Macrophages From Cancer Patients: Analysis of TRAIL, TRAIL Receptors, and Colon Tumor Cell Apoptosis

Jean-Philippe Herbeuval, Claude Lambert, Odile Sabido, Michèle Cottier, Pierre Fournel, Michel Dy, Christian Genin

Background: Tumor-infiltrating macrophages secrete cytokines, including Fas ligand, tumor necrosis factor-α (TNF-α), and TNF-related apoptosis-inducing ligand (TRAIL). TRAIL induces apoptosis in tumor cells but not in normal cells; however, regulation of TRAIL and its receptors in cancer patients is relatively uncharacterized. We investigated whether macrophages from cancer patients produce TRAIL and whether apoptosis in cultured colon adenocarcinoma cells involves TRAIL and its receptors. Methods: Macrophages isolated from pleural effusions of nine cancer patients and five control patients with congestive heart failure (whose effusions contained no tumor cells) were cultured. Levels of TRAIL, TNF-α, interferon α, and Fas ligand in conditioned medium were measured by enzyme-linked immunosorbert assays. Apoptosis of human colon adenocarcinoma cell lines, including Colo 205, was determined by the Annexin V method and terminal deoxynucleotidyltransferase-mediated deoxyuridine 5′-triphosphate nick-end labeling (TUNEL). Cell-surface TRAIL receptors were measured by flow cytometry. Results: Conditioned culture medium from macrophages isolated from pleural effusions containing 1%–5% tumor cells (CM-A) contained TRAIL at 980–1300 pg/mL, whereas that from macrophages from pleural effusions containing more than 50% tumor cells or containing no tumor cells (CM-B) contained TRAIL at 0–50 pg/mL. When cultured with medium containing 50% CM-A, 40% (95% confidence interval [CI] = 30% to 50%) of Colo 205 cells underwent apoptosis; when cultured with 50% CM-B, 8% (95% CI = 3% to 13%) underwent apoptosis. When Colo 205 cells were cultured with 50% CM-A, cell-surface expression of TRAIL death receptors DR5 and DR4 increased 13-fold and sixfold, respectively, compared with that of untreated Colo 205 cells. Recombinant TRAIL induced 90% (95% CI = 85% to 95%) of Colo 205 cells to undergo apoptosis and acted synergistically with TNF-α to induce apoptosis. Conclusion: Macrophages from cancer patients appear to be activated by tumor cells to produce TRAIL and to increase the expression of TRAIL death receptors DR4 and DR5 on tumor cells. [J Natl Cancer Inst 2003;95:611–21]

Tumor infiltration by macrophages directly influences cancer cell growth in vitro and in vivo (1–3). Tumor-infiltrating macrophages appear to be able to distinguish between malignant and normal cells and can attack neoplastic cells in patients through contact-dependent and cytotoxin-mediated mechanisms (4); the presence of tumor-infiltrating macrophages is associated with good prognosis for patients with colorectal adenocarcinoma (5). However, the story is complicated because such macrophages can secrete a variety of cytokines and thus can promote or inhibit tumor growth, depending on which cytokines are secreted (6). When cytokines of the tumor necrosis factor-α (TNF-α) family are secreted, as is frequently observed in macrophages that have been exposed to human lung cancer cells (7) or colorectal cancer cells (8–10), apoptosis is induced.

TNF-α and Fas ligand (FasL), members of the TNF-α family, induce apoptosis by binding to their corresponding cell-surface receptors. The receptor for TNF-α is TNF-R1, and the receptor for FasL is Fas. These receptors contain intracytoplasmic domains that, after ligand binding, induce apoptosis in tumor cells (11). TNF-related apoptosis-inducing ligand (TRAIL), another member of the TNF-α family (12), does not induce apoptosis in normal cells (13) but does induce apoptosis in several human tumor cell lines (14). Several melanoma cell lines (e.g., WM9, WM35, WM98-1, WM164, WM793, WM1205-Ln, WM1791-C, and WM3211) are resistant to apoptosis induced by FasL or TRAIL but are sensitive to TRAIL-mediated apoptosis (15,16). The following five TRAIL receptors have been described: TRAIL-R1 or death receptor 4 (DR4), TRAIL-R2 or DR5, TRAIL-R3 or decoy receptor 1 (DcR1), TRAIL-R4 or DcR2, and osteoprotegerin (OPG) (17). DR4 and DR5 contain cytoplasmic death domains and induce apoptosis after ligand binding (18,19); DcR1, DcR2, and OPG lack such death domains and do not induce apoptosis (20,21). TRAIL is secreted by immune cells, including lymphocytes, natural killer cells (22), monocytes, dendritic cells (23), and macrophages (24). The expression of decoy receptors for TRAIL in normal tissues, but not in many tumor cell lines, may account for the resistance of normal tissues and for the broad sensitivity of tumor cell lines to TRAIL-induced apoptosis (25). However, the molecular mechanisms involved in the production and regulation of TRAIL and its receptors in vivo remain to be elucidated.

An inverse association between the number of macrophages in pleural effusions from cancer patients and the extent of malignant disease has been reported (26–28). Our objective in this study was to investigate the cytotoxic effects of macrophages on human tumor cells. We sampled pleural effusions from nine patients with cancer and from five control patients with congestive heart failure to obtain large numbers of macrophages that had been in contact with large or small numbers of tumor cells in pleural effusions from the cancer patients or no tumor cells in pleural effusions from patients with congestive heart failure. We then used human Colo 205, Colo 320, and Caco-2 colon adenocarcinoma cell lines to examine whether macrophages secrete TRAIL and whether apoptosis of tumor cells induced by TRAIL is inhibited by macrophages. We also analyzed whether macrophages isolated from pleural effusions of patients with cancer contain TRAIL.

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See “Notes” following “References.”

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carcinoma cells as target cell lines to develop an *in vitro* model to test whether macrophages that had been activated by tumor cells in pleural effusions produced TRAIL, whether macrophages induced increased expression of TRAIL death receptors on target tumor cells, and whether TRAIL induced target colon adenocarcinoma cell lines to undergo apoptosis.

**Materials and Methods**

**Materials**

Human recombinant TNF-α was purchased from PreproTech, Inc. (Rocky Hill, NJ). Human recombinant TRAIL, interferon α (IFN α), and FasL were purchased from R&D Systems (Abingdon, U.K.). We used recombinant DR5 (R&D Systems) at 1, 5, 10, and 20 μg/mL to selectively block TRAIL-mediated apoptosis. Membrane TRAIL was detected with a mouse anti-human monoclonal antibody from Diaclone Research (Besancon, France). Goat anti-human antibodies against TRAIL receptors DR4, DR5, DcR1, and DcR2 (all polyclonal antibodies) were purchased from R&D Systems. The tetrapeptide caspase inhibitor benzoylcarbonyl-Val-Ala-Asp(OMe)-CH₂-fluoromethyl ketone (zVAD-fmk) was purchased from France Biochem (Meudon, France). Stock solutions of the inhibitor were prepared in dimethyl sulfoxide (Life Technologies, Gaithersburg, MD) and stored at 4 °C.

**Tumor and Control Cell Collection**

Pleural fluids containing tumor cells and leukocytes were collected by drainage thoracentesis from nine patients with effusions related to various cancers (three with pleura mesothelioma, four with lung adenocarcinoma, one with breast adenocarcinoma, and one with colon adenocarcinoma). All cancer patients had stage IV metastatic disease. As a tumor-unrelated control, pleural fluids were also collected from five patients with congestive heart failure, as previously described (29). This *in situ* control was considered appropriate because the macrophages had not been in contact with tumor cells. Although *in vitro* adherence of macrophages to a culture dish might have induced the macrophages to produce TRAIL, we did not detect any TRAIL after a 48-hour culture of macrophages from control donors, whereas we did detect the production of TRAIL by macrophages from cancer patients after a 48-hour culture. If the pleural effusion was bloody, red cells (which represented less than 1% of total cells) were removed by Ficoll centrifugation (at room temperature for 20 minutes at 800 g) and, essentially, none of the macrophages in the effusions came from the small amount of contaminating blood. After collection, pleural fluids were placed in sterile, screw-cap 2-L ethylene containers (Plastiques Gosselin, Hazebrouck, France) with 2000 IU of sodium heparin (Laboratoires LEO S.A., Saint Quentin en Yvelines, France) at room temperature for 1–2 hours, for transportation to the laboratory. The thoracentesis procedure was approved by the Institutional Review Board at the University Hospital Center, Saint-Etienne, France, and signed informed consent was obtained from all the cancer patients and patients with congestive heart failure.

**Cytologic Evaluation of Cells Present in the Pleural Fluid**

After the pleural fluids were centrifuged at 400 g at room temperature, the cells were transferred to slides (Merck Eurolab, Strasbourg, France) and spread thinly. At least four smears were prepared from each sample. Air-dried smears were processed with May–Grünewald–Giemsa staining, and wet-fixed smears were processed with Papanicolaou staining.

The cytologic features that we used to distinguish malignant cells were their relatively large size, high nuclear-to-cytoplasmic volume ratio, nuclear hyperchromasia with irregularities of the chromatin pattern, large nucleoli, and abnormal mitotic figures. Tumor cells were classified as adenocarcinoma, poorly differentiated carcinoma, oat-cell carcinoma, squamous cell carcinoma, and malignant mesothelioma. Macrophages showed an irregular nuclear shape and a voluminous cytoplasm with numerous vacuoles.

**Preparation of Conditioned Medium**

Pleural fluids were centrifuged at 400 g for 10 minutes at 4 °C, and cell pellets were resuspended in RPMI-1640 medium containing 20 mM HEPES buffer (Life Technologies, Paisley, Scotland), 5% type AB human serum (Western States Plasma-SeraCare, Inc., Oceanside, CA), 2 mM l-glutamine (Eurobio, Les Ulis, France), and antibiotics (penicillin G at 100 U/mL, streptomycin at 100 μg/mL, and amphotericin B at 0.25 μg/mL; Life Technologies). Cells were plated at 2 × 10⁶ cells per mL, and macrophage-like cells were allowed to adhere to 75-cm² tissue culture flasks (Falcon, Oxnard, CA) at 37 °C in an incubator in a humidified atmosphere of 5% CO₂/95% air for 2 hours. Nonadherent cells were removed by two washings with Dulbecco’s phosphate-buffered saline (PBS) containing 0.02% EDTA (Eurobio) at room temperature. Macrophages accounted for 95% of the remaining adherent cells, as determined by light microscopy counting of May–Grünewald–Giemsa stained cells (30). After the 2-hour incubation, macrophages were removed from culture dishes by scraping and pipetting and plated in 75-cm² culture dishes (Merck Eurolab) at 500,000 cells per milliliter of culture medium.

Adherent cells were cultured for 48 hours in the same medium at 37 °C in an incubator in a humidified atmosphere of 5% CO₂/95% air. This time was chosen because a kinetic analysis indicated that all cytokines tested were maximal produced between 24 and 48 hours of culture, and TRAIL was maximally produced by 48 hours. Conditioned culture medium was centrifuged at 400 g for 10 minutes at 4 °C, and the supernatant was stored in aliquots at −80 °C until use. After thawing, supernatants were filtered through 0.22-μm (pore size) filters. Culture supernatants from macrophages isolated from pleural effusions of patients with small numbers of tumor cells (1%–5%) were designated as conditioned medium A (CM-A). Culture supernatants from macrophages from pleural effusion of patients with large numbers of tumor cells (50%) or from patients with congestive heart failure with no tumor cells were designated as CM-B. Each of the five CM-A and nine CM-B samples were prepared and studied individually. It should be noted that no patient pleural effusions contained tumor cells in the range of 6%–50%.

**Tumor Cell Culture Conditions**

The human colon cell lines Colo 205, Colo 320, and Caco-2 were purchased from the European Collection of Cell Cultures (Salisbury, U.K.). Tumor cells were cultured under the same conditions used for macrophages, except that RPMI-1640 medium was supplemented with 10% heat-inactivated fetal calf serum (Life Technologies). The appropriate cell density for plat-
ing was first determined under basal conditions to avoid contact inhibition of cell growth or depletion of nutrients from the medium (data not shown). Different numbers of Colo 205, Colo 320, and Caco-2 cells were plated in 24-well plates (Falcon) and stained with trypan blue, and viable cells were counted with a hemocytometer. Exponential growth was observed at a plating density of 50 000 cells per well. Cells were cultured for an additional 4 days with 50% conditioned medium (CM-A or CM-B). A concentration–response experiment, in which 0.1%–100% CM-A and CM-B were added to culture medium, determined that 50% CM-A or 50% CM-B was the optimal concentration for the production of TRAIL and the induction of apoptosis.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokines in Conditioned Medium

Levels of FasL, TNF-α, IFN-α, and TRAIL were measured in each conditioned medium and cell culture supernatant with commercial enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer’s instructions: FasL and TNF-α, Quantikine from R&D Systems; trimeric TRAIL, Diacalone; and IFN-α, Biosource International (Camarillo, CA). Positive controls were supplied in each kit. The optical density was measured at 492 nm on a Titertek Multiskan reader (Flow Labs, Irvine, U.K.). Detection ranges of the various ELISA kits were as follows: 15–1000 pg/mL for TNF-α; 12–500 pg/mL for IFN-α; 0.1–500 pg/mL for TRAIL; and 4–250 pg/mL for FasL. The cytokine content of each of the 14 conditioned media was tested three times. Levels of cytokines were expressed as picograms per milliliter per 50 000 macrophages. Cytokines were not detected in the medium used to culture the macrophages.

Measurement of TRAIL mRNA by Reverse Transcription–Polymerase Chain Reaction (RT–PCR)

Macrophages isolated from cancer patient pleural effusions with small numbers of tumor cells and from pleural effusions from patients with congestive heart failure were tested directly for TRAIL mRNA, without being cultured. mRNA was extracted from 50 000 cultured macrophages with TRIzol reagent (Life Technologies), according to the manufacturer’s instructions. RT was performed with a SuperScript preamplification system for first-strand cDNA synthesis (Life Technologies) in a Perkin-Elmer thermocycler. To quantify total cDNA synthesis, first-strand cDNA synthesis (Life Technologies) in a Perkin-Elmer thermocycler. To quantify total cDNA synthesis, reverse transcription–polymerase chain reaction (RT–PCR) was performed with a SuperScript preamplification system for first-strand cDNA synthesis (Life Technologies), according to the manufacturer’s instructions. The RT reaction was inactivated by heating at 70 °C for 15 minutes. RT efficiency was estimated by the radioactivity associated with synthesized cDNA, and the number of copies was measured with RT–PCR. The following oligonucleotide primers were used to standardize subsequent PCRs.

The production of TRAIL mRNA was semiquantitatively measured with RT–PCR. The following oligonucleotide primers for human TRAIL (874 base pairs [bp]) and β₂-microglobulin (280 bp) mRNAs were synthesized by Life Technologies: TRAIL 5’ primer = TATTCACATCTCATCGGGGGA and 3’ primer = AGTGGTCTGTGCGTCTGCT; β₂-microglobulin 5’ primer = CACGAGAATGGGAGG and 3’ primer = CATGGTCTGTGCGTCTC. The housekeeping gene β₂-microglobulin was also amplified in parallel as a reference for the quantification of TRAIL transcripts. Each cycle in the 30-cycle amplification was as follows: 1 minute at 95 °C for denaturation, 1 minute at 55 °C for annealing, and 1 minute at 72 °C for primer extension. All PCR-amplified products were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide (0.5 μg/mL) and visualized by UV irradiation.

Detection of Membrane-Bound TRAIL on Macrophages by Flow Cytometry

Macrophages were cultured 24 hours or 48 hours, and membrane TRAIL expression was determined by incubating the macrophages for 15 minutes at room temperature with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human TRAIL monoclonal antibody or with relevant control isotype-matched antibodies (each at 5 μg/mL; Diaclone) in PBS containing 3% bovine serum albumin. After two washes in ice-cold PBS, the cells were analyzed by flow cytometry (BD, San Jose, CA).

Aptosis Studies

Morphologic examination of tumor cells treated with conditioned medium or recombinant cytokines showed the loss of membrane asymmetry and condensation of cytoplasm and nucleus, which are characteristics of apoptotic cells. Cells were tested further for apoptosis by using the Annexin V test and the terminal deoxynucleotidyltransferase-mediated deoxyuridine 5’-triphosphate (dUTP) nick-end labeling (TUNEL) assay. In early apoptosis, phosphatidylserine is exposed to the outer leaflet of the plasma membrane. Annexin V preferentially binds to the exposed phosphatidylserine and thus detects early events in apoptosis (25). We cultured Colo 205, Colo 320, and Caco-2 cells with 50% CM-A or CM-B or various recombinant cytokines for 24 hours. As a control, cells were grown in fresh RPMI-1640 medium supplemented with 10% fetal calf serum. To determine the combination of cytokines that induces the optimal level of apoptosis, we tested several combinations of TRAIL, IFN-α, TNF-α, and FasL (each at 0.1, 10, 25, 50, and 100 ng/mL). Cells were then centrifuged, washed with the buffer from the Annexin kit (Coger, Paris, France), and incubated for 20 minutes with medium only (negative control) or with FITC-conjugated Annexin V (Annexin V-FITC) (Coger) in the dark at room temperature. To eliminate necrotic cells and membrane fragments that might be Annexin V-FITC-positive, we added propidium iodide and selected propidium iodide-negative cells as follows. After two washes, propidium iodide (100 μL/mL) was added directly to the cell pellet, and tubes were placed on ice. Labeled cells were quantitated by flow cytometry with FACSStar Plus cell sorter (BD). Apoptotic cells were visualized on a cytogram showing the number of propidium iodide-negative cells versus the number of Annexin V-FITC-positive cells.

To test for later stages of apoptosis in Colo 205, Colo 320, and Caco-2 cells, we used flow cytometry to measure DNA fragmentation detected with the TUNEL assay (31). The TUNEL assay labels DNA strand breaks in apoptotic cells; these breaks are characteristic of later stages of apoptosis. Propidium iodide was used as a probe for total DNA content. For the TUNEL assay, 10⁶ Colo 205 cells were washed twice in PBS and suspended in a buffer containing terminal deoxynucleotidyltransferase and biotin-conjugated dUTP (biotin-dUTP) (ApopDETEK, Enzo Dako, Trappes, France), according to the manufacturer’s instructions. The cells were incubated at 37 °C for 60 minutes and washed twice in PBS. Colo 205 cells that incorporated biotin-dUTP in the terminal deoxynucleotidyltransferase reaction were suspended in 200 μL of staining solution containing Avdin-FITC (6 μg/mL; Immunotech-Coulter, Marseille, France),
5% nonfat dry milk (Boehringer Mannheim, Meylan, France), RNase (20 μg/mL; Boehringer Mannheim), and 0.1% Triton X-100 (Sigma-Aldrich, L’Isle d’Abeau Chesnes, France) in standard saline citrate buffer (Sigma-Aldrich). Cells were incubated for 30 minutes at 37°C in the dark, washed twice with PBS, resuspended in 200 μL of propidium iodide (100 μg/mL in PBS), and analyzed by flow cytometry. To verify the activity of terminal deoxynucleotidyltransferase as a positive control, Colo 205 cells were treated with DNase (1 μg/mL; Boehringer Mannheim), which fragments the DNA, and then subjected to the TUNEL assay. Apoptotic cells were visualized on a cytogram for a dual parameter analysis plotting propidium iodide intensity versus Avidin-FITC intensity. Information on at least 10,000 events was acquired for each TUNEL assay.

To determine whether the caspase cascade pathway is involved in Colo 205 cell death, we used the caspase inhibitor zVAD-fmk to specifically block caspase-mediated apoptosis. Colo 205 cells were incubated with 50 μM zVAD-fmk for 4 hours at 37°C and then cultured for 24 hours in 50% CM-A or with recombinant TRAIL or TNF-α (each at 50 ng/mL). Cells were then analyzed for apoptosis by the Annexin V method, as described above.

Recombinant DR5 binds TRAIL and prevents TRAIL from binding to its cell-surface receptors, resulting in inhibition of TRAIL-associated pathways. To confirm that TRAIL was involved in Colo 205 cell death, cells were cultured for 24 hours with 50% CM-A and recombinant DR5 at 1, 5, 10, and 20 μg/mL or with recombinant TRAIL (at 50 ng/mL) and DR5 at 1, 5, 10, and 20 μg/mL, and apoptosis was assessed by the Annexin V method, as described above.

Detection of TRAIL. Receptors on Colo 205 Cell Membranes by Flow Cytometry

Colo 205 cells were cultured alone or with CM-A, and the surface expression of TRAIL receptors DR4, DR5, DcR1, and DcR2 was examined by incubating Colo 205 cells with goat anti-human DR4, DR5, DcR1, DcR2, or relevant control isotype-matched antibodies (each at 5 μg/mL; R&D Systems) in PBS containing 3% bovine serum albumin for 45 minutes on ice. After three washes in PBS, the cells were incubated for 45 minutes on ice with an FITC-conjugated mouse anti-goat antibody diluted 1:200 in PBS containing 3% bovine serum albumin (R&D Systems). Cells were washed twice in ice-cold PBS and analyzed by flow cytometry (BD).

Table 1. Quantification of cytokine production by macrophages obtained from pleural effusions of cancer patients and control subjects*

<table>
<thead>
<tr>
<th>Type of macrophage-conditioned medium</th>
<th>% of malignant cells in pleural fluids</th>
<th>Production of cytokines by macrophages (pg/mL)</th>
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<td></td>
<td></td>
<td>TRAIL</td>
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<tr>
<td>CM-A</td>
<td>1–5†</td>
<td>980–1300</td>
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<tr>
<td>CM-B</td>
<td>&gt;50‡</td>
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<td>CM-B</td>
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*TRAIL = TNF-related apoptosis-inducing ligand; TNF-α = tumor necrosis factor α; INF-α = interferon-α; FasL = Fas ligand; CM-A = conditioned medium A (culture supernatants from macrophages isolated from pleural effusions of patients with small numbers of tumor cells [1%–5%]); CM-B = conditioned medium B (culture supernatants from macrophages from pleural effusion of patients with large numbers of tumor cells (>50%) or from patients with congestive heart failure with no tumor cells); UD = undetectable.

†Pleural effusions from cancer patients (one with adenocarcinoma of the colon, one with adenocarcinoma of the breast, one with adenocarcinoma of the lung, and two with pleural mesothelioma). All patients had stage IV metastatic disease.

‡Pleural effusions from cancer patients (three with adenocarcinoma of the lung and one with pleural mesothelioma). All patients had stage IV metastatic disease.

§Pleural effusions from five patients with congestive heart failure.

Statistical Analysis

All experiments were repeated a minimum of three times. All comparisons of data were made with a two-sided Student’s t test (data meet the requirements for a Student’s t test).

To test the effects of combined treatment (TNF-α plus TRAIL or TNF-α plus IFN-α) for synergistic effects, combinations of cytokines were evaluated, and dose–response curves for single cytokines and combinations of cytokines were compared. Characteristics of the effects of the combined treatment were then analyzed by the isobole method (32) for combinations of molecules A and B: \( A_a / A_a + B_b / B_b = D \), where \( A_a \) and \( B_b \) are the concentrations of cytokines used in combination to induce an effect, and \( A_a \) and \( B_b \) are the concentrations of cytokines able to produce the same magnitude of effect if used individually. If the combination index \( D \) is less than 1, the combination of cytokines act synergistically; if \( D \) is greater than or equal to 1, the respective effects were antagonistic or additive, respectively. Each experiment was performed in triplicate, and the statistical significance (\( P \)) of the combination indices compared with the additive combination index (\( D = 1 \)) was determined by a two-sided Student’s t test.

RESULTS

Production of TRAIL, TNF-α, and IFN-α by Macrophages From Cancer Patients and Patients With Congestive Heart Failure

Macrophages were isolated from cancer patient pleural effusions with small numbers of tumor cells (1%–5% of total cells), cancer patient pleural effusions with large numbers of tumor cells (>50% of total cells), and control pleural effusions from patients with congestive heart failure (control for no tumor cells). These macrophages were cultured for 2 days, and the levels of TRAIL and TNF-α secreted into culture supernatants were determined. High levels of TRAIL (980–1300 pg/mL) were detected in culture supernatants from macrophages exposed \( \text{in vivo} \) to pleural effusions with 1%–5% malignant cells (CM-A; Table 1). Low levels of TRAIL (0–50 pg/mL) were detected in culture supernatants from macrophages exposed \( \text{in vivo} \) to pleural effusions with greater than 50% malignant cells or from macrophages exposed \( \text{in vivo} \) to pleural effusions from patients with congestive heart failure containing no tumor cells (CM-B; Table 1). In contrast, no statistically significant difference was observed in the levels of TNF-α and IFN-α in CM-A compared with CM-B.
or CM-B. FasL was not detected in any macrophage-conditioned medium.

TRAIL mRNA expression was observed in freshly isolated macrophages only from cancer patients whose pleural effusions contained a low level of tumor cells (<5%). This level of mRNA expression was directly related to the amount of TRAIL protein produced after 48 hours of culture by macrophages isolated from the same pleural effusion (Fig. 1, lane 1). We used flow cytometry to assess macrophage membrane-associated TRAIL and found that TRAIL was bound to the membranes of macrophages that produced CM-A but not to the membranes of macrophages that produced CM-B from control patients with congestive heart failure. Membrane-associated TRAIL was weakly detected on macrophages that produced CM-A from three of the five cancer patients (data not shown).

### Analysis of Apoptosis Mediated by Cytokines and by Conditioned Medium

Colo 205, Colo 320, and Caco-2 cells were cultured in medium containing 50% CM-A or CM-B. As shown in Fig. 2, A, after 4 days of culture with CM-A, the number of cells in untreated cultures was greater than that in untreated controls: for Colo 205 cells, 590,000 untreated cells versus 230,000 treated cells (inhibition of 62%, 95% confidence interval [CI] = 57% to 67%); for Colo 320 cells, 550,000 untreated cells versus 330,000 treated cells (inhibition of 41%, 95% CI = 36% to 46%); for Caco-2 cells, 590,000 untreated cells versus 350,000 treated cells (inhibition of 40%, 95% CI = 37% to 43%). The numbers of cells in untreated cultures and in cultures treated with medium containing 50% CM-B were similar: for Colo 205 cells, 590,000 untreated cells versus 545,000 treated cells (8% of inhibition, 95% CI = 3% to 12%); for Colo 320 cells, 550,000 untreated cells versus 530,000 treated cells (inhibition of 3%, 95% CI = 1% to 5%); for Caco-2 cells, 590,000 treated cells versus 572,000 treated cells (inhibition of 4%, 95% CI = 2% to 6%).

To determine whether apoptosis was responsible for the decreased number of Colo 205 cells cultured with CM-A, we used Annexin V and flow cytometry to assay for apoptosis in cells cultured with 50% CM-A or 50% CM-B or in control cells cultured in medium alone. After 24 hours of culture, 40% (95% CI = 30% to 50%) of Colo 205 cells cultured with CM-A were apoptotic (Fig. 2, B, 1), but essentially no Colo 205 cells cultured with CM-B (8%, 95% CI = 3% to 13%) or medium alone (3%, 95% CI = 1% to 5%) were apoptotic (Fig. 2, B, 2). After 3 days of culture, 66% (95% CI = 60% to 72%) of Colo 205 cells cultured with CM-A were in the final stage of apoptosis, as determined by the TUNEL method (Fig. 2, C, lower left panel), whereas 12% (95% CI = 7% to 17%) of cells cultured with CM-B (Fig. 2, C, lower right panel) and 8% (95% CI = 6% to 10%) of control cells cultured with medium alone (Fig. 2, C, upper right panel) were in the final stage of apoptosis. As a positive control, when cells were treated with DNase to fragment the DNA, as observed in the final stage of apoptosis, 98% (95% CI = 96% to 100%) of cells were apoptotic. Similar results were obtained with Colo 320 and Caco-2 cells (data not shown).

As shown in Fig. 3, A, recombinant TRAIL and recombinant TNF-α induced apoptosis in Colo 205 cells in a dose-dependent manner. Recombinant TRAIL (100 ng/mL) induced 90% (95% CI = 85% to 95%) of Colo 205 cells to undergo apoptosis. Recombinant TRAIL (100 ng/mL) induced apoptosis in 80% (95% CI = 69% to 91%) of Colo 320 cells and 75% (95% CI = 64% to 86%) of Caco-2 cells. Recombinant TRAIL induced apoptosis more effectively and more rapidly than recombinant TNF-α. With recombinant TRAIL (50 ng/mL), apoptosis was observed after only 4 hours of culture, but with recombinant TNF-α (50 ng/mL), apoptosis was observed after 12 hours of culture (data not shown). Colo 205 cells were moderately sensitive to recombinant IFN-α but were resistant to recombinant Fasl at all concentrations tested.

Because only high concentrations of cytokines used individually induced apoptosis, we determined whether combinations of recombinant TRAIL, TNF-α, and IFN-α at lower concentrations could induce apoptosis. As shown in Fig. 3, B, the combination of recombinant TNF-α and IFN-α (each at 10 ng/mL) or the combination of recombinant TNF-α and TRAIL (each at 10 ng/mL) induced 53% (95% CI = 43% to 63%) and 57% (95% CI = 51% to 64%), respectively, of Colo 205 cells to undergo apoptosis, whereas recombinant TNF-α, IFN-α, or TRAIL (each at 10 or 20 ng/mL) used alone did not induce apoptosis. Recombinant TNF-α and TRAIL acted synergistically because the combination index D was 0.45 (P <.001). The combination of recombinant TRAIL, TNF-α, and IFN-α (each at 10 ng/mL) induced 65% (95% CI = 50% to 80%) of cells to undergo apoptosis. When we tested recombinant TRAIL at 1.3 ng/mL and TNF-α at 200 pg/mL (which are the concentrations detected in CM-A), we did not observe any apoptosis of Colo 205 cells.

To determine whether the caspase cascade pathway is involved in Colo 205 cell death, we used the caspase inhibitor zVAD-fmk to specifically block caspase-mediated apoptosis. As shown in Fig. 4, A, zVAD-fmk at 50 μM dramatically reduced the number of apoptotic cells that were induced by CM-A (63% reduction, 95% CI = 53% to 73%), recombinant TRAIL (82%, 95% CI = 76% to 90%), or TNF-α (73%, 95% CI = 60% to 86%).

Recombinant DR5 at 5 μg/mL, which competitively binds to TRAIL and blocks the interaction between TRAIL and DR5 on target cells, inhibited apoptosis mediated by CM-A and recombinant TRAIL (Fig. 4, B). Only 35% (95% CI = 28% to 42%) of Colo 205 cells underwent apoptosis when cultured with re-
combinant TRAIL (50 ng/mL) and recombinant DR5, compared with 88% (95% CI = 77% to 99%) when cultured with TRAIL alone. The inhibition was greater when cells were cultured with CM-A and DR5—only 15% (95% CI = 8% to 22%) of cells were apoptotic—compared with 40% (95% CI = 30% to 50%) when cells were cultured with CM-A alone. Recombinant DR5 could also inhibit CM-A-mediated apoptosis in Colo 320 cells (65% inhibition, 95% CI = 61% to 69%) and Caco-2 cells (71% inhibition, 95% CI = 68% to 74%). Use of higher concentrations of recombinant DR5 (10 and 20 mg/mL) did not increase the inhibition of CM-A-mediated apoptosis.

**Effect of CM-A on the Expression of TRAIL Receptor in Colo 205 Cells**

Colo 205 cells were cultured alone (control) or with 50% CM-A for 24 hours, and then TRAIL receptors were measured.
The levels of TRAIL receptors DR4 and DR5 on Colo 205 cell membranes were approximately 13-fold and sixfold, respectively, higher in treated cells than in untreated control cells (Fig. 5). As measured by the mean fluorescence intensity (MFI), levels of DR4 and DR5 were 3.22 (95% CI = 1 to 5.5) and 4 (95% CI = 32 to 54) and 25 (95% CI = 20 to 30), respectively, for CM-A-treated cells. The expression of DR4 and DR5 was unchanged (Fig. 5): 8 (95% CI = 5 to 11) and 3 (95% CI = 1 to 5), respectively, for untreated cells and 9 (95% CI = 4 to 14) and 3 (95% CI = 1 to 5), respectively, for CM-A-treated cells.

We also investigated the combination of recombinant TNF-α and IFN-α and the combination of recombinant TNF-α and TRAIL on Colo 205 cells. Recombinant IFN-α alone moderately increased the expression of DR4 by 3.5-fold, but increased DR5 expression by only 1.33-fold (Table 2). The combination of recombinant TNF-α and IFN-α induced a 5.6-fold increase in DR4 expression and a 1.22-fold increase in DR5 expression. Recombinant TNF-α, TRAIL, and IFN-α, alone or in combination, did not alter the expression of DcR1 and DcR2 on Colo 205 cells, i.e., the increase of DcR1 or DcR2 expression was always less than 1.1.

**DISCUSSION**

To investigate the possible effects that macrophages may have on colorectal cancer cells, we developed a human cell culture model that approximates physiologic conditions. Culture supernatants from macrophages that were isolated from pleural effusions of patients with benign or malignant diseases were used to stimulate Colo 205, Colo 320, and Caco-2 colon adenocarcinoma cell lines. Our findings indicate that macrophages consistently produced similar amounts of TNF-α and IFN-α, irrespective of the patient’s disease. However, macrophages from some cancer patients produced higher levels of TRAIL than macrophages from other cancer patients or from noncancer patients, suggesting that the former macrophages may have been activated by tumor cells in the pleural space. We also showed that TRAIL was the most active of the three apoptosis-associated cytokines secreted by macrophages into conditioned medium.

We found that, for macrophages isolated from pleural effusions, the level of TRAIL produced by macrophages was independent of the number of tumor cells in the corresponding pleural effusion. High levels of TRAIL were produced by macrophages that had been in contact with small numbers of malignant cells (CM-A), and low levels of TRAIL were produced by macrophages that had been in contact with large numbers of malignant cells or no malignant cells (CM-B). Thus, the number of tumor cells to which macrophages were exposed in the pleural effusion appeared to directly affect the production of TRAIL by macrophages. Macrophages may need to be exposed to malignant cells to produce TRAIL, but exposure to a large number of cancer cells appears to reduce the production of TRAIL. A similar effect has been described for the production of TNF-α, and this effect is inhibited by exposure of the macrophages to immunosuppressive molecules such as interleukin 10 that are secreted by tumor cells [33,34].

We investigated whether conditioned medium from macrophages had antitumor activity against Colo 205, Colo 320, and Caco-2 colon tumor cells and found that CM-A containing high levels of TRAIL induced apoptosis in tumor cells but that CM-B containing low levels of TRAIL did not induce apoptosis, despite similar concentrations of TNF-α and IFN-α in both CM-A and CM-B. Thus, TRAIL appears to play a predominant role in the induction of apoptosis in Colo 205 cells. Ligand-bound death receptors of the TNF receptor family, such as TRAIL receptors, activate a signaling pathway that activates the caspase cascade. This caspase cascade can be blocked by the caspase inhibitor zVAD-fmk [35]. In our model, apoptosis was blocked by zVAD-fmk, suggesting that apoptosis in our model involved the caspase cascade pathway. Conditioned medium-mediated apoptosis was complete, as demonstrated by DNA fragmentation detected by the TUNEL method. Finally, recombinant DR5 inhibited 62.5% of CM-A-induced apoptosis, demonstrating that TRAIL participated in CM-A-mediated cell death. However, because the inhibition was not complete, we cannot exclude the possibility that other factors produced by

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**Fig. 3.** Recombinant tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), TNF-α, interferon-α (IFN-α), Fas ligand (FasL), and Colo 205 cells apoptosis. A) Dose-response experiments with individual cytokines. Colo 205 cells were cultured for 24 hours with increasing amounts of recombinant TRAIL, TNF-α, IFN-α, or FasL (from 0 to 100 ng/mL). Apoptosis was quantified by the Annexin V method and flow cytometry. Results are expressed as the percentage of apoptotic Colo 205 cells (mean ± 95% confidence intervals [CIs] of five experiments). B) Combination cytokine treatment. Colo 205 cells were cultured for 24 hours alone (control) or with recombinant TRAIL, TNF-α, and/or IFN-α at 10 ng/mL, separately or in combinations, as indicated. Apoptosis was analyzed by the Annexin V method and flow cytometry. Results are presented as the mean ± 95% CIs of five experiments.
Macrophages may play a role in conditioned medium-induced apoptosis. We showed that only TRAIL and TNF-α of the cytokines tested were able to induce Colo 205 cells to undergo apoptosis, with TRAIL being more efficient and exerting a more rapid effect than TNF-α. Colo 205 cells contain high levels of DR4 and DR5 cell-surface TRAIL death receptors but contain TNF-R1, the only known TNF-α death receptor, which may explain this observation. TNF-α can induce apoptosis in tumor cells and also promote tumor invasion and metastasis (36). In breast cancer, TNF-α produced by tumor-infiltrating macrophages increases angiogenesis (37). However, the severe toxicity of TNF-α in normal tissues has ruled it out for cancer therapy (38,39). Consequently, we tested recombinant TRAIL in combination with recombinant TNF-α and/or IFN-α. TRAIL and TNF-α acted synergistically to induce Colo 205 cells to undergo apoptosis, perhaps because TRAIL death receptors and TNF-α death receptors use similar signaling pathways to induce apoptosis. DR4, DR5, and TNF-R1 activate the adapter proteins TRADD (TNF receptor-associated death domain) and FADD (Fas-associated death domain) (45). FADD in turn activates caspase 8, thereby initiating a series of caspase-dependent events that lead to cell death. Therefore, low levels of TNF-α and TRAIL may stimulate death receptors enough to activate FADD protein, which in turn activates caspase 8.

Griffith et al. (14,15) showed that a wide variety of human tumor cell lines are sensitive to TRAIL in vitro, and the antitumor activity of TRAIL has been demonstrated against human thyroid and colon cancer cells transplanted into nude mice (41–43). Moreover, the tumor doubling times of established Colo 205 tumors in mice receiving daily intravenous injections of soluble recombinant human TRAIL are statistically significantly longer in a TRAIL dose-dependent manner (44). We also demonstrate that, although Colo 205 cells were sensitive to TRAIL-mediated apoptosis, they were resistant to FasL-mediated apoptosis, as observed with human melanoma cell lines (16).

Oligomeric TRAIL is more biologically active than monomeric TRAIL. The fact that recombinant TRAIL forms oligomers less efficiently than natural TRAIL (information provided by R&D Systems) could account for our findings that only high levels of recombinant TRAIL (50 ng/mL) induced apoptosis in our Colo 205 cell model and that the TRAIL concentration in conditioned medium was quite low (1 ng/mL). Consequently, we tested recombinant TRAIL in combination with recombinant TNF-α and/or IFN-α. TRAIL and TNF-α acted synergistically to induce Colo 205 cells to undergo apoptosis, perhaps because TRAIL death receptors and TNF-α death receptors use similar signaling pathways to induce apoptosis. DR4, DR5, and TNF-R1 activate the adapter proteins TRADD (TNF receptor-associated death domain) and FADD (Fas-associated death domain) (45). FADD in turn activates caspase 8, thereby initiating a series of caspase-dependent events that lead to cell death. Therefore, low levels of TNF-α and TRAIL may stimulate death receptors enough to activate FADD protein, which in turn activates caspase 8.
Because the concentration of TRAIL in our conditioned medium was too low to induce apoptosis, even in combination with TNF-α/H9251 or IFN-α/H9251, we investigated the expression of TRAIL receptors DR4 and DR5 on Colo 205 cell membranes. We found that treatment with CM-A increased the expression of TRAIL death receptors DR4 and DR5 but did not affect the expression of decoy receptors. Consequently, we hypothesize that increasing the expression of TRAIL death receptors increases the sensitivity of Colo 205 cells to TRAIL, even to low concentrations of TRAIL.

Regulation of the TRAIL receptors could be important for optimizing the therapeutic use of TRAIL. TNF-α/H9251 and TRAIL act synergistically to increase DR5 expression on epithelial tumor cells and to induce these cells to undergo apoptosis (46). In epithelial tumor cells, the binding of TNF-α to its receptor TNF-R1 increases the expression of DR5 and thereby enhances the ability of TRAIL to induce apoptosis. In our study, we observed that, in Colo 205 cells, the combination of TNF-α and IFN-α or the combination of TNF-α and TRAIL moderately increased the expression of DR4 but not of DR5. These findings raise the possibility that other macrophage-secreted molecules mediate the expression of DR5.

In summary, we have demonstrated for the first time, to the best of our knowledge, that human macrophages harvested from a metastatic site can, presumably, be activated by tumor cells located at that site to produce large amounts of TRAIL. We showed that TRAIL could induce Colo 205 tumor cells to un-

Table 2. Effect of recombinant TRAIL, TNF-α, and IFN-α molecules on expression of death and decoy receptors on Colo 205 cell membrane, as determined by flow cytometry

<table>
<thead>
<tr>
<th>Treatment of Colo 205 cells</th>
<th>DR4</th>
<th>DR5</th>
<th>DcR1</th>
<th>DcR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>4.5</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>CM-A</td>
<td>43</td>
<td>24</td>
<td>9</td>
<td>2.6</td>
</tr>
<tr>
<td>TRAIL</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
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<td>11</td>
<td>6</td>
<td>7.5</td>
<td>2.5</td>
</tr>
<tr>
<td>TNF-α + IFN-α</td>
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<td>2.5</td>
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<td>7</td>
<td>5</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>TRAIL + IFN-α</td>
<td>11</td>
<td>6</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

*TRAIL = TNF-related apoptosis-inducing ligand; TNF-α = tumor necrosis factor α; IFN-α = interferon α; DR = death receptor; DcR = decoy receptor; CM-A = conditioned medium A (culture supernatants from macrophages isolated from pleural effusions of patients with small numbers of tumor cells [1%–5%]). Data presented are representative of three experiments.
dergo apoptosis and that TRAIL and TNF-α act synergistically to induce apoptosis in these cells. The synergistic effect of TRAIL and TNF-α may have implications for subsequent clinical applications, i.e., TRAIL might act as a coactivator of tumor cell death that would permit the use of subtoxic doses of another anticancer drug. We also showed that macrophages produce molecules that increased the expression of death receptors DR4 and DR5 on tumor cells, which may make such cells susceptible to TRAIL-induced apoptosis at lower concentrations of TRAIL.

References


NOTES

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