Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells

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Ishimura, Norihisa, Hajime Isomoto, Steven F. Bronk, and Gregory J. Gores. Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells. Am J Physiol Gastrointest Liver Physiol 290: G129–G136, 2006. First published September 15, 2005; doi:10.1152/ajpgi.00242.2005.—Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising agent for cancer therapy; however, many cholangiocarcinoma cells are resistant to TRAIL-mediated apoptosis. Resistance to apoptosis may unmask TRAIL signaling cascades favoring tumor biology. Thus our aim was to examine whether TRAIL is expressed by human cholangiocarcinomas, and if so, to determine whether it promotes a malignant phenotype. To address this objective, TRAIL expression in human liver specimens was evaluated by immunohistochemistry. The effect of TRAIL on tumor cell migration, invasion, and proliferation was examined in three human cholangiocarcinoma cell lines. TRAIL expression was upregulated by cholangiocytes in preneoplastic disease, primary sclerosing cholangitis, and human cholangiocarcinoma specimens. TRAIL promoted tumor cell migration and invasion but did not induce cell proliferation. TRAIL-mediated cell migration and invasion was NF-kB dependent. These data demonstrate that TRAIL promotes cell migration and invasion via a NF-kB-dependent pathway in human cholangiocarcinoma cell lines, an observation that has a potential negative implication for TRAIL in cancer therapy.

CHOLANGIOCARCINOMA (CCA) is a highly malignant, generally fatal neoplasm originating from the bile duct epithelial cells or cholangiocytes of the intra- and extrahepatic biliary system (33). The incidence of this neoplasm is increasing in Western countries (12, 25, 37). Chronic inflammation of the bile duct, such as that occurring in primary sclerosing cholangitis (PSC), is a known risk factor for the development of this cancer (4, 5, 12, 38), and PSC can be considered a preneoplastic disease. CCA is a devastating disease that shows little response to most treatment modalities, including chemotherapy, radiation therapy, antihormonal treatment, or immunotherapy (9, 15, 45). Therefore, new treatment modalities will be necessary to effectively control and palliate this cancer.

Recently, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) has been shown to be a potential candidate for cancer therapy (46). TRAIL induces apoptosis in various cancer cells but not in normal tissues (1, 49). TRAIL, a type 2 transmembrane protein, was initially identified based on homology of its extracellular domain with Fas ligand (FasL) and TNF-α (50). TRAIL binds to its cognate death receptors (TRAIL-R1 and -R2) as a trimeric structure (29, 48). The binding results in receptor aggregation permitting recruitment of Fas-associated death domain (FADD) and pro-caspase 8 and 10 to the receptor complex. When recruited to this death-inducing signaling complex (DISC), these proteases self-activate by induced proximity, initiating apoptosis. When apoptotic pathways are blocked, TRAIL has also been demonstrated to activate additional signaling cascades including those involving NF-κB and c-Jun NH2 terminal kinase (JNK) pathways independent of its cytotoxic signaling. By activating these pathways, TRAIL can also promote cell differentiation and cell proliferation (6, 36).

CCA cells can be resistant to TRAIL-mediated apoptosis (41), likely due to enhanced expression of Mcl-1, a potent antiapoptotic protein of the Bcl-2 family (42). Apoptosis resistance may lead to unmasking of alternative TRAIL-initiated signaling pathways that potentially could favor tumor biology. For example, recent studies have shown that FasL, another death ligand of the TNF-α superfamily, promotes tumor progression by facilitating cell invasion and migration in apoptosis-resistant cells (3). Likewise, it has been shown that TRAIL induces a proangiogenic phenotype in nontransformed human endothelial cells, including an increase in both cell migration and invasion (35). TRAIL has also been found to be paradoxically expressed in human non-small cell lung cancer tumors and pancreas cancer (28, 39). The paradoxical expression of TRAIL in apoptosis-resistant human cancers suggests a potential role for TRAIL in cancer biology. However, the expression and function of TRAIL in apoptosis-resistant cancer cells remains obscure.

The objective of this study was to determine whether TRAIL promotes cell migration and/or invasion in human CCA cells. To address this objective, the following questions were formulated: 1) Is TRAIL expression upregulated in the preneoplastic biliary tract disease PSC and CCA? 2) Does TRAIL expression in human CCA cell lines promote cell migration and invasion in vitro? and 3) If so, by what signaling cascade does TRAIL promote tumor progression? Results of the present study demonstrate that TRAIL induces tumor cell migration and invasion via a NF-κB pathway in human CCA cells.

MATERIALS AND METHODS

Human liver tissue. This minimal risk study was approved by the Institutional Review Board. Archival liver specimens were obtained from patients who had undergone liver transplantation for PSC (N = 15) and primary biliary cirrhosis (PBC; N = 9) and surgical resection for CCA (N = 11). Normal liver specimens were obtained from 12 liver biopsy samples of 5 living and 7 deceased liver transplant donors. Patients with other potential etiologies for their liver disease were excluded from the study by examination of the medical record.
and rereview of the pathological specimens. Five frozen human CCA specimens with paired nontumor tissue were also obtained from a frozen tissue bank of surgical specimens.

**Immunohistochemistry.** Immunolabeling was performed using formalin-fixed paraffin-embedded blocks (5 μm thick). Freshly cut sections were deparaffinized in xylenes and rehydrated through sequential graded ethanol steps for 60 min. Slides were next incubated in 3% hydrogen peroxide for 10 min at room temperature and then incubated in blocking buffer [5% normal goat serum in PBS-0.05% Tween (PBS-T)] for 30 min also at room temperature. Samples were incubated in appropriate antibody dilution (1:1,000) in blocking buffer overnight at 4°C. After being washed three times with PBS-T, slides were incubated with the peroxidase-conjugated secondary antibody, which was obtained from the DAKO Envision™ system, horseradish peroxidase-conjugated 3,3'-diaminobenzidine tetrachloride (DAB) kit (DAKO; Carpinteria, CA), for 30 min at room temperature. Peroxi-
dase activity was detected by incubating the samples with 3,3'-DAB as the chromogen. Slides were subsequently counterstained with hematoxylin. Primary antibodies recognizing TRAIL was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mounted slides were examined by light microscopy, and immunoreactivity was assessed by two independent observers using a three-grade system, where 0 denotes the absence of staining; 1 denotes minimal and variable staining; and 2 denotes obvious, uniform, and intense staining. Only specimens with grade 2 immunoreactivity were considered positive.

**Cell lines and culture.** Three human CCA cell lines, KMBC-1, KMBC, and Mz-ChA-1, were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and gentamicin (100 mg/L) as previously described in detail (22, 27, 51). H69 cells, an immortalized but nonmalignant human cholangiocyte cell line, were cultured as previously described (14).

**Adenovirus 5 inhibitor of kB superrepressor.** The recombinant replication-deficient adenovirus (Ad) 5 inhibitor of kB (Ad-IκBx), containing an IκB in which serines 32 and 36 are mutated to alanines (19) (generous gift of D. A. Brenner, Columbia University, New York, NY), and adenovirus expression green fluorescent protein (Ad-GFP) were grown and purified as described previously [17, 34]. The mutated IκBx, referred to as the IκB superrepressor, cannot be phosphorylated and therefore remains complexed to NF-κB despite activation of the IKK complex, preventing NF-κB activation by the canonical pathway. The titer of Ad-IκBx and Ad-GFP was ~1 × 109 plaque-forming units/ml. For the determination of viral transduction efficiency, 1 × 105 cells were plated in six-well plates for 24 h. Cells were infected with Ad-GFP at a variety of multiplicities of infection (MOIs) from 1 to 500. Six hours after infection, the virus-containing medium was aspirated and replaced with normal growth medium for various MOIs. Cell viability was determined 15 min at 16,000 × g (4°C); the supernatant was collected, and the protein concentration determined using Bradford reagent (Sigma Chemical; St. Louis, MO) with BSA as the standard. Samples were resolved by 12.5% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with appropriate primary antibodies at a dilution of 1:1,000. Horseradish peroxidase-conjugated secondary antibodies (Biosource; Camarillo, CA) were incubated at a dilution of 1:3,000 for 1 h at room temperature. Bound antibodies were visualized using chemiluminescent substrate (ECL, Amersham; Arlington Heights, IL) and exposed to Kodak X-OMAT film (Kodak; New Haven, CT). The primary antibodies included goat anti-IκBx and goat anti-actin (obtained from Santa Cruz Biotechnology; Santa Cruz, CA) and rabbit anti-phospho-IκBx (specific for the phosphorylated forms of IκBx, obtained from Cell Signaling Technology; Beverly, MA).

**NF-κB DNA binding assay.** Cells were incubated in the presence or absence of TRAIL (2.5 ng/ml). The nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce; Rockford, IL) according to the manufacturer’s instructions. Nuclear protein extracts were screened for NF-κB DNA binding activity using the Trans AM assay (Active Motif; Carlsbad, CA) employing the NF-κB consensus binding motif 5'-GGGACTTTCTC-3' nucleotide sequence and p65 antiserum in a quantitative ELISA-like assay. Results are expressed as mean optical density (OD).

**Immunocytochemistry.** Cells were cultured on coverslips and fixed with PBS containing 4% paraformaldehyde for 20 min at 37°C. After being washed with PBS, cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature and then blocked with PBS containing 1% BSA for 60 min at 37°C. Cells were subsequently incubated with anti-NF-κB (p65) antibody (Santa Cruz Biotechnology) diluted 1:1,000 in blocking buffer at 4°C for 16 h. After being washed, coverslips were incubated with Cy3-conjugated goat anti-rabbit Ig (Jackson ImmunoResearch Laboratories; West Grove, PA) in blocking buffer. Cells were then washed three times in PBS and mounted using a Prolong Antifade Kit (Molecular Probes). Slides examined by fluorescent microscopy (LSM 510, Zeiss; Jena, Germany).

**Real-time PCR.** Total RNA was extracted from cells or human tissue using TRIzol Reagent (Invitrogen) and was reverse transcribed into cDNA with Moloney leukemia virus reverse transcriptase and random primers (both from Invitrogen). Quantification of the cDNA template was performed with a real-time PCR (LightCycler, Roche Molecular Biochemicals; Mannheim, Germany) using SYBR green (Molecular Probes). The PCR primers for SNF1/AMP kinase-related kinase (SNARK) were forward 5'-CACCTC-ACGGGAAGGTGAGA-3' and reverse 5'-CAATGATGTAGG-GGTTTGG-3'; the primers for human TRAIL were forward 5'-ACCAACGAGCTGAACGACAT-3' and reverse 5'-CAAGTGCA-AGTGCTCAGGA-3' (40). As an internal control, primers for 18S rRNA were purchased from Ambion (Austin, TX). After the electrophoresis in 1% agarose gel, each expected base pair PCR product was cut out and eluted into Tris-κCI using a DNA elution kit (Gel extraction kit, Qiagen; Valencia, CA). The concentrations of extracted PCR products (copies/μl) were measured using a spectrophotometer at 260 nm and used to generate standard curves. The inverse linear relationship between copy and cycle numbers was then determined. Each resulting standard curve was then used to calculate the copy number per microliter in experimental samples. The target mRNA expression level was calculated as the ratio of the target mRNA to 18S rRNA for each sample. All PCR conditions and primers were optimized to produce a single product of the correct base pair size.

**Migration and invasion assay.** Migration of tumor cells was evaluated using a Transwell chamber (Corning Coster; Cambridge, MA) equipped with a filter membrane with 8-μm pores. Human CCA cells were plated at 1 × 105 cells/well in DMEM containing 10% fetal bovine serum onto the upper compartment of the chamber. For invasion assays, cells (1 × 105 cells/well) were seeded onto the upper compartment of Matrigel-coated chambers with a filter membrane containing 8-μm pores (Beckton Dickinson; Bedford, MA). Cells were cultured in the absence or presence of TRAIL for 24–48 h. The filter membrane was removed, fixed with methanol, and stained with hematoxylin. The number of cells that had migrated to the lower surface of the filter membrane was counted in five random fields under a light scope (×200).

**Cell proliferation assay.** Proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) tetrazolium assay (Promega; Madison, WI), which measures the number of viable cells. Cells were plated at a density of 2.5 × 105 cells/well on 96-well plates. The assay was
initiated by adding 20 µL MTS solution reagent to 100 µL culture medium for each well. After being incubated for 4 h at 37°C, plates were read in a microplate autoreader at 490-nm wavelength. Results are expressed as the mean OD for selected paradigms performed in duplicate (n = 6).

Apoptosis assay. Apoptotic cells were counted by assessing the characteristic nuclear changes of apoptosis (i.e., chromatin condensation and nuclear fragmentation) using the nuclear binding dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Chemical) and fluorescence microscopy (24).

TRAIL ELISA. Cells were plated at a density of 1 x 10^5 cells/well on six-well plates. After 24 h, medium was replaced in the absence or presence of reagents. Cells were collected using cell lysis buffer at an appropriate time point and incubated as 37°C for 30 min. Cell lysate was then subjected to centrifugation for 15 min at 12,000 g (4°C); the supernatant was collected and stored at -80°C. TRAIL concentrations were measured using a Quantikine human TRAIL/TNFSF10 immunoassay kit (R&D Systems; Minneapolis, MN) according to the manufacturer’s instructions. Picograms of TRAIL per microgram of protein were determined by the calculation of total TRAIL production and quantity of cell protein.

Statistical analysis. All data represent at least three independent experiments and are expressed as means ± SD unless otherwise indicated. Differences between groups were compared using ANOVA for repeated measures and a post hoc Bonferroni test to correct for multiple comparisons. Significance differences between categorical variables were assessed by contingency table analysis. Differences with P < 0.05 were considered as significant.

Reagents. The NF-kB inhibitor BAY11-7082 was purchased from Calbiochem (San Diego, CA). Recombinant human TRAIL was purchased from R&D Systems.

RESULTS

TRAIL protein expression is paradoxically enhanced in PSC and CCA. TRAIL protein expression was assessed by immunohistochemistry in PSC, PBC, CCA, and normal human liver specimens. There was intense immunoreactivity for TRAIL in the biliary epithelia of PSC and CCA specimens but minimal immunoreactivity in PBC and normal livers (Fig. 1A). In normal liver specimens, moderate- to high-level expression (defined as a score of 2 on scale of 0–2) was only observed in 17% (2/12) of the normal liver specimens and 22% (2/9) of PBC specimens. In contrast, high-level TRAIL expression was observed in the majority of PSC and CCA liver specimens,

![Fig. 1. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression in the human liver. A: immunohistochemistry for TRAIL in normal liver tissue and tissue with primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and cholangiocarcinoma (CCA). Magnification: ×200. Weak expression was observed in normal and PBC ductal epithelium. In contrast, upregulated expression of TRAIL was frequently observed in PSC and CCA. B: quantitation of TRAIL immunoreactivity. High-level immunoreactivity for TRAIL cases (defined as a score of 2 on a scale of 0–2) relative to the total number of patient specimens was calculated as a percentage. High-level expression of TRAIL was only observed in PSC and CCA specimens. *P < 0.01 and **P < 0.001 compared with the normal liver.](http://ajpgi.physiology.org/10.220.33.1/2017/AJP-GastrointestLiverPhysiol-VOL290-JANUARY2006-SS471.html)
Fig. 2. TRAIL induces NF-κB activation in KMCH cells. A: immunoblot analysis of IκBα and phosphorylated (p)-IκBα. Cells were infected with adenoviruses (Ad) expressing IκBα (Ad-IκBα) at a multiplicity of infection of 100 for 6 h. Twenty-four hours after infection, cells were treated with recombinant TRAIL (2.5 ng/ml) for 8 h. Expression of Ad-IκBα inhibited phosphorylation of IκBα by TRAIL. B: TRAIL induces nuclear translocation of NF-κB (p65) in human CCA cells. Immunocytochemistry for NF-κB (p65) was performed before and after TRAIL treatment. Nuclear translocation of NF-κB (p65) was observed in TRAIL-treated KMCH cells. Nuclear translocation of NF-κB was inhibited by both the pharmacological NF-κB inhibitor BAY11-7082 (1 μM) and the transduction with Ad-IκBα superrepressor. C: TRAIL induced NF-κB DNA binding activity. Nuclear protein was extracted and screened for NF-κB DNA binding activity using the Trans AM assay (Active Motif). Data were expressed as the mean optical density (OD) for selected paradigms performed in duplicate (N = 3). Stimulation of TRAIL for 4 h significantly increased NF-κB activity, which was blocked by Ad-IκBα. *P < 0.01 compared with control.
80% (12/15) and 91% (10/11) of the specimens, respectively (Fig. 1B). We were able to confirm enhanced expression of TRAIL mRNA in five human CCA specimens relative to paired nontumor tissue; indeed, TRAIL mRNA was twofold or greater (range 2- to 16-fold) in CCA tissue than in paired nontumoral tissue from the same patient. These data suggest that TRAIL is paradoxically expressed in biliary epithelia of patients with the neoplastic disease PSC and in CCA.

**TRAIL induces NF-κB activation in apoptosis-resistant CCA cell lines.** Although TRAIL was not expressed in any of the CCA cell lines examined, its expression could be induced 11-fold by incubation with 5 ng/ml of interferon (IFN)-γ for 24 h (data not shown). Apparently, TRAIL expression is not an inherent characteristic of CCA but requires an external stimulus. Nonetheless, this paradigm is relevant to PSC, where the majority of the lymphocytes in the portal tracts are Th1-polarized, CD4-positive lymphocytes, which are known to secrete IFN-γ (7). Therefore, we next assessed the cellular response to exogenous TRAIL. Consistent with our prior observation (23, 42), all three cell lines examined were resistant to apoptosis at TRAIL concentrations of 0–5 ng/ml (data not shown). Therefore, to determine whether TRAIL activates nonapoptotic signaling pathways, CCA cells were incubated in the presence of exogenous TRAIL (2.5 ng/ml). Because TRAIL can activate NK-κB in other cell types and this transcription factor has been implicated in tumor biology (8, 47), we next determined whether TRAIL activated NF-κB in CCA cells. The exogenous administration of TRAIL resulted in NF-κB activation as assessed by multiple complementary experiments (Fig. 2). Initially, we observed that TRAIL treatment of KMCH cells was associated with phosphorylation and a reduction in IκBα, a prerequisite for NF-κB activation. More importantly, direct translocation of the NF-κB p65 subunit from the cytoplasm to the nucleus and an increase in NF-κB binding activity in nuclear extracts could be readily demonstrated (Fig. 2, A–C). Although the depicted results were obtained in the presence of serum, an equal magnitude of NF-κB activation was observed in cells deprived of serum for 24 h (data not shown). As assessed by all three assays, TRAIL-mediated NF-κB activation was blocked by transduction with the Ad-IκB superrepressor. In addition, the pharmacological NF-κB inhibitor BAY11-7082 (1 μM) inhibited nuclear translocation of the p65 subunit of NF-κB. Although the data depicted are for KMCH cells, virtually identical data were observed for all these paradigms in the other human CCA cell lines. mRNA expression of the the NF-κB target gene SNARK was measured in the presence and absence of TRAIL in KMCH cells. This NF-κB target gene was selected because it has been implicated in Fas/NF-κB-mediated cell motility (26). After cells were treated with TRAIL (2.5 ng/ml) for 4 h, SNARK mRNA expression was increased 1.7-fold (P < 0.05). Finally, NF-κB activation, as assessed by measuring DNA binding activity in nuclear extracts, was not observed in H69 cells, which undergo rapid and extensive apoptosis induction when incubated with TRAIL (data not shown). Taken together, these data demonstrate that TRAIL selectively activates NF-κB in apoptosis-resistant human CCA cell lines.

**TRAIL promotes tumor cell migration and invasion.** To test the hypothesis that TRAIL may function as a tumor-promoting ligand, we assessed the effect of TRAIL on CCA cell migration and invasion using the Transwell cell migration assay and Matrigel-coated chamber invasion assay, respectively. These two assays are complementary because the Transwell assay assesses cell migration, whereas the Matrigel-based assay requires invasion of the cells through the Matrigel layer plus migration. Incubation with recombinant TRAIL (0.5 ng/ml) increased Mz-Cha-1 cell migration almost twofold (Fig. 3, A and B). We next tested whether the TRAIL-induced cell migration was dependent on activation of NF-κB. Transduction of cells with the Ad-IκBα superrepressor or incubation with 1 μM BAY11-7082 blocked TRAIL-induced cell migration. Likewise, tumor invasion was promoted by TRAIL in Matrigel-coated Transwell chambers (Fig. 4A and B). Tumor cell invasion was also blocked by transduction with the Ad-IκBα superrepressor or treatment with 1 μM BAY11-7082. TRAIL treatment could potentially increase cell proliferation, which would interfere with quantification of the invasion assay (if the absolute cell numbers increased, the number but not the percentage of cells migrating across the chamber would also increase). To determine whether tumor cells responded to

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**Fig. 3.** TRAIL promotes tumor cell migration. A: migration of Mz-Cha1 cells was evaluated using a Transwell chamber (Corning Coster) equipped with a filter membrane with 8-μm pores. Cells were plated at 1 × 10⁵ cells/well in DMEM containing 10% fetal bovine serum onto the upper compartment of the chamber. Cells were cultured in the absence or presence of TRAIL (0.5 ng/ml) for 24 h. The filter membrane was removed, fixed with methanol, and stained with hematoxylin. B: the number of cells that had migrated to the lower surface of the filter membrane was counted in 5 random fields under a light scope (×200). TRAIL increased cell migration almost 2-fold for all cell lines, which was blocked by BAY11-7082 or Ad-IκBα. *P < 0.01 and **P < 0.001 compared with control.
TRAIL with increased proliferation, we performed a MTS assay (Fig. 4C). Exposure to TRAIL, however, did not increase cell proliferation. Thus TRAIL promotes cell migration and invasion of the human CCA cell line, a cell function necessary for cancer invasion and metastasis.

**Inhibition of NF-κB did not sensitize cells to TRAIL-induced apoptosis.** NF-κB activation can render selective cell types resistant to apoptosis, including TRAIL-mediated apoptosis. Therefore, we assessed the effect of NF-κB inhibition on potential TRAIL-mediated CCA cell apoptosis by transducing cells with the Ad-IκBα superrepressor (Fig. 5). The sensitivity of human CCA cells to TRAIL was examined morphologically. Incubation with human recombinant TRAIL (5 ng/ml for 24 h) did not sensitize KMCH cells to TRAIL-mediated cell death (Fig. 5). However, H69 cells readily underwent TRAIL-mediated cytotoxicity under these conditions (Fig. 5). These data demonstrate that NF-κB does not inhibit TRAIL-mediated apoptosis of human CCA cells.

**DISCUSSION**

The principal findings of this study relate to the cellular mechanisms by which TRAIL promotes a malignant phenotype.

**Fig. 4.** TRAIL promotes cell invasion and migration. A and B: invasion assay of Mz-ChA1 cells incubated with TRAIL (0.5 ng/ml) using Matrigel-coated chambers with a filter membrane containing 8-μm pores (Beckton Dickinson). Stimulation of TRAIL on CCA cells increased invasiveness, which was blocked by BAY11-7082 or Ad-IκBα. *P < 0.001 compared with the normal liver. C: TRAIL did not significantly alter cell proliferation. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. The OD 490-nm value was measured by a microplate autoreader. Recombinant TRAIL (0.5 ng/ml) did not significantly alter cell proliferation rates compared with nontreated KMCH cells.

**Fig. 5.** Inhibition of NF-κB activity did not sensitize KMCH or H69 cells to TRAIL-induced apoptosis. Cells were transduced with Ad-IκBα or Ad-green fluorescent protein (GFP) for 24 h. Subsequently, cells were treated with 5 ng/ml TRAIL for 24 h. Apoptosis was quantitated using 4',6-diamidino-2-phenylindole dihydrochloride staining and fluorescence microscopy. Data are expressed as means ± SD; n = 3 for each experiment.
in CCA cells. The observations demonstrate the following: 1) TRAIL is paradoxically upregulated by cholangiocytes in PSC, a preneoplastic biliary tract disease, and this expression pattern was retained by human CCA; 2) TRAIL induces NF-κB activation in apoptosis-resistant CCA cell lines; 3) TRAIL promotes tumor cell migration and invasion but does not alter cell proliferation; and 4) inhibition of NF-κB reduces TRAIL-mediated cell migration and invasion but does not sensitize cells apoptosis. Each of these findings is discussed in greater detail below.

TRAIL was observed to be paradoxically upregulated by cholangiocytes in the preneoplastic, inflammatory biliary tract disease PSC. More importantly, this expression pattern was retained by human CCA. Consistent with these observations, increased expression of TRAIL also has been observed in other human cancers, including human non-small cell lung cancer tumors and pancreas cancer (28, 39). Although TRAIL was not expressed by the three human CCA cell lines examined, its expression was induced by IFN-γ, a cytokine present in chronic biliary tract inflammatory disorders (7, 44). TRAIL expression, therefore, may not be an intrinsic property of malignant transformation of CCA cells but instead likely represents a response to external cytokines and inflammation. Because chronic inflammatory disorders of the biliary tract such as PSC predispose to the development of CCA, our results suggest a potential role for TRAIL in this process.

NF-κB is a transcription factor that has been implicated in cellular immune and inflammatory responses, proliferation, tumor migration and invasion, and protection of cells from apoptosis (2, 11, 16). The current observations, demonstrating TRAIL-induced NF-κB activation in human CCA cell lines, are consistent with reports that TRAIL has been reported to activate NF-κB in hepatocellular carcinoma cells (21) and colon carcinoma cell lines (18). Because aberrant activation of NF-κB has been implicated in the development and progression of human and murine cancers (8, 31), TRAIL-induced NF-κB activation may play a role in the pathogenesis of CCA. Our data suggest that TRAIL-mediated NF-κB activation promotes CCA cell migration and invasion. Indeed, another death ligand, FasL, which is also expressed in CCA cells (30, 32), has also been shown to promote tumor cell invasion in apoptosis-resistant cells (26). In this prior study (26), FasL induced the NF-κB target gene serine/threonine kinase SNARK, which stimulated cell migration and invasion. Indeed, in the current study, we also observed that TRAIL increased expression of this kinase in CCA cells. More recently, another death ligand, TNF-α, has been also reported to promote invasiveness of CCA cell lines through NF-κB activation (43). Collectively, these data demonstrate that NF-κB activation is required for the novel nonapoptotic function of death receptor ligands including FasL, TNF-α, and now TRAIL as tumor-promoting agents in apoptosis-resistant cells.

TRAIL-induced NF-κB activation could potentially inhibit TRAIL-induced apoptosis via the induction of genes whose products provide resistance to apoptosis. For example, inhibition of NF-κB signaling augments TRAIL-mediated apoptosis in neuroblastoma cells (20) and melanoma cells (10). However, our data demonstrate that NF-κB inhibition did not sensitize CCA cells to TRAIL-induced apoptosis. Although our data are at odds with those reports, this discrepancy is likely explained by the upregulated expression of Mcl-1, an antiapoptotic Bcl-2 protein in CCA cells. Indeed, Mcl-1 regulates the sensitivity to TRAIL-mediated cytotoxicity in CCA cells (42). Moreover, we have recently demonstrated that Mcl-1 was regulated by Akt (23), which has been shown to promote CCA cell growth (13). Thus Mcl-1, but not NF-κB, mediates TRAIL resistance in CCA cells.

In the current study, TRAIL promoted cell migration without further enhancing cell proliferation. Cancer cell invasion requires the suppression of anoikis, stimulation of cell motility, and the ability to breakdown the stromal matrix, whereas cell proliferation requires the complex interplay between various cell cycle genes. The two processes, tumor invasion and proliferation, although both features of advanced cancer, are regulated by different cellular processes. Our data demonstrating that TRAIL enhances cell migration in a proliferating cell line without further enhancing cell growth is consistent with these current concepts in tumor biology.

In conclusion, TRAIL potently stimulates cell migration and invasion in CCA cells and is likely to be involved in invasion, dissemination, and metastasis of CCA through a NF-κB dependent pathway. Given the expression of TRAIL in the inflammation, preneoplastic disease PSC, TRAIL may also participate in NF-κB-driven neoplasia development and progression. Although TRAIL is being promoted as a promising anticancer drug, TRAIL-mediated invasiveness of apoptosis-resistant cancer cells may restrict its use to apoptosis-sensitive tumors. Indeed, as a cautionary note, our data suggest TRAIL therapy could represent a potential risk for patients with TRAIL-resistant cancers.

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