes them to curcumin-induced apoptosis. (**A**) Cells were seeded on to cover slips, treated with or without curcumin (25 μM) for 24 h, fixed using 3% paraformaldehyde and stained with DAPI. Cells with condensed and fragmented chromatin were counted in five different fields and the mean values of triplicate samples expressed in percentage are shown and these results were confirmed in another independent experiment. (**B**) Bax$^{+/-}$ and Bax$^{-/-}$ cells were pretreated with 50 μM of IETD-fmk or Z-VAD-fmk followed by curcumin for 24 h and then processed as described before for DAPI staining. Cells with condensed and fragmented chromatin were counted in five different fields and the mean values of triplicate samples expressed in percentage are shown and another independent experiment confirmed these results. (**C**) Bax$^{+/-}$ or Bax$^{-/-}$ cells were stably transfected with vector alone or Bax–GFP construct using Lipofectamine as per manufacturer's instructions (Life Technologies, Inc.) and whole cell extracts prepared from G418-resistant clones were analyzed for GFP expression by western blot (12% gel) and β-actin was used as a loading control. The experiment was repeated at least two times with similar results. (**D**) Cells after transfection with control vector or Bax–GFP were treated with or without curcumin and the migration of GFP-tagged Bax protein was monitored under fluorescent microscope and representative micrographs are shown. (**E**) 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 in percentage are shown and another independent experiment confirmed these results.

uniform diffuse staining with DAPI (data not shown) whereas upon 10, 25 and 50 μM curcumin treatment, nuclear condensation and fragmentation of Bax$^{+/-}$ cells were observed in 20, 45 and 65% at 24 h, 25, 60 and 78% at 48 h and 30, 70

and 85% at 72 h, respectively (Figure 4A). Bax$^{-/-}$ cells treated with 10, 25 and 50 μM curcumin were found to exhibit apoptotic nuclei about 3, 7 and 12% at 24 h, 4, 10 and 18% at 48 h and 6, 14 and 10% at 72 h, respectively (Figure 4A). These

results clearly show that Bax is required for curcumin-induced nuclear condensation and fragmentation in human colon cancer cells. To determine whether the rate of cell death in Bax$^{+/-}$ and Bax$^{-/-}$ cells is sensitive to caspase inhibition, we used caspase 8 inhibitor and a pan-caspase inhibitor with curcumin treatment. The cells were then stained with DAPI to observe the difference in the rate of cell death. It was found that curcumin treatment together with IETD-fmk (caspase 8 inhibitor) showed no significant difference in cell death between Bax$^{+/-}$ and Bax$^{-/-}$ cells compared to those treated with curcumin alone. However, in Bax$^{+/-}$ cells treated with Z-VAD-fmk (pan-caspase inhibitor) and curcumin ~28% of cells were apoptotic as compared with 10% in Bax$^{-/-}$ cells (Figure 4B). These results suggest that curcumin-induced apoptosis is mediated at least in part through caspases other than caspase 8.

The above data suggest that the cells deficient in Bax resist curcumin-induced apoptosis and to see how the addition of Bax into Bax$^{+/-}$ or its reintroduction into Bax$^{-/-}$ cells, would affect curcumin-induced translocation of Bax from the cytosol into mitochondria, we have established stable clones expressing Bax–GFP and used one of them for further experiments. Western blotting with an antibody to GFP confirmed the presence of GFP in the stable clone (Bax–GFP) and its absence in the vector-transfected cells, while the β-actin levels were unaltered in both the cells (Figure 4C). Upon curcumin treatment granular mitochondrial GFP staining was observed whereas the untreated cells showed cytosolic pattern of staining indicating the migration of overexpresed Bax–GFP fusion protein to the mitochondria in Bax$^{+/-}$ or Bax$^{-/-}$ cells (Figure 4D). Bax$^{+/-}$ cells (containing one allele of *Bax*) transfected either with the control vector or with Bax–GFP responded to curcumin whereas the Bax$^{-/-}$ cells responded well to curcumin only when transfected with Bax–GFP but not to the control vector (Figure 4E). These experiments confirm the important role of Bax in the induction of apoptosis in human colon cancer cells by curcumin.

Effects of transient transfection of Smac on the changes in morphology, PARP cleavage and caspase 3 activation induced by curcumin

To find out whether proapoptotic factors such as Smac can substitute for Bax in sensitizing the human colon cancer cells to curcumin-induced apoptosis, we transiently transfected both Bax$^{+/-}$ and Bax$^{-/-}$ cells with full-length Smac by Lipofectamine 2000 and exposed them to curcumin (25 μM) for 24 h. Western blot confirmed the overexpression of Smac in cells transfected with Smac compared with those transfected only with the vector (Figure 5A). When Smac was overexpressed, cells were sensitized to curcumin even in the absence of Bax, and 40–50% Bax$^{-/-}$ cells showed condensed chromatin as determined by DAPI staining (Figure 5B), consistent with the transfection efficiency of ~50–70%. Smac significantly enhanced the apoptotic potential of curcumin in Bax$^{+/-}$ cells as >65% cells showed intensely condensed chromatin compared with the corresponding vector transfected cells, which showed 40% (Figure 5B). We also analyzed caspase 3 activation and PARP cleavage using antibodies specific for the products of cleavage by immunofluorescence. All the untreated cells showed negative staining but Smac over-expressing cells showed intense staining for the cleaved products of both caspase 3 and PARP (Figure 5C) substantiating that Smac overexpression can bypass Bax deficiency-mediated resistance against curcumin
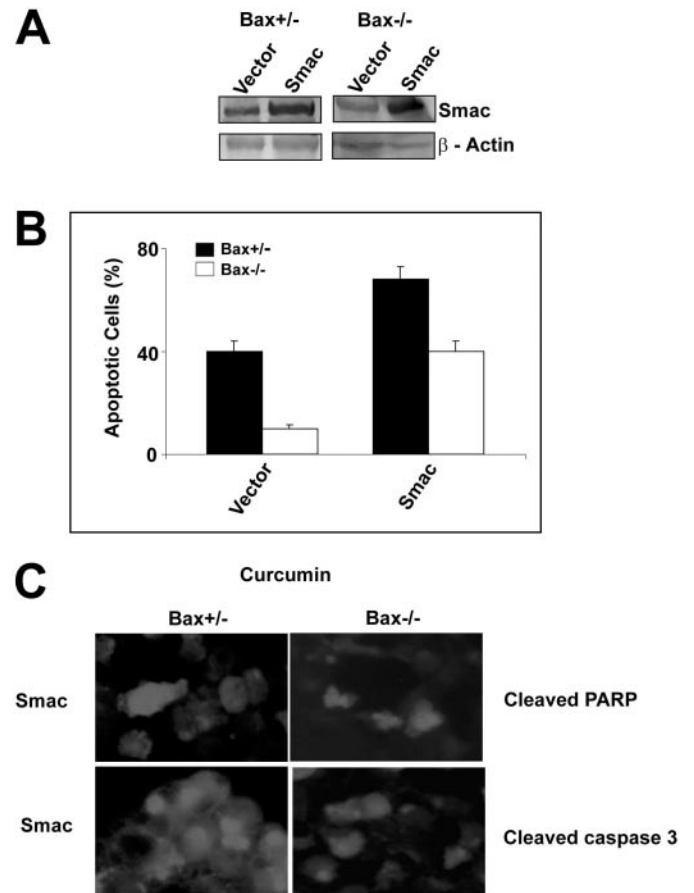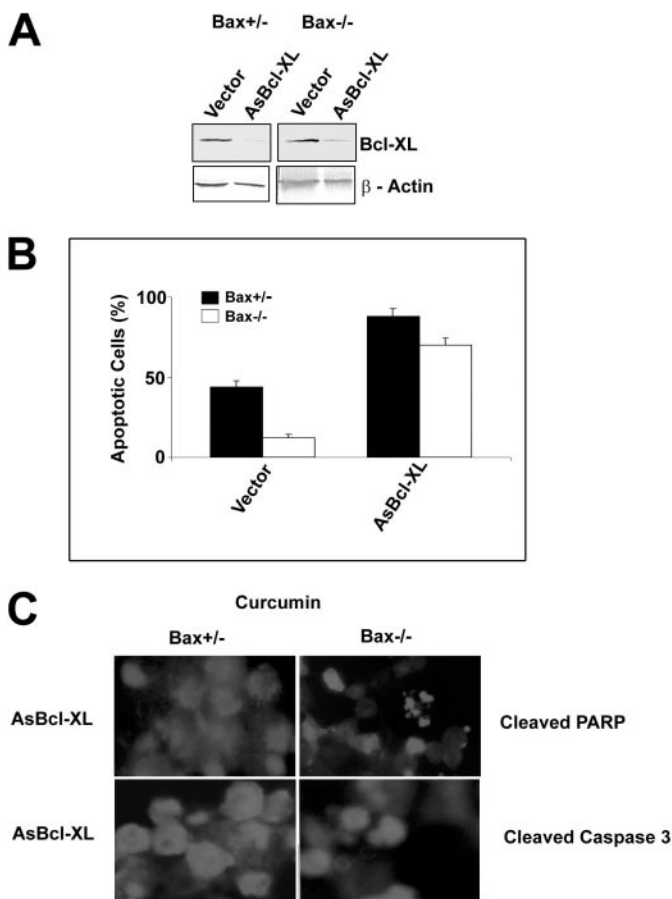

Fig. 5. Transient transfection of Smac reverses the resistance of Bax$^{-/-}$ cells to curcumin. (**A**) Cells were transiently transfected with pcDNA3 Smac or control vector using Lipofectamine 2000 as per the manufacturer's instructions (Life Technologies, Inc.) and whole cell extracts prepared were analyzed for Smac expression by western blot (12% gel) using a polyclonal antibody and β-actin was used as a loading control. The experiment was repeated at least two times with similar results. (**B**) Bax$^{+/-}$ or Bax$^{-/-}$ cells after transfection for 24 h with control vector or Smac were treated with or without curcumin for another 24 h and stained with DAPI as described above. Cells with condensed and fragmented chromatin were counted in five different fields and the mean values of triplicate samples expressed in percentage are shown and these results were confirmed by another independent experiment. (**C**) Bax$^{+/-}$ or Bax$^{-/-}$ cells transfected with control vector or Smac were processed for immunofluorescence as described above before or after treatment with curcumin and incubated with the polyclonal primary antibody for cleaved products of caspase 3 or PARP for 2 h at 37°C and then incubated with rhodamine-conjugated secondary antibody and visualized under a fluorescent microscope. The experiment was repeated three times with similar results.

treatment by accelerating the processing of caspase 3 and its substrates.

Transient transfection of antisense Bcl-XL and its effect on the expression of Bax and curcumin-induced changes in chromatin fragmentation, cleaved PARP and caspase 3

Bcl-2 family protein, Bcl-XL, prevents cell death induced by the overexpression of Bax (19), and curcumin-induced apoptosis is also known to be prevented by Bcl-2/Bcl-XL (34). Hence it was of interest to know whether relieving the inhibitory function of Bcl-XL by antisense Bcl-XL down-regulation could restore the sensitivity of cells to curcumin in the absence of Bax. Transient transfection of an antisense

Fig. 6. Downregulation of Bcl-XL is sufficient to overcome the resistance of Bax$^{-/-}$ cells to curcumin. (A) Cells were transiently transfected with pcDNA3 AsBcl-XL or control vector with Lipofectamine 2000 as per the manufacturer's instructions (Life Technologies, Inc.) and whole cell extracts prepared were analyzed for Bcl-XL expression by western blot (12% gel) using a polyclonal antibody and β-actin was used as a loading control and the experiment repeated at least two times with similar results. (B) Bax$^{+/-}$ or Bax$^{-/-}$ cells (24 h after transfection with pcDNA3 AsBcl-XL or control vector) were treated with or without curcumin for 24 h and stained with DAPI as described above. Cells with condensed and fragmented chromatin from the experiment described were counted in five different fields and the mean values of triplicate samples expressed in percentage are shown and these results were confirmed by another independent experiment. (C) Bax$^{+/-}$ or Bax$^{-/-}$ cells (24 h after transfection with pcDNA3 AsBcl-XL or control vector) were treated with or without curcumin for 24 h, processed as described previously and incubated with a polyclonal primary antibody for cleaved caspase 3 or PARP for 2 h at 37°C and then incubated with rhodamine-conjugated secondary antibody and visualized under fluorescent microscope. The experiment was repeated three times with similar results.

construct of Bcl-XL (pcDNA3 AsBcl-XL) significantly downregulated the level of expression of Bcl-XL in Bax$^{+/-}$ or Bax$^{-/-}$ cells while it had no effect on β-actin levels as confirmed by western blot analysis (Figure 6A). DAPI staining indicated that ~60% of Bax$^{-/-}$ cells showed condensed nuclear morphology when Bcl-XL is downregulated compared with 12% of vector-transfected cells, and 85% of antisense Bcl-XL transfected Bax$^{+/-}$ cells were highly apoptotic compared with 45% in the corresponding vector-transfected cells (Figure 6B). Immunofluorescent detection of active caspase 3 and cleaved PARP also supported the finding that antisense inhibition of Bcl-XL restored the responsiveness to curcumin in Bax$^{-/-}$ cells while enhancing
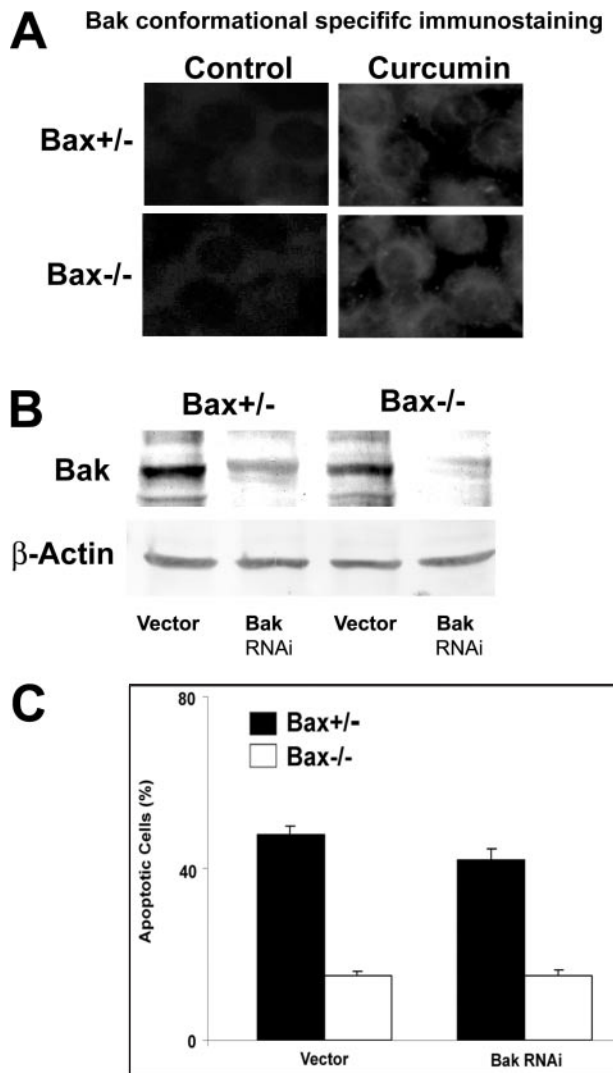
Fig. 7. Bax is more important than Bak in curcumin-induced apoptosis. (A) Bax$^{+/-}$ or Bax$^{-/-}$ cells were treated with or without curcumin for 24 h, processed for activated Bak as previously described. The cells were incubated with a monoclonal primary antibody for conformational specific Bak for 2 h at 37°C and then incubated with rhodamine-conjugated secondary antibody and visualized under fluorescent microscope. The experiment was repeated three times with similar results. (B) Cells were transiently transfected with pBS/U6 Bak RNAi or control vector with Lipofectamine 2000 as per the manufacturer's instructions (Life Technologies, Inc.) and whole cell extracts prepared were analyzed for Bcl-XL expression by western blot (15% gel) using a monoclonal antibody and β-actin was used as a loading control and the experiment repeated at least two times with similar results. (C) Bax$^{+/-}$ or Bax$^{-/-}$ cells (24 h after transfection with pBS/U6 Bak RNAi or control vector) were treated with or without curcumin for 24 h, processed for DAPI as described earlier. Apoptotic cells with condensed and fragmented chromatin were counted in five different fields and the mean values of triplicate samples expressed in percentage are shown and these results were confirmed by another independent experiment.

it in Bax$^{+/-}$ cells (Figure 6C). These results suggest that downregulation of Bcl-XL is an effective option to sensitize human colon cancer cells deficient in Bax.

Effect of Bak RNAi on curcumin-induced apoptosis of Bax$^{+/-}$ and Bax$^{-/-}$ cells

To understand the role of Bak in curcumin-induced apoptosis, Bax$^{+/-}$ or Bax$^{-/-}$ cells were treated with or without curcumin

for 24 h and immunostained with conformational specific antibody to detect activated Bak. Both Bax$^{+/-}$ and Bax$^{-/-}$ cells were positive for activated Bak upon curcumin treatment but not the untreated control cells (Figure 7A). To understand the relative importance of Bax and Bak in curcumin-induced apoptosis Bax$^{+/-}$ or Bax$^{-/-}$ cells were transiently transfected with Bak RNAi vector by Lipofectamine 2000 to downregulate Bak. Furthermore, western blotting confirmed the downregulation of Bak in RNAi transfected cells (Figure 7B). After 24 h of transfection with Bak RNAi vector, the cells were treated with curcumin (25 μM) for 24 h, and there was no considerable difference in the percentage of apoptotic cells in Bak RNAi transfected Bax$^{+/-}$ or Bax$^{-/-}$ cells compared with their corresponding vector-transfected cells treated with curcumin (Figure 7C). In Bcl-XL downregulated or Smac overexpressed Bax$^{+/-}$ and Bax$^{-/-}$ cells, there was no difference in the staining for activated Bak (data not shown).

The above results when considered together, demonstrate the requirement of Bax but not Bak in curcumin-induced apoptosis, but this requirement is dispensable if the cells can express additional amounts of alternative proapoptotic molecules such as Smac or downregulate antiapoptotic molecules like Bcl-XL under Bax-deficient conditions.

Discussion

Deficiency of Bax is a frequent cause for resistance against therapy in at least 15% of human colon, gastric and endometrial cancers (35–37). In this study we demonstrate for the first time that Bax is required for curcumin-induced apoptosis, particularly for the release of apoptogenic molecules, cytochrome c, Smac and AIF from the mitochondria. However, the requirement for Bax can be bypassed with ectopic expression of Smac or downregulation of Bcl-XL to sensitize the human colon adenocarcinoma cells to curcumin-induced apoptosis. The Bax-deficient derivative of HCT116 human colon cancer cells used in our study is partially resistant to the apoptotic effects of the chemotherapeutic agent 5-fluorouracil and totally resistant to the chemopreventive agent sulindac and other non-steroidal antiinflammatory drugs (26). In addition, this cell line is resistant to apoptosis induced by a variety of agents including UV, bortezomib, staurosporine, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and thapsigargin (18,24,38,39). Proapoptotic drugs such as staurosporine and actinomycin D require the presence of Bax to induce cytochrome c release and cell death (40). Curcumin acts through both mitochondrial- and receptor-mediated apoptotic pathways (34) and we have shown earlier that it induces the release of cytochrome c, Smac and AIF in human colon cancer cells (30,31). Partial but not complete activation of caspase 8 by curcumin observed in Bax$^{-/-}$ cells is consistent with the notion that although caspase 8 activation is upstream of Bax the complete activation of caspase 8 requires the interaction of Bax with Bid, a known substrate of caspase 8 (14,15).

Curcumin-induced release of Smac into the cytosol from Bax$^{+/-}$ and Bax$^{-/-}$ cells transfected with Smac is likely to have contributed to the sequestering of IAPs and the consequent activation of caspases 9 and 3, and PARP cleavage (41). Under the conditions of Smac transfection, sufficient amount of Smac may be available in the cytosol to inhibit the IAPs thereby enhancing caspase activity which in turn acts on the mitochondria to amplify the release of cytochrome

c and such a feedback amplification loop has been reported earlier (42). Smac enhances the sensitivity of Jurkat cells to TRAIL and Epothilone B derivative-induced apoptosis (43) and stable expression of Smac in neuroblastoma cells sensitized them to apoptosis induced by TRAIL, cisplatin, doxorubicin and etoposide by the activation of caspases and cleavage of caspase substrates (44). Overexpression of Smac restores the apoptotic sensitivity of Bax knockout cells to thapsigargin-induced apoptosis (24). Infection of ovarian carcinoma cells with a recombinant adenovirus encoding Smac leads to cell death at multiplicities of infection in a concentration-dependent manner independent of cytochrome c release (45).

Bcl-XL and Bak predominantly reside on the mitochondrial outer membrane surface (46,47) and accumulation of Bax multimers in mitochondria is essential to neutralize the function of Bcl-XL and to activate Bak-induced release of cytochrome c (19). From the present study it is apparent that Bak conformational specific change in response to curcumin treatment is independent of Bax, Smac overexpression or Bcl-XL downregulation. The active Bak alone cannot induce cytochrome c release or other downstream events leading to cell death in the absence of Bax. Our findings suggest that Bak conformational change may be an early step in curcumin-induced apoptosis. In the absence of Bax the effect of Bak is neutralized by Bcl-XL thus preventing Bak-mediated cytochrome c release. It has been reported that the truncated Bid can serve as a death ligand which moves from the cytosol to the mitochondria to activate the Bak- or Bax-induced cytochrome c release (40,48). Once Bcl-XL is downregulated, Bid can probably induce the release of cytochrome c by oligomerizing Bak, independent of Bax. Even in the absence of Bax, under the condition of downregulated levels of Bcl-XL, curcumin may have induced conformational changes in Bak thereby inducing the release of cytochrome c and cell death. It is possible that mitochondrial Bak is always kept in an inactive conformation by its close association with the antiapoptotic protein Bcl-XL which is relatively more abundant than Bcl-2 in Bax$^{-/-}$ cells (R.Rashmi, S.Kumar and D.Karunagaran, unpublished data). Mouse embryonic fibroblasts defective in both Bax and Bak were resistant to apoptosis induced by overexpression of various BH3 proteins such as Bid, Bim and Noxa (20). However, cells deficient in either Bax or Bak were not significantly defective when apoptosis was induced by various agents, suggesting the importance of relative expression of proapoptotic and antiapoptotic proteins in chemoresistance. Presumably the proteins of Bcl-2 family interact with each other to set a survival threshold for the cell.

Although many studies suggest that AIF is caspase-independent (12,49) some reports suggest that caspase inhibitors prevent the effects of AIF favoring the idea that at least, part of its action is caspase-dependent (50,51). Further studies are needed to clarify the mechanisms by which AIF is released from mitochondria in response to curcumin. The present study has highlighted Bax and not Bak as a critical regulator of curcumin-induced apoptosis and the results implicate Bax reintroduction, Smac transfection and Bcl-XL downregulation as novel and potential strategies to sensitize Bax-deficient tumors resistant to curcumin therapy. Our findings support the notion that the relative ratio of proapoptotic and antiapoptotic proteins in a cell determines their sensitivity to drug-induced apoptosis highlighting the contribution of

relative expression of proapoptotic and antiapoptotic proteins to chemoresistance.

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