Human Melanoma Cells Selected for Resistance to Apoptosis by Prolonged Exposure to Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Are More Vulnerable to Necrotic Cell Death Induced by Cisplatin

Xu Dong Zhang, Jing Jing Wu, Susan Gillespie, Jodie Borrow, and Peter Hersey

Abstract

**Purpose:** Heterogeneous sensitivity of melanoma cells to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptosis may lead to outgrowth of TRAIL-resistant cells and limit successful treatment by TRAIL. The present study aims to better understand the biological characteristics of melanoma cells resistant to TRAIL-induced apoptosis.

**Experimental Design:** We generated TRAIL-resistant melanoma cells by prolonged exposure to TRAIL and characterized the cells in terms of their sensitivity to killing induced by a panel of cytotoxic agents using biological and biochemical methods.

**Results:** TRAIL-resistant melanoma cells are cross-resistant to apoptosis induced by another death ligand FasL, the DNA-damaging agent cisplatin, the histone deacetylase inhibitor suberic bishydroxamate, and the antimicrotubule Vinca alkaloid, vincristine. The apoptotic signaling seemed to be inhibited upstream of mitochondrial apoptotic events and was associated with decreased expression of multiple apoptotic mediators, including pro-caspase-8, Fas-associated death domain, Bid, Bim, p53, and the products of its proapoptotic target genes. Despite being resistant to apoptosis, TRAIL-resistant melanoma cells were more vulnerable to cisplatin-induced nonapoptotic cell death. This was characterized by lack of DNA fragmentation, delayed externalization of phosphatidylserine, caspase and p53 independence, and severe mitochondrial disruption, and was preceded by poly(ADP)ribose polymerase (PARP) activation and depletion of intracellular ATP, indicative of necrotic cell death. Inhibition of PARP activity partially converted the mode of cell death from necrosis to apoptosis.

**Conclusions:** TRAIL-resistant melanoma cells are cross-resistant to apoptosis induced by various apoptotic stimuli but are more sensitive to nonapoptotic cell death induced by cisplatin. Exploration of chemotherapy-induced nonapoptotic cell death may provide an alternative strategy in overcoming resistance of melanoma cells to TRAIL-induced apoptosis.

There has been little progress in the medical treatment of metastatic melanoma because of the absence of effective systemic therapies. This is believed to be primarily due to resistance of melanoma cells to apoptosis induced by therapeutic agents, such as chemotherapy, irradiation, and immunotherapy (1, 2). We have previously shown that a tumor necrosis factor (TNF) family member, TNF-related apoptosis-inducing ligand (TRAIL), induces apoptosis in approximately two thirds of melanoma cell lines (3, 4). 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 (5–7). Heterogeneous sensitivity of tumor cells to TRAIL-induced apoptosis, however, may lead to persistent growth of TRAIL-resistant cells, and limit successful treatment of melanoma by TRAIL.

Two major apoptotic pathways have been identified as the death receptor–mediated “extrinsic apoptotic pathway” and the mitochondrion-mediated “intrinsic apoptotic pathway” (8, 9). Although each pathway is initially mediated by different mechanisms, they share a common final phase of apoptosis, consisting of activation of the executioner caspases and dismantling of substrates critical for cell survival (10, 11). Induction of apoptosis by chemotherapeutic agents is believed to be largely mediated by the intrinsic apoptotic pathway (1, 2). This involves release of mitochondrial apoptotic proteins, such as cytochrome c, and second mitochondrial-derived activator of caspase/direct IAP-binding protein with low isoelectric point (Smac/DIABLO; refs. 12–14). The Bcl-2 family members play a
central role in regulating changes in mitochondrial outer membrane permeability (15, 16). Although TRAIL-induced apoptosis is initiated by the extrinsic apoptotic pathway, past studies have shown that the intrinsic apoptotic pathway plays a critical role in regulating sensitivity of melanoma cells to TRAIL-induced apoptosis (4).

In addition to inducing apoptosis, a number of chemotherapeutic agents have been reported to induce nonapoptotic forms of cell death (17–21). For example, DNA-alkylating agents kill cells resistant to apoptosis by inducing necrosis (20). In regard to melanoma, we and others have shown that ingenol 3-angelate, one of the active ingredients in an extract from Euphorbia peplus, induces caspase-independent nonapoptotic cell death (18, 22). In addition, cisplatin (cis-diaminedichloroplatinum, CDDP) has also been shown to induce necrosis in a melanoma cell line when used at relatively high concentrations (23). The significance of nonapoptotic forms of cell death in chemotherapy and the mechanism(s) by which they are induced by chemotherapeutic drugs remain largely unclear; it is, however, noteworthy that nonapoptotic cell death is often observed under conditions in which apoptosis is inhibited.

We have previously reported that prolonged exposure to TRAIL resulted in stable populations of melanoma cells resistant to TRAIL-induced apoptosis, which were able to be passaged in the presence of TRAIL (24, 25). We report in the present study that these TRAIL-resistant melanoma cells expressed lower levels of the major components of the apoptotic pathways, including procaspase-8, Fas-associated death domain (FADD), Bid, Bim, procaspase-3, p53, and Bak, and the products of its proapoptotic target genes, and were cross-resistant to apoptosis induced by a number of apoptotic stimuli, including Fasl, the DNA-damaging agent CDDP, the histone deacetylase inhibitor suberibishydroxamate (SBHA), and the antimicrotubule Vinca alkaloid vincristine. Nevertheless, treatment of the apoptosis-resistant melanoma cells with CDDP induced massive nonapoptotic cell death, which is characterized by lack of DNA fragmentation, delayed externalization of phosphatidylserine, caspase and p53 independence, plasma membrane perturbations, severe mitochondrial disrup-

### Materials and Methods

**Cell culture and reagents.** Human melanoma cell lines Mel-FH, Mel-RM, and MM200 have been described previously (3, 4). The cell lines were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Australia). Recombinant human TRAIL and Fasl were supplied by Immunex (Seattle, WA). The rabbit polyclonal antibodies against caspase-3, caspase-8, and Bid, and the mouse monoclonal antibodies (mAb) against PARP and the rabbit mAb against the active form of caspase-3 were purchased from PharMingen (San Diego, CA). The mAb against wild-type p53 was from Upstate Biotechnology (Waltham, MA). The rabbit polyclonal antibody against Bak and the mouse mAbs against Bcl-2, Bcl-Xl, Mel-1, and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse mAb against XIAP was purchased from Transduction Laboratories (Lexington, KY). The rabbit polyclonal antibody against Bim and the mAb against Noxa were from Imgenex (San Diego, CA). The rabbit polyclonal antibody against PUMA was a kind gift from Dr. J. Yu (University of Pittsburgh Cancer Center, Pittsburgh, PA). The cell-permeable pan caspase inhibitor Z-Val-Ala-Asp (OMe)-CH2F (z-VAD-fmk) was purchased from Calbiochem (La Jolla, CA). The histone deacetylase inhibitor SBHA and the PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isouquinolinone (DPQ) were purchased from Sigma (Castle Hill, Australia). JC-1 was from Molecular Probes (Eugene, OR). CDDP and vincristine were supplied by Upjohn (Sydney, NSW, Australia).

**Generation of TRAIL-selected cells.** Generation of TRAIL-selected resistant cells was done as described previously (25). Briefly, Mel-RM, Mel-FH, and MM200 cells were seeded in 25 cm² culture flasks and cultured in DMEM containing TRAIL at 200 ng/mL. The surviving cells were fed every 3 days for 5 to 6 weeks with culture medium containing TRAIL (200 ng/mL) until they reached 70% to 80% confluence. The resulting cells were then passaged and cultured in the presence of TRAIL for at least 20 weeks. The resulting cell lines were designated as Mel-RM.S, Mel-FH.S, and MM200.S, respectively.

**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 (25). Briefly,
cells were seeded at 5,000 per well onto flat-bottomed 96-well culture plates and allowed to grow for 24 hours followed by the desired treatment. Cells were then labeled with MTT from the Vybrant MTT Cell Proliferation Assay kit (Molecular Probes) according to the instruction of the manufacturer.

**Flow cytometry.** Immunostaining on intact and permeabilized cells was carried out as described previously (3, 4). Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer.

**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 (3, 22).

**Propidium iodide uptake assay.** Tumor cells were seeded at $1 \times 10^5$ per well in 24-well plates and allowed to reach exponential growth for 24 hours before treatment. The propidium iodide uptake assay was done as described previously. Briefly, adherent cells and nonadherent cells were collected and washed with ice-cold PBS. Cells were then resuspended in 100 μl binding buffer at $1 \times 10^6$/ml and stained with propidium iodide. After incubation at room temperature for 15 minutes, an additional 400 μl of binding buffer was added and cells were analyzed by flow cytometry within 1 hour.

**Mitochondrial membrane potential ($\Delta \Psi_m$).** Tumor cells were seeded at $1 \times 10^5$ per well in 24-well plates and allowed to reach exponential growth for 24 hours before treatment. JC-1 staining was done according to the instructions of the manufacturer (Molecular Probes). Briefly, adherent cells and nonadherent cells were collected and washed with PBS. Cells were then incubated with 10 μg/ml of JC-1 in warm PBS at 37°C for 15 minutes. After washing with PBS, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). Cells with polarized mitochondria presented in the upper right quadrant of the dot plot due to the formation of JC-1 aggregates, which emit orange color (590 nmol/L) when excited at 488 nmol/L.

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Fig. 2. Expression levels of multiple intracellular apoptotic mediators in the TRAIL-selected cells. A to D, whole cell lysates from the parental and TRAIL-selected melanoma cells were subjected to Western blot analysis. Representative of three individual experiments.
Cells with depolarized mitochondria emit green color (530 nmol/L) and are visualized in the lower right quadrant of the dot plot.

**Measurement of intracellular ATP content.** Intracellular ATP of melanoma cells was measured by a luciferin-luciferase bioluminescence assay using the ATP determination kit (Molecular Probes). Cells were washed and collected in ice-cold PBS. Ten microliters of cell lysate were added to 90 μL of working buffer containing 0.5 mmol/L luciferin, 1.25 μg/mL luciferase, 25 mmol/L tricine buffer (pH 7.8), 5 mmol/L MgSO4, 100 μmol/L EDTA, and 1 mmol/L DTT. Luminescence was then analyzed with a luminometer. A standard curve was generated from known concentrations of ATP supplied with the kit and was used to calculate the concentration of ATP in each sample.

**Western blot analysis.** Western blot analysis was carried out as described previously (3, 4).

**Results**

Low expression of key components of the apoptotic pathways in the TRAIL-selected cells. We have previously shown that prolonged exposure to TRAIL resulted in stable populations of melanoma cells resistant to TRAIL-induced apoptosis, which were able to be passaged in the presence of TRAIL (25). This was further evidenced by lack of DNA fragmentation and caspase-3 activation in the presence of TRAIL (Fig. 1A and B). The TRAIL-selected cells expressed markedly lower levels of the TRAIL death receptors, TRAIL-R1 and TRAIL-R2 (25), but the levels of decoy receptors for TRAIL, TRAIL-R3, and TRAIL-R4 remained as low as those in their parental counterparts (ref. 3; data not shown). Figure 2A shows that the levels of expression of pro-caspase-8 and pro-caspase-3 were markedly lower in the TRAIL-selected cells, whereas the levels of pro-caspase-9 remained similar between the TRAIL-selected and parental cells. The BH3-only proteins of the Bcl-2 family—Bid, Bim, PUMA, Noxa, and Bad—were also expressed at markedly lower levels in the TRAIL-selected cells (Fig. 2B). In contrast, there was no apparent difference in the levels of expression of the multidomain proapoptotic proteins, Bax and Bak (Fig. 2B). Similarly, no difference was found in the expression of the antiapoptotic members of the Bcl-2 family—Bcl-xl, Bcl-2, Bcl-xL, Bcl-w, and the IAP family members, ML-IAP and XIAP (Fig. 2C). The expression of the adaptor protein of the extrinsic apoptotic pathway, FADD, was decreased in the selected cells, but the adaptor protein of the intrinsic apoptotic pathway, Apaf-1, was expressed at similar levels between the selected cells and their parental counterparts (Fig. 2D). As reported before, the FLIP protein was expressed at low levels in all the parental melanoma cell lines except for a moderate level of FLIPS in Mel-RM cells (3). Unexpectedly, FLIP was hardly detectable in the TRAIL-selected melanoma cells (Fig. 2D).
The TRAIL-selected melanoma cells are cross-resistant to apoptosis induced by various apoptotic stimuli, but are more sensitive to nonapoptotic cell death induced by CDDP. As shown in Fig. 3A, the sensitivity of the TRAIL-selected cells to apoptosis induced by the clinically relevant chemotherapeutic drugs, CDDP and vincristine, was markedly decreased in comparison with that of the parental counterparts. Mel-RM.S cells were also much less sensitive than Mel-RM cells to apoptosis induced by the histone deacetylase inhibitor SBHA (Fig. 3A). As reported before, MM200 and Mel-FH cells were resistant to SBHA-induced apoptosis (26). The sensitivity of the TRAIL-selected cells to FasL-induced apoptosis was also significantly decreased (Fig. 3C), which was associated with a decrease in the expression of Fas on the surface (Fig. 3D).

Despite resistance to CDDP-induced apoptosis, the majority of the TRAIL-selected cells were found to be rounded, irregular, and detached from the culture plates after exposure to CDDP for 24 hours, indicating nonapoptotic cell death (Fig. 4A). This was further confirmed in assays with 4',6-diamidino-2-phenylindole (DAPI) staining of the floating cells collected from Mel-RM and Mel-RM.S treated with CDDP. Figure 4B shows that DNA fragmentation and/or chromatin condensation were common in the parental cells after exposure to CDDP, but a large proportion of the nuclei from the TRAIL-selected cells did not exhibit the apoptotic characteristics.

We quantitated viable versus apoptotic Mel-RM and Mel-RM.S cells treated with CDDP at a range of doses for 24 hours. Figure 4C shows that Mel-RM.S cells were more sensitive to CDDP-induced cell death, with a decrease in cell viability being observed when CDDP was used as low as 1 μg/mL, and only ~15% of the cells remained alive when it was used at 10 μg/mL. In contrast, a decrease in viability of Mel-RM cells was only seen when the drug was used at 2.5 μg/mL, and ~50% of the cells were still alive when treated with 10 μg/mL of CDDP. Cell death induced by CDDP in Mel-RM seemed to be largely accounted for by apoptosis as shown in Fig. 4D. However, only 15% of the Mel-RM.S cells died through apoptosis. Figure 4E shows that, in contrast to CDDP, vincristine and SBHA induced little cell death other than apoptosis, which was measured by MTT assays in both Mel-RM and Mel-RM.S cells. A summary of studies on CDDP-induced apoptosis in relation to non-apoptotic cell death in Mel-FH, Mel-FH.S, MM200, and MM200.S is shown in Fig. 4F.

Treatment with CDDP, but not FasL, SBHA, or vincristine, resulted in disruptions of mitochondria in the TRAIL-selected cells. As shown in Fig. 5A, FasL and vincristine induced a reduction in ΔΨm measured by JC-1 dye in both Mel-RM and MM200 cells. In contrast, SBHA only caused a reduction in ΔΨm in Mel-RM cells. This is consistent with the resistance of MM200 to SBHA-induced apoptosis (26). As expected, FasL, SBHA, and vincristine did not induce a reduction in ΔΨm in Mel-RM.S and MM200.S cells. In contrast, CDDP induced an even more marked reduction in ΔΨm in Mel-RM.S and MM200.S cells. Although a reduction in ΔΨm was detectable as early as 6 hours after exposure to CDDP in Mel-RM.S cells with a marked increase at 16 hours, there was only minimal reduction in ΔΨm in Mel-RM cells even at 16 hours after treatment (Fig. 5B).

CDDP induces perturbations of plasma membrane integrity. Uptake of propidium iodide is a test for plasma membrane integrity and indicates primary necrosis when observed in the absence of evidence for apoptosis (18, 27). As shown in Fig. 5C, uptake of propidium iodide was seen in 20% of Mel-RM.S and 15% of MM200.S cells 6 hours after treatment with CDDP when there was no uptake in the parental counterparts. By 16 hours, 52% of Mel-RM.S cells and 36% of MM200.S cells seemed to be propidium iodide positive, and by 24 hours uptake of propidium iodide was seen in the majority of the TRAIL-selected cells; however, there was much less propidium iodide uptake in the parental cells.

We analyzed the relationship between the uptake of propidium iodide induced by CDDP and an early apoptotic event, externalization of phosphatidylserine, detected by FITC-conjugated Annexin V. As shown in Fig. 5D, the percentage of propidium iodide–positive cells in Mel-RM.S was higher than that of Annexin V–positive cells at 6 hours after treatment with CDDP. Kinetic studies indicated that the majority of CDDP-treated Mel-RM.S cells did not become Annexin V positive unless they first became propidium iodide positive.
CDDP-induced nonapoptotic cell death of the TRAIL-selected cells is caspase and p53 independent. Figure 6A shows that the levels of processed caspase-3 induced by CDDP were much lower in Mel-RM.S than Mel-RM cells. Inhibition of caspase activation by the pan caspase inhibitor, z-VAD-fmk, before the addition of CDDP was found to efficiently inhibit apoptosis induced by CDDP in both Mel-RM and Mel-RM.S cells; however, it had little effect on cell death induced by CDDP in Mel-RM.S cells (Fig. 6B).

As shown in Fig. 6C, CDDP induced a marked increase in the levels of p53 in the parental cells but the levels of p53 in the TRAIL-selected cells remained barely detectable after treatment with CDDP. These results are consistent with our previous studies showing a marked reduction in p53 levels in the TRAIL-selected cells (25).

Inhibition of PARP activity partially converts nonapoptotic cell death to apoptosis. The intracellular depletion of ATP mediated by PARP activation is believed to play a determining role in bioenergetic failure and subsequently necrosis (20, 21). As shown in Fig. 6D, CDDP induced a rapid decrease in the ATP levels that was observed as soon as 1 hour after exposure to the drug in both Mel-RM and Mel-RM.S cells. At 3 and 6 hours after the addition of CDDP, the levels of ATP were 42% and 10% of the control, respectively, in Mel-RM.S. In contrast, the levels of ATP in Mel-RM cells remained relatively stable with 54% of the control level at 6 hours after treatment with CDDP. PARP activation induced by CDDP in the TRAIL-selected but not in the parental cells was confirmed by Western blot analysis of the production of PAR (Fig. 6E).

We treated the parental and TRAIL-selected melanoma cells with the PARP inhibitor, DPQ, 2 hours before adding CDDP. Figure 6F shows that the percentages of apoptotic cell death induced by CDDP in Mel-RM.S, but not in Mel-RM cells, were markedly increased, but there was no change in the overall levels of cell death induced by CDDP.

Discussion

We found in this study that TRAIL-selected melanoma cells were cross-resistant to apoptosis induced by either FasL that initiates the extrinsic apoptotic pathway or vincristine and SBHA that initiate the intrinsic apoptotic pathway. This was associated with decreased expression levels of multiple key apoptotic mediators in the TRAIL-selected cells, including the components of the extrinsic apoptotic pathway, Fas, procaspase-8, FADD, and Bid. The decrease in the levels of these molecules may account for the cross-resistance of the TRAIL-selected melanoma cells to FasL-induced apoptosis in that this may result in deficiency in formation of the DISC and in cross-talk between the two apoptotic pathways mediated by Bid (28, 29). This is supported by the finding that FasL-induced reduction in ΔΨm was inhibited in the TRAIL-selected cells, as was caspase-3 activation (data not shown). FLIP, an important inhibitor of the extrinsic apoptotic pathway in many cellular systems, did not seem to play a role in cross-resistance to either TRAIL- or FasL-induced apoptosis in that the expression levels were even decreased in the TRAIL-selected melanoma cells (30, 31).
Both SBHA and vincristine induced a reduction in $\Delta \Psi_m$ and activation of caspase-3 in the sensitive parental but not in the TRAIL-selected melanoma cells. This suggests that inhibition of apoptosis induced by these agents also occurred upstream of mitochondria. The BH-3 only proteins PUMA, Noxa, Bim, and Bad, which may act as intrinsic apoptotic initiators, seemed to be at much lower levels in the TRAIL-selected cells. Among them, Bim plays an important role in initiating apoptosis induced by SBHA (26). Bim is transcriptionally regulated by the forkhead transcription factors (32–34), which are phosphorylated by Akt resulting in their retention in the cytosol and thereby rendering them inactive (33, 35). Activation of the MAP kinase extracellular signal-regulated kinase 1/2 (ERK1/2) pathway was also reported to phosphorylate the BimEL protein and target it for proteasome degradation (36). The TRAIL-selected melanoma cells are known to have higher levels of activation of Akt and ERK1/2 (25). This may account for the low levels of Bim expression in these cells. The decrease in the expression of PUMA and Noxa in the TRAIL-selected cells may be

Fig. 5: CDDP induces reduction in $\Delta \Psi_m$ and primary perturbations of plasma membrane in the TRAIL-selected cells. A, cells were treated with CDDP (10 $\mu$g/mL), SBHA (30 $\mu$g/mL), or vincristine (1 ng/mL) for 24 hours followed by measurement of $\Delta \Psi_m$ using JC-1 in flow cytometry. Representative of three individual experiments. B, Mel-RM and Mel-RM.S cells were treated with CDDP at 10 $\mu$g/mL for the indicated time periods followed by measurement of $\Delta \Psi_m$ using JC-1 in flow cytometry. Representative of three individual experiments. C, cells were treated with CDDP (10 $\mu$g/mL) for the indicated time periods followed by staining with propidium iodide. The propidium iodide positive cells were quantitated by flow cytometry analysis. Points, mean of three individual experiments; bars, SE. D, Mel-RM.S cells were treated with CDDP (10 $\mu$g/mL) for the indicated time periods followed by either quantitation of uptake of propidium iodide or externalization of phosphatidylserine using FITC-conjugated Annexin V staining by flow cytometry analysis. Points, mean of three individual experiments; bars, SE.
accounted for by the low levels of p53 expression, and failure of p53 to be up-regulated in response to DNA damage by treatment with CDDP. The mechanism by which p53 and its targets were reduced in the TRAIL-selected cells remains unclear, but as discussed previously, evidence suggests that this was the result of selection of previously existing, characteristically distinct cells (25). Explanations for the low levels of pro-caspase-3, pro-caspase-8, FADD, and Bid are not

**Fig. 6.** CDDP-induced nonapoptotic cell death of the TRAIL-selected cells is independent of caspases and p53 and is associated with PARP activity. A, cells were treated with CDDP at 10 μg/mL for 16 hours followed by quantitation of the cells with processed caspase-3 using a mAb that specifically recognized the processed caspase-3 in permeabilized cells by flow cytometry analysis. Columns, mean of three individual experiments; bars, SE. B, z-VAD-fmk inhibits CDDP-induced apoptosis, but not nonapoptotic cell death of the TRAIL-selected cells. Mel-RM and Mel-RM.S cells were treated with z-VAD-fmk at 30 μmol/L 1 hour before addition of CDDP (10 μg/mL) for another 24 hours. Apoptotic cells were quantitated by the propidium iodide method using flow cytometry. Nonapoptotic cell death was quantitated using MTT assays. Columns, mean of three individual experiments; bars, SE. C, cells were treated with CDDP (10 μg/mL) for 16 hours. Whole cell lysates were subjected to Western blot analysis. Representative of three individual experiments. D, cells treated with CDDP (10 μg/mL) for the indicated time periods were subjected to quantitation of ATP. Y axis, percentages of the control value. Points, mean of three individual experiments; bars, SE. E, whole cell lysates from Mel-RM and Mel-RM.S cells treated with CDDP (10 μg/mL) for the indicated time periods were subjected to Western blot analysis. Representative of three individual experiments. F, cells were treated with DPQ (20 μmol/L) 2 hours before adding CDDP (10 μg/mL) for another 24 hours. Apoptotic cells were quantitated by measurement of sub-G1 DNA content using flow cytometry. Overall cell death was quantitated using MTT assays. Columns, mean of three individual experiments; bars, SE.
so readily apparent. The low levels of these proteins persisted for 2 weeks in the absence of TRAIL and were not changed by the addition of an antagonistic antibody against TRAIL into the culture medium containing TRAIL (data not shown). These results would again be consistent with selection of preexisting variants of melanoma cells. Taken together, the deficiency expression of multiple apoptotic mediators, in particular BH3-only proteins Bim, PUMA, and Noxa, may be collectively responsible for resistance of the TRAIL-selected cells to apoptosis induced by CDDP, SBHA, or vincristine. Consistent with this, we found that none of the compounds were able to up-regulate Bim, PUMA, or Noxa in the TRAIL-selected melanoma cells (data not shown).

Several lines of evidence suggest that the cytotoxic effect of CDDP in the TRAIL-selected cells was largely due to induction of necrosis. These included lack of DNA fragmentation, delayed externalization of phosphatidylserine, caspase and p53 independence, plasma membrane perturbations, severe mitochondrial disruption, rapid PARP activation, and depletion of intracellular ATP. Although CDDP has been reported to induce necrotic cell death when used at relatively high concentrations (23), our study is the first to show that CDDP induced predominantly necrosis in melanoma cells selected for resistance to apoptosis at the same doses at which it induced primarily apoptosis in the corresponding parental counterparts.

In contrast to SBHA and vincristine, CDDP induced an increased reduction in ΔΨm, indicative of more severe mitochondrial damage. Mitochondria are well known as targets for chemotherapeutic drugs for their central role in the induction and regulation of apoptosis (1, 2). However, it has recently become evident that mitochondria can also play a critical role in primary necrosis (37, 38). Damage to mitochondria may result in disruption to the mitochondrial electron transport chain, which would in turn result in reduced ATP production and consequently in a disruption of the bioenergetic state of the cells leading to necrotic cell death (39).

Although DNA damage can initiate apoptosis, it has been recently reported that DNA-damaging agents could kill Bax−/−Bak−/− or p53−/− cells by induction of primary necrosis, indicating an intact apoptotic pathway is not required for killing of cells with DNA damage (20, 21). In fact, it has also been reported that the initiation of apoptosis might actually suppress necrotic cell death because activated caspases may degrade proteins required for necrosis (20, 21, 40). Our results support these views in that apoptotic pathways in the TRAIL-selected cells seemed to be severely impaired. The initiating factor for induction of necrosis by CDDP in the TRAIL-selected cells remains to be elucidated, but increased PARP activity may play an important role in the process (20, 21). Evidence for this was the rapid PARP activation and depletion of ATP, which preceded features of necrosis in the TRAIL-selected cells induced by CDDP. Furthermore, inhibition of PARP activity by DPQ partially converted the mode of cell death from necrosis to apoptosis.

An important question is whether these in vitro studies are relevant to melanoma in patients. We and others have shown that TRAIL is expressed on CD4 and CD8 T lymphocytes (41, 42), natural killer cells (43, 44), monocytes (45), and dendritic cells (46). Interaction of these effector cells during the evolution of melanoma would seem highly likely, particularly at the primary melanoma stage in the skin. Some melanoma cells also express TRAIL in vivo (47). Fresh isolates of melanoma are often resistant to TRAIL-induced apoptosis and it is therefore likely that exposure to TRAIL in vivo may result in resistance to apoptosis induced by other apoptotic stimuli in vitro (24). The induction of necrotic cell death by CDDP may therefore be an important alternative mechanism to induce cell death in melanoma cells resistant to apoptosis due to prior exposure to TRAIL.

In conclusion, we have shown that (a) TRAIL-resistant melanoma cells generated from prolonged exposure to TRAIL are cross-resistant to both intrinsic and extrinsic apoptotic signaling due to decreases in expression levels of multiple key apoptotic mediators, including pro-caspase-8, pro-caspase-3, Bid, Bim, p53, and the products of its pro-apoptotic genes, such as PUMA and Noxa; (b) the TRAIL-selected melanoma cells were more vulnerable to necrosis induced by CDDP, which seemed to be caspase and p53 independent and to be mediated, at least in part, by activation of PARP. These results suggest that agents that can induce necrosis, such as CDDP, may still be effective in treating melanoma cells that have acquired resistance to apoptosis, especially when used following apoptosis inducers such as TRAIL. Moreover, exploration of chemotherapy-induced nonapoptotic cell death may provide an alternative strategy in overcoming resistance of melanoma cells to apoptosis.

Acknowledgments

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References

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CDDP Induces Necrosis of Apoptosis-Resistant Melanoma

Human melanoma cells selected for resistance to apoptosis by prolonged exposure to tumor necrosis factor-related apoptosis-inducing ligand are more vulnerable to necrotic cell death induced by cisplatin.

Xu Dong Zhang, Jing Jing Wu, Susan Gillespie, et al.