Inhibition of Endoplasmic Reticulum Stress–Induced Apoptosis of Melanoma Cells by the ARC Protein

Li Hua Chen, Chen Chen Jiang, Ralph Watts, Rick F. Thorne, Kelly A. Kiejda, Xu Dong Zhang, and Peter Hersey

Immunology and Oncology Unit, Calvary Mater Newcastle Hospital, Newcastle, New South Wales, Australia

Abstract

We have shown previously that most melanoma cell lines are insensitive to endoplasmic reticulum (ER) stress–induced apoptosis, but resistance can be reversed through activation of caspase-4 by inhibition of the MEK/ERK pathway. We report in this study that apoptosis was induced by the ER stress inducer thapsigargin or tunicamycin via a caspase-8–mediated pathway in the melanoma cell line Me1007, although the MEK/ERK pathway was activated in this cell line. The high sensitivity of Me1007 to ER stress–induced apoptosis was associated with low expression levels of the apoptosis repressor with caspase recruitment domain (ARC) protein that was expressed at relatively high levels in the resistant melanoma cell lines. Transfection of cDNA encoding ARC into Me1007 cells inhibited both caspase-8 activation and apoptosis induced by thapsigargin or tunicamycin. In contrast, inhibition of ARC by small interfering RNA knockdown sensitized the resistant melanoma cell lines to ER stress–induced apoptosis, which was inhibitable by blockage of caspase-8 activation. Both exogenous and endogenous ARC seemed to predominantly locate to the cytoplasm and mitochondria and could be coimmunoprecipitated with caspase-8. Taken together, ER stress can potentially activate multiple apoptosis signaling pathways in melanoma cells in a context-dependent manner. Whereas the MEK/ERK signaling pathway plays an important role in inhibiting ER stress–induced caspase-4 activation, ARC seems to be critical in blocking activation of caspase-8 in melanoma cells subjected to ER stress. [Cancer Res 2008;68(3):834–42]

Introduction

A number of cellular stress conditions, such as nutrient deprivation, hypoxia, alterations in glycosylation status, and disturbances of calcium flux, lead to accumulation and aggregation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen and cause so-called ER stress (1–3). The ER responds to the stress conditions by activation of a range of stress-response signaling pathways to alter transcriptional and translational programs, which couples the ER protein folding load with the ER protein folding capacity and is termed the unfolded protein response (1–3).

The unfolded protein response is fundamentally a cytoprotective response. However, excessive or prolonged unfolded protein response results in apoptotic cell death. Although caspase-12 in rodents and its human homologue caspase-4 are thought to be key mediators (4–6), activation of other caspases, including caspase-2, caspase-3, caspase-7, caspase-8, and caspase-9, has been reported to be involved in ER stress–induced apoptosis (4, 7–9). Several recent studies have challenged the role of caspase-12 and caspase-4 as ER stress was found to induce similar degrees of apoptosis regardless of the presence or absence of these caspases (10, 11). Nevertheless, we have shown that ER stress induces apoptosis in human melanoma cell lines by activation of caspase-4 when the MEK/ERK signaling pathway is inhibited (12). The majority of melanoma cell lines are otherwise relatively resistant to ER stress–induced apoptosis, except that one line, Me1007, seems to be exceptionally sensitive to apoptosis induced by ER stress inducers although the MEK/ERK pathway is also constitutively activated in the cells (12). This suggests that, besides activation of caspase-4, ER stress can potentially initiate other apoptotic mechanism(s), which is less sensitive to the inhibitory effect of the MEK/ERK pathway, but nevertheless blocked in most melanoma cell lines.

Apoptosis repressor with caspase recruitment domain (ARC) is an endogenous inhibitor of apoptosis that was initially thought to be primarily expressed in terminally differentiated cells, such as cardiac and skeletal myocytes and neurons (13). ARC seems to be distinct from most endogenous apoptosis inhibitors in that it cannot only inhibit death receptor–mediated apoptotic signaling by binding to and inhibiting caspase-8 but also blocks the mitochondrial apoptotic pathway by physically interacting with Bax and inhibiting its activation (13–15). Moreover, ARC can also bind to FADD, Fas, and caspase-2, which protects cells from apoptosis induced by varying stimuli (13, 14). Recently, ARC was reported to be expressed by various human cancer cells and to be largely located in nuclei (16, 17). However, the functional significance of endogenous ARC in cancer cells remains to be fully elucidated.

In the present report, we examined the mechanisms by which ER stress induces apoptosis in the melanoma cell line Me1007 and explored the potential role of ARC in protection of the majority of melanoma lines from ER stress–induced apoptosis. We show that the ER stress inducer thapsigargin or tunicamycin induced apoptosis of Me1007 cells by activation of caspase-8, which was inhibitable by overexpression of ARC. In addition, relatively high levels of endogenous ARC expression seemed to play a role in inhibition of caspase-8 activation and apoptosis induced by ER stress in most melanoma cell lines. In contrast to previous reports, both endogenous and exogenous ARC in
melanoma was predominantly located in the cytoplasm and mitochondria.

**Materials and Methods**

**Cell lines and fresh melanoma isolates.** Human melanoma cell lines Mel-RM, MM200, IgR3, Mel-CV, Me4405, Sk-Mel-28, Mel-FH, and Me1007 have been described previously (12, 18). They were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories). Melanocytes were kindly provided by Dr. P. Parsons (Queensland Institute of Medical Research) and cultured in medium supplied by Clonetics (Edward Kellar).

**Antibodies, recombinant proteins, and other reagents.** Tunicamycin and thapsigargin were purchased from Sigma Chemical Co. They were dissolved in DMSO and made up in stock solutions of 1 mmol/L, respectively. The cell-permeable general caspase inhibitor Z-Val-Ala-Asp(OMe)-CH$_2$F (z-VAD-fmk), the caspase-3 specific inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH$_2$F (z-DEVD-fmk), the caspase-9 specific inhibitor Z-Leu-Glu(Ome)-His-Asp(Ome)-CH$_2$F (z-LEHD-fmk), the caspase-8 specific inhibitor Z-Ile-Glu(Ome)-Thr-Asp(OMe)-CH$_2$F (z-IETD-fmk), and the caspase-2 specific inhibitor Z-Val-Ala-Asp(OMe)-Val-Ala-Asp(OMe)-CH$_2$F (z-VDVAD-fmk) were purchased from Calbiochem. The caspase-4 specific inhibitor Z-Leu-Glu-Val-Asp-FMK (z-LEVD-fmk) was from BioVision. The rabbit polyclonal antibody against caspase-3, caspase-8, caspase-2, and caspase-9 were from Stressgen. The mouse monoclonal antibody (mAb) against caspase-4 was from Abcam. The rabbit mAb against GRP78 was purchased from Santa Cruz Biotechnology. The rabbit polyclonal antibody against ARC was from Cayman. The mouse mAb against Flag M2 was purchased from Sigma. Isotype control antibodies used were the ID4.5 (mouse IgG2a) mAb against Salmonella typhi supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science), the 107.3

**Figure 1.** ER stress–induced apoptosis of Me1007 cells is caspase-dependent. A, left, thapsigargin (TG) or tunicamycin (TM) induces the ER stress response. Whole-cell lysates from Me1007 and Mel-RM cells treated with thapsigargin (1 μmol/L) or tunicamycin (3 μmol/L) for indicated periods were subjected to Western blot analysis of GRP78 expression. The data shown are representative of three individual experiments. Right, kinetics of thapsigargin-induced or tunicamycin-induced apoptosis of Me1007 cells. Me1007 and Mel-RM cells treated as above were subjected to measurement of apoptosis by the propidium iodide method using flow cytometry. Points, mean of three individual experiments; bars, SE. B, Me1007 cells were treated with the general caspase inhibitor z-VAD-fmk (20 μmol/L), the caspase-2 inhibitor z-VDVAD-fmk (50 μmol/L), the caspase-3 inhibitor z-DEVD-fmk (20 μmol/L), the caspase-4 inhibitor Z-LEVD-fmk (30 μmol/L), the caspase-8 inhibitor z-IETD-fmk (20 μmol/L), or the caspase-9 inhibitor z-LEHD-fmk (20 μmol/L) for 1 h before adding thapsigargin (1 μmol/L) or tunicamycin (3 μmol/L) for a further 96 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE. C, whole-cell lysates from Me1007 cells treated with thapsigargin (1 μmol/L) or tunicamycin (3 μmol/L) for indicated periods were subjected to Western blot analysis of caspase-3, caspase-4, caspase-8, and caspase-9. The lower parts of the caspase-8 and caspase-3 graphs were obtained from the same membranes with longer exposure, respectively, to better visualize the cleaved forms of caspase-8 and caspase-3. The arrow-pointed bands in figures for caspase-4 and caspase-3 are presumably either nonspecific bands or intermediately cleaved caspase-4 and caspase-3, respectively. The data shown are representative of three individual experiments.
mouse IgG1 mAb purchased from PharMingen, and rabbit IgG from Sigma Chemical Co.

**Apoptosis.** Quantitation of apoptotic cells by measurement of sub-G1 DNA content using the propidium iodide method was carried out as described elsewhere (12, 18). Melanoma cells were seeded onto sterile glass coverslips in 24-well plates (Falcon 3047; Becton Dickinson) for 16 to 24 h. MitoTracker Red CMXRos (50 nmol/L; Molecular Probes) was added to the culture medium for 30 min before washing cells with PBS, followed by fixation with 2% paraformaldehyde for 10 min. Cells were then permeabilized in 0.05% saponin diluted in PBS containing 10% human antibody serum, followed by incubation with primary antibodies, which were then detected with Alexa Fluor 488–conjugated antimouse or antirabbit immunoglobulin. Coverslips were mounted with SlowFade Gold medium (Invitrogen), and confocal images were acquired using a Zeiss LSM 510 scanner fitted to an Axiovert 100 M microscope. Dual-color analysis was performed with appropriate single-color controls with individual channels recorded sequentially. Instrument settings were adjusted to obtain minimal saturated pixels, and the settings were kept constant on where semiquantitative comparisons were performed.

**Caspase activity assay.** Measurement of caspase activities by fluorometric assays was performed as described previously (12). The specific substrates z-DEVD-afc, Ac-LEVD-afc, and z-LEHD-afc were used to measure caspase-3, caspase-4, and caspase-8 activities, respectively (Calbiochem). The generation of free AFC was determined using Fluostar OPTIMA (LABTECH) set at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

**Western blot analysis.** Western blot analysis was carried out as described previously (12, 18). Labeled bands were detected by Immun-Star horseradish peroxidase chemiluminescent kit, and images were captured and the intensity of the bands was quantitated with the Bio-Rad VersaDoc image system (Bio-Rad).

**Immunoprecipitation.** Methods used were as described previously with minor modification (12). Briefly, 100 µL of lysates were precleared by incubation with 20 µL of a mixture of protein A and protein G Sepharose packed beads (Santa Cruz) in a rotator at 4°C for 2 h and then with 20 µL of fresh packed beads overnight. Twenty micrograms of anti-Flag M2 antibody, anticaspase-8 antibody, or control immunoglobulin was then added to the lysates and rotated at 4°C for 2 h. The beads were then pelleted by centrifugation and washed five times with ice-cold lysis buffer before elution of the proteins from the beads in lysis buffer at room temperature for 1 h. The resulted immunoprecipitates were then subjected to SDS-PAGE and Western blot analysis.

**Reverse transcription–PCR.** Total RNA was extracted from cells by using SV total RNA isolation system (Promega). One microgram of RNA was subjected to reverse transcription. PCR was carried out in a two-step protocol using Moloney murine leukemia virus transcriptase (Invitrogen) and Taq DNA polymerase (Promega) according to the manufacturers’ instruction. The primer sequences for caspase-8 are forward, 5′-TGCCCTCAAGTTCCTGTGCTTGGA-3′; reverse, 5′-GGATGGATGATAAGTTTCATGATTCT-3′. Twenty-five microliters of mixture were used for reaction, which contains 100 ng cDNA sample, 3 µL MgCl2, 400 nmol/L deoxynucleotide triphosphate, 400 nmol/L primer mix, and 5 units/µL Taq DNA polymerase for 35 cycles with the annealing temperature of 60°C.

**Plasmid vector and transfection.** The pcDNA3-ARC-Flag was kindly provided by Dr G. Nunez (University of Michigan Medical School) and was described previously (12). Melanoma cells were seeded at 1 × 10⁴ cells per well in 24-well plates 24 h before being transfected with 0.8 µg of plasmid in Opti-MEM medium (Invitrogen) with 5% FCS using Lipofectamine reagent (Invitrogen) according to the manufacturer’s transfection protocol. Efficiency of siRNA was measured by Western blot analysis 24 h after transfection.

**Small RNA interference.** Melanoma cells were seeded at 3.5 × 10⁴ cells per well in 24-well plates and allowed to reach ~50% confluence on the day of transfection. The small interfering RNA (siRNA) constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon), the siGENOME SMARTpool ARC (D-015682-00), and the siGENOME SMARTpool caspase-8 (M-003466-01-04). The nontargeting siRNA control, SiControl siGENOMESMART pool ARC (D-001206-13-20), was also obtained from Dharmacon. Cells were transfected with 50 to 100 nmol/L siRNA in Opti-MEM medium (Invitrogen) with 5% FCS using OligoFectamine reagent (Invitrogen) according to the manufacturer’s transfection protocol. Efficiency of siRNA was measured by Western blot analysis 24 h after transfection.

**Results**

**ER stress–induced apoptosis in the melanoma cell line Me1007 is caspase dependent.** Our previous studies have shown that, in contrast to most melanoma cell lines that are resistant to ER stress–induced apoptosis, Me1007 is highly sensitive to apoptosis induced by the ER stress inducers thapsigargin and tunicamycin (12). To further study the susceptibility of this melanoma line to apoptosis induced by ER stress, we treated the cells with thapsigargin at 1 µmol/L or tunicamycin at 3 µmol/L for varying periods. The resistant melanoma line Mel-RM was used as a control. Figure 1A shows that whereas thapsigargin and tunicamycin induced rapid up-regulation of the ER chaperon GRP78 in both cell lines, indicative of activation of the ER stress response, they only induced significant apoptosis in Me1007 cells, which could be observed by 6 h and peaked at 36 h after treatment. This difference in sensitivities between the two cell lines was further evidenced in assays with DAPI staining (Supplementary Fig. S1).

We examined involvement of caspases in ER stress–induced apoptosis in Me1007 cells by treating the cells with the general caspase inhibitor z-VAD-fmk and specific inhibitors z-VDVAD-fmk against caspase-2, z-DEVD-fmk against caspase-3, z-LEHD-fmk against caspase-4, z-IEHD-fmk against caspase-8, and z-LEHD-fmk against caspase-9, 1 h before the addition thapsigargin or tunicamycin. As shown in Fig. 1B, z-VAD-fmk and the inhibitors against caspase-8, caspase-4, caspase-9, or caspase-3 inhibited thapsigargin-induced or tunicamycin-induced apoptosis to varying degrees. In contrast, the inhibitor against caspase-2 exhibited only minimal inhibitory effects on thapsigargin-induced or tunicamycin-induced apoptosis in Me1007 cells but blocked apoptosis of MM200 cells induced by TRAIL, which was used as a positive control for the caspase-2 inhibitor (Fig. 1B and data not shown).

Consistent with results in assays with the caspase inhibitors, Western blot analysis showed that caspase-8, caspase-4, caspase-9, and caspase-3 were activated by thapsigargin or tunicamycin in Me1007, but not Mel-RM, cells (Fig. 1C and data not shown). Whereas activation of caspase-8, caspase-4, and caspase-3 could be detected as early as at 6 h, caspase-9 activation could only be observed at or after 24 h. Of note, the proenzyme of caspase-8 was detected at very low levels in Me1007 cells before treatment but was markedly induced by thapsigargin or tunicamycin. Up-regulation of procaspase-8 by the ER stress inducers was not observed in Mel-RM cells (data not shown). Activation of caspase-8 and caspase-4 by thapsigargin or tunicamycin in Me1007 cells was also shown in fluorometric assays detecting activities of the caspases by specific substrates in whole-cell lysates (Supplementary Fig. S2).

**Caspase-8 plays an important role in ER stress–induced apoptosis of Me1007 cells.** We tested if activation of capasse-8 or
caspase-4 is an upstream factor in ER stress–induced apoptosis in Me1007 cells by monitoring caspase activation in the cells treated with the caspase-8 inhibitor z-IETD-fmk or the caspase-4 inhibitor z-LEVD-fmk, followed by the addition of thapsigargin or tunicamycin. Figure 2A shows that the caspase-8 inhibitor significantly blocked activation of both caspase-8 and caspase-4, but the caspase-4 inhibitor inhibited only caspase-4 activity with minimal effects on caspase-8 activation.

To further confirm the role of caspase-8 in ER stress–induced apoptosis of Me1007 cells, we silenced caspase-8 by a specific siRNA pool in the cells. As shown in Fig. 2B, the caspase-8 siRNA significantly inhibited up-regulation of caspase-8 by thapsigargin but had no effect on the levels of caspase-4. Figure 2C shows that siRNA knockdown of caspase-8 not only inhibited thapsigargin-induced apoptosis but also blocked activation of caspase-4 and caspase-3. Thus, activation of caspase-8 seemed to be an initiating factor in ER stress–induced apoptosis in Me1007 cells.

We studied the mechanism(s) by which procaspase-8 is up-regulated by examining caspase-8 mRNA expression in Me1007 cells.
before and after exposure to thapsigargin or tunicamycin. The results show that caspase-8 mRNA seemed to be low in Me1007 before treatment, but after exposure to thapsigargin or tunicamycin, there was an increase in the levels of caspase-8 mRNA, which could be detected as soon as 3 h and reached a peak by 6 h (Supplementary Fig. S3). This suggests that caspase-8 in Me1007 cells could be up-regulated by ER stress at the transcriptional level.

Endogenously expressed ARC contributes to resistance of melanoma to ER stress–induced apoptosis. To understand the mechanism(s) by which ER stress–induced caspase-8 activation is inhibited in most melanoma cell lines but not in Me1007, we studied the expression of ARC in a panel of melanoma cell lines. As shown in Fig. 3A, ARC was detected at varying but generally higher levels in melanoma cell lines in comparison with those in

Figure 3. Endogenously expressed ARC contributes to resistance of melanoma to ER stress–induced apoptosis. A, top, ARC is expressed in melanoma cell lines. Whole-cell lysates from melanocytes and a panel of melanoma cell lines were subjected to Western blot analysis of ARC expression. The data shown are representative of three individual experiments. Bottom, ER stress induces a decrease in ARC expression in Me1007 cells. Whole-cell lysates from Me1007 and Mel-RM cells treated with thapsigargin (1 μmol/L) or tunicamycin (3 μmol/L) for indicated periods were subjected to Western blot analysis of ARC expression. The data shown are representative of three individual experiments. B, top, Mel-RM and MM200 cells were transfected with the control or ARC siRNA. Twenty-four hours later, whole-cell lysates were subjected to Western blot analysis of ARC expression. The data shown are representative of three individual experiments. Bottom, Mel-RM and MM200 cells were transfected with the control or ARC siRNA. Twenty-four hours later, the cells were treated with thapsigargin (1 μmol/L) or tunicamycin (3 μmol/L) for a further 36 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± SE of three individual experiments. C, siRNA knockdown of ARC enhances thapsigargin-induced or tunicamycin-induced caspase-8 activation. Twenty-four hours later after transfection with the control or ARC siRNA, the cells were treated with thapsigargin (1 μmol/L) or tunicamycin (3 μmol/L) for a further 16 h. Whole-cell lysates were subjected to Western blot analysis of the expression of caspase-8. The data shown are representative of three individual experiments. D, the caspase-8 inhibitor z-IETD-fmk partially inhibits thapsigargin-induced or tunicamycin-induced apoptosis in Mel-RM and MM200 cells with ARC being knocked down. Twenty-four hours later, after transfection with the control or ARC siRNA, the cells were treated with z-IETD-fmk (30 μmol/L) for 1 h before the addition of thapsigargin (1 μmol/L) or tunicamycin (3 μmol/L) for a further 36 h. Apoptosis was measured by the propidium iodide method. Columns, mean of three individual experiments; bars, SE.
melanocytes. Of interest, the levels of ARC in Me1007 seemed to be the lowest among the melanoma cell lines. Figure 3A also shows that exposure to thapsigargin or tunicamycin did not cause any notable change in the levels of ARC in Mel-RM cells but resulted in a moderate decrease in ARC expression in Me1007 cells that could be observed as soon as 6 h after exposure to thapsigargin or tunicamycin.

To study if endogenous ARC plays a role in protection of melanoma cells from ER stress–induced apoptosis, we inhibited ARC with a specific siRNA pool in Mel-RM and MM200 cells. As shown in Fig. 3B, siRNA knockdown of ARC markedly inhibited its expression in both cell lines and resulted in an increase in apoptosis induced by thapsigargin or tunicamycin. This was associated with induction of caspase-8 activation as shown in both Western blot analysis and fluorometric assays (Fig. 3C and Supplementary Fig. S4). Figure 3D shows that pretreatment with the caspase-8 inhibitor z-IETD-fmk partially inhibited induction of apoptosis by thapsigargin or tunicamycin in Mel-RM and MM200 cells with ARC being knocked down by siRNA.

**Exogenous ARC inhibits ER stress–induced apoptosis in Me1007 cells.** To study if the high sensitivity of Me1007 cells to ER stress–induced apoptosis is associated with the low levels of ARC expression, we transfected cDNA encoding Flag-tagged ARC into Me1007 cells (Fig. 4A). The levels of thapsigargin-induced or
tunicamycin-induced apoptosis in ARC-transfected cells were significantly decreased in comparison with those in vector alone-transfected cells ($P < 0.05$, two-tailed Student's $t$ test; Fig. 4A). As shown in Fig. 4B, thapsigargin-induced or tunicamycin-induced caspase-8 activation was also reduced in ARC-transfected cells.

**ARC is physically associated with caspase-8 in melanoma cells.** To understand the mechanism(s) by which exogenous ARC protects Me1007 cells from ER stress-induced apoptosis, we carried out immunoprecipitation using the anti-Flag M2 antibody in whole-cell lysates from Me1007 cells transfected with Flag-tagged ARC before and after exposure to thapsigargin or tunicamycin. As shown in Fig. 4C, ARC, but not caspase-8, was detected in protein complexes precipitated with the anti-Flag antibody before treatment. In contrast, caspase-8 was readily seen along with ARC in the precipitates obtained from cells treated with thapsigargin or tunicamycin. The antibody M2-matched isotype control mouse IgG did not produce precipitates containing either ARC or caspase-8 (data not shown).

We next studied if the endogenous ARC was associated with caspase-8 in Mel-RM and MM200 cells. As shown in Fig. 4D, ARC was detected in caspase-8 complexes obtained with an antibody against caspase-8 in the whole lysates from Mel-RM and MM200 cells before and after exposure to thapsigargin or tunicamycin. In contrast, the ARC protein was not precipitated from whole-cell lysates using the isotype-matched control mouse IgG (data not shown). These results indicate that ARC can bind to caspase-8 in melanoma cells.

**ARC in melanoma is located to the cytoplasm and mitochondria.** We studied the subcellular localization of ARC using confocal microscopy as shown in Fig. 5A. The endogenous ARC in Mel-RM and MM200 cells stained with an anti-ARC antibody seemed to locate to the cytoplasm, with a proportion of the protein showing colocalization with mitochondria labeled with Mitotracker. A similar distribution was shown for exogenous ARC in Me1007 cells transfected with ARC-Flag and detected by the anti-Flag M2 antibody. No nuclear staining pattern of ARC was observed in the cell lines. Exposure to thapsigargin or tunicamycin did not cause any notable change in the subcellular localization of ARC (data not shown).

We confirmed absence of ARC in the nucleus and its mitochondrial localization in melanoma cells by Western blot analysis of isolated nuclear and mitochondrial fractions in Mel-RM cells and

![Figure 5.](cancerres.aacrjournals.org)
Me1007 cells expressing ARC-Flag. As shown in Fig. 5B, ARC was observed in the mitochondrial fractions, but not in the nuclear fractions from both cell lines. This further confirms the presence of ARC in mitochondria but not in nuclei in melanoma cells.

Discussion

As reported elsewhere, most melanoma lines are resistant to ER stress–induced apoptosis, although cultured melanocytes and fibroblasts undergo apoptosis under the same conditions (12). It was therefore of much interest that the melanoma cell line Me1007 was highly sensitive to apoptosis induced by the ER stress inducers thapsigargin or tunicamycin. This led us to a closer look at the mechanisms involved. In the present study, apoptosis of Me1007 cells induced by thapsigargin or tunicamycin seemed to depend on activation of caspase-8 that in turn gave rise to activation of caspase-4 and caspase-9 as shown by kinetic studies, experiments with specific caspase inhibitors, and knockdown of caspase-8 by siRNA.

Involvement of factors known to inhibit caspase-8 led to studies on ARC, which was found to be expressed at relatively high levels in most melanoma lines but was at low levels in Me1007 cells and cultured melanocytes. Moreover, when ARC was transfected into Me1007 cells, they became resistant to ER stress–induced apoptosis and activation of caspase-8 was also inhibited. The more general importance of the role of ARC in protection of melanoma cells from ER stress–induced apoptosis was shown by siRNA knockdown of ARC in two typical resistant melanoma lines, which showed that these cell lines became sensitive to ER stress–induced apoptosis that was dependent on activation of caspase-8. Immunoprecipitation studies clearly showed that both exogenous and endogenous ARC was physically associated with caspase-8. These results indicate that ARC is involved in protection of melanoma cells against ER stress–induced apoptosis.

ARC was identified as an endogenous CARD containing protein that inhibits apoptosis by selectively binding to and inhibiting caspase-8 and caspase-2, but not caspase-1, caspase-9, and caspase-3 (13). It can also interact with Bax and inhibits its activation and subsequently mitochondrial apoptotic events (14, 15). In addition, binding of ARC with Fas and FADD has been reported to play a role in inhibiting death receptor-mediated apoptosis (14). However, most of these observations were made in terminally differentiated cells, such as cardiac and skeletal myocytes, and neurons, which were initially believed to be the only cell types that express ARC (13–15, 21–26). It was not until recently that ARC has been found to be expressed in various human cancer cells (16, 17). The present study seemed to be the first to show that endogenous ARC has a functional role in most melanoma cell lines.

The levels of ARC expression in the apoptosis sensitive Me1007, but not those in the resistant melanoma lines, were rapidly decreased by treatment with thapsigargin or tunicamycin. This suggests that removal of the inhibitory effect of ARC may be required to ensure optimal induction of apoptosis by ER stress (14, 24, 27, 28). In support of this, degradation of ARC by the ubiquitin-proteasome pathway has been reported to be an initiating event in apoptosis induced by varying stimuli (27, 28). Alternatively, the decrease in ARC may be a consequence of induction of apoptosis. ARC was found to be phosphorylated at Thr149 by protein kinase CK2 and that this was required to target ARC to mitochondria (29). We found in this study that ARC in melanoma cells was predominantly located to the cytoplasm and mitochondria, but it is not yet known whether ARC is phosphorylated by protein kinase CK2 in melanoma cells. The cytoplasmic and mitochondrial localization of ARC in melanoma cells seemed to be different from previous reports that showed ARC to be mainly localized to nuclei in cancer cells (16). This difference may be due to the different types of cells used in the studies.

Importance of the role of ARC in protection of melanoma cells from ER stress–induced apoptosis was shown by siRNA knockdown of ARC in two typical resistant melanoma lines, which showed that these cell lines became sensitive to ER stress–induced apoptosis which was dependent on activation of caspase-8. Immunoprecipitation studies clearly showed that both exogenous and endogenous ARC was physically associated with caspase-8. These results indicate that ARC is involved in protection of melanoma cells against ER stress–induced apoptosis.

Many other mechanisms have been implicated in the induction of apoptosis by ER stress, such as CHOP-mediated up-regulation...
of TRAIL-R2 and/or down-regulation of Bel-2 (8, 35), up-regulation of the BH3-only protein PUMA, Noxa, and Bim (36–38), and activation of caspase-12 in murine systems and its counterpart caspase-4 in human cells (4–6). The present finding that ER stress induces apoptosis of melanoma cells by activation of caspase-8 when ARC is deficient, along with our previous studies showing that ER stress can induce apoptosis of melanoma cells by activation of caspase-4 when the MEK/ERK pathway is inhibited (12), indicates that ER stress can potentially activate multiple apoptotic signaling pathways. Other mechanisms involved may include calcium flux and activation of BH3-only proteins, such as PUMA, Noxa, and Bim (9, 25, 36, 38). It is conceivable that melanoma cells may have adapted to ER stress by inhibiting multiple apoptotic pathways as illustrated schematically in Fig. 6. The present study reveals that expression of the ARC protein is another adaptive mechanism by which melanoma cells are protected from ER stress–induced apoptosis and suggests that targeting ARC may improve treatment results of clinically available chemotherapeutic drugs and those in development for clinical use that can induce ER stress in melanoma cells, such as cisplatin and sorafenib (39, 40).

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