Effects of Tumor Necrosis Factor-related Apoptosis-inducing Ligand Alone and in Combination with Chemotherapeutic Agents on Patients’ Colon Tumors Grown in SCID Mice

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been reported to induce apoptosis in a variety of malignant cell lines, but it shows little or no toxicity in most normal cells. We examined the response of three human colon tumors to TRAIL alone and in combination with chemotherapy, using SCID mice engrafted with intact patient surgical specimens. These tumors, taken from fresh surgical specimens, contained the heterogeneous tumor cell population characteristic of patient tumors and showed differential sensitivity to TRAIL alone. We also investigated the effect of TRAIL in combination with chemotherapy, using one tumor that showed moderate sensitivity to TRAIL alone. Combinig TRAIL with either 5-fluorouracil (5-FU) or CPT-11 (irinotecan hydrochloride) produced a greatly enhanced antitumor effect over that of either agent alone, with 50% of the animals achieving complete tumor regression with a combination of TRAIL and CPT-11. By histological analysis, tumors treated with TRAIL plus either 5-FU or CPT-11 were seen to consist mainly of connective tissue and fibrotic areas with only a few scattered tumor cells encapsulated in the connective tissue. Several markers were assessed to investigate the basis for the observed therapeutic effect, and significant induction of apoptosis was observed in tumors treated with curative combinations. Cytoplasmic and cell surface expression of the TRAIL receptors DR4 and DR5 was observed in this patient’s tumor by immunohistochemistry. Tumors treated with CPT-11 showed increased membrane expression of DR5, suggesting that CPT-11 may increase sensitivity to TRAIL by up-regulation of DR5. These results obtained in a relevant preclinical model support the idea that the use of TRAIL in combination with either 5-FU or CPT-11 may be an effective strategy in controlling human colon cancer.

INTRODUCTION

A type II membrane protein belonging to the TNF family that appears to induce apoptotic cell death selectively in a variety of tumor cells was independently discovered by two groups and named TRAIL or Apo-2 ligand (1, 2). Other proapoptotic members of this family include TNF-α and FasL. However, TNF infusion causes a lethal inflammatory response that resembles septic shock (3, 4), and infusion of agonistic anti-Fas antibody causes lethal liver damage (5, 6). It has been reported that TRAIL (Apo2L) can preferentially induce apoptosis in a variety of malignant cell lines, but most normal cells tested do not appear to be TRAIL sensitive. Importantly, TRAIL can suppress tumor growth of these cell lines in vivo without exhibiting systemic toxicity (7, 8). For a recent review, see Ashkenazi et al. (9).

The issue of possible TRAIL toxicity to normal tissues is an important concern because a previous report showed that although hepatocytes from rat, mouse, and rhesus monkey were not sensitive to TRAIL, apoptosis was seen in normal human hepatocytes in vitro (10). This caused concern that substantial liver toxicity might result if TRAIL were used in human cancer therapy. However, a recent study (11) resolved this discrepancy by demonstrating that different recombinant versions of Apo2L/TRAIL show different levels of toxicity. Although the polyhistidine-tagged recombinant version of human Apo2L/TRAIL was in fact toxic to hepatocytes, the version of human Apo2L/TRAIL that is being developed as a clinical candidate was not (11). In fact, this version shows little or no toxicity to isolated human or cynomolgus monkey hepatocytes in vitro or to any major organs when administered systemically to two relevant nonhuman primates. On the basis of this work (11), it appears that this formulation of Apo2L/TRAIL would not be expected to cause major toxicity to the liver or other organs if administered clinically.

TRAIL induces apoptosis by its interaction with two death domain-containing receptors, referred to as TRAIL-R1 (DR4) and TRAIL-R2 (DR5; Refs. 12–15). Binding of TRAIL to DR4 and DR5 leads to the activation of caspase-8 and -10. These, in turn, activate downstream effector caspases such as caspase-3, -6, and -7, thereby inducing apoptosis (16). Activated caspase-8 can also cleave and activate the proapoptotic molecule Bid, which then translocates to the mitochondria and induces changes in mitochondrial membrane potential. Subsequently, cytochrome c released from mitochondria and dATP bind to Apaf-1, which activates caspase-9; eventually caspase-3 is activated to induce apoptosis (17, 18). These death receptors were found to be widely expressed on malignant cells as well as normal tissues (13, 14). Two additional receptors were identified as TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2); these have incomplete or no cytoplasmic death domains and have been proposed to inhibit TRAIL-induced apoptosis by acting as decoy receptors (19–21). These putative decoy receptors are highly expressed in normal tissues, whereas they have substantially lower expression in malignant cell lines (19). The expression of these decoy receptors for TRAIL in normal tissues, but not in many tumor cell lines, suggests one explanation for the resistance of normal tissues and the broad sensitivity of tumor cell lines to TRAIL-induced apoptosis.

The combination of TRAIL and a chemotherapeutic agent, such as 5-FU, cis-diaminedichloroplatinum(II), doxorubicin, and CPT-11 was shown to augment TRAIL-induced apoptosis in some human cancer cells (22–26). This synergy was also observed in multidrug-resistant cell lines (27) or TRAIL-resistant cell lines (22, 27, 28). It has been proposed that chemotherapeutic agents augment TRAIL-induced apoptosis by up-regulating DR5 (25, 26). Ionizing radiation may also enhance TRAIL-induced apoptosis by up-regulation of DR5 (29, 30).

Nearly all previous work on the effects of TRAIL has involved the

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4 The abbreviations used are: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; 5-FU, 5-fluorouracil; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling.
use of long-term cultured cell lines either in vitro or grown as tumors in animal models; unfortunately, cell lines often do not respond the way that either primary or metastatic patient tumors may respond to various treatments. Tumors growing within patients are known to be remarkably heterogeneous in terms of their malignant phenotypes and response to treatment; moreover, impaired vascular delivery of effective doses of drugs to the interior of tumors can often be a barrier not encountered during in vitro studies. Using a SCID mouse xenograft model to evaluate the response of patients’ tumors to TRAIL in vivo, we investigated the antitumor effect of TRAIL on three patients’ primary colon tumors. These xenografts resemble the original lesion more closely in histology and growth patterns than do cell lines that have been in culture for years. The effect of TRAIL in combination with the chemotherapeutic agents 5-FU or CPT-11 was investigated using one of these tumors. We demonstrate here that TRAIL is at least as effective as 5-FU at controlling colon tumor growth (at the doses used); moreover, the combination of TRAIL with 5-FU or CPT-11 produced the highest percentages of apoptotic cells and the greatest tumor growth inhibition. We also show that CPT-11 increases membrane expression of DR5, supporting the proposal that drug induced up-regulation of DR5 is one mechanism by which chemotherapy may augment TRAIL-induced apoptosis.

MATERIALS AND METHODS

Colon Tumor Model. These studies were carried out using a patient-derived tumor/SCID mouse xenograft model. Pieces (2–3 mm) of freshly obtained surgical specimens were implanted s.c. into three to four SCID mice. These mice were monitored until the tumor reached 1 cm in diameter; the tumor was then recovered, and 2–3-mm pieces were passaged into new mice. After the second passage, a mouse bearing a 1–2-cm tumor was used as a donor of tumor to implant experimental mice. Mice were assigned randomly to control or treatment groups of six mice each. Histologically, these tumors were moderately well-differentiated adenocarcinomas; this phenotype was closely maintained through several passages.

SCID Mice. CB.17 SCID/SCID mice were used at 6–8 weeks of age and kept under sterile conditions in a pathogen-free environment. The mice were provided with sterile water and food ad libitum, and all manipulations were carried out aseptically inside a laminar flow hood.

Materials. LZ-zhuTRAIL was obtained from Immunex Corp (Seattle, WA), 5-FU was from Roche Laboratories Inc. (Nutley, NJ), and irinotecan hydrochloride (CPT-11) was from The Upjohn Company (Kalamazoo, MI); rabbit polyclonal antibodies for DR4 and goat polyclonal antibodies for DR5 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Treatment Protocol. Pieces of human colon tumors were implanted s.c. into SCID mice. Treatment was initiated when the tumor reached 6–7 mm in diameter (a mean volume of 150 mm³). TRAIL (100 or 500 μg/200 μl) was administered daily by i.p. injection for 14 days. 5-FU (40 or 80 mg/kg) was administered weekly by i.p. injection. These dosages were selected based on the observed effects of several doses: toxicity was observed at doses >80 mg/kg, whereas at these doses, no side effects (such as weight loss) were observed. CPT-11 (25 or 50 mg/kg; Ref. 23) was administered three times per week by i.v. injection. Mice in control groups received saline (200 μl) by i.p. injection.

The tumors were measured every 2 days, and the relative tumor volumes were calculated. At the time points indicated, mice were sacrificed by cervical dislocation, and the tumors were dissected out, fixed in neutral-buffered formalin, and embedded in paraffin for further study.

TUNEL Assay. To detect apoptotic cells, the ApopTag Plus peroxidase in situ Apoptosis Detection Kit (Intergen Company, Purchase, NY) was used. Five-μm-thick paraffin sections of tumor were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Tissue sections were incubated in 20 μg/ml proteinase K (DAKO Corporation, Carpinteria, CA) for 15 min. After sections were rinsed in distilled water, endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide in PBS (0.05 M phosphate buffer containing 0.145 M sodium chloride, pH 7.4) for 5 min. After being washed with PBS, the sections were incubated with equilibration buffer and then TdT enzyme in a humidified chamber at 37°C for 60 min. They were subsequently put into prewarmed working solution step wash buffer for 10 min. After being rinsed in PBS, the sections were incubated with antidigoxigenin-peroxidase conjugate for 30 min. Peroxidase activity in each section was demonstrated by the application of diaminobenzidine (Peroxidase Substrate Kit; Vector Laboratories). Hematoxylin was used as a counterstain.

At least 1000 cells were counted under a microscope in several random fields of each section. The number of apoptotic cells was divided by the total number of cells counted, and the result was expressed as percentage of apoptotic cells.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized and rehydrated, and antigen retrieval was accomplished by heating in 0.01 M citrate buffer in a microwave oven. The solution was brought to a boil and boiled for 20 min. After the endogenous peroxidase activity was quenched for 30 min in 3% hydrogen peroxide in PBS, nonspecific binding was blocked by treatment for 10 min with a biotin blocking system (DAKO Biotin Blocking System; DAKO Corporation) and by incubation with 0.03% casein in PBS for 30 min at room temperature. The primary antibodies of rabbit anti-DR4 polyclonal antibody (1:100 dilution) or goat anti-DR5 polyclonal antibody (1:40 dilution) were then applied, and the sections were incubated for 1 h at room temperature in a moist chamber. After the sections had been washed in PBS, a secondary biotinylated antibody (Vectorstain Universal Quick Kit; Vector Laboratories, Burlingame, CA) was applied, and the sections were again incubated for 30 min. Peroxidase activity was demonstrated by applying diaminobenzidine (Peroxidase Substrate Kit; Vector Laboratories). Hematoxylin was used as a counterstain. Normal rabbit IgG was used in place of primary antibody as a negative control for immunostaining.

Statistical Analysis. The unpaired Student t test was used to evaluate the significance of differential tumor growth rates. The difference was considered significant when P was < 0.05.

RESULTS

Antitumor Activity of TRAIL. Using a patient tumor-SCID mouse xenograft model, we evaluated three patient-derived colon tumors for TRAIL sensitivity (tumors 10642, 11124, and 11712). Treatment with a 500-μg dose of TRAIL for 14 days significantly inhibited growth of all three tumors (Fig. 1). Comparison of the growth rates of these tumors revealed great variability between tumors. Whereas tumor 10642 (Fig. 1C) underwent an ~70-fold increase in tumor volume, tumor 11712 (Fig. 1A) increased only 7-fold. Tumor 11124 (Fig. 1B) exhibited an intermediate rate of growth. This heterogeneity in growth rate is typical of patient tumors engrafted into SCID mice and probably reflects inherent patient-to-patient differences.

Antitumor Activity of TRAIL in Combination with Chemotherapy. Tumor 10642 was selected for further investigation of the antitumor activity of TRAIL, when used in combination with chemotherapeutic agents, because this was the most aggressive of the three adenocarcinomas based on the rapid rate of growth of this tumor. Mice were treated with either 100- or 500-μg doses of TRAIL alone and in combination with either 40 or 80 mg/kg 5-FU for 14 days, and tumor growth was observed during the treatment period and for 14 days after cessation of treatment (Fig. 2). Dose-dependent growth inhibition was seen with TRAIL alone, and this inhibition was statistically significant at both doses. The dosages of 5-FU that were chosen produced only partial growth inhibition so that any possible increase in antitumor effect achieved by the addition of TRAIL could be recognized (inhibition by 5-FU alone was statistically significant at 80 mg/kg). It was observed that the 100-μg dose of TRAIL combined with 40 mg/kg 5-FU treatment produced greater suppression of the tumors than was achieved with either single agent. Furthermore, the
results of this experiment indicate that the greatest therapeutic benefit was achieved with the higher doses of TRAIL and 5-FU in combination. Of particular importance is the fact that the combination of a 500 μg/ml dose of TRAIL combined with either dose of 5-FU led to the partial regression of tumors. However these effects were not durable, and after cessation of the treatment tumor growth resumed in all groups.

The antitumor activity of TRAIL in combination with CPT-11 was also investigated using colon tumor 10642 (Fig. 3). Significant growth inhibition was again seen with either dose of TRAIL treatment alone. At the doses selected (either 25 or 50 mg/kg), CPT-11 treatment alone also significantly inhibited tumor growth. Treatment with combinations of 500 μg/dose TRAIL and CPT-11 led not only to tumor growth suppression but also to tumor regression. By day 15, all of the tumors in the combination groups had undergone regression, and notably, 3 of 6 tumors treated with 500 μg/dose TRAIL + 25 mg/kg CPT-11 and 4 of 6 tumors treated with 500 μg/dose TRAIL + 50 mg/kg CPT-11 completely disappeared. One mouse was then sacrificed in each group, and the tumor was removed for histology. The remaining five mice in each group were observed for 14 days after cessation of treatment, and regrowth of a tumor that had apparently undergone complete regression was observed in only one mouse in each of these two groups (two of five and three of five showed no signs of regrowth). The mice were sacrificed for histological evaluation at day 28.

Histological Analysis of Tumors from TRAIL and Chemotherapy-treated Mice. Histology of the original surgical specimens indicated that these tumors were moderately well-differentiated adenocarcinomas (Fig. 4A). The histology of this tumor during passage in SCID mice maintained that seen in the original specimen. Although smaller than the control tumors, a tumor treated with 500 μg/ml TRAIL alone for 15 days consisted mainly of viable tumor cells and relatively little stroma (Fig. 4B). In contrast, a tumor treated with 500 μg/dose TRAIL and 50 mg/kg CPT-11 was significantly smaller and, at day 15, consisted of mainly stromal elements with only a few scattered tumor cells (Fig. 4C).

Histological examination of various murine organs (including liver

Fig. 1. Effects of TRAIL alone on surgical specimens of three patients’ colon adenocarcinomas grown in SCID mice [plotted as the mean relative tumor volume of 5 mice/group ± SD (bars)]. Mice bearing tumors with a starting volume of 150 mm³ were treated with i.p. injections of 500 μg/dose TRAIL (●) or saline (○) daily for 14 days. Tumor volume was monitored during the 2-week treatment period and for 2 weeks after cessation of treatment. Although the growth rate of these patients’ tumors varied, the growth of each was significantly inhibited by TRAIL. A, tumor 11712 (P = 0.02); B, tumor 11124 (P < 0.01); C, tumor 10642 (P < 0.01).

Fig. 2. Effect of TRAIL alone and in combination with 5-FU on a patient’s colon tumor grown in SCID mice (horizontal bar indicates 14-day treatment cycle). A, the mean of the relative tumor volumes of each group plotted over time. B, plot of the individual tumor sizes at days 15 and 29 to illustrate the size distribution of these tumors. Dose-dependent inhibition of tumor growth was observed with either agent alone compared with the control group ( ). Although the tumors of the 40 mg/kg 5-FU-treated group (E) were smaller than those of the control group, this difference was not statistically significant (P = 0.07). However, a significant degree of inhibition was seen after treatment with 100 μg/dose TRAIL (●; P = 0.05), 80 mg/kg 5-FU (●; P = 0.01), or 500 μg/dose TRAIL ( ●; P = 0.01). More effective tumor inhibition was achieved with the combination therapies: 100 μg/dose TRAIL + 40 mg/kg 5-FU inhibited tumor growth more effectively than either agent alone ( ●; P = 0.001), and 500 μg/dose TRAIL in combination with 40 ( ●) or 80 mg/kg ( ○) 5-FU produced measurable regression of tumors (P = 0.001) by day 15. However, after cessation of treatment, tumor regrowth was observed in these groups.
In vivo, TRAIL must gain access to the tumor via the vascular system. In this study, we also carried out a detailed investigation of the antitumor effects of TRAIL in combination with the chemotherapeutic agents 5-FU or CPT-11 on one selected surgical specimen of colon tumor that showed moderate sensitivity to TRAIL alone. We chose to examine TRAIL in combination with these chemotherapeutic agents because of the clinical importance of these particular chemotherapeutics in the treatment of colon cancer. We also examined protein expression of DR4 and DR5 by immunohistochemistry.

Our results demonstrate that although these patients’ colon tumors exhibited great heterogeneity in their growth rates in SCID mice, TRAIL significantly inhibited the growth of all three. This may be important clinically because there is also great variability in the growth of tumors in patients. The antitumor activity of TRAIL could be greatly augmented by its use in combination with either of the chemotherapeutic agents 5-FU or CPT-11. Treatment with TRAIL combined with 5-FU led to increased suppression of tumor growth and even regression of some tumors. On the other hand, treatment with the combination therapy of TRAIL and CPT-11 led not only to significant increase of these tumors to illustrate the size distribution of these tumors. Significant growth inhibition was seen with treatment with either 100 μg/dose TRAIL (□; P = 0.05) or 500 μg/dose TRAIL (●; P = 0.01) alone. Slightly higher inhibition was observed with either 25 mg/kg (●; P = 0.01) or 50 mg/kg CPT-11 (●; P = 0.001) alone. It is particularly noteworthy that combinations of TRAIL and CPT-11 led to regression of all tumors by day 15 (P = 0.001 in each case) with each of the three combinations tested (□, 100 μg/dose TRAIL + 25 mg/kg CPT-11; ●, 500 μg/dose TRAIL + 25 mg/kg CPT-11; ♦, 500 μg/dose TRAIL + 50 mg/kg CPT-11) and apparent complete regression of three of six and four of six tumors, respectively in the higher dose TRAIL groups. These mice were observed for 14 days after cessation of treatment, and regrowth of a tumor that had undergone complete regression was observed in only one mouse in each of these two groups. The values plotted include all mice in each group. The mice were sacrificed for histological evaluation at day 28.

**DISCUSSION**

TNF and Fas ligand have been shown to induce apoptosis in many types of tumor cells; however, systemic administration of these ligands for therapeutic purposes has been hampered by severe side effects (3–6). TRAIL has been shown to exert potent cytotoxic activity against many tumor cell lines but not most normal cells (1, 2). In animal models, TRAIL has also suppressed tumor growth in vivo without affecting normal tissues (7, 8). We examined the effects of TRAIL on patients’ colon tumors grown in SCID mice to help predict the in vivo sensitivity of human primary tumors to TRAIL. These tumor xenographs more closely resemble the original lesion than do cell lines that have been in culture for years and may have the advantage of potentially exhibiting clinically relevant tumor heterogeneity. Furthermore, in this in vivo system, TRAIL must gain access to the tumor via the vascular system. In this study, we also carried out a detailed investigation of the antitumor effects of TRAIL in combination with the chemotherapeutic agents 5-FU or CPT-11 on one selected surgical specimen of colon tumor that showed moderate sensitivity to TRAIL alone. We chose to examine TRAIL in combination with these chemotherapeutic agents because of the clinical importance of these particular chemotherapeutics in the treatment of colon cancer. We also examined protein expression of DR4 and DR5 by immunohistochemistry.

Our results demonstrate that although these patients’ colon tumors exhibited great heterogeneity in their growth rates in SCID mice, TRAIL significantly inhibited the growth of all three. This may be important clinically because there is also great variability in the growth of tumors in patients. The antitumor activity of TRAIL could be greatly augmented by its use in combination with either of the chemotherapeutic agents 5-FU or CPT-11. Treatment with TRAIL combined with 5-FU led to increased suppression of tumor growth and even regression of some tumors. On the other hand, treatment with the combination therapy of TRAIL and CPT-11 led not only to the greatest tumor suppression and regression but also the complete elimination of some tumors. It should be noted that these results were obtained with the most aggressive of these three TRAIL-sensitive and lung) from mice treated with TRAIL and/or CPT-11 did not show any changes indicative of toxicity; in addition, we found no evidence of serum markers indicative of liver damage (data not shown).

After only 5 days of combination therapy with 500 μg/dose TRAIL and 50 mg/kg CPT-11, large numbers of apoptotic cells with nuclear condensation and eosinophilic cytoplasm were observed being shed into the glandular lumen (Fig. 4D). Correspondingly, the density of viable tumor cells was substantially decreased. Apoptotic cells were found mainly in the center of the tumor (Fig. 4E), whereas there was proportionately more connective tissue in the tumor’s periphery, even at this early time point. (Fig. 4F).

**Analysis of Apoptosis after TRAIL Treatment.** To analyze the degree to which these treatments induced apoptosis, representative tumors were removed from mice after 5 days of treatment, and the number of apoptotic cells was quantified by the TUNEL assay. The degree of apoptosis in the tumors treated with either CPT-11 or TRAIL alone was significantly higher than that seen in the control group. The largest number of apoptotic cells was seen in the tumors treated with the 500 μg/dose TRAIL and 80 mg/kg CPT-11 combination (Fig. 4, G–J). The extent of apoptosis in these groups was as follows: (a) control group, 2.1 ± 0.6%; (b) 80 mg/kg CPT-11 group, 5.3 ± 1.0%; (c) 500 μg/dose TRAIL group, 7.0 ± 1.5%; and (d) the group receiving CPT-11 plus TRAIL, 13.9 ± 2.8% (Fig. 5). After 2 weeks of treatment, the degree of apoptosis in the tumor treated with 500 μg/dose TRAIL was much higher than that seen in the control group. In contrast, there were only a few apoptotic cells in tumors treated with the TRAIL/CPT-11 combination (data not shown). We hypothesize that because the size of these tumors was so small at this point, most tumor cells had already been killed and that this is the reason that the extent of apoptosis was low. We also analyzed the groups for apoptosis after 4 weeks by TUNEL assay, but there was no significant difference among the groups at this point (data not shown).

**Expression of DR4 and DR5.** We examined these tumors for expression of DR4 and DR5 by immunohistochemistry. Immunostaining of both DR4 and DR5 was consistent with cytoplasmic and cell surface expression, although strong membrane localization was not seen (Fig. 4, K and L). However, in a tumor that had been treated with CPT-11, there appeared to be increased membrane expression of DR5 (Fig. 4M). On the other hand, treated tumors did not show a similar increase in DR4.

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**Fig. 3. Effects of TRAIL alone and in combination with CPT-11 on a patient’s colon tumor grown in SCID mice (horizontal bar indicates 14-day treatment cycle). A, mean of the relative tumor volumes of each group plotted over time. B, plot of the individual tumor sizes at days 15 and 29 to illustrate the size distribution of these tumors.**

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**Fig. 4. Effects of TRAIL on patients’ colon tumors grown in SCID mice to help predict the in vivo sensitivity of human primary tumors to TRAIL.** These tumor xenographs more closely resemble the original lesion than do cell lines that have been in culture for years and may have the advantage of potentially exhibiting clinically relevant tumor heterogeneity. Furthermore, in this in vivo system, TRAIL must gain access to the tumor via the vascular system. In this study, we also carried out a detailed investigation of the antitumor effects of TRAIL in combination with the chemotherapeutic agents 5-FU or CPT-11 on one selected surgical specimen of colon tumor that showed moderate sensitivity to TRAIL alone. We chose to examine TRAIL in combination with these chemotherapeutic agents because of the clinical importance of these particular chemotherapeutics in the treatment of colon cancer. We also examined protein expression of DR4 and DR5 by immunohistochemistry.
tumors. TRAIL alone or in combination with either 5-FU or CPT-11 at the doses indicated did not produce any observable toxic effects in the normal tissues examined (in should be noted, however, that the human TRAIL used in this study does not significantly react with murine tissue).

By histological analysis, the combination of TRAIL with 5-FU or CPT-11 led to substantial fibrosis and connective tissue replacement. Only a few scattered tumor cells encapsulated by connective tissue could be found; these isolated colonies of tumor cells were probably responsible for the tumor regrowth seen in some of our experiments. Thus, we feel that the major cellular outcome of combination therapy (and to a lesser extent of TRAIL treatment alone) is a nearly complete replacement of tumor tissue by stromal elements. Because of the extent of connective tissue replacement observed, the true volume of actual cancer cells may be much smaller than the tumor size would indicate. We assume from these distinctive histological observations that shortly after TRAIL-induced apoptosis occurs, dead cancer cells and apoptotic bodies are rapidly phagocytosed by adjacent stromal cells or macrophages (31) and the space becomes filled with infiltrating fibroblasts. We assessed apoptosis after 2 weeks of treatment, but at this time point, there were only a few apoptotic cells in tumors treated with the TRAIL/CPT-11 combination. Apparently, by this point most tumor cells had already been killed, so that few additional apoptotic cells could be detected. In contrast, if the assay for apoptosis was done after just 5 days of treatment, large numbers of apoptotic cells were found primarily in the center of the tumor (E), whereas the periphery of the tumor contained increased proportions of connective tissue (F). G–J, apoptosis was examined by TUNEL assay: G, control tumor; H, 50 mg/kg CPT-11; I, 500 μg/dose TRAIL; J, combination of 500 μg/dose TRAIL and 50 mg/kg CPT-11. After 5 days of treatment, the degree of apoptosis in either the 50 mg/kg CPT-11- or 500 μg/dose TRAIL-treated tumor was significantly higher than in the control group (arrows). Noticeably higher numbers of apoptotic cells are present in the tumor treated with the combination of 500 μg/dose TRAIL and 50 mg/kg CPT-11 (see also Fig. 5).

K–M, protein expression of DR4 and DR5 by immunohistochemistry. Immunostaining of both DR4 (K) and DR5 (L) was consistent with cytoplasmic and cell surface expression. M, increased membrane expression of DR5 was seen in the tumor treated with 50 mg/kg CPT-11 (arrow). Bars, 50 μm in all panels except F, in which the bar is 100 μm.

**Fig. 4.** Histological features of tumor 10642 after treatment with TRAIL and/or CPT-11 for 15 days. A, the histological appearance of the surgical specimen indicates that it is a moderately differentiated adenocarcinoma consisting of undulating ducts of malignant cells surrounded by stroma. B, a tumor treated with 500 μg/dose of TRAIL is significantly smaller than control tumors and consists mainly of tumor cells and stroma. C, in contrast, a tumor treated with the combination of 500 μg/dose TRAIL and 50 mg/kg CPT-11 is significantly smaller and consists of mainly stromal elements (arrow) with only a few scattered tumor cells. D–F, after just 5 days of combination therapy with 500 μg/dose TRAIL and 50 mg/kg CPT-11, large numbers of apoptotic cells with nuclear condensation and eosinophilic cytoplasm are observed being shed into the glandular lumen (D, arrows). These apoptotic cells were found primarily in the center of the tumor (E), whereas the periphery of the tumor contained increased proportions of connective tissue (F). G–J, apoptosis was examined by TUNEL assay: G, control tumor; H, 50 mg/kg CPT-11; I, 500 μg/dose TRAIL; J, combination of 500 μg/dose TRAIL and 50 mg/kg CPT-11. After 5 days of treatment, the degree of apoptosis in either the 50 mg/kg CPT-11- or 500 μg/dose TRAIL-treated tumor was significantly higher than in the control group (arrows). Noticeably higher numbers of apoptotic cells are present in the tumor treated with the combination of 500 μg/dose TRAIL and 50 mg/kg CPT-11 (see also Fig. 5).

K–M, protein expression of DR4 and DR5 by immunohistochemistry. Immunostaining of both DR4 (K) and DR5 (L) was consistent with cytoplasmic and cell surface expression. M, increased membrane expression of DR5 was seen in the tumor treated with 50 mg/kg CPT-11 (arrow). Bars, 50 μm in all panels except F, in which the bar is 100 μm.

**Fig. 5.** Analysis of the degree of apoptosis present in tumors on day 5 of treatment with TRAIL and CPT-11 alone and in combination: control group, 2.1 ± 0.6%; 25 mg/kg CPT-11, 4.0 ± 1.3%; 50 mg/kg CPT-11, 5.3 ± 1.0%; 100 μg/dose TRAIL, 3.5 ± 0.7%; 500 μg/dose TRAIL, 7.0 ± 1.5%; 25 mg/kg CPT-11 + 100 μg/dose TRAIL, 10.1 ± 2.1%; 25 mg/kg CPT-11 + 500 μg/dose TRAIL, 12.5 ± 2.3%; and 500 μg/dose CPT-11 + 500 μg/dose TRAIL, 13.9 ± 2.8%. Bars, SD.
cells with nuclear condensation and eosinophilic cytoplast were observed being shed into the glandular lumen in the tumor treated with TRAIL and CPT-11. There was obvious fibrosis and connective tissue at the periphery of the tumor at this time point, but less was seen in the middle of the tumor.

Previous reports have demonstrated that chemotherapeutic agents and ionizing radiation can induce DR5 expression (25–30), and it has been proposed that this may be one mechanism by which enhanced killing by TRAIL could occur. CPT-11 has been shown to be an inhibitor of the nuclear enzyme topoisomerase I and is believed to block DNA transcription and replication through the inhibition of this enzyme (32). Previous reports showed that the combination of CPT-11 with TRAIL could augment TRAIL-induced apoptosis in colon cancer cell lines not only by inducing up-regulation of DR5 (20) but also by suppressing TRAIL-induced nuclear factor-κB activation (24). Compared with TNF-α and Fas ligand, however, activation of nuclear factor-κB induced by TRAIL is not as strong (19). In the present study, we examined the effect of the chemotherapeutic agent CPT-11 on DR4 and DR5 expression in a patient’s colon cancer by immunohistochemistry. Immunostaining of the control tumor (and the surgical specimen) revealed both DR4 and DR5 in the cytoplasm and cell membrane. This result is consistent with recent studies using thyroid cancer and hepatocellular carcinoma (24, 33). In our study, we also observed an increase in membrane staining of DR5 in tumors treated with CPT-11. However, tumors treated with CPT-11 did not show a similar increase in DR4. This observation is supportive of the suggestion that CPT-11 treatment may increase DR5 expression in a patient-derived colon tumor, leading to an increased sensitivity to TRAIL as observed in colon cell lines. However, this point remains to be further clarified by quantifying expression levels of these receptors. It will also be important to evaluate the expression levels of other molecules found to be important in the sensitivity of malignant cells to Apo2L/TRAIL to further define how the antitumor efficacy of these agents is improved by their use in combination therapies.

In light of recent reports, the role of Bax will be of particular interest. Although many factors have been suggested to control sensitivity to TRAIL in various cells, two recent reports have demonstrated that in the human colon cell line HCT116, TRAIL/Apo2L sensitivity is dependent on the expression of the proapoptotic molecule Bax. Whereas the Bax+/- cell line is sensitive to Apo2L/TRAIL, the Bax-deficient sister clone is not (34, 35). Reconstituation of Bax expression by transfection restores sensitivity to Apo2L/TRAIL (34). Additionally, Leblanc et al. (34) showed that Apo2L/TRAIL treatment of the Bax+/- cell line (which is also mismatch repair deficient) either in vitro or in vivo with Apo2L/TRAIL induces the outgrowth of clones that are resistant by virtue of Bax mutations. These authors also showed that these cell lines both express Bak, which is usually insufficient to substitute for Bax. However, pretreatment with a chemotherapeutic drug up-regulates DR5 (etoposide) and/or Bak [etoposide or camptothecin (CPT-11)], restoring sensitivity to Apo2L (34). We are currently investigating the relationship between Bax and Bak expression in the patient-derived tumors and their sensitivity to Apo2L/TRAIL in the SCID mouse xenograft model. Additionally, it will be important to characterize both the histology and expression levels of these molecules in the tumors that regrow after cessation of TRAIL treatment to determine whether treatment leads to the outgrowth of resistant clones as was seen in the HCT116 cell line.

In summary, TRAIL showed significant antitumor activity against three patients’ colon tumors grown in SCID mice. The combination of TRAIL with either 5-FU or CPT-11 led to greater tumor growth inhibition than that seen with either drug alone. The antitumor activity of the combination with CPT-11 was particularly impressive: half of the tumors underwent complete regression. This combination of TRAIL with CPT-11 also produced a high percentage of apoptotic cells and substantial fibrosis and connective tissue replacement. These results suggest that TRAIL combined with doses of either 5-FU or CPT-11, which are not by themselves able to suppress tumor growth, may be an effective new strategy in controlling human colon cancer. Importantly, it may be possible to achieve therapeutic benefit with lower doses of chemotherapeutic drugs when they are used in combination with TRAIL. This could reduce the toxic side effects caused by these drugs as well as retard the development of drug resistance. The dosages of chemotherapeutic agents used were selected to facilitate our ability to demonstrate an augmentation of effect by the combination therapy, and this enhanced effect was seen to be dosage dependent. We did not investigate the effect of TRAIL in combination with dosages of chemotherapeutic agents that could, alone, suppress tumor growth. A comprehensive study is necessary to identify the optimal dosages and combinations needed to achieve the maximal antitumor effect with minimal toxicity. Another issue that will be important to resolve is the effect of prior chemotherapy on this antitumor activity, although none of these three patients received chemotherapy before surgical resection of their tumor. It remains to be determined whether neoadjuvant chemotherapy before TRAIL treatment will interfere with the antitumor effect of TRAIL in colon or other tumor types.

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