A Newly Identified Member of Tumor Necrosis Factor Receptor Superfamily (TR6) Suppresses LIGHT-mediated Apoptosis

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TR6 (decoy receptor 3 (DecR3)) is a new member of the tumor necrosis factor receptor (TNFR) family. TR6 mRNA is expressed in lung tissues and colon adenocarcinoma, SW480. In addition, the expression of TR6 mRNA was shown in the endothelial cell line and induced by phorbol 12-myristate 13-acetate/ionomycin in Jurkat T leukemia cells. The open reading frame of TR6 encodes 300 amino acids with a 29-residue signal sequence but no transmembrane region. Using histidine-tagged recombinant TR6, we screened soluble forms of TNF-ligand proteins with immunoprecipitation. Here, we demonstrate that TR6 specifically binds two cellular ligands, LIGHT (herpes virus entry mediator (HVEM)-L) and Fas ligand (FasL/CD95L). These bindings were confirmed with HEK 293 EBNA cells transfected with LIGHT cDNA by flow cytometry. TR6 inhibited LIGHT-induced cytotoxicity in HT29 cells. It has been shown that LIGHT triggers apoptosis of various tumor cells including HT29 cells that express both lymphotxin β receptor (LTβR) and HVEM/TR2 receptors. Our data suggest that TR6 inhibits the interactions of LIGHT with HVEM/TR2 and LTβR, thereby suppressing LIGHT-mediated HT29 cell death. Thus, TR6 may play a regulatory role for suppressing in FasL- and LIGHT-mediated cell death.

The members of the tumor necrosis factor (TNF) family are involved in regulating diverse biological activities such as regulation of cell proliferation, differentiation, cell survival, cell death, cytokine production, lymphocyte co-stimulation, and isotype switching (1, 2). Receptors in this family share a common structural motif in their extracellular domains consisting of multiple cysteine-rich repeats of approximately 30–40 amino acids (3). While TNFR1, CD95/Fas/APO-1, DR3/TRAMP/APO-3, DR4/TRAIL-R1/APO-2, DR5/TRAIL-R2, and DR6 receptors contain a conserved intracellular motif of ~80 amino acids called death domain, associated with the activation of apoptotic signaling pathways, other members, which contain a low sequence identity in the cytoplasmic domains, stimulate the transcription factors NF-κB and AP-1 (1–3).

Most TNF receptors contain a functional cytoplasmic domain. However, some members of the TNFR superfamily do not have cytoplasmic domains and are secreted, such as osteoprotegerin (OPG) (4), or linked to the membrane through a glycosylphospholipid tail, such as TRID/Drk1/TRAIL-R3 (5, 6). Viral open reading frames encoding soluble TNFRs have also been identified, such as SFV-T2 (7), Va53 (8), G4RG (9), and csmB (3).

By searching an expressed sequence tag (EST) data base, a new member of the TNFR superfamily was identified, named TR6, and was characterized as a soluble cognate receptor for LIGHT and FasL/CD95L. LIGHT and FasL mediate the apoptosis, which is the most common physiological form of cell death and occurs during embryonic development, tissue remodeling, immune regulation, and tumor regression.

LIGHT is highly induced in activated T lymphocytes and macrophages. LIGHT was characterized as a cellular ligand for HVEM/TR2 and LTβR (10). HVEM/TR2 is a receptor for herpes simplex virus type 1 (HSV-1) entry into human T lymphoblasts. The soluble form of HVEM/TR2-Fc and antibodies to HVEM/ TR2 were shown to inhibit a mixed lymphocyte reaction, suggesting a role for this receptor or its ligand in T lymphocyte proliferation (10–12). The level of LTβR expression is prominent on epithelial cells but is absent in T and B lymphocytes. Signaling via LTβR triggers cell death in some adenocarcinomas (13). LIGHT produced by activated lymphocytes could evoke immune modulation from hematopoietic cells expressing only HVEM/TR2 and induce apoptosis of tumor cells, which express both LTβR and HVEM/TR2 receptors (14, 15).

FasL is one of the major effectors of cytotoxic T lymphocytes and natural killer cells. It is also involved in the establishment of peripheral tolerance in the activation-induced cell death of lymphocytes. Moreover, expression of FasL in nonlymphoid and tumor cells contributes to the maintenance of immune privilege of tissues by preventing the infiltration of Fas-sensitive lymphocytes (16). FasL is also processed and shed from the surface of human cells (17).

Here we demonstrate that TR6 (DecR3), a new member of the TNFR superfamily, binds LIGHT and FasL. Therefore TR6 may act as an inhibitor in LIGHT-induced tumor cell death by blocking LIGHT interaction with its receptors.

The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; LTβR, lymphotxin β receptor; DecR3, decoy receptor 3; HVEM, herpes virus entry mediator; HUVEC, human umbilical vein endothelial cell; OPG, osteoprotegerin; TR6-(His), C-terminal hexahistidine-tagged TR6; EST, expressed sequence tag; RT-PCR, reverse transcription-polymerase chain reaction; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; IFN, interferon.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF134240. || To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202, Tel.: 317-274-3950; Fax: 317-274-4096; E-mail: kkwon@sunflower.bio.indiana.edu.

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MATERIALS AND METHODS

Identification and Cloning of New Members of the TNFR Superfamily—An EST cDNA database, obtained from more than 600 different cDNA libraries, was screened for sequence homology with the cysteine-rich motif of the TNFR superfamily, using the blastn and tblastn algorithms. Three EST clones containing an identical open reading frame, the amino acid sequence of which showed significant homology to TNFR-I, TNFR-II, and FasL, were identified from cDNA libraries of human normal prostate and pancreas tumor. A full-length TR6 cDNA clone encoding an intact N-terminal signal peptide was obtained from a human normal prostate library.

RT-PCR Analysis—For RT-PCR analysis, total RNA was isolated using TriZOL (Life Technologies, Inc.) from various human cell lines before and after stimulation with phorbol 12-myristate 13-acetate/oncolysin or lipopolysaccharide. RNA was converted to cDNA by reverse transcription and amplified for 35 cycles by PCR. Primers used for amplification of the TR6 fragment are according to the sequence of TR6.

β-Actin was used as an internal control for RNA integrity. PCR products were run on 2% agarose gel, stained with ethidium bromide, and visualized by UV illumination.

Recombinant Protein Production and Purification—The recombinant TR6 protein was produced with hexahistidine at the C terminus. TR6-(His) encoding the entire TR6 protein was amplified by PCR. For correctly oriented cloning, a (His) encoding the entire TR6 protein was amplified by PCR. For orientation confirmation, a (His) encoding the entire TR6 protein was amplified by PCR. For orientation confirmation, a (His) encoding the entire TR6 protein was amplified by PCR.

Production of HVEM/TR2-Fc, LTβR-Fc, and FLAG-tagged soluble LIGHT (sLIGHT) fusion proteins were previously described (14). FLAG-tagged sLIGHT proteins were purified with anti-FLAG mAb affinity column. FLAG-tagged LIGHT (sLIGHT) fusion proteins were previously described (14). Fc-fusion protein, LTβR-Fc, and FLAG-tagged soluble LIGHT (sLIGHT) were incubated overnight with various FLAG-tagged ligands of the TNF superfamily and anti-Fc agarose in binding buffer (150 mM NaCl, 0.1% Nonidet P-40, 0.25% Procion Red) and a BanHI site on the 5′ end of the primer (5′-AGACCGGACCTTCGCTGCTCAAGGCAGTCG-3′) were generated. The amplified fragment was cut with BanHI/HindIII and cloned into a mammalian expression vector, pCEP4 (Invitrogen). The TR6-(His)/pCEP4 plasmid was stably transfected into HEK 293 EBNA cells to generate recombinant TR6-(His). Serum-free culture media from cells transfected TR6-(His)/pCEP4 were passed through Ni-column (Novagen). The column eluents were fractionated by SDS-PAGE, and TR6-(His) was detected by Western blot analysis using the anti-poly(His)6 antibody (Sigma).

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For RT-PCR analysis, total RNA was isolated using TriZOL (Life Technologies, Inc.) from various human cell lines before and after stimulation with phorbol 12-myristate 13-acetate/oncolysin or lipopolysaccharide. RNA was converted to cDNA by reverse transcription and amplified for 35 cycles by PCR. Primers used for amplification of the TR6 fragment are according to the sequence of TR6.

β-Actin was used as an internal control for RNA integrity. PCR products were run on 2% agarose gel, stained with ethidium bromide, and visualized by UV illumination.

Cell-binding Assay—For cell-binding assays, HEK 293 EBNA cells were stably transfected using the calcium phosphate method with pCEP4/pCEP4 vector alone. After selection with hygromycin B, cells were harvested and incubated with TR6-(His), HVEM/TR2-Fc, or LTβR-Fc for 20 min on ice. For detecting Fc-fusion protein, cells were stained with FITC-conjugated goat anti-human IgG. To detect TR6 binding, cells were stained with anti-poly(His)6 and FITC-conjugated goat anti-mouse IgG consecutively. The cells were analyzed by FACScan (Becton Dickinson).

Cytotoxicity Assay—Cytotoxicity assays using HT29 cells were carried out as described previously (13). Briefly, 5000 HT29 cells were seeded in 96-well plates with 1% fetal bovine serum, Dulbecco’s modified Eagle’s medium, and treated with sLIGHT (10 ng/ml) and 10 units/ml human recombinant interferon-γ (IFN-γ) (supplied from NIAID, NIH Repository). Serial dilutions of TR6-(His) were added in quadruplicate to microtiter wells. Cells treated with IFN-γ and sLIGHT were incubated with various amounts of TR6-(His) for 4 days before the addition of [3H]thymidine for the last 6 h of culture. Cells were harvested, and thymidine incorporation was determined using a liquid scintillation counter.

RESULTS AND DISCUSSION

TR6 Is a New Member of the TNFR Superfamily—TR6 was identified by searching an EST data base. Three clones containing an identical open reading frame were identified from cDNA libraries of human normal prostate and pancreas tumor. A full-length TR6 cDNA encoding an intact N-terminal signal peptide was obtained from a human normal prostate library.

TR6 has a soluble, secreted form, and TR6 exerts pro-apoptotic activity on HEK 293 EBNA cells and HT29 cells, but not on normal human prostate cells. TR6 is a member of the TNFR superfamily, and its amino acid sequence shares 30% sequence homology with OPG and TNFR-II. TR6 contains two perfect and two imperfect cysteine-rich motifs, and its amino acid sequence was remarkably similar to the TR1/OPG amino acid sequence. TR6 shows ~30% sequence homology with OPG and TNFR-II.

mRNA Expression—We analyzed expression of TR6 mRNA in human multiple tissue blots by Northern hybridization. Northern blot analyses indicated that TR6 mRNA was ~1.3 kilobases in length and was expressed predominantly in lung
tissue and the colorectal adenocarcinoma cell line SW480 (data not shown). RT-PCR analyses were performed to determine the expression patterns of TR6 in various cell lines. TR6 transcript was detected weakly in most hematopoietic cell lines. The expression of TR6 was induced upon activation in Jurkat T cells, monocyte THP-1 cells, and endothelial HUVEC cells. Amplification of β-actin was used as an internal control.

Identification of the Ligand for TR6—To identify the ligand for TR6, several FLAG-tagged soluble proteins of TNF ligand family members were screened for binding to recombinant TR6-(His) protein by immunoprecipitation. TR6-(His) selectively bound LIGHT-FLAG and FasL-FLAG among FLAG-tagged soluble TNF ligand members tested (Fig. 3). This result indicates that TR6 binds at least two ligands, LIGHT and FasL. LIGHT exhibits significant sequence homology with the C-terminal receptor-binding domain of FasL (31%), but sLIGHT is unable to bind to Fas (10, 14). They may have a similar binding epitope for TR6 binding.

Previously, Zhai et al. (14) and Harrop et al. (15) reported the biological functions of LIGHT and its possible mechanisms of action as a ligand for HVEM/TR2 and/or LTβR. LIGHT is expressed in activated T cells. LIGHT, in conjunction with serum starvation or addition of IFN-γ, inhibits the cell proliferation in tumor cells, MDA-MB-231 and HT29.

To determine whether TR6 might act as an inhibitor to LIGHT interactions with HVEM/TR2 or LTβR, TR6-(His) was used as a competitive inhibitor in LIGHT-HVEM/TR2 interaction. When LIGHT was immunoprecipitated with HVEM/TR2-Fc in the presence of TR6-(His), HVEM/TR2-Fc binding to LIGHT was decreased competitively by TR6-(His), but TR6-(His) binding to LIGHT was not changed by HVEM/TR2-Fc (Fig. 3B). Furthermore, the binding of HVEM/TR2-Fc (6 nM) or LTβR (6 nM) was completely inhibited by 20 nM TR6-(His) protein in immunoprecipitation assays (Fig. 3C). These results support the notion that TR6 may act as a strong inhibitor of LIGHT function through HVEM/TR2 and LTβR.

Binding of TR6-(His) to LIGHT-transfected Cells—To determine whether TR6 binds to LIGHT expressed on the cell surface, we performed a binding assay using LIGHT-transfected HEK 293 EBNA cells by flow cytometry. LIGHT-transfected HEK 293 EBNA cells were stained significantly by TR6-(His) as well as by HVEM/TR2-Fc and LTβR-Fc. No binding was detected by HVEM/TR2-Fc or LTβR-Fc on pCEP4 vector-transfected HEK 293 EBNA cells. (Fig. 4). Furthermore, control isotype did not bind to LIGHT-transfected HEK 293 EBNA cells, and none of the above fusion proteins bound to vector-transfected cells, confirming the specificity of these bindings. These bindings indicate that TR6 can bind to both soluble and membrane-bound forms of LIGHT.

TR6 Inhibits LIGHT-induced Cytotoxicity in HT29 Cells—Brown et al. (13) have shown that Fas activation leads to rapid cell death (12–24 h) whereas LTβR takes 2–3 days in induction of apoptosis for the colorectal adenocarcinoma cell line, HT29. Zhai et al. (14) also reported that LIGHT leads to the death of the cells expressing both LIGHT and HVEM/TR2 but not the cells expressing only the LTβR or HVEM/TR2 receptor. Both HVEM/TR2 and LTβR are involved cooperatively in LIGHT-mediated killing of HT29 cells (14). To determine whether binding of TR6 inhibits LIGHT-mediat
that Fasl could itself transduce signals, leading to cell cycle arrest and cell death in CD4\(^+\) T cells but cell proliferation in CD8\(^+\) T cells. Therefore, TR6 may be involved in signaling through FasL and LIGHT.

HUVEC cells constitutively expressed TR6 in RT-PCR analysis. LIGHT and FasL have been known to be expressed in activated T cells. Therefore it is speculated that TR6 and its ligands are important for interactions between activated T lymphocytes and endothelium. TR6 may be involved in activated T cell trafficking as well as endothelial cell survival.

In this paper we have identified a novel soluble member of the TNFR superfamily, TR6, which is constitutively expressed in lung tissue, tumor cells, and in endothelial cells. We have also identified the ligands for TR6, LIGHT, and FasL, which are involved in the cell death pathway. TR6 bound specifically to LIGHT and FasL and inhibited their activities. Like DcR1, DcR2, and another soluble member of the TNFR superfamily, OPG, TR6 may act as an inhibitor of signaling through TNF family members, FasL and LIGHT. Hence, TR6 may have important roles in the inhibition of apoptosis and tumor modulation.

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**Addendum—**During the preparation of this manuscript, Petti et al. (18) published the DcR3 that is identical to TR6. Our finding that TR6 interacts with FasL is in line with their observation.

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**FIG. 5.** TR6 inhibits LIGHT-induced cell death in HT29 cells. A, HT29 cells were incubated in 96-well plates with control medium, 10 units/ml IFN-\(\gamma\) alone, purified sLIGHT protein (10 ng/ml) in the absence or presence of IFN-\(\gamma\) (10 units/ml), purified sLT\(\beta\)R-Fc (200 ng/ml), or TR6-His (200 ng/ml) in the presence of IFN-\(\gamma\) (10 units/ml) and sLIGHT (10 ng/ml). B, cells were incubated with various doses of TR6-His and IFN-\(\gamma\) (10 units/ml) with (open circle) or without (filled circle) sLIGHT (10 ng/ml). In all assays, cells were cultured for 4 days, and proliferation was detected during the last 6 h of culture by the addition of 1 \(\mu\)Ci of \(\text{l}^{3}\hbox{H}\)thymidine. Cells were harvested, and thymidine incorporation was determined using a liquid scintillation counter.