Up-Regulation of Mcl-1 Is Critical for Survival of Human Melanoma Cells upon ER Stress

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ABSTRACT

We have previously shown that most melanoma cell lines are insensitive to endoplasmic reticulum (ER) stress-induced apoptosis, and this involves activation of the MEK/ERK signaling pathway and expression of the apoptosis repressor with caspase recruitment domain (ARC) protein in the cells. In the present study we show that up-regulation of the anti-apoptotic Bcl-2 family member Mcl-1 is another mechanism critical for protection of melanoma cells against ER stress-induced apoptosis. Inhibition of Mcl-1 by small interference RNA (siRNA) rendered melanoma cells sensitive to apoptosis induced by the ER stress inducers thapsigargin and tunicamycin, but this sensitization was partially reversed by siRNA knockdown of PUMA or Noxa, as demonstrated in Mcl-1-deficient melanoma cells. Both PUMA and Noxa were increased by ER stress through transcriptional up-regulation, but only up-regulation of Noxa was dependent on p53, whereas up-regulation of PUMA appeared to be mediated by a p53-independent mechanism(s). Up-regulation of Mcl-1 was also due to increased transcription that involved the IRE1α and ATF6 signaling pathways of the unfolded protein response. In addition, activation of the MEK/ERK signaling pathway appeared to be necessary for optimal up-regulation of Mcl-1. Taken together, these results reveal the mechanisms of resistance of melanoma cells to apoptosis induction mediated by BH3-only proteins upon ER stress, and identify Mcl-1 as a target for the treatment of melanoma in combination with therapeutics that induce ER stress.

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 (UPR) (1-3).

The UPR of mammalian cells is initiated by three ER transmembrane proteins - activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and double-stranded RNA-activated protein kinase-like ER kinase (PERK) that act as proximal sensors of ER stress. Under unstressed conditions, the luminal domains of these sensors are occupied by the ER chaperon glucose-regulated protein 78 (GRP78) (1-3). Upon ER stress, sequestration of GRP78 by unfolded proteins activates these sensors by inducing phosphorylation and homodimerization of IRE1 and PERK, and relocalization of ATF6 to the Golgi where it is cleaved by Site 1 and Site 2 proteases (S1P and S2P) leading to its activation as a transcriptional factor (1-3).

The UPR is fundamentally a cyto-protective 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 (4-7). Among them, Bcl-2 family proteins appear to be critical, as ER stress-induced apoptosis can be inhibited by over-expression of Bcl-2 or its anti-apoptotic homologs, suggesting that activation of pro-apoptotic proteins of this family is important in initiating ER stress-mediated apoptotic signaling (5, 6). In support of this, Bax- and Bak-deficient cells are resistant to ER stress-induced apoptosis (5). A number of BH3-only proteins, including PUMA, Noxa, Bim, and BIK, have been shown to be up-regulated/activated (8-13), whereas Bcl-2 and Mcl-1 have been reported to be

down-regulated (14, 15), thus contributing to induction of apoptosis by ER stress. In addition, various other mechanisms have been shown to play roles in initiating apoptotic signaling by ER stress, such as activation of caspase-8, -2, and caspase-12 in murine systems and its counterpart caspase-4 in human cells (5, 6, 16-19).

Most cultured human melanoma cell lines are not sensitive to ER stress-induced apoptosis (17, 18). While the MEK/ERK signaling pathway is important for inhibiting ER stress-induced caspase-4 activation (18), the apoptosis repressor with caspase recruitment domain (ARC) protein is critical in blocking activation of casapse-8 in melanoma cells subjected to ER stress (17). It seems that ER stress can potentially activate multiple apoptosis signaling pathways, but they are apparently inhibited, conceivably also by various mechanisms such as activation of MEK and ARC, in melanoma cells. However, the role of Bcl-2 family of pro- and anti-apoptotic proteins in determining sensitivity of melanoma cells to ER stress-induced apoptosis remains undefined.

We show in this report that up-regulation of the anti-apoptotic Bcl-2 family member Mcl-1 is critical for survival of melanoma cells by neutralizing the BH3-only proteins PUMA and Noxa, which are also increased by ER stress. Up-regulation of Mcl-1 is due to increased transcription that is mediated, at least in part, by activation of the IRE1α and ATF6 signaling pathways of the UPR. In addition, the MEK/ERK pathway also plays a role in ER stress-induced increases in Mcl-1 levels. These results reveal the mechanisms by which melanoma cells are refractory to apoptosis mediated by BH3-only proteins under ER stress, and identify Mcl-1 as a potential target for the treatment of melanoma in combination with therapeutics that induce ER stress.

MATERIALS AND METHODS

Cell Lines

Human melanoma cell lines Mel-RM, MM200, IgR3, Mel-CV, Sk-Mel-28, and Mel-FH have been described previously (17, 18). They were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Australia).

Antibodies, Recombinant Proteins, and Other Reagents

Tunicamycin (TM) and thapsigargin (TG) were purchased from Sigma Chemical Co. (Castle Hill, Australia). They were dissolved in DMSO and made up in stock solutions of 1mM. Actinomycin D was also purchased from Sigma Chemical Co. (Castle Hill, Australia). The MEK inhibitor, U0126, was purchased from Promega Corporation (Madison, WI). The mouse MAbs against Bcl-2, Bcl-X_L, and Mcl-1, and the rabbit polyclonal Ab against Smac, GRP78, BIK, IRE1α, ATF6 and PERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The MAb against Noxa and the polyclonal Ab against Bim were purchased from Imgenex (San Diego, CA). The rabbit polyclonal Ab against PUMA was from Cell Signalling Technology (Beverly, MA). The mouse MAb against cytochrome c was from Pharmingen (Bioclone, Marrickville, Australia). The rabbit polyclonal anti-Bax against amino acids 1 through 20 was purchased from Upstate Biotechnology (Lake Placid, NY). The mouse MAb against Bak (Ab-1) was purchased from Calbiochem (La Jolla, CA). Isotype control Abs used were the ID4.5 (mouse IgG2a) MAb against *Salmonella typhi* supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, Australia), the 107.3 mouse IgG1 MAb purchased from PharMingen (San Diego, CA), and rabbit IgG from Sigma Chemical Co. (Castle Hill, Australia).

Apoptosis

Quantitation of apoptotic cells by measurement of sub-G1 DNA content using the propidium iodide method was carried out as described elsewhere (17, 18).

Flow Cytometry

Immunostaining on intact and permeabilized cells was carried out as described previously (17, 18). Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer.

Mitochondrial Membrane Potential (ΔΨm)

Melanoma cells were seeded at $1x10^5$ cells/well in 24-well plates and allowed to reach exponential growth for 24 hours before treatment. Changes in ΔΨm were studied by staining the cells with the cationic dye, JC-1, according to the manufacture's instructions (Molecular Probes, Eugene, OR) as described previously (20).

Western Blot Analysis

Western blot analysis was carried out as described previously (17, 18). 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 VersaDoc™ image system (Bio-Rad, Regents Park, NSW, Australia).

Plasmid Vector and Transfection

Stable Mel-RM and MM200 transfectants of *Bcl-2* were established by electroporation of the pEF-puro vector carrying human Bcl-2 provided by Dr. David Vaux (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia) and described elsewhere (20, 21). Mcl-1 cDNA cloned into p3XFLAG-CMV-10 was provided by Dr. Xiaodong Wang (Howard Hughes Medical Institute, Texas, USA) and described elsewhere (22). Melanoma cells were seeded at $1x10^5$ cell per well in 24-well plate 24 hours before transfection. Cells

were transfected with 0.8μg plasmid as well as the empty vector (Sigma-Aldrich, Castle Hill, NSW, Australia) in Opti-MEM medium (Invitrogen, Carlsbad, CA) with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Six hours after transfection, the cells were switched into antibiotic-free medium containing 5% FCS for a further 24 hours. Cells were then passaged at 1: 10 into fresh medium for further 24hours followed with G418 (Sigma-Aldrich, Castle Hill, NSW, Australia) selection.

Real-Time PCR

Real-time RT-PCR was performed using the ABI Prism 7700 sequence detection system (PE Applied Biosystems) as described previously (18). Twenty-five μl mixture was used for reaction, which contains 5μl cDNA sample (0.5-1μg/μl), 300nM forward primers for Mcl-1 (GGAAGGCGCTGGAGACCT TA), PUMA (GCATGCCTGCCTCACCTT), or Noxa (TGGAAGTCGAGTGTGCTACTCAA), 300nM reverse primers for Mcl-1 (CAACGATTTCACATCGTCTTCGT), PUMA (TCACACGTCGCTCTCTCTAAACC), or Noxa (CAGAAGAGTTTGGATATCAGATTCAGA), 200nM probes for Mcl-1 (VIC-TTG ATG TCC AGT TTC CGA AGC ATG CCT- TAMRA), PUMA (6FAM-CCC CGC CCC ATC AAT CCC AT-TAMRA), or Noxa (VIC-TTTCTGCCGGAAGTTCAGTTTGTCTCCAA-TAMRA), and 9mM $MgCI_2$. For Bcl-2, assay-on-demand for Bcl-2 (Assay ID: Rn99999125_m1) was used according to manufacturer's protocol (Applied Biosystems, Foster City, CA). Analysis of cDNA for β-actin was included as a control. After incubation at 50°C for 2 min followed by 95°C for 10 min, the reaction was carried out for 40 cycles of the following: 95°C for 15 sec and 60°C for 1 min. The threshold cycle value (Ct) was normalized against β-actin cycle numbers. The relative abundance of mRNA expression of a control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly.

Small RNA Interference (siRNA)

The siRNA constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, CO), the siGENOME SMARTpool Bcl-2 (M-003307-04-0010), the siGENOME SMARTpool Mcl-1 (M-004501-04-0010), the siGENOME SMARTpool PUMA (M-004380-01-0010), the siGENOME SMARTpool Noxa (M-026087-00-0010), the siGENOME SMARTpool Bim (M-004383-01-0010), the siGENOME SMARTpool MEK1 (M-003571-01-0010), the siGENOME SMARTpool IRE1α (M-004951-01-0010), the siGENOME SMARTpool ATF6 (M-009917-01-0010), the siGENOME SMARTpool PERK (M-004883-01-0010), and the ON-TARGETplus SMARTpool p53 (L-003329-00-0005). The non-targeting siRNA control, SiConTRolNon-targeting SiRNA pool (D-001206-13-20) was also obtained from Dharmacon. Transfection of siRNA pools was carried out as described previously (18).

Short Hairpin RNA (shRNA) Knockdown of Mcl-1

Melanoma cell lines were seeded at $1x10^4$ per well in 96 well plates and left to attach overnight. Sigma MISSION® Lentiviral Transduction Particles for shRNA-mediated knockdown of Mcl-1 (clone IDs TRCN0000005514-18) were applied to ~70% confluent cells in the presence of polybrene (4 or 8 μg/ml) at MOIs of 0.5, 1 or 5 in 100 μl DMEM. After 16 - 24 hours, the culture medium was replaced and cells were left another 24 hours. Cells were selected with 2 μg/ml puromycin for 3 days until mock-transduced controls (polybrene only) were completely dead. For each transduced melanoma cell line, up to 4 wells of cells per lentiviral clone were tested for Mcl-1 knockdown via Western Analysis. Cells with lowest Mcl-1 levels were expanded for experimental use.

RESULTS

ER Stress Induces Transcriptional Up-Regulation of Bcl-2 and Mcl-1 in Melanoma Cell Lines

Most melanoma cell lines are not sensitive to ER stress-induced apoptosis (17, 18). To study the role of anti-apoptotic Bcl-2 family members in the resistance, we monitored the levels of Bcl-2, Mcl-1 and Bcl-X_L proteins in Mel-RM and MM200 cells treated with the classic ER stress inducers TG and TM, respectively, for varying periods. Figure 1A shows that treatment with TG or TM resulted in up-regulation of Bcl-2 and Mcl-1, along with the ER chaperon GRP78, a commonly used indicator of activation of the UPR. The increase in Bcl-2 appeared to be transient that was observed at 16-24 hours and followed by a decrease, whereas the elevated levels of Mcl-1 persisted for up to 48 hours after treatment. Notably, neither TG nor TM induced notable up-regulation of Bcl-X_L in Mel-RM and MM200 cells. Up-regulation of the Bcl-2 and Mcl-1 proteins by ER stress was observed in another 4 melanoma cell lines but not in cultured melanocytes (Supplementary Figure 1).

Treatment with TG or TM resulted in a marked increase (up to 5-6-fold) in the levels of the Bcl-2 transcript that was detected as early as 1-3 hours in both Mel-RM and MM200 cells (Figure 1B). Similarly, a substantial increase (up to 4-5-fold) in the Mcl-1 mRNA levels was also observed (Figure 1C), although the kinetics of this increase varied between the two cell lines. Up-regulation of Bcl-2 and Mcl-1 mRNA levels could be inhibited by pretreatment with actinomycin D (Figure 1D), suggesting that this was due to a transcriptional increase, rather than a change in the mRNA stability.

Inhibition of Mcl-1 Renders Melanoma Cells Sensitive to ER Stress-Induced Apoptosis

Having established that Bcl-2 and Mcl-1 are up-regulated by ER stress, we tested if inhibition of these pro-survival proteins sensitizes melanoma cells to ER stress-induced apoptosis by transfecting siRNA pools against Mcl-1 and Bcl-2 into Mel-RM and MM200 cells, respectively (Figure 2A). siRNA knockdown of Mcl-1 resulted in a marked increase in sensitivity to TG- and TM- induced apoptosis (Figure 2B). In contrast, inhibition of Bcl-2 caused only a minimal increase in induction of apoptosis by TG or TM (Figure 2B). The different effects of inhibition of Mcl-1 and Bcl-2 on ER stress-induced apoptosis were also shown by differences in activation of caspase-3 induced by TG or TM (Supplementary Figure 2). These results demonstrate that Mcl-1 plays a critical role in protection of melanoma cells against ER stress-induced apoptosis.

Overexpression of Bcl-2 Does Not fully Compensate for Inhibition of Mcl-1 in Protection of Melanoma Cells against ER Stress-Induced Apoptosis

To further study the roles of Mcl-1 and Bcl-2 in protection against ER stress-induced apoptosis of melanoma cells, we inhibited Mcl-1 by siRNA in Mel-RM and MM200 cells that had been stably transfected with cDNA encoding Bcl-2 (Figure 2C & Supplementary Figure 3). Overexpression of Bcl-2 inhibited enhancement to TG- and TM-induced apoptosis by siRNA knockdown of Mcl-1 at 24 hours. However, by 48 hours, the levels of apoptosis were comparable between cells overexpressing Bcl-2 and those containing only the vector (Figure 2C & Supplementary Figure 3). These results suggest that Bcl-2 can delay ER stress-induced apoptosis in melanoma cells with deficient Mcl-1 expression. When Bcl-2 was inhibited by siRNA in Mel-RM and MM200 cells that had been stably transfected with cDNA encoding Mcl-1, there was no appreciable increase in the levels of apoptosis induced by TM and TG (Figure 2D & Supplementary Figure 4). These results further emphasize the importance of Mcl-1 in protection of melanoma cells when submitted to ER stress.

Inhibition of Mcl-1 Promotes Activation of Bax and Bak by ER Stress

Mcl-1 protects against apoptosis by inhibiting directly or indirectly activation of the pro-apoptotic proteins Bax and Bak (23, 24). We therefore examined if ER stress induces activation of Bax and Bak when Mcl-1 is inhibited by monitoring their activation status in permeabilized Mel-RM and MM200 cells with antibodies that specifically recognize activated Bax and Bak, respectively, in flow cytometry (25). siRNA inhibition of Mcl-1 resulted in an increase in activation of Bax, and to a lesser extent, activation of Bak induced by TG or TM in both cell lines (Figure 3A). Consistently, inhibition of Mcl-1 caused an increase in reduction of $\Delta\Psi m$ induced by TG or TM (Supplementary Figure 5). Moreover, TG and TM induced cytosolic expression of cytochrome C and Smac/DIABLO in cells transfected with the Mcl-1 siRNA (Supplementary Figure 6), indicating mitochondrial release of these apoptogenic proteins. Therefore, induction of apoptosis by ER stress in melanoma cells when Mcl-1 is inhibited is closely coupled to activation of Bax and Bak.

The BH3-Only Proteins PUMA and Noxa Are Up-regulated at the Transcriptional Level in melanoma cells by ER Stress

BH3-only proteins of the Bcl-2 family are the sensors of activation of Bax and Bak (23, 24). We analyzed their expression levels in Mel-RM and MM200 cells before and after induction of ER stress. Treatment with TG or TM caused increases in PUMA and Noxa protein levels in both cell lines (Figure 3B). In contrast, neither TG nor TM induced increases in the expression levels of another two BH3 only proteins Bim (Bim_{EL}) and BIK. The Bim isofoms Bim_L and Bim_S were not detected before and after treatment with TG or TM. Up-regulation of PUMA but not Bim (Bim_{EL}) by ER stress was also observed in cultured melanocytes (supplementary Figure 7).

Treatment with TG or TM also resulted in up to 4-5-fold increases in PUMA and Noxa mRNA levels in Mel-RM and MM200 cells, respectively, by 1-3 hours (Figure 3C). Similar to up-regulation of Bcl-2 and Mcl-1 mRNA, the increase in PUMA and Noxa

mRNA levels was inhibited by pretreatment with actinomycin D (Supplementary Figure 8), indicating that this was the consequence of an increase in PUMA and Noxa transcription.

PUMA and Noxa are both transcriptional targets of p53 and play important roles in p53-mediated apoptosis (26, 27). The expression levels of p53 were transiently up-regulated by TG and TM in both Mel-RM and MM200 cells (Supplementary Figure 9), suggesting that up-regulation of PUMA and Noxa by ER stress in melanoma cells may be associated with p53 activation. To test this, we silenced p53 by transfecting a p53 siRNA pool into Mel-RM cells. Strikingly, while up-regulation of Noxa by TG or TM was inhibited, up-regulation of PUMA was not reduced by siRNA knockdown of p53 (Figure 3D). Therefore, ER stress-induced up-regulation of Noxa is largely p53-dependent, whereas up-regulation of PUMA by ER stress is mediated primarily by a p53-independent mechanism(s) in melanoma cells.

PUMA and Noxa Mediate ER Stress-Induced Apoptosis of Melanoma Cells when Mcl-1 Is Inhibited

To elucidate roles of PUMA and Noxa in ER stress-induced apoptosis of melanoma cells when Mcl-1 is inhibited, we transfected PUMA and Noxa siRNA pools into MM200 cells in which Mcl-1 had been stably knocked down with shRNA by lentiviral infections (Figure 4A & B). Inhibition of PUMA or Noxa partially blocked TG- or TM-induced apoptosis (approximately 40-50% of inhibition) in MM200 cells with Mcl-1 being also knocked down by shRNA (Figure 4C). In contrast, siRNA inhibition of Bim had only minimal effects on TG- or TM-induced apoptosis in the Mcl-1-deficient cells (Figure 4D). These results demonstrate that PUMA and Noxa play important roles in ER stress-induced apoptosis of melanoma cells when Mcl-1 is inhibited.

The IRE1α and ATF6 Pathways of the UPR Are Involved in Transcriptional Up-Regulation of Mcl-1

Because Mcl-1 is crucial in protection of melanoma cells from ER stress-induced apoptosis, we examined the signaling pathway(s) of the UPR responsible for transcriptional up-regulation of Mcl-1 upon ER stress in Mel-RM and MM200 cells by transfecting siRNA pools for IRE1α, ATF6 and PERK into Mel-RM and MM200 cells, respectively (Figure 5A). Inhibition of either IRE1α or ATF6 partially inhibited up-regulation of the Mcl-1 protein induced by TG or TM in Mel-RM and MM200 cells (Figure 5B). In contrast, siRNA knockdown of PERK did not have any notable effect on the levels of Mcl-1 protein expression. However, it is notable that the PERK siRNA efficiency was not as high as that of IRE1α or ATF6 siRNA (Figure 5A). Knockdown of IRE1α or ATF6 also inhibited up-regulation of Mcl-1 mRNA induced by TG or TM (Figure 5C). These observations indicate that both IRE1α and ATF6 signaling pathways of the UPR play roles in transcriptional up-regulation of Mcl-1 in melanoma cells when submitted to ER stress.

Inhibition of MEK Partially Blocks Up-Regulation of Mcl-1 by ER Stress

Activation of the MEK/ERK signaling pathway plays an important role in protection of melanoma cells from ER stress-induced apoptosis (18). This is partially attributable to its effect on induction of GRP78 that in turn prevents activation of caspase-4 (18). On the other hand, we have also found that inhibition of MEK down-regulates Mcl-1 in melanoma cells cultured in medium with a sub-optimal concentration (0.5%) of serum (20). We therefore studied if the MEK/ERK pathway plays a part in up-regulation of Mcl-1 upon ER stress. As expected, treatment with the MEK inhibitor U0126 attenuated the increase in the Mcl-1 protein levels induced by TG or TM in both Mel-RM and MM200 cells (Figure 6A). Consistently, inhibition of the MEK/ERK pathway by a MEK1 siRNA pool also blocked up-regulation of Mcl-1 by ER stress (Figure 6B & C).

Moreover, blockage of ER stress-induced Mcl-1 up-regulation by U0126 appeared to occur at the transcriptional level as shown by Real time PCR analysis (Figure 6D). Collectively, these results indicate that activation of the MEK/ERK pathway is necessary for optimal induction of Mcl-1 transcription in melanoma cells by ER stress.

DISCUSSION

The results above reveal that, in contrast to down-regulation of Bcl-2 and Mcl-1 by ER stress in a number of cell types (7, 15), these anti-apoptotic proteins of the Bcl-2 family are transcriptionally up-regulated by ER stress in human melanoma cell lines. They demonstrate that the increase in Mcl-1 plays an essential role in antagonizing the pro-apoptotic BH3-only proteins PUMA and Noxa, which are also up-regulated by transcriptional mechanisms in melanoma cell lines when subjected to ER stress.

Although up-regulated, Bcl-2 did not appear to be critical for protection of melanoma cells from ER stress-induced apoptosis. This was evidenced by the minimal effect of siRNA inhibition of Bcl-2 on sensitivity of melanoma cells to apoptosis induced by TG or TM, and the inability of over-expression of Bcl-2 to rescue melanoma cells with deficient Mcl-1 expression upon ER stress, although it did delay the onset of apoptosis. In contrast, siRNA inhibition of Mcl-1 readily enhanced ER stress-induced apoptosis, and over-expression of Mcl-1 efficiently protected melanoma cells from apoptosis induced by TG or TM, even when Bcl-2 was inhibited by siRNA. These results indicate that Mcl-1, but not Bcl-2, plays a determining role in survival of melanoma cells under ER stress conditions.

We have previously shown that Mcl-1 expression is associated with melanoma progression, whereas Bcl-2 expression decreases during progression of melanoma (28). Interestingly, we have found in a separate study that GRP78 is highly expressed on most melanoma tissue sections and the expression levels increase with melanoma progression (data not shown). This indicates that melanoma cells in vivo may have adapted to ER stress conditions that are conceivably caused by the rapid growth rate and perhaps inadequate vascularization that result in hypoxia, glucose deprivation, and acidosis (29, 30). These results, along with our current findings, suggest that Mcl-1 may contribute to adaptation of melanoma cells to ER stress in vivo. Consistent with this, hypoxia and ER

stress have been reported to select for highly metastatic Lewis lung carcinoma cells over-expressing Mcl-1 (31).

Up-regulation of Mcl-1 by ER stress in melanoma cells appeared to occur at the transcription level and both the IRE1α- and ATF6- mediated signaling pathways of the UPR played roles in this increase. On activation, the RNase activity of IRE1α cleaves XBP1 mRNA, generating a splicing variant of XBP1 mRNA that encodes a potent transcription factor (1-3). ATF6 itself is a transcription factor that on activation relocates to Golgi where it is cleaved into the smaller active form that activates transcription of UPR target genes (1-3). XBP1 and ATF6 may act directly to activate transcription of Mcl-1 in melanoma cells but the UPR element (UPRE) or ER stress response element (ERSE) consensus sequence, which is necessary for activation of the UPR target gene by XBP1 and ATF6 (1-3), could not be identified in the promoter region of the Mcl-1 gene (data not shown). It is conceivable that the IRE1α and ATF6 signaling pathways may activate Mcl-1 transcription indirectly via other transcription factors such as PU.1 and STAT-3 (32, 33), both of which can be activated by the MAPK p38 and the latter can be activated by ER stress (32, 34, 35).

Significantly, inhibition of the MEK/ERK pathway blocked up-regulation of Mcl-1 by ER stress at the transcriptional level. This indicates that a MEK/ERK-regulated transcriptional mechanism(s) is required for optimal up-regulation of Mcl-1 by ER stress. The MEK/ERK signaling pathway is known to be constitutively activated in virtually all melanomas, which is a common cause for resistance of melanoma cells to induction of apoptosis (36-38). Although induction of ER stress did not result in further activation of ERK in melanoma cells, the constitutively activated MEK/ERK pathway plays an important role in protection of melanoma cells from ER stress-induced apoptosis (18). This has been previously shown to be partially attributable to its effect on induction of GRP78 that in turn prevents activation of caspase-4 (18). Notably, inhibition of MEK by

either U0126 or the MEK1 siRNA did not reduce the basal levels of Mcl-1 expression in the current study, but inhibited its basal levels in a previous study when the cells were cultured with medium containing a sub-optimal concentration (0.5%) of serum (20). This is presumably due to stronger activation of MEK/ERK in the cells cultured with the optimal concentration of serum (5%) as in this study (20 & data not shown). Regardless, the current results identify up-regulation of Mcl-1 as another mechanism by which the MEK/ERK pathway underlies adaptation of melanoma cells to ER stress.

Increases in the Mcl-1 mRNA levels have been recently noted in Hela cells after exposure to TG (15). However, the Mcl-1 protein levels in Hela cells were markedly reduced by TG due to translational repression mediated by phosphorylation of the α-subunit of eukaryotic translation initiation factor (eIF2α) downstream of PERK (15). We cannot entirely exclude the involvement of eIF2α in regulation of Mcl-1 in melanoma cells under ER stress but inhibition of PERK by siRNA did not have any significant effect on the Mcl-1 protein expression. Similarly, we cannot exclude that changes in Mcl-1 protein turnover may play a role in determining the Mcl-1 levels during ER stress in melanoma cells (15, 39). Regardless, our results indicate that up-regulation of Mcl-1 transcription is the dominant regulatory mechanism governing Mcl-1 expression in melanoma cells under ER stress.

Induction of apoptosis of melanoma cells by ER stress when Mcl-1 was inhibited was associated with activation of Bax/Bak, reduction in mitochondrial membrane potential, and mitochondrial release of apoptogenic proteins. This pointed to the involvement of one or more pro-apoptotic BH3-only proteins. Among them, PUMA, Noxa, Bim and BIK have all been reported to play roles in induction of apoptosis by ER stress in diverse cell types (8-11, 13). For example, siRNA library screening showed that PUMA and Noxa were induced in HCT116 cells, and siRNA inhibition of PUMA partially protected the cells from ER stress-induced apoptosis (10). In addition, PUMA and Noxa were shown to

mediate apoptosis induced by ER stress in mouse embryo fibroblasts (MEFs) (8). On the other hand, Bim was believed to be critical in ER stress-induced apoptosis in thymocytes, macrophages, and epithelium cells from breast and kidney (13). It seems that induction/activation of BH3-only proteins by ER stress is highly cell type-specific. We found in this study that PUMA and Noxa, but not Bim and BIK are transcriptionally up-regulated in melanoma cells by ER stress, and contribute to induction of apoptosis when Mcl-1 is inhibited, further emphasizing the cell type-dependent specificity in regulation of BH3-only proteins by ER stress. It is conceivable that PUMA and Noxa may cooperate with each other in ER stress-induced apoptosis of melanoma cells when Mcl-1 is inhibited, in that inhibition of either PUMA or Noxa resulted only in partial inhibition of apoptosis. Although PUMA can be bound to by both Bcl-2 and Mcl-1, Noxa can only be antagonized by Mcl-1 (40). This may be one of the reasons why Bcl-2 is relatively ineffective against ER stress-induced apoptosis.

PUMA and Noxa are both transcriptional targets of p53 (26, 27), which was up-regulated by ER stress, albeit transiently, in melanoma cells. However, only the increase in Noxa was dependent on p53, whereas up-regulation of PUMA was mediated largely by a p53-independent mechanism(s). Although p53-dependent up-regulation of PUMA and Noxa by ER stress was shown in MEFs (8), p53-independent up-regulation of PUMA and Noxa has also been reported (10, 41-43). It seems that mechanisms that regulate PUMA and Noxa expression by ER stress may be cell type-dependent. The p53-independent transcriptional mechanism(s) responsible for up-regulation of PUMA by ER stress in melanoma cells remains to be elucidated. The transcription factor E2F1 has been shown to up-regulate PUMA by ER stress in HCT116 and NIH3T3 cells (10). The same transcription factor has also been shown to activate Noxa transcription (44).

The present results, 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 (18), and by activation of caspase-8 when the ARC protein is deficient (17),

demonstrate that ER stress can potentially activate multiple apoptosis signaling pathways in melanoma cells, which are however inhibited by various survival mechanisms, including activation of the MEK/ERK pathway, up-regulation of Mcl-1, and expression of ARC. Among them, the MEK/ERK pathway appears of particular importance in that it is not only required for optimal up-regulation of Mcl-1, but also plays a role in up-regulation of GRP78 in melanoma cells under ER stress (18). Whether this pathway is also involved in regulation of ARC in melanoma cells remains to be studied. Our past studies indicated, however, that ER stress does not increase the levels of ARC in melanoma cell lines (17). It is also unknown whether Mcl-1, GRP78, and ARC may directly or indirectly crosstalk with each other in protection of melanoma cells from ER stress-induced apoptosis. Nevertheless, our studies suggest that melanoma cells may have adapted to ER stress by inhibiting multiple apoptotic pathways as illustrated previously (17). The present study identifies Mcl-1 as another adaptive mechanism by which melanoma cells survive ER stress conditions. This may be developed during melanoma evolution since ER stress does not up-regulate Mcl-1 in melanocytes. Collectively, these results suggest that targeting Mcl-1 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 (45, 46).

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LEGENDS TO FIGURES

Figure 1 – ER stress induces transcriptional up-regulation of Bcl-2 and Mcl-1 in melanoma cells.

A. Mel-RM and MM200 cells were treated with TG (1μM) or TM (3μM) for indicated periods. Whole cell lysates were subjected to Western blot analysis of Bcl-2, Mcl-1, Bcl-X_L and GRP78 expression. The data shown are representative of three individual experiments.

B & C. Mel-RM and MM200 cells were treated with TG (1μM) or TM (3μM) for the indicated periods before total RNA was isolated and subjected to Real-time PCR analysis for Bcl-2 (B) and Mcl-1 (C) mRNA expression. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments.

D. Mel-RM and MM200 cells were treated actinomycin D (3μg/ml) for 1 hour before the addition of TG (1μM) or TM (3μM) for a further 6 hours. Total RNA was isolated and subjected to Real-time PCR analysis for Bcl-2 (upper panel) and Mcl-1 (lower panel) mRNA expression. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments.

Figure 2 – Mcl-1 is critical for protection of melanoma cells from ER stress-induced apoptosis.

A. Mel-RM and MM200 cells were transfected with the control, Bcl-2 (upper panel), or Mcl-1 (lower panel) siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of Bcl-2 and Mcl-1 expression. The data shown are representative of three individual experiments.

B. Mel-RM and MM200 cells were transfected with the control, Bcl-2, or Mcl-1 siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM)

for a further 48 hours. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± SE of three individual experiments.

C. Upper left panel: Whole cell lysates from Mel-RM cells transfected with the cDNA encoding Bcl-2 or the vector alone were subjected to Western blot analysis of Bcl-2 expression. Lower left panel: Mel-RM cells over-expressing Bcl-2 and those carrying the vector alone were transfected with the control or Mcl-1 siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of Mcl-1 expression. Right panel: Mel-RM cells containing the vector alone and those over-expressing Bcl-2 were transfected with the Mcl-1 siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for a further 24 or 48 hours. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are either representative of three individual experiments (left panels), or the mean ± SE of three individual experiments (right panel).

D. Upper left panel: Whole cell lysates from Mel-RM cells transfected with with the cDNA encoding Mcl-1 or the vector alone were subjected to Western blot analysis of Mcl-1 expression. Lower left panel: Mel-RM cells over-expressing Mcl-1 and those carrying the vector alone were transfected with the control or Bcl-2 siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of Bcl-2 expression. Right panel: Mel-RM cells containing the vector alone and those over-expressing Mcl-1 were transfected with the Bcl-2 siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for a further 24 or 48 hours. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are either representative of three individual experiments (left panels), or the mean ± SE of three individual experiments (right panel).

Figure 3 – ER stress-induced apoptosis of melanoma cells when Mcl-1 is inhibited is associated with activation of Bax/Bak, and up-regulation of PUMA and Noxa.

A. Mel-RM and MM200 cells were transfected with the control or Mcl-1 siRNA as shown in Figure 2A. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for another 36 hours, followed by measurement of activation of Bax and Bak in flow cytometry using antibodies that specifically recognize activated Bax and Bak, respectively. The filled histograms and open histograms were generated from cells transfected with the control (C) and Mcl-1 (M) siRNA , respectively. The numbers represent mean fluorescence intensities (MFIs). The data shown are representative of three individual experiments.

B. Mel-RM and MM200 cells were treated with TG (1μM) or TM (3μM) for indicated periods. Whole cell lysates were subjected to Western blot analysis of PUMA, Noxa, Bim, and BIK expression. The data shown are representative of three individual experiments.

C. Mel-RM (left) and MM200 (right) cells were treated with TG (1μM) or TM (3μM) for the indicated time periods before total RNA was isolated and subjected to Real-time PCR analysis for PUMA and Noxa mRNA expression. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments.

D. Effects of siRNA knockdown of p53 on up-regulation of PUMA and Noxa by ER stress. Mel-RM cells were transfected with the control or p53 siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for another 24 hours. Whole cell lysates were subjected to Western blot analysis of p53, PUMA, and Noxa expression. The data shown are representative of three individual experiments.

Figure 4 – siRNA knockdown of PUMA or Noxa partially inhibited ER stress-induced apoptosis in MM200 cells with Mcl-1 being inhibited by shRNA.

A & B. MM200 cells with Mcl-1 being stably knocked down by shRNA (A; clone 14 & clone 18) were transfected with the control, PUMA, or Noxa siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of PUMA (B, left panel, clone 14) and Noxa (B, right panel, clone 14) expression. The data shown are representative of three individual experiments.

C. MM200 cells with Mcl-1 being stably knocked down by shRNA (clone 14) were transfected with the control, PUMA, or Noxa siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for a further 48 hours. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± SE of three individual experiments.

D. siRNA knockdown of Bim has a minimal effect on ER stress-induced apoptosis when Mcl-1 is inhibited. Upper panel: MM200 cells with Mcl-1 being stably knocked down by shRNA (clone 14) were transfected with the control or Bim siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of Bim expression. Lower panel: MM200 cells with Mcl-1 being stably knocked down by shRNA (clone 14) were transfected with the control or Bim siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for a further 48 hours. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are either representative of three individual experiments (upper panel) or the mean ± SE of three individual experiments (lower panel).

Figure 5 – The IRE1α and ATF6 pathways of the UPR are involved in ER stress-induced up-regulation of Mcl-1.

A. Mel-RM and MM200 cells were transfected with the control, IRE1α, ATF6, or PERK siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of IRE1α, ATF6 and PERK expression. The relative expression levels of IRE1α, ATF6 and PERK were inhibited by 82%, 86%, and

56%, respectively, in Mel-RM cells, and by 80%, 85% and 76%, respectively in MM200 cells. The data shown are representative of three individual experiments.

B. Mel-RM and MM200 cells were transfected with the control, IRE1α, ATF6, or PERK siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for a further 24 hours. Whole cell lysates were subjected to Western blot analysis of Mcl-1 expression. The data shown are representative of three individual experiments.

C. Mel-RM and MM200 cells were transfected with the control, IRE1α, or ATF6 siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for a further 6 hours. Total RNA was isolated and subjected to Real-time PCR analysis for Mcl-1 mRNA expression. The relative abundance of mRNA expression in cells transfected with the control siRNA without any further treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments

Figure 6 – Inhibition of MEK blocks up-regulation of Mcl-1 by ER stress.

A. Mel-RM and MM200 cells were treated with U0126 (20μM) for 1 hour before the addition of TG (1μM) or TM (3μM) for 36 hours. Whole cell lysates were subjected to Western blot analysis of Mcl-1 expression. The data shown are representative of three individual experiments.

B & C. Mel-RM and MM200 cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for a further 24 hours. Whole cell lysates were subjected to Western blot analysis of MEK1, phosphorylated ERK1/2, and ERK1/2(B), and Mcl-1 (C) expression. The data shown are representative of three individual experiments.

D. Mel-RM and MM200 cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, cells were treated with TG (1μM) or TM (3μM) for a

further 6 hours. Total RNA was isolated and subjected to Real-time PCR analysis for Mcl-1 mRNA expression. The relative abundance of mRNA expression in cells transfected with the control siRNA before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments.