Tumor-derived Mutations in the TRAIL Receptor DR5 Inhibit TRAIL Signaling through the DR4 Receptor by Competing for Ligand Binding*

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TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a cytokine that preferentially induces apoptosis in tumor cells compared with normal cells through two receptors (DR4 and DR5). Somatic mutations in these receptors have been found in different kinds of cancer; however, it is poorly understood how the mutations affect signaling. We found that point mutations (L334F, E326K, E338K, and K386N) that were identified in human tumors result in the DR5 receptor losing its ability to form a functional death-inducing signaling complex and induce apoptosis. The mutant receptors also have a “dominant negative” effect whereby they inhibit the ability of TRAIL to induce apoptosis through functional DR4 receptors. This dominant negative mechanism is achieved through competition for TRAIL binding as shown by experiments where the ability of the mutant DR5 receptor to bind with the ligand was abolished, thus restoring TRAIL signaling through DR4. The inhibitory effect on signaling through the wild-type DR4 protein can be overcome if the inhibitory mechanism is bypassed by using a DR4-agonistic antibody that is not subject to this competition. This study provides a molecular basis for the use of specific therapeutic agonists of TRAIL receptors in people whose tumors harbor somatic DR5 mutations.

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2 The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DISC, death-inducing signaling complex; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; IP, immunoprecipitation; PLAD, pre-ligand assembly domain.

TRAIL2 (tumor necrosis factor-related apoptosis-inducing ligand) (1, 2) and agonistic antibodies that recognize TRAIL receptors (3, 4) preferentially kill tumor cells and produce potent anti-tumor activity in a variety of experimental models. It is therefore hoped that these agents may be useful to treat cancer (5–9), and several clinical trials are ongoing with different TRAIL receptor agonists alone or in combination with other anti-cancer drugs. TRAIL induces apoptosis through two death domain-containing receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). Upon receptor activation, DR4 and DR5 recruit FADD (10–12) and caspase-8 to form the death-inducing signaling complex (DISC), which activates caspase-8, subsequently leading to the activation of executioner caspases such as caspase-3 that induce apoptosis (8, 13). In addition, other signaling pathways leading to NFκB activation and activation of the ERK, JNK, and p38 MAP kinase pathways are generated by a second complex that forms after the receptor-containing DISC (14). DR4 and DR5 form homotrimers and heterotrimers (10), although the exact role of a DR4–DR5 receptor complex is unclear. TRAIL also binds to three other receptors, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin. DcR1 is a glycoporphatidylinositol-anchored membrane protein without a death domain, and DcR2 contains a truncated death domain; DcR1 and DcR2 are unable to recruit FADD or signal apoptosis and inhibit apoptosis by sequestering TRAIL or by forming complexes with DR4 and DR5 to create heteromeric receptor complexes that are unable to activate signaling (15, 16). Osteoprotegerin is a soluble receptor that also binds receptor activator of nuclear factor κ B ligand and may have a more prominent role in bone and myeloid cell development than in regulating TRAIL-induced apoptosis.

DR4 and DR5 map to human chromosome 8p21–22, a site of frequent allelic loss in tumors. This led to the suggestion that as potential tumor suppressors TRAIL receptors may also harbor somatic mutations in human tumors. Mutations in DR4 and DR5 have been identified in various human tumors (17–21). Most mutations identified so far are in DR5 and affect the intracellular domain of the receptor, i.e. the region that binds FADD. For example, in a study of non small cell lung cancer, ~10% of tumors had mutations in the death domain of DR5 (17) and similar mutations were found in 20% of breast cancers from patients with lymph node involvement (20). Despite the small number of patients studied so far and uncertainties about how common these mutations are in different populations of cancer patients, some mutations have been found repeatedly in different tumor types and in different patients with the same disease, suggesting that TRAIL receptor mutations are selected during tumorigenesis and, therefore, may have important functional effects in tumor cells. A mutagenesis study from El-Deiry and...
Dominant Negative Mechanism of Mutant TRAIL Receptors

coworkers (22) showed that some of the tumor mutations in DR5 produced a receptor that was compromised in its ability to induce apoptosis when exogenously expressed. This study also suggested that the mutant receptors were unable to properly bind FADD; however, these experiments were performed in cells with non-physiological levels of the mutant receptor alongside the endogenous wild-type DR5 protein. It was also unclear whether the mutant DR5 receptor had any effect on signaling by DR4.

Here, we studied tumor-derived somatic mutations in DR5 by expressing them at physiological levels in DR5-deficient cells and found that these receptors lost their ability to recruit FADD and caspase-8 or to induce apoptosis. Surprisingly, we found that TRAIL-induced apoptosis was blocked in the DR5 mutant cells even in the presence of wild-type DR4. The mechanism of this “dominant negative” effect is that mutant DR5 receptors prevent TRAIL from binding to DR4. Importantly, this effect can be overcome by directly activating the wild-type DR4 receptors with an agonistic antibody instead of the ligand. This provides a rationale for choosing among TRAIL receptor therapeutic agonists to maximize tumor cell killing even in cells where somatic mutations in one of the receptors create a situation where cells are unable to respond to the physiological ligand.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies and reagents were purchased from the following sources: Caspase-8 and FADD antibodies, Cell Signaling, Beverly, MA; anti-green fluorescent protein, Chemicon, Temecula, CA; mapatumumab (previously known as HGS-ETR1) and lexatumumab (previously known as HGS-ETR2), Human Genome Sciences. Untagged and His-tagged recombinant DR5 mutants were also inserted in-frame with mCherry into the pcDNA3.1-Puro(−) expression vector, which was used for further experiments. All the cell lines expressed equivalent expression levels on the cell surface (Fig. 1A).

Cells and Culture Conditions—BJAB cells were maintained in RPMI 1640 plus 10% fetal bovine serum. DR5 wild-type and mutant cell lines were generated by electroporating mammalian constructs into the cells and selecting for stable transformants as previously described (12). To assess expression levels of the receptors, cells were prepared using the manufacturer’s protocol, stained with antibodies against DR5 and DR4 (R&D Systems), and analyzed using a FACS Calibur flow cytometer.

DISC IP—1 × 10^7 cells were suspended in 10 ml of culture medium, incubated with lexatumumab at 1 μg/ml in 4 °C for 30 min, transferred to 37 °C for another 1 h, washed in phosphate-buffered saline three times, and then lysed in IP buffer (150 mM NaCl, 20 mM Tris+Cl, pH 7.5/1% Triton X-100) supplemented with complete protease inhibitors (Roche Applied Science). After the lysates were centrifuged (15 min at 13,000 rpm), antibodies were precipitated at 4 °C overnight. The beads were washed three times with IP buffer supplemented with 0.5 mM NaCl, and samples were subjected to Western blotting analysis.

Cytotoxicity Assay—Cells were seeded in 96-well plates at a density of 1.0 × 10^4 cells/ml in growth medium. Agonistic DR5 or DR4 antibody was cross-linked with an equal amount of anti-human IgG Fc for 30 min before serial dilution. TRAIL was prepared according to the manufacturer’s instructions before addition to the cells. After 24 h, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent (Promega, Madison, WI) was added to each well and incubated for an additional 2 h. Percent survival was calculated compared with control wells containing no agonists.

Subcellular Fractionation—1.0 × 10^7 cells were pelleted at 1,200 × g for 10 min at room temperature and washed with cold phosphate-buffered saline three times before resuspension in 500 μl of ice-cold hypotonic buffer (10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM MgCl_2, 10 μg/ml aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride) and incubation on ice for 20 min. Cells were homogenized by passing through a 30-gauge needle and centrifuged at 200 × g for 10 min to remove nuclei and cell debris. The supernatant was spun at 13,000 × g for 60 min at 4 °C, and this supernatant was collected as the cytosolic fraction. The pellet was washed in ice-cold hypotonic buffer containing 1% Triton X-100, incubated on ice for 30 min, and then centrifuged at 13,000 × g for 60 min at 4 °C; the supernatant represented the membrane fraction.

RESULTS

To investigate the tumor-derived somatic mutations under normal signaling conditions without overexpression, mammalian expression constructs of wild-type DR5 and the point mutations E326K, L334F, E338K, and K386N were transfected into the previously described (12) DR5-deficient BJAB cells (BJAB^DR5,DEF). After selection with puromycin, we used an anti-DR5 antibody to measure cell surface expression of wild-type and mutant DR5 by flow cytometry. Cells stably expressing wild-type or mutant DR5 at approximately physiological expression levels on the cell surface (Fig. 1A) were sorted and used for further experiments. All the cell lines expressed equivalent amounts of the wild-type endogenous DR4 molecule; these lines therefore represent an isogenic set of cells that all express TRAIL receptors at physiologically relevant levels but differ in the particular tumor-derived point mutation in DR5 that is expressed. To test whether the DR5 mutants were functional we used a DR5-specific agonistic antibody that is in clinical development (lexatumumab). Cells were treated with increasing amounts of lexatumumab, and cytotoxicity was measured after 24 h. As expected based on our previous work (12), BJAB^DR5,DEF cells were completely resistant to lexatumumab whereas cells expressing wild-type DR5 died in a dose-dependent manner (Fig. 1B). Cells expressing DR5 molecules with each of the mutations were also resistant to lexatumumab-induced death, indicating that these point mutations abolish the ability of DR5 to induce apoptosis. We hypothesized that DR5 mutants could not induce apoptosis because they fail to assemble the DISC. To test this hypothesis, we performed a DISC IP experiment using lexatumumab. FADD and caspase-8 were detected in wild-type DR5 receptor complex, while the four mutants that were resistant to lexatumumab-induced cell
death were unable to recruit FADD and caspase-8 to the receptor complex (Fig. 1C).

To test whether the other proteins required for death receptor-induced apoptosis were intact and functional, we tested whether DR5 mutant cells responded to Fas ligand and TRAIL. The DR5-deficient cells, all the DR5 mutant cells, and the cells expressing wild-type DR5 were equally sensitive to Fas ligand-induced cell death (Fig. 2, left), indicating that FADD, caspase-8, and other downstream signaling proteins involved in the strongest inhibitory effect on TRAIL signaling through DR4 (Fig. 2B) and has been identified in different kinds of cancer and in different patients with the same kind of cancer (19, 20), suggesting that it has a functional effect on tumor cell behavior that is selected for during tumor development and progression.

To test whether ligand competition is important for the dominant negative effect, we made mutant receptors that cannot bind TRAIL. If the dominant negative mechanism is due to ligand competition, then a DR5 molecule that contains the...
Dominant Negative Mechanism of Mutant TRAIL Receptors

![Diagram](image)

**FIGURE 3.** Disruption of ligand binding ability of DR5 mutant can restore cells responding to TRAIL triggering death signal. A, DR5E338K-Cherry, DR5E338KM152-Cherry, and DR5E338KΔPLAD-Cherry are all expressed in the cell membrane fraction at similar levels. DR5E338KΔPLAD-Cherry was also found in the cytosolic fraction. B, DISC IP using His-tagged TRAIL probed with anti-Cherry. TRAIL precipitates DR5E338K-Cherry but not DR5E338KΔPLAD-Cherry and DR5E338KM152-Cherry, indicating that the two mutations in the extracellular domain abolish binding to TRAIL. C, cells were treated with His-tagged TRAIL and cell viability determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt (MTS) assay. Only the E338K mutant cells displayed a rightward shift in the dose-response curve. Cells with DR5E338KΔPLAD-Cherry and DR5E338KM152-Cherry receptors were killed at the same extent as BJABDR5DEF cells, while the wild-type DR5-Cherry caused a leftward shift, indicating that the mutations that prevent ligand binding also prevent the inhibition of TRAIL-induced apoptosis through DR4. Error bars represent mean ± S.D. from triplicates.

E338K tumor mutation in the intracellular domain along with a second mutation in the extracellular domain that prevents TRAIL binding should prevent the dominant negative effect and the DR4 signaling should occur as in the cells that lack any DR5. Methionine 152 of DR5 is critical for TRAIL binding (23). Another important structure in the DR5 extracellular domain is the pre-ligand assembly domain (PLAD) from amino acids 81 to 97, and deletion of these amino acids has been reported to prevent ligand binding (16). We made three constructs (all fused with an mCherry fluorescence protein tag to facilitate detection) that contain the E338K mutation in the intracellular death domain along with wild-type extracellular domain (DR5E338K), the M152A mutation (DR5E338K/M152A), or the deletion of the PLAD (DR5E338K/ΔPLAD). All the mutant DR5 receptors were expressed at similar levels in the cell membrane fraction (Fig. 3A) although the ΔPLAD mutant was also found in the cytoplasm. This was confirmed by immunofluorescence microscopy (data not shown), suggesting that this mutant DR5 protein to be unable to bind the ligand.

We asked whether mutant DR5 was physically interacting with wild-type DR4 by precipitating DR5 from cells treated with or without TRAIL and asking whether DR4 was present in the complex. Consistent with a previous report showing that although DR4 and DR5 can form complexes together, the relative amount of the heterocomplex is small (10), we found little DR4 interacting with either mutant of wild-type DR5 in our cells (data not shown). These data further support the idea that the mutant DR5 molecules work by competing for TRAIL binding. If this hypothesis is correct, it should be possible to avoid the dominant negative mechanism by activating DR4 with an agonist that cannot bind to DR5. Such an agonist (the mapatumumab antibody) has been developed and is in clinical trials (24, 25). Therefore, if this mechanism is applicable, it should be feasible to activate TRAIL receptor-induced apoptosis in tumor cells that have been selected during tumor development and progression to block TRAIL signal-
show here that at least some of the tumor mutations in DR5 have a more severe phenotype. The DR5 mutations not only block signaling by the mutant receptor itself (confirming previous results from Dr. El-Deiry’s group) (22) but also have a dominant negative phenotype whereby expression of the mutant DR5 at the normal physiological levels can also prevent signaling by TRAIL through DR4. This leads to a situation where the presence of the mutant DR5 actually leads to a more severe inhibition of TRAIL signaling than loss of the protein. Therefore, in tumor cells that have such mutations all TRAIL signaling would be inhibited even though the wild-type DR4 receptor is present, and this would be expected to confer a stronger selective advantage to the tumor cell.

We conclude that the mechanism by which signaling through DR4 is inhibited involves competition for ligand binding but not the formation of significant amounts of heterocomplexes that contain DR4 and DR5. TRAIL receptors can also activate NFκB and MAPK pathways (14), which can inhibit apoptosis. Therefore, we also considered the possibility that the mutated DR5 receptors might still be able to activate these cell survival signal pathways and thus overcome apoptosis signaling through DR4. However, lexatumumab could not activate NFκB and p38 pathways in the DR5 (E338K) mutant cell line, and upon TRAIL stimulation these cells showed less activation of NFκB, p38, JNK, and ERK compared with wild-type DR5 cells (data not shown). Therefore, the mutant receptors do not send survival signals that block DR4-induced apoptosis, providing further support for our idea that the inhibitory effect is mediated by the ligand competition mechanism. Understanding this mechanism led us to predict that we should be able to avoid the dominant negative mechanism by activating DR4 with an antibody that does not recognize DR5. Our data (Fig. 4) with mapatumumab show that this is indeed feasible. Moreover, because this antibody is in clinical development (24, 25), this result shows that such an approach might be used to provide a rational basis for choosing particular TRAIL receptor therapeutic agonists for treatment of particular patients. For example, we predict that a person whose tumor has the E338K mutation would not benefit from treatment with either recombinant TRAIL or an anti-DR5 antibody but would be more likely to benefit from treatment with mapatumumab. It may also be feasible to avoid the inhibitory effect of mutant TRAIL receptors using modified versions of TRAIL that target only one of the receptors (35–37).

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