

Tunicamycin Sensitizes Human Melanoma Cells to Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Induced Apoptosis by Up-regulation of TRAIL-R2 via the Unfolded Protein Response

Chen Chen Jiang, Li Hua Chen, Susan Gillespie, Kelly A. Kiejda, Nizar Mhaidat, Yu Fang Wang, Rick Thorne, Xu Dong Zhang, and Peter Hersey

Immunology and Oncology Unit, Newcastle Misericordiae Hospital, Newcastle, NSW, Australia

Abstract

We have reported previously low expression of death receptors for tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) in fresh isolates and tissue sections of melanoma. This seemed to correlate with relative resistance of freshly isolated melanoma cells to TRAIL-induced apoptosis. We show in this study that the endoplasmic reticulum (ER) stress inducer, tunicamycin, selectively up-regulated the cell surface expression of TRAIL-R2, but not other members of the TNF receptor family, and enhanced TRAIL-induced apoptosis in cultured melanoma cells and fresh melanoma isolates. Tunicamycin-mediated sensitization of melanoma cells to TRAIL-induced apoptosis was associated with increased activation of the caspase cascade and reduction in mitochondrial membrane potential and was inhibited by a recombinant TRAIL-R2/Fc chimeric protein. Up-regulation of TRAIL-R2 on the melanoma cell surface was associated with increased transcription of TRAIL-R2 and its total protein levels. Two signaling pathways of the ER stress-induced unfolded protein response mediated by inositol-requiring transmembrane kinase and endonuclease 1 α (IRE1 α) and activation of transcription factor 6 (ATF6), respectively, seemed to be involved. In one melanoma line, there was clear evidence of activation of the IRE1 α pathway, and small interfering RNA (siRNA) knockdown of IRE1 α substantially reduced the up-regulation of TRAIL-R2. Similarly, there was evidence for the activation of the ATF6 pathway, and siRNA knockdown of ATF6 had a delayed effect on TRAIL-R2 expression in one but not another melanoma cell line. Moreover, the transcription factor CCAAT/enhancer-binding protein homologous protein seemed to be involved in the up-regulation of TRAIL-R2 by tunicamycin, but its role varied between different melanoma lines. Taken together, our results suggest that agents that induce ER stress may enhance TRAIL-R2 expression and increase the therapeutic response to TRAIL in melanoma. [Cancer Res 2007;67(12):5880–8]

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a member of the TNF family that seems to be a promising candidate for cancer therapeutics because of its

selective cytotoxicity against malignancies (1–3). The potential significance of TRAIL as an anticancer agent has been supported by studies in animal models showing selective toxicity to human tumor xenografts but not normal tissues (4, 5). Induction of apoptosis by TRAIL is mediated by its interaction with two death domain-containing receptors, TRAIL-R1 and TRAIL-R2. This, in turn, orchestrates the assembly of the death-inducing signaling complex that contains adapter components such as Fas-associated death domain that activates initiator caspases, caspase-8 and caspase-10, leading eventually to the activation of effector caspases such as caspase-3 and apoptosis (1–3).

We have previously shown that the sensitivity of cultured melanoma cells to TRAIL-induced apoptosis is, in general, correlated with the levels of the cell surface expression of TRAIL death receptors, in particular, TRAIL-R2 (6, 7). Subsequent studies showed that fresh melanoma isolates are relatively resistant to TRAIL-induced apoptosis due to low levels of TRAIL-death receptor expression (8). Moreover, melanoma cells selected for TRAIL resistance by prolonged exposure to TRAIL express substantially reduced levels of TRAIL-R2 on their surface (9, 10). Studies on melanoma tissue sections revealed that reduced TRAIL-R2 expression is associated with disease progression and a poor prognosis (11). Taken together, these studies indicate that melanoma may not respond to treatment with TRAIL unless given with agents that increase the cell surface expression of TRAIL death receptors, in particular, TRAIL-R2.

The cellular response to endoplasmic reticulum (ER) stress, the unfolded protein response (UPR), consists of three distinct yet coordinated signaling pathways initiated, respectively, by inositol-requiring transmembrane kinase and endonuclease 1 α (IRE1 α), activation of transcription factor 6 (ATF6), and protein kinase-like ER kinase (PERK; refs. 12–14). As an adaptive response, the UPR is activated to alleviate the stress condition imposed on the ER and is orchestrated by transcriptional activation of multiple genes mediated by IRE1 α and ATF6, a general decrease in translation initiation, and selective translation of specific mRNAs mediated by PERK (12–14). However, if the stress on ER remains unresolved, prolonged activation of the UPR can lead to apoptosis (12–14). Tunicamycin, a naturally occurring antibiotic that induces ER stress by inhibiting the first step in the biosynthesis of N-linked oligosaccharides in cells (15), was reported to sensitize human colon and prostate cancer cells to TRAIL-induced apoptosis via the up-regulation of TRAIL-R1 and/or TRAIL-R2 (16, 17). However, a potential role of the UPR in the regulation of TRAIL death receptor expression by ER stress inducers has not been fully studied. In addition, the effect of ER stress on the expression of TRAIL death receptors in melanoma cells remains unknown.

In the present report, we show that tunicamycin selectively up-regulated the cell surface expression of TRAIL-R2 and enhanced

Requests for reprints: Peter Hersey or Xu Dong Zhang, Room 443, David Maddison Clinical Sciences Building, Cnr. King & Watt Streets, Newcastle, NSW 2300, Australia. Phone: 61-2-49-236828; Fax: 61-2-49236184; E-mail: Peter.Hersey@newcastle.edu.au or Xu.Zhang@newcastle.edu.au.

©2007 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-07-0213

TRAIL-induced apoptosis in cultured melanoma cells and fresh melanoma isolates. Up-regulation of TRAIL-R2 expression on the cell surface was associated with enhanced TRAIL-R2 gene transcription and elevated TRAIL-R2 total protein levels. We show, for the first time, that the IRE1 α - and ATF6-mediated signaling pathways of the UPR contribute to the up-regulation of TRAIL-R2 by tunicamycin in melanoma cells in a cell line-dependent manner.

Materials and Methods

Cell lines. Human melanoma cell lines Mel-RM, MM200, Igr3, Mel-CV, Me4405, Sk-Mel-28, and Mel-FH have been described previously and were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories; refs. 6, 18). Melanocytes were kindly provided by Dr. P. Parsons (Queensland Institute of Medical Research, Brisbane, Australia) and cultured in medium supplied by Clonetics (Edward Kellar). Human umbilical vascular endothelial cells (HUVEC) were kindly supplied by D. Clark (Transplantation Unit, John Hunter Hospital, Australia) and were cultured as described elsewhere (19). The cell line of human embryonic fibroblasts, FLOW 2000, was cultured in DMEM containing 5% FCS as described previously (20).

Fresh melanoma isolates. Isolation of melanoma cells from fresh surgical specimens was carried out as described previously (8).

Antibodies, recombinant proteins, and other reagents. Tunicamycin was purchased from Sigma Chemical Co.. It was dissolved in DMSO to make up a stock solution of 1 mmol/L. Recombinant human TRAIL and the TRAIL-R2/Fc chimera were supplied by Immunex. The mouse monoclonal antibodies (mAb) against TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, Fas, TNF-R1, and TNF-R2 were also supplied by Immunex. The cell-permeable general caspase inhibitor Z-Val-Ala-Asp(OMe)-CH₂F (z-VAD-fmk) and the caspase-8-specific inhibitor Z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH₂F (z-IETD-fmk) were purchased from Calbiochem. The rabbit polyclonal Abs against caspase-3 and caspase-8 were from Stressgen. The mouse mAbs against poly(ADP-ribose) polymerase (PARP) and Bid were from PharMingen. The rabbit mAbs against immunoglobulin-binding protein (Bip), eukaryotic initiation factor 2 α (eIF2 α), phosphorylated eIF2 α , x-box-binding protein 1 (XBP1), IRE1 α , ATF6, PERK, and CCAAT/enhancer-binding protein-homologous protein (CHOP), were purchased from Santa Cruz Biotechnology. Isotype control Abs used were the ID4.5 [mouse immunoglobulin G₂ α (IgG₂ α) 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, and rabbit IgG from Sigma Chemical Co.

Flow cytometry. Immunostaining on intact and permeabilized cells was carried out as described previously (6, 18). Analysis was carried out using a Becton Dickinson FACScan flow cytometer.

Apoptosis. Quantitation of apoptotic cells by measurement of sub-G₁ DNA content using the propidium iodide method was carried out as described elsewhere (6, 18).

Mitochondrial membrane potential ($\Delta\Psi_m$). Melanoma cells were seeded at 1×10^5 cells per well in 24-well plates and allowed to reach exponential growth for 24 h before treatment. Changes in $\Delta\Psi_m$ were studied by staining the cells with the cationic dye, JC-1, according to the manufacturer's instructions (Molecular Probes) as described previously (9, 21).

Confocal microscopy. Melanoma cells seeded onto glass coverslips were subject to formaldehyde fixation before indirect immunofluorescent staining. Primary antibodies were detected with Alexa Fluor 488-conjugated anti-mouse Ig, and the Golgi apparatus was decorated using Alexa Fluor 594-labeled wheat germ agglutinin (WGA; Invitrogen). After staining, coverslips were mounted with SlowFade Gold medium (Invitrogen), and confocal images were acquired using a Zeiss LSM 510 scanner fitted to an Axiocvert 100M microscope. Alexa-488 images were acquired using a BP500-550 IR filter with excitation at 488 nm using an argon laser. Alexa-594 images were acquired using a LP560 filter with excitation at 543 nm using a helium neon laser. Dual-color analysis was done with

appropriate single-color controls with individual channels recorded sequentially. Instrument settings were adjusted to obtain minimal saturated pixels, and where semiquantitative comparisons were done, the settings were kept constant.

Western blot analysis. Western blot analysis was carried out as described previously (9, 21). Labeled bands were detected by Immuno-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).

Real-time PCR. Total RNA was isolated with spin or vacuum (SV) total RNA isolation system (Promega). Reverse transcription-PCR was carried out using Moloney murine leukemia virus (MMLV) transcriptase and Oligo d(T), and the resulting cDNA products were used as templates for real-time PCR assays. Real-time PCR was done using the ABI Prism 7700 sequence detection system (Applied Biosystems). For TRAIL-R2, 25 μ L mixture was used for reaction, which contains 5 μ L cDNA sample (0.5–1 μ g/ μ L), 300 nmol/L forward primers for TRAIL-R2 (CGCTGCACCAGGTGTGATT), 300 nmol/L reverse primers for TRAIL-R2 (GTGCCGGCTTCGACTGACA), 200 nmol/L probes for TRAIL-R2 (6FAM-CCCTGCACCAGCAGCAAACAG-TAMRA), and 9 mmol/L MgCl₂. After incubation at 50°C for 2 min followed by 95°C for 10 min, the reaction was carried out for 45 cycles of the following: 95°C for 15 s and 60.6°C for 45 s. For TRAIL-R1, assay-on-demand for TRAIL-R1 (Assay ID: Hs01560092-g1) was used according to the manufacturer's protocol (Applied Biosystems). Analysis of cDNA for β -actin was included as a control. The threshold cycle value (C_t) 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 interfering RNA. Melanoma cells were seeded at 3.5×10^4 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 IRE1 α (M-004951-01-0010), the siGENOME SMARTpool ATF6 (M-009917-01-0010), the siGENOME SMARTpool CHOP (M-004819-01-0010). The nontargeting siRNA control, SiConTRoNontargeting siRNA pool (D-001206-13-20) was also obtained from Dharmacon. Cells were transfected with 50–100 nmol/L siRNA in Opti-MEM (Invitrogen) with 5% FCS using the OligofectAMINE reagent (Invitrogen) according to the manufacturer's transfection protocol. Efficiency of siRNA was measured by Western blot analysis 24 h after transfection.

Results

Tunicamycin selectively up-regulates TRAIL-R2 on the melanoma cell surface. In the search for agents that increase TRAIL death receptor expression on the melanoma cell surface, we found that the ER stress inducer tunicamycin markedly up-regulated the cell surface expression of TRAIL-R2 in both Mel-RM and MM200 cells, with a significant increase being detected at 16 h, and further increases at 24 and 36 h after exposure to the compound (Fig. 1A and B). In contrast, tunicamycin did not induce any change in the expression of the other TNF receptor family members, TRAIL-R1, TRAIL-R3, TRAIL-R4, Fas, TNF-R1, and TNF-R2 on the cell surface; neither did it cause up-regulation of TRAIL-R2 on the surface of normal cells, including melanocytes, fibroblasts, and HUVECs (Fig. 1A and C, and data not shown). Studies on a panel of melanoma cell lines revealed that tunicamycin could up-regulate TRAIL-R2 on the cell surface in all but Sk-Mel-110 (Fig. 1C).

Up-regulation of the cell surface expression of TRAIL-R2 in melanoma cells by tunicamycin was confirmed in studies using confocal microscopy (Fig. 1D). As was reported before (22), TRAIL-R2 in melanoma cells was predominantly located in the Golgi network with a small proportion of the protein being detected on the cell surface before treatment. However, after exposure to

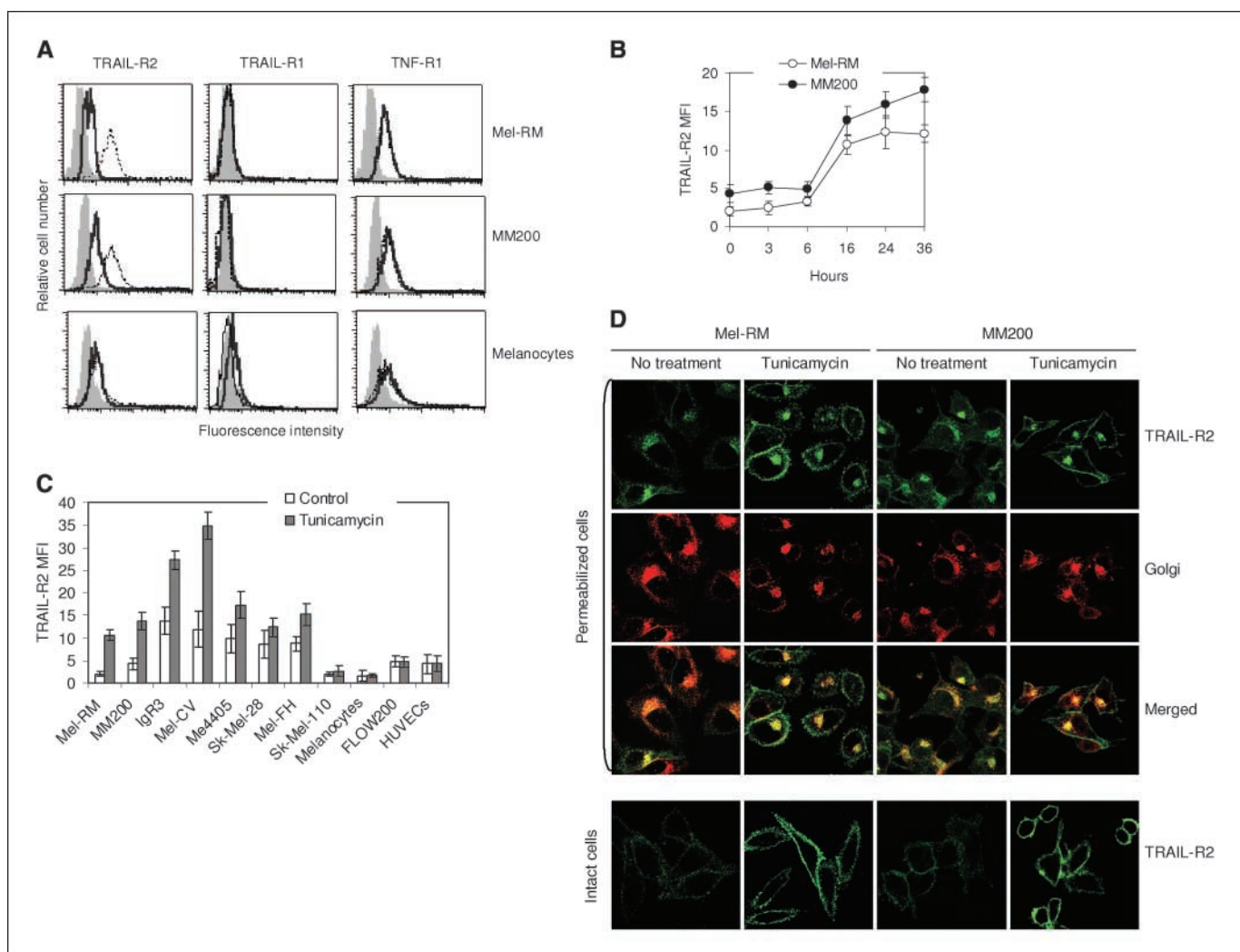


Figure 1. Tunicamycin selectively up-regulates TRAIL-R2 on the melanoma cell surface. *A*, melanoma cells (Mel-RM and MM200) and melanocytes with (*dotted lines*) or without (*thick lines*) treatment with tunicamycin ($3 \mu\text{mol/L}$) for 16 h were subjected to measurement of the cell surface expression of TRAIL-R2, TRAIL-R1, and TNF-R1 in flow cytometry. The filled histograms are isotype controls. The data shown are representative of three individual experiments. *B*, Mel-RM and MM200 cells with or without treatment with tunicamycin ($3 \mu\text{mol/L}$) for indicated time periods were subjected to measurement of the cell surface TRAIL-R2 expression in flow cytometry. The data shown are the MFIs of TRAIL-R2. *Points*, mean of three individual experiments; *bars*, SE. *C*, a summary of studies on a panel of melanoma cells and normal cells (melanocytes, FLOW 2000 fibroblasts, and HUVECs). Cells with or without treatment with tunicamycin ($3 \mu\text{mol/L}$) for 16 h were subjected to measurement of the cell surface TRAIL-R2 expression in flow cytometry. The data shown are the MFIs of TRAIL-R2. *Columns*, mean of three individual experiments; *bars*, SE. *D*, Mel-RM and MM200 cells with or without treatment with tunicamycin ($3 \mu\text{mol/L}$) for 16 h were either permeabilized or left intact before staining for TRAIL-R2 with (permeabilized cells) or without (intact cells) colabeling for the Golgi. Stained cells were then subjected to analysis in confocal microscopy. Magnification, $\times 594$. The data shown are representative of three individual experiments.

tunicamycin for 24 h, there was a marked increase in the cell surface expression of TRAIL-R2.

Tunicamycin sensitizes melanoma cells to TRAIL-induced apoptosis. To study if up-regulation of TRAIL-R2 by tunicamycin results in increased sensitivity of melanoma cells to TRAIL-induced apoptosis, we treated Mel-RM and MM200 cells with tunicamycin at $3 \mu\text{mol/L}$ for 16 h before the addition of TRAIL (200 ng/mL) for a further 24 h. Figure 2A shows that tunicamycin alone did not induce significant apoptosis, whereas TRAIL induced apoptosis in 31.2% of Mel-RM and 46.5% of MM200 cells. As expected, pretreatment with tunicamycin markedly enhanced TRAIL-induced apoptosis in both cell lines (Fig. 2A). In association with this, pretreatment with tunicamycin enhanced TRAIL-induced activation of caspase-8 and Bid, reduction in $\Delta\Psi\text{m}$, activation of caspase-3, and cleavage of its substrate PARP (Fig. 2B).

The role of up-regulation of TRAIL-R2 in the sensitization of melanoma cells to TRAIL-induced apoptosis by tunicamycin was further studied by the inhibition of the interaction between TRAIL and TRAIL-R2 using a TRAIL-2/Fc chimeric protein. Figure 2C shows that the TRAIL-R2/Fc chimera significantly inhibited TRAIL-induced apoptosis in the absence or presence of tunicamycin. Similarly, tunicamycin-mediated sensitization of melanoma cells to TRAIL-induced apoptosis was blocked by either the general caspase inhibitor z-VAD-fmk or the caspase-8-specific inhibitor z-IETD-fmk (Fig. 2C). Taken together, these results indicate that the up-regulation of TRAIL-R2 expression on the cell surface is responsible for the sensitization of melanoma cells to TRAIL-induced apoptosis by tunicamycin.

Tunicamycin-mediated sensitization to TRAIL-induced apoptosis was confirmed in a large panel of melanoma cell lines as shown in

Fig. 2D, with the exception of Sk-Mel-110. It is of interest that TRAIL alone did not induce apoptosis in normal cells, but treatment with tunicamycin alone resulted in varying degrees of apoptosis in melanocytes, fibroblasts, and HUVECs. Pretreatment with tunica-

mycin followed by TRAIL led to enhanced induction of apoptosis in FLOW 2000 fibroblasts but not melanocytes and HUVECs.

Tunicamycin increases transcription of TRAIL-R2 and its total protein levels. To understand the mechanism by which

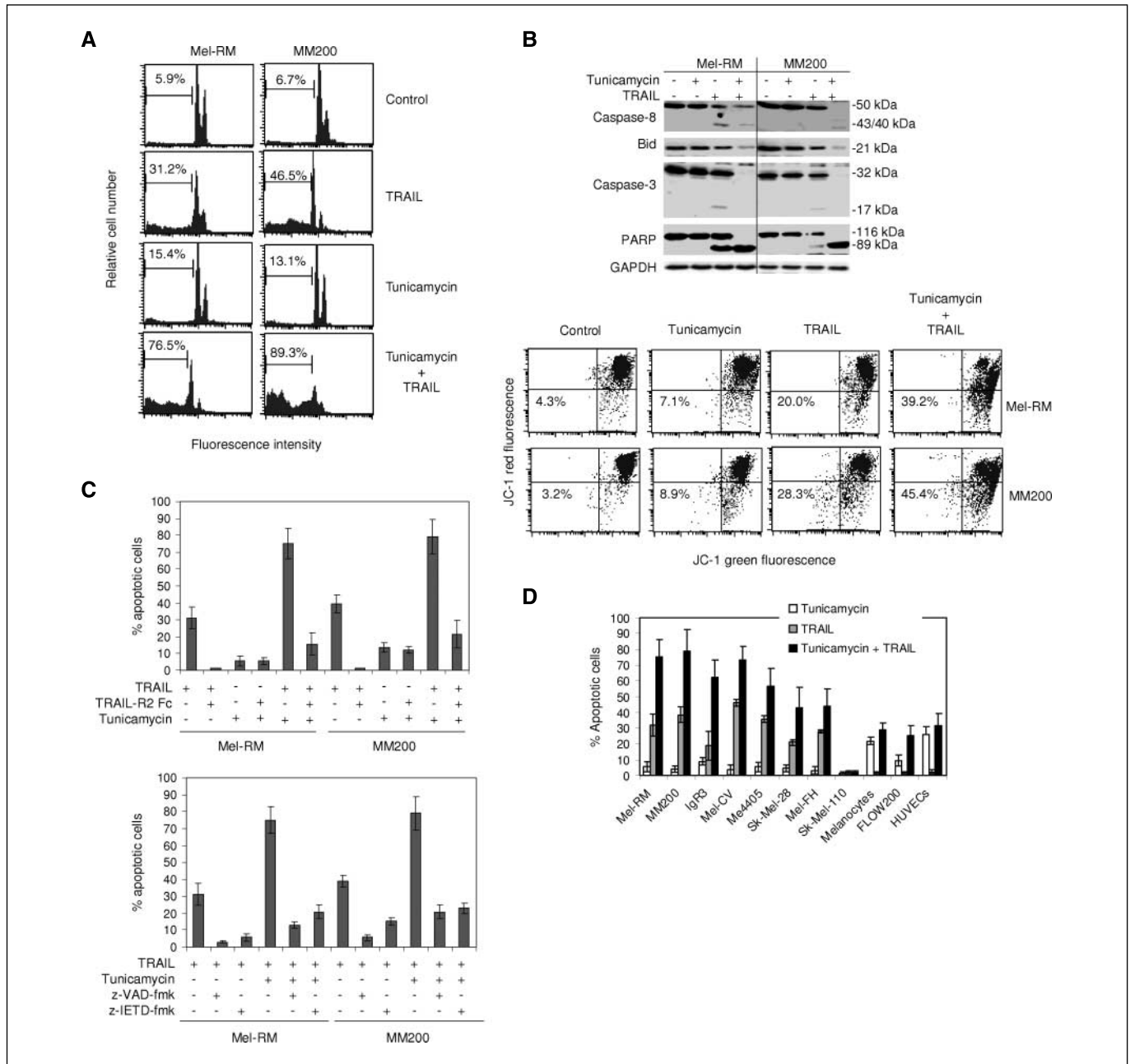


Figure 2. Tunicamycin sensitizes melanoma cells to TRAIL-induced apoptosis. *A*, Mel-RM and MM200 cells with or without treatment with tunicamycin alone (3 μ mol/L) for 40 h, TRAIL (200 ng/mL) alone for 24 h, or tunicamycin (3 μ mol/L) for 16 h, followed by the addition of TRAIL (200 ng/mL) for another 24 h, were subjected to measurement of apoptosis by the propidium iodide method using flow cytometry. The data shown are representative flow-cytometric histograms of three individual experiments. *B*, tunicamycin potentiates TRAIL-induced caspase activation and reduction in $\Delta\Psi_m$. Mel-RM and MM200 cells were treated with tunicamycin alone (3 μ mol/L) for 16 h, TRAIL (200 ng/mL) alone for 3 h, or tunicamycin (3 μ mol/L) for 16 h, followed by the addition of TRAIL (200 ng/mL) for another 3 h. *Top*, whole cell lysates were subjected to Western blot analysis. *Bottom*, $\Delta\Psi_m$ was measured using JC-1 by flow cytometry. *Number in each bottom left quadrant plot*, percentage of cells with reduction in $\Delta\Psi_m$. The data shown are representative of three individual experiments. *C*, a TRAIL-R2/Fc chimera or caspase inhibitors inhibit sensitization of melanoma cells to TRAIL-induced apoptosis by tunicamycin. Mel-RM and MM200 cells with or without pretreatment with a TRAIL-R2/Fc chimera (10 μ g/mL; *top*), the general caspase inhibitor z-VAD-fmk (30 μ mol/L), or the caspase-8-specific inhibitor, z-IETD-fmk (30 μ mol/L; *bottom*) for 1 h, followed by the addition of tunicamycin (3 μ mol/L) for 40 h, TRAIL (200 ng/mL) for 24 h, or tunicamycin (3 μ mol/L) for 16 h before adding TRAIL (200 ng/mL) for another 24 h, were subjected to measurement of apoptosis by the propidium iodide method using flow cytometry. *Columns*, mean of three individual experiments; *bars*, SE. *D*, a summary of studies on a panel of melanoma cells and normal cells (melanocytes, FLOW 2000 fibroblasts, and HUVECs). Cells treated with tunicamycin alone (3 μ mol/L) for 40 h, TRAIL (200 ng/mL) alone for 24 h, or tunicamycin (3 μ mol/L) for 16 h, followed by the addition of TRAIL (200 ng/mL) for another 24 h, were subjected to measurement of apoptosis by the propidium iodide method using flow cytometry. *Columns*, mean of three individual experiments; *bars*, SE.

Downloaded from <http://cancerres.aacrjournals.org/cancerres/article-pdf/67/12/5880/2568135/5880.pdf> by guest on 24 April 2024

tunicamycin up-regulates TRAIL-R2 expression in melanoma cells, we quantitated TRAIL-R2 mRNA expression in Mel-RM and MM200 cells before and after exposure to the compound for varying intervals. As shown in Fig. 3A, treatment with tunicamycin up-regulated the levels of TRAIL-R2 mRNA in both cell lines with a marked increase being observed at 16 h and further increases at 24 and 36 h. In contrast, there was no alteration in the levels of TRAIL-R1 mRNA after exposure to tunicamycin (Fig. 3A).

We next studied if the up-regulation of TRAIL-R2 mRNA leads to increased TRAIL-R2 total protein levels by measuring TRAIL-R2 expression in permeabilized Mel-RM and MM200 cells in flow cytometry. Figure 3B shows that tunicamycin induced a marked increase in the levels of the TRAIL-R2 total protein with a similar kinetics to that of the up-regulation of TRAIL-R2 mRNA (Fig. 3A). However, it was of note that the levels of the TRAIL-R2 total protein experienced a transient decrease at early stages after exposure to tunicamycin at 6 h in both cell lines. Up-regulation of the TRAIL-R2 total protein levels by tunicamycin was confirmed by Western blot analysis of TRAIL-R2 expression in whole cell lysates from Mel-RM and MM200 cells treated with tunicamycin for varying intervals (Fig. 3C).

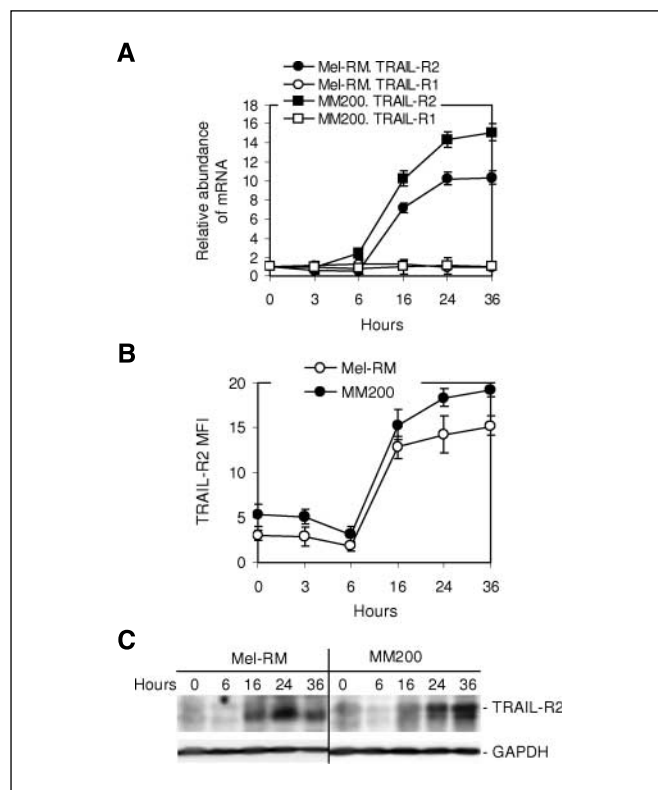


Figure 3. Tunicamycin increases TRAIL-R2 gene transcription and its total protein levels. **A**, Mel-RM and MM200 cells were treated with tunicamycin (3 $\mu\text{mol/L}$) for the indicated time periods before total RNA was isolated and subjected to real-time PCR analysis for TRAIL-R2 and TRAIL-R1 mRNA expression. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. *Points*, mean of three individual experiments; *bars*, SE. **B**, Mel-RM and MM200 with or without treatment with tunicamycin (3 $\mu\text{mol/L}$) for indicated time periods were permeabilized and subjected to measurement of TRAIL-R2 expression in flow cytometry. The data shown are the MFIs of TRAIL-R2 in permeabilized cells. *Points*, mean of three individual experiments; *bars*, SE. **C**, Mel-RM and MM200 cells were treated with tunicamycin (3 $\mu\text{mol/L}$) for indicated time periods. Whole cell lysates were subjected to Western blot analysis. The data shown are representative of three individual experiments.

The role of the IRE1 α -mediated signaling pathway of the UPR in up-regulation of TRAIL-R2 by tunicamycin in melanoma cells. As shown in Fig. 4A, exposure of either Mel-RM or MM200 cells to tunicamycin resulted in the up-regulation of the ER chaperone protein Bip, induction of the UPR effector XBP1, phosphorylation of the translation initiator eIF2 α increases in the expression of the UPR transducers, IRE1 α and ATF6, and appearance of the cleaved form of ATF6 (12–14). Moreover, another UPR transducer, PERK was phosphorylated as evidenced by the appearance of an extra band with reduced electrophoretic motility, which corresponds to phosphorylated PERK (23). These observations confirmed that treatment with tunicamycin initiated the UPR in both cell lines. However, the changes in XBP1, IRE1 α , ATF6, and PERK occurred more rapidly and were more pronounced in Mel-RM than in MM200 cells.

To elucidate if any of the UPR signaling pathways plays a role in the up-regulation of TRAIL-R2 by tunicamycin in melanoma cells, we silenced the UPR transducer IRE1 α by transfecting an IRE1 α -specific siRNA construct into Mel-RM and MM200 cells. As shown in Fig. 4B, siRNA knockdown of IRE1 α markedly inhibited its basal expression and its up-regulation by tunicamycin in both cell lines. Figure 4C shows that the inhibition of IRE1 α efficiently blocked the up-regulation of TRAIL-R2 by tunicamycin in Mel-RM cells, which could be observed at either 16 or 36 h after treatment with tunicamycin. However, IRE1 α siRNA had no effect on tunicamycin-mediated up-regulation of TRAIL-R2 in MM200 cells (Fig. 4C).

The role of the ATF6-mediated signaling pathway of the UPR in the up-regulation of TRAIL-R2 by tunicamycin in melanoma cells. We also examined if the ATF6-mediated signaling pathway of the UPR plays a role in tunicamycin-mediated up-regulation of TRAIL-R2 in melanoma cells by transfecting an ATF6-specific siRNA construct into Mel-RM and MM200 cells. As shown in Fig. 4B, siRNA knockdown of ATF6 significantly reduced its expression levels as either the native p90 form or as the cleaved p50 form in cells before and after treatment with tunicamycin. Figure 4D shows that the inhibition of ATF6 blocked up-regulation of TRAIL-R2 by tunicamycin in Mel-RM cells. However, the inhibitory effect could only be observed by 36 h after exposure to the compound, whereas at 16 h, the increase was minimally affected. Similar to the inhibition of IRE1 α , inhibition of ATF6 by siRNA had no effect on tunicamycin-induced up-regulation of TRAIL-R2 in MM200 cells (Fig. 4D).

CHOP is involved in the up-regulation of TRAIL-R2 by tunicamycin in melanoma cells. We studied the expression of the transcription factor CHOP, which is also a UPR effector (12–14), in Mel-RM and MM200 cells before and after treatment with tunicamycin. As shown in Fig. 5A, CHOP was constitutively expressed at relatively high levels in both cell lines, but surprisingly, was not up-regulated by tunicamycin. Instead, treatment with tunicamycin resulted in a marked decrease in CHOP expression.

To clarify a potential role of CHOP in the up-regulation of TRAIL-R2 by tunicamycin, we inhibited CHOP by transiently transfecting a CHOP siRNA construct into Mel-RM and MM200 cells. Figure 5B shows that siRNA knockdown of CHOP significantly inhibited its expression in both cell lines. As shown in Fig. 5C, the CHOP siRNA moderately inhibited the increase in TRAIL-R2 at 16 h after treatment with tunicamycin. However, by 36 h, the increase in TRAIL-R2 expression in MM200 cells was markedly reduced with the mean fluorescence intensity (MFI) being approximately the same as that in cells without treatment with

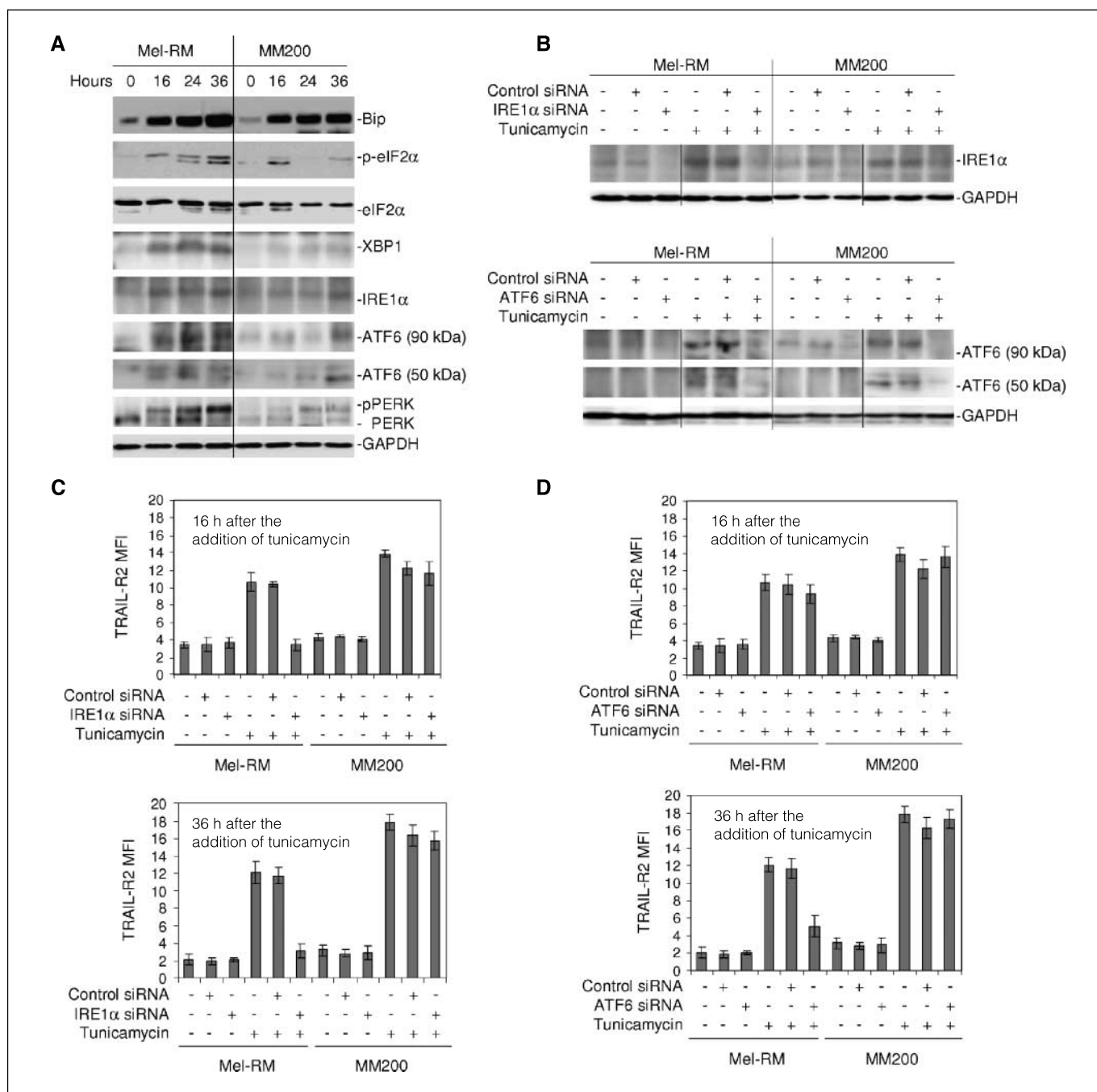


Figure 4. The IRE1 α - and ATF6-mediated signaling pathways of the UPR contribute to the up-regulation of TRAIL-R2 by tunicamycin in melanoma cells. **A**, tunicamycin induced the UPR in melanoma cells. Whole cell lysates from Mel-RM and MM200 cells with or without treatment with tunicamycin (3 μ mol/L) for indicated time periods were subjected to Western blot analysis. The data shown are representative of three individual experiments. **B**, knockdown of IRE1 α and ATF6 by siRNA decreases the levels of expression of IRE1 α and ATF6, respectively, in the absence or presence of tunicamycin. Mel-RM and MM200 cells were transfected with the control, IRE1 α (*top*) or ATF6 (*bottom*) siRNA. Twenty-four hours later, the cells were treated with tunicamycin (3 μ mol/L) for 36 h. Whole cell lysates were then subjected to Western blot analysis. The data shown are representative of two individual experiments. **C**, inhibition of IRE1 α by siRNA blocks up-regulation of TRAIL-R2 by tunicamycin in Mel-RM but not MM200 cells. Mel-RM and MM200 cells were transfected with the control, or IRE1 α siRNA. Twenty-four hours later, the cells were treated with tunicamycin (3 μ mol/L) for either 16 (*top*) or 36 h (*bottom*) before measurement of TRAIL-R2 expression in flow cytometry. The data shown are the MFIs of TRAIL-R2. *Columns*, mean of duplicate assays in two individual experiments; *bars*, SE. **D**, inhibition of ATF6 by siRNA blocks up-regulation of TRAIL-R2 by tunicamycin in Mel-RM but not MM200 cells. Mel-RM and MM200 cells were transfected with the control or ATF6 siRNA. Twenty-four hours later, the cells were treated with tunicamycin (3 μ mol/L) for either 16 (*top*) or 36 h (*bottom*) before measurement of TRAIL-R2 expression in flow cytometry. The data shown are the MFIs of TRAIL-R2. *Columns*, mean of duplicate assays in two individual experiments; *bars*, SE.

tunicamycin. In contrast, the increase in Mel-RM cells remained only partially inhibited, albeit to a greater extent than that at 16 h.

Tunicamycin up-regulates TRAIL-R2 and enhances TRAIL-induced apoptosis in fresh melanoma isolates. We studied if

tunicamycin also up-regulated TRAIL-R2 in fresh melanoma isolates. Freshly isolated melanoma cells, Mel-NW and Mel-RT, were treated with tunicamycin for 24 h. As shown in Fig. 6A and B, treatment with tunicamycin markedly increased the levels of

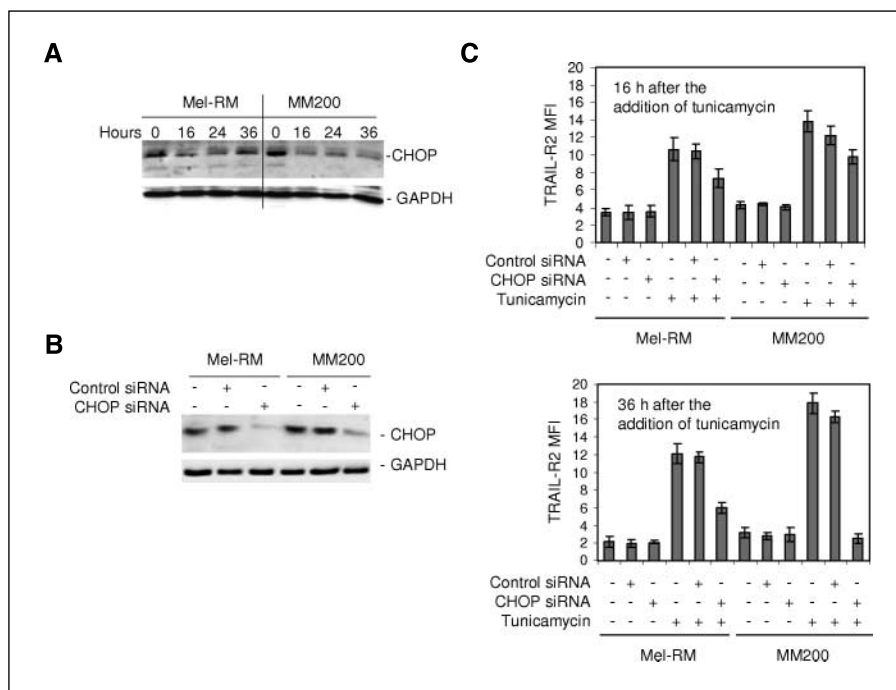


Figure 5. CHOP is involved in the up-regulation of TRAIL-R2 by tunicamycin in melanoma cells. *A*, tunicamycin decreases the CHOP expression in melanoma cells. Whole cell lysates from Mel-RM and MM200 cells with or without treatment with tunicamycin (3 μmol/L) for the indicated time periods were subjected to Western blot analysis. The data shown are representative of three individual experiments. *B*, inhibition of CHOP expression by siRNA. Mel-RM and MM200 cells were transfected with the control or CHOP siRNA. Twenty-four hours later, the cells were harvested, and whole cell lysates were subjected to Western blot analysis. The data shown are representative of two individual experiments. *C*, inhibition of CHOP by siRNA blocks up-regulation of TRAIL-R2 by tunicamycin in melanoma cells. Mel-RM and MM200 cells were transfected with the control or CHOP siRNA. Twenty-four hours later, the cells were treated with tunicamycin (3 μmol/L) for either 16 (*top*) or 36 h (*bottom*) before measurement of TRAIL-R2 expression in flow cytometry. The data shown are the MFIs of TRAIL-R2. *Columns*, mean of duplicate assays in two individual experiments; *bars*, SE.

TRAIL-R2 on the cell surface as measured in flow cytometry, and the TRAIL-R2 total protein levels as detected in Western blot analysis, in both Mel-NW and Mel-RT cells. Figure 6C shows that neither tunicamycin nor TRAIL induced significant levels of apoptosis in a panel of fresh melanoma isolates. However,

pretreatment with tunicamycin followed by the addition of TRAIL resulted in a marked increase in the percentages of apoptotic cells. As shown in Fig. 6D, sensitization of fresh melanoma isolates to TRAIL-induced apoptosis by tunicamycin was substantially inhibited by a recombinant TRAIL-R2/Fc chimera, indicating that

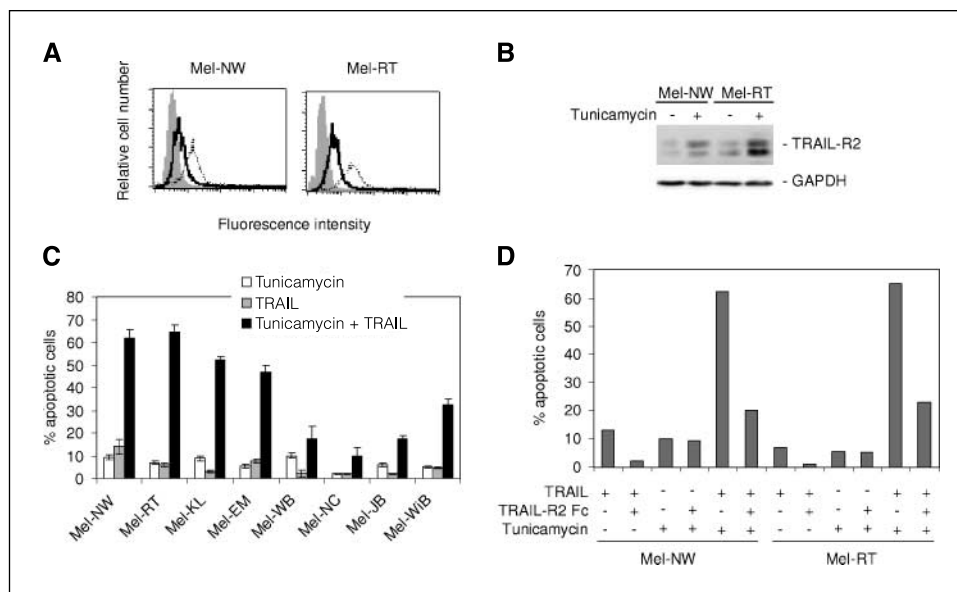


Figure 6. Tunicamycin up-regulates TRAIL-R2 and enhances TRAIL-induced apoptosis in fresh melanoma isolates. *A*, Mel-NW and Mel-RT cells with (*dotted lines*) or without (*thick lines*) treatment with tunicamycin (3 μmol/L) for 16 h were subjected to measurement of the cell surface TRAIL-R2 expression in flow cytometry. The filled histograms are isotype controls. The data shown are representative of three individual experiments. *B*, tunicamycin up-regulates the TRAIL-R2 total protein levels in fresh melanoma isolates. Whole cell lysates from Mel-NW and Mel-RT cells with or without treatment with tunicamycin (3 μmol/L) for 24 h were subjected to Western blot analysis. The data shown are representative of two individual experiments. *C*, tunicamycin sensitizes fresh melanoma isolates to TRAIL-induced apoptosis. Cells with or without treatment with tunicamycin alone (3 μmol/L) for 40 h, TRAIL (200 ng/mL) alone for 24 h, or tunicamycin (3 μmol/L) for 16 h, followed by the addition of TRAIL (200 ng/mL) for another 24 h, were subjected to measurement of apoptosis by the propidium iodide method using flow cytometry. *Columns*, mean of duplicate assays in two individual experiments; *bars*, SE. *D*, a TRAIL-R2/Fc chimera inhibits sensitization of fresh melanoma isolates to TRAIL-induced apoptosis by tunicamycin. Mel-NW and Mel-RT cells with or without pretreatment with a TRAIL-R2/Fc chimera (10 μg/mL) for 1 h were treated with tunicamycin (3 μmol/L) for 40 h, TRAIL (200 ng/mL) for 24 h, or tunicamycin (3 μmol/L) for 16 h before adding TRAIL (200 ng/mL) for another 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are representative of two individual experiments.

the effect of tunicamycin on TRAIL-induced apoptosis in fresh melanoma isolates is accounted for by the increase in TRAIL-R2 expression on the cell surface.

Discussion

The present study shows that tunicamycin, a naturally occurring antibiotic that induces ER stress by inhibition of glycosylation (15), can potently enhance TRAIL-induced apoptosis in cultured melanoma cell lines and fresh melanoma isolates by selectively up-regulating TRAIL-R2 on the cell surface. We show, for the first time, that the IRE1 α - and ATF6-mediated signaling pathways of the UPR contribute to the up-regulation of TRAIL-R2 in a cell line-dependent manner.

Although TRAIL seems to be a promising candidate for cancer therapeutics (1–5), our past studies indicated that fresh isolates of melanoma and melanoma in tissue sections frequently had low TRAIL death receptor expression and, therefore, may be unresponsive to TRAIL (8, 11). However, unlike studies in many other solid cancers, in which TRAIL death receptors could be up-regulated by other therapeutic drugs (24–27), we have not found these to increase TRAIL death receptor expression in melanoma. Agents tested have included DNA-damaging agents, microtubulin-targeting agents, histone deacetylase inhibitors, and mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitors (ref. 21, and data not shown). The ability of tunicamycin to up-regulate TRAIL-R2 in melanoma is therefore of particular interest. Importantly, up-regulation of TRAIL-R2 by tunicamycin seemed to be highly selective. This is because tunicamycin did not up-regulate the expression of the other TNF receptor family members, TRAIL-R1, TRAIL-R3, and TRAIL-R4, TNF-R1 and TNF-R2, and Fas. Moreover, tunicamycin did not up-regulate TRAIL-R2 expression in normal cells, including melanocytes, fibroblasts, and HUVECs. This melanoma-specific selectivity of tunicamycin in the up-regulation of TRAIL-R2 would be an advantage for its potential clinical use.

Up-regulation of TRAIL-R2 by tunicamycin was associated with enhanced apoptotic signaling induced by TRAIL. This was evidenced by increased activation of caspase-8 and Bid, reduction in $\Delta\Psi_m$, activation of caspase-3, and cleavage of its substrate PARP. Caspase-8 and caspase-3 are the major initiator and effector caspase, respectively, in TRAIL-induced apoptosis of melanoma cells, whereas Bid is the essential mediator that links the death receptor apoptotic pathway to the mitochondrial apoptotic pathway (27, 28–30). The latter is known to play an important role in TRAIL-induced apoptosis of melanoma (7, 31). The finding that a TRAIL-R2/Fc chimera or a caspase-8-specific inhibitor efficiently blocked TRAIL-induced apoptosis in the presence of tunicamycin suggests that enhanced apoptotic signaling was due to the increased interactions between TRAIL and TRAIL-R2.

Tunicamycin-mediated up-regulation of TRAIL-R2 on the melanoma cell surface was associated with increased TRAIL-R2 gene transcription and elevated TRAIL-R2 total protein levels. However, TRAIL-R2 total protein levels experienced a rapid but transient decrease after exposure to tunicamycin. This may reflect reduced initiation of translation that is the most immediate response of the UPR to ER stress (14, 32). Indeed, the translation initiator eIF2 α was phosphorylated by tunicamycin in melanoma cells. Similarly, a number of the UPR components, including the ER chaperone protein Bip, the UPR effector XBP1, and the UPR transducers PERK, IRE1 α , and ATF6 were up-regulated/activated

by tunicamycin (12–14). These results indicate that the UPR was activated by tunicamycin in melanoma cells and suggest that the increase in TRAIL-R2 gene transcription may be the outcome of selective up-regulation of transcription of UPR target genes.

Both the IRE1 α - and ATF6-mediated signaling pathways of the UPR seemed to play roles in tunicamycin-mediated up-regulation of TRAIL-R2 in Mel-RM cells as shown by the inhibition of TRAIL-R2 up-regulation when IRE1 α or ATF6 was knocked down by siRNA. IRE1 α seemed particularly important in that inhibition IRE1 α markedly inhibited the increase in TRAIL-R2 even at a relatively early stage (16 h) after treatment with tunicamycin. In contrast, the inhibitory effect of ATF6 siRNA was only evident at 36 h. Both IRE1 α and ATF6 are ER membrane-localized proteins that act as UPR transducers (12–14). On activation of the UPR, IRE1 α displays its RNase activity that cleaves XBP1 mRNA, generating a splicing variant of XBP1 mRNA that encodes a potent transcription factor, which in turn activates transcription of many UPR target genes. 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. It is conceivable that both the effector of IRE1 α , XBP1, and ATF6 may act directly or indirectly via other transcription factors such as CHOP to activate transcription of TRAIL-R2 in Mel-RM cells (12–14).

It is unclear why IRE1 α and ATF6 did not play a role in the up-regulation of TRAIL-R2 by tunicamycin in MM200 cells. However, tunicamycin treatment resulted in greater up-regulation of IRE1 α and ATF6 in Mel-RM than in MM200 cells, and XBP1 was just above the detectable level in MM200 cells. It is therefore likely that the IRE1 α - and ATF6-mediated signaling pathways of the UPR were less activated by tunicamycin in MM200 cells and, thus, did not play a role in tunicamycin-mediated up-regulation of TRAIL-R2. Similarly, phosphorylation of PERK and its substrate eIF2 α was less in MM200 compared with Mel-RM cells. In addition to the attenuation of translation, in general, the PERK-mediated pathway of the UPR can cause selective promotion of translation of a small subset of mRNAs, including the ATF4 mRNA (12–14, 32). The latter encodes a transcription factor that activates transcription of UPR target genes including CHOP, which has been shown to be responsible for the up-regulation of TRAIL-R2 induced by tunicamycin in human prostate cancer cells (17).

A paradox arising from the current study was that CHOP was constitutively expressed at relatively high levels, but was decreased by tunicamycin in melanoma cells. Nevertheless, CHOP was involved in the up-regulation of TRAIL-R2 as shown by knockdown experiments with CHOP siRNA. The TRAIL-R2 promoter is known to contain a CHOP binding site, which was shown to play a role in the up-regulation of TRAIL-R2 upon the activation of the UPR (17, 33). In Mel-RM cells, CHOP siRNA but not ATF6 siRNA blocked tunicamycin-induced up-regulation of TRAIL-R2 at 16 h, suggesting that CHOP may act independently of ATF6 in these cells. In MM200 cells, inhibition of IRE1 α or ATF6 by siRNA did not block the increase in TRAIL-R2 induced by tunicamycin, suggesting that the up-regulation of TRAIL-R2 by CHOP in these cells was independent of IRE1 α or ATF6. The high expression of CHOP in melanoma may indicate that the UPR is constitutively activated in melanoma cells as reported in other cancer cells (34–36). The decrease in CHOP expression after tunicamycin treatment may be due to the attenuation of translation during the UPR (33). Further studies are needed to test this hypothesis.

Our finding that tunicamycin could sensitize fresh melanoma isolates to TRAIL-induced apoptosis by the up-regulation of

TRAIL-R2 is of particular importance because it is known that fresh melanoma isolates are relatively resistant to TRAIL-induced apoptosis due to low levels of TRAIL death receptor expression (8). This may reflect more closely the *in-vivo* status of TRAIL death receptor expression in melanoma cells and their susceptibility to TRAIL-induced apoptosis. However, although tunicamycin alone did not induce significant apoptosis in melanoma cells, it induced varying degrees of apoptosis in normal cells, such as melanocytes, fibroblasts, and HUVECs. These results suggest that careful evaluation of low doses of tunicamycin or other ER stress-inducing agents in combination with low concentrations of TRAIL is

required before *in vivo* investigations are carried out. They point, however, to agents which may sensitize melanoma to TRAIL and increase its therapeutic potential in melanoma.

Acknowledgments

Received 1/17/2007; revised 3/15/2007; accepted 4/16/2007.

Grant support: NSW State Cancer Council and the National Health and Medical Research Council, Australia. X.D. Zhang is a Cancer Institute NSW Fellow.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

- Nagata S. Apoptosis by death factor. *Cell* 1997;88:355–65.
- Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998;281:1305–8.
- Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp Cell Res* 2000;256:58–66.
- Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* 1999;5:157–63.
- Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999;104:155–62.
- Zhang XD, Franco A, Myers K, Gray C, Nguyen T, Hersey P. Relation of TNF-related apoptosis-inducing ligand (TRAIL) receptor and FLICE inhibitory protein expression to TRAIL-induced apoptosis of melanoma. *Cancer Res* 1999;59:2747–53.
- Hersey P, Zhang XD. How melanoma cells evade trail-induced apoptosis. *Nat Rev Cancer* 2001;1:142–50.
- Nguyen DT, Zhang XD, Hersey P. Regulation of TRAIL induced apoptosis of fresh melanoma isolates. *Clin Cancer Res* 2001;7:966–73s.
- Zhang XD, Wu JJ, Gillespie SK, Borrow JM, Hersey P. Human melanoma cells selected for resistance to apoptosis by prolonged exposure to TRAIL are more vulnerable to non-apoptotic cell death induced by cisplatin. *Clin Cancer Res* 2006;12:1335–64.
- Wu JJ, Zhang XD, Gillespie S, Hersey P. Selection for TRAIL resistance results in melanoma cells with high proliferative potential. *FEBS Lett* 2005;579:1940–4.
- Zhuang L, Lee CS, Scolyer RA, et al. Progression in melanoma is associated with decreased expression of death receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *Hum Pathol* 2006;37:1286–94.
- Harding HP, Calton M, Urano F, Novoa I, Ron D. Transcriptional and translational control in the mammalian unfolded protein response. *Annu Rev Cell Dev Biol* 2002;18:575–99.
- Zhang K, Kaufman RJ. Signaling the unfolded protein response from the endoplasmic reticulum. *J Biol Chem* 2004;18:279:25935–8.
- Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem* 2005;74:739–89.
- Elbein AD. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu Rev Biochem* 1987;56:497–534.
- Jin Z, McDonald ER III, Dicker DT, El-Deiry WS. Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. *J Biol Chem* 2004;279:35829–39.
- Shiraishi T, Yoshida T, Nakata S, et al. Tunicamycin enhances tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human prostate cancer cells. *Cancer Res* 2005;65:6364–70.
- Zhang XD, Gillespie SK, Hersey P. Staurosporine induces apoptosis of melanoma by both caspase-dependent and -independent apoptotic pathways. *Mol Cancer Ther* 2004;3:187–97.
- Zhang XD, Nguyen T, Thomas WD, Sanders JE, Hersey P. Mechanisms of resistance of normal cells to TRAIL induced apoptosis vary between different cell types. *FEBS Lett* 2000;482:193–9.
- Bates RC, Rankin LM, Lucas CM, Scott JL, Krissansen GW, Burns GF. Individual embryonic fibroblasts express multiple β chains in association with the α v integrin subunit. Loss of β 3 expression with cell confluence. *J Biol Chem* 1991;266:18593–9.
- Gillespie S, Borrow J, Zhang XD, Hersey P. Bim plays a crucial role in synergistic induction of apoptosis by the histone deacetylase inhibitor SBHA and TRAIL in melanoma cells. *Apoptosis* 2006;11:2251–65.
- Zhang XD, Franco AV, Nguyen T, Gray CP, Hersey P. Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. *J Immunol* 2000;164:3961–70.
- Nishitoh H, Matsuzawa A, Tobiume K, et al. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 2002;16:1345–55.
- Sheikh MS, Burns TF, Huang Y, et al. p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor α . *Cancer Res* 1998;58:1593–8.
- Singh TR, Shankar S, Chen X, Asim M, Srivastava RK. Synergistic interactions of chemotherapeutic drugs and tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand on apoptosis and on regression of breast carcinoma *in vivo*. *Cancer Res* 2003;63:5390–400.
- Nakata S, Yoshida T, Horinaka M, Shiraishi T, Wakada M, Sakai T. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. *Oncogene* 2004;19:6261–71.
- Chinnaiyan AM, Prasad U, Shankar S, et al. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proc Natl Acad Sci U S A* 2000;97:1754–9.
- Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J Immunol* 1998;161:2833–40.
- Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998;94:491–501.
- Luo X, Budihardjo I, Zou H, Slaughter C, Wang X, Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998;94:481–90.
- Zhang XD, Zhang XY, Gray CP, Nguyen T, Hersey P. Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of human melanoma is regulated by Smac/DIABLO release from mitochondria. *Cancer Res* 2001;61:7339–48.
- Kaufman RJ. Regulation of mRNA translation by protein folding in the endoplasmic reticulum. *Trends Biochem Sci* 2004;29:152–8.
- Yamaguchi H, Wang HG. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. *J Biol Chem* 2004;279:45495–502.
- Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? *Nat Rev Cancer* 2004;4:966–77.
- Jamora C, Dennert G, Lee AS. Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME. *Proc Natl Acad Sci U S A* 1996;93:7690–4.
- Shuda M, Kondoh N, Imazeki N, et al. Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. *J Hepatol* 2003;38:605–14.