The Deubiquitinase Inhibitor PR-619 Sensitizes Normal Human Fibroblasts to Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL)-mediated Cell Death*

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TNF-related apoptosis-inducing ligand (TRAIL) is a potential cancer therapy that selectively targets cancer cell death while non-malignant cells remain viable. Using a panel of normal human fibroblasts, we characterized molecular differences in human foreskin fibroblasts and WI-38 TRAIL-resistant cells and marginally sensitive MRC-5 cells compared with TRAIL-sensitive human lung and colon cancer cells. We identified decreased caspase-8 protein expression and protein stability in normal fibroblasts compared with cancer cells. Additionally, normal fibroblasts had incomplete TRAIL-induced caspase-8 activation compared with cancer cells. We found that normal fibroblasts lack the ubiquitin modification of caspase-8 required for complete caspase-8 activation. Treatment with the deubiquitinase inhibitor PR-619 increased caspase-8 ubiquitination and caspase-8 enzymatic activity and sensitized normal fibroblasts to TRAIL-mediated apoptosis. Therefore, posttranslational regulation of caspase-8 confers resistance to TRAIL-induced cell death in normal cells through blockade of initiation of the extrinsic cell death pathway.

TNF-related apoptosis inducing ligand (TRAIL) is a member of the TNF family and has cytotoxic effects against various cancer cells but causes little or no toxicity to non-malignant normal cells (1, 2). The selective killing of cancer cells has made TRAIL an exciting cancer target, and the clinical use of TRAIL is currently being explored (3, 4). TRAIL can bind to five receptors. Death receptor 4 (DR4) (5) and death receptor 5 (DR5) (6) are death domain-containing, proapoptotic TRAIL receptors. Decay receptor 1 (DcR1) and decay receptor 2 (DcR2) are decay receptors that are incapable of TRAIL-induced cell death signaling because of truncated or nonfunctional death domains (7–10). Osteoprotegerin is a soluble decoy TRAIL receptor (11). TRAIL/TRAIL receptor ligation causes receptor trim- erization and recruitment of the intracellular adaptor protein Fas-associated death domain. Caspase-8 is also recruited and interacts with Fas-associated death domain in a complex referred to as the death-inducing signaling complex (12). Death-inducing signaling complex activation of caspase-8 leads to activation of effector caspases, including caspase-3, resulting in cell death (12). TRAIL can also activate the intrinsic death pathway and lead to mitochondrial permeabilization and release of proapoptotic factors, including cytochrome c, and activation of initiator caspase-9. Activated caspase-9 can then activate caspase-3, causing cell death (12).

Not all cancer cells are susceptible to TRAIL-mediated cell death. TRAIL sensitivity in cancer cells has been studied extensively, and several resistance mechanisms have been defined. Altered DR4 and DR5 posttranslational modifications, including glycosylation (13, 14), abnormal TRAIL receptor transport to the cell surface (15), TRAIL receptor endocytosis (16), recruitment of the enzymatically inactive caspase-8 homologue FLICE-inhibitory protein (FLIP) to the death-inducing signaling complex (17), and decreased expression of oncogenic c-myc, have been described as TRAIL resistance mechanisms (18). Other mechanisms of TRAIL resistance to cell death include decreased caspase expression (19), increased NF-κB activation (20), increased Bcl-2 family expression, including Bcl-XL (21) and Mcl-1 (22), and increased inhibitor of apoptosis protein family expression (23). Although studies have been done to determine why some cancer cells are resistant to TRAIL, why non-malignant normal cells are TRAIL-resistant remains largely unexplored. The expression of TRAIL decay receptors has been implicated as a TRAIL resistance mecha- nism for normal cells (24), although it is not clear why tumor cells express fewer decay receptors. Some tumor cells express elevated decay receptors (25), and decay receptor expression has also been associated with cellular senescence (26).

In this study, we investigated TRAIL resistance mechanisms in human non-malignant fibroblasts. We report that TRAIL-resistant normal fibroblasts express the proapoptotic TRAIL receptor DR5 but little DR4. Normal fibroblasts have decreased initiator caspase-8 protein expression compared with TRAIL-sensitive colon cancer cells. Our studies show that TRAIL-mediated caspase-8 activity is reduced in normal fibroblasts. TRAIL-induced caspase-8 ubiquitination, required for com-
plete caspase-8 activation, is diminished in normal fibroblasts, and this suggests that incomplete caspase-8 ubiquitination and, therefore, activation leads to minimal activation of downstream effector caspases, resulting in normal cell survival. Addition of a deubiquitine inhibitor sensitizes normal fibroblasts to TRAIL-induced cell death. We report deficient caspase-8 ubiquitination as a mechanism through which normal cells escape TRAIL-induced apoptosis.

Experimental Procedures

Cell Culture and Reagents

WI-38 (embryonic lung) fibroblasts, human foreskin fibroblasts (HFFs), MRC-5 (lung) fibroblasts, SW480 (human colon cancer), DLD1 (human colon cancer), HCT116 (human colon cancer), and H460 (human lung cancer) cells were purchased from the ATCC. WI-38 and MRC-5 cells were grown in Eagle’s minimum essential medium (ATCC) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HFF cells were grown in Dulbecco’s modified Eagle’s medium (ATCC) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. WI-38 (embryonic lung) fibroblasts, human foreskin fibroblasts (HFFs), MRC-5 (lung) fibroblasts, SW480 (human colon cancer), DLD1 (human colon cancer), HCT116 (human colon cancer), and H460 (human lung cancer) cells were purchased from the ATCC. WI-38 and MRC-5 cells were grown in Eagle’s minimum essential medium (ATCC) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Fibroblasts were used at early passages. All cells were maintained at 37 °C with 5% CO₂.

Western Blotting and Antibodies

Cells were lysed in cell lysis buffer (1% Nonidet P-40, 10% glycerol, 50 mM Tris, 150 mM NaCl, and 2 mM EDTA) and cleared by centrifugation. To observe caspase-8 ubiquitination, an alternative cell lysis buffer (1% SDS, 10% glycerol, 20 mM Tris, 150 mM NaCl, and 2 mM EDTA) was used. Cellular proteins were resolved on polyacrylamide gels and transferred onto PVDF membranes (Millipore). Membranes were blocked with 4% nonfat milk before adding primary antibody. Caspase-8 antibodies were purchased from BD Biosciences (catalog no. 551242) and Enzo Life Sciences (catalog no. ADI-AAM-118-E). β-Actin and tubulin antibodies were purchased from Sigma-Aldrich. Caspase-9 (catalog no. 9502), caspase-3 (catalog no. 9664), DR5 (catalog no. 3696), Bcl-xL (catalog no. 2764), and Mcl-1 (catalog no. 4572) antibodies were purchased from Cell Signaling Technology. DR4 (catalog no. sc-7863) and SP1 (catalog no. sc-59) antibodies were purchased from Santa Cruz Biotechnology. DcR1 (catalog no. 550622) and DNMT1 (catalog no. sc-880 plate reader). Coomassie Blue Staining—Cells were seeded in 12-well plates. After TRAIL treatment, cells were washed twice with PBS supplemented with 0.1 mM CaCl₂. Cells were fixed for 45 min with 4% formaldehyde diluted in PBS supplemented with 0.1 mM CaCl₂. Cells were washed with Coomassie Blue staining solution for 3 min and then washed twice with PBS supplemented with 0.1 mM CaCl₂ (32).

Caspase-8 Enzymatic Activity—Caspase-8 activity was examined using a fluorescent caspase-8 enzymatic activity kit (BioVision) according to the instructions of the manufacturer. Fluorescent samples were analyzed using a Beckman Coulter DTX 880 plate reader.

Cleaved Caspase-3—Cells were washed once with 1% FBS diluted in PBS. Cells were then fixed and permeamblized with Cytofix/Cytoperm (BD Biosciences). Cells were stained with a cleaved active caspase-3 antibody (BD Biosciences). Cells were washed in Cytoperm wash buffer (BD Biosciences) and stained with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes). Cells were then analyzed on a Beckman Coulter EPICS Elite flow cytometer.

Cell Death Assays

Propidium Iodide Staining—After TRAIL treatment, cells were trypsinized and washed once with 1% FBS diluted in PBS. Cells were permeamblized with ethanol and stained with propidium iodide (30). Cells were analyzed on a Beckman Coulter EPICS Elite flow cytometer.

CellTiter Glo Cell Viability—Cells were seeded in 60-mm dishes or 6-well plates. Cell-Titer Glo (Promega) was used to measure TRAIL-induced ATP reduction (31). Bioluminescence was analyzed on a Xenogen IVIS 100 (31).

Trypan Blue Cell Viability—After TRAIL and PR-619 co-treatment, cells were trypsinized and washed once with PBS. Cells were resuspended in a 1:1 dilution of 0.4% trypan blue, and cell viability was calculated using a Nexcelom Cellometer Auto T4.

Cycloheximide was purchased from Sigma-Aldrich. The caspase-8 inhibitor Z-IETD-fmk and the pan-caspase inhibitor Z-VAD-fmk were purchased from R&D Systems. Cycloheximide was purchased from Sigma-Aldrich.
Results

MRC-5 Lung Fibroblasts Are Mildly Sensitive to TRAIL-induced Cell Death—We examined TRAIL sensitivity in three human non-malignant fibroblast cell lines (WI-38, HFF, and MRC-5). Two colon cancer cell lines (DLD1 and HCT116) and one lung cancer cell line (H460) were used as positive controls for TRAIL-induced cell death. No marked decrease in Coomassie Blue-stained, viable WI-38, HFF, and MRC-5 cells was observed with overnight treatment of increasing amounts of TRAIL (Fig. 1A). At a dose of 25 ng/ml, TRAIL greatly reduced cell viability in HCT116 and DLD1 colon cancer cells, whereas H460 cells required higher doses of TRAIL to decrease cell viability (Fig. 1A). Because Coomassie Blue staining of viable cells may not reveal small changes in cell survival after TRAIL treatment, we examined TRAIL sensitivity in human normal fibroblasts using more quantitative techniques. Normal fibroblasts and colon and lung cancer cells were treated with 50 ng/ml TRAIL for 18 h and stained with propidium iodide to detect TRAIL-induced DNA fragmentation. As expected, sub-G1+ cells increased considerably after TRAIL treatment in DLD1, HCT116, SW480, and H460 cells (Fig. 1B). 2.7-Fold and 1.57-fold increases in sub-G1+ cells were found in WI-38 and HFF cells, respectively, after TRAIL treatment. MRC-5 fibroblasts had a 6.13-fold increase after TRAIL treatment, revealing that

![Table 1](image-url)

**FIGURE 1. Normal fibroblasts are resistant to TRAIL-induced cell death.** A, Coomassie Blue-stained viable cells after TRAIL treatment with different doses (25, 50, and 100 ng/ml) in a panel of non-malignant normal fibroblasts or in TRAIL-sensitive cancer cells (t = 18 h). B, percentage of sub-G1+ cells by flow cytometric analysis of DNA content in a panel of non-malignant normal fibroblasts or TRAIL-sensitive cancer cells (50 ng/ml TRAIL, t = 18 h). C, bioluminescence imaging of ATP after TRAIL treatment (100 ng/ml, t = 18 h). *, p < 0.0005; **, p < 0.0001.
MRC-5 cells are mildly susceptible to TRAIL-induced cell death (Fig. 1B). The absolute percent of sub-G1, content indicative of cell death for normal cells was less than 5%, whereas, for TRAIL-sensitive cancer cells, it was greater than 40%. To further determine whether MRC-5 fibroblasts are sensitive to TRAIL-mediated cell death, we treated normal fibroblasts with 50 ng/ml TRAIL overnight and examined bioluminescent cellular ATP levels. Intracellular reduction in ATP is a characteristic of cells undergoing cell death (33, 34). ATP levels remained similar in untreated and TRAIL-treated WI-38 and HFF cells (Fig. 1C). However, a decrease in ATP was observed in TRAIL-treated MRC-5 fibroblasts (Fig. 1C), and, although statistically significant, the decrease was fairly small compared with what was observed with colon cancer SW480 cells after TRAIL treatment under the same experimental conditions.

Normal Fibroblasts Express the Proapoptotic TRAIL Receptors DR4 and DR5—Because WI-38 and HFF fibroblasts were found to be largely TRAIL-resistant and MRC-5 fibroblasts were slightly TRAIL-sensitive, we examined TRAIL receptor expression to determine whether TRAIL-resistant normal cells had reduced proapoptotic death receptor expression or elevated decoy receptor expression. Normal fibroblasts had decreased DR5 protein expression compared with TRAIL-sensitive cancer cells (Fig. 2B). DR4 expression was detectable but down-regulated in normal fibroblasts compared with colon and lung cancer cells (Fig. 2A). No DcR1 protein expression was observed in normal fibroblasts. Normal fibroblast DcR2 protein levels were similar compared with TRAIL-sensitive cancer cells (Fig. 2C). Therefore, in these experiments, the TRAIL resistance of the normal cells is not adequately explained by the commonly held understanding that normal cells express greater levels of TRAIL decoy receptors as a protective mechanism from TRAIL-induced apoptosis. WI-38 cells do appear to express higher levels of DcR2 that may contribute to TRAIL resistance.

Normal Fibroblasts Have Diminished Caspase-8 and Expression—Several mechanisms have been identified that confer TRAIL resistance in malignant cells, including increased expression of the inhibitor of caspase activation and caspase-8 homolog c-FLIP (17) as well as increased expression of the anti-apoptotic Bcl-2 family member Mcl-1 (32). Additionally, c-myc expression has been shown to represent a major determinant of TRAIL sensitivity in malignant cells (18). We examined the protein levels of c-FLIP, Mcl-1, and c-myc in non-malignant normal cells and TRAIL-sensitive cancer cells. We found that normal fibroblasts had similar protein expression of c-myc, c-FLIP, and Mcl-1 compared with colon and lung cancer cells (Fig. 3, A and B). Expression of the Bcl-2 family member Bcl-xL was also examined. No differences were observed between normal fibroblasts and TRAIL-sensitive cancer cells (Fig. 3C).

We next investigated the expression of initiator and effector caspases involved in TRAIL-mediated cell signaling to determine whether normal fibroblasts lack a crucial TRAIL signaling protein. Markedly reduced expression of initiator caspase-8 was observed in normal fibroblasts compared with TRAIL-sensitive cancer cells (Fig. 3, D and E). MRC-5 fibroblasts had less caspase-8 than TRAIL-sensitive cancer cells but more caspase-8 than WI-38 and HFF cells. Decreased caspase-9 protein expression was also observed (Fig. 3D). Alternatively, caspase-10 expression was up-regulated in normal fibroblasts compared with colon and lung cancer cells (Fig. 3D). Similar expression levels of effector caspase-3 in normal fibroblasts and colon and lung cancer cells were noted (Fig. 3D).
We also examined DcR1 and caspase-8 mRNA levels in non-malignant normal cells and TRAIL-sensitive cancer cells to see whether decreased protein levels correlated with decreased mRNA levels. Diminished caspase-8 mRNA levels were also observed in normal fibroblasts compared with colon and lung cancer cells (Fig. 3F). DcR1 mRNA was barely detected in the majority of normal and cancer cells examined (Fig. 3F).

**Normal Fibroblasts Have Decreased Caspase-8 Expression in the Absence of New Protein Synthesis**—To determine whether the reduced caspase-8 protein expression observed in normal fibroblasts was due to epigenetic silencing, we incubated normal fibroblasts with non-lethal doses of the histone deacteylase inhibitor trichostatin A (TSA) or the DNA methyltransferase inhibitor 5-azacytidine (5-Aza) for 24, 48, or 72 h. TSA and 5-Aza activity was first confirmed by induction of apoptosis (Fig. 4A) and disappearance of DNA methyltransferase 1 protein (35) (Fig. 4B), respectively. No increase in caspase-8 protein expression was observed in treated HFF cells (Fig. 4C). Treatment with TSA or 5-Aza caused no change in WI-38 caspase-8 protein levels (Fig. 4D), suggesting that epigenetic silencing may not be responsible for the reduced caspase-8 protein expression observed in normal fibroblasts.

To determine whether decreased normal fibroblast caspase-8 expression was due to reduced caspase-8 protein stability, we examined caspase-8 expression in MRC-5 normal lung fibroblasts, DLD1 colon cancer cells, and H460 lung cancer cells after treatment with the protein synthesis inhibitor cycloheximide (CHX). MRC-5 caspase-8 expression was decreased compared with DLD1 and H460 cells (Fig. 4E). After 18 h of cycloheximide treatment, the DLD1 and H460 caspase-8 band density values were 0.73 and 0.51, respectively. The MRC-5 caspase-8 band density value after 18 h of cycloheximide treatment was 0.17, suggesting a possible decrease in caspase-8 stability in non-malignant normal cells in the absence of new protein synthesis (Fig. 4, E and F).

**Incomplete TRAIL-induced Caspase-8 Activation in Normal Fibroblasts**—To investigate potential blocks in the initiation of TRAIL signaling, specifically incomplete caspase activation, in TRAIL-resistant normal cells, we performed Western blotting analysis on normal cells after treatment with TRAIL. Normal fibroblasts treated with 50 ng/ml TRAIL had no visible processed caspase-8 after 12 h, unlike DLD1 and SW480 TRAIL-treated cells, where this was observed readily (Fig. 5A). A stronger ECL reagent was applied to determine whether cleaved products existed but were difficult to visualize. No cleaved caspase-8 was observed in TRAIL-treated MRC-5 or WI-38 cells (Fig. 5A). No disappearance of procaspase-3 or cleaved poly adenosine diphosphate ribose polymerase (PARP) was noted in TRAIL-treated normal fibroblasts. Disappearance of procaspase-3 and PARP cleavage was observed in TRAIL-treated DLD1 and SW480 colon cancer cells (Fig. 5A).

Enzymatic caspase-8 activity was explored in TRAIL-treated normal fibroblasts and colon cancer cells. After 4 h of TRAIL treatment, caspase-8 activity increased in H460 cells but only increased marginally in MRC-5 and WI-38 cells after TRAIL treatment (Fig. 5B). Little change in caspase-8 activity was observed in TRAIL-treated HFF fibroblasts (Fig. 5B).

**FIGURE 4.** Normal fibroblasts do not respond to inhibition of epigenetic silencing of caspase-8 but appear to have decreased caspase-8 stability. A, MRC-5 and HFF cells were treated for 60 h with increasing concentrations of TSA (0.1, 0.25, 0.50, 1.0, and 2.0 μM) and subjected to bioluminescence imaging of intracellular ATP to confirm TSA-mediated cell death and activity. B, nuclear extracts were obtained from MRC-5 cells treated with 0.5 μM 5-Aza-C for 96 h and analyzed by Western blotting for DNTM1 protein expression to confirm 5-Aza-C activity. The nuclear transcription factor Sp1 was used as a loading control. C, cells were treated for 24, 48, or 72 h with 0.05 μM TSA or 0.5 μM 5-Aza-C. Caspase-8 protein expression was analyzed by Western blotting. D, WI-38 cells were treated for 48 h with 0.05 μM TSA or 0.5 μM 5-Aza-C and then analyzed for caspase-8 protein expression. β-Actin was used as a loading control. E, MRC-5 fibroblasts, DLD1 colon cancer cells, and H460 lung cancer cells were treated with 30 μg/ml cycloheximide for 4, 8, 12, and 18 h. Cells were lysed, and caspase-8 protein levels detected by Western blotting analysis using a Becton Dickinson (BD) caspase-8 antibody. F, densitometric analysis of caspase-8 protein expression. For each cell line, the caspase-8 protein band density in untreated cells was considered baseline and assigned a 1.0 value.
FIGURE 5. Normal fibroblasts have incomplete TRAIL-induced caspase-8 activation. A, MRC-5, WI-38, DLD1, and H460 cells were treated with 50 ng/ml TRAIL for 12 h. Cell lysates were collected, and caspase-8, caspase-3, and PARP proteins were examined by Western blotting. Tubulin was used as a loading control. B, HFF, WI-38, MRC-5, and H460 cells were treated with 50 ng/ml TRAIL for 0.5, 1, 2, or 4 h and analyzed for caspase-8 enzymatic activity. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
The Deubiquitinase Inhibitor PR-619 Increases Normal Fibroblast Caspase-8 Ubiquitination—Posttranslational modifications have been found to regulate caspase-8 activity. Jin et al. (36) have reported previously that caspase-8 ubiquitination is required for complete TRAIL-induced caspase-8 activation in H460 and H2122 lung cancer cells. Furthermore, deubiquitinated caspase-8 reduced TRAIL-mediated cell death (36). We investigated caspase-8 ubiquitination in normal fibroblasts compared with TRAIL-sensitive cancer cells. Normal fibroblasts had fewer high molecular weight caspase-8 bands associated with polyubiquitination compared with SW480 and DLD1 colon cancer cells (Fig. 6A). Densitometry was performed to investigate the ratio of caspase-8 ubiquitination to procaspase-8 to determine whether the decrease in normal cellular caspase-8 ubiquitination was due to reduced caspase-8 protein levels in normal fibroblasts. High molecular weight band densitometry revealed a 0.011 high molecular weight to pro-caspase-8 expression ratio in HFF cells. SW480 and DLD1 had a ratio of 0.030 and 0.023, respectively.

Because caspase-8 ubiquitination is necessary for efficient TRAIL-mediated apoptosis and normal fibroblasts have reduced caspase-8 ubiquitination, we investigated whether a deubiquitinase inhibitor, PR-619, could increase normal fibroblast caspase-8 ubiquitination. MRC-5 and WI-38 cells were treated with 8 μM PR-619 for 15 or 30 min. WI-38 treatment with PR-619 caused an increase in caspase-8 ubiquitination after 15 min (Fig. 6B). A significant increase in caspase-8 ubiquitination was seen in MRC-5 fibroblasts after 30 min of PR-619 treatment (Fig. 6B).

We also investigated caspase-8 protein stability after PR-619 treatment. H460 and MRC-5 cells were treated with CHX and/or PR-619 for 14 h. H460 and MRC-5 caspase-8 protein expression was similar in CHX-treated cells and cells co-treated with CHX and PR-619 (Fig. 6C). These data suggest that PR-619 does not affect caspase-8 protein stability in human nonmalignant normal fibroblasts.

The Deubiquitinase Inhibitor PR-619 Sensitizes Normal Fibroblasts to TRAIL-induced Cell Death—Because normal fibroblast caspase-8 ubiquitination is enhanced after addition of PR-619 (Fig. 6B), we next investigated TRAIL sensitivity after combined treatment with TRAIL and PR-619. Normal fibroblasts were pretreated with PR-619, and then TRAIL was added to the culture medium. WI-38 and MRC-5 normal cell viability decreased with combined treatment of TRAIL and PR-619 (Fig. 7A). TRAIL and TRAIL plus PR-619 cell viabilities were found to be similar in H460 cells. A slight decrease in SW480 cell viability was observed with combined treatment of PR-619 and TRAIL compared with TRAIL treatment alone (Fig. 7A).

We next sought to characterize the increased cell death observed in normal fibroblasts after combined treatment of PR-619 and TRAIL. MRC-5 cells were pretreated with the pan-caspase inhibitor Z-VAD-fmk before addition of PR-619 and TRAIL. PR-619 plus TRAIL showed a marked increase in cleaved caspase-3-positive cells. However, Z-VAD-fmk reduced cleaved caspase-3-positive cells, demonstrating that the cell death observed in normal fibroblasts after co-treatment of TRAIL and PR-619 is caspase-mediated (Fig. 7B).

Western blotting analysis was performed on MRC-5 cells after treatment with PR-619 and TRAIL. Disappearance of procaspase-8 can be observed after treatment with TRAIL and PR-619. Cleaved PARP and disappearance of procaspase-3 was also noted after treatment with PR-619 and TRAIL (Fig. 7C).
We next examined the role of caspase-8 in TRAIL plus PR-619 normal fibroblast cell death. MRC-5 cells were pretreated with the caspase-8 inhibitor Z-IETD-fmk, treated with TRAIL and PR-619, and analyzed for cleaved caspase-3 using flow cytometry. Pretreatment with Z-IETD-fmk significantly reduced the percentage of cleaved caspase-3-positive cells after co-treatment, suggesting that caspase-8 is necessary for TRAIL plus PR-619-induced cell death in normal human fibroblasts (Fig. 7D).

We next investigated whether normal fibroblast caspase-8 activity increases with combined treatment with TRAIL and PR-619. MRC-5 caspase-8 enzymatic activity increased in a time-dependent manner after treatment with PR-619 and TRAIL (Fig. 7E).

**Discussion**

The unique ability of TRAIL to preferentially kill tumors cells but leave non-malignant cells uninjured makes TRAIL and the TRAIL pathway an exciting potential cancer therapy. TRAIL-resistant tumor cells have been described previously, and many resistance mechanisms have been identified. However, the mechanism(s) that lead to normal cell TRAIL resistance have yet to be fully characterized. Van Dijk et al. (37) have previously investigated TRAIL resistance in non-transformed primary dermal fibroblasts and umbilical artery smooth muscle cells. They found that multiple pathways control TRAIL resistance in normal cells. c-FLIP, Bcl-2, and X-linked inhibitor of apoptosis protein have all been found to provide resistance to normal cells and act in a redundant manner (37).

Here we report a previously unrecognized mechanism of TRAIL resistance in human non-malignant normal cells involving incomplete activation of initiator caspase-8. Caspase-8 expression has been noted previously to be under epigenetic control, although we did not observe reactivation of its expression after treatment of normal cells with an histone deacetylase inhibitor or a DNA methyltransferase inhibitor. Our results...
suggest a posttranslational mechanism for the control of caspase-8 expression that may be relevant to the resistance to TRAIL. Because caspase-8 activation is an apical event in the extrinsic cell death pathway, this is likely to be a pivotal determinant of the resistance of normal cells to TRAIL-mediated cell death.

Normal fibroblasts express the TRAIL receptor DR5 but express little DR4 and DcR1 (Fig. 2, A–C). Our data are consistent with those of Zhang et al. (38), who found marginal protein expression of DR4 and DcR1 in MRC-5 fibroblasts by flow cytometry. Expression of c-myc was comparable in TRAIL-resistant normal cells and TRAIL-susceptible cancer cells (Fig. 3A). Expression of c-myc has been reported previously to positively correlate with TRAIL sensitivity (18). Ricci et al. (18) have noted diminished c-myc protein expression in WI-38 fibroblasts. They were able to induce TRAIL sensitivity in serum-starved WI-38 cells after adenoviral c-myc overexpression, highlighting the ability of c-myc to sensitize normal cells to TRAIL (18). We conclude that c-myc expression alone cannot predict and elucidate TRAIL resistance in normal fibroblasts but may play a role in such cells in concert with a number of other downstream molecules in the cell death pathway.

Initial studies to confirm normal fibroblast TRAIL resistance revealed marginal TRAIL sensitivity in MRC-5 lung fibroblasts but not in WI-38 cells and HFFs (Fig. 1, A–C). We noted that MRC-5 fibroblasts have less caspase-8 protein expression compared with TRAIL-sensitive H460 and SW480 cells (Fig. 3, D and E). However, MRC-5 cells have more caspase-8 expression than WI-38 cells and HFFs, suggesting that the increase in caspase-8 may translate into increased MRC-5 TRAIL sensitivity compared with WI38 and HFF cells (Fig. 3E). WI-38 and HFF cells are not deficient in caspase-8 protein. Caspase-8 protein levels can be detected by Western blotting analysis when using an enhanced chemiluminescent substrate (data not shown). The link between caspase-8 and TRAIL sensitivity has been well documented (19, 39, 40). Decreased caspase-8 expression has been noted in a variety of malignancies that are resistant to TRAIL-mediated cell death, and decreased caspase-8 is also a poor prognosis marker for head and neck squamous cell carcinoma (41). Treatment with histone deacetylase inhibitors and/or DNA methyltransferase inhibitors have been shown to increase caspase-8 expression and improve TRAIL sensitivity in a variety of tumor cells (40, 42, 43). Normal fibroblast treatment with TSA or 5-Aza failed to increase caspase-8 protein expression, suggesting that caspase-8 epigenetic silencing is not the cause of decreased caspase-8 expression in normal fibroblasts (Fig. 4, C and D). Accelerated degradation of caspase-8 has been described previously in TRAIL-resistant DLD1 colon cancer cells (44). We treated normal fibroblasts with cycloheximide to stop de novo protein synthesis and examined caspase-8 protein levels. Cycloheximide treatment of TRAIL-sensitive cancer cells displayed a caspase-8 stability and half-life profile similar to A2780 ovarian cancer cells treated with comparable CHX concentrations and incubation periods (45). Normal fibroblasts had decreased caspase-8 stability compared with TRAIL-sensitive colon and lung cancer cells (Fig. 4, E and F). We believe that decreased caspase-8 protein stability may contribute to diminished caspase-8 expression in normal fibroblasts.

Examination of TRAIL-induced caspase-8 activity in normal fibroblasts revealed limited caspase-8 enzymatic activity and no indication of cleaved products by Western blotting analysis after TRAIL exposure (Fig. 5, A and B). Insufficient caspase-8 activity and activation in TRAIL-treated normal cells suggests that caspase-8 is not fully active. Caspase-8 posttranslational modifications that affect caspase-8 activity have been described previously and include phosphorylation and ubiquitination (36, 46–48). Jin et al. have found previously that caspase-8 polyubiquitination is necessary for complete TRAIL-induced caspase-8 activation and cell death in H460 and H2122 lung cancer cells (36). Caspase-8 ubiquitination has been found to be comprised of both Lys-63 and Lys-48 chains. A20-mediated deubiquitination of caspase-8 resulted in reduced TRAIL-induced cell death (36). Conversely, caspase-8 Lys-63 linked polyubiquitination by the E3 ubiquitin ligase HECTD3 has been found to decrease caspase-8 activation and reduce TRAIL-mediated viability in breast cancer cells (49). In this study, we investigated the caspase-8 ubiquitination status in HFFs and found that HFF cells displayed decreased basal caspase-8 ubiquitination compared with SW480 and DLD1 colon cancer cells (Fig. 6A). Normal fibroblast treatment with the deubiquitinase inhibitor PR-619 caused a significant increase in caspase-8 ubiquitination (Fig. 6B), and TRAIL plus PR-619 co-treatment caused increased cell death in WI-38 and MRC-5 fibroblasts (Fig. 7A). Increased WI-38 and MRC-5 fibroblast TRAIL-mediated apoptosis after pretreatment with PR-619 suggests that, although these cells have reduced caspase-8 protein expression, TRAIL susceptibility is possible with complete caspase-8 activation.

PR-619 has a broad specificity that inhibits multiple deubiquitinating enzymes (50). A specific A20 inhibitor is needed to confirm that A20 is responsible for removing polyubiquitin chains from caspase-8 in normal fibroblasts. A20 is a unique protein in which the N terminus has deubiquitinase properties, whereas the C terminus acts as an E3 ligase (51, 52). The dual role of A20 as an E3 ligase and deubiquitinase further complicates its role in TRAIL-mediated cell death. A20 has been shown to enhance TRAIL-induced cell signaling in hepatocellular carcinoma cells (53, 54), but it protects against TRAIL-mediated cell death in glioblastoma cells (55). We believe that A20 is responsible for normal fibroblast caspase-8 deubiquitination. A20 has been found previously to protect endothelial cells from death receptor-mediated cell death by inhibiting caspase-8 activation (55). Moreover, Wang et al. (56) found A20 expression to be increased in peripheral blood mononuclear cells isolated from healthy individuals compared with samples isolated from lymphoma patients. Our data suggest that deubiquitination and, therefore, inactivation of the key initiator caspase-8, needed for cell death, may be a regulation mechanism to prevent unintentional initiation of the cell death pathway.

The appeal of TRAIL as a potential cancer therapy lies in its ability to selectively kill cancer cells while leaving normal cells intact. Our findings reveal normal cell cytotoxicity with PR-619 and TRAIL co-treatment. Deubiquitinase regulation of apopto-
sis has recently led to deubiquitinating enzymes becoming cancer therapy targets (57). Clinical studies with PR-619 have not been performed. However, preclinical work with the small-molecule deubiquitinase inhibitor b-AP15 is underway (58, 59). b-AP15 induced tumor cell apoptosis and inhibited tumor progression in several solid tumor models (58). Therapies combining TRAIL and a deubiquitinase inhibitor may cause normal cell toxicity and should be examined carefully. Modulation of deubiquitinase activity emerges from this study as a potentially important nodal point for modulation of the therapeutic index of TRAIL-pathway-based cancer therapy.

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