Glucose-regulated protein 78 antagonizes cisplatin and adriamycin in human melanoma cells

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Resistance of melanoma cells to chemotherapy remains a major obstacle to successful treatment of melanoma once it has spread beyond locoregional sites. We report in this study that activation of the unfolded protein response (UPR) is involved in resistance of melanoma cells to two chemotherapeutic drugs, cisplatin (CDDP) and adriamycin, and this is associated with glucose-regulated protein 78 (GRP78)-mediated inhibition of activation of caspase-4 and -7. The UPR was constitutively activated in cultured melanoma cell lines and fresh melanoma isolates as evidenced by elevated expression levels of the GRP78 protein and the active form of x-box-binding protein 1 messenger RNA. Treatment with CDDP or adriamycin further increased the levels, indicative of induction of endoplasmic reticulum stress and activation of the UPR by the drugs. Inhibition of GRP78 by small-interference RNA (siRNA)-sensitized melanoma cells to CDDP- and adriamycin-induced apoptosis. This was associated with enhanced caspase-4 and -7 activation as siRNA knockdown of the caspases blocked induction of apoptosis. In contrast, overexpression of GRP78 attenuated activation of caspase-4 and -7 and induction of apoptosis by the drugs. CDDP- and adriamycin-induced activation of caspase-4 and -7 appeared to be mediated by calpain activity in that it was blocked by the calpain inhibitors calpeptin and PD150606 even when GRP78 was inhibited by siRNA. These results provide new insights into resistance mechanisms of melanoma cells to CDDP and adriamycin and identify GRP78 as a potential target for enhancing chemosensitivity in melanoma.

Introduction

Melanoma continues to increase in incidence in many parts of the world, but there is currently no curative treatment once the disease has spread beyond the primary site because of the absence of effective systemic therapies. This is believed to be largely due to resistance of melanoma cells to induction of apoptosis by available chemotherapeutic drugs and biological reagents (1,2). Inappropriate activation of survival signaling pathways such as those mediated by extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK and phosphoinositide 3-kinase (PI3K)/Akt, either as consequences of genetic alterations or resulting from environmental stimulations, is believed to play a central role in resistance of melanoma to apoptosis (1,2). However, the potential effect of signaling pathways initiated by the endoplasmic reticulum (ER) upon stress stimuli remains undefined.

Abbreviations: CDDP, cisplatin; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GRP78, glucose-regulated protein 78; MAb, monoclonal antibody; MEK, ERK kinase; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small-interference RNA; TM, tunicamycin; UPR, unfolded protein response; XBP1, x-box-binding protein 1.

The ER responds to stress conditions by activation of a range of signaling pathways that couple the ER protein folding load with the ER protein folding capacity and is termed the unfolded protein response (UPR) (3–5). The UPR of mammalian cells is initiated by three ER transmembrane proteins—activating transcription factor 6, inositol-requiring enzyme 1 and double-stranded RNA-activated protein kinase-like ER kinase that act as proximal sensors of ER stress (3–5). Under unstressed conditions, the luminal domains of these sensors are occupied by the ER chaperone glucose-regulated protein 78 (GRP78). Upon ER stress, sequestration of GRP78 by unfolded proteins activates these sensors by inducing phosphorylation and homodimerization of inositol-requiring enzyme 1 and protein kinase-like ER kinase and relocation of activating transcription factor 6 to the Golgi where it is cleaved by site 1 and site 2 proteases, leading to its activation as a transcriptional factor (3–5).

The UPR is fundamentally a cytoprotective response, but excessive or prolonged UPR can result in apoptosis. This involves many of the same molecules that have important roles in other apoptotic cascades (6–10). Among them, caspase-12 in rodents and its human homologue caspase-4 are thought to be key mediators, whereas activation of other caspases, including caspase-2, -3, -7, -8 and -9, may also be involved (6,7,9). Although several recent studies have questioned the role of caspase-12 and -4 (11,12), we have found that the pharmacological ER stress inducers tunicamycin (TM) and thapsigargin can induce caspase-4-mediated apoptosis in human melanoma cell lines when the MEK/ERK pathway is inhibited (13). Caspase-4 is otherwise bound to and inhibited by GRP78 (13). The latter may also protect cells from apoptosis by a number of other mechanisms such as maintenance of ER calcium homeostasis and prevention of caspase-7 activation (14,15).

There is increasing evidence that the UPR is activated in various solid tumors, e.g. elevated expression of GRP78 has been reported in a number of cancers such as breast cancer and prostate cancer (14,15). It seems that some cancer cells may have adapted to ER stress by activation of the UPR without resulting in cell death. Persistent expression of proteins that facilitate cell survival such as GRP78 may be a central feature of adaptation to ER stress (16,17). In support of this, GRP78 expression is known, in some cases, to be associated with tumor development and growth and correlated with resistance to certain forms of chemotherapy (14,15). In particular, GRP78 is known to protect against various DNA-damaging agents, including the topoisomerase II inhibitors etoposide and adriamycin, the topoisomerase I inhibitor camptothecin and the alkylating agent temozolomide (18,19). On the other hand, induction of ER stress has been reported to be involved in induction of apoptosis in enucleated melanoma cells by the alkylating agent cisplatin (CDDP) (20).

We have studied the activation status of the UPR and its role in responses to CDDP and adriamycin in human melanoma cells. We show in this report that the GRP78 protein and the active form of x-box-binding protein 1 (XBP1) messenger RNA (mRNA) are expressed at elevated levels in cultured melanoma cell lines and fresh melanoma isolates, and the expression levels can be further increased by CDDP and adriamycin. We demonstrate that the UPR plays a role in protection of melanoma cells against the drugs, and this is, at least in part, due to GRP78-mediated inhibition of the activation of caspase-4 and -7. These results provide new insights into resistance of melanoma to CDDP and adriamycin and identify GRP78 as a potential target for sensitizing melanoma cells to chemotherapy.

Materials and methods

Cell lines

Human melanoma cell lines Mel-RM, MM200, IgR3, Mel-CV, Me4405, Sk-Mel-28, Mel-FH and Me1007 have been described previously (13,21). Among them, MM200, Me4405, Me1007 and IgR3 were from primary...
melanoma. Mel-RM, Mel-CV, Sk-Mel-28 and Mel-FH were from metastatic melanoma (21). They were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia). The cultured human melanocyte line HEMn-MP was purchased from Bankia Scientific (Bulintha, Queensland, Australia) and the cells were cultured in medium supplied by Clonetics (Edward Kellar, Victoria, Australia).

**Fresh melanoma isolates**

Isolation of melanoma cells from fresh surgical specimens was carried out as described previously (22,23). All fresh melanoma isolates were from metastatic melanomas.

**Antibodies, recombinant proteins and other reagents**

CDDP and adriamycin were supplied by Pharmacia Upjohn (Sydney, New South Wales, Australia). TM was purchased from Sigma Chemical Co. (Castle Hill, Australia). The caspase-3-specific inhibitor Z-Val-Ala-Asp(OMe)-CH2F (z-VAD-fmk) and the caspase-3-specific inhibitor Z-Val-Ala-Asp(OMe)-CH2F (z-VAD-fmk) were purchased from Calbiochem (La Jolla, CA). The caspase-4-specific inhibitor Z-Leu-Glu-Val-Asp-fmk (z-LEVD-fmk) was from BioVision (Mountain View, CA). The mouse monoclonal antibody (MAB) against caspase-4 was from Abcam (Cambridge, UK). The rabbit MAB against GRP78 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Isotype control antibodies used were the ID.45 (mouse IgG2a) MAB against Salmonella typhi supplied by Dr L. Ashman (Institute for Medical and Veterinary Science, Adelaide, Australia), and the 107.3 mouse IgG1 MAB was purchased from PharMingen (San Diego, CA) and rabbit IgG from Sigma Chemical Co.

**Cell viability assays**

The cytotoxic effect of CDDP on melanoma cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as described previously (24). Briefly, cells were seeded at 5000 per well onto flat-bottomed 96-well culture plates and allowed to grow for 24 h followed by the desired treatment. Cells were then labeled with MTT from the Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instruction.

**Apoptosis**

Quantitation of apoptotic cells by measurement of sub-G1 DNA content using the propidium iodide method or by Annexin-V staining was carried out as described elsewhere (13,24).

**Western blot analysis**

Western blot analysis was carried out as described previously (23,25). Labeled bands were detected by Immun-Star® HRP Chemiluminescent Kit, and images were captured and the intensity of the bands was quantitated with the Bio-Rad VersaDocTM image system (Bio-Rad, Regents Park, New South Wales, Australia).

**Detection of XBP1 mRNA splicing**

The method used for detection of unspliced and spliced XBP1 mRNAs was as described previously (13). Briefly, reverse transcription–polymerase chain reaction products of XBP1 mRNA were obtained from total RNA extracted using primers 5'-ggtagctgactcggctgtgggtagctc-3' (antisense). Because a 26 bp fragment containing an Apa-LI site is spliced upon activation of XBP1 mRNA, the reverse transcription–polymerase chain reaction products were digested with Apa-LI to distinguish the active spliced form from the inactive unspliced form. Subsequent electrophoresis revealed the inactive form at two cleaved fragments and the active form as a non-cleaved fragment.

**Flow cytometry**

Immunostaining on intact and permeabilized cells was carried out as described previously. Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer (21,22).

**Caspase activity assay**

Measurement of caspase activities by fluorometric assays was performed as described previously (13). The specific substrates Z-DEVD-AFC, Ac-LEVD-AFC and z-LEHD-AFC were used to measure caspase-3, -4 and -9 activities, respectively (Calbiochem). The generation of free AFC was determined using Fluostar OPTIMA (LABTECH, Offenburg, Germany) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

**Calpain activity assay**

Measurement of calpain activities by fluorometric assays was performed as described previously (26). Briefly, cells were suspended in cold lysis buffer (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, 5 mM MgCl2, 42 mM KCl and 0.32 M sucrose) and lysed by repeated passage through a 27 gauge syringe. For each sample, 40 μg of protein was incubated with the calpain substrate N-Succ-Leu-Tyr-AMC (Suc) (Sigma Chemical Co.) in assay buffer (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, 1% Triton X-100 and 100 μM CaCl2) at 37°C for 2 h. The generation of free AMC was determined using Fluostar OPTIMA (LABTECH) set at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

**Small-interference RNA**

Melanoma cells were seeded at 4 × 104 cells per well in 24-well plates and allowed to reach ~50% confluence on the day of transfection. The small-interference RNA (siRNA) constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, CO), the siGENOME SMARTpool GRP78 (M-008198-01), the siGENOME SMARTpool caspase-4 (M-004404-00) and the siGENOME SMARTpool caspase-7 (M-004407-02). The non-targeting siRNA control SiConTRolNon-targeting SiRNA pool (D-001206-13-20) was also obtained from Dharmacon. Cells were transfected with 50–100 nM siRNA in Opti-MEM Medium (Invitrogen, Carlsbad, CA) with 5% fetal calf serum using Lipofectamine Reagent (Invitrogen) according to the manufacturer’s transfection protocol. Twenty-four hours after transfection, the cells were switched into medium containing 5% fetal calf serum and were treated as designed before quantitation of apoptotic cells by measurement of sub-G1 DNA content using the propidium iodide method in flow cytometry. Efficiency of siRNA was measured by western blot analysis.

**Results**

**Expression of GRP78 in melanoma cell lines and fresh melanoma isolates**

To study if melanoma cells may express increased levels of GRP78, we examined GRP78 expression in a panel of melanoma cell lines in western blot analysis. These include four melanoma cell lines established from primary (MM200, Me4405, Me1007 and Igr3) and another four from metastatic melanomas (Mel-RM, Mel-CV, Sk-Mel-28 and Mel-FH). A cultured melanocyte line was included as a control. The results showed that in comparison with the melanocyte line, melanoma cell lines expressed varying but generally higher levels of GRP78 (Figure 1A). Quantitation of western blot band densities indicated that the relative levels of GRP78 expression differed by up to four times among the melanoma cell lines (supplementary Figure 1 available at Carcinogenesis Online), but there was no significant difference in the levels of GRP78 between melanoma cell lines from primary and those from metastatic melanomas (P > 0.05, two-tailed student’s t-test). Similarly, there was no apparent association between the levels of GRP78 expression and p53 or BRAF status in the melanoma cell lines (data not shown).

We examined if melanoma cells in vivo also express elevated levels of GRP78. Five metastatic melanoma specimens from patients undergoing surgery before adjunctive treatment were processed, and the resulting fresh melanoma isolates were tested for GRP78 expression. Compared with melanocytes, all the fresh isolates expressed increased levels of GRP78, which were even higher than those in most cultured melanoma cell lines (Figure 1A and supplementary Figure 1 is available at Carcinogenesis Online). These results indicate that GRP78 is commonly expressed at increased levels in melanoma cells, conceivably as a consequence of activation of the UPR due to chronic ER stress. To confirm this, expression of another indicator of UPR activation, the spliced XBP1 mRNA (27,28), was analyzed by polymerase chain reaction. As shown in Figure 1B, the spliced XBP1 mRNA could not be detected in the melanocyte line but was observed to varying degrees in all the melanoma cell lines and fresh melanoma isolates.

**CDDP and adriamycin upregulate GRP78 in melanoma cells**

We studied whether CDDP and adriamycin impinge on ER stress and GRP78 expression in melanoma cells. Mel-RM and MM200 cells were treated with the drugs for varying periods ranging from 6 to 36 h. Figure 2A shows that, while the established ER stress inducer TM induced marked increases in the levels of GRP78 in both cell lines (13,23), CDDP and adriamycin also caused upregulation of GRP78 with similar kinetics, albeit to a lesser extent. We also monitored the levels of the...
The UPR in chemosensitivity of melanoma

GRP78 protects melanoma cells against killing induced by CDDP and adriamycin

GRP78 is known to be an important prosurvival factor in cells under ER stress (14,29). We examined whether it also protects melanoma cells against cytotoxicity mediated by CDDP and adriamycin. First, the cytotoxic potentials of the drugs were tested in the panel of melanoma cell lines. As shown in Figure 3A, exposure to CDDP or adriamycin for 48 h reduced cell viability to varying degrees as measured in MTT assays. This was associated with externalization of phosphatidylserine, activation of caspase-3 and accumulation of sub-G1 DNA content (supplementary Figure 2 is available at Carcinogenesis Online) and could be inhibited by the general caspase inhibitor z-VAD-fmk (Figure 3B), indicating that the cytotoxicity of the drugs against melanoma cells was largely mediated by induction of apoptosis. We analyzed the relationship between the GRP78 levels and sensitivities of melanoma cell lines to CDDP and adriamycin but did not find any significant correlation (regression analysis, $X^2 = 0.2161$ and $0.0176; P > 0.05$, respectively) (supplementary Figure 3 is available at Carcinogenesis Online).

To study the role of GRP78 in regulating sensitivity of melanoma cells to the drugs, we transfected a siRNA pool for GRP78 into Mel-RM and MM200, two melanoma cell lines with moderate levels of GRP78 expression. Western blot analysis showed that the levels of GRP78 were markedly reduced in cells transfected with the GRP78 siRNA in comparison with those with the control siRNA (Figure 3C). Assessment of apoptosis induction indicated that inhibition of GRP78 by siRNA resulted in significant increases in sensitivity of both cell lines to killing induced by CDDP or adriamycin ($P < 0.01$, two-tailed student’s t-test) (Figure 3C).

To confirm the role of GRP78 in protection of melanoma cells against CDDP and adriamycin, Mel-RM and MM200 cells were transfected with complementary DNA encoding GRP78. Western blot analysis verified that GRP78 expression was markedly increased in cells transfected with GRP78 complementary DNA but not in those transfected with the vector alone (Figure 3D). Overexpression of GRP78 markedly inhibited CDDP- or adriamycin-induced killing in both cell lines ($P < 0.01$, two-tailed student’s t-test) (Figure 3D). Taken together, these results indicate that GRP78 antagonizes cytotoxic effects of cisplatin and adriamycin in melanoma cells.

GRP78 inhibits activation of the caspase cascade induced by CDDP and adriamycin

We have shown previously that GRP78 can bind to caspase-4 and inhibit its activation in melanoma cells submitted to ER stress (13). In addition, GRP78 is also known to interact with caspase-7 and suppress its activation (18,19). We therefore studied whether GRP78-mediated protection of melanoma cells against CDDP and adriamycin is associated with inhibition of activation of caspase-4 and -7. First, we examined if CDDP and adriamycin induce activation of caspase-4 and -7 in melanoma cells by western blot analysis of whole-cell lysates from Mel-RM and MM200 cells treated with the drugs. Figure 4A shows that both drugs induced activation of caspase-4 and -7, as evidenced by reduction in the proenzyme levels and appearance of smaller cleaved forms of the caspases. Activation of caspase-4 and -7 was also confirmed in fluorometric assays using caspase-4-specific substrate Ac-LEVD-AFC and the caspase-7 substrate Ac-DEVD-AFC, respectively (Figure 4A).

To further study the role of caspase-4 and -7 in CDDP- and adriamycin-mediated killing of melanoma cells, we silenced
caspase-4 and -7 by specific siRNA pools in Mel-RM and MM200 cells, respectively. Figure 4B shows that while the caspase-4 siRNA significantly reduced the levels of caspase-4 but not caspase-7 expression, the caspase-7 siRNA similarly decreased the levels of caspase-7 but not caspase-4 expression. CDDP- and adriamycin-mediated killing of Mel-RM and MM200 cells were significantly inhibited in cells transfected with the caspase-4 and -7 siRNA, respectively, in comparison with those transfected with the control siRNA ($P < 0.01$, two-tailed student’s $t$-test) (Figure 4B).

We next examined the role of GRP78 in regulating activation of caspase-4 and -7 induced by CDDP or adriamycin. As shown in Figure 4C, inhibition of GRP78 by siRNA knockdown (Figure 3C)
enhanced, whereas overexpression of GRP78 (Figure 3D) attenuated caspase-4 and -7 activities induced by CDDP and adriamycin as measured in fluorometric assays. Inhibiting activation of caspase-4 and -7 by overexpression of GRP78 was also confirmed in western blot analysis (supplementary Figure 4 is available at Carcinogenesis Online). Collectively, these results demonstrate that GRP78 protects...
Corresponding parental cells and are expressed as the fold increase. The data shown are the mean ± SE of three individual experiments. (A) Whole-cell lysates from Mel-RM and MM200 cells treated with CDDP (10 μg/ml) or adriamycin (1 μM) for 36 h in the presence or absence of calpeptin (20 μM) were incubated with N-Suc-Leu-Tyr-AMC (Suc) for 20 min and subjected to fluorometric assays. The values of calpain activity in cells without any treatment were arbitrarily designated as 1. The values of calpain activity in cells of each experimental group were compared with those in the corresponding control cells and are expressed as the fold increase. The data shown are the mean ± SE of three individual experiments. (B) Inhibition of calpain by PD150606 partially blocks activation of caspase-4 and -7 induced by CDDP and adriamycin. Whole-cell lysates from Mel-RM and MM200 cells treated with CDDP (10 μg/ml) or adriamycin (1 μM) for 36 h in the presence or absence of PD150606 (40 μM) were subjected to measurement of caspase-4 and -7 activity with specific substrates in fluorometric assays. The values in the corresponding parental cells without treatment were arbitrarily designated as 1 (data not shown). The values of activity in cells of each experimental group were compared with those in the corresponding parental cells and are expressed as the fold increase. The data shown are the mean ± SE of three individual experiments. (C) Inhibition of calpain by PD150606 partially blocks apoptosis of melanoma cells induced by CDDP and adriamycin. Whole-cell lysates from Mel-RM and MM200 cells treated with CDDP (10 μg/ml) or adriamycin (1 μM) for 48 h in the presence or absence of PD150606 (40 μM). Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± SE of three individual experiments.

**Calpain activity contributes to activation of caspase-4 and -7 induced by CDDP or adriamycin**

Calpain activity has been reported to contribute to CDDP-induced caspase-7 activation in melanoma cells (26). We examined if calpain activity similarly plays a role in caspase-4 activation induced by CDDP and if it is involved in activation of the caspases by adriamycin in melanoma cells. As expected, treatment with CDDP resulted in increased calpain activity (Figure 5A). Similarly, exposure to adriamycin also led to increases in calpain activity in melanoma cells (Figure 5A). Inhibition of calpain activity by the inhibitor PD150606 or calpeptin (Figure 5A) partially blocked activation of caspase-4 and -7 and apoptosis induced by CDDP and adriamycin (Figure 5B and C). These results indicate that calpain activity is an initiating factor for activation of ER-associated caspases in melanoma cells induced by CDDP and adriamycin.

**Discussion**

Resistance of melanoma cells to chemotherapeutics is a major obstacle to successful treatment of melanoma once it has spread beyond locoregional sites. In the present study, we show that activation of the UPR contributes to resistance of melanoma cells against the chemotherapeutic drugs CDDP and adriamycin, and this is, at least in part, due to inhibition of activation of the ER-associated caspases, caspase-4 and -7, by GRP78. These results provide new insights into resistance mechanisms of melanoma cells to chemotherapy and may have important therapeutic applications in the treatment of melanoma.

Although elevated GRP78 expression levels have been reported in a number of solid cancers (14,15), the present study appears to be the first to show that GRP78 is expressed at relatively high levels in both cultured melanoma cell lines and fresh melanoma isolates. The elevated levels of the GRP78 protein, along with the increased levels of the spliced XBP1 mRNA, suggest that the UPR in melanoma cells is constitutively activated (27,30). In support of this, we found in a separate study on tissue sections from a large panel of melanocytic tumors demonstrated that GRP78 was expressed at relatively high levels on most melanoma tissue sections (data not shown). Given the highly malignant nature of melanoma, it is conceivable that the rapid growth rate and perhaps inadequate vascularization would create a microenvironment with hypoxia, glucose deprivation and acidosis, which in turn results in chronic ER stress. In addition, increased glycolytic activity in melanoma cells may also contribute to ER stress (14,15,29). In support of this, increased lactate dehydrogenase levels, indicative of increased glycolytic activity, are common in metastatic melanomas (31,32). Whether there are other properties of melanoma cells that predispose to ER stress remains unknown. It has recently been reported that ER stress was induced at early stages of melanoma initiation by transfection of melanocytes with oncogenic forms of HRAS (HRAS(G12V)) (33).

GRP78 appeared to protect melanoma cells against cytotoxic effects of CDDP and adriamycin. This was largely due to inhibition of induction of apoptosis, as killing of melanoma cells by the drugs was associated with hallmarks of apoptosis such as externalization of phosphatidylserine, accumulation of sub-G1 DNA content and activation of caspase-3, which could be efficiently inhibited by a general caspase inhibitor. Although DNA adducts are generally believed to be
the key toxic lesions induced by CDDP (34), a number of recent studies have shown that CDDP can exert cytotoxicity independently of its DNA-damaging activity (20,26,35). Particularly, CDDP induced ER stress-mediated apoptotic signaling in enucleated cells of the human melanoma cell line 224 (20). We observed in this study that CDDP induced increases in the GRP78 protein and the spliced XBP1 mRNA levels, indicating that induction of ER stress and activation of the UPR is a general effect of CDDP on melanoma cells. Similarly, we found, for the first time, that the topoisomerase II inhibitor adriamycin also caused ER stress and activation of the UPR in melanoma cells. Together, these results suggest that CDDP and adriamycin, two chemotherapeutic drugs that were conventionally regarded as DNA-damaging agents, can exert their cytotoxicity in melanoma cells by inducing ER stress. Their cytotoxicity is, however, attenuated by GRP78 in human leukemia and bladder carcinoma cells via induction of calpain activity (26). The present study demonstrated that GRP78 can bind to and inhibit caspase-4 in human neuroblastoma cells (10). The physical association between GRP78 and caspase-4 suggests that caspase-4 may also be present in the ER in melanoma cells. This was supported by the punctate staining pattern of caspase-4 in immunofluorescence studies, indicative of organelle localization as shown previously (13).

Another caspase that appeared to involve in CDDP- and adriamycin-induced apoptosis was caspase-7. This was shown by activation of caspase-7 by the drugs and inhibition of apoptosis by siRNA knockdown of the caspase. Although caspase-7 is an executor caspase (38), it has been shown to be localized to the ER membrane where it was bound to and inhibited by GRP78 in human leukemia and bladder carcinoma cell (18). Similarly, we found that inhibition of GRP78 by siRNA increased, whereas overexpression of GRP78 inhibited caspase-7 activity. This is consistent with our previous finding that GRP78 can bind to and inhibit caspase-4 (13). Caspase-4 has been shown to be localized to the ER membrane and mitochondria in human neuroblastoma cells (10). The physical association between GRP78 and caspase-4 suggests that caspase-4 may also be present in the ER in melanoma cells (13). This was supported by the punctate staining pattern of caspase-4 in immunofluorescence studies, indicative of organelle localization as shown previously (13).

CDDP has been reported to activate caspase-7 in human melanoma cells via induction of calpain activity (26). The present study demonstrated that calpain activity was also responsible for caspase-4 activation induced by CDDP and adriamycin in melanoma cells. In the murine system, a number of mechanisms have been suggested to be responsible for caspase-12 activation (7,39–41). For example, caspase-12 could be activated by a direct association with the ER stress transducer inositol-requiring enzyme 1α and the adapter protein TRAF2 (40). In addition, caspase-12 could be directly activated downstream of caspase-7 upon ER stress (7,39). Our observations did not support a role of caspase-7 in activation of caspase-4 by CDDP and adriamycin in melanoma cells, as caspase-7 and -4 were activated with similar kinetics, and siRNA inhibition of caspase-7 did not block caspase-4 activation (data not shown). It is possible that these caspases may be simultaneously activated by calpain activity, at least in the case of treatment with CDDP or adriamycin.

Besides inhibition of caspase-4 and -7, GRP78 may also protect melanoma cells against CDDP and adriamycin by other mechanisms (14,29). For example, as a protein chaperone, GRP78 can bind to unfolded/misfolded protein and thereby protecting cells against ER stress (14,29). GRP78 is also known to act as a calcium-binding protein in the ER, thus preventing calcium efflux into the cytosol and inhibiting apoptosis (14,29). Similarly, activation of the UPR may provide other protective mechanisms against CDDP and adriamycin apart from induction of GRP78 (42,43).

In summary, we demonstrate that activation of the UPR plays an important part in protection of melanoma cells against cytotoxic effects of CDDP and adriamycin. This is mediated, at least in part, by GRP78-mediated inhibition of caspase-4 and -7. Therefore, agents that target GRP78, such as the macrocyclic compound versipelostat and the green tea extract epigallocatechin, both of which are natural products and in development for clinical use (14,44,45), could be expected to have a significant role for sensitizing melanoma cells to these chemotherapeutic drugs.

Supplementary material

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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