Repeated Treatment with Subtoxic Doses of TRAIL Induces Resistance to Apoptosis through Its Death Receptors in MDA-MB-231 Breast Cancer Cells

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Abstract
Recombinant human tumor necrosis factor–related apoptosis–inducing ligand (rhTRAIL) is being evaluated clinically in treating various malignancies. Previous studies have shown that repeated application of high doses of rhTRAIL results in a subpopulation of parental cells that is unresponsive to the death ligand. However, it is not clear whether TRAIL-sensitive cancer cells could acquire resistance to TRAIL treatment. Here, we found that MDA-MB-231 breast cancer cells, which are highly sensitive to TRAIL-induced apoptosis, became resistant to TRAIL killing after a prolonged exposure to subtoxic doses of rhTRAIL. The resulting TRAIL-resistant cells were cross-resistant to antibodies against its death receptors (DR4 and DR5); however, they retained sensitivity to several clinically relevant chemotherapies. Surface expression of DR4 and DR5 was significantly reduced in the selected cells, resulting in failure in death-inducing signaling complex formation and caspase activation. In addition, real-time PCR analysis revealed an upregulation in multiple apoptosis-regulator genes, including c-FLIP, Stat5α, and Stat5β. Inhibition of Janus-activated kinase, an upstream activator of signal transducer and activator of transcription 5 (Stat5), or knockdown of Stat5 itself partially restored cellular sensitivity to TRAIL-induced apoptosis, suggesting that Stat5 signaling is also involved in the development of TRAIL resistance. Furthermore, we showed that acquired TRAIL resistance was effectively eliminated by combination with etoposide, doxorubicin, or paclitaxel. These results suggest that tumor cells could acquire resistance to TRAIL therapy especially when they are repeatedly exposed to low levels of the death ligand, highlighting the necessity of combination with therapies that target the resistance mechanisms. (Mol Cancer Res 2009;7(11):1835–44)

Introduction
Drug resistance is a major cause of cancer treatment failure. Today, most cancer therapies involve multiple agents, as it is almost universally the case that single drugs or single-target drugs will encounter resistance. In developing new combination drug therapies, a key goal is to identify agents that target or bypass the resistance mechanisms.

Recombinant human tumor necrosis factor (TNF)–related apoptosis–inducing ligand (rhTRAIL) is emerging as a potential anticancer agent due to its selective cytotoxicity to various cancer cells, but not normal cells (1, 2). TRAIL induces apoptosis through its death receptors (DR4 and DR5). These death receptors are characterized by an intracellular death domain that facilitates assembly of the death-inducing signaling complexes (DISC) and subsequent activation of a caspase cascade (3-5). Upon ligand binding, the death receptors recruit the adaptor protein Fas-associated death domain and initiator caspase-8 and/or caspase-10 to form a DISC, where caspase-8 is self-cleaved into a 10-kDa fragment that is released from the DISC and an intermediate 43/41-kDa species that remains tethered to the DISC. The 43/41-kDa intermediate is subsequently processed to give an 18-kDa fragment that associates with the 10-kDa fragment to form the active caspase-8 pro tease, which is released into the cytosol. The active caspase-8 cleaves the effector caspases such as caspase-3, caspase-6, and caspase-7, leading to degradation of multiple cellular proteins and execution of apoptosis. Activated caspase-8 can also cleave a pro-apoptotic protein Bid that links to the mitochondrial pathway for amplifying caspase activation (6). TRAIL sensitivity is dependent not only on the levels of DR4 and DR5 on cell surface but also on the intracellular proteins regulating caspase activities. At the level of the DISC, the cellular FLICE-like protein (c-FLIP) competes with the initiator caspases for Fas-associated death domain binding sites, thereby inhibiting the activation of caspase-8 or caspase-10 (7). Caspase activity is negatively regulated by antiapoptotic proteins, including Bcl2, Bcl-XL, and members of the inhibitors of apoptosis (IAP) family (XIAP, cIAP1, cIAP2, and survivin; ref. 8). TRAIL sensitivity is also dependent on the levels of decoy receptors such as DcR1 and DcR2 that lack a death domain but possess a comparable binding affinity to TRAIL (4).

Unlike conventional cancer therapeutics, TRAIL induces apoptosis independently of the p53 tumor suppressor gene, which is frequently inactivated in cancer cells. Thus, rhTRAIL may be effective against tumors that have acquired resistance to conventional therapy. Preclinical studies have shown that TRAIL displays single-agent activity and cooperates with...
Prolonged Application of Subtoxic Doses of TRAIL Induces Acquired Resistance to TRAIL-Induced Apoptosis in Breast Cancer Cell Lines

Previous studies have shown that repeated exposure of TRAIL-sensitive human cancer cell lines to high doses of TRAIL (50-500 ng/mL) induced apoptosis. We investigated whether TRAIL-sensitive cancer cells could acquire resistance to TRAIL-induced apoptosis. MDA-MB-231 human breast cancer cells, which are highly sensitive to TRAIL killing, were treated repeatedly with subtoxic doses of rhTRAIL (0.1-2 ng/mL). The selection process was associated with a basal cell death (<10%), thereby giving rise to a cell population that is representative of the majority of parental cells. The resistant cells were found to be resistant to apoptosis by rhTRAIL as well as antibodies against its death receptors. We showed that acquired TRAIL resistance involves distinct mechanisms such as down-regulation of DR4 and DR5 on the cell surface and sustained activation of signal transducer and activator of transcription 5 (Stat5) signaling. Interestingly, acquired TRAIL resistance can be overcome by combination with the chemotherapeutics etoposide, doxorubicin, or Taxol. Our findings suggest that tumor cells could develop resistance to TRAIL-induced apoptosis when they are exposed to low concentrations of the ligand.
MDA-MB-231 cellswere sensitive to both antibodies (∼50% apoptotic cells seen after 24 hours at 10 μg/mL). By contrast, MDA-231/TR cells only displayed a basal level of apoptosis (<10%) under the same conditions (Fig. 3A and B). To determine whether the mitochondrial apoptosis pathway was also affected in MDA-231/TR cells, we evaluated cellular responses to three clinically relevant chemotherapeutic drugs. As shown in Fig. 3C, MDA-231/TR cells maintained similar sensitivities as parental cells to etoposide (VP-16), doxorubicin, and Taxol (data not shown), respectively. These results indicate that TRAIL-resistant cells that resulted from prolonged exposure to subtoxic doses of TRAIL were cross-resistant toward apoptosis by agonistic anti-DR4 or anti-DR5 antibodies while retaining sensitivity to some chemotherapeutic drugs. The results also suggest that the intrinsic mitochondrial apoptotic pathway used by those chemotherapeutics was intact and fully functional in the acquired TRAIL-resistant cells.

Surface Expression of DR4 and DR5 Was Downregulated in the Selected TRAIL-Resistant Cells

The surface expression of TRAIL death receptors is essential for transmitting a death signal from their ligands. To investigate the mechanism of acquired TRAIL resistance, we first evaluated the total protein expression of TRAIL receptors in parental and MDA-231/TR cells by Western blotting. As shown in Fig. 4A, total expression of DR4, DR5, and decoy receptors DcR1 and DcR2 was similar in parental and selected TRAIL-resistant cells. To assess the expression of DR4 and DR5 on the cell surface, surface proteins were biotinylated, purified by streptavidin agarose, and analyzed by immunoblotting using antibodies to DR4 or DR5. This approach is advantageous over flow cytometry in detecting cell surface protein expression because it provides a direct measure for potential posttranslational modifications such as truncation and glycosylation in the receptor proteins (21, 22). Strikingly, cell surface DR4 and DR5 were found to be significantly decreased in MDA-231/TR cells compared with parental cells (Fig. 4B). As a control, surface expression of β1-integrin, Her-2, and epidermal growth factor receptor (data not shown) was not changed between the two cell lines. Consistent results were obtained by flow cytometry analysis using phycoerythrin (PE)-conjugated antibodies specific to DR4 or DR5 (Fig. 4C). In line with the reduction in surface DR4 and DR5, DISC assembly was also impaired in TRAIL-resistant cells (Fig. 4D). In parental MDA-MB-231
cells, TRAIL efficiently induced a time-dependent assembly of DISC complexes that contain DR4, DR5, procaspase-8, and its cleaved product p43/41 (Fig. 4C). At 60 minutes, p43/p41 proteins were significantly detected. In contrast, the recruitment of DISC components was diminished in MDA-231/TR cells. Similar results were obtained after treatment with biotinylated anti-DR5 antibodies (Fig. 4E). These results show that DR4 and DR5 were selectively downregulated from the cell surface during repeated application of subtoxic doses of rhTRAIL. Although the surface expression of death receptors does not necessarily predict TRAIL sensitivity due to potential defects in downstream signaling components (8, 13, 23), the above results are in agreement with our previous data that loss of surface death receptors is sufficient to account TRAIL resistance (12).

Stat5 Signaling Is Implicated in the Development of TRAIL Resistance

The TRAIL-induced apoptosis pathway is regulated by multiple intracellular proteins, including cFLIP, XIAP, and members of the Bcl-2 family. These proteins are key regulators of caspase activity and have been implicated in intrinsic TRAIL resistance in some tumor cell lines (8, 13). The repeated application of TRAIL may alter the expression of caspase regulators, thereby contributing to acquired TRAIL resistance. To explore this possibility, we performed real-time PCR analysis using a human PCR array containing a total of 384 apoptosis-related genes. The mRNA expression levels were normalized to five housekeeping genes (GAPDH, RPL13A, B2M, ACTB, and HPRT1) and presented as fold changes in Supplement I. Overall, ~80% of the genes examined, including DR4 and DR5, showed no difference or <1.5-fold difference in their mRNA levels between parental and MDA-231/TR cells. Interestingly, c-FLIP, Stat5a, and Stat5b were upregulated in TRAIL-resistant cells when compared with parental cells, showing 1.6- and 2.3-fold increase, respectively. Their protein levels were also increased by a similar magnitude (Fig. 5A and B). Consistently, Bcl-XL and cyclin D1, target genes of Stat5 (24), were also elevated in TRAIL-resistant cells. In parental cells, c-FLIP undergoes a rapid degradation in response to rhTRAIL treatment (Fig. 5C), which was blocked by a general caspase inhibitor Z-VAD (data not shown). By contrast, c-FLIP and Stat5 protein expressions were sustained in the selected TRAIL-resistant cells. The sustained expression of c-FLIP has been shown to render TRAIL resistance by inhibiting caspase-8 activation (7, 25). In agreement, knockdown of c-FLIP by RNA interference increased TRAIL-induced apoptosis in both parental and MDA-231/TR cells (Fig. 5D). Stat5 proteins are key regulators of cell growth and survival (17, 18). Given the significant increase in Stat5b mRNA (2.3-fold) in the selected TRAIL-resistant cells, we examined whether knockdown of its expression could sensitize TRAIL-induced apoptosis. This was achieved by a validated stealth small interfering RNA (siRNA) specific to the Stat5b transcript (Invitrogen). As shown in Fig. 5E, knockdown of Stat5b also partially restored sensitivity of MDA-231/TR cells to TRAIL-induced apoptosis. This effect was associated with a reduction in Bel-XL and cyclin D1 expression (Fig. 5E, top). Consistent with Stat5b knockdown
data, selective inhibition of Janus-activated kinase (JAK) kinase, an upstream regulator of Stat5 signaling, showed a similar effect (Fig. 5F). These results suggest that the upregulated c-FLIP and Stat5 (at least Stat5b) signaling is also involved in the development of TRAIL resistance.

Acquired TRAIL Resistance Is Overcome by Combination with Clinically Relevant Chemotherapies

We asked whether acquired TRAIL resistance can be overcome by combination with chemotherapies. We first examined the effect of the anticancer agent VP-16. Cells were exposed to increasing concentrations of VP-16 for 24 hours in the presence or absence of a fixed dose of rhTRAIL (10 ng/mL). As shown in Fig. 6A, treatment with VP-16 resulted in a dose-dependent increase in TRAIL-induced apoptosis in MDA-231/TR cells. Strikingly, a comparable level of apoptosis (∼60%) was achieved in the two cell lines after treatment with VP-16 (10 μmol/L) and rhTRAIL. This synergistic effect was confirmed by enhanced cleavage of caspase-8 and caspase-3 in MDA-231/TR cells (Fig. 6B). To understand how VP-16 restored TRAIL sensitivity, we measured the surface levels of DR4 and DR5 in cells treated with VP-16. As shown in Fig. 6C, VP-16 treatment increased the surface expression of DR4 and DR5 in the TRAIL-resistant MDA-MB-231 cells. VP-16 is a topoisomerase II inhibitor that induces double strand breaks in DNA, thus leading to p53 activation (26). In addition, p53 has been implicated in the regulation of DR4 (27) and DR5 (28) transcriptions. Thus, VP-16 seems to sensitize TRAIL-resistant cells to TRAIL-induced apoptosis by upregulating surface expression of TRAIL death receptors.

Additionally, VP-16 also sensitized the selected cells to apoptosis induced by anti-DR4 or anti-DR5 monoclonal antibodies (Fig. 6D). Furthermore, we observed that acquired TRAIL resistance was effectively abolished by combining with two other chemotherapies that are currently being used in treating breast cancers—doxorubicin or Taxol (Fig. 6E). These results are consistent with the data in Fig. 3C, which demonstrates that the mitochondrial pathways used by those therapies was not affected in the selection to TRAIL resistance. Collectively, our data suggest that tumor cells could develop resistance to apoptosis by rhTRAIL when they are repeatedly treated with low doses of the death ligand. However, acquired TRAIL resistance can be eliminated by combining with therapies that induce apoptosis through a death receptor–independent pathway.

Discussion

Drug resistance is a shared phenomenon to most cancer therapies, TRAIL without exception. The underlying mechanisms are generally either innate, in which they are intrinsic to cancer cells, or acquired, which occurs due to adaptive changes in response to therapy and due to the selection of survival phenotypes. Intrinsic TRAIL resistance has been seen in some human cancer cell lines (29-31), including breast cancer (12, 20, 32, 33). In this study, we showed that TRAIL-sensitive breast cancer cell lines (MDA-MB-231 and MD-MB-468) became resistant to TRAIL-induced apoptosis after prolonged treatments with subtoxic doses of rhTRAIL (0.1-2 ng/mL). The resulting TRAIL-resistant cells were cross-resistant to anti-DR4 or anti-DR5 antibodies while maintaining sensitivity to several clinically relevant anticancer drugs. Acquired TRAIL resistance seemed to arise from at least two distinct mechanisms: (a) diminished surface expression of DR4 and DR5 and (b) upregulated c-FLIP and Stat5 expression. These results suggest that
tumor cells could develop resistance to apoptosis through TRAIL receptors, especially when exposed to low levels of the death ligand as a result of administration or barrier of tumor microenvironment. The information gathered from this study may contribute to the design of therapeutic strategies aimed to counteract the acquired TRAIL resistance mechanisms in the clinical setting.

Several groups have attempted to study acquired TRAIL resistance in human cancer cell lines of colon (11), leukemia (15), ovarian (16), melanoma (17), and breast carcinoma (18). However, all these studies used high doses of TRAIL (50-500 ng/mL) that killed most of the parental cells during the selection for stable clones. Based on the inherent heterogeneity of cancer cells in terms of response to TRAIL-induced apoptosis (19), the selected stable clones were most likely derived from a subset (<1%) of parental cells rather than truly acquired resistance. To our knowledge, the present study is the first to show that TRAIL-sensitive cancer cells evolved into TRAIL-resistant cells in response to TRAIL treatment at repeated and subtoxic doses. The selected resistant cells

FIGURE 4. Surface expression of DR4 and DR5 is selectively downregulated in TRAIL-resistant cells. A. Total protein levels of TRAIL receptors were determined by Western blot analysis using antibodies specific to DR4, DR5, DcR1, or DcR2. Actin was shown as a loading control. B. Determination of surface DR4 and DR5 by immunopurification. Cell surface proteins were biotinylated, isolated by streptavidin agarose, and analyzed by Western blotting. Right, blots for 5% of starting cell lysates (5% input) used in the pull-down assays. β1-Integrin was measured as a control. C. FACS analysis of surface expression of DR4 and DR5. Cells were stained with PE-conjugated antibodies specific to individual receptor. Left, representative histograms for DR4. Right, the normalized surface expression of DR4 and DR5 as determined by the differences between the mean values of PE-anti-DR4 or PE-anti-DR5 antibodies and their corresponding PE-IgG controls. D. TRAIL DISC formation. Cells were treated with (His)6-TRAIL (1 μg/mL) at 37°C for 0, 15, 30, and 60 min, respectively. Equal amounts of cell lysates were incubated with Ni2+-agarose at 4°C for 1 h, and purified proteins were analyzed by immunoblotting for the presence of DR5 and caspase-8. The full-length and processed forms of caspase-8 were seen in parental MDA-MB-231 cells but not in MDA-231/TR cells. E. DISC assembly induced by anti-DR5 antibodies. Cells were treated with biotinylated anti-DR5 antibodies (10 μg/mL) at 37°C for 60 min, and DISC complexes were isolated by streptavidin agarose and blotted for the presence of caspase-8 (C-8) and DR5.
remained fully responsive to etoposide, doxorubicin, and Taxol—the cytotoxicity of which is exclusively achieved through the mitochondrial pathway (Fig. 3C)—suggesting that defects occurred within the death receptor apoptosis pathway. Interestingly, acquired TRAIL resistance was effectively eliminated by combination with clinically relevant chemotherapies. These results provide a rationale for combining rhTRAIL with established chemotherapies to ensure killing of a tumor population that have acquired resistance to either therapy. The agonistic antibodies to TRAIL death receptors DR4 or DR5 are also currently being tested in treating different malignancies. It would be of interest to determine whether tumor cells could develop resistance to these agents when they are administered at subtoxic doses in clinical settings.

TRAIL resistance has been associated with various mechanisms, including (a) defects in TRAIL receptors such as loss of cell surface expression of DR4 and DR5 (11, 12) or inhibitory binding of TRAIL by decoy receptors DcR1 and DcR2 (34, 35) and (b) overexpression of antiapoptosis proteins such as c-FLIP (7), Bcl-2/Bcl-XL(36), and the inhibitors of apoptosis (IAP) family members (37). We found that surface expression of DR4 and DR5 was downregulated in the selected TRAIL-resistant cells, whereas their total protein levels were not changed (Fig. 4A and B). Like TNFα (38), TRAIL has been shown to induce internalization of its death receptors in colon (39, 40) and breast cancer cells (41). Therefore, it is likely that DR4 and DR5 were internalized and retained in the cytosol of the selected cells, as a result of repeated exposure to low doses

FIGURE 5. Altered expression of multiple intracellular proteins in the selected TRAIL-resistant cells. A. Western blot analysis revealed a significant increase in c-FLIP and Stat5, and Stat5-regulated proteins Bcl-XL and cyclin D1, but not c-IAP1 or Bcl-2 in MDA-231/TR cells versus parental cells. B. Fold changes in protein expressions in MDA-231/TR cells relative to parental cells was estimated by densitometry analysis of blots as in A from three independent experiments. C. Protein expression of c-FLIP and Stat5 in response to rhTRAIL. Cells were treated with rhTRAIL (10 ng/mL) for the indicated times, lysed, and analyzed by immunoblotting. D. Effect of c-FLIP knockdown on TRAIL-induced apoptosis. Cells were transiently transfected with a control siRNA or a mixture of three siRNA sequences (see Materials and Methods) targeting different regions of the human c-FLIP transcript. After 24 h, cells were analyzed by immunoblotting for c-FLIP protein expression (top) or treated with rhTRAIL (20 ng/mL) at 37°C for an additional 4 h followed by FACS analysis for apoptosis (bottom). E. The effect of Stat5 knockdown was assessed similarly as in D (top, immunoblots for Stat5 using an anti–pan Stat5 antibody recognizing both Stat5a and Stat5b, Bcl-XL, and cyclin D1; bottom, apoptosis after incubation with TRAIL for 4 h at 20 ng/mL). F. Cells were pretreated with a cell-permeable JAK kinase inhibitor at the indicated concentrations for 1 h, followed by rhTRAIL (20 ng/mL) for an additional 4 h. The results are expressed as JAK inhibition–induced apoptosis (apoptosis % induced by JAK inhibitor combining with rhTRAIL – apoptosis % by rhTRAIL alone; n = 3, mean ± SD).
of TRAIL. Loss of surface DR4 and DR5 resulted in failure of DISC assembly and caspase activation (Fig. 4D and E), which seems to be a critical mechanism in acquired TRAIL resistance. The results also suggest that tumor cells could lose their surface death receptors when continuously exposed to low levels of physiologic TRAIL, leading to naturally occurring TRAIL resistance (12).

Stat5 proteins are members of the STAT family of transcription factors that mediate cytokine and growth factor–induced signals that culminate in various cellular responses, including cell growth and apoptosis (42, 43). Stat5a and Stat5b are selectively expressed in the mammary gland and other epithelial cells. Interestingly, a repeated application of subtoxic doses of rhTRAIL upregulated Stat5a and Stat5b expression at both the mRNA and protein levels; subsequently, the expression of their target genes, including Bcl-XL and cyclin D1 (Fig. 5A-C), was also upregulated. Knockdown of Stat5b or inhibition of its upstream regulator JAK kinase partially restored sensitivity of MDA-231/TR cells to TRAIL killing (Fig. 5E and F). Given that Stat5 is a principal target of JAK2, these results suggest that JAK2/Stat5 signaling may play a significant role in acquired TRAIL resistance. Our results are in agreement with those of Fuke et al. (44), who showed that the JAK inhibitor AG490 augmented TRAIL-induced apoptosis in liver carcinoma cell lines. In addition, expression of a Stat5 dominant-negative mutant resensitized heat shock protein 70–mediated resistance of leukemia cell lines to TRAIL-induced apoptosis (45). However, it is not clear how subtoxic doses of TRAIL upregulate Stat5 expression. Stat5 activation has been implicated in the progression of human prostate cancers (24, 46). TRAIL-mediated upregulation of Stat5 signaling should be taken into consideration in TRAIL therapy especially when drug resistance occurs. It will be of significance to find out the link between TRAIL and the JAK/Stat5 signaling pathway.

In summary, our data suggest that repeated exposure of TRAIL-sensitive cancer cells to subtoxic doses of rhTRAIL can lead to the development of resistance to the death ligand. A critical mechanism is the reduced surface expression of DR4 and DR5, likely through ligand-induced internalization (41). We also show that the mitochondrial pathway is intact in the selected cells. These findings have an important implication in targeting TRAIL receptors with established anticancer drugs as new regimens for treating human breast cancers.

**FIGURE 6.** Sensitization of TRAIL-resistant cells by combination with clinically relevant chemotherapies. A. Pretreatment with VP-16 abolished acquired TRAIL resistance. Cells were pretreated with etoposide (VP-16) at 0, 1, or 10 mmol/L for 24 h followed by incubation without (−TRAIL) or with (+TRAIL) 10 ng/mL TRAIL for additional 6 h. Apoptosis was measured by FACS as in Fig. 1A. B. Western blots for caspase-8 (C-8) and caspase-3 (C-3) in the indicated cell lines after treatment with VP-16 (10 μmol/L) and rhTRAIL (10 ng/mL). C. VP-16 treatment upregulates surface expression of TRAIL death receptors. Cells were treated with VP-16 (100 μmol/L) for 24 h and analyzed by surface biotinylation as described in Fig. 4B. Transferrin receptor (TfR) was used as a control. D. VP-16 sensitized MDA-231/TR cells to apoptosis by anti-DR4 (DR4 mAb) and anti-DR5 (DR5 mAb) antibodies, respectively. Cells were sequentially treated with VP-16 for 24 h and the indicated antibodies for another 24 h. E. Effects of doxorubicin (25 nmol/L) and Taxol (1 μmol/L) on TRAIL-induced apoptosis. Cells were treated with the indicated drugs for 24 h followed by 10 ng/mL rhTRAIL for 6 h (n = 3, mean ± SD).
Materials and Methods

Antibodies and Reagents

rhTRAIL, monoclonal antibodies specific to the respective extracellular domains of DR4 and DR5, and their PE-conjugated forms were purchased from R&D Systems. Antibodies against human caspase-3 and caspase-8, and JAK kinase inhibitor I, were from Calbiochem. Monoclonal antibodies to c-IAP1, c-IAP2, and FLIP were from Cell Signaling. Anti–pan Stat5 was from Invitrogen. Antibodies to Bel-2, Bel-XL, cyclin D1, and phospho-Stat5 (Tyr694) were from BD Biosciences. Anti-actin, anti-survivin, horseradish peroxidase–conjugated goat anti-rabbit IgG and anti-mouse IgG1 were from Santa Cruz Biotechnology. Etoposide (VP-16) and doxorubicin were from Sigma. c-FLIP and Stat5b siRNA oligonucleotides and their corresponding negative controls with similar GC content were purchased from Invitrogen. c-FLIP siRNA sequences were 5′-CCCUGGGCUAUGAAGUCCUCAUUA-3′ (designated as siFLIP-I), 5′-CCCAGAAACUGAGACAAGAAAGAAA-3′ (II), and 5′-GGGAAAAUCUGUGGUGCUCUAUUA-3′ (III). The sequence for a validated stealth siRNA against Stat5b (Invitrogen) was 5′-CCCCUCACUGAGACAGCGGCUUUAAU-3′. Transient transfections of RNAi duplexes were carried out using Lipofectamine RNAiMAX (Invitrogen).

TRAIL Treatment

The MDA-MB-231 and MDA-MB-468 human breast cancer lines were obtained from the American Type Culture Collection. Both cell lines are highly sensitive to TRAIL–induced apoptosis (12, 20). Cells were grown in six-well plates and exposed continuously to increasing doses of rhTRAIL (0.1, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 ng/mL) for 4 wk. Cell density was maintained at 70% to 80% confluency and subcultured as needed. Each dose was repeated four times by feeding fresh medium every 24 h. After each exposure, cell death was assessed by flow cytometry (see below). By day 28, the resulting cells (designated MDA-231/TR) were harvested and kept frozen. For the assays, cells were maintained in a complete DMEM/F-12 50/50 medium containing 1 ng/mL rhTRAIL at 37°C and 5% CO₂.

Apoptosis Assay

Apoptosis was determined by flow cytometry as previously described (12). Briefly, cells were grown on six-well plates to 70% to 80% confluence and treated with rhTRAIL or other agents. At the indicated time points, cells were harvested, stained with FITC-conjugated Annexin V and PI, and analyzed by FACS Calibur and CellQuest software (Becton Dickinson). Cell viability was measured by the MTS assay (47).

Detection of Cell Surface TRAIL Death Receptors

The cell surface expression of DR4 and DR5 was detected by biotinylation and extraction of the total cell surface proteins followed by Western blot analysis. Cell surface biotinylation was done using the Pierce Cell Surface Protein Isolation kit (Pierce) per manufacturer’s instruction. Briefly, cells were grown in T75 cm² flasks to 80% to 90% confluence. After washing twice with ice-cold PBS, cells were incubated with the Sulfo-NHS-SS-Biotin reagent for 30 min at 4°C. The reaction was terminated by the addition of quenching solution and washed thrice with TBS buffer [25 mmol/L Tris, 150 mmol/L NaCl (pH 7.2)]. The resulting cells were lysed in 0.5 mL lysis buffer. Cell debris was removed by centrifugation at 10,000 × g for 2 min at 4°C. The cleared supernatant was loaded to Neutra/Avdin agarose column for capturing biotinylated proteins. After extensive washes, the bound proteins were eluted by a buffer containing 50 mmol/L DTT. The purified cell surface proteins were analyzed by immunoblotting using antibodies specific to DR4 or DR5. Surface β₃-integrin or transferrin receptor was detected as a control.

DISC Formation

TRAIL DISC formation was analyzed as described previously (11). In brief, 1 × 10⁷ cells were incubated with 1 μg/mL of (His)₆-TRAIL at 37°C for 15, 30, or 60 min. Cells were washed twice with ice-cold PBS and lysed for 30 min on ice in a lysis buffer [30 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100] supplemented with protease inhibitors and 10 mmol/L imidazole. The lysates were cleared twice by centrifugation at 4°C. Protein concentration was determined by a BCA protein assay (Pierce). Equal amounts of extracts were incubated with nickel-agarose at 4°C for 1 h. The affinity complexes were subsequently washed four times with lysis buffer and eluted with 1 mol/L imidazole. The isolation of DISC complexes induced by anti-DR5 antibodies was done using biotinylated anti-DR5 antibody (10 μg/mL) in combination with streptavidin as previously described (12). DISC components were detected by Western blot analysis.

Quantitative Real-time PCR Array

Quantitative real-time PCR (qRT-PCR) was done using an apoptosis PCR Array containing 384 apoptosis-related genes (SuperArray Biosciences). Briefly, total RNA samples were isolated using the Trizol reagent. All samples had 260/280 ratios above 2.0 and 260/230 ratios above 1.7. An equal amount of RNA (2 μg) was used for reverse transcription using RT² First Strand Kit from SuperArray Biosciences. PCR reactions were done using the RT² profiler PCR array PAHS-3012 E (Human Apoptosis PCR Array 384 HT) on the ABI Fast 7900 using RT² Real-time SYBR Green PCR master mix PA-012. The total volume of the PCR reaction was 10 μL. The thermocycler parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative changes in gene expression were calculated using the ΔΔCt (threshold cycle) method. Five housekeeping genes were included on the array (B2M, Hprt1, Rpl13a, Gapdh, and Actb) to normalize the RNA amounts.

Western Blotting

Cells (1 × 10⁶) were lysed in SDS lysis buffer containing 50 mmol/L Tris-HCl (pH 7.0), 2% SDS, and 10% glycerol, and incubated for 20 min at 95°C. Protein concentrations were estimated using the BCA protein assay (Pierce). Equal amounts of cell lysates (20 μg per lane) were resolved by electrophoresis using a 4% to 12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to polyvinylidene fluoride membranes (Millipore) for immunoblot analysis with an appropriate dilution of antibodies (1:1,000 to 1:2,000). When necessary, the membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) and reprobed with appropriate antibodies. Immunocomplexes were visualized by chemiluminescence using ECL (Santa Cruz Biotechnology).
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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