Soluble Decoy Receptor 3 Induces Angiogenesis by Neutralization of TL1A, a Cytokine Belonging to Tumor Necrosis Factor Superfamily and Exhibiting Angiostatic Action

Chia-Ron Yang,1 Shie-Liang Hsieh,2 Che-Ming Teng,1 Feng-Ming Ho,3 Wen-Ling Su,2 and Wan-Wan Lin1

Department of Pharmacology, College of Medicine, National Taiwan University, Taipei; 2Department of Microbiology and Immunology, and Immunology Research Center, National Yang-Ming University, Taipei; and 3Taoyuan General Hospital, Department of Health, Executive Yuan, Taoyuan, Taiwan

ABSTRACT

TL1A is a member of the tumor necrosis factor superfamily and plays an important role in regulating endothelial cell apoptosis. A previous study shows TL1A is able to interact with death receptor 3 and decoy receptor 3 (DcR3). Here, we demonstrate that DcR3 is able to induce angiogenesis in human umbilical vein endothelial cells (HUVECs). DcR3 promotes HUVEC proliferation and migration and up-regulates matrix metalloproteinase-2 mRNA expression and enzyme activity. Furthermore, DcR3 enhances EC differentiation into cord-like structures in vitro, as well as neovascularization in vivo. The effects of DcR3 on HUVECs are also mimicked by anti-TL1A and anti-death receptor 3 antibodies. In contrast, human aortic endothelial cells, which do not express TL1A, are not responsive to DcR3 treatment, including cell proliferation, migration, and angiogenic differentiation. These data demonstrate DcR3 might not only help tumor cells to escape immune surveillance but also induce angiogenesis by blocking TL1A action in endothelial cells. The pathological role of DcR3 in promoting cancer progression raises the possibility to target DcR3 for antiangiogenic therapy in the future.

INTRODUCTION

Angiogenesis, a process that involves the endothelial cells sprouting from preexisting postcapillary venules to form new blood vessels, is a fundamental step in a variety of physiological and pathological conditions. These include rheumatoid arthritis (1), diabetic retinopathy (2), tumor growth (3), wound healing, and the female reproductive system (4). Angiogenesis is a multistep process that involves cell proliferation, migration, tube formation of endothelial cells, remodeling of the extracellular matrix, and functional maturation of the newly assembled vessels (5, 6). The angiogenic process is tightly regulated by a variety of positive factors or negative molecules. Several cytokines belonging to tumor necrotic factor (TNF) family were recently identified to play important roles in angiogenesis.

TL1A/vascular endothelial cell growth inhibitor (VEGI)-L, a novel member of TNF superfamily, contains 251 amino acids in length and is a longer variant of VEGI (also called TL1). TL1A and VEGI are abundantly expressed in endothelial cells as well as in kidney, lung, prostate, placenta, and liver (7–9). It has been reported that recombinant soluble forms of VEGI and TL1A are potent inhibitors to suppress endothelial cell proliferation, angiogenesis, and tumor growth (9, 10–12). However, overexpression of the full-length VEGI (174 amino acids) did not give rise to a VEGI peptide in cell-conditioned media and did not show angiogenic activity in vivo (9). This strongly suggested that TL1A, but not VEGI/TL1, is the predominant, full-length gene product. Previous studies demonstrate that TL1A and soluble VEGI are able to induce endothelial cells apoptosis via an autocrine manner (8, 9, 13), and overexpression of TL1A was shown to inhibit tumor neovascularization and progression in a mouse xenograft tumor model (9, 12). As to action mechanism, multiple signaling pathways, including stress protein kinases (p38 mitogen-activated protein kinase, c-Jun NH2-terminal kinase), as well as certain caspases, contribute to VEGI-induced apoptosis (13).

Death receptor-3 (DR3; also known as TRAMP, Apo-3, WSL-1, LARD) is a member of the TNF receptor (TNFR) superfamily that contains a death domain in its cytoplasmic tail and can induce apoptosis (14–16) or nuclear factor-kB activation (16). However, unlike the broad tissue distribution of TNFRI, DR3 appears to be preferentially expressed by lymphocytes and is efficiently induced after T-cell activation (17, 18). TL1A was shown as a ligand of DR3 in T cells, where the engagement leads to stimulate T-cell proliferation (19). Nevertheless, as we know, the exact membrane receptor responsible for the ability of TL1A to induce endothelial cells apoptosis has not been studied yet. Moreover, the possible expression of DR3 at its mRNA and protein levels in endothelial cells has not been reported.

The second receptor for TL1A is the soluble decoy receptor 3 (DcR3). DcR3, also known as TR6 or M68, is a soluble receptor belonging to the TNFR superfamily and is a decoy receptor for FasL, LIGHT, and TL1A (19–22). DcR3 can apparently neutralize the biological effects of FasL, LIGHT, and TL1A by inhibiting the FasL-Fas (20), LIGHT-LTβR (21), LIGHT-HVEM (23), and TL1A-DR3 (19) interaction. These actions implicate an important role of DcR3 in immune regulation elicited by FasL, LIGHT, and TL1A (24).

Previous studies indicated that DcR3 is overexpressed in malignant tumors arising from esophagus, stomach, glioma, lung, colon, and rectum (20, 22, 25–27). Tumor cells engineered to release high amounts of DcR3 are protected from FasL-induced apoptotic cell death and chemotaxis, which in turn results in a decreased immune cell infiltration in glioma xenografts (26). DcR3-treated dendritic cells were shown to down-regulate T-cell proliferation and skew immune response to Th2 phenotype (28). By using a sandwich ELISA, high serum levels of DcR3 were detected in many cancer patients (29). All these observations suggest that DcR3 is involved in the progression and immune evasion of malignant tumors.

In this study, we are interested to know whether DcR3, in addition to modulate immune response by down-regulating T-cell proliferation and dendritic cell differentiation, has pathological relevance in TL1A-induced angiostatic effect. To address this question, a soluble DcR3 is generated to interfere with the autocrine function of TL1A. We found that treatment of human umbilical vein endothelial cells (HUVECs) with DcR3 is sufficient to induce cell proliferation, migration, MMP-2 expression, tube formation, and angiogenesis. The existence of TL1A and DR3 gene expression in HUVECs, as well as the mimic of anti-TL1A and anti-DR3 antibodies to DcR3 action, indicate that the angiostatic function of TL1A autocrine in HUVECs can be masked by DcR3. All these results suggest tumor cells might use DcR3 to promote angiogenesis and thus facilitate tumor growth.
MATERIALS AND METHODS

Materials. Matrigel basement membrane matrix was purchased from BD Biosciences (Bedford, MA). Endothelial cell growth supplement was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Blind well chamber and 8-μm polycarbonate membrane were purchased from Costar (Cambridge, MA). Collagenase type I and total hemoglobin kit (no. 525) were purchased from Sigma Diagnostics (St. Louis, MO). M199 medium and 0.25% trypsin/1 mM EDTA 4Na were purchased from Life Technologies, Inc. (Grand Island, NY). Antihuman DR3 antibody (Ab) and monoclonal antihuman VEGF Ab were purchased from R&D Systems (Minneapolis, MN). Polyclonal anti-CD31 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human vascular endothelial growth factor (VEGF) was purchased from PeproTech EC (London, United Kingdom). FCS was obtained from Biological, Inc. (Kibbutz Beit Haeneek, Israel). TL1A Ab was prepared as follows: female BALB/c mice strain was immunized with peptide sequence (TL1A amino acids 61–80, AQGEACVQFQALKGQEFAPS), predicted to be extracellular domain of TL1A protein without sequence homology with VEGF. Repeated boosts were carried out every 2 weeks for 10 times. After fusion experiment, monoclonal Ab 6E6 belonging to IgG1 was generated. LTBR.Flag was prepared as we described previously (30). Lipopolysaccharide from Escherichia coli was obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. HUVECs were obtained by treating human umbilical cord veins with 0.05% collagenase for 8 min and cultured in 75 cm² flasks in M199 containing 20% fetal bovine serum, 15 mg/ml endothelial cell growth supplement, 5 units/ml heparin, and 20 mM HEPES. Human aortic endothelial cells (HAECs) were obtained from BioWhittaker, Inc. (Walkersville, MD) and cultured in EGM-2 medium. Prostate cancer PC-3, adenocarcinoma HT-29, and monocytic THP-1 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Peripheral mononuclear cells were isolated as we described previously (28), PC-3 and HT-29 cell line cultures were cultured in DMEM. Human THP-1 cells and monocytes were cultured in RPMI 1640. All of the culture media were supplemented with 10% (volume for volume) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Generation of Recombinant DeR3.Flag and DeR3 Proteins. Recombinant DeR3.Fc fusion protein was produced as described previously (28). To generate the DeR3.Flag, the open reading frame of the human DeR3 gene was isolated by reverse transcription (RT)-PCR using the forward primer 5’-GGATAATCTTCGACCATGAGGCCGTG-3’ and the reverse primer 5’-GGATTCTGTGACAGGGAGGAAGGC-3’. The amplified product was ligated in-frame into the EcoR1-cut pCMV-Flag 4a (Clontech Laboratories, Palo Alto, CA) vector containing the cDNA of the Flag Tag. The DeR3.Flag fusion gene was then subcloned into the pBacPAK9 vector (Clontech Laboratories) and cotransfected with linearized BacPAK6 DNA (Clontech Laboratories) into Sf21 cells. Plaque assays were performed on the cotransfection supernatant to obtain individual viral plaques and followed by PCR examination to confirm the incorporation of DeR3.Flag DNA. Recombinant virus was amplified to obtain working stocks and used to infect Sf21 cells. The supernatant from recombinant virus-infected Sf21 cells was filtered and purified on an anti-Flag M2 affinity gel. The bound DeR3.Flag protein was then eluted with 0.1 M glycine buffer (pH 3.0), followed by dialysis against PBS. Recombinant DeR3 protein was cleaved from the recombinant DeR3.Fc fusion protein by papain. Briefly, 2.5 mg/ml DeR3.Fc was transferred into a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) and dialyzed against 100 mM sodium acetate (pH 5.3) at 25°C for 2 h followed by further dialysis at 4°C overnight. EDTA and cysteine were added to the reaction at final concentrations of 1 and 50 mM, respectively, followed by the addition of papain-conjugated agarose; this was incubated at 37°C for 4 h. The papain-conjugated agarose was removed by centrifugation, and the cleaved Fc portion was removed by incubation of the Fc and DeR3 mixture with protein A-Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech, Piscataway, NJ). The DeR3 proteins we generated did not have a problem with lipopolysaccharide contamination, because of the baculovirus generating system, and no effect of DeR3 proteins on nitric oxide (NO) production from murine macrophage RAW264.7 cell line. This cell line is a very sensitive and useful model for assessing lipopolysaccharide-induced inducible NO synthesis expression and NO production.

Immunoblotting Assay. Cells were lysed in a lysis buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 0.1% Triton X-100, 10%
glycerol, 1 mM DTT, 1 μg/ml leupeptin, 5 μg/ml aprotime, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate]. Whole cell extracts were prepared by centrifugation at 12,000 × g for 15 min at 4°C. Whole cell extracts (120 μg) were electrophoresed on a 10% SDS-polyacrylamide gel and blotted onto nitrocellular membranes. Immunoblot detection was performed with the corresponding rabbit antisera or mouse monoclonal Ab using an enhanced chemiluminescence detection kit and exposure to photographic film.

**Immunoprecipitation Assay.** TL1A-Ab and DcR3-Fc (5 μg each) with protein A/G agarose beads were added to 1 ml of concentrated conditioned media obtained from confluent HUVECs cultured for 24 h in a 75T flask. Immunoprecipitation proceeded at 4°C overnight. The precipitated beads were washed three times with 1 ml of ice-cold cell lysis buffer, and then the immune complex was resolved by 12% SDS-PAGE gel electrophoresis, followed by immunoblotting assay against TL1A Ab.

**RT-PCR.** Total RNA was isolated from HUVECs and human cell lines using RNAzol B Reagent (TEL-TEST, Inc., Friendswood, TX). Single-strand cDNA for a PCR template was synthesized from 10 μg of total RNA using random primers and M-MLV reverse transcriptase (Promega Corp., Madison, WI). The oligonucleotide primers used for the amplification are as follows. Human TL1A (GenBank accession no. AF520785) sense (123–146), 5′-ATG GCC GAG GAT CTG GGA CTG AGC-3′ and antisense (843–878), 5′-CTA TAG TAA GAA GGC TCC AAA GAA GGT TTT TTC TTT-3′; produced size 756 bp. Human DR3 (GenBank accession no. U72763) sense (515–536), 5′-ATG GCC GAT GTG CCT GGT GAG TCT GCT GCT-3′ and antisense (852–875), 5′-AGC GCC TCC TGG GTC TCG GGG TAG-3′; produced size 361 bp. Human FasL (GenBank accession no. NM000639) sense (456–475), 5′-GGA TGG TTC ATG TGT CCC AGC TTT GCA ATG GCT GCG TGT CCT GCC TCC TGA TGC TCC TGG GTC TCG GGG TAG-3′; produced size 597 bp. Human LIGHT (GenBank accession no. NM_014798) sense (182–211), 5′-CTT CAC ATG GAG GAT TGT GTG GAT GGA-3′ and antisense (536–575), 5′-CTT CAC ATG GAG GAT TGT GTG GAT GGA-3′; produced size 536 bp. Human DR3 (GenBank accession no. U72763) sense (515–536), 5′-ATG GCG ATG GCT GCG TGT CCT GCC TCC TCA TCA CC-3′ and antisense (1122–1124), 5′-TCT TCC CCT CCA TCA TCA CC-3′; produced size 387 bp. Human MMP-2 (GenBank accession no. J03210) sense (157–182), 5′-GTC ATG GAC ACA CTA AAG AAG A-3′ and antisense (732–736), 5′-TTG CCA TCC TTC TCA AAG TTG TAG G-3′; produced size 510 bp. Equal amounts of each reverse transcription product (1 μg) were PCR amplified using Taq polymerase in 35 cycles consisting of 1 min at 95°C, 1 min at 66°C (for FasL and LIGHT) or 58°C (for TL1A, DR3, and MMP-2), and 1 min at 72°C. The amplified cDNA was run on 1% agarose gels and visualized by ethidium bromide.

**[3H]Thymidine Incorporation Assay.** Cells were seeded in 96-well plates (10^3 cells/well) and incubated in 2% FCS medium for 24 h. Drugs were then added, and after 20 h, 0.5 mCi of [methyl-3H]thymidine was added to each well. Culture media were removed, and the cells were washed three times with PBS, treated with 4 N sodium hydroxide and 50% trichloroacetic acid, and then processed by harvesting Filter-Mate (Packard). Incorporated radiolabel was quantified in a gamma counter and compared with the corresponding control.

**Detection of Apoptosis.** Apoptosis of treated HUVECs was detected by the ELISA method (using mouse monoclonal antibodies directed against DNA and histones) of Cell Death Detection ELISAPLUS kit (Roche Diagnostics, Germany). Each group of apoptosis assay was performed at the manufacturer’s protocol.

**Cell Migration Assay.** The migration assay was measured with a modified Boyden chamber assay (Costar). Chemotactoactants were loaded into the bottom wells of the chamber. The wells were covered with a polycarbonate filter with 8-μm pores coated with 10 μg/ml gelatin. HUVECs (1 × 10^5 cells in 200 μl of M199 medium) were added into the top wells of the chamber. The chambers were incubated for 6 h at 37°C in an atmosphere of 95% air and 5% CO_{2}. At the end of incubations, nonmigrated cells on top of the filters were wiped off with cotton swabs, and migrated cells attached to the bottom of filters were fixed and stained with 1% crystal violet and then measured at an absorbance of 550 nm.

**Zymography.** For metalloproteinase (MMP) analysis by zymography, HUVECs were seeded in 24-well plates (2 × 10^5 cells/well) and incubated in 2% FCS medium for 24 h. Supernatants were collected and electrophoresed for analysis in 10% SDS-PAGE copolymerized with 1 mg/ml gelatin. The gels were then washed for 30 min at 22°C in 2.5% Triton X-100 to remove SDS and then incubated in 50 mM Tris (pH 7.6), 1 mM ZnCl₂, and 5 mM CaCl₂ for 18 h at 37°C. After incubation, the gels were stained with 0.1% Coomassie Blue. Enzyme-digested regions were identified as white bands on a blue background.

**Tube Formation Assay.** HUVECs (1 × 10^5 cells/ml) were cultured into slide chambers, which were precoated with the 5 mg/ml Matrigel basement matrix. 300 μl of conditioned media for a PCR template was synthesized from 10 μg of total RNA using random primers and M-MLV reverse transcriptase (Promega Corp., Madison, WI). The oligonucleotide primers used for the amplification are as follows. Human TL1A (GenBank accession no. AF520785) sense (123–146), 5′-ATG GCC GAG GAT CTG GGA CTG AGC-3′ and antisense (843–878), 5′-CTA TAG TAA GAA GGC TCC AAA GAA GGT TTT TTC TTT-3′; produced size 756 bp. Human DR3 (GenBank accession no. U72763) sense (515–536), 5′-ATG GCC GAT GTG CCT GGT GAG TCT GCT GCT-3′ and antisense (852–875), 5′-AGC GCC TCC TGG GTC TCG GGG TAG-3′; produced size 361 bp. Human FasL (GenBank accession no. NM000639) sense (456–475), 5′-GGA TGG TTC ATG TGT CCC AGC TTT GCA ATG GCT GCG TGT CCT GCC TCC TGA TGC TCC TGG GTC TCG GGG TAG-3′; produced size 597 bp. Human LIGHT (GenBank accession no. NM_014798) sense (182–211), 5′-CTT CAC ATG GAG GAT TGT GTG GAT GGA-3′ and antisense (536–575), 5′-TTG CCA TCC TTC TCA AAG TTG TAG G-3′; produced size 510 bp. Equal amounts of each reverse transcription product (1 μg) were PCR amplified using Taq polymerase in 35 cycles consisting of 1 min at 95°C, 1 min at 66°C (for FasL and LIGHT) or 58°C (for TL1A, DR3, and MMP-2), and 1 min at 72°C. The amplified cDNA was run on 1% agarose gels and visualized by ethidium bromide.

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membrane matrix. Cells were treated with vehicle or stimulators for 24 h, and then the tube formation was observed by microscopy.

**In Vivo Angiogenesis Model.** The 9.9 mg/ml/0.5-ml Matrigel basement membrane matrix containing vehicle or stimulators were s.c. injected into the nude mice. After 6-day incubation, animals were euthanatized with i.p. injection of 50 mg/kg pentobarbital. The Matrigel plug was carefully clipped for histological examination and the determination of angiogenesis using a hemoglobin assay kit. For histological examination, the plug was put into 4% paraformaldehyde and embedded in paraffin. The embedded tissues were sectioned at 6 μm thick, stained with H&E, and analyzed using a microscope. Matrigel plug with the same weight was homogenized, and hemoglobin content was assayed at the manufacturer’s instruction and then measured at absorbance 550 nm. For immunostaining, the paraffin-embedded slices were deparaffinized with xylene, and endogenous peroxidase was blocked by 3% H2O2 and methanol (1:4 volume for volume) for 10 min. After washing with PBS, slices were incubated with antimouse CD31 Ab and then developed by DAKO LSAB + kit (DAKO Corp., Carpinteria, CA) at the manufacturer’s instructions. Sections were counterstained with H&E and analyzed using a microscope. The handling of mice follows the regulation of National Taiwan University Guide for the Care and Use of Laboratory Animals.

**Chicken Chorioallantoic Membrane (CAM) Assay.** Fertilized chick embryos were incubated for 10 days at 37°C with 70% humidity. A small hole was made with a drill directly over the air sac at the end of the egg. The embryos were candled to determine a location to drill a second hole directly over embryonic blood vessels. The CAM was separated from the egg’s shell by applying vacuum to the original hole. A 1 × 1 cm² window was cut in the egg’s shell over the dropped CAM with a grinding wheel, exposing the CAM to direct access for experimental manipulation. Cortisone acetate-treated filter disks were soaked with 10 μl of stimulators in PBS and added directly to the CAM. The windows were sealed with tape and incubated at 37°C. After 72-h incubation, sections of CAM tissue were cut and fixed in 4% paraformaldehyde for 2 min. The images were collected on a microscope.

**Data Analysis and Statistics.** Data are expressed as means ± SE of at least three experiments and represented as folds of control. Statistical comparisons between groups were performed using Student’s t test. P < 0.05 was considered statistically significant.

**RESULTS**

**TL1A and DR3 Expression in HUVECs.** Before we performed experiments to understand the effects of DcR3 in angiogenesis relating to binding and neutralization of TL1A, we first checked the expression of TL1A in cells and culture supernatant. Immunoblotting (Fig. 1A, top) and RT-PCR analyses (Fig. 1A, bottom) demonstrated the expression of TL1A in HUVECs and prostate cancer cell line PC3 but not in HAEC. On the basis of the same amounts of total protein and RNA quantification, TL1A protein and mRNA in HUVECs were 1.6- and 2.3-fold of those in PC3. Consistent with these findings, immunoprecipitation by DcR3.Fc and TL1A-Ab, followed by anti-TL1A Ab as probe in Western blot analysis, revealed the presence of a M̅ 32,000 protein from the culture medium of HUVECs, indicating TL1A is not only expressed in HUVECs but also secreted to the culture medium (Fig. 1B). In addition, using Western blotting (Fig. 1C, top) and RT-PCR (Fig. 1C, bottom) approaches, we detected the

![Fig. 3](image-url) Induction of HUVEC migration by DcR3 (1 × 10⁶ cells in 200 μl of M199 medium) was placed in the top compartment of Boyden chamber. DcR3.Flag, DcR3, LTαR.Flag, VEGF, TL1A-Ab, DR3-Ab, or mouse IgG1-Ab at the concentrations indicated was added into the bottom compartment of Boyden chamber. After 6 h, cells migrated through the filter were fixed and stained with H&E (A). Staining with 1% crystal violet and measured absorbance at 550 nm were determined (B and C). Images in A represent ×100 magnification. The data in B and C represent the mean ± SE from at least three independent experiments performed in duplicate. *P < 0.05 as compared with the corresponding control.
presence of DR3, the membrane form receptor for TL1A, in HUVECs, HAEC, and PC3 but not in colon epithelial cell line HT29. Compared with the approximate equivalence of DR3 levels in HUVECs and PC3, DR3 expression in HAECs was much less. The presence of TL1A and DR3 simultaneously in the same cell type raised the question whether TL1A acts as a negative regulator for HUVECs by binding to DR3 receptor on cell membrane. Except determining TL1A expression, we further measured the expression of the other ligands of DcR3, FasL and LIGHT. RT-PCR results shown in Fig. 1D indicated that FasL can be expressed in PC3 and primary human monocytes, but not in HUVECs, whereas LIGHT was expressed in monocytic THP-1 cells but not in HUVECs and PC3.

Proapoptotic and Antiapoptotic Effect of DcR3 in HUVECs. We next examined the effect of DcR3 fusion proteins (DcR3.Fc, Fc-free Flag-tagged DcR3.Flag) and DcR3 itself on endothelial cell proliferation. As shown in Fig. 2A, the addition of DcR3.Flag induced HUVEC proliferation from 0.1 to 3 μg/ml in a dose-dependent manner and reached plateau at 3 μg/ml. Likewise, DcR3 and DcR3.Fc, each at 3 μg/ml, had similar effect on cell proliferation as DcR3.Flag, suggesting the specific effect of DcR3 rather than the fused tag proteins. Results also indicated an additive response between DcR3.Flag and VEGF. Observing the noneffectiveness of LTβR.Flag protein on cell proliferation (Fig. 2A), we again confirmed the specific effect of DcR3 unrelated to Flag protein. To assess whether TL1A neutralization contributes to the specific effect of DcR3, two approaches were carried out, i.e., anti-TL1A Ab (3 μg/ml) to deplete sTL1A in the supernatant and anti-DR3 Ab (3 μg/ml) to block the binding of sTL1A to DR3. We found that both anti-TL1A and anti-DR3 antibodies have similar effect as DcR3 to induce cell proliferation (Fig. 2B), indicating the effect of DcR3 and DcR3 fusion proteins on EC proliferation is via blocking the negative regulatory role of TL1A on cell growth. Except HUVECs, we also assessed the DcR3 effect in HAECs. Nonetheless, in contrast to the action in HUVECs, we did not observe the increased cell proliferation after DcR3 treatment, regardless of the same response caused by VEGF in HAECs (data not shown). Given that HAECs did not express TL1A (Fig. 1A), this striking result in HAECs provides strong evidence supporting DcR3 action through TL1A binding.

We next investigated whether DcR3 could influence cell apoptosis. As shown in Fig. 2C using apoptotic kit assay, the antiapoptotic effect of VEGF, DcR3 markedly inhibited the apoptosis of HUVECs induced by serum deprivation for 12 h, incubation with TNF-α (50 ng/ml) plus IFN-γ (100 units/ml) for 24 h, and high glucose (33 mM) for 48 h. In addition, results from an assay of another apoptotic parameter, phosphatidylserine externalization stained by annexin V also showed that the percentages of cell viability were increased by DcR3 (data not shown).

DcR3 Induces HUVEC Migration. We next examined the effect of DcR3 on migratory motility of HUVECs. After incubation with DcR3.Flag in a Boyden chamber for 6 h, the migration of HUVECs in response to DcR3.Flag was in a dose-dependent manner, and the maximal effect was observed when HUVECs were treated with 10 μg/ml DcR3.Flag (Fig. 3, A and B). Similar efficacy on cell migration was observed for DcR3, whereas LTβR.Flag was without effect (Fig. 3B). The migratory activity of DcR3 at 3 μg/ml increased 2.5-fold over the control and was comparable with that of the VEGF at 10 ng/ml. In addition, the treatment with anti-TL1A Ab and anti-DR3 Ab induced similar effects as DcR3 on HUVEC migration (Fig. 3C). Accordingly, the TL1A-absent HAECs did not respond to DcR3 and DcR3.Flag (data not shown). All these results extend our previous argument that the DcR3-mediated HUVEC migration is via the blocking of negative regulator TL1A binding to DR3 on cell membrane.

DcR3.Flag Enhances Gelatinolytic Activity Released by HUVECs. MMP-2 is a major extracellular matrix proteolytic enzyme and secreted by endothelial cells to enhance endothelial cell migration across the matrix. SDS-PAGE gelatin zymography of the conditioned media from HUVECs demonstrated a characteristic gelatinase activity at Mr 72,000 corresponding to proMMP-2 before the treatment of DcR3.Flag or VEGF. When cells were incubated for 24 h in the medium supplemented with various concentrations of DcR3.Flag, the secretion of the Mr 62,000 and 64,000 active forms of MMP-2 was increased in a dose-dependent manner (Fig. 4A). DcR3.Flag also up-regulated the expression of MMP-2 mRNA by 7.5-, 8.3-, and 6.1-fold at 2, 4, and 6 h, respectively (Fig. 4B). The increased gelatinase activity by DcR3 was also mimicked by anti-TL1A and -DR3 antibodies (Fig. 4C). This supports that the up-regulation of MMP-2 by DcR3 might be via the neutralization of endogenous sTL1A.

DcR3 Induces Tube Formation of HUVECs. We further investigated whether the removal of endogenous sTL1A would affect the tube formation of HUVECs, a characteristic of angiogenesis. No significant morphological change was observed when HUVECs were placed on Matrigel for 24 h or were treated with LTβR.Flag. In contrast, incubation of HUVECs with DcR3, anti-TL1A monoclonal Ab, anti-DR3 monoclonal Ab, or VEGF on Matrigel for 24 h led to the formation of elongated and robust tube-like structures (data not shown).

DcR3 Induces Angiogenesis in Vivo. All of the evidences shown above suggest the endogenous TL1A acts as a negative regulator in the TL1A/DR3 autocrine loop, and blockade of the negative feedback...
Fig. 5. Identification of DcR3-induced angiogenesis by mouse Matrigel plug assay and chorioallantoic membrane (CAM) assay. In A, nude mice were injected s.c. with 0.5 ml of Matrigel with or without DcR3.Flag (30 μg/ml) and VEGF (150 ng/ml). After 6 days, animals were euthanized. In B, the Matrigel plug was embedded in paraffin. The embedded tissue was stained with H&E and analyzed by histological examination. In C, deparaffinized sections were immunohistochemically stained using an antimouse CD31 Ab and analyzed by microscopy. Image represents ×200 magnification. In D, quantitative effect of DcR3.Flag and VEGF on neovessel formation in Matrigel plug was analyzed by hemoglobin assay kit. The data represent the mean ± SE from eight mice in each group. *P < 0.05 as compared with the corresponding control. In E, 10-day-old chick CAMs were exposed to filter paper disks saturated with DcR3.Flag (3 μg) or VEGF (25 ng) and then incubated for 72 h. Photomicrographs were taken at ×20 with a stereomicroscope. The data are representative of three independent experiments.
loop seems to be able to induce angiogenesis. Herein, we further asked whether DcR3.Flag has the ability to induce angiogenesis in vivo. To answer this question, an in vivo angiogenesis assay was used to evaluate the in vivo angiogenic activity of DcR3. Matrigels alone, or in conjunction with DcR3.Flag (30 μg/ml) or VEGF (150 ng/ml), were injected s.c. into nude mice for 7 days. The solid gel plug was removed from the mice at day 7 after implantation for histological examination. As shown in Fig. 5A, both DcR3.Flag and VEGF induced significant neovessels within Matrigels than control group. The effect of DcR3.Flag (30 μg/ml) was comparable with that of VEGF (150 ng/ml). Histological analysis demonstrated the induction of cellularity and formation of cords, tubules, and several blood-filled channels containing RBCs on Matrigel pellets by DcR3.Flag and VEGF (Fig. 5B). Immunohistochemical analysis with endothelial marker CD31 (31) also revealed the appearance of endothelium in Matrigels treated with DcR3.Flag (30 μg/ml) and VEGF (150 ng/ml; Fig. 5C). In contrast, only few infiltrating single elongated cells were observed in Matrigel pellets without angiogenic stimuli. Quantitation of angiogenesis by hemoglobin content showed that the addition of DcR3 and VEGF to the Matrigel, compared with Matrigel alone, could increase angiogenic response ~7- and 8-fold, respectively (Fig. 5D). This suggests that DcR3 might have potent angiogenic activity in vivo via removing the suppressive effect of endogenous TL1A.

Meanwhile, the identification of DcR3-induced angiogenesis by chicken CAM assay was carried out. As shown in Fig. 5E, treating 3 μg of DcR3.Flag and 25 ng of VEGF in 10-day fertilized chick embryos, CAM could induce a significant neovascularization compared with the control group.

**DISCUSSION**

Angiogenesis is an essential step for many physiological and pathological processes, especially cancer formation (3). The process of angiogenesis is the outcome of an imbalance between positive and negative angiogenic factors (3, 6). However, the relative expression of the individual angiogenesis factors that contribute to this process has to be investigated more thoroughly. In the present study, by using a decoy receptor DcR3 to reflect the potential autocrine or paracrine role of endogenous TL1A in angiogenesis, we, for the first time demonstrate a novel action of this decoy receptor. We suggest DcR3 might act as an angiogenic factor via blocking the negative regulator TL1A.

During the formation of new blood vessels, endothelial cells are stimulated to migrate, proliferate, and invade surrounding tissue to form capillaries. In this study, we demonstrated that DcR3 induces a proangiogenic phenotype in human endothelial cells. This phenotype includes both early (i.e., increase in cell proliferation, migration, and MMP-2 expression) and late angiogenic events (differentiation into vascular cords; Refs. 6, 31, and 32). In the in vitro angiogenesis models, we have revealed that the addition of DcR3 induces HUVEC proliferation, up-regulates migratory motility, and induces the formation of tube network. In the mouse Matrigel plug and chicken CAM assays, the ability of DcR3 to promote neo-vessel formation in vivo was comparable with the well-established angiogenic factor VEGF. The angiogenic effect of DcR3 was independent of VEGF, because DcR3 could not induce any VEGF increase by ELISA assay (data not shown). In addition, the effect of DcR3 and VEGF on cell proliferation is additive. Meanwhile, ELISA assay and NO detection rule out the involvement of TNF-α and NO in DcR3 action (data not shown). Thus, based on these results, we propose a novel action of DcR3 in support of tumor survival and growth. This newly identified angiogenic action of DcR3, together with its suppressive functions as identified previously in immune responses, which help malignant cells escape host immune attack (9, 26–28, 33), strengthen the crucial role of DcR3 in tumor progress. Because DcR3 is highly expressed in several tumor cells (20, 22, 25–27), and detected in serum of cancer patients (29), understanding the regulation and pathological actions of DcR3 would provide a new strategy in tumor therapy.

It had been described that TL1A is the predominant, full-length gene product in endothelial cells, and an overexpression study showed the antiangiogenic activity of TL1A in vivo (8, 19). On the basis of these findings, although TL1A is assumed to be a putative angiogenic inhibitor, its physiological significance and receptor identity are poorly unknown. Addressing these points in the present study, we firstly demonstrated that cytokine TL1A and its receptor DR3 are both expressed in HUVECs. This conclusion is based on the detection of their mRNA transcription and protein expression. Moreover, we identified the existence of secreted TL1A in culture medium. Meanwhile, we observe that anti-TL1A and -DR3 antibodies could lead to an increase in cell proliferation, migratic motility, and induction of the formation of tube network. These entire angiogenic effects of TL1A and DR3 antibodies are similar to those induced by DcR3. As a result, we suggest the secreted TL1A at physiological concentrations is sufficient to exert its apoptotic function in endothelial cells, and this action is mediated by DR3-related signaling pathway. Although currently we do not show the signaling transduction mediated by DR3 leading to inhibition of endothelial cells, previous studies have supported this notion. DR3 is a death domain containing receptors belonging to TNFR superfamily. Like signaling cascades of TNFRI, it was described capable of inducing caspase-dependent cell apoptosis (7–9). Consistently, according to the study of Yue et al. (13), activation of caspases contributes to VEGI-induced apoptosis.

Intriguingly, our study indicates the angiogenic action of DcR3 is endothelial cell type specific. Unlike the increased angiogenesis in HUVECs, DcR3, however, did not affect proliferation, migration, and differentiation-dependent tube formation of HAECs. These differences in biological effects between these two types of endothelial cells are mainly explained by the expression or not of TL1A. In HAECs, TL1A mRNA and protein are undetected, and this notion is in line with a previous study by Migone et al. (19). This cell type-dependent phenomenon again supports our conclusion, i.e., the action of DcR3 is mediated by abrogating the negative regulatory role of TL1A.

In conclusion, we for the first time provide evidence to demonstrate that the decoy receptor for TNF family cytokines, DcR3, can induce angiogenesis in both in vitro and in vivo systems. We also define the mechanism by which DcR3 achieves this action, which is mediated through the neutralizing ability of DcR3 on the negative regulator of endothelial cells, TL1A. These results suggest important roles of DcR3 in the pathogenesis of cancers and might be helpful to develop better strategies for cancer treatment in the future.

**REFERENCES**


Soluble Decoy Receptor 3 Induces Angiogenesis by Neutralization of TL1A, a Cytokine Belonging to Tumor Necrosis Factor Superfamily and Exhibiting Angiostatic Neutralization of TL1A, a Cytokine Belonging to Tumor Necrosis Factor Superfamily and Exhibiting Angiostatic Action

Chia-Ron Yang, Shie-Liang Hsieh, Che-Ming Teng, et al.


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