

TRAIL Resistance of Breast Cancer Cells Is Associated with Constitutive Endocytosis of Death Receptors 4 and 5

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its agnostic antibodies, which are being evaluated clinically as anticancer therapies, selectively kill cancer cells through the death receptors DR4 and DR5. However, their therapeutic potential is limited by occurring resistance in tumor cells. Here, we compared the apoptotic response of a panel of six human breast cancer cell lines with recombinant human TRAIL and antibodies to DR4 or DR5. Despite their total mRNA and protein expression, TRAIL death receptors, with a higher frequency in DR4, are absent on cell surface in some cell lines. Loss of cell surface expression of DR4 or DR5 accounts for resistance to their corresponding antibody and, importantly, correlates with a decreased sensitivity to TRAIL. TRAIL resistance occurs when both receptors are absent on cell surface regardless of alterations in Bcl-2 family proteins or caspases. Furthermore, inhibition of endocytosis by pharmacologic inhibitors or disruption of clathrin-dependent endocytosis signaling components (adaptor protein 2 and clathrin) restores cell surface expression of the death receptors and sensitize TRAIL-resistant cells to TRAIL-induced apoptosis. DR4 endocytosis appears to be mediated by its cytoplasmic domain EAQC³³⁷LL. The results show that TRAIL death receptors undergo constitutive endocytosis in some breast cancer cells. Loss of cell surface expression of DR4 and DR5 could be evaluated as a biomarker for TRAIL resistance in breast tumors. Moreover, the clathrin-mediated endocytosis pathway could be a potential target for therapeutics to overcome tumor resistance to TRAIL receptor-targeted therapies. (Mol Cancer Res 2008;6(12):1861–71)

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis through the death receptors DR4 and DR5 expressed on the surface of target cells (1-3). Recombinant human TRAIL (rhTRAIL) preferentially induces apoptosis in cancer cells over normal cells and shows little or no overt toxicity when systemically administered to animals (4, 5). This has led to multiple clinical trials with biological agents targeting TRAIL receptors, including rhTRAIL and agonistic antibodies to DR4 and DR5. However, a significant portion of tumor cells (6-8), including breast cancer (9-11), are resistant to these agents. Nonresponsive patients will not benefit from the treatments but may still suffer from the potential side effects, highlighting the need for biomarkers that can help predict tumor sensitivity to TRAIL receptor-targeted therapies.

On ligand binding, the death receptors assemble a death-induced signaling complex (DISC) that contains the adaptor protein Fas-associated death domain (FADD) and the apoptosis-initiating protease pro-caspase-8 (or pro-caspase-10; ref. 1). In the DISC, pro-caspase-8 is processed, releasing active caspase-8 into the cytoplasm, where it cleaves and activates effector caspases, such as caspase-3, -6, and -7. Caspase-8 can also stimulate the cleavage of Bid, thus amplifying caspase activation through mitochondria. This apoptotic machinery is shared by TRAIL and antibodies against DR4 or DR5. TRAIL resistance, in some cases, is caused by the simultaneous expression of decoy receptors (DcR1, DcR2, and OPG) that lack a death domain but possess a comparable binding affinity to TRAIL. However, the presence of decoy receptors cannot explain the lack of response of many cancer cells to antibodies specifically targeting DR4 or DR5. Other defects along the pathway, including down-regulation of caspase-8 or -10 and overexpression of antiapoptotic molecules cellular FLICE-inhibitory protein (FLIP; ref. 12) and Bcl-2 and Akt (2), have been associated with TRAIL resistance in individual cell lines. However, none of these factors showed a consistent correlation with TRAIL resistance in multiple cancers (13). A better understanding of the mechanisms of TRAIL resistance may lead to the identification of predictive biomarkers for tumor responsiveness and identification of better targets for drugs to overcome the resistance.

Although the mRNA expression of both DR4 and DR5 is found in a wide variety of tumor cells including breast cancer cells (9, 10), there is no correlative link between total receptor expression levels and the sensitivity of tumors to rhTRAIL. Recent studies have suggested that the mRNA expression of TRAIL receptors does not necessarily reflect their functional

Received 7/2/08; revised 8/8/08; accepted 8/28/08.

Grant support: Food and Drug Administration Critical Path Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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doi:10.1158/1541-7786.MCR-08-0313

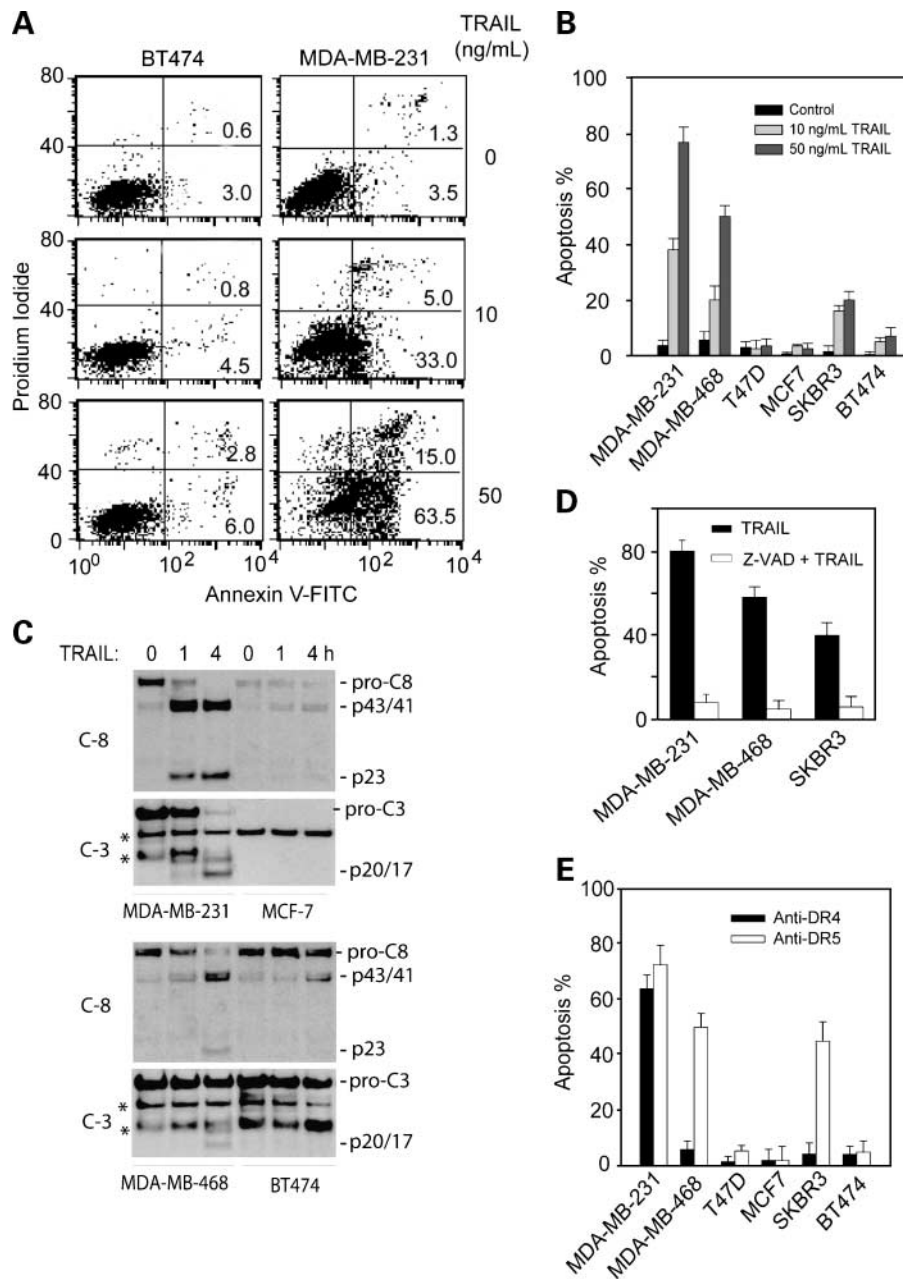


FIGURE 1. Apoptotic response of human breast cancer cell lines to rhTRAIL and the DR4 and DR5 antibodies. **A.** Fluorescence-activated cell sorting analysis of apoptosis after staining with FITC-Annexin V and propidium iodide. Cells at 70% to 80% confluence were treated with 10 or 50 ng/mL rhTRAIL for 6 h and then were harvested. Bottom right quadrant, percentage of early apoptotic cells with exposed phosphatidylserine (Annexin V-FITC positive) but intact membrane (propidium iodide negative). Top right quadrant, necrotic or apoptotic cells in terminal stages with positive staining of both Annexin V-FITC and propidium iodide. **B.** Quantification of TRAIL-induced apoptosis (as described for **A**) in the indicated cell lines. Mean \pm SD total percentage of the cells in the right quadrants ($n = 3$). **C.** Activation of caspase-8 and -3. Cells were treated with 10 ng/mL rhTRAIL for 1 or 4 h as indicated. Caspase activity is indicated by the decrease of pro-caspases (*pro-C8* and *pro-C3*) and appearance of the p23 and the p20/p17 cleaved forms of caspase-8 (*C-8*) and caspase-3 (*C-3*), respectively. MCF7 cells express relatively low levels of pro-caspase-8 and are deficient in caspase-3 protein. Representative of three independent experiments. *, nonspecific bands. **D.** Caspase inhibitor Z-VAD blocks TRAIL-induced apoptosis. Cells were pretreated without or with a general caspase inhibitor Z-VAD at 10 μ M and then incubated with 50 ng/mL rhTRAIL for an additional 6 h. The extent of apoptosis was measured as described in **A**. **E.** Apoptotic responses to DR4 and DR5 antibodies. Cells were treated with 10 μ g/mL anti-DR4 or anti-DR5 monoclonal antibodies for 24 h and apoptosis was analyzed as in **A**. Mean \pm SD ($n = 3$).

protein expression due to post-translational regulation. DR4, but not DR5, was shown to be *N*-glycosylated in prostate cancer cells (14). Both receptors undergo *O*-glycosylation in various tumor cell lines, with a higher level in TRAIL-sensitive

cells (13). They are also subject to regulation of endocytosis pathways (15-17). In line with these findings, the cell surface DR4 protein was down-regulated in some TRAIL-resistant cancer cells, including those of colon (18) and leukemia (19).

However, little is known about the functional status of TRAIL death receptors in breast cancer cells.

Breast cancer cells are generally resistant to TRAIL, but the mechanism is unresolved (9, 10). In the present study, we found that the two TRAIL death receptors, with a higher incidence in DR4, are functionally deficient in some breast cancer cell lines due to down-regulation of cell surface expression. DR4 deficiency, which may result from defects in its protein sorting machinery, is consistent with the cellular resistance to anti-DR4 antibody and a decreased sensitivity to rhTRAIL. The results highlight the importance of determining the functionality of death receptors in predicting TRAIL sensitivity of tumor cells. In addition, the data show that the ability of a combinational therapy in restoring cell surface expression of death receptors is crucial to overcome resistance to TRAIL-related therapies.

Results

TRAIL Requires Both DR4 and DR5 for Maximal Killing in Breast Cancer Cells

To investigate the mechanisms of TRAIL resistance in breast cancer cells, we first compared the induction of apoptosis by rhTRAIL and agonistic antibodies to DR4 and DR5 in a panel of six human breast cancer cell lines. As shown previously (9, 10), rhTRAIL was a potent inducer of apoptosis in MDA-MB-231 cells. At a concentration as low as 10 ng/mL, rhTRAIL induced substantial and rapid apoptosis, indicated by Annexin V binding (Fig. 1A and B), and time-dependent cleavage of caspase-8 and -3 (Fig. 1C). By contrast, other cell lines were either less sensitive (MDA-MB-468 and SKBR3) or resistant (T47D, MCF7, and BT474) to rhTRAIL-induced apoptosis. The resistance of BT474 and MCF7 cells was maintained even with higher doses of rhTRAIL (up to 1 µg/mL) or longer incubation (48 h; data not shown). In TRAIL-resistant cells, no activation of caspase-8 and -3 was detected (Fig. 1C; data not shown for T47D). MCF7 cells only express minimal caspase-8 protein and are deficient in caspase-3 expression. Consistently, a general caspase inhibitor Z-VAD completely blocked TRAIL-induced apoptosis in MDA-MB-231 and two other cell lines (Fig. 1D). These results indicate that caspase activation is an essential step in rhTRAIL-induced cell killing. TRAIL-resistant cells appeared to have defective stimulation of

caspase-8 processing and correspondingly less or no cleavage of caspase-3.

When treated with the agonist antibodies to DR4 and DR5, these cell lines showed remarkable difference in sensitivity (Fig. 1E). Anti-DR5 induced apoptosis in cell lines, except T47D, MCF7, and BT474 cells. However, anti-DR4 showed apoptosis-inducing activity only in MDA-MB-231 cells and was inactive on the other cell lines. The sensitivities appeared to correlate with those observed for rhTRAIL (Fig. 1B; Table 1). For example, the hypersensitivity of MDA-MB-231 cells correlates with its susceptibility to both anti-DR4 and anti-DR5. Loss of response to either antibody correlates well with a decreased sensitivity to rhTRAIL (MDA-MB-468 and SKBR3). The cell lines (BT474, T47D, and MCF7) that are nonresponsive to both anti-DR4 and anti-DR5 are completely resistant to rhTRAIL. These results indicate that both DR4 and DR5 are required for rhTRAIL to achieve maximal killing effect of breast cancer cells. In addition, the failure of anti-DR4 or anti-DR5 to induce apoptosis raises the possibility that the death receptor may be functionally deficient in those cells.

Deficiency in DR4 and DR5 Cell Surface Expression Correlates with Resistance to Respective Antibody and Reduced Sensitivity to TRAIL

To identify molecular determinants that control the rhTRAIL sensitivity in breast cancer cells, we first examined total protein expression of TRAIL receptors by Western blotting (Fig. 2A). Both DR4 and DR5 were expressed across the cell lines, showing no direct relationship with the observed patterns of sensitivity. For example, DR4 protein was expressed in all the cell lines, with a higher level in MDA-MB-231 cells. DR5 was detected as two bands corresponding in sizes to the two splicing variants (20), but its overall protein level is even higher in TRAIL-resistant cells (T47D and MCF7) compared with TRAIL-sensitive cells (MDA-MB-231 and MDA-MB-468). Likewise, DcR1 and DcR2 were comparable in these cell lines irrespective of their sensitivity to rhTRAIL, suggesting that the pattern of decoy receptor expression may not be responsible for the observed resistance.

Because only cell surface DR4 and DR5 are able to bind TRAIL and mediate apoptosis, we next examined the cell surface expression of TRAIL receptors by flow cytometry using phycoerythrin (PE)-conjugated antibodies. The presence of these receptors on the cell surface is indicated by a right shift of the peak after binding to a specific antibody relative to a control IgG-PE (Fig. 2B and C). DR4 was expressed on the surface of MDA-MB-231 and MCF7 cells but not those of MDA-MB-468, T47D, SKBR3, and BT474 cell lines. In contrast, DR5 was detected on the cell surface in most of the cell lines, except T47D and BT474. Only a slight shift in DR5 occurred in MDA-MB-468 and SKBR3 cells, which correlated with sensitivity to anti-DR5. The cell surface expression was confirmed by confocal microscopy analysis of endogenous proteins after immunostaining with FITC-conjugated anti-DR4 or anti-DR5 antibodies (not shown). As shown in Fig. 2D, DR4 protein was localized in the plasma membrane in MDA-MB-231 but was found predominantly in the cytosol of BT474 cells.

TRAIL-resistant cell lines (T47D, MCF7, and BT474) were no longer able to efficiently assemble a DISC after rhTRAIL treatment as shown by weak signals of the adaptor molecule

Table 1. Correlation between Cell Surface Expression of Death Receptors DR4 and DR5 and Sensitivity to rhTRAIL and Its Agonistic Antibodies in Breast Cancer Cell Lines

Cell lines	DR4 surface*	DR5 surface*	Relative sensitivity †		
			Anti-DR4	Anti-DR5	rhTRAIL
MDA-MB-231	✓	✓	+++++	+++++	+++++
MDA-MB-468	x	✓	—	+++	++++
T47D	x	x	—	—	—
BT549	x	✓	—	+++	+++
MCF7	✓	✓	—	—	—
SKBR3	x	✓	—	+++	+
BT474	x	x	—	—	—

*Presence (✓) or absence (x) on cell surface.

† Apoptosis relative to MDA-MB-231 cells after treatment with the indicated agent (+, sensitive; —, resistant; data from Fig. 1A and B and data not shown for BT549).

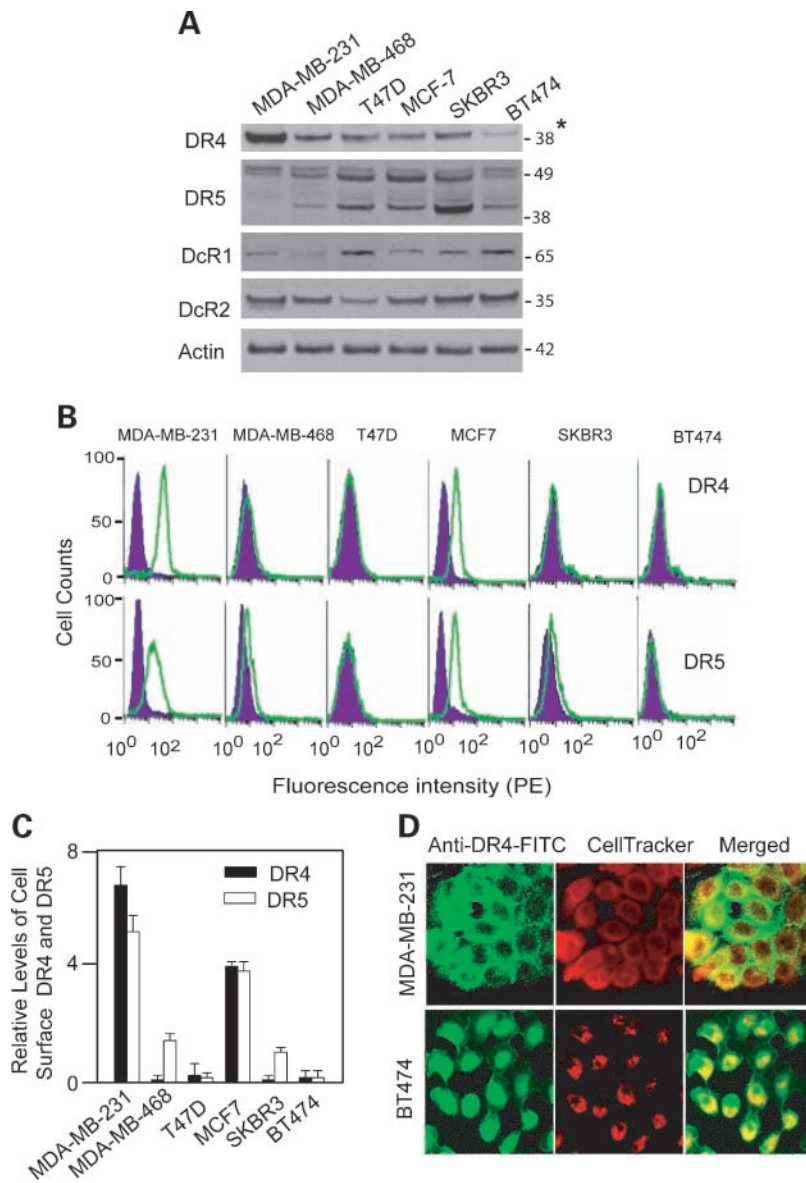


FIGURE 2. DR4 and DR5 cell surface expression is down-regulated in TRAIL-resistant cells. **A.** Total protein expression levels of TRAIL receptors. Equal amounts of whole-cell lysates from the indicated cell lines were analyzed by Western blotting using antibodies specific to DR4 and DR5 and the decoy receptors DcR1 and DcR2, respectively. Anti-DR4 antibody recognizes a peptide corresponding to the NH₂-terminal amino acids 1 to 20 of human DR4 mature protein (purchased from Imgenex). Actin was shown as a loading control. *, markers of molecular weight. **B.** Flow cytometry analysis of cell surface expression of DR4 and DR5. Cells were stained with PE-conjugated monoclonal anti-DR4 or anti-DR5 (open histograms). Shaded histograms, control cells stained with isotype-matched control IgG. A single experiment representative of six independent experiments with similar results. Right shift, presence of DR4 or DR5 on cell surface. **C.** Quantification of results in **B** reveals the relative levels of cell surface DR4 and DR5. Folds of increase in fluorescence intensity compared with the corresponding IgG-PE. **D.** Confocal microscope images showing the subcellular localization of endogenous DR4 protein. Cells were permeabilized with 0.2% Triton X-100 and labeled with anti-DR4-FITC (green); cytoplasm was stained with CellTracker Red (red). Representative of three independent experiments.

FADD and pro-caspase-8 in the immunocomplexes of biotinylated rhTRAIL (Fig. 3A). DR4 and DR5 proteins were also virtually undetectable in the coimmunoprecipitates from those cell lines, except for MCF7 cells. This was in contrast to MDA-MB-231 cells in which treatment with rhTRAIL recruited FADD into DISC and the processing of caspase-8. Taken together, the results suggest that TRAIL death receptors become functionally deficient in rhTRAIL-resistant cells as a result of down-regulation of cell surface expression. Importantly, loss of cell surface expression of the death receptors, with a higher incidence in DR4, appears to be sufficient to account for the relative sensitivity of some breast cancer cells to apoptotic induction by either rhTRAIL or its agonistic antibodies (Table 1).

We also characterized expression of the regulatory proteins that are involved in the death receptor-mediated apoptosis signaling pathway. The antiapoptotic molecules FLIP, Bcl-2, and Bcl-x have been implicated in the resistance of cancer cells

to apoptosis and are found to be differentially expressed in breast cancer cell lines (Fig. 3B). For instance, the long form of FLIP (FLIP_L) and its p43 cleaved product were more under-expressed in MDA-MB-231 cells than other lines. In addition, MCF7 cells were deficient in caspase-3 and expressed little pro-caspase-8 protein compared with other cell lines (Fig. 1C). These factors might contribute to the observed TRAIL sensitivity in individual cell lines, but none showed a consistent correlation. Likewise, the expression levels of proapoptotic proteins Bad, Bax, and Bid were not directly related to the observed sensitivities to rhTRAIL and the agnostic antibodies.

DR4 and DR5 Undergo Constitutively Endocytosis through Clathrin-Dependent Pathway in TRAIL-Resistant Cells

Like other transmembrane proteins (21), TRAIL receptors appear to subject to membrane transport and endocytic sorting

signals (15-18, 22, 23). The absence of DR4 or DR5 cell surface expression promoted us to identify defects within the translational modification processes. We first tested whether exogenous DR4 can be expressed on the plasma membrane in TRAIL-resistant cells. Cells were transfected with a plasmid expressing green fluorescent protein (GFP)-tagged wild-type DR4 (GFP-DR4-wt) and its cellular localization was detected by confocal microscopy. In MDA-MB-231 cells, DR4-GFP-wt was expressed on the cell surface but was found exclusively in the cytoplasm of BT474 cells (Fig. 4A). These data suggest that the sorting machinery of TRAIL death receptors is defective in TRAIL-resistant cells, resulting in failure of expression on plasma membrane.

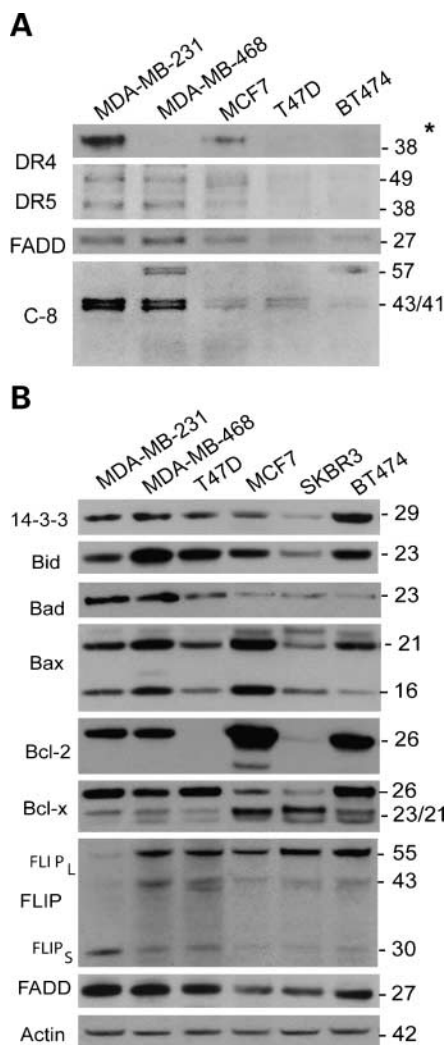


FIGURE 3. TRAIL DISC does not assemble in TRAIL-resistant cell lines. **A.** Cells were treated with biotinylated TRAIL (200 ng/mL) at 4°C for 30 min and then at 37°C for an additional 30 min. Equal amounts of cell lysates were incubated with streptavidin agarose beads overnight at 4°C, and the coimmunoprecipitates were analyzed by immunoblotting for the presence of DR4, DR5, FADD, and caspase-8. The full-length and processed forms of caspase-8 associated with the DISC formation are seen in MDA-MB-231 and MDA-MB-468 cells. **B.** Comparison of protein expression of relevant TRAIL signaling components (14-3-3, Bid, Bad, Bax, Bcl-2, Bcl-x, FLIP, and FADD) between TRAIL-sensitive and TRAIL-resistant cell lines. Representative blots of three separate experiments.

We then tested whether endocytosis inhibitors could affect TRAIL receptor expression. All three inhibitors tested had no effect on the total protein levels of the receptors (Fig. 4B, *top*). Strikingly, phenylarsine oxide (PAO), a general inhibitor of endocytosis (24, 25), induced a dose-dependent restoration of receptor cell surface expression in BT474 cells (Fig. 4B, *bottom*). At threshold, the levels of surface DR4 and DR5 were comparable with those of MDA-MB-231 cells (Fig. 2B). Treatment with chlorpromazine, an inhibitor of clathrin-mediated endocytosis (26, 27), also increased the cell surface receptor expression. However, no effect was observed with the raft/caveolae endocytosis inhibitor filipin (28, 29). Consistent with these data, sequential treatment with PAO or chlorpromazine, but not filipin, followed by TRAIL induced massive apoptosis in T47D (data not shown) and BT474 (Fig. 4D) cells. Pretreatment with PAO also increased cell surface expression of the epidermal growth factor receptor, which is undetectable on the surface of wild-type BT474 cells. In contrast, Her-2/erbB-2 cell surface expression was not affected (Fig. 4D). These results suggest that clathrin-mediated endocytosis pathways may be responsible for the deficiency in DR4 and DR5 cell surface expression in TRAIL-resistant cells.

We further examined the role of clathrin pathway in TRAIL receptor endocytosis by targeted gene silencing. As shown in Fig. 5A and B, knockdown of adaptor protein 2 (AP2) or clathrin significantly increased DR4 and DR5 cell surface expression without changing their total protein levels in BT474 cells. The resultant cells became highly susceptible to TRAIL-induced apoptosis (Fig. 5A, *bottom*). Similar to endocytosis inhibitor (Fig. 4D), disruption of AP2 or clathrin did not alter the surface expression of Her-2/erbB-2 or transferrin receptor but remarkably increased epidermal growth factor receptor surface levels (Fig. 5C). Taken together, these results suggest that DR4 and DR5 may undergo constitutive endocytosis through clathrin-dependent pathways in TRAIL-resistant cells and experimental inhibition of endocytosis restores DR4- and DR5-mediated signaling in response to TRAIL. The results also suggest that different receptors are regulated by distinct endocytosis pathways.

DR4 Endocytosis Is Mediated by Its Dileucine Sorting Signal

Sequence analysis of DR4 protein reveals two potential sorting signals within the cytoplasmic domain: EAQC³³⁷LL and ⁴⁰⁹YAML. These signals are present in many other receptors and have been implicated in mediating the rapid internalization of proteins and targeting to the endosomal-lysosomal compartments (21). To test whether these signals are responsible for DR4 sorting, we generated two DR4 mutants, LL³³⁷⁻³³⁸AA and Y⁴⁰⁹A, using site-directed mutagenesis. When overexpressed in BT474 cells, the DR4(Y⁴⁰⁹A) mutant, like the wild-type DR4 protein, was found in the cytoplasm (Fig. 5D, *top*) and remained resistant to rhTRAIL and anti-DR4 antibody (data not shown). However, the DR4(LL³³⁷⁻³³⁸AA) mutant showed expression almost exclusively on the cell surface. The resulting cells underwent considerable apoptosis when treated with rhTRAIL or anti-DR4 (Fig. 5D, *bottom*). Interestingly, restoration of DR4 cell surface expression did not alter resistance of BT474 cells to anti-DR5 antibody. These results show that the dileucine-based sorting signal (EAQC³³⁷LL) is

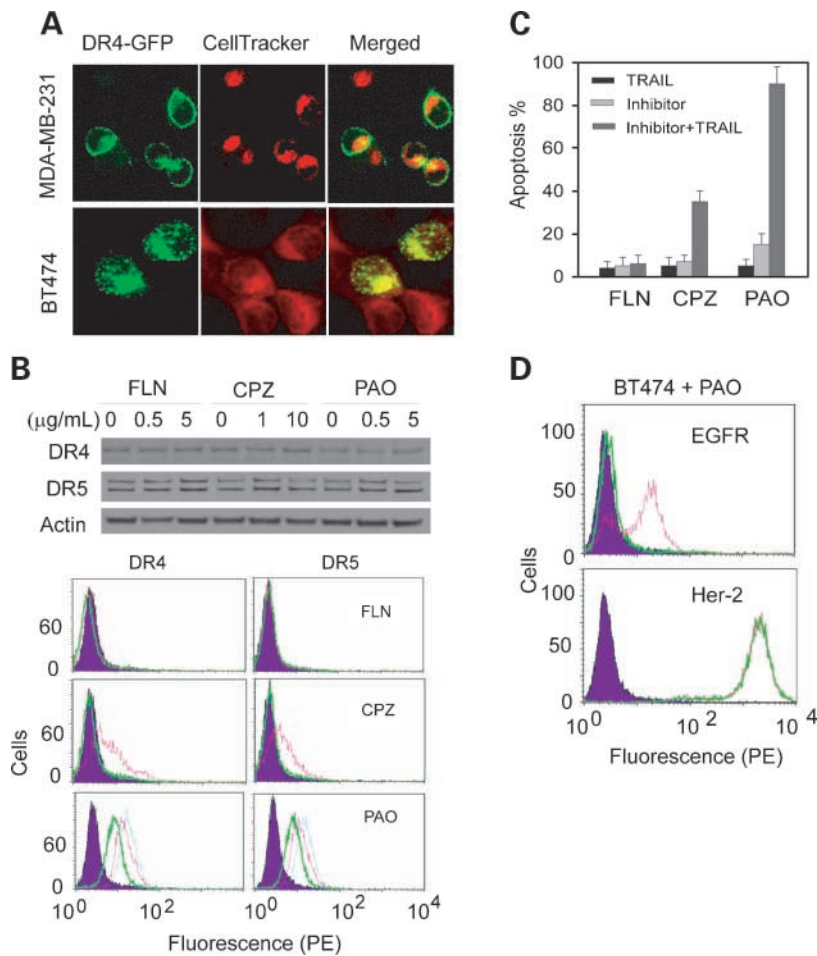


FIGURE 4. Inhibitors of endocytosis restore DR4 and DR5 cell surface expression and sensitize TRAIL-resistant cells to TRAIL-induced apoptosis. **A.** Wild-type DR4 ectopically expressed in BT474 cells fail to be translocated to the cell surface. MDA-MB-231 and BT474 cells were transfected with a plasmid expressing wild-type DR4 tagged with GFP (DR4-GFP), counterstained with CellTracker Red, and analyzed by confocal microscopy. **B.** BT474 cells were treated for 20 h with filipin III (FLN; an inhibitor of raft/caveolae endocytosis; left to right, 0, 0.5, and 5 $\mu\text{g}/\text{mL}$), chlorpromazine (CPZ; an inhibitor of clathrin-mediated endocytosis; left to right, 0, 1, and 5 $\mu\text{g}/\text{mL}$), or PAO (a general inhibitor of endocytosis; left to right, 0, 0.1, 0.5, and 5 $\mu\text{g}/\text{mL}$). The protein expressions of DR4 and DR5 were analyzed by Western blotting (top) and flow cytometry using PE-conjugated antibodies (bottom). **C.** Cells were sequentially treated with the indicated inhibitors (5 $\mu\text{g}/\text{mL}$) for 20 h and rhTRAIL (20 ng/mL) for an additional 6 h. Apoptosis was measured as in Fig. 1A. **D.** Effects of PAO on epidermal growth factor receptor and Her-2/erbB-2 cell surface expression. Cells were treated with 5 $\mu\text{g}/\text{mL}$ PAO for 20 h and analyzed for surface expression of the indicated receptors by fluorescence-activated cell sorting using PE-conjugated antibodies.

a key structural determinant in DR4 for its sorting through the clathrin-mediated endocytosis machinery.

DR4 Mutations in Breast Cancer Cells

The observation on DR4 sorting signal promoted us to examine whether TRAIL resistance resulted from DR4 gene mutations. To this end, reverse transcription-PCR (RT-PCR) analyses were done using total RNA isolated from the individual cell lines and using primers specific to the different regions of DR4 mRNA. All cell lines showed a single amplification product for each portion of the gene (Fig. 6). DNA sequence analysis confirmed the absence of deletion or splicing variants of DR4 gene. No mutations were found in the sorting signal regions of DR4. Instead, several other mutations were identified, including A422G (His¹⁴¹Arg), G626C (Arg²⁰⁹Thr), A683C (Glu²²⁸Ala), and C853A (Leu²⁸⁵Ile), in different cell lines (Table 2). However, there is no apparent correlation between the DR4 mutational profiles and the observed cell surface expression of DR4 and DR5 and TRAIL sensitivities. These data suggest that genetic mutations in DR4 may only play a small role, if any, in TRAIL resistance of breast cancer cells.

Discussion

Resistance of tumor cells to death receptor-induced apoptosis is a limiting factor for the clinical use of rhTRAIL

and its agonistic antibodies. Predicting whether a tumor will respond to these agents is still an empirical process because no biomarker for tumor susceptibility has been identified. In this study, we show that TRAIL death receptors, particularly DR4, are functionally deficient in several breast cancer cell lines due to down-regulation of cell surface expression. Regardless of total DR4 and DR5 protein levels, their absence on cell surface is sufficient to account for the failure in forming TRAIL DISC and later events of apoptosis. Thus, loss of cell surface expression of TRAIL death receptors may be evaluated as a potential predictive biomarker for TRAIL sensitivity in breast cancer cells.

Several different mechanisms have been identified to block TRAIL-induced apoptosis in individual cancer cell lines, including the simultaneous expression of decoy receptors (30), overexpression of antiapoptotic molecules (e.g., FLIP, Bcl-2, and Bcl-X_L; refs. 12, 31), and loss of caspase-8 expression (32). However, none of these factors showed a consistent correlation with TRAIL resistance in multiple cancers. A recent study showed that mRNA expression of the peptidyl *O*-glycosyltransferase GALNT14, which is responsible for the *O*-glycosylation of death receptors, correlated with TRAIL sensitivity in pancreatic carcinoma, non-small cell lung carcinoma, and melanoma cell lines. However, GALNT14 mRNA was only detected in a small portion (~10%) of breast cancers (13).

As death receptors, DR4 and DR5 must be properly expressed on cell surface to transmit an apoptotic signal from their ligands. DR4 protein is absent on the surface in most (4 of 6) of the breast cancer cell lines studied despite its comparable levels of total mRNA (Supplementary Data) and protein. Importantly, DR4 deficiency correlated well with the cellular resistance to anti-DR4 antibody and a decreased sensitivity to rhTRAIL. As with our results, down-regulation of DR4 cell surface expression was associated with TRAIL

resistance in leukemia (19) and colon cancer cells (18). The cell lines (BT474 and T47D) lacking both DR4 and DR5 cell surface expression are completely resistant to rhTRAIL and the corresponding antibody. However, the cell surface expression of DR4 and DR5 does not necessarily reflect the cellular sensitivity to TRAIL-induced apoptosis. This is the case for MCF7 cells, which express both DR4 and DR5 on cell surface but are resistant to rhTRAIL and antibodies. This may be attributed to the defects downstream of TRAIL death receptors,

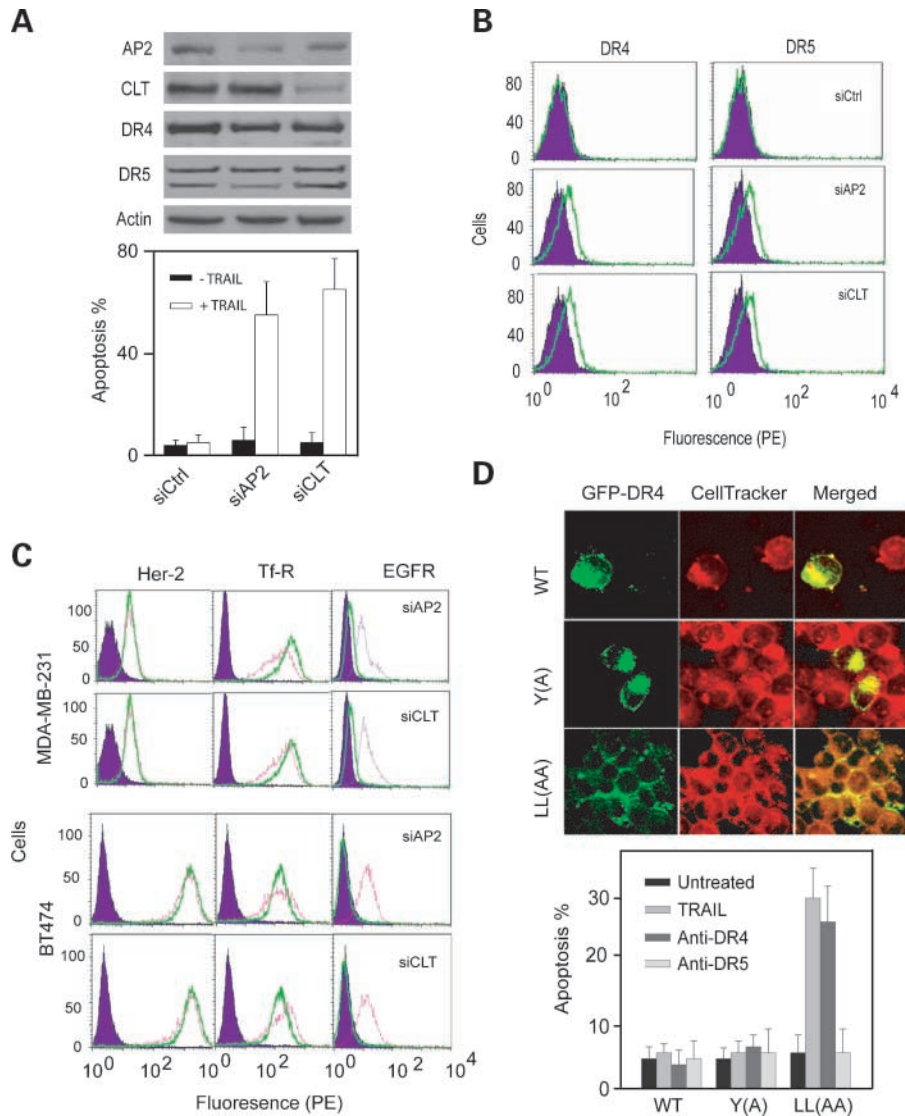


FIGURE 5. Clathrin-dependent, constitutive endocytosis of DR4 and DR5 in TRAIL-resistant cells. **A.** Knockdown of AP2 or clathrin sensitizes BT474 cells to TRAIL-induced apoptosis. Cells were transfected with a RNA interference-negative control duplex (*siCtrl*) or siRNA against AP2 (*siAP2*) and clathrin heavy chain (*siCLT*). Top, Western blots of the indicated proteins after 48 h post-transfection. As determined by densitometry, AP2 and clathrin proteins were reduced by 60% and 80%, respectively, in cells transfected with the corresponding siRNA. The levels of DR4 and DR5 proteins were not affected. Representatives of three independent transfections with siRNA sequence 5'-CCUGGGCCGCAUGUAUCUCUUAU-3' (AP2) or 5'-CCGAAAUUUGAUGUCAAUACUUA-3' (clathrin). Similar results were obtained with other two siRNA sequences (see Materials and Methods). Bottom, apoptosis of cells after treatments with 20 ng/mL rhTRAIL for an additional 6 h. **B** and **C.** Effects of AP2 or clathrin knockdown on cell surface expression of the indicated receptors. Transfection of siRNA was as in **A**. Fluorescence-activated cell sorting analyses were done using PE-conjugated antibodies specific to DR4, DR5, Her-2/erbB-2, transferrin receptor, or epidermal growth factor receptor. **D.** DR4 endocytosis is mediated by its cytoplasmic domain EAQC³³⁷LL. Top, mutation in the sorting sequence of DR4 restores its cell surface expression in BT474 cells. BT474 cells were transiently transfected with plasmids for GFP fusion proteins of wild-type DR4 or mutants containing the ⁴⁰⁹Y(A) or ³³⁷LL(AA) mutations. Representative of three independent experiments. Bottom, expression of DR4-³³⁷LL(AA) but not DR4-⁴⁰⁹Y(A) restores BT474 cell sensitivity to apoptotic induction by rhTRAIL or anti-DR4. Mean \pm SD ($n = 3$). After transfection, EGFP-positive cells were isolated by fluorescence-activated cell sorting and treated with TRAIL (10 ng/mL) or the indicated antibodies (10 μ g/mL) for 24 h.

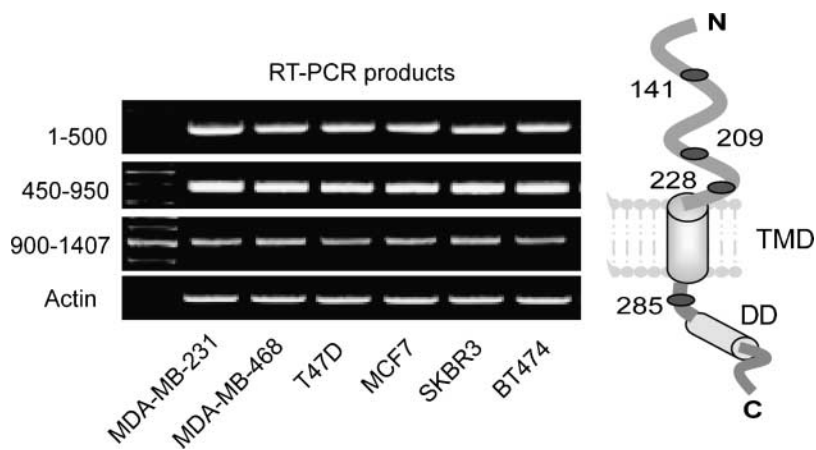


FIGURE 6. DR4 gene expression and mutations. Semiquantitative RT-PCR analyses were done using equal amount of total RNA (0.4 μ g) from the indicated cell lines using primers for the different regions of DR4 mRNA (1-1407 bp). Amplified products corresponding to regions 1 to 500, 450 to 950, and 900 to 1,407 bp of the DR4 gene. Right, schematic view of the locations of the mutated residues in DR4 protein (1-468 amino acids). TMD, transplasma membrane domain (240-268 amino acids). DD, death domain (379-445 amino acids). DR4 mutations are summarized in Table 2.

including lack of caspase-3 protein expression (Fig. 1B) due to gene depletion (33), lower caspase-8 protein, and higher levels of Bcl-2 relative to the proapoptotic Bad protein (Fig. 3B). The cellular FLIP, in its long form (FLIP_L) and p43 cleaved form, is remarkably underexpressed in MDA-MB-231 cells compared with other cell lines, which presumably facilitate the activation of caspase-8 and execution of apoptosis by TRAIL. Nonetheless, loss of cell surface expression of death receptors appears to be sufficient to avoid apoptosis by their ligands regardless of the defects in the intracellular signaling components. Our results warrant additional studies on the functionality of TRAIL receptors in other cancer cell lines and patient specimens. If it is confirmed in clinic, the absence of cell surface expression of DR4 and/or DR5 could be a useful biomarker for TRAIL sensitivity. Furthermore, the results suggest that certain tumors may use DR4 or DR5 deficiency as a mechanism of evading death receptor-mediated immune surveillance (34).

TRAIL appears to require both DR4 and DR5 for a maximal killing in breast cancer cells (Fig. 1; Table 1). The two death receptors may act synergistically by forming heteroreceptor complexes (35). This observation is in contrast to previous studies showing that DR5 is the primary receptor leading to apoptosis in various types of cancer cells (36-39). Based on these previous results, DR5 has been the primary target for development of antibody therapy. There are currently five anti-DR5 antibody products, compared with one anti-DR4 product, in clinical trials. However, there is also evidence that TRAIL induces apoptosis exclusively through DR4 in cancer cell lines from skin (40), ovary (41), and leucocytes (19) as well as primary cells from chronic lymphocytic leukemia and mantle

cell lymphoma (42). The molecular basis for this preference of TRAIL is not clear but may be due to difference in the functional status of death receptors in a specific tumor. We propose that TRAIL may have to use one receptor, whereas the other is defective. Our results suggest that DR4 may be deficient at a higher frequency than DR5 in breast cancer cells.

The molecular mechanism for TRAIL death receptor deficiency remains unclear, but it may involve defects in the protein sorting machinery. Recent studies suggest that protein transport and endocytosis pathways may play an important role in the regulation of death receptor expression (15-18, 22, 23). On ligation, TRAIL induces internalization/endocytosis of its receptors through clathrin-dependent pathways (15, 43). This process is accompanied by simultaneously activation of caspases, which in turn cleave clathrin pathway components such as AP2 or clathrin itself in cells from BJAB and colon carcinoma (15). AP2 cleavage was also observed in MDA-MB-231 but not BT474 cells (Supplementary Data). Austin et al. (15) suggested that caspase-mediated cleavage of clathrin or AP2 may provide a positive feedback loop, thereby halting the endocytic machinery and reinforcing caspase activation and apoptosis execution through TRAIL receptors. In agreement, blockade of receptor internalization rather amplified the apoptotic signaling of TRAIL (43). Moreover, Jin et al. (18) showed that a prolonged TRAIL treatment of colon cancer cells resulted in down-regulation of cell surface DR4 and TRAIL resistance. Consistent with these reports, TRAIL failed to induce apoptosis in breast cancer cell lines (T47D and BT474) lacking surface expression of DR4 and DR5 (Fig. 2; Table 1). Restoration of surface expression of TRAIL receptors, which was achieved by either endocytosis inhibitors or knockdown of clathrin or AP2, restored cellular sensitivity to TRAIL-induced apoptosis (Figs. 4 and 5). In contrast, Her-2/erbB-2 and transferrin receptor were not affected by disruption of clathrin pathway. These results suggest that DR4 and DR5 may undergo a constitutive endocytosis through clathrin-dependent pathways, resulting in failure of cell surface expression and resistance to TRAIL-induced apoptosis. However, the total protein levels of clathrin or AP2 was not directly associated with the cell surface DR4 and DR5 in the cell lines studied (Supplementary Data), suggesting that other clathrin pathway components may also contribute to the receptor deficiency. Mutational analysis reveals

Table 2. DR4 Mutations in Breast Cancer Cell Lines

Mutation	Cell lines
A422G (His ¹⁴¹ Arg)	MDA-MB231 MCF7 SKBR3
G626C (Arg ²⁰⁹ Thr)	MDA-MB-231 MCF7 SKBR3
A683C (Glu ²²⁸ Ala)	BT474
C853A (Leu ²⁸⁵ Ile)	T47D

that the dileucine-based sorting signal (EAQC³³⁷LL) is a key structural determinant in DR4 for its endocytosis (Fig. 5D). However, no mutations were found in the sorting signal regions of DR4 gene in the cell lines studied (Table 2). Additional studies are required to identify molecular alterations in both endocytosis and transport pathways that are responsible for the deficiency of TRAIL death receptors. The acquired information may contribute to the selection of appropriate patient population for treatment and design of strategies to overcome tumor resistance to TRAIL-related therapies.

In summary, our results show that breast cancer cells may use cell surface down-regulation of DR4 and/or DR5 as a mechanism to escape apoptosis through TRAIL receptors. These data may help to predict tumor resistance likely to be found in patient samples and provide a rational approach to improve TRAIL receptor-targeted therapies.

Materials and Methods

Cell Lines

Human breast cancer cell lines including MDA-MB-231, MDA-MB-468, T47D, MCF7, SKBR3, and BT474 were obtained from the American Type Culture Collection. MDA-MB-231 cells were grown in DMEM/F-12 (1:1 mix; Mediatech) supplemented with 5% fetal bovine serum, 4 mmol/L glutamine, 50 μ mol/L β -mercaptoethanol, and 1 mmol/L sodium pyruvate at 37°C and 5% CO₂ in air. Other cell lines were cultured as recommended by the American Type Culture Collection.

Antibodies and Reagents

rhTRAIL, expressed and purified as homotrimeric protein from *Escherichia coli*, and monoclonal antibodies specific to the extracellular domains of human death receptors DR4 and DR5 and their PE-conjugated forms, decoy receptors DcR1 and DcR2, and PE-conjugated antibodies to transferrin receptor, epidermal growth factor receptor, and Her-2/erbB-2 were purchased from R&D Systems. Anti-DR4 conjugated with Alexa Fluor 488 was from AbD Serotec. Rabbit polyclonal antibody against a peptide corresponding to amino acids 1 to 20 of human DR4 mature protein was from Imgenex. Antibodies against human caspase-3 and -8 were from Calbiochem. Antibodies to FADD, Bcl-2, Bcl-x, Bid, Bad, Bax, and 14-3-3 were from BD Pharmingen. Anti-FLIP was from Cell Signaling Technology, and anti-actin was from Santa Cruz Biotechnology. Stealth RNA interference-negative control duplex and the validated siRNA duplexes targeting different regions of AP2 (5'-CCUGGCGCGCAUGUAUCUCUUAU-3', 5'-AUAGAAGAGAUACAUGCGGCCAGG-3', and 5'-CCGGAAGCCGAUGAGUUGCUGAAUA-3') and clathrin heavy chain (5'-CCGAAAUUUGAUGUCAUACUUA-3', 5'-UGAAGUAUUGACAUCAAAUUUCCGG-3', and 5'-AAAUUCUUAACUCUGCAAGGCGG-3') were purchased from Invitrogen. Inhibitors of endocytosis, PAO and chlorpromazine, were from Calbiochem; filipin III was from Cayman Chemical.

Apoptosis Assays

Cells were grown on 6-well plates to 70% to 80% confluence and treated with rhTRAIL or anti-DR4 or

anti-DR5 antibodies at the indicated concentrations. As indicated, cells were pretreated with the endocytosis inhibitors. At the selected time points, cells were collected and analyzed by flow cytometry after staining with Annexin V-FITC (BD Pharmingen) and propidium iodide (44).

Flow Cytometric Analysis of Cell Surface Expression of Death Receptors

Fluorescence-activated cell sorting analysis of cell surface expression of DR4 and DR5 was done using the PE-conjugated antibodies (R&D Systems) as per the manufacturer's recommendation. In brief, cells at 70% to 80% confluence were harvested by incubation with EDTA-trypsin and washed twice in PBS. Cells (1×10^5) were incubated in 25 μ L PBS containing 1% goat serum for 15 min at room temperature. Afterwards, cells were incubated with 10 μ g/mL anti-DR4-PE or anti-DR5-PE (mouse IgG1-PE and IgG2b-PE as respective control) for 45 min at 4°C in the dark. Cells were then washed twice with PBS and resuspended in 0.5 mL PBS for final analysis.

Plasmids and Transfection

The DR4 cDNA was amplified by RT-PCR using total RNA from MDA-MB-231 cells and primers specific to human DR4 gene and subcloned into a pEGFP-N1 vector (BD Bioscience) for expression of DR4-GFP protein (EGFP fused to the intracellular COOH terminus of DR4). Mutations were introduced using the Quick Change Site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. All constructs were verified by DNA sequencing. Plasmids were transfected using FuGENE reagent (Roche Diagnostics). The reverse transfections of siRNA duplex (either single siRNA sequence or a mixture of the three duplexes) were done using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence and Microscopy

Cells were cultured on glass chamber slides at 70% to 80% confluency and stained with CellTracker Red before harvest. Cells were fixed in 3% paraformaldehyde for 30 min in PBS (pH 7.4). To stain the endogenous DR4, cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min. After incubation in a blocking buffer containing 10% goat serum for 20 min, cells were incubated with FITC-conjugated anti-DR4 for 1 h and washed three times with the blocking buffer. Finally, the slides were detached from the medium chamber and mounted with Antifade reagent (Invitrogen). DR4-GFP-transfected cells were directly visualized after CellTracker Red staining. Confocal microscopy acquisitions were done on a Zeiss LAM 5 PASCAL confocal laser scanning microscope. All images are representative of three to five independent experiments.

Reverse Transcription-PCR

Total RNA was isolated from different cells using Trizol reagent (Invitrogen). RT-PCR was carried out using High-Fidelity One-Step RT-PCR kit (Invitrogen) as per the manufacturer's instructions. Primer pairs used for detection

different regions of human DR4 are as follows: 1 to 500 bp (5'-ATGGGCGCCACCACCAGCTA and 5'-CATGGGAGG-CAAGCAAACA), 450 to 950 bp (a primer pair from R&D Systems), and 900 to 1,407 bp (5'-GAGCAACGCA-GACTCGCT and 5'-TCACTCCAAGGACACGCGC). β -Actin was used as an internal control. The RT-PCR products were resolved on 1% agarose gel and purified using a QIAquick Gel Extraction kit (Qiagen). DNA sequencings were done with Applied Biosystems 3100 automated sequencer (Food and Drug Administration Core Facility).

TRAIL DISC Formation

The assays were done by using biotinylated TRAIL in combination with streptavidin beads (Pierce) as described previously (16). In brief, rhTRAIL was labeled with EZ-Link Sulfo-NHS-LC-Biotin according to the manufacturer's instructions (Pierce). Cells were precooled on ice, incubated with biotinylated TRAIL (200 ng/mL) for 30 min on ice and then 30 min at 37°C, washed extensively, and lysed in a lysis buffer (Pierce) supplemented with protease inhibitors (Calbiochem). Protein concentration was determined by a BCA protein assay (Pierce). Equal amounts of extracts were incubated with streptavidin-Sepharose beads overnight at 4°C and the associated proteins were detected by immunoblotting.

Western Blotting

Western blot analyses were done as described previously (45). In brief, cells (1×10^6) 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 μ g/lane) were resolved by electrophoresis using a 4% to 12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membranes (Millipore) for immunoblot analysis with an appropriate dilution of antibodies (1:1,000-1:2,000). When necessary, the membranes were stripped by Restore Western Blot Stripping Buffer (Pierce) and reprobed with appropriate antibodies. Immunocomplexes were visualized by chemiluminescence using ECL (Santa Cruz).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Emily Shacter for critical reading of the article and Dr. Tatsushi Yoshida for useful discussion and comments.

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