Silencing of Prion Protein Sensitizes Breast Adriamycin-Resistant Carcinoma Cells to TRAIL-Mediated Cell Death

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Abstract

We investigated the relationship between the resistance to the proapoptotic action of tumor necrosis factor–related apoptosis inducing ligand (TRAIL) and cellular prion protein (PrPc) function, using the TRAIL-sensitive MCF-7 human breast adenocarcinoma cell line and two TRAIL-resistant sublines: 2101 and MCF-7/ADR. All of the cell lines tested expressed TRAIL-R1 and TRAIL-R2. TRAIL decay receptors were not detected, suggesting that the resistance of 2101 and MCF-7/ADR cells, strongly expressing PrPc, to TRAIL-mediated cell death was independent from the expression of TRAIL receptors and death-inducing signaling complex formation. Down-regulation of PrPc by small interfering RNA increased the sensitivity of Adriamycin- and TRAIL-resistant cells to TRAIL, but not to epirubicin/Adriamycin. TRAIL-mediated apoptosis in PrPc knocked-down cells was associated with caspase processing, Bid cleavage, and Mcl-1 degradation. In addition, an increased sensitivity of apoptosis-resistant cells to TRAIL after PrPc silencing was not associated with the increased recruitment of receptors and intracellular signaling molecules to the death-inducing signaling complex. Bel-2 expression was substantially decreased after PrPc knock-down but the levels of Bel-Xl and Mcl-1 were not affected. The down-regulation of Bel-2 was concomitant with Bax delocalization. Our findings support the notion that silencing of PrPc facilitates the activation of proapoptotic Bax by down-regulation of Bel-2 expression, thereby abolishing the resistance of breast cancer cells to TRAIL-induced apoptosis. [Cancer Res 2007;67(22):10910–9]

Introduction

Despite promising early results, the treatment of breast cancer with modulators has remained problematic. This may be because there is an array of alternate resistance mechanisms, controlled by different families of genes, including those involved in apoptosis. These alternative pathways may influence drug resistance, leading to diminished cell killing by chemotherapeutic drugs. The effector molecules are poorly understood, and their relative contributions in any one disease remain to be elucidated. Generally, there is a need to develop new anticancer drugs and novel regimens that are capable of killing drug-resistant cells. Chemoresistance has been attributed to a failure of the drug to interact with its target, either due to a reduction of the effective concentration of the drug because of enhanced drug efflux pumps (1), or to detoxification enzymes or to changes to the drug’s target(s) themselves (2). Classic multidrug resistance is attributed to the elevated expression of ATP-dependent drug-efflux pumps ABCB1 (also known as P-glycoprotein), ABCG1 (also known as multidrug resistance–associated protein) and ABCG2 (also known as breast cancer–resistance protein and mitoxantrone-resistance protein), all of which belong to the superfamily of ATP-binding cassette transporters (for review, see ref. 3). P-glycoprotein protects cells from chemotherapy-induced apoptosis, and also from other death stimuli including death receptor ligands and UV radiation. Activation of death receptor pathways is a possible approach to breast cancer treatment because of the ability of death receptors to induce apoptosis directly, thus bypassing cellular drug resistance. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; also called Apo2L) is a member of the tumor necrosis factor (TNF) ligand family. It rapidly induces apoptosis in a variety of transformed cell lines. TRAIL interacts with five receptors, i.e., TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and osteoprotegerin. Ligation of TRAIL to TRAIL-R1 and TRAIL-R2 induces apoptosis as these receptors contain functional death domains, whereas TRAIL interaction with the other three receptors does not induce apoptosis. The interaction between TRAIL and the death receptors (TRAIL-R1 and TRAIL-R2) leads to the recruitment of Fas-associated death domain kinase (FADD) and activates caspase-8 to promote cell death without the involvement of mitochondria. Caspase-10 is recruited to and activated at the TRAIL and CD95 death-inducing signaling complexes (DISC) in a FADD-dependent manner but cannot functionally substitute caspase-8 (4). TRAIL can also activate the cleavage of Bid by caspase-8, and this drives cells to apoptosis through a mitochondrial amplification loop (for review, see refs. 5, 6).

There have been reports that TRAIL induces apoptotic activity in some normal human cells (7–9). However, TRAIL suppresses the growth of TRAIL-sensitive human mammary adenocarcinoma in mice and non–human primates without any significant toxic effects; in particular, TRAIL has none of the effects seen with TNF-α and Fasl. (10, 11), indicating that the apoptotic effect of TRAIL on nontransformed human cells depends on the TRAIL preparations used. Although TRAIL can induce apoptosis in tumor cells of diverse origins (12–14), several studies have shown that most breast cancer cell lines are resistant to its apoptotic effects (15, 16), suggesting that TRAIL alone might be ineffective for breast cancer therapy. Nevertheless, several studies have shown that TRAIL-resistant breast cancer cells in vitro and in vivo can be sensitized by chemotherapeutic drugs, indicating that combination therapy might be possible (15, 17, 18). Therefore, understanding the...
molecular mechanisms of TRAIL resistance and identifying ways to sensitize these cells to undergo apoptosis upon TRAIL treatment may help the search for effective cancer therapy.

The cellular prion protein (PrPc) is a glycosylphosphatidylinositol-anchored protein, expressed by all known mammals, predominantly in the brain. Several intriguing lines of evidence have recently emerged indicating that PrPc may protect human neurons from various kinds of internal or environmental stress (for reviews, see ref. 19). PrPc may participate in apoptosis: it shares significant similarity with the Bcl-2 homology domain 2 and binds Bcl-2 in yeast double-hybrid tests (20, 21). Indeed, PrPc can protect human neurons from serum withdrawal (22) and Bax-induced apoptosis (23–25). Stress-induced protein 1 is a cell surface ligand for PrPc that transduces neuroprotective signals (26). PrPc protects a human breast carcinoma cell line from TNF-induced cell death: ectopic expression of human PrPc converted TNF-sensitive MCF-7 cells into TNF-resistant cells by a mechanism involving the alteration of cytochrome c release from mitochondria and nuclear condensation (27). Du et al. recently reported a study with Adriamycin-sensitive gastric carcinoma cell lines indicating that PrPc is also involved in multidrug resistance of tumors (28).

We investigated whether PrPc overexpression in breast carcinomas is associated with resistance to TRAIL-induced apoptosis. We used TRAIL-resistant breast carcinoma cell lines and small interfering RNA (siRNA) technology to attenuate PrPc expression. Our findings suggest that selective PrPc down-regulation sensitizes human Adriamycin-resistant cells to TRAIL-mediated apoptosis independently of DISC formation, and also shows that Bcl-2 expression was abolished following PrPc knock-down.

**Materials and Methods**

**Reagents and antibodies.** Recombinant human soluble Apo2L/TRAIL was purchased from PeproTech, Inc. Adriamycin was purchased from Sigma. Protease inhibitors were purchased from Roche Applied Science. BCA protein assay reagent was obtained from Pierce. CHAPS was obtained from Sigma. Anti–TRAIL-R monoclonal antibody (mAb; clones M27, M413, M430, and M445 directed against TRAIL-R1 (DR4), -R2 (DR5), -R3 (DecR1) and -R4 (DecR2), respectively) were obtained from Immunex. Antibodies against cleaved caspase-3 (9661 clone), Bcl-2 (mAb, 100 clone), Bax (N-20 clone), actin (c-11 clone), Bid (FL-195), Mcl-1 (clone S-19), Bcl-XL (clone M430, and M445 directed against TRAIL-R1 (DR4), -R2 (DR5), -R3 (DecR1) and -R4 (DecR2), respectively) were obtained from Immunex. Antibodies against cleaved caspase-3 (9661 clone), Bcl-2 (mAb, 100 clone), Bax (N-20 clone), actin (c-11 clone), Bid (FL-195), Mcl-1 (clone S-19), Bcl-XL (clone S-18), and TRADD (C-20) were purchased from Santa Cruz Biotechnology. Antibody against PrPc (mAb, Pri-308) was a gift from Dr. Jacques Grassi (CEA, France). Antibody against GM1-210, a cis-Golgi network–associated protein, was a gift from Dr. Michel Borenens (Institut Curie, France; ref. 29).

**Cell lines.** TNF-resistant 2101 cells were obtained from PeproTech, Inc. Adriamycin was purchased from Sigma. Protease inhibitors were purchased from Roche Applied Science. BCA protein assay reagent was obtained from Pierce. CHAPS was obtained from Sigma. Anti–TRAIL-R monoclonal antibody (mAb; clones M27, M413, M430, and M445 directed against TRAIL-R1 (DR4), -R2 (DR5), -R3 (DecR1) and -R4 (DecR2), respectively) were obtained from Immunex. Antibodies against cleaved caspase-3 (9661 clone), Bcl-2 (mAb, 100 clone), Bax (N-20 clone), actin (c-11 clone), Bid (FL-195), Mcl-1 (clone S-19), Bcl-XL (clone S-18), and TRADD (C-20) were purchased from Santa Cruz Biotechnology. Antibody against PrPc (mAb, Pri-308) was a gift from Dr. Jacques Grassi (CEA, France). Antibody against GM1-210, a cis-Golgi network–associated protein, was a gift from Dr. Michel Borenens (Institut Curie, France; ref. 29).

**Cell lines.** TNF-resistant 2101 cells were established from TNF-resistant RA-1 cells transfected by p55 TNF receptor cDNA. Parental RA-1 cells were derived from TNF-sensitive human breast carcinoma MCF-7 cells after continuous exposure to increasing doses of recombinant TNF as previously described (30). Adriamycin-resistant MCF-7 cells with a multidrug-resistant phenotype were established as described in ref. (31). All the cells were maintained and propagated in RPMI 1640 containing 10% FCS. OligofectAMINE reagent was used according to the manufacturer's instructions (Invitrogen) to transfected subconfluent cells with siRNA PrPc.

**Confocal scanning immunofluorescence microscopy.** After appropriate transfection and treatment with various apoptotic stimuli, cells were plated at 5 × 10^4 cells/well, washed with PBS and fixed by incubation with 4% paraformaldehyde in PBS for 60 min. They were then incubated for 5 min with methanol (only used for Bax detection) and rinsed thrice with PBS, SDS (0.1% in PBS) was used for 10 min to permeabilize the cells. The cells were washed thrice with PBS and nonspecific binding sites were blocked by incubation with 10% FCS in PBS for 20 min. Cells were then incubated for 60 min with antibodies against Bax, Bcl-2, or PrPc and washed thrice with PBS. They were then incubated with Alexa 488–conjugated goat anti-mouse IgG secondary antibody and Alexa 546–conjugated anti-rabbit antibody (Molecular Probes), and washed thrice with PBS. The nuclei were stained by incubation with To-Pro-3 iodide (Molecular Probes) for 5 min.
Cells were then mounted with antifading Vectashield from Vector and examined under an LSM 510 confocal microscope (Zeiss), as previously described (27, 32).

**Results**

**Resistance of 2101 and MCF-7/ADR to TRAIL and Adriamycin.** We studied the sensitivity of MCF-7 cells and derivatives (2101 and MCF-7/ADR) to Adriamycin and TRAIL-mediated cell death: the cell lines 2101 and MCF-7/ADR were resistant to the cytotoxic action of various concentrations of TRAIL which were present for various times (12, 24, 48, and 72 h). Cell viability was measured by an MTT assay done in triplicate. Cell viability (%) = 100 × (A1/A0), where A1 and A0 are the absorbance of treated and untreated cell samples, respectively. B. cells were treated with the indicated concentration of Adriamycin (ADR) and TRAIL for 24 h. Apoptosis was assessed by Annexin V-FITC/propidium iodide staining. Columns, means of three independent determinations; bars, SD.

**Resistance to TRAIL-mediated apoptosis was not due to a lack of expression of TRAIL receptors.** Next, we sought to ascertain whether the resistance of the multidrug-resistant MCF-7/ADR and 2101 cell lines to TRAIL-mediated cell death was due to their not expressing TRAIL-R1 and TRAIL-R2, respectively, or to enhanced expression of the TRAIL decoy receptors (TRAIL-R3 and TRAIL-R4). Immunofluorescence analysis (Fig. 2) indicated that all of the cell lines expressed TRAIL-R1 and TRAIL-R2. The TRAIL decoy receptors were not detected. These data suggest that the resistance of 2101 and MCF-7/ADR cells to TRAIL-mediated cell death was independent of the expression of TRAIL receptors.

**Resistance of 2101 and MCF-7/ADR to Adriamycin correlates with high expression of PrPc.** Ectopic expression of human PrPc protects mammary tumor cells from cell death induced by TNF (27). Du et al. recently reported a study with Adriamycin-sensitive gastric carcinoma cell lines indicating that PrPc is also involved in multidrug resistance of tumors (28). We investigated the relationship between PrPc expression and resistance to TRAIL or Adriamycin. We first checked the expression levels of PrPc in 2101 and MCF-7/ADR cell lines. Real-time PCR analysis (Fig. 3A) and immunofluorescence analysis by fluorescence-activated cell sorting (FACS; Fig. 3B) and by confocal microscopy (Fig. 3C) indicated that both of the cell lines contained more PrPc than the parental MCF-7 cell line, and that the protein was present in the Golgi apparatus and at the cell surface. Thus, overexpression of PrPc seems to coincide with the resistance of breast carcinoma cell lines to Adriamycin and TRAIL-induced cell death.

**Knock-down of PrPc is sufficient to sensitize resistant human breast carcinoma cell lines to TRAIL-induced apoptosis.** We used two siRNA duplexes (siRNA PrPc 465 and siRNA PrPc 545) complementary to both transcript variants of Prnp to determine the effect of silencing of PrPc in both 2101 and MCF-7/ADR.
ADR cells. One of these siRNA duplexes (siRNA PrPc 465) corresponds to the unstructured domain, and the other to the structured domain. We did transient siRNA transfections of the 2101 and MCF-7/ADR cell lines using these two kinds of PRNP siRNA duplexes, and real-time quantitative RT-PCR analysis to evaluate mRNA down-regulation. PrPc mRNA levels were significantly lower in cells transfected with these siRNA molecules than in cells transfected with the scramble control siRNA (Fig. 4A). Immunofluorescence analysis by FACS (Fig. 4B, top) and by confocal microscopy (Fig. 4B, bottom) also indicated that PrPc levels were significantly lower in cells transfected with these siRNA molecules than in cells transfected with the scramble control siRNA. Transfection with these siRNA molecules for 72 h without any treatment did not significantly increase cell sensitivity to cell death.

We then analyzed the sensitivity of siRNA-transfected cells to Adriamycin and to TRAIL-mediated cytotoxicity by MTT assay (Fig. 4C). PrPc knock-down partly restored the sensitivity of MCF-7/ADR and 2101 cells to the cytotoxic action of TRAIL, but it failed to significantly increase the sensitivity of cells to Adriamycin. The PRNP siRNA did not significantly increase the apoptosis of either cell line in response to Adriamycin/epirubicin. In contrast, the PrPc down-regulation enhanced TRAIL-induced apoptosis, resulting in the apoptosis of 25% of MCF-7/ADR cells (Fig. 4D, right) and almost 60% of 2101 cells (Fig. 4D, left). Western blot analysis indicated that TRAIL treatment did not affect the amount of PrPc in siRNA-transfected cells (data not shown). These various findings strongly suggest that PrPc knockdown by siRNA is sufficient to sensitize MCF-7/ADR and 2101 cell lines to TRAIL-induced apoptosis. In accordance with the presumed function of PrPc as an inhibitor of death receptor--induced Bid cleavage and caspase-3 activation, Western blot analyses indicated that down-regulation of PrPc by siRNAs enhanced TRAIL-induced Bid cleavage and caspase-3 processing. Bid cleavage and caspase-3 processing were not enhanced following Adriamycin treatment, confirming that the down-regulation of PrPc by siRNAs did not increase the sensitivity of the cells to Adriamycin-induced cell death (Fig. 5A).

**Knock-down of PrPc down-regulates Bcl-2 expression.** We investigated whether the enhancement of TRAIL-induced apoptosis associated with PrPc down-regulation involved the modulation of Bcl-2 family members. Western blot analyses indicated that in both cell lines, Bcl-2 was less abundant in the PRNP siRNA 545–transfected than control-transfected cells; other members of the Bcl-2 family, including Bax, Bcl X L, and Mcl-1 were largely unaffected. TRAIL treatment decreased the abundance of Bcl-2 and Mcl-1, but not Bcl-Xl in the PRNP siRNA 545–transfected cells (Fig. 5A). Similar findings were obtained with PRNP siRNA 465–transfected cells. Thus, inhibiting PrPc expression resulted in the down-regulation of Bcl-2 expression.

Knock-down of PrPc down-regulates Bcl-2 expression. Western blot analyses in both cell lines indicated that in the presence of PrPc following TRAIL treatment, Bid is not cleaved and Mcl-1 is not down-regulated. In the absence of PrPc, Bid cleavage and down-regulation of Mcl-1 were observed after 5 h of TRAIL treatment, and peaked after 10 h of treatment (Fig. 5A).

**Figure 2.** TRAIL receptor expression at the surface of MCF-7 and derivative cells (2101 and MCF-7/ADR cells). TRAIL receptors on cells were determined by indirect immunofluorescence analysis by FACS, using anti–TRAIL-R1 (M271 clone), anti–TRAIL-R2 (M413 clone), anti–TRAIL-R3 (M430 clone), and anti–TRAIL-R4 (M445 clone; gray histogram). Isotypic IgG 1 (negative controls) were included (open histogram). The level of cell surface expression is indicated by the shift of the gray histogram to the right from the open control curve. Numbers, percentages of positive cells; numbers in parentheses, mean fluorescence intensities. Results are representative of three independent experiments.
Next, we tested whether silencing PrPc by siRNAs, which resulted in the down-regulation of Bcl-2 protein, also affects Bcl-2 mRNA. Real-time quantitative RT-PCR analysis showed that in the absence of PrPc, Bcl-2 mRNA was slightly down-regulated in both cell lines (Fig. 5C). These observations are consistent with the absence of PrPc down-regulating Bcl-2 at both mRNA and protein levels. However, the down-regulation at the protein level is more pronounced than that at the mRNA level.

A characteristic feature of Bcl-2 proteins is that they form heterodimers with Bax or other proapoptotic molecules, and this may block their proapoptotic activity. PrPc prevents Bax-mediated cell death by preventing the Bax proapoptotic conformational change in human neurons and in MCF-7 cells (24). PrPc partially inhibits Bax translocation to the mitochondria in MCF-7 cells. The requirement for Bax in TRAIL-induced apoptosis of type II cells has been mapped to the mitochondrial amplification cycle (33, 34). We therefore tested whether silencing PrPc by siRNAs, which resulted in the down-regulation of Bcl-2, also affects the localization of Bax. Immunofluorescence staining of untreated 2101 cells (Fig. 5D, top) and untreated MCF-7/ADR cells (Fig. 5D, bottom) showed a diffuse cytosolic localization for Bax. As expected, after silencing of PrPc, Bax immunoreactivity became punctuate, but there was no evidence of significant nuclear condensation. After incubation with TRAIL for 4 h, more of the PrPc siRNA-transfected cells (+ siRNAs + TRAIL; Fig. 5D) than untransfected cells (+ TRAIL, Fig. 5D) became apoptotic. To check that the silencing of PrPc by siRNAs results in the observed Bcl-2 down-regulation, Bcl-2 staining was also examined and the findings confirmed those obtained by Western blotting. Bcl-2 down-regulation was concomitant with the change affecting the localization of Bax. These various findings suggest that inhibiting PrPc expression may facilitate the cleavage of Bid, which is concomitant with Mcl-1 down-regulation, and also activate the proapoptotic Bax by down-regulation of Bcl-2 expression levels.

Knock-down of PrPc does not affect TRAIL DISC formation.

We sought to ascertain whether the knockdown of PrPc expression by RNA interference modulates the expression of TRAIL receptors on the cell surface. Immunofluorescence analysis suggests that knockdown of PrPc does not affect the expression of TRAIL receptors (data not shown). However, this observation did not rule out the possibility that knockdown of PrPc expression by RNA interference could facilitate the formation of the DISC in response to TRAIL exposure. To test this possibility, we did immunoprecipitation experiments in MCF-7/ADR and 2101 cells in which PrPc was down-regulated by siRNA for 72 h. Then, cells were incubated for 30 min or 1 h at 37°C with Flag-TRAIL crosslinked to an anti-Flag antibody. In the presence or absence of PrPc, the same amount of recruitment of TRAIL-R1 and TRAIL-R2, FADD, procaspase-8, and FLIP-L to the DISC was observed (Fig. 6). PrPc down-regulation therefore does not affect this DISC formation, and the increased sensitivity of Adriamycin- and TRAIL-resistant cells to TRAIL after PrPc silencing, is not associated with increased recruitment of TRAIL-R1 and TRAIL-R2, FADD, procaspase-8, or FLIP-L to the DISC. In addition, TRADD is not recruited in the presence or absence of PrPc. Thus, TRAIL DISC formation is not involved in the resistance of MCF-7/ADR and 2101 to TRAIL-induced apoptosis.
Figure 4. Knock-down of endogenous PrPc sensitizes MCF-7/ADR cells and 2101 cells to TRAIL-induced cell death. MCF-7/ADR and 2101 cells were transfected with 28 nmol/L of siRNA PrPc 465, siRNA PrPc 545, or scramble control siRNA (Control). A, expression of the PRNP gene in cell lines was examined by quantitative RT-PCR. Columns, means of three independent determinations; bars, SD (***, P < 0.0001). B, PrPc protein was determined by immunofluorescence staining using Pri-308 mAb, and analyzed either by FACS analysis (top) or confocal microscopy (bottom). Columns, means of at least three independent experiments; bars, SD. Nuclei were counterstained with To-Pro-3. The confocal scanning fluorescence micrographs are representative of the vast majority of the cells analyzed (blue, nucleus; green, PrPc). C, 72 h after transfection, 2101 and MCF-7/ADR cells were treated or not treated with TRAIL (300 ng/mL) and with Adriamycin (500 ng/mL) for 24 h. Cell mortality was measured by an MTT assay done in triplicate. Cell mortality (%) = [1 – (A1/A0)] × 100, where A1 and A0 are the absorbance of treated and untreated cell samples, respectively. Columns, means of three independent determinations; bars, SD (*, P < 0.05). D, cells were treated as described in C, then apoptosis was evaluated by Annexin V-FITC/propidium iodide staining and FACS analysis. Columns, means of three independent determinations; bars, SD (*, P < 0.05).
The principal findings of this article concern the molecular mechanisms by which breast carcinoma cells are resistant to apoptosis. We compared breast carcinoma MCF-7 cells and two derivative clones (2101 and MCF-7/ADR) and found that MCF-7 cells are more sensitive than both 2101 and MCF-7/ADR cells to anthracycline-based treatment and death domain receptor-mediated apoptosis. The resistance of 2101 cells and multidrug-resistant MCF-7/ADR cells to apoptosis was independent of TRAIL receptor expression. Our experimental system might therefore be an appropriate in vitro model for investigating whether PrPc mediates apoptosis resistance in breast carcinoma cells. We have previously shown that PrPc protects a human breast carcinoma cell line from TNF-induced cell death (27). However, the question of whether down-regulation of PrPc sensitizes the resistant breast carcinoma cell line to apoptosis remained to be answered. Here, we

**Discussion**

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report that resistance to Adriamycin- and TRAIL-induced apoptosis was strongly correlated with PrPc overexpression, as revealed by assaying both PrPc mRNA and protein.

This is the first demonstration that down-regulation of PrPc sensitizes Adriamycin-resistant human breast carcinoma cell lines to TRAIL-mediated cell death. In addition, the increased sensitivity of Adriamycin- and TRAIL-resistant cells to TRAIL after PrPc silencing was not associated with increased recruitment of receptors and intracellular signaling molecules to the DISC. These data are consistent with previous reports. Indeed, PrPc protects human neurons and MCF-7 cells against Bax-mediated apoptosis by affecting the Bax conformation (24). In yeast, this effect seems to be related to the interactions between PrPc and endogenous yeast proteins that act downstream from Bax in a cellular stress or toxicity pathway (25). Down-regulation of PrPc by siRNA correlated with a change in Bax localization and a large decrease in the amount of Bcl-2 such that the Bax/Bcl-2 ratio increased. The ratio between Bax or Bak and Bcl-2 or Bcl-Xl is believed to be important in determining sensitivity to cell death. A characteristic feature of Bcl-2 proteins is the formation of homodimers and heterodimers with other members of the family. For example, Bcl-2 could form heterodimers with Bax, or other proapoptotic molecules, and may thus block their proapoptotic activity. Our data is the first to suggest that down-regulation of PrPc sensitizes human Adriamycin-resistant breast carcinoma cell lines to TRAIL-mediated apoptosis through Bcl-2 down-regulation. This observation is in agreement with other reports indicating that the transfection of MCF-7 with the Bcl-2 gene did not reduce TNF sensitivity (35). Bcl-2 was overexpressed in the TNF-sensitive MCF-7 cell line, but was down-regulated in the TNF-resistant MCF-7 cell line (36). The observed decline in Bcl-2 levels following PrPc silencing is also consistent with previous findings indicating that PrPc acts through Bcl-2. Indeed, PrPc interacts with Bcl-2 in the yeast two-hybrid system (21). Like Bcl-2, PrPc prevents Bax conformational change (24, 37) and Bax-mediated cell death (23). Both PrPc and Bcl-2 expression protect against cell death associated with serum deprivation (22) and oxidative stress (38). The signals activating Bax are not well known, but one current model is that death receptor–mediated activation of caspase-8 triggers Bid processing (39, 40). Truncated Bid (tBid) then signals translocation of cytosolic Bax to the mitochondria, where it forms homodimers or heterodimers with other Bcl-2 family members. The resultant conformational changes in Bax can result in channel formation allowing the release of apoptogenic proteins, including cytochrome c, from the mitochondrial intermembrane space, thereby activating downstream execution (40–42). Bid cleavage observed in breast carcinoma cell sensitization to TRAIL by PrPc silencing (Fig. 5) implicates Bax insertion into the mitochondrial membrane triggered by tBid (43). Consistent with these results, and with data suggesting that TRAIL induces Mcl-1 degradation (44), we observed that inhibition of PrPc expression did not affect Mcl-1 expression, but that TRAIL induced a substantial decrease of Mcl-1 degradation which is concomitant with the cleavage of Bid. This does not occur, however, when cells are treated with Adriamycin. This may be because tBid interferes only weakly with the antiapoptotic function of Bcl-2, whereas it may be able to neutralize the antiapoptotic function of Mcl-1 (45). This does not seem to be the case for Adriamycin, which seems to induce a BH3-only family member which apparently cannot overcome the apoptosis-inhibitory effect exerted by Mcl-1 in spite of the Bcl-2 down-regulation. However, several different mechanisms for the development of Adriamycin resistance have been described.

Silencing of PrPc Sensitizes Human Breast Carcinoma to Apoptosis

Figure 6. Influence of PrPc knockdown on the formation of the DISC in response to TRAIL. 2101 and MCF-7/ADR cells were either untreated (Medium) or transfected with 28 nmol/L of siRNA PrPc 545, siRNA PrPc 465, or scramble control siRNA (siRNA scramble) for 72 h, then treated for the times indicated with TRAIL (500 ng/mL with 2 μg/mL of anti-Flag antibody). DISCs were immunoprecipitated with protein G and analyzed for the proteins indicated by immunoblotting, with the exception that TRAIL was not added to lysates of control cells. Lysates prepared from 2101 and MCF-7/ADR cells (Lys.) are shown as positive controls for the TRADD antibody. Numbers are molecular weights in kDa.

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including reduced drug uptake and increased drug efflux (1). Recent studies have shown that breast neoplasms can be classified into two very distinct entities according to the expression profile of the gene for the estrogen receptor α (ER; ref. 46). Troester et al. reported that the proteins involved in the response to anthracycline-based chemotherapy may differ according to cell lineage and ER-status (47). Here, we show that down-regulation of PrPc by siRNA was insufficient to sensitize ER-positive 2102 and ER-positive MCF-7/ADR to Adriamycin-mediated apoptosis. This is consistent with our recent data suggesting that the absence of PrPc expression is associated with high sensitivity to adjuvant chemotherapy only in ER-negative tumors (48).

Our observations are not consistent with the data reported by Du et al. showing the partial induction of cell apoptosis by PrPc knock-down expression in the Adriamycin-resistant gastric carcinoma SGC7901/ADR cell line (28). This discrepancy could be due to the different in vitro models used. Note also that our observation is consistent with data suggesting that PrPc cannot prevent staurosporine- or thapsigargin-mediated cell death, both of which are mediated through tBid/Bak (19).

Although our data suggest that knock-down of PrPc expression increased the sensitivity of Adriamycin-resistant human breast carcinoma cells to TRAIL-induced apoptosis by down-regulation of Bel-2 and altering Bax localization, our study presented several limitations. Our experiments are uninformative about how PrPc down-regulation induces the down-regulation of Bel-2. We intend to study this issue subsequently. However, real-time PCR analysis indicated that this down-regulation involves both mRNA and protein levels. The difference at the protein level is more pronounced than that at the mRNA level. Various Bel-2 antibodies all gave the same pattern in Western blot analyses (data not shown) ruling out the possibility that Bel-2 down-regulation was due to the cleavage of Bel2.

In conclusion, it has been established that Bel-2 and Mcl-1 antagonize cell death by sequestering members of the proapoptotic family including the BH3-only family and multidomain proapoptotics. We report that in the presence of PrPc and under TRAIL-apoptotic conditions, small amounts of tBid were produced. tBid has a much higher affinity for Mcl-1 than Bel-2, and consequently, tBid interferes only weakly with the antiapoptotic function of Bel-2 (45). Mcl-1, which has high affinity with tBid, interacts with and blocks tBid-mediated cell death. In the absence of PrPc and under TRAIL-apoptotic conditions, Bel-2 expression was abolished. We propose that as a result, more tBid was available to interact with Mcl-1 and thus neutralized the antiapoptotic function of Mcl-1 more strongly. In addition, degradation of Mcl-1 by TRAIL-activated caspase-3 may produce proapoptotic forms of Mcl-1 that mediate a Bax-dependent apoptotic cascade. In addition, the apoptotic signal leads to caspase-3 activation, which activates caspase-8 that further increases tBid formation, independently of DISC formation. This accelerates the cross-talk between the extrinsic and intrinsic pathways via an amplification loop, thereby abolishing the resistance of breast cancer cells to TRAIL-induced apoptosis.

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