Cloning and apoptosis-inducing activities of canine and feline TRAIL

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Abstract

The apoptosis process is crucial to various biological processes including embryo development and organism homeostasis. Inducing apoptosis of cancer cells has become a very attractive field for cancer therapy in the recent years. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; also called Apo2L, TNFSF10, CD253, or TL2) is a member of tumor necrosis factor family. Preclinical studies showed that human TRAIL induced apoptosis of various tumor cell lines, whereas nontransformed normal cell lines were not affected. We have cloned both canine and feline TRAIL full-length genes by using Rapid Amplification of cDNA Ends-PCR technology. Truncated soluble versions of the canine and feline TRAIL genes were also constructed. The degree of identity between canine TRAIL protein and the human, mouse, chicken, porcine, and rat homologues is 81.3%, 61.7%, 54.3%, 82.9%, and 63.2%, respectively. The degree of identity between the feline TRAIL protein and the human, mouse, chicken, porcine, and rat homologues is 84.2%, 64.2%, 54.4%, 86.8% and 65.7%, respectively. The identity between the canine and feline TRAIL proteins is 93.2%. The canine and feline soluble TRAIL proteins were expressed in both mammalian and bacterial expression systems. Western immunoblot assays with TRAIL-specific antibody confirmed the identity of expressed protein. Both canine and feline TRAIL proteins were shown to specifically induce apoptosis and inhibit cell growth of cancer cells at a level comparable with their human counterpart. [Mol Cancer Ther 2008;7(7):2181–91]

Introduction

Cancer is not only devastating to humans but also is the most common cause of natural death in dogs (1–4). Dogs develop tumors twice as frequently as humans do, and it has been reported that 45% to 50% of dogs that live to ≥10 years succumb to cancer regardless of age and that 23% of dogs that present for necropsy died of cancer. Combination treatment involving surgical removal of the tumor and adjuvant chemotherapy is the most common treatment, but the prognosis for dogs having an invasive/metastatic tumor is poor, with median survival time ranging from weeks to months. Other treatments, such as radiation therapy and palliative treatment, have only limited success (5, 6).

Apoptosis is involved in very important physiologic roles such as elimination of virus-infected cells, deletion of activated lymphocytes at the end of immune responses, and removal of abnormal cells in the body (7). Apoptosis can be induced by a variety of stimuli that include anticancer drugs as well as death factors (8). The mechanisms relating to death factors involve the interactions between cell-surface death receptors and death ligands including tumor necrosis factor, Fas/CD95L, and LT-α. However, the potential use of these three proteins for cancer treatment is limited because of their acute toxic effect on normal tissues in vivo. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; also called Apo2L, TNFSF10, CD253, or TL2) is a type II transmembrane protein that was initially identified because of its extracellular domain homology with tumor necrosis factor, CD95L, and LT-α (9). The TRAIL protein has been reported to induce apoptosis in various tumor cell lines but not in nontransformed, normal cells (9–11). In addition, preclinical studies in mice and nonhuman primates have shown that administration of TRAIL can induce apoptosis in human tumors but that no cytotoxicity in normal organs or tissues is found (10, 12, 13). The reported normal hepatocyte and keratinocyte toxicity (14–16) was attributed to the presence of a polyhistidine tag and lack of the optimum amount of zinc (17, 18).

Unlike other tumor necrosis factor family members whose expressions are tightly regulated and are often only transiently expressed on activated cells, TRAIL mRNA is constitutively expressed in a wide range of tissues (9). However, the TRAIL protein was not found on the surface of a variety of cells of immune system and its presence could only be detected after stimulation with IFN (19–22). Work with mice in which TRAIL is blocked with neutralizing antibodies and in TRAIL−/− transgenic knockout mice suggested that TRAIL contributes to host immunosurveillance against tumor development (20, 23). Furthermore, various roles of TRAIL have been proposed in autoimmune (24) as well as preventing breast cancer–induced bone destruction in a mouse model (25).
TRAIL can bind to five receptors: four membrane bound and one soluble (26–34). Two of the membrane receptors, death receptor 4 (DR4 or TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2, KILLER, or TRICK2), contain a cytoplasmic death domain through which TRAIL transmits an apoptotic signal. Two other membrane receptors, decoy receptor 1 (Dr(1), TRAIL-R3, or TRID) and decoy receptor 2 (Dr(2), TRAIL-R4, TRUNDD, or LIT), do not have an intact death domain. The fifth soluble receptor, osteoprotegerin, only binds TRAIL with low affinity. The existence of decoy receptors and their widespread expression on normal cells was initially seen as the explanation for protection of normal cells against TRAIL-induced apoptosis. However, there was no correlation between TRAIL resistance and the levels of its decoy receptors in some cancer cell lines (35). The alterations in expression and function of Bcl-2 family members may contribute to TRAIL sensitivity and resistance (26).

This report describes the cloning of both canine and feline full-length TRAIL genes, encoding canine and feline full-length TRAIL proteins, as well as truncated soluble versions. The TRAIL protein sequences were compared with published human, mouse, chicken, porcine, and rat TRAIL sequences. We have expressed both forms of TRAIL proteins in mammalian cells as well as in bacterial cells. Apoptosis-inducing activities were shown with the expressed proteins. These activities suggest that canine and feline TRAIL may be useful as an anticancer therapeutic tool for dogs and cats.

Materials and Methods
Isolation of RNA from Canine and Feline Cell Lines and Reverse Transcription-PCR Amplification

Three cell lines were obtained from the American Type Culture Collection: CRL-6130 (Fc28.Lu, feline lung), CRL-6252 (ECF50.HT, canine heart), and CRL-6569 (Fc2.Lu, feline lung). Total RNA was purified using a 5' Total RNA Isolation System following instructions from the manufacturer (Promega).

Two pairs of primers were designed to amplify internal regions of canine and feline TRAIL cDNA based on consensus sequences from human (accession no. U37518) and mouse (accession no. U37522). The 5' primer of both sets, N4066E09 (Table 1), corresponded to nucleotides 382 to 408 of human TRAIL cDNA and nucleotides 354 to 380 of mouse TRAIL cDNA. The 3' primer of set 1, N4066E06, corresponded to nucleotides 634 to 669 of human TRAIL cDNA. The 3' primer of set 2, N4066E11, corresponded to nucleotides 841 to 866 of human TRAIL cDNA. The reverse transcription-PCR was done using a Ready-to-Go Reverse Transcription-PCR Kit from Amersham Pharmacia (Illinois).

Rapid Amplification of cDNA Ends-PCR of Canine and Feline TRAIL Fragments

Due to the lack of DNA sequence homology between human and murine TRAIL, three internal TRAIL oligo primers were used in Rapid Amplification of cDNA Ends (RACE)-PCR. They were N4066E06 and N4066E07, corresponding to nucleotides 634 to 669 of human TRAIL cDNA as well as nucleotides 605 to 640 of mouse TRAIL cDNA and N4066E09. The RACE-PCR was done using SMART RACE cDNA Amplification Kit from Clontech Laboratories. The first-strand cDNA synthesis was carried out to prepare 5'-RACE-Ready cDNA and 3'-RACE Ready cDNA, respectively. RACE was carried out according to Clontech’s instruction manual.

Southern Analysis

The DNA transfer for Southern analysis was carried out with a Turboblotter of Schleicher & Schuell. Human TRAIL DNA probe was made with a DIG kit from Boehringer Mannheim. Human spleen marathon-ready cDNA (Clontech) was mixed together with TRAIL-specific primers N4066E12 and N4066F02, PCR buffer, DIG mix, and enzyme mix. Prehybridization was carried out at 42°C for 3 h. Hybridization with DIG-labeled TRAIL PCR DNA was carried out overnight at 42°C. Enhanced chemiluminescence detection was done according to Amersham’s instruction.

Cloning of TRAIL RACE DNA and Assembly of Full-Length Canine and Feline TRAIL Sequences

The canine and feline RACE PCR products were cloned into Eukaryotic TA cloning vector pUni/V5-His-TOPO (Invitrogen). DNA sequences were obtained for the clones and full-length canine and feline sequences were assembled using DNAsStar Seqman program (DNAsStar).

The TRAIL protein sequence alignment and the phylogenetic tree were generated using DNAsStar MegAlign (ClustalW, Slow/Accurate, Gonnet).

Cloning of Full-Length Canine and Feline TRAIL

To clone full-length canine TRAIL gene, dog heart total RNA (CRL-6252) was used for the first-strand cDNA synthesis reaction in the presence of oligo pdN6 and AMV reverse transcriptase. The DNA was then used as templates for PCR. The primers used were N7429A12 for the 5' primer and N9294G07 for the 3' primer. Pfx polymerase (Life Technologies) was used in the PCR. An abundant band (0.9 kb) was further confirmed by Southern analysis using DIG-labeled human TRAIL DNA as a probe.

To obtain full-length feline TRAIL gene, 5'-RACE-Ready DNA and 3'-RACE-Ready DNA were used as templates for PCR, respectively. The primers used were N7974C01 for the 5' primer and N7974C02 for the 3' primer. Possible full-length TRAIL DNA was further analyzed by Southern analysis as described above using DIG-labeled human TRAIL DNA as the probe. The PCR products (0.9 kb) were excised and isolated from the agarose gel using a QIAquick Gel Extraction Kit. The purified band was then cloned into pUni/V5-His-TOPO cloning vector.

Cloning of Soluble Canine and Feline TRAIL

Human soluble TRAIL gene was used as a positive control. Human spleen marathon-ready DNA, as well as canine and feline RACE-Ready DNA and 3'-RACE-Ready DNA, was used in the PCR with Advantage HF (high-fidelity DNA polymerase; Invitrogen). The 5' primer for human soluble TRAIL used was N7974C06. The 3' primer,
His-TOPO cloning vector. The above PCR product was then cloned into pUni/V5-His-TOPO cloning vector: 5
malian Expression Vector soluble TRAIL the V5tag and His tag in the vector. The 5
primer to terminate translation; the vector-encoded PDGFR transmembrane domain downstream of the insert was not translated in the final plasmid construct.

Cloning of Soluble Human, Canine, and Feline TRAIL into Bacterial Expression Vectors

Human, canine, and feline TRAIL genes in pUni/V5-His-TOPO cloning vectors were recombined with pBAD/Thio-E, in the presence of Cre recombinase, according to Invitrogen’s manufacturer instructions. There was a Thio protein (thioredoxin) fusion at the NH2 termini of all the proteins.

Soluble canine and feline TRAIL were also cloned into bacterial expression vector pBAD/His A. Only the native TRAIL proteins were expressed with no Thio protein fusion at the NH2 terminus. The PCR was carried out with Pfu polymerase and the primers used were canine TRAIL 5’ primer, R1886B08, and 3’ primer, 43114-002, to generate a V5-His tag fusion. The feline 5’ primer was 43114-004 and the feline TRAIL 3’ primer was 43114-006 to generate a V5-His tag fusion. The PCR DNA was digested with NeoI and PsI and cloned into the pBAD/Myc-His vector.

Human, canine, and feline soluble TRAIL plasmids were also recombined with pCRT7-E, in the presence of Cre Recombinase, according to Invitrogen’s manufacturer instructions. The new bacterial expression plasmids put the TRAIL genes under bacterial promoter pT7, which is inducible by isopropyl-L-thio-β-D-galactopyranoside.

Transfection of TRAIL

Human 293 cells were transfected with mammalian expression plasmids encoding soluble canine or feline TRAIL using LipofectAMINE Plus per manufacturer’s instructions (Life Technologies).

Expression of Soluble TRAIL-Thio and TRAIL Proteins Under pBAD-Thio and pBAD Promoters in Bacteria

Bacterial expression plasmids (human, canine, or feline soluble TRAIL under pBAD-Thio or pBAD promoters) were transformed into Escherichia coli TOP 10 cells (Invitrogen). Single colonies were inoculated in L broth containing 50 μg/mL kanamycin and three different

Table 1. Oligos used in the study

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<tr>
<th>Name</th>
<th>Use</th>
<th>Sequence</th>
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R4289C10, created a fusion with the V5 tag and His tag in the vector. The 5’ primer for canine soluble TRAIL was N8071H11. The 3’ primer, R1886B08, created a fusion with the V5 tag and His tag in the vector. The 5’ primer for feline soluble TRAIL was N8071H10. The 3’ primer, R1886B10, created a fusion with the V5 tag and His tag in the vector. The above PCR product was then cloned into pUni/V5-His-TOPO cloning vector.

Cloning of Soluble Canine and Feline TRAIL into Mammalian Expression Vector

The soluble portion of the canine and feline TRAIL genes was cloned into pDisplay vector (Invitrogen) by PCR amplification of soluble canine and feline TRAIL genes in pUni/V5-His-TOPO cloning vector: 5’ primer for canine TRAIL gene, S2633B10; 5’ primer for feline TRAIL gene, S2633B09; and 3’ primer, S2633B11. To facilitate cloning, two restriction enzyme sites Bgl II (5’ primer) and Pst I (3’ primer) were incorporated into the primer sequences as shown by italics (Table 1). The insert was fused in-frame to the signal peptide and HA epitope sequences present in the vector. The stop codon TAA (shown in bold) of TRAIL was included in the 3’ primer to terminate translation; therefore, the vector-encoded PDGFR transmembrane domain downstream of the insert was not translated in the final plasmid construct.

Cloning of Soluble Human, Canine, and Feline TRAIL into Bacterial Expression Vectors

Human, canine, and feline TRAIL genes in pUni/V5-His-TOPO cloning vectors were recombined with pBAD/Thio-E, in the presence of Cre recombinase, according to Invitrogen’s manufacturer instructions. There was a Thio protein (thioredoxin) fusion at the NH2 termini of all the proteins.
concentrations of arabinose were added to each culture: 0.2%, 0.02%, and 0.002%. The cultures were incubated for an additional 4 h at 37°C with agitation; 1 mL of each culture was then centrifuged and lysed in 100 µL of 1× sample buffer and separated with 10% Bis-Tris gel (Invitrogen) stained with 0.25% (w/v) Brilliant Blue R.

The solubility of TRAIL-Thio and TRAIL expressed proteins was examined by two cycles of freeze/thaw of the bacterial lysate followed by sonication (Branson Sonifier 250) at 20% duty cycle with output control of 3. The whole lysate was then centrifuged for 1 min at 12,000 rpm to separate the soluble fraction from the insoluble fraction (pellet). Equivalent volumes of both fractions were loaded onto a protein gel and stained with 0.25% Brilliant Blue R.

To purify TRAIL proteins expressed in bacterial cultures, the bacterial cells were lysed with an Anderson Emulsi FlexC5 mechanical lysis devise. The lysates were centrifuged and supernatant was loaded onto the pre-wet His-band Quick 900 cartridges following the manufacturer’s instructions (Novagen). The initial flow-through was reloaded to the columns to further enhance the binding of TRAIL protein to the columns. Each eluted fraction was loaded onto a protein gel and stained with Silver stain (Invitrogen). Duplicate samples were analyzed and confirmed with Western analysis using anti-TRAIL polyclonal AF375 (R&D Systems). Fractions that were positive for canine or feline TRAIL were pooled together and dialyzed (Slide-A-Lyzer cassette, 7000 MWCO, 3-12 mL; Pierce) overnight.

**Western Analysis**

The culture supernatants of transfected cells or purified bacterial fractions were separated by 10% Bis-Tris gel and transferred to a nitrocellulose membrane (Novex). The initial flow-through was reloaded to the columns to further enhance the binding of TRAIL protein to the columns. Each eluted fraction was loaded onto a protein gel and stained with Silver stain (Invitrogen). Duplicate samples were analyzed and confirmed with Western analysis using anti-TRAIL polyclonal AF375 (R&D Systems). Fractions that were positive for canine or feline TRAIL were pooled together and dialyzed (Slide-A-Lyzer cassette, 7000 MWCO, 3-12 mL; Pierce) overnight.

**Results**

Identification of Canine and Feline TRAIL Genes

Total RNA was isolated from three cell lines (one canine heart cell line and two feline lung cell lines). To test the gene expression of TRAIL in those cell lines, two pairs of primers were designed to amplify internal regions of canine and feline TRAIL cDNA based on consensus sequences from human and mouse. One pair of primers (N4066E09 + N4066E06) generated a 504-bp PCR fragment, whereas the other (N4066E09 + N4066E11) generated a 288-bp fragment. The PCR product was easily amplified in all three cell lines (data not shown).

**Cloning of TRAIL RACE DNA and Assembly of Full-Length Canine and Feline TRAIL Sequences**

The RACE PCR products were cloned into eukaryotic TA cloning vector pUni/V5-His-TOPO. Four independent clones containing inserts of the expected size (0.7 kb for RACE primer E06, 1.1 kb for RACE primer E07, and 0.7 kb for RACE primer E09) were sequenced. The sequences were assembled and analyzed using DNAStar Seqman. The nucleotide sequence of canine TRAIL and the corresponding protein sequence have been deposited at GenBank (EU036633). Similarly, the nucleotide sequence of feline TRAIL and the corresponding protein sequence can be found at GenBank (EU036634).

Figure 1 shows the alignment of all known amino acid sequences of TRAIL. The degree of identity between canine TRAIL protein and that of human (GenBank P50591, ref. 9), mouse (GenBank NP_033451; ref. 9), chicken (GenBank BAC79267 and CAG31850; refs. 36, 37), porcine (GenBank AA154594), and rat (GenBank EFO30546) is 81.3%, 61.7%, 54.3%, 82.9%, and 63.2%, respectively. The degree of identity between feline TRAIL protein and that of human, mouse, chicken, porcine, and rat is 84.2%, 64.2%, 54.4%, 86.8%, and 63.7%, respectively. The identity between
The canine and feline TRAIL protein is 93.2%. Position 126 to 301 shows the alignment of all known amino acid sequences of soluble TRAIL (Fig. 1). The degree of identity between soluble canine TRAIL protein and that of human, mouse, chicken, porcine, and rat homologues is 82.1%, 70.3%, 61.6%, 85.4%, and 70.7%, respectively. The degree of identity between soluble feline TRAIL protein and that of human, mouse, chicken, porcine, and rat homologues is 85.8%, 72.3%, 62.2%, 90.1%, and 72.6%, respectively. The identity between soluble canine and feline TRAIL proteins is 93.2%. The phylogenetic tree of the full-length TRAIL proteins is shown in Fig. 2.

Cloning of Full-Length Canine and Feline TRAIL Genes

To clone full-length canine TRAIL gene, dog heart total RNA was used for the first-strand cDNA synthesis reaction, which then was used as a template for PCR using primers N7429A12 and N9294G07. An abundant band (0.9 kb) was further confirmed by Southern analysis using DIG-labeled human TRAIL DNA as a probe. This PCR product was then cloned into a pUni/V5-His-TOPO cloning vector. The full-length feline TRAIL gene was obtained by PCR using N7974C01 and N7974C02. Possible full-length feline TRAIL DNA was further analyzed by Southern analysis using DIG-labeled human TRAIL DNA as the probe. This PCR product (0.9 kb) was cloned into pUni/V5-His-TOPO cloning vector.

Cloning of Soluble Canine and Feline TRAIL Genes

The soluble version of human TRAIL protein, which contains the extracellular portion of the gene, was shown to possess apoptosis-inducing activity for cancer cells (tumor-killing activity; ref. 10). This form of protein can be conveniently produced in bacteria (12). To clone the soluble versions of canine and feline TRAIL genes, primers were designed based on the sequence homology with the human protein sequence and first-strand cDNA was used as a template. The soluble extracellular portion of each gene (human, canine, and feline TRAIL) was cloned into pUni/V5-His-TOPO cloning vector. Two versions of the clones were designed: one possessing a COOH-terminal V5-His tag and the other contained only the native protein without tags.

Cloning of Soluble Human, Canine, and Feline TRAIL Genes into a Mammalian Expression Vector

The soluble portion of the canine and feline TRAIL genes was subcloned into the pDisplay vector by PCR amplification of soluble canine and feline TRAIL genes in pUni/V5-His-TOPO cloning vectors. The insert was fused in-frame to the signal peptide and the HA epitope sequences present in the vector. The stop codon TAA of TRAIL was included in the primer to terminate translation; therefore, the vector-encoded PDGFR transmembrane domain downstream of the insert was not translated in the final plasmid construct.

Cloning of Soluble Human, Canine, and Feline TRAIL Genes into a Bacterial Expression Vector

The soluble portion of the canine and feline TRAIL genes was subcloned into the pDisplay vector by PCR amplification of soluble canine and feline TRAIL genes in pUni/V5-His-TOPO cloning vector. The insert was fused in-frame to the signal peptide and the HA epitope sequences present in the vector. The stop codon TAA of TRAIL was included in the primer to terminate translation; therefore, the vector-encoded PDGFR transmembrane domain downstream of the insert was not translated in the final plasmid construct.
presence of Cre recombinase. The pBAD/Thio-E vector is inducible by arabinose and contains a Thio protein fusion at the NH2 terminals. With the pCRT7-E vector, soluble TRAIL genes were expressed under bacterial phage promoter T7, which is inducible by isopropyl-β-D-thio-β-D-galactopyranoside. To clone native soluble TRAIL genes without the Thio fusion at the NH2 terminal, canine and feline TRAIL genes in pUni/V5-His-TOPO cloning vectors were used as the DNA template in PCR. The PCR was carried out using canine primers 43114-001 and 43114-004 (creating V5-His tag fusion). Feline primers were 43114-002 and 43114-006 (creating V5-His tag fusion). The purified PCR bands were cloned into vector, pBAD/Myc-His.

Expression of Soluble Canine and Feline TRAIL Genes in Mammalian Cells

Human 293 cells were transfected with plasmids encoding canine or feline soluble TRAIL genes. Supernatants as well as cells from the transfection were analyzed by Western using both HA antibody against HA epitope (Fig. 3A) and anti-human TRAIL antibody, AF375 (Fig. 3B).

Five different commercial anti-human TRAIL antibodies were tested for their cross-reactivity with soluble canine or feline TRAIL proteins. AF375, anti-human TRAIL goat antisera (R&D Systems), showed the strongest reactivity with both canine and feline TRAIL proteins (data not shown). The antibodies from Sigma and Upstate Biotechnology did not have visible cross-reactivity for both canine and feline TRAIL.

Expression of Soluble TRAIL Proteins in Bacteria

Bacterial expression plasmids with the pBAD-Thio promoter were transformed into E. coli TOP 10 cells and gene expression under the induction of arabinose was analyzed. There was very clear induction of TRAIL-Thio protein expression for all six constructs (human with and without tag, canine with and without tag, and feline with and without tag) with all the concentrations of arabinose (data not shown). The identity of these TRAIL-Thio proteins was further confirmed by Western analysis using anti-human TRAIL antibody as well as anti-V5 antibodies (data not shown). For soluble TRAIL proteins without Thio fusion, there was a very clear induction of TRAIL protein expression for feline constructs; the level for canine TRAIL protein expression was relatively low (data not shown). However, the expression of soluble canine TRAIL protein was clearly visible in Western analysis using anti-human TRAIL antibody (data not shown).

Expression of soluble TRAIL under the T7 promoter was also explored. Bacterial T7 expression plasmids were
transformed into *E. coli* BL21 cells and gene expression under induction of isopropyl-β-D-thiogalactopyranoside was analyzed. There was no clear induction of TRAIL protein expression for all constructs (data not shown). The same samples were further analyzed by Western immunoblot analysis using anti-V5 (Invitrogen) and anti-human TRAIL antibody (AF375) as probes. There was a slight induction of feline and human TRAIL protein expression with the presence of isopropyl-β-D-thiogalactopyranoside (data not shown). There was no canine TRAIL protein expressed despite several attempts on several independent clones. There was no sequence error found with the two canine TRAIL constructs.

The solubility of expressed TRAIL proteins was examined. Although >50% of TRAIL-Thio proteins were present in the insoluble fractions, there was a significant amount of TRAIL-Thio in the soluble fractions (data not shown). For the TRAIL protein without Thio fusion, the majority of proteins were present in the soluble fractions at either 30°C or 37°C. Furthermore, the level of soluble TRAIL proteins was more abundant at 37°C than at 30°C (data not shown).

In addition, the solubility of soluble human TRAIL proteins under pT7 promoter in bacteria was also explored and found to be very poor. The majority of the TRAIL proteins were present in lysate pellets (insoluble fractions; data not shown).

Bacterial cultures expressing canine and feline TRAIL-Thio as well as TRAIL proteins (with V5-His tag) were lysed and loaded onto the pre-wet His-band Quick 900 cartridges. For TRAIL proteins without Thio fusion, canine TRAIL proteins were clearly visible in fractions 2 to 4 and its identity was confirmed by Western analysis with anti-TRAIL polyclonal AF375 (data not shown). The feline TRAIL proteins were eluted in fractions 2 to 5 and its identity was confirmed by Western analysis with anti-TRAIL polyclonal AF375 (data not shown). The fractions that were positive for TRAIL proteins (fractions 2-4 for canine TRAIL and fractions 2-5 for feline TRAIL) were pooled together and dialyzed against PBS. Five fractions that were negative for TRAIL proteins were similarly pooled and dialyzed and were used as a negative control for the following assays.

**Figure 4.** Apoptosis assays for mammalian (caTRAIL-m and feTRAIL-m) and bacteria expressed (caTRAIL-b and feTRAIL-b) TRAIL proteins. U937 human tumor cells were grown in the presence or absence of TRAIL proteins produced from mammalian or bacterial cells. Apoptosis-inducing activity was measured in a MTT growth inhibition assay (A), Cell Death ELISA apoptosis assay (B), and Annexin V FITC apoptosis assay (C). PBS, staurosporin (0.4 μmol/L), and commercial human TRAIL (huTRAIL; 100 ng/mL) were used as positive controls. Vector is the conditioned medium transfected with plasmid vector control. Control is the bacterial expressed column fractions that do not contain TRAIL.
MTT Assay for Cell Growth Inhibition

One important feature of TRAIL is its ability to specifically induce apoptosis and inhibit cell growth of tumor cells. U937 human tumor cells were grown in the presence or absence of TRAIL proteins produced from conditioned medium of transfected 293 cells. The proliferation rate was normalized against control cells that were treated with normal growth medium. Another negative control used was conditioned medium from green fluorescent protein–transfected cells. Both staurosporin (0.4 μmol/L; a potent apoptosis inducer) and commercial human TRAIL (100 ng/mL) were assayed at the same time as positive controls. Also used in the assay were both mammalian and bacterial expressed canine and feline TRAIL proteins. The results suggested that the addition of soluble canine and feline TRAIL expressed from mammalian cells inhibited the cell growth of U937 cells over 35% of that of the control (Fig. 4A). The bacterial purified samples also appeared to inhibit cell growth, but the effect did not seem to be TRAIL specific, because bacterial fractions without TRAIL caused a similar effect (Fig. 4A).

Cell Death Detection ELISA Plus Assay for Apoptosis

The apoptosis-induced cell death was measured in this assay. Human tumor cells (U937) were grown in the presence or absence of TRAIL proteins produced from both mammalian and bacterial expression systems. The addition of mammalian-expressed canine and feline TRAIL induced apoptotic cell death of U937 cells. This inhibitory activity was comparable with that of the human commercial TRAIL proteins (seven times that of controls; Fig. 4B). The bacterially expressed canine and feline TRAIL also induced apoptosis in this test (Fig. 4C).

Apoptosis-Inducing Activity of Canine and Feline SolubleTRAIL for Various Human Tumor Cell Lines

Five human tumor cell lines (HeLa, cervix epitheloid carcinoma; PT-3 and HT-29, colon adenocarcinoma; SW480, colon adenocarcinoma; and U937, histiocytic lymphoma) were assayed for their sensitivity to mammalian-expressed canine and feline TRAIL proteins. In the MTT cell inhibition assay, the U937 and PT-3 human tumor cell lines were both very sensitive to canine and feline TRAIL-induced apoptosis (Fig. 5A). However, HeLa and HT-29 cell lines did not seem to respond to TRAIL treatment, which included all three forms (human, canine, and feline TRAIL).

The Cell Death ELISA assay was also done for the five human tumor cell lines. All five cell lines showed sensitivity to TRAIL-induced apoptosis in this assay (Fig. 5B). Furthermore, both canine and feline TRAIL induced apoptosis very effectively. This result further confirmed that TRAIL is a potent anticancer therapeutic agent with broad therapeutic spectrum.

![Graph A](image)

![Graph B](image)

**Figure 5.** Apoptosis assays of mammalian expressed TRAIL proteins for various human cancer cell lines. Human tumor cells (HeLa, PT-3, HT-29, SW480, and U937) were grown in the presence or absence of mammalian expressed TRAIL protein supernatants. Apoptosis-inducing activity was measured in MTT growth inhibition assay (**A**) and Cell Death ELISA apoptosis assay (**B**). Staurosporin (0.4 μmol/L) and commercial human TRAIL (100 ng/mL) were used as positive controls. The supernatant from vector plasmid-transfected cells was used as a negative control. Mean of eight replicates.
Apoptosis-Inducing Activity of Canine and Feline TRAIL for Various Canine Tumor Cell Lines

Eight canine tumor cell lines (D22, canine osteosarcoma; D17, canine osteosarcoma; CF21.T, canine arm/shoulder cancer; CF11.T, canine osteosarcoma; MDCK, canine kidney cell line; DH82, canine histiocytosis; 0309 and 030E, both independent canine histiocytosis clones) were assayed for their sensitivity against mammalian expressed canine and feline TRAIL proteins. The U937 human tumor cell line was assayed at the same time and used as positive control. For the MTT cell growth inhibition assay, canine TRAIL-containing culture supernatant induced apparent apoptosis for all the cell lines tested. The effect of feline TRAIL was less clear for cell lines MDCK, CF21.T, D17, and D22 (Fig. 6A). This result shows the efficacy of canine TRAIL as a potential therapeutic for canine tumor cell lines.

For the Cell Death ELISA apoptosis assay, canine TRAIL induced apparent apoptosis for D22, D17, and, to a lesser extent, CF21.T and 0309 (Fig. 6B). Feline TRAIL also had an effect on D17, CF21.T, 0309, and 030E. This conclusion was supported by the results of the MTT assay (data not shown). It is well known that the Fas ligand causes liver toxicity in vivo (15, 38). The fact that TRAIL proteins do not cause apoptosis of liver cells in vitro indicates that there likely to be little, if any, liver toxicity of TRAIL proteins when employed in vivo.

Discussions

We have reported here the cloning of the full-length canine and feline apoptosis inducer TRAIL genes by using RACE-PCR based on known human and mouse TRAIL sequences. Soluble truncated versions were also constructed. Expression in both mammalian and bacterial expression systems was confirmed via Western immunoblot assays using an anti-TRAIL antibody. We have also shown that both canine and feline soluble proteins were secreted into conditioned medium when expressed in mammalian cells. In bacterial cells, the majority of the soluble canine and feline TRAIL proteins remain in the soluble fraction. Furthermore, both canine and feline TRAIL proteins, whether expressed in mammalian cells or bacterial cells, were shown to specifically inhibit tumor cell growth and induce apoptosis of cancer cells at a level comparable with their human counterpart. Taken together, those properties make canine and feline TRAIL proteins attractive therapeutic targets for dogs and cats with cancer.

Human TRAIL has been shown to inhibit the growth of a wide variety of primary and metastatic tumors (13, 27, 39).
The treatment with TRAIL can be repeated many times without inducing drug resistance or side effects. TRAIL can also be conveniently combined with other cancer therapies such as surgery, chemotherapy, radiation therapy, and immunotherapy to achieve superior therapeutic effects (28, 40–43). The cloning and identification of canine and feline TRAIL allows for the treatment of dog and cat tumors using species-specific apoptosis inhibitors, thereby minimizing the risk of evoking immune responses under repeated administration. Furthermore, spontaneous canine tumors are very similar to their human correlates in histopathologic and biological behavior (3, 44). Experimental results obtained from canine tumors will also provide valuable information for human cancer biology and treatment.

Normal hepatocytes and keratinocytes displayed signs of toxicity when treated with human TRAIL (14–16). This toxicity was attributed to the presence of a polyhistidine tag and lack of the optimum amount of zinc (17, 18). In this study, we treated normal canine hepatocytes with mammalian cell-expressed canine soluble HA-tagged TRAIL and did not detect any TRAIL-induced apoptosis, whereas Fas ligand induced potent apoptosis. It is possible that HA-tagged TRAIL protein preserves its basic protein conformation similarly to nontagged TRAIL protein. If this is the case, it has a distinct advantage over the His-tagged version and will offer a very useful tool in TRAIL research.

The TRAIL genes from seven species have been cloned and sequenced: human, mouse, rat, porcine, chicken, canine, and feline. The TRAIL protein homology between canine and feline is quite high (93.2% identity), and this homology is higher than the homology between mouse and rat TRAIL protein (86.2% identity). As expected, human TRAIL has higher protein homology with feline, porcine, and canine (84.2%, 82.2%, and 81.3% identity, respectively) than with TRAIL proteins of rat, mouse, and chicken (67.9%, 64.2%, and 55.8%, respectively). Furthermore, the TRAIL protein homology among the truncated soluble TRAIL protein homology between species is higher than with TRAIL proteins of rat, mouse, and chicken (86.2% identity). As expected, human TRAIL has higher protein homology with feline, porcine, rat TRAIL protein (86.2% identity). As expected, human TRAIL has higher protein homology with feline, porcine, and canine (84.2%, 82.2%, and 81.3% identity, respectively) than with TRAIL proteins of rat, mouse, and chicken (67.9%, 64.2%, and 55.8%, respectively). Furthermore, the TRAIL protein homology among the truncated soluble proteins for the seven species is higher.

It is interesting to note that although commercial human TRAIL induced potent apoptosis for human U937 cells, its effect on canine cell lines was very minimal (Fig. 6B; data not shown). Canine and feline TRAIL showed potent apoptosis-inducing activity for various human tumor cell lines (Fig. 5A and B). The species specificity of TRAIL for its susceptible cell lines seems to be unidirectional (canine and feline TRAIL induce apoptosis of human tumor cell lines, whereas human TRAIL cannot effectively induce apoptosis of canine tumor cell lines).

The results of the three apoptosis assays we described in this study (MTT Growth Inhibition Assay, Cell Death Elisa Apoptosis Assay, and Annexin V FITC Apoptosis Assay) are, in general, consistent but not in every single case. For example, human HeLa and HT-29 cells showed little growth inhibition by any of the TRAIL proteins (human, canine, and feline TRAIL), whereas they displayed apoptosis induced by various TRAIL proteins. In contrast, for canine cell line 030E, various TRAIL proteins did not induce much of apoptosis in Cell Death ELISA Apoptosis Assay, whereas cell growth inhibition was observed in MTT growth inhibition assay. There does not seem to be one assay that is consistently superior over the other assays.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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