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Coupling endoplasmic reticulum stress to the cell death program in mouse melanoma cells: effect of curcumin

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Abstract

The microenvironment of cancerous cells includes endoplasmic reticulum (ER) stress the resistance to which is required for the survival and growth of tumors. Acute ER stress triggers the induction of a family of ER stress proteins that promotes survival and/or growth of the cancer cells, and also confers resistance to radiation and chemotherapy. Prolonged or severe ER stress, however, may ultimately overwhelm the cellular protective mechanisms, triggering cell death through specific programmed cell death (pcd) pathways. Thus, downregulation of the protective stress proteins may offer a new therapeutic approach to cancer treatment. In this regard, recent reports have demonstrated the roles of the phytochemical curcumin in the inhibition of proteasomal activity and triggering the accumulation of cytosolic Ca^{2+} by inhibiting the Ca^{2+} -ATPase pump, both of which enhance ER stress. Using a mouse melanoma cell line, we investigated the possibility that curcumin may trigger ER stress leading to programmed cell death. Our studies demonstrate that curcumin triggers ER stress and the activation of specific cell death pathways that feature caspase cleavage and activation, p23 cleavage, and downregulation of the anti-apoptotic Mcl-1 protein.

Keywords

Endoplasmic reticulum; Curcumin; ER stress; Caspase; Apoptosis; Programmed cell death

Introduction

The efficient functioning of the endoplasmic reticulum (ER) is essential for proper cellular activities and survival. Any condition that interferes with ER functions triggers the accumulation and aggregation of misfolded or unfolded proteins and induction of an ER stress response to restore normal ER function [1–3]. The induction of a family of ER signaling proteins is required to block ER stress signals, maintain ER function and integrity, ensure protein folding, and protect cells from misfolded protein toxicity [1–6]. Prolonged ER stress impairs the protective mechanisms designed to promote correct folding and degrade faulty proteins, ultimately leading to organelle dysfunction and programmed cell death (pcd) [7–9].

The microenvironment of cancerous cells and solid tumors is characterized by an inherent physiological ER stress response that provides an overall protective role in tumor development. The levels of a family of ER proteins are elevated correlating with tumor progression, metastasis and drug resistance. It is believed that this family of ER stress proteins is anti-apoptotic and confers protection to cancer cells against the natural immune defense mechanisms of the host and anti-cancer drugs [10,11]. This suggests that down-regulation of the protective stress proteins, or the provocation of an ER stress-mediated cell death process by small molecules, may retard tumor development, growth, and invasion, and potentially improve treatment outcome [10–12].

Curcumin, the main biologically active phytochemical isolated from turmeric root, is a powerful antioxidant and anti-inflammatory compound, and is a potent inhibitor of the proliferation of cancer cells [12–15]. Numerous studies have demonstrated its role in suppressing the expression of cyclin D1 and other transcription factors that are implicated in carcinogenesis, blocking the activation of nuclear factor kappa B (NF- κ B) and activator protein 1 (AP-1), inducing apoptosis in tumor cells by activating caspases and down-regulating anti-apoptotic Bcl-2 family proteins [13,16]. Recent reports have also demonstrated that curcumin (a) inhibits proteasomal activity [17], (b) triggers the accumulation of cytosolic Ca^{2+} by inhibiting the Ca^{2+} -ATPase pump [18,19], and (c) disrupts protein disulfide bond formation [12], all of which trigger an ER stress response. Therefore, we evaluated the possibility that curcumin may trigger ER stress-induced apoptosis in mouse melanoma cells. Our studies demonstrate that curcumin triggers ER stress and activates the intrinsic apoptotic pathway, affecting both Bcl-2 family proteins and caspases.

Material and methods

Cells, culture conditions and cell extracts

Murine melanoma B16–F10 cells, human embryonic kidney HEK293 and NIH3T3 mouse fibroblasts were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. Curcumin (Sigma Chemical Co, St. Louis MO, USA) was dissolved in absolute alcohol at a stock concentration of 10 mM and was diluted to the required concentration immediately before use with cell culture media. A 10 mM stock solution of BAPTA/AM (Sigma Chemical Co, St. Louis MO, USA) was prepared in DMSO and was diluted in cell culture media just before addition to the cells. Salubrinal (Sal; EMD Biosciences) was dissolved in DMSO and diluted in cell culture media before addition to cells. Q-VD-OPH (MP Biomedicals, Aurora, Ohio, USA), a cell permeable irreversible caspase inhibitor, was dissolved in DMSO according to manufacturer's data sheet and further diluted with cell culture media.

Total cell extracts were prepared as described [7,9,20,21]. Briefly, cells from untreated or curcumin-treated cells were resuspended in RIPA buffer (50 mM Tris, pH 7.5, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl) containing protease inhibitors

(complete Mini; Roche, Penzberg, Germany). 100–200 µg protein from total extracts was used for Western blotting.

Western blotting

SDS-PAGE and Western blot analyses were performed as described earlier [7–9,22]. Membranes were probed with 1:500 dilutions of mouse-specific anti-caspase-9, anti-caspase-12, anti-caspase-7, anti-caspase-3 and anti-Bax antibodies (all from Cell Signaling Laboratories), a 1:1,000 dilution of anti-p23 monoclonal antibody (BD Biosciences), a 1:500 dilution of anti-KDEL monoclonal antibody (Stressgen), a 1:500 dilution of anti-Mcl-1 antibody (Santa Cruz), and a 1:50,000 dilution of anti-GAPDH rabbit polyclonal antibody (Research Diagnostics, Inc).

Flow cytometry

Flow cytometry analysis was performed as described [21]. Media and cells from untreated and treated samples were collected by trypsinization, stained with 3 µg/ml GFP-Annexin-V and 2.5 µg/ml propidium iodide in Annexin-V binding buffer and incubated at room temperature for 15 min, and read on a BD LSR flow cytometer (BD Biosciences, San Jose, CA, USA). Data were processed with CellQuest Pro (BD Biosciences).

Immunoprecipitation

Cell lysis and immunoprecipitation were performed as previously described [8,9,22]. Cell extracts from untreated and curcumin-treated cells were prepared as described [7,22]. A total of 200 µg protein from the total extract was subjected to immunoprecipitation. Following an overnight incubation at 4°C with the antibodies, protein A/G-Sepharose was added to the samples and incubated at 4°C for an additional 6 h. Samples were spun briefly to pellet the protein A/G-Sepharose conjugate. The supernatant was subjected to SDS-PAGE and Western blotting.

Caspase activity assay

The fluorogenic substrate benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin was purchased from Enzyme Systems Products and dissolved in dimethyl formamide as a 10 mM stock solution. Cell extracts (50–100 µg of protein) from untreated or curcumin-treated cells were incubated with 100 µM peptide substrate. Caspase activity was determined by measuring the release of amino-4-trifluoromethylcoumarin from the synthetic substrate using continuous recording instruments as described earlier [7]. Caspase activity was analyzed using a SpectraMAX 340 plate reader (Molecular Devices) at excitation and emission wavelengths of 444 and 538 nm, respectively, and expressed as DEVDase activity/mg protein.

Evaluation of cell death

Assessment of cell death was carried out by pelleting floating and adherent cells (after trypsinization) as previously described [7,9,20]. The cell pellet was resuspended in 1 × PBS/0.4% Trypan blue and cells were counted using a hemocytometer. Cell death was determined as the percentage of dead cells over the total number of cells. Statistical significance was determined by two-way analysis of variance (ANOVA).

Alternatively, assessment of cell death was carried out by the MTT assay as previously described [23]. Briefly, cells were seeded in a 24-well plate at a density of 50,000 per well. Following treatment with curcumin, viable cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, 50 µl of 1 mg/ml MTT was added to the cells (500 µl) and incubated at 37°C for 2 h. The medium was discarded,

the dark blue formazan crystalline product was dissolved in dimethyl sulfoxide and the absorbance was analyzed in a Spectramax plate reader (Molecular Devices) at 570 nm. Cell death was determined as the percentage of live cells over the total number of cells.

Results

Curcumin triggers ER stress-induced cell death in melanoma cells

Several studies have reported the use of curcumin at various concentrations varying from 1 to 100 μ M [12,13,17,24]. In order to test whether melanoma cells are susceptible to ER stress and cell death, B16 mouse melanoma cells were treated with different concentrations of curcumin. As shown in Fig. 1a, exposure of cells to curcumin led to a decrease in cell viability in a time and dose-dependent manner. This is similar to results reported earlier [13] demonstrating induction of apoptosis by curcumin in human melanoma cells. In contrast, NIH3T3 mouse fibroblasts and HEK-293 cells were resistant to curcumin treatment and displayed minimal cell death at 100 μ M (Fig. 1b) suggesting curcumin's specificity and its ability to distinguish cancer cells from transformed cells (HEK293) or spontaneously immortalized cells (3T3).

A high level of glucose regulated protein (GRP) expression is indicative of ER stress [7–9, 25], and curcumin treatment of melanoma cells resulted in the induction of GRP78 and GRP94 expression (Fig. 1a). Interestingly, despite showing minimal cell death toxicity, curcumin treatment of both NIH3T3 mouse fibroblasts and HEK293 cells resulted in the induction of GRP expression (Fig. 1b) suggesting that in contrast to melanoma cells, upregulation of GRP family of proteins in NIH3T3 and HEK293 cells may confer survival advantage through their anti-apoptotic properties [11,26].

Cells exposing phosphatidylserine, which serves as a marker of apoptosis, can be labeled with a His-GFP-Annexin-V fusion protein [21]. Loss of plasma membrane integrity can be followed by uptake of propidium iodide [21]. After 24 h of ER stress, fluorescence-activated cell-sorting analysis revealed that 60% or more of the cells were GFPAn-V-positive with curcumin treatment compared to 18% in untreated cells (Fig. 2). This method seems more sensitive and reliable for measuring dying cells as opposed to the trypan blue method (Fig. 1) that involves manual counting of dead cells and may explain the discrepancy in the viability of melanoma cells at 24 h of curcumin treatment as measured by the above two methods (Fig. 1 and Fig. 2).

GADD 153/CHOP is a 30 kDa protein that triggers growth arrest and DNA damage. While GADD 153 is ubiquitously expressed at very low levels in basal conditions, it is robustly expressed by perturbations that induce ER stress in a wide variety of cells [4,5,27]. Similarly, phosphorylation of the alpha subunit of the eukaryotic initiation factor-2 (eIF2 α) is a well documented mechanism of down-regulating protein synthesis triggered by agents that induce ER stress and protein misfolding [4,5]. As shown in Fig. 3a, curcumin treatment resulted in increased phosphorylation of eIF2 α and increased protein expression of GADD 153.

Salubrinal (Sal) is an inhibitor of serine/threonine phosphatase PP1 and inhibits eIF2 α dephosphorylation that in turn blocks ER stress-induced cell death [28,29]. To study the effect of Sal on curcumin-treated melanoma cells, we added Sal in combination with curcumin. As shown in Fig 3b, addition of Sal to cells significantly decreased the number of dying cells and increased survival by more than 50%.

Processing of caspases in ER stress-induced cell death triggered by curcumin

Caspase-dependent apoptotic cell death features caspase activation that, in the case of executioner caspases (caspases-3 and -7) requires cleavage into a large and small subunit. Cleavage and activation of downstream caspases is promoted by proximal initiator caspases

(caspases-12, -9 and -8) which themselves undergo dimerization and cleavage [30]. In order to determine whether caspases are cleaved during ER stress-induced apoptosis in melanoma cells, cell-free extracts from curcumin-treated cells were analyzed by Western blotting. As shown in the time course in Fig. 4a, curcumin treatment resulted in the processing and cleavage of caspases-12, -9, -7 and -3. Processing of caspase-12 was seen initially at 12 h of curcumin treatment. The antibody we employed for caspase-9 recognized full length (50 kDa) and an amino-terminally cleaved product (39 kDa, prodomain + large subunit) of caspase-9 [7]. Cleavage of caspase-7 was very similar to that of caspase-9, with more of the cleaved form present at 24 h of treatment. Processing of the pro-form of caspase-3 (32 kDa) to the p28 form was also observed at 24 h of curcumin treatment.

Caspase activity measurements on cell extracts were performed using Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin as substrate. As shown in Fig. 4b, melanoma cells treated with curcumin activated caspases as demonstrated by increased DEVDase activity. Q-VD-OPH (QVD), a broad spectrum cell-permeable caspase inhibitor that irreversibly binds to active caspases, blocks cell death with minimal toxicity. As shown in Fig. 4b, the DEVDase activity triggered by curcumin was significantly suppressed by QVD. QVD not only inhibited DEVDase activity but also enhanced melanoma cell viability (data not shown).

BAPTA-AM blocks GADD 153 induction and caspase cleavage

Recent reports have demonstrated the role of curcumin in triggering the accumulation of cytosolic Ca^{2+} by several mechanisms including inhibiting Ca^{2+} -ATPase pump [18,19,31, 32] that in turn could trigger an ER stress response leading to cell death. The calcium chelator BAPTA-AM blocks Ca^{2+} release from the ER, prevents mitochondrial ROS accumulation and blocks apoptosis [29,33–35]. This meant that blocking Ca^{2+} release by the calcium chelator BAPTA-AM could potentially inhibit ER stress-induced cell death triggered by curcumin. We therefore analyzed the effects of BAPTA-AM on cell death, GADD153 induction and caspase cleavage after exposing mouse melanoma cells to curcumin. As shown in Fig. 5, preincubation of melanoma cells with 50 μM BAPTA-AM followed by curcumin treatment not only inhibited cell death (Fig. 5a) but also blocked induction of GADD 153 expression and caspase-7 cleavage (Fig. 5b), suggesting that a combination of ER stress and cytosolic Ca^{2+} influx facilitates and accelerates curcumin-induced apoptosis in melanoma cells. Interestingly, BAPTA-AM did not block the induction of expression of GRP family proteins triggered by curcumin (Fig. 5b) suggesting that Ca^{2+} release may be downstream of GRP accumulation. BAPTA-AM alone did not have any effect on GADD153 expression or caspase processing.

Expression of PARP and p23 in curcumin-treated melanoma cells

PARP, a 116 kDa nuclear poly (ADP-ribose) polymerase that is involved in DNA repair predominantly in response to environmental stress, is cleaved by caspases in vitro and is one of the main cleavage targets of caspase-3 in vivo [36]. As shown in Fig. 6 (top panel), ER stress-induced cell death triggered by curcumin resulted in cleavage of PARP to yield an 85 kDa cleaved fragment, as described earlier [7]. In an earlier study, we found that p23, an HSP90 co-chaperone protein, plays a role in mediating ER stress-induced cell death [37]. p23 is cleaved to a 19 kDa product during ER stress-induced cell death, irrespective of triggering agent. As shown in Fig. 6, curcumin treatment of melanoma cells also resulted in the processing of p23 and formation of the 19 kDa cleaved fragment [37]. The antibody we employed recognized full length p23 and the 19 kDa cleaved product (Fig. 6, middle panel). In addition, we also generated a neo-epitope antibody that recognized only the cleaved fragment (19 kDa) in curcumin-treated samples and not the parent p23; this confirmed p23 cleavage (Fig. 6, bottom panel).

Effect of curcumin on the expression of pro and anti-apoptotic proteins of the Bcl-2 family

Recent studies have disclosed the role of several ER stress-induced cell death effectors, including pro and anti-apoptotic members of the Bcl-2 family [1–3,38–41]. Mcl-1 is an anti-apoptotic member of the Bcl-2 family that localizes to the mitochondria and inhibits apoptosis [42–44]. Recent studies have shown that expression of Mcl-1 protein is downregulated in cells undergoing pcd [42,43,45,46]. Similarly, proapoptotic Bax undergoes a conformational change and accumulates on the mitochondrial surface triggering the release of cytochrome c to activate the final steps of pcd [47–49].

To further understand the role of curcumin in ER stress-induced apoptosis in mouse melanoma cells, we asked whether curcumin has any effect on the expression of these two proteins. Exposure of melanoma cells to curcumin led to a time-dependent decrease in Mcl-1 expression (Fig. 7a). The antibody that we employed failed to detect the expression of Mcl-1S (short chain, a pro-apoptotic protein). For detecting Bax expression before and after curcumin treatment, immunoprecipitation was performed with an anti-Bax antibody that recognizes conformationally-altered Bax, and the resulting immunoprecipitate was analyzed by immunoblotting using non-conformation-dependent antisera specific for Bax [27,37,50]. As shown in Fig. 7b, while Bax expression in total cell lysates decreased with time of curcumin treatment, immunoprecipitation with the conformation-specific antibody revealed increased levels of conformationally altered Bax only in the curcumin-treated cell lysates, suggesting that a population of pro-apoptotic Bax is present as an active conformer (i.e., with the N-terminal region exposed) in curcumin-treated cells.

Discussion

The endoplasmic reticulum (ER) is extremely sensitive to changes that affect its structure, integrity and function. Changes in calcium homeostasis, inhibitors of protein disulfide bond formation, oxidative stress, inhibition of proteasomal activity can all disrupt protein synthesis and folding, resulting in unfolded or misfolded proteins [4,11,51,52]. The cell responds by initiating a cascade of quality control signaling mechanisms (termed “ER stress response”) that restore normal ER function [51]. Several of these signaling molecules, which include the GRP family of chaperone proteins, also play critical roles in cytoprotection by triggering survival signals [4]. Cancerous cells adapt to local cellular environments by triggering protective ER stress responses [10]. Therefore, approaches to down-regulate the protective ER stress response in cancerous cells may significantly improve treatment outcome [11]. Prolonged or severe ER stress overwhelms cellular protective mechanisms, ultimately triggering cell death. ER stress-induced cell death is coupled to specific independent death pathways, as well as demonstrating cross-talk with the classic intrinsic (Apaf-dependent) and extrinsic apoptotic pathways [7–9, 53–55]. This implies that the use of small molecules to provoke a chronic ER stress-mediated cell death process may also retard tumor development, growth, and invasion, thus providing novel targets for therapeutic intervention.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), the major yellow pigment isolated from turmeric root (*curcuma longa*) and commonly used as a flavoring agent in food, has drawn much attention because of its effect on several biochemical pathways. It has been demonstrated to have anti-inflammatory, antioxidant, and anti-proliferative properties [15]. Its in vivo actions include suppression of carcinogenesis of the skin, stomach, colon, breast, and liver in mice [15], and in vitro, it has been shown to inhibit the growth of a wide variety of tumor cells [14,56]. Curcumin’s anti-cancer effects appear to be due to its ability to block the transformation, proliferation, and invasion of tumor cells by suppressing the activation of certain transcription factors and promoting the activation of cell death proteases [12,14,16,17]. Similarly, topical application of curcumin normally used to accelerate wound healing [57], markedly inhibits tumor progression by blocking DNA and RNA synthesis [58–

60]. Several of these effects were demonstrated using 5,000–12,000 mg of curcumin in clinical trials and up to 100 μM curcumin for in vitro studies with no obvious toxicity [61]. The advantage of using curcumin over other compounds stems from the fact that it is relatively non-toxic even at high concentrations, and can be taken orally or applied topically without causing any significant undesirable side effects [15].

Recent evidence also suggest that curcumin inhibits cellular proteasome activity thus triggering the accumulation of proteins destined for degradation [17], disrupts protein disulfide bond formation [12,19], and also inhibits the Ca^{2+} -ATPase activity, resulting in cytosolic Ca^{2+} accumulation [12,19]. All of these events disrupt the balance between ER protein synthesis and degradation, resulting in ER stress and pcd [12]. Our data confirm that melanoma cells are sensitive to curcumin treatment at concentrations ranging from 50 to 100 μM . Elevation in the expression of the GRP family of proteins, phosphorylation of eIF2 α , inhibition of cell death by salubrinal, induction of GADD 153 protein expression, and cleavage of downstream caspases including ER specific caspase-12 and caspase-7, all indicate the involvement of ER stress in triggering cell death. Blockage of GADD 153 protein expression and caspase-7 cleavage by the calcium chelator BAPTA-AM suggests that a combination of ER stress and accumulation of cytosolic Ca^{2+} accelerates curcumin-induced apoptosis in melanoma cells. Failure to block the induction of expression of GRP family proteins by BAPTA-AM suggests the involvement of multiple ER associated signaling pathways triggered by curcumin that finally converge on the caspase/Bcl-2 pathway to trigger melanoma cell death. The mechanism by which curcumin exhibits specific toxicity towards cancerous cells is still not clear. Our studies on a class of transformed cells (HEK293 and 3T3) indicate that these cells are resistant to curcumin treatment. The plausible explanation is that cancer cells including melanomas may be selectively vulnerable due to their inability to cope with prolonged ER stress due to the accumulation of misfolded proteins and Ca^{2+} leakage [10,11]. While prolonged ER stress eliminates the protective properties of ER stress and GRP family of proteins and drives the melanoma cells to death pathways, upregulation of anti-apoptotic GRP family of proteins in 293 and 3T3 cells may actually trigger survival signaling pathways [10,11]. The present study warrants further investigation into the anti-apoptotic roles of the GRP family of proteins and the survival pathways that they regulate.

Our earlier studies demonstrated the role of p23, a small chaperone protein, in ER stress-induced cell death [37]. Our present results suggest that in addition to PARP, p23 is also susceptible to cleavage following curcumin treatment of melanoma cells. A specific neo-epitope antibody recognizing only the cleaved fragment (19 kDa) confirmed this finding. While the significance of p23 cleavage is still not clear, it is possible that ER stress-induced cleavage of p23 resulting in the 19 kDa product abolishes the ability of p23 to act as an anti-apoptotic protein, thus rendering the melanoma cells more susceptible to pcd [37]. It is also possible that the 19 kDa cleaved product may display proapoptotic activity analogous to proteins such as Bcl-2 and Bid [62,63].

Our results also indicate that downregulation of the anti-apoptotic Mcl-1 protein expression and increased expression of pro-apoptotic Bax facilitates curcumin-mediated cell death in mouse melanoma cells. A short splice variant of Mcl-1 mRNA encoding a protein termed Mcl-1 short was recently identified [64–66]. Furthermore, it was suggested that Mcl-1S dimerizes with Mcl-1, downregulates its expression, blocks its anti-apoptotic properties and triggers pcd [42,44]. While we were unable to detect the expression of Mcl-1S, exposure of melanoma cells to curcumin triggered a time-dependent decrease in Mcl-1 expression that could lead to its inactivation. ER stress inducers including cellular stress inducers have also been shown to induce a change in Bax conformation resulting in its accumulation on the mitochondria and inducing the release of cytochrome c to activate the final steps of pcd [27,50,67]. Thus the balance between anti-apoptotic Bcl-2 family proteins (in this case Mcl-1) and pro-apoptotic

Bcl-2 proteins (in this case, Bax) modulates ER—mitochondrial-dependent melanoma cell survival versus death.

In summary, the present work suggests that curcumin triggers ER stress and induces apoptosis in melanoma cells featuring the up-regulation of GRP family proteins, activation of caspases, cleavage of p23, and disruption of the balance between anti- and pro-apoptotic Bcl-2 in favor of pro-apoptotic Bax activity. Aspects of the present study that are appealing are the facts that curcumin is a commonly used food spice, it can be classified as a small molecule, it can be used for topical application, and it does not trigger serious side effects, thus making it an attractive candidate for possible treatment and prevention of melanoma.

Abbreviations

ER, Endoplasmic reticulum; pcd, Programmed cell death; eIF2 α , Eukaryotic initiation factor-2 alpha; GRP, Glucose regulated protein.

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Fig. 1.

Curcumin triggers ER stress induced cell death in melanoma cells. GRP78 and GRP94 expression in (a) mouse melanoma cells treated with different concentrations of curcumin for different time periods and (b) in HEK293 cells and NIH3T3 mouse fibroblasts treated with different concentrations of curcumin for 24 h. Cell extracts (150 μg protein) were analyzed by Western blot analysis for GRP78, GRP94 and GAPDH (loading control) protein expression. Surviving versus dead cells were quantified as described in Section 'Methods'. Data (mean ± SE) are from at least three independent experiments

Fig. 2. Curcumin triggers ER stress-induced cell death in melanoma cells. Representative flow cytometry dot plots from curcumin-treated melanoma cells stained with GFPAn-V/Propidium iodide after 24 h of treatment. The average cell death counts are listed under each dot-plot and are expressed as a percentage of total dead cells

Fig. 3.

GADD 153 and Phospho-eIF2 α protein expression during ER stress. **(a)** Cell extracts (150 μ g protein) from untreated or curcumin-treated (75 μ M) melanoma cells were analyzed by Western blot analysis with Phospho-eIF2 α antibody, eIF2 α antibody or GADD 153 antibody. Membranes were also probed with anti-GAPDH antibody to serve as a loading control. **(b)** Melanoma cells were either left untreated or exposed to curcumin (75 μ M) in the presence or absence of salubrinal (Sal, 50 μ M), a specific inhibitor of phospho-eIF2 α phosphatase. Surviving versus apoptotic cells were analyzed by MTT assay as described in Section 'Methods'

Fig. 4.

Curcumin triggered ER stress-induced cell death involves cleavage of caspases. **(a)** Melanoma cells were treated with 75 μM curcumin for the indicated times. Cell-free cytosolic extracts (150 μg of protein) from untreated and curcumin-treated cells were prepared as described under Section "Experimental Procedures" and analyzed by Western blot analysis. Membranes were probed with anti-caspase-12, anti-caspase-9, anti-caspase-7 or anti-caspase-3 antibodies. All caspase antibodies were capable of detecting the pro and active forms. Cell extracts were also probed with GAPDH as a loading control. Each Western blot is representative of three independent experiments. **(b)** Melanoma cells were either left untreated or exposed to curcumin (75 μM) in the presence or absence of the cell permeable caspase specific inhibitor Q-VD-OPH (25 μM). Surviving versus apoptotic cells were analyzed by MTT assay as described in Section "Methods". Data are from three independent experiments

Fig. 5.

Effects of BAPTA-AM on curcumin-treated melanoma cells. **(a)** Melanoma cells were either left untreated or preincubated with BAPTA-AM (50 μ M) for 3 h followed by treatment with 75 μ M curcumin for 24 h. Surviving versus apoptotic cells were analyzed by MTT assay as described in Section 'Methods'. Data are from three independent experiments. **(b)** Melanoma cells were preincubated with BAPTA-AM (50 μ M) for 3 h followed by treatment with 75 μ M curcumin for 24 h. Cell extracts (150 μ g of protein) were prepared as described under Section "Experimental Procedures" and analyzed by Western blot analysis. Membranes were probed with anti KDEL antibody, anti-GADD 153 antibody or anti-caspase-7 antibody

Fig. 6. Curcumin triggers cleavage of caspase substrates. Melanoma cells were treated with 75 μ M curcumin for the indicated times. Cell extracts (150 μ g of protein) from untreated and curcumin-treated cells were prepared as described under Section “Experimental Procedures” and analyzed by Western blot analysis. Membranes were probed with anti-PARP, anti-p23, or neo specific anti-p19 antibody. Cell extracts were also probed with GAPDH as a loading control. Each Western blot is representative of three independent experiments

Fig. 7.

Effect of curcumin treatment on Bcl-2 family of proteins. Melanoma cells were treated with 75 μM curcumin for the indicated times. Cell extracts (150 μg of protein) from untreated and curcumin-treated cells were prepared as described under Section “Experimental Procedures” and analyzed by Western blot analysis. **(a)** Membranes were probed with anti-Mcl-1 antibody for detection of the anti apoptotic Mcl-1 protein. **(b)** Immunoprecipitation was performed with anti-Bax6A7 antibody that recognizes conformationally changed Bax, and the resulting immunoprecipitate was analyzed by immunoblotting using antisera specific for Bax protein