

Ectopic expression of Bcl-XL or Ku70 protects human colon cancer cells (SW480) against curcumin-induced apoptosis while their down-regulation potentiates it

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Curcumin, the yellow pigment derived from *Curcuma longa*, is known to induce apoptosis of several cancer cells. However, many cancer cells protect themselves by over-expressing antiapoptotic proteins such as Bcl-XL or Ku70. To study their role in curcumin-induced apoptosis, human colon cancer cells (SW480) were made to over-express or under-express Bcl-XL (by stable transfection) and Ku70 (by transient transfection) using plasmid constructs that express their genes in sense or antisense orientation, respectively. Stable cells that express Bax [Bax-GFP (green fluorescent protein)], a proapoptotic member of the Bcl-2 family, were also established. Curcumin-induced cell death and nuclear condensation was more in AsBcl-XL and AsKu70 cells that under-express Bcl-XL and Ku70, respectively, compared with the vector-transfected cells. Bcl-XL and Ku70 protected the cells by inhibiting the release of cytochrome c, Smac (second mitochondria derived activator of caspase) and apoptosis inducing factor (AIF), and the activation of caspases 9, 8 and 3 triggered by curcumin. AsBcl-XL and AsKu70 cells were more sensitive to curcumin through enhanced activation of caspases 9 and 3 and release of cytochrome c, Smac and AIF. Curcumin-induced activation of caspase 8 was blocked by Ku70 but not by Bcl-XL. However, caspase 8 activation by curcumin was accelerated in both AsBcl-XL and AsKu70 cells suggesting a possible feedback activation of caspase 8 by caspase 3. Bax-GFP cells were highly sensitized when Ku70 was down-regulated supporting the reported role of Ku70 in the retention of Bax within the cytosol. The study reveals the potential of antisense inhibition of antiapoptotic proteins as an effective strategy to tackle chemoresistant cancers with curcumin.

Introduction

Curcumin, the yellow pigment derived from *Curcuma longa*, is a pharmacologically safe compound that possesses antioxidant, anti-inflammatory and anticancer activities (1–3). Curcumin inhibits cell proliferation and induces apoptosis of several cancer cells (3–5). Curcumin-mediated inhibition of

Abbreviations: AFC, 7-amino-4-trifluoromethyl coumarin; AIF, apoptosis inducing factor; $\Delta\Psi_m$, mitochondrial membrane potential; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent 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.

cell proliferation, however, is not always associated with apoptosis (6,7) and differential over-expression of certain antiapoptotic proteins may account for the failure of curcumin to induce apoptosis. It is documented that over-expression of antiapoptotic members of the Bcl-2 (8,9), inhibitor of apoptosis protein (10) or heat shock protein families (hsp 70, hsp 27 and hsp 90) leads to the development of therapy-resistant tumors (11,12). Cancer cells of different histological origin are said to be protected by at least one of the two antiapoptotic proteins, Bcl-2 or Bcl-XL (13). Bcl-XL expression in breast and colorectal carcinomas is associated with advanced stages (14,15). Bcl-2 and Bcl-XL proteins mediate Ras-induced resistance to anoikis in the intestinal epithelial cells (16,17). Antisense oligonucleotides complementary to either Bcl-2 or Bcl-XL mRNA are known to act against tumor cells either singly or in combination with chemotherapeutic drugs (18,19). Bax, a proapoptotic protein of the Bcl-2 family, is translocated into mitochondria during apoptosis promoting the release of cytochrome c and other apoptogenic proteins. Recently it has been shown that Ku 70, a subunit of Ku protein complex involved in DNA repair, plays a major role in keeping the Bax protein in an inactive conformation during apoptosis (20). High expression of Ku70 in cancer cells suggests a possible role for this protein in cancer progression and chemoresistance (21,22). It is hypothesized that silencing of such antiapoptotic proteins may be an attractive strategy for sensitizing cancer cells to curcumin-induced apoptosis.

Our laboratory has demonstrated the antiapoptotic effects of NF- κ B against curcumin-induced apoptosis (23) and we have also shown that heat shock can inhibit curcumin-induced apoptosis in human colon cancer cells and suggested that silencing the expression of hsp 70 is an effective approach to augment curcumin-based therapy (24,25). Herein, we show that ectopic expression of Bcl-XL or Ku70 in human colon cancer cells (SW480) blocks the release of cytochrome c, apoptosis inducing factor (AIF) and second mitochondria derived activator of caspase (Smac) from mitochondria and the activation of caspases 9 and 3 induced by curcumin. Antisense inhibition of Bcl-XL or Ku70 sensitizes the human colon cancer cells by enhancing these apoptotic events.

Materials and methods

Cell culture

Human colon cancer cell line SW480 (provided by Ajit Kumar, Indian Institute of Science, Bangalore, India) was maintained on DMEM (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma) in an atmosphere of 95% air and 5% CO₂.

Reagents and antibodies

Curcumin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], DAPI (4,6-diamidino-2-phenylindole) and rhodamine-conjugated anti-rabbit and anti-goat secondary antibodies were procured from Sigma. A goat polyclonal antibody to Smac (sc-12683) and rabbit polyclonal antibodies to cytochrome c (sc-7159), AIF (sc-5586), poly (ADP) ribose polymerase (PARP) (sc-7150), Bcl-XL (sc-7195), Bax (sc-7480) and β -actin (sc-7210)

were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to caspase 3 (# 9662) and caspase 9 (# 9502), and a mouse monoclonal antibody to caspase 8 (# 9746) were obtained from Cell Signaling Technology (Beverly, MA). A mouse monoclonal antibody to Ku70 (no. 61193) was obtained from BD Pharmingen.

Stable transfection

SW480 cells grown on 60 mm dishes were transfected with 8 μ g of pEmd-C1 vector containing the cDNA for full-length human Bcl-XL or Bax-GFP (green fluorescent protein) (gifted by Dr C.Diestelhorst, Case Western Reserve University Medical School, Cleveland, OH) or pcDNA3 Bcl-XLAS, an expression construct for Bcl-XL in antisense direction (a kind gift from Dr George Filmus, University of Toronto, Canada), by Lipofectamine 2000 method (Life Technologies) as per manufacturer's instructions. The transfected cells were selected by growing the cells in 1 mg/ml of G418 (Life Technologies) in DMEM for 2 months. The resistant clones formed were picked up and maintained separately with 100 μ g/ml G418 and analyzed for protein expression. For transient transfection of Ku70 or AsKu70, cells were transfected with pCMV-2B Ku70 or pcDNA3 Ku70AS (from Dr Shigemi Matsuyama, Blood Research Institute, Milwaukee), respectively, using Lipofectamine 2000.

MTT assay

Bcl-XL, AsBcl-XL or neo cells were seeded at a density of 5×10^3 cells/well in 96-well plates and allowed to grow for 24 h. Subsequently, the cells were incubated with curcumin (25 μ M) for 16, 24 or 48 h and cell viability was determined by MTT assay and expressed as percentage over untreated control (26). For some experiments involving transient transfection, the cells were seeded in to 96 wells and after 24 h curcumin treatment was given before determining the cell viability by MTT assay.

Western blotting

Cells were harvested, washed thrice in phosphate-buffered saline (PBS) and lysed in RIPA lysis 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, and 10 mM dithiothreitol and 1 μ g/ml each of leupeptin and aprotinin]. The cell lysates (50 μ g of protein) were resolved by SDS-PAGE and the separated proteins were transferred to nitrocellulose membrane by wet transfer method using Bio-Rad electro-transfer apparatus. After blocking with 10% non-fat milk in Tris-buffered saline containing 0.2% Tween 20, the membrane was incubated with the primary antibody followed by horseradish peroxidase conjugated secondary antibody. Proteins were visualized by (3,3'-diamino benzidine) ($\text{DAB}/\text{H}_2\text{O}_2$) (Sigma). For analyzing the released proteins from mitochondria, the cells (untreated or after treatment) were harvested, washed twice with PBS and the cell pellet was re-suspended in digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4 , 8 mM Na_2HPO_4 , 250 mM sucrose, 190 μ g/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 with appropriate secondary antibodies.

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 lipophilic dye (ApoAlert mitochondrial membrane sensor kit, Clonetech) as per the manufacturer's protocol and analyzed by fluorescence microscopy using a band-pass filter. The cells with intact $\Delta\Psi_m$ stained red while green fluorescence indicated the loss of $\Delta\Psi_m$.

Dapi staining

The cells grown on 12 mm coverslips were incubated with or without 25 μ M of curcumin for 24 h, 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 μ g/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 coverslips, after appropriate treatments for different time periods, were fixed and permeabilized as above and then incubated with the respective primary antibody for overnight. After extensive washing with Tris-buffered saline containing 0.2% Tween 20, the cells were incubated with rhodamine-conjugated secondary antibody at 1:200 dilution for 45 min in the dark. The coverslips were mounted with 50% glycerol-PBS, and viewed under fluorescent microscope and photographed. The cells with perinuclear punctate mitochondrial staining have been scored as intact mitochondria and

those with diffuse cytoplasmic staining have been scored as mitochondria, which released proteins. The percentage of cells with appropriate staining was calculated from the total number of cells from four different fields by at least two investigators.

Determination of caspase activities

The subconfluent cells growing on 100-mm dishes treated with or without curcumin (25 μ M) for 8, 16 or 24 h were assayed spectrofluorimetrically for the enzymatic activities of caspases 3, 9 and 8 (27). Briefly, the whole cell lysate was incubated with 50 μ M of fluorimetric substrates of caspase 3 (Ac-DEVD-AFC), caspase 9 (Ac-LEHD-AFC) or caspase 8 (Z-IETD-AFC) in a total volume of 500 μ l of reaction buffer [50 mM HEPES-KOH, pH 7.0, 10% glycerol, 0.1% 3-(cholamidopropyl)-dimethylammonio-1-propane sulfonate, 2 mM EDTA, 2 mM dithiothreitol] at 37°C for 1 h. The released 7-amino-4-trifluoromethyl coumarin (AFC) was quantified 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. In addition, western blotting was also used to assess the activation of caspases 3, 9 and 8 in cells treated with or without curcumin by detecting the intact mother band as well as the corresponding cleaved fragments.

Results

Curcumin does not alter the expression levels of antiapoptotic proteins, Bcl-2, Bcl XL and Ku70 but induces the expression of proapoptotic protein, Bax, in SW480 cells

We first checked whether curcumin treatment alters the expression of antiapoptotic proteins, Bcl-XL, Bcl-2 and Ku70, and the proapoptotic protein, Bax, by western blotting in SW480 cells before or after 25 μ M curcumin treatment for 8, 16 or 24 h. As shown in Figure 1, there was no marked difference in the expression of Bcl-XL, Bcl-2 or Ku70 proteins after treatment with curcumin but there was a slight up-regulation of Bax after curcumin treatment for 8 h and there

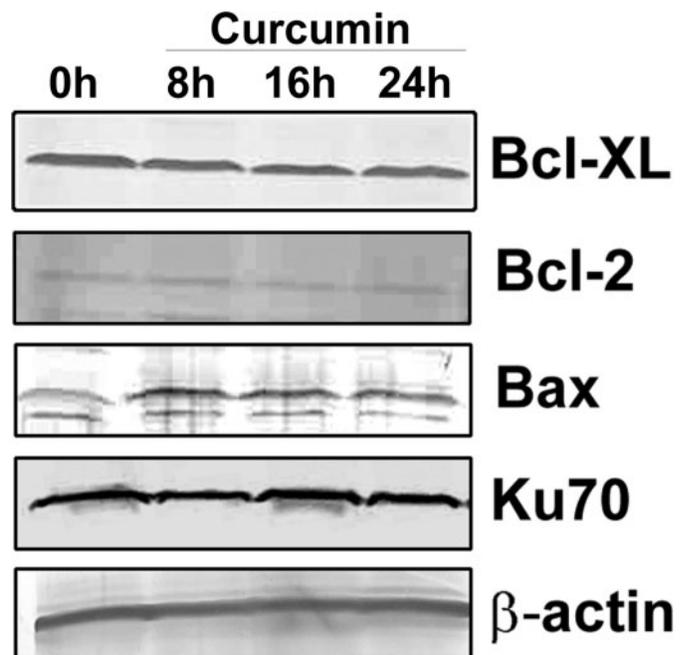


Fig. 1. Relative expression of Bcl-2, Bcl-XL, Bax and Ku70 in SW480 cells upon curcumin treatment. SW480 cells were exposed to curcumin (25 μ M) for 8, 16 or 24 h or left untreated and whole cell extracts were analyzed for the expression of Bcl-2, Bcl-XL, Bax, Ku70 or β -actin using appropriate antibodies and western blotting was carried out as described in the Materials and methods and the results were confirmed in another independent experiment.

was no further increase of Bax expression up to 24 h. The results show that curcumin by itself does not bring about marked alterations in the basal expression levels of Bcl-XL, Bcl-2 and Ku70 although it induces the expression of Bax initially.

Stable clones of SW480 cells ectopically express Bcl-XL or Bcl-XLAS

Antiapoptotic proteins of the Bcl-2 family such as Bcl-XL are over-expressed in certain solid tumors and consequently the traditional chemotherapy often fails (13,14). Blocking the expression of an antiapoptotic protein may be ideal for bypassing chemoresistance and sensitizing such tumor cells. We transfected SW480 human colon cancer cells with N-terminally GFP-tagged pEmd-C1 Bcl-XL (in sense orientation) or pcDNA3 Bcl-XLAS (in antisense orientation) plasmid constructs to develop stable cell lines that over-express and under-express Bcl-XL, respectively. Transfected clones were selected with G418 (1 mg/ml) and western blot analysis was done for the expression of Bcl-XL using a polyclonal antibody to Bcl-XL. The vectors, pEmd-C1 and pcDNA3, contain neomycin-resistant genes and in comparison with pcDNA3 vector-transfected cells (neo) all the Bcl-XL-transfected clones (Bcl-XL) expressed higher levels of Bcl-XL (Figure 2A) and all the three Bcl-XLAS-transfected clones (AsBcl-XL) showed lower levels of Bcl-XL (Figure 2B). Clone 1 of Bcl-XL cells with 6-fold higher levels of Bcl-XL (Figure 2A), and clone 3 of AsBcl-XL cells showing very low expression of Bcl-XL (Figure 2B) were used for subsequent experiments. β -Actin was used as loading control for western blot analyses.

Bcl-XL cells resist curcumin-induced cell death whereas AsBcl-XL cells succumb to it

The cytotoxicity assay was done by MTT, after neo, Bcl-XL or AsBcl-XL cells were incubated with or without 25 μ M curcumin for 16, 24 or 48 h. For neo cells, the cell viability was 66% at 16 h, 53% at 24 h and 35% at 48 h, whereas 85% at 16 h, 75% at 24 h and 68% at 48 h of Bcl-XL cells survived with significant changes in viability at all these time-points compared with neo cells ($P < 0.0001$), but for AsBcl-XL cells the viability was 40% ($P < 0.0001$) at 16 h, 30% ($P < 0.001$) at 24 h and 20% ($P < 0.001$) at 48 h (Figure 2C). These results indicate that curcumin induces cell death progressively in neo cells with an increase in time whereas Bcl-XL reduces the cytotoxicity in all the time periods studied and quite remarkably antisense inhibition of Bcl-XL sensitizes SW480 cells to curcumin-induced cytotoxicity.

Transient transfection of Ku70 inhibits cell death induced by curcumin and its down regulation sensitizes both SW480 and Bax-GFP cells to curcumin

Many anticancer drugs induce a conformational change in the proapoptotic protein, Bax, followed by its insertion and oligomerization at mitochondrial membrane, an essential event required for the release of cytochrome c and other apoptogenic molecules (28). To understand the roles of Bax and Ku70 (a Bax-interacting protein suppressing its action) in curcumin-induced apoptosis we established a stable clone of SW480 cells expressing Bax (Bax-GFP) or the empty vector (neo) and then transiently transfected SW480 or Bax-GFP cells with Ku70 in sense (Ku70) or antisense (AsKu70) orientation and compared the sensitivity of these cells to curcumin treatment. Bax-GFP cells showed the expression of the fusion

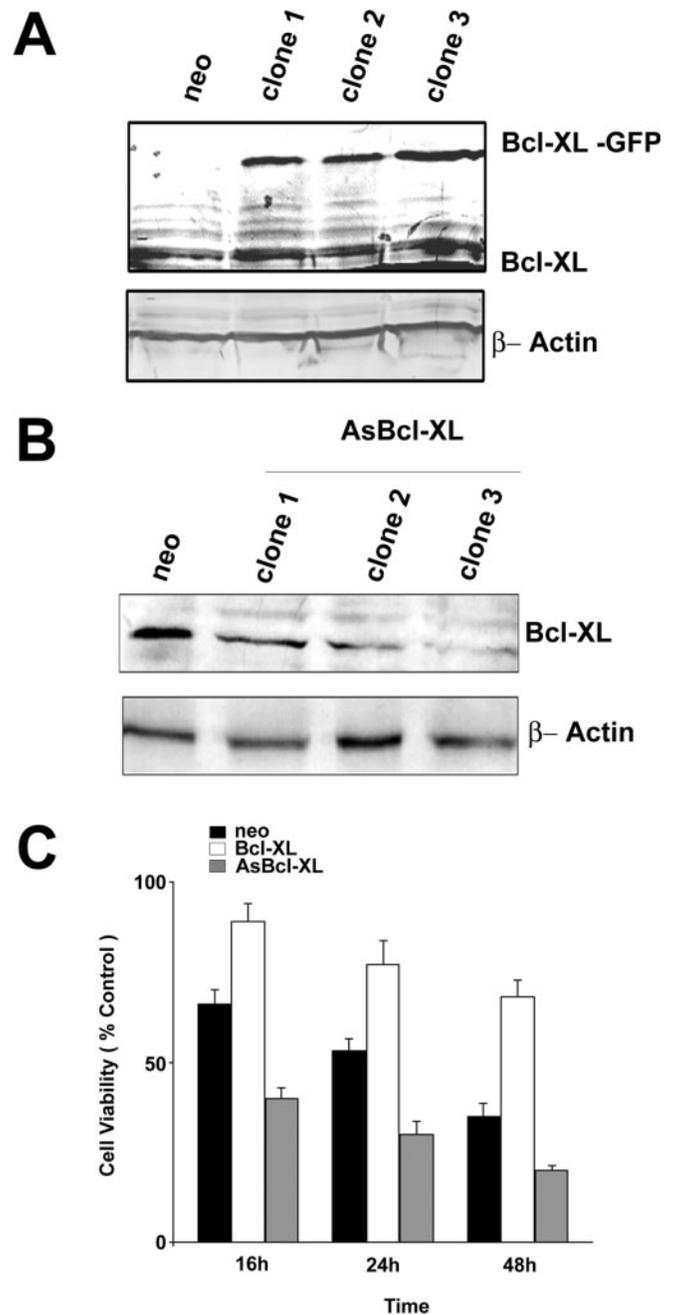


Fig. 2. Changes in Bcl-XL expression and viability in neo, Bcl-XL or AsBcl-XL cells. (A) Stable clones of SW480 cells expressing pcDNA3 vector (neo) or Bcl-XL-GFP (clones 1, 2 and 3) were established using Lipofectamine 2000 as per the manufacturer's instructions (Life Technologies) and cell lysates were analyzed by western blot analysis (12% gel) using a specific rabbit polyclonal antibody against Bcl-XL or β -actin (loading control) as described in the Materials and methods. (B) Neo or clones of AsBcl-XL were analyzed for Bcl-XL or β -actin expression by western blot as described above. (C) Neo, Bcl-XL or AsBcl-XL cells grown on 96-well plates were treated with or without curcumin (25 μ M) for 16, 24 or 48 h and the cell viability (expressed as percentage over untreated control) was determined by MTT assay as described in the Materials and methods. The values represent means of triplicate samples \pm SD and the above experiments were repeated twice with similar results.

protein (54-kDa) and the native Bax protein while the expression of Bax alone was observed in the neo cells (Figure 3A). Figure 3B shows that the expression level of Ku70 was significantly reduced in AsKu70 transfected cells whereas Ku70 transfected cells showed a 3-fold increase in

Ku70 expression compared with the neo cells whereas the actin levels remained unchanged in these cells. Nearly 48% of neo and 68% ($P < 0.003$) of Ku70 cells were viable after 8 h of curcumin treatment and only 28% ($P < 0.0001$) of AsKu70 cells were viable and similarly in Bax-GFP cells the viability was 40% ($P < 0.002$) while it was only 18% ($P < 0.0001$) in AsKu70 cells (Figure 3C). These results suggest that while Bax expression is not very effective in potentiating curcumin-induced cell death even the basal level of Ku70 present in SW480 cell is effective in preventing curcumin induced cell death and thus silencing its expression is an effective approach to kill these cells by curcumin.

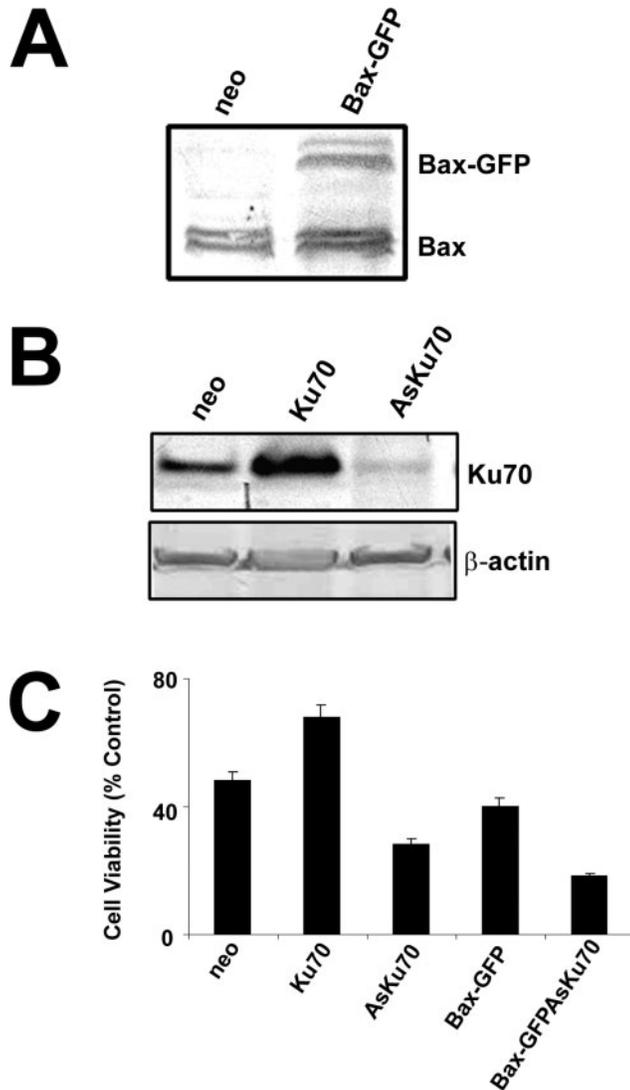


Fig. 3. Relative expression of Bax in neo and Bax-GFP cells and that of Ku70 in neo, Ku70 or AsKu70 cells and the changes in viability in all these cells induced by curcumin. (A) Stable clones of SW480 cells expressing pEGFP vector (neo) or the pEmd-C1 containing the cDNA for full-length human Bax-GFP were established and western blotting was done using a specific polyclonal antibody to Bax as described in the Materials and methods. (B) SW480 cells were transiently transfected with the empty vector (neo) or expression vectors for Ku70, or AsKu70 by Lipofectamine 2000 and after 24 h, whole cell extracts were prepared for western blot analysis for the expression of Ku70, Bax or β -actin (loading control) as described in the Materials and methods. (C) Transfected cells from the above experiments (24 h after transfection) were treated with or without curcumin for 24 h and cell viability was determined by MTT assay and all the above experiments were repeated at least twice with similar results.

Bcl-XL prevents curcumin-induced nuclear condensation and fragmentation, but its antisense potentiated these effects

To understand whether the changes in cell viability induced by curcumin in Bcl-XL and AsBcl-XL cells were mediated by inhibition of apoptosis, DAPI staining was done since nuclear condensation and fragmentation are notable features of apoptosis. It was found that curcumin induces nuclear condensation and fragmentation in 60% of neo cells, whereas almost 70% ($P < 0.001$) of Bcl-XL cells showed healthy appearance, but 98% ($P < 0.001$) of antisense-inhibited cells showed apoptotic morphology (Figure 4A and B) indicating the potential of

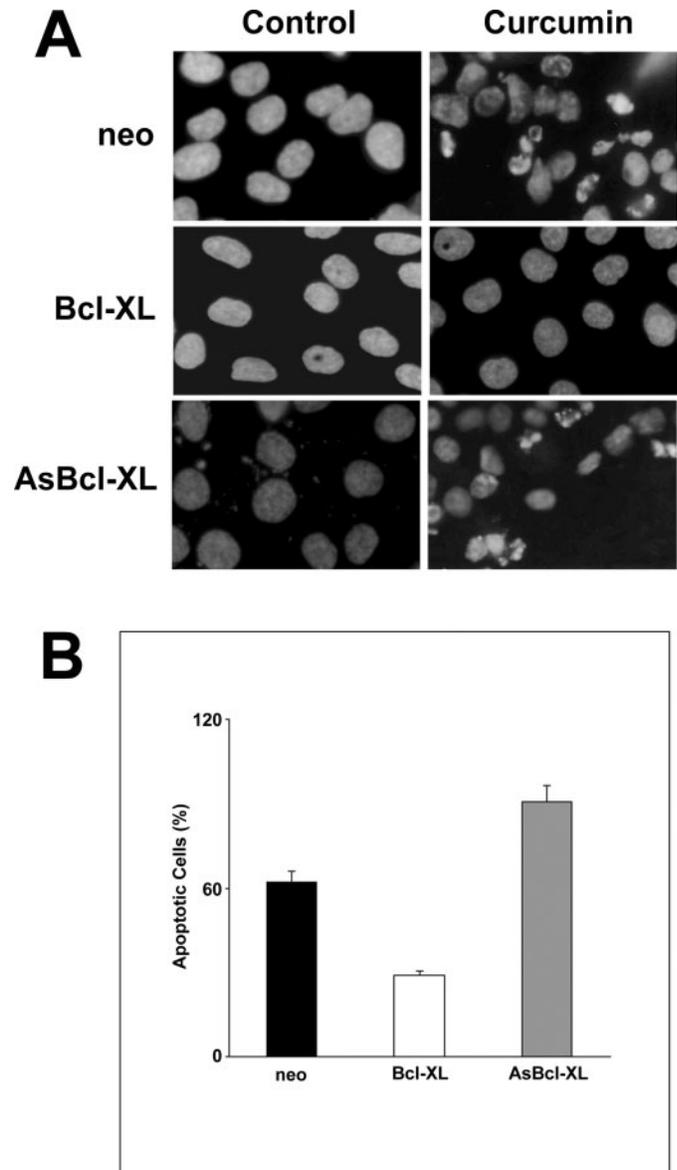


Fig. 4. Changes in nuclear morphology of neo, Bcl-XL or AsBcl-XL cells induced by curcumin. (A) Neo, Bcl-XL or AsBcl-XL cells grown on glass coverslips, treated with or without curcumin (25 μ M) for 24 h were fixed, permeabilized and then incubated with DAPI as described in the Materials and methods and representative fluorescent micrographs from untreated (control) and curcumin-treated cells are shown. (B) Cells with condensed and fragmented nuclei were counted in five different fields and the mean values of triplicate samples were expressed in percentage \pm SD and these results were confirmed in another independent experiment. See online Supplementary Material for a color version of this figure.

antisense inhibition to accelerate curcumin-induced nuclear apoptotic changes.

Bcl-XL protects SW480 cells against the loss of $\Delta\Psi_m$ and blocks the release of cytochrome c, AIF and Smac induced by curcumin

One of the earliest changes of apoptosis, the loss of $\Delta\Psi_m$, was assessed using a cationic mitochondrial dye that accumulates within the mitochondria if the $\Delta\Psi_m$ is intact (red color). If the $\Delta\Psi_m$ is altered, the dye in its monomeric form remains in the cytoplasm and it fluoresces green. Upon curcumin treatment (25 μM) for 12 or 24 h, Bcl-XL cells (94–98%) retained

$\Delta\Psi_m$ whereas AsBcl-XL cells (88–90%) lost their $\Delta\Psi_m$ and the neo cells retaining $\Delta\Psi_m$ were 50 and 28% at 12 and 24 h, respectively (Table I). These results indicate that Bcl-XL transfection significantly protects SW480 cells against the loss of $\Delta\Psi_m$ induced by curcumin and antisense inhibition of Bcl-XL enhances the loss of $\Delta\Psi_m$.

Apoptotic stimuli induce the loss of $\Delta\Psi_m$ and release of cytochrome c, AIF and Smac stored within mitochondria into the cytosol, but Bcl-XL (localized at the outer mitochondrial membrane) is known to block the release of cytochrome c and AIF (29). To understand whether Bcl-XL has a similar role in curcumin-induced apoptosis and to find its temporal relation

Table I. Changes in the retention of $\Delta\Psi_m$, cytochrome c, AIF and Smac in mitochondria induced by curcumin

Cell type	Time	Retention of $\Delta\Psi_m$ %	Retention in mitochondria (% control)		
			Cyt c	AIF	Smac
neo	12 h	50 \pm 5	26 \pm 2	36 \pm 5	40 \pm 3.9
	24 h	28 \pm 4	18 \pm 2	30 \pm 3	28 \pm 2
Bcl-XL	12 h	98 \pm 11 ($P < 0.007$)	97 \pm 7 ($P < 0.002$)	97 \pm 10 ($P < 0.005$)	96 \pm 6 ($P < 0.001$)
	24 h	94 \pm 6 ($P < 0.003$)	93 \pm 5 ($P < 0.001$)	92 \pm 5 ($P < 0.001$)	94 \pm 5 ($P < 0.001$)
As-Bcl-XL	12 h	12 \pm 1.6 ($P < 0.0001$)	12 \pm 2 ($P < 0.0001$)	14 \pm 2 ($P < 0.0001$)	20 \pm 2.5 ($P < 0.0001$)
	24 h	10 \pm 2 ($P < 0.0001$)	8 \pm 1.4 ($P < 0.0001$)	8 \pm 0.9 ($P < 0.0001$)	18 \pm 4 ($P < 0.0001$)

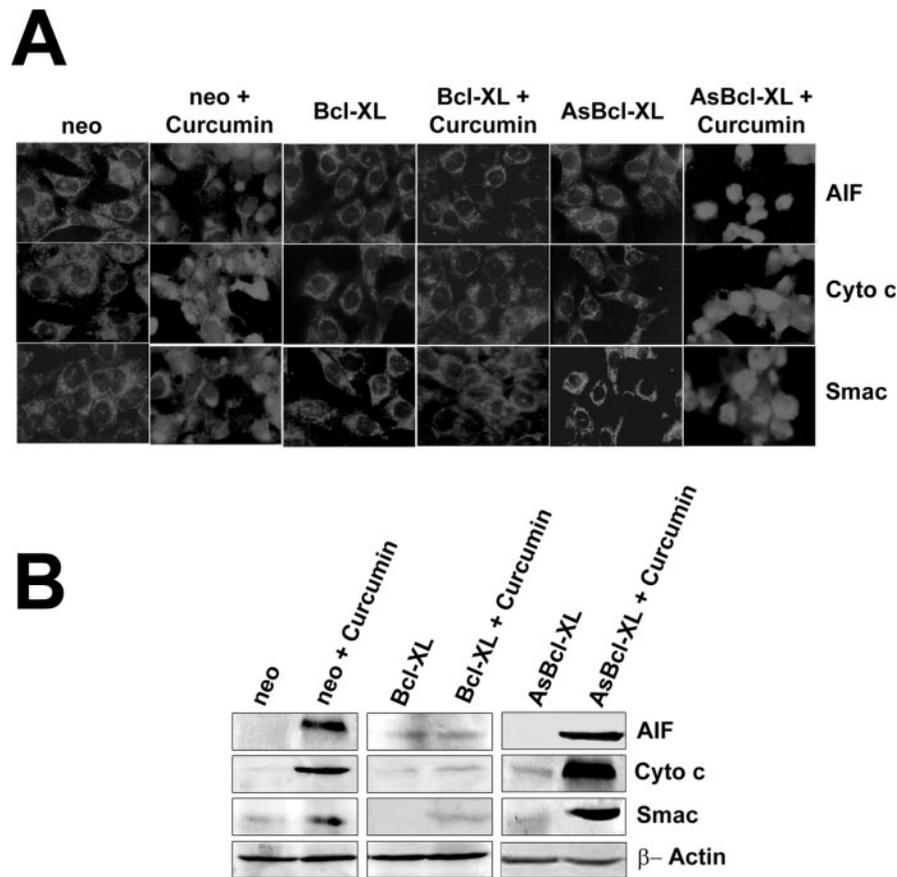


Fig. 5. Curcumin-induced mitochondrial release of cytochrome c, AIF or Smac by immunofluorescent staining and western blotting in neo, Bcl-XL or AsBcl-XL cells. (A) The cells grown on coverslips were left untreated or treated with curcumin (25 μM) for 24 h, fixed with 3.7% paraformaldehyde, permeabilized and incubated overnight with a primary antibody to Smac, cytochrome c or AIF and then incubated again with rhodamine-conjugated secondary antibody and visualized under fluorescent microscope as described in the Materials and methods. (B) For western blot analysis, the cells treated with or without curcumin 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 using respective antibodies for AIF or Smac or cytochrome c or β -actin (control) and appropriate secondary antibodies as described in the Materials and methods. All the above experiments were repeated at least two times with similar results. See online Supplementary Material for a color version of this figure.

with $\Delta\Psi_m$ loss, we used a semi-quantitative cell population-based study for the release of cytochrome c, AIF and Smac at 12 or 24 h after treatment with curcumin by immunofluorescence. The percentage of cells showing intact punctate perinuclear staining with rhodamine was calculated from the total number of cells counted from at least four different areas. All the results of immunofluorescence experiments are tabulated (Table I) and the results from a representative experiment done after 24 h with or without curcumin are shown in Figure 5A. All the untreated neo, Bcl-XL and AsBcl-XL cells showed a more granular appearance for cytochrome c, Smac and AIF around the nucleus and upon curcumin treatment, almost 70–80% of neo cells showed a diffuse staining pattern in the cytoplasm for cytochrome c, indicating its release from the mitochondria (Table I and Figure 5A). Granular pattern was more in Bcl-XL cells and very less in AsBcl-XL cells for the release of cytochrome c, AIF and Smac for both time periods after curcumin treatment (Table I and Figure 5A). These observations led us to the conclusion that curcumin induces the release of cytochrome c, AIF and Smac, but Bcl-XL effectively blocks curcumin-induced release of AIF and cytochrome c and Smac whereas the antisense inhibition of

Bcl-XL remarkably enhanced the release of all three molecules from mitochondria. It appears that $\Delta\Psi_m$ loss is a later event than cytochrome c release or both are independently regulated as even after 12 h of curcumin treatment only 50% of neo cells showed loss of $\Delta\Psi_m$ while almost 74% showed cytochrome c release (Table I). Similarly the release of AIF and Smac also appears to be independent of the changes in $\Delta\Psi_m$ (Table I).

To specifically look at the proteins released from mitochondria into the cytosol, western blot analysis of the digitonin-permeabilized cell lysates from cells treated with or without curcumin for 24 h was done. Untreated neo, Bcl-XL and AsBcl-XL cells showed no or faint bands for cytochrome c, AIF and Smac whereas in curcumin-treated neo and more so in AsBcl-XL cells intense bands were notable for all these proteins, but in Bcl-XL cells the bands for cytochrome c and AIF and Smac were less intense (Figure 5B). These results suggest that curcumin induces the release of cytochrome c, AIF and Smac and while Bcl-XL blocks the release of these proteins, antisense inhibition of Bcl-XL effectively releases these molecules during curcumin-induced apoptosis.

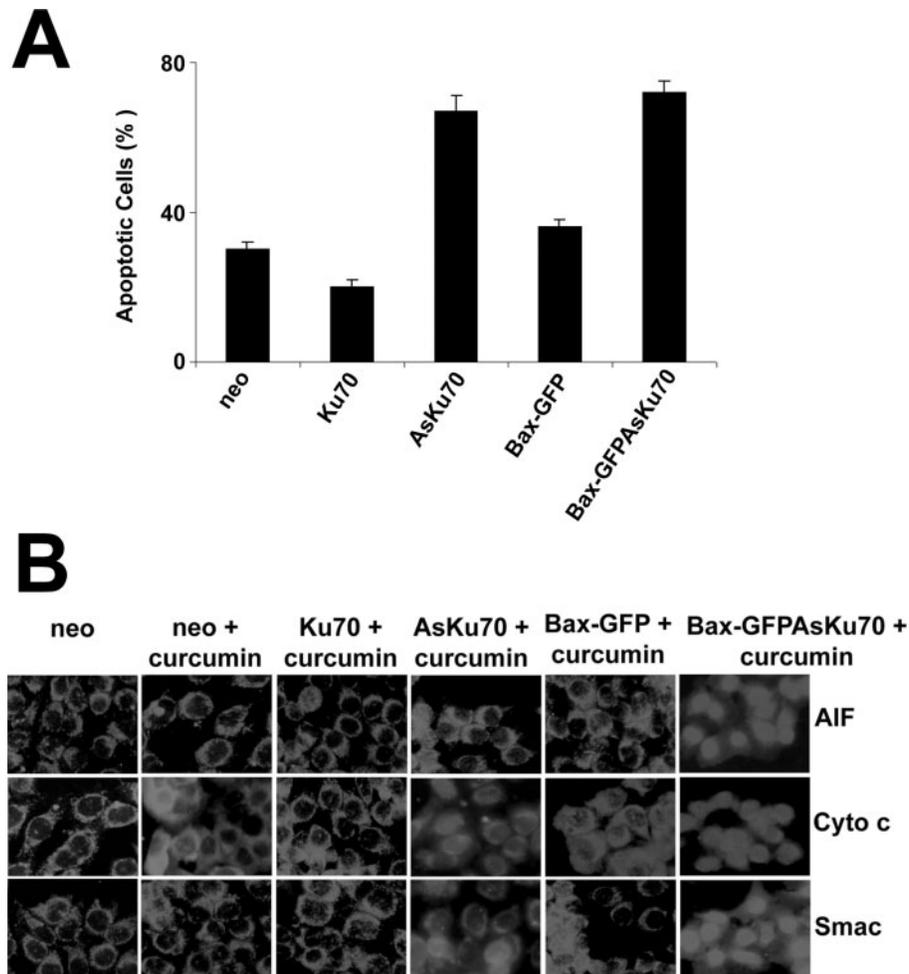


Fig. 6. Curcumin-induced changes in the nuclei of neo, Ku70, AsKu70, Bax-GFP or Bax-GFPAsKu70 cells. (A) Cells, 24 h after transient transfection with the indicated genes, were treated with or without curcumin for 8 h followed by paraformaldehyde fixation and staining with DAPI as described in the Materials and methods. Cells with condensed and fragmented nuclei were counted in five different fields and the mean values of triplicate samples were expressed in percentage \pm SD and these results were confirmed in another independent experiment. In comparison with neo cells treated with curcumin the results were significant for Ku70 ($P < 0.011$), AsKu70 ($P < 0.0001$), Bax-GFP ($P < 0.108$) and Bax-GFPAsKu70 ($P < 0.0001$) cells upon curcumin treatment. (B) Release of cytochrome c, AIF and Smac was visualized in neo, Ku70, AsKu70, Bax-GFP or Bax-GFPAsKu70 cells under fluorescent microscope before and after curcumin treatment as described in Figure 5A. See online Supplementary Material for a color version of this figure.

Ku70 suppresses curcumin-induced apoptosis whereas its antisense potentiates curcumin-mediated apoptotic changes in both Neo and Bax-GFP cells

The cells transiently transfected with Ku70 or AsKu70 were exposed to curcumin (25 μ M) for 8 h and nuclear condensation was assessed by DAPI staining and the release of cytochrome c, Smac or AIF was analyzed by immunofluorescence as described earlier. Apoptotic cells were more in AsKu70 cells compared with neo cells upon treatment with curcumin and the marginal effect of Bax-GFP cells in enhancing curcumin-induced apoptosis was potentiated by AsKu70 (Figure 6A). Curcumin treatment induced the release of cytochrome c in nearly 50% of neo cells and the release of AIF and Smac was moderately affected and ectopic expression of Ku70 prevented the release of cytochrome c, Smac and AIF whereas their release was accelerated in AsKu70 cells (Figure 6B). In Bax-GFP cells only the release of cytochrome c was accelerated compared with neo cells upon curcumin treatment but the

release of AIF and Smac was unaffected (Figure 6B). Curcumin treatment for 8 h was enough to trigger massive apoptosis in Bax-GFPAsKu70 cells as evident from the release of cytochrome c, AIF, Smac (Figure 6B). Overall these results suggest the importance of Ku70 in blocking the release of apoptogenic proteins from mitochondria and the potential of its antisense inhibition as an approach to restore the apoptotic program initiated by Bax-mediated mitochondrial permeabilization in the presence of curcumin.

Bcl-XL blocks curcumin-induced caspase 9 activation and antisense blocking potentiates the processing of caspase 9

In the mitochondria-mediated pathway once cytochrome c is released into the cytosol it activates procaspase 9 by complexing with Apaf-1 and finally procaspase 3 is activated. The processing of procaspase 9 was assessed using specific synthetic fluorimetric substrates and western blot. The relative fluorescent intensity calculated as percentage from control of different cytosols was plotted (Figure 7) and the western blot

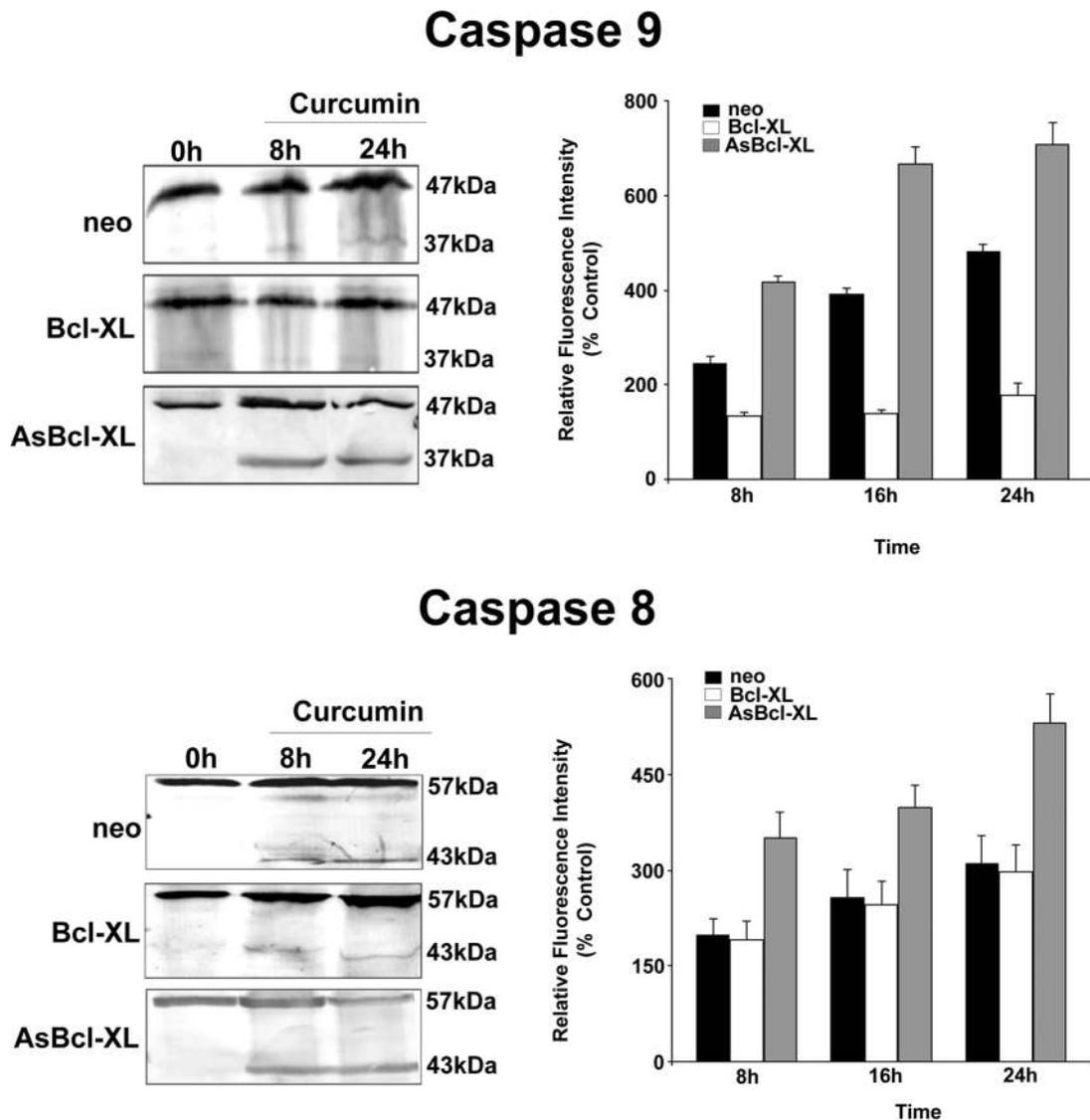


Fig. 7. Curcumin-mediated changes in the activities of caspases 9 and 8 determined by spectrofluorimetric and western blot methods in neo, Bcl-XL or AsBcl-XL cells. Cell lysates from curcumin-treated or untreated cells were incubated with a fluorimetric substrate (Ac-LEHD-AFC) of caspase 9 in a reaction buffer at 37°C for 1 h as described in the Materials and methods. Caspase 9 activation was also assessed by western blotting as described in the Materials and methods. Caspase 8 activation was determined by using a specific fluorimetric substrate (Z-IETD-AFC) essentially as described above. Caspase 8 activation was also confirmed by western blot analysis as described above. These experiments were repeated twice with similar results and the error bars denote the standard deviations.

of the corresponding samples are shown in Figure 7. Consistent with the release of cytochrome c, Bcl-XL blocks curcumin-induced activation of caspase 9 significantly compared with neo cells treated with curcumin. The antisense inhibition of Bcl-XL increased the caspase 9 processing and an intense cleaved fragment was noted at 12 h itself in antisense-inhibited cells.

Bcl-XL fails to prevent curcumin-induced caspase 8 activation, but antisense inhibition accelerates caspase 8 processing

A progressive activation of caspase 8 upon curcumin treatment was found in neo as well as Bcl-XL cells in all the time periods studied (Figure 7). Bcl-XL over-expression did not block curcumin-induced caspase 8 activation. However, AsBcl-XL cells showed increased activity of caspase both by western blotting and fluorimetric assays. By 8 h marked cleavage fragment was evident in AsBcl-XL cells substantiating even though Bcl-XL cannot prevent the processing of caspase 8 its

down-regulation could potentiate caspase 8 activation induced by curcumin.

Bcl-XL prevents curcumin-induced caspase 3 activation and cleavage of PARP

The processing of procaspase 3 was assessed using a specific synthetic fluorimetric substrate or by western blot using an antibody to caspase 3 that detects both the cleaved and intact fragments. The relative fluorescent intensity values calculated as percentages from control of different cytosols were plotted and the western blot of the same samples are depicted (Figure 8). PARP, a caspase 3 cleavage substrate, is a 116-kDa modular nuclear protein involved in DNA repair, usually in response to environmental stress (30). In neo cells treated with curcumin PARP was cleaved starting from 16 h onwards, but in Bcl-XL curcumin-treated cells PARP cleavage was completely prevented (Figure 8). The silencing of Bcl-XL potentiated curcumin-mediated caspase 3 processing and subsequent PARP cleavage.

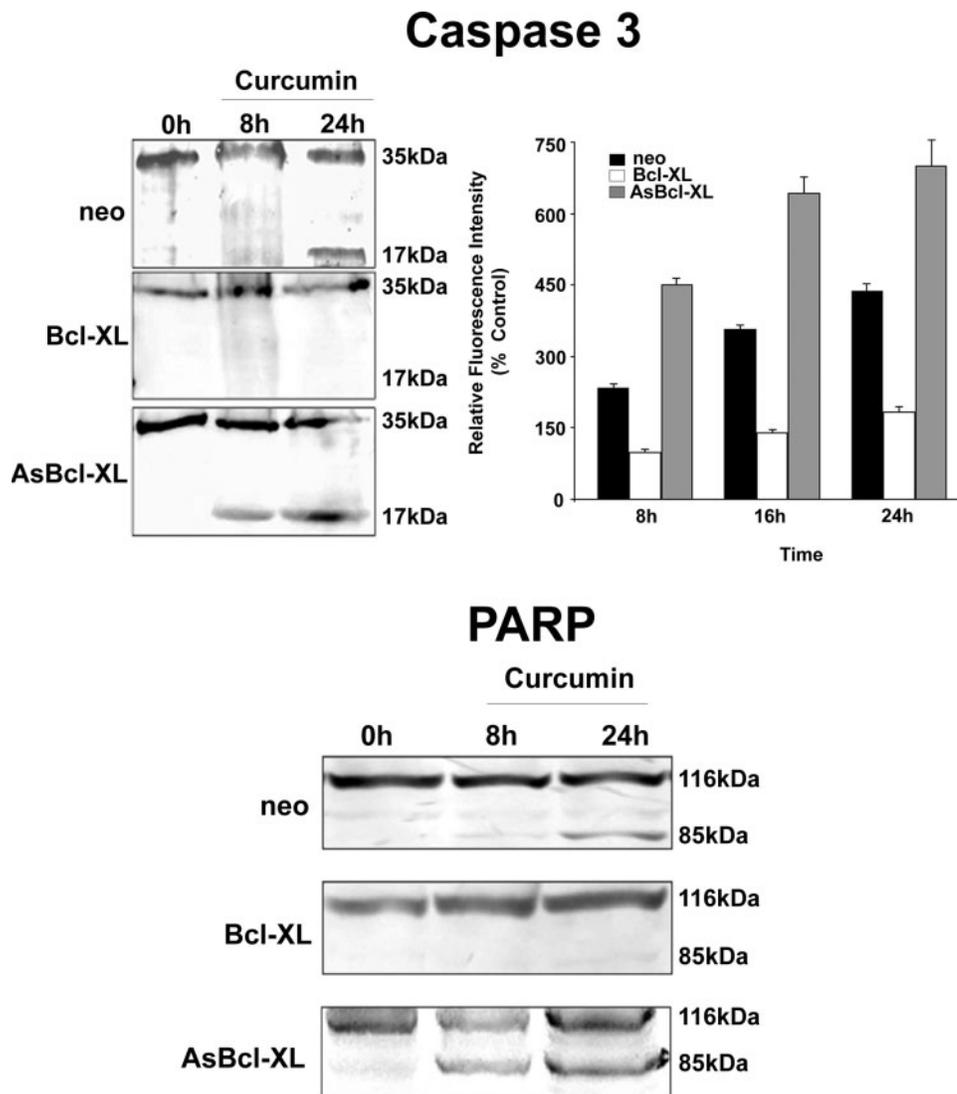


Fig. 8. Changes in caspase 3 activity and cleavage of PARP induced by curcumin in neo, Bcl-XL or AsBcl-XL cells. Curcumin-induced activation of caspase 3 at the indicated periods of time was determined using a fluorimetric substrate (Ac-DEVD-AFC) as described in the Materials and methods. Caspase 3 activation was confirmed by western blot analysis as described earlier. Cell lysates (60 μ g of protein) prepared after treating the cells with or without curcumin (25 μ M) for the indicated time periods were subjected to 8% SDS-PAGE and PARP was detected with antiPARP polyclonal antibody by western blotting as described in the Materials and methods. All these experiments were repeated at least twice with similar results and the error bars denote standard deviations.

Ku70 inhibits curcumin-induced activation of caspases 9, 8 and 3 while its down-regulation accelerates caspases 9, 8 and 3 activation in neo, Bax-GFP and Bax-GFPAsKu70 cells

Curcumin (25 μ M) treatment for 8 h activated the caspases 9, 8 and 3 in neo cells and their activation was relatively less in Ku70 cells although this effect was minimum for caspase 8 (Figure 9A–C). Curcumin-induced activation of caspases 9, 8

and 3 was accelerated in AsKu70 cells whereas in Bax-GFP cells their activation remained comparable with that of neo cells (Figure 9A–C). In Bax-GFPAsKu70 cells 8 h of curcumin treatment accelerated the activation of caspases 9, 8 and 3 in Ku70 cells (Figure 9A–C).

Discussion

In the present study we have demonstrated that Ku70 and Bcl-XL have the potential to protect human colon cancer cells against curcumin-induced apoptosis. Consistent with earlier reports showing that Bcl-XL predominantly blocks apoptosis induced by various stimuli at the level of mitochondria, the processing of caspase 9, caspase 3 and cleavage of a caspase substrate like PARP induced by curcumin is prevented in Bcl-XL cells in the present study (9,31). Expression of Bcl-XL prevents the redistribution of cytochrome c from mitochondria to cytosol, a prominent requisite for caspase activation and apoptosis (32–34). Increased expression of Bcl-XL in colorectal cancer plays a key role in the induction of resistance to anoikis during the progression of this disease (35). Bcl-2 and Bcl-XL over-expression in HL-60 cells confers protection against curcumin-mediated apoptosis (36). In agreement with our results showing an antiapoptotic role for Ku70 in curcumin-induced apoptosis, Ku70-deficient cells were reported to be more sensitive to bleomycin-induced apoptosis whereas cells over-expressing Ku70 were resistant (37). Our observation that Ku70 prevents the release of cytochrome c, AIF and Smac in curcumin-mediated cell death is consistent with the function of Ku70 as an inhibitor of Bax by preventing its translocation to mitochondria, which is an essential step, required for cell death (20). Since the Bax/Bcl-2 ratio is an important regulator of apoptosis, it is relevant to note that in SW480 cells the Bax/Bcl-2 ratio remains unaltered by curcumin though it affected this initially (Figure 1). Similar to our results, curcumin treatment does not alter the expression levels of Bcl-2 and Bax proteins in hepatoblastoma cells (38). However, curcumin is said to down-regulate the antiapoptotic Bcl-2, Bcl-XL and IAP proteins in human renal cells (39) and promyelocytic leukemia HL-60 cells (3) suggestive of cell-type-dependent changes. Curcumin has been reported to induce the expression of Bax in breast cancer cells through a p53-dependent pathway acting downstream of p53 (40). The failure of Bax-GFP cells to potentiate curcumin-induced apoptosis is presumably because of high basal level of Ku70 that was not altered by curcumin treatment. This is further substantiated by our observation that antisense inhibition of Ku70 markedly improves the release of cytochrome c in Bax-GFP cells.

Since Bcl-XL cells treated with curcumin exhibit caspase 8 activation although DNA condensation and PARP cleavage were not detected, the protective action of Bcl-XL presumably lies downstream of caspase 8 activation. Recently it has been reported that a bifunctional apoptosis regulator present in Bcl-XL over-expressing MCF-7-Fas cells sequesters active caspase 8 on mitochondrial surface by preventing the cleavage of substrates of caspase 8 (41). However, apoptosis in certain cell types requires caspase 8-mediated cleavage and activation of a Bcl-2 family member, Bid, the truncated form of which amplifies the mitochondrial release of cytochrome c (42). Presumably in human colon cancer cells Bcl-XL suppresses caspase 8-mediated cell death by preventing the release of cytochrome c. The observation that the expression of Ku70

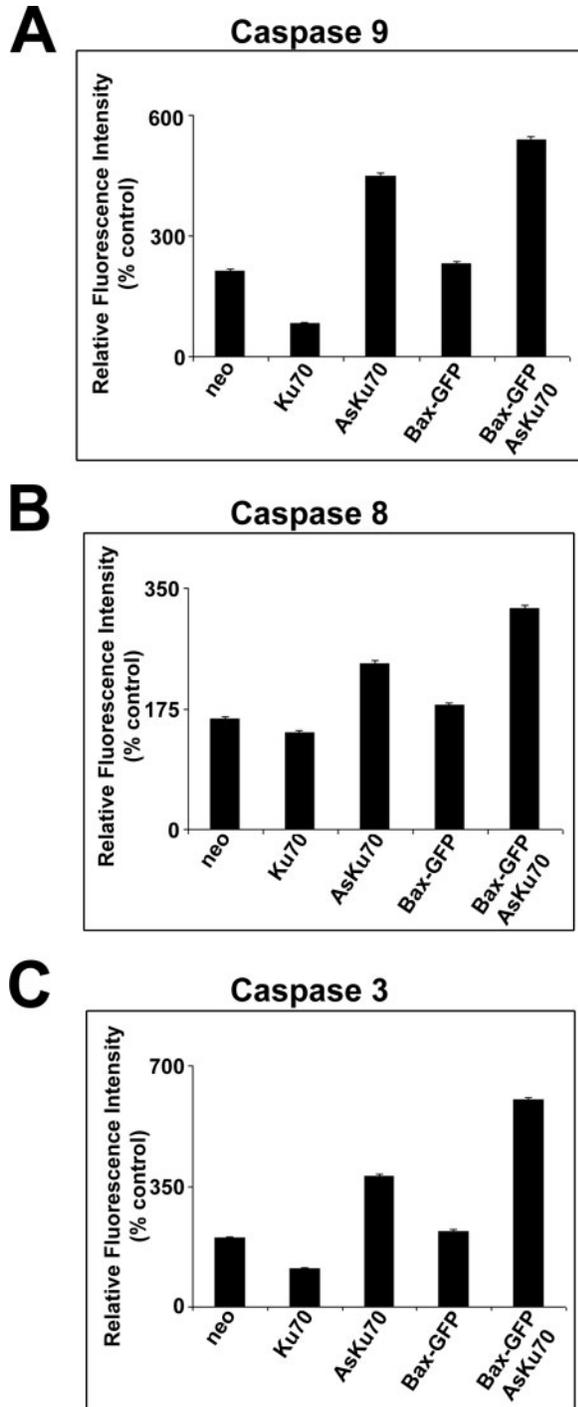


Fig. 9. Curcumin-induced changes in the activities of caspases 8, 9 and 3 in neo, Ku70, AsKu70, Bax-GFP or Bax-GFPAsKu70 cells. Neo, Ku70, AsKu70, Bax-GFP or Bax-GFPAsKu70 cells were treated with or without curcumin (25 μ M) for 8 h and the cytosolic extracts were prepared and analyzed for caspase 9 (A), caspase 8 (B) or caspase 3 (C) activities using a spectrofluorimeter as described in the Materials and methods. Each experiment was repeated at least once again with similar results.

but not that of Bcl-XL inhibits caspase 8 activation induced by curcumin in spite of the acceleration of curcumin-induced activation of caspase 8 in both AsBcl-XL and AsKu70 cells is suggestive of secondary caspase 8 activation by caspase 3. This is consistent with the finding that caspase 3 can serve as a feedback loop by cleaving other caspases to amplify the apoptotic process (43–45).

The notion that the release of cytochrome c, AIF and Smac from mitochondria is subsequent to the loss of $\Delta\Psi_m$ does not appear to be the case in curcumin-induced apoptosis of human colon cancer cells as cytochrome c is released before substantial loss of $\Delta\Psi_m$ occurs and simultaneous release of AIF and Smac is also unlikely. In support of this it has been shown that the release of AIF occurs downstream of cytochrome c release and requires caspase activation (46). Although our findings support the idea that release of AIF requires caspase activation, further studies are needed to confirm this. Recently it has been reported that mitochondrial outer membrane permeabilization induced by proapoptotic drugs results in the release of cytochrome c, Smac, HtrA2/Omi, but subsequent caspase activation is required to induce the translocation of endonuclease G or AIF (47).

Activation of caspases 9 and 3, cleavage of PARP, release of cytochrome c, AIF and Smac in AsBcl-XL cells treated with curcumin implies that antisense inhibition is an important strategy to overcome chemoresistance. Increased release of Smac in AsBcl-XL and AsKu70 cells suggest that curcumin-induced apoptosis is favored by sequestering inhibitor of apoptosis protein by Smac (24,48,49). In addition, accelerated release of curcumin-induced AIF in AsBcl-XL cells may lead to caspase-independent cell death and it is reported that the release of AIF and endonuclease G is under the regulatory control of Bcl-2/Bcl-XL (50).

Our findings demonstrate, for the first time, the efficacy of antisense inhibition of Bcl-XL or Ku70 in sensitizing human colon cancer cells to mitochondrial apoptotic signaling induced by curcumin. It is to be seen whether antisense oligonucleotides to Bcl-XL and Ku70 in combination with curcumin would be a powerful strategy to tackle chemoresistant Bcl-XL or Ku70 over-expressing cancer cells.

Supplementary material

Supplementary material can be found at: <http://www.carcin.oupjournals.org/>.

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