TNFSF15 Modulates Neovascularization and Inflammation

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Abstract Tumor necrosis factor superfamily-15 (TNFSF15; also known as VEGI or TL1A) is a unique cytokine that functions in the modulation of vascular homeostasis and inflammation. TNFSF15 is expressed abundantly in established vasculature but is down-regulated at sites of neovascularization such as in cancers and wounds. TNFSF15 inhibits endothelial cell proliferation and endothelial progenitor cell differentiation. Additionally, TNFSF15 stimulates T cell activation, Th1 cytokine production, and dendritic cell maturation. Some of the functions of TNFSF15 are mediated by death receptor-3. We review the experimental evidences on TNFSF15 activities in angiogenesis, vasculogenesis, inflammation, and immune system mobilization.

Keywords TNFSF15 · VEGI · TL1A · Neovascularization · Cytokine

Introduction

Tumor necrosis factor superfamily (TNFSF) is a group of structurally related cytokines with a wide range of functions in the modulation of immunity, inflammation, and the differentiation, proliferation, and programmed death of many different types of cells [1, 2]. TNF family members exert their activities through specific cell-surface receptors that belong to the TNF receptor superfamily (TNFRSF). Thus far, 19 TNFSF members and 27 TNFRSF members have been identified. Most of them have been implicated in a wide variety of disease conditions, including cancer [3, 4], arthritis [5, 6], bone remodeling [7], allergy [8], diabetes [9,

10], atherosclerosis [11], myocardial infarction [12], graft versus host disease [13], and acquired immune deficiency [14, 15]. A subset of these receptors, known as the death receptors, contain a segment of amino acid residues within the cytoplasmic region named the death domain, and are responsible for the initiation of intracellular signals leading to cell death [1, 15, 16].

Tumor necrosis factor superfamily-15 (TNFSF15) is a more recently discovered TNFSF member. It is also known as vascular endothelial growth inhibitor (VEGI) [17], TNF-like ligand-1 (TL1) [18], or TL1A [19]. The role of TNFSF15 in physiology and pathology is only beginning to be elucidated. Similar to other TNFSF members, TNFSF15 is multifunctional. However, TNFSF15 functions appear to be uniquely associated with vascular endothelial cell activities. TNFSF15 is predominantly produced by vascular endothelial cells and specifically inhibits endothelial cell growth [17, 18, 20, 21]. TNFSF15 is also a T-cell co-activator [19, 22–24] and a stimulator of dendritic cell maturation [25], which is a critical initial step in the activation of the host immune system [26–28]. TNFSF15 thus plays a key role in the maintenance of vascular and immune system homeostasis (Fig. 1).

Discovery of TNFSF15

TNFSF15 was initially identified from the human umbilical vein endothelial cell cDNA library and named TNF-like ligand 1 (TL1) because of a high degree of sequence homology to TNF- α [29]. The primary structure of TNFSF15 exhibits a 20–30 % overall homology to human TNF- α , TNF- β and the Fas ligand, typical of the degree of conservation among TNFSF members. TNFSF15 was simultaneously discovered as a specific inhibitor of vascular endothelial cell growth, and thus named VEGI [17]. This newly discovered cytokine is later given the Human Gene Mapping Workshops approved nomenclature of TNF superfamily member 15 [30]. Interestingly, phylogenically TNFSF15 appears to represent one of the earliest members

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Fig. 1 Schematic presentation of TNFSF15 functions. TNFSF15 inhibits endothelial cell proliferation and endothelial progenitor cell differentiation, co-activates T-cells, and stimulates dendritic cell maturation

of the TNF family, as TNF itself has not been found in the avian genome, but the cloning of TNFSF15 from chickens has been reported [31], implying that TNFSF15 is part of the early immune system in evolution.

TNFSF15 Isoforms

TNFSF15 gene is mapped to human chromosome 9q32. It spans about 17 kb and consists of four exons. Three different TNFSF15 transcripts generated by differential splicing have been described [32]. The initially characterized TNFSF15 isoform consists of 174 amino acid residues, thus designated VEGI-174, containing a putative transmembrane domain and an extracellular domain common to all three isoforms. The other two transcripts of 7.5 and 2.0 kb encode isoforms of 251 (VEGI-251) and 192 amino acid residues (VEGI-192), respectively. All three isoforms are present in endothelial cells and, when secreted as soluble proteins or prepared as recombinant proteins, are able to induce apoptosis to proliferating endothelial cells [20, 32, 33]. The carboxyl terminal segment of 151 amino acid residues common to all three isoforms is the TNF homology domain (THD). VEGI-251 and VEGI-192 differ in the first exon. VEGI-251 possesses a secretion signal peptide, and is found in the conditioned media when the VEGI-251 expression vector is transfected into mammalian cells [32], while the other two isoforms remain associated with the cells under similar experimental conditions. As we will discuss below, secretion is necessary for TNFSF15 to exert its functions. The significance of the cytosolic presence of the two non-secreted isoforms is currently not apparent.

Preparations as Research Reagents

From TNFSF15 a number of reagents have been derived. VEGI-174 is a truncated form of initially discovered VEGI cDNA consisting of 174 amino acids [21], which is also known as TL1 [18]. TL1A is another recombinant protein

consisting of 180 amino acids, from residues 72 to 251 of VEGI-251 [19]. VEGI-192 is yet another recombinant protein consisting of the full length of isoform VEGI-192 [33]. Most of TL1A studies are related to its function as a co-stimulator of T cells [34–36] and its role in inflammation [37]. On the other hand, VEGI-174 and VEGI-192 are reported mainly in the context of angiogenesis inhibition and tumor growth inhibition [33, 38]. However, these two lines of activities are closely related. For example, an anti-TL1A antibody was shown to induce angiogenesis [39], and VEGI-192 was demonstrated to stimulate the maturation of dendritic cells [25], one of the essential components of the immune system. It will be interesting to reveal how the two lines of TNFSF15 activities intertwine mechanistically.

Tertiary Structure

The crystal structure of human TNFSF15 is recently reported [40, 41]. The tertiary structure is composed of three TNFSF15 monomers, resembling the tertiary structure of other TNF family members. Each of the monomeric unit is a sandwich of two β -sheets. A loop between strands C and D in TNFSF15 is the longest among the TNF ligand members, while the AA' loop, part of which is disordered, in TNFSF15 is the second longest after that in TRAIL [42]. Similar loops in TNF- β /LT- α are known to participate in receptor binding [42].

One of the TNFSF15 receptors is known as decoy receptor-3 (DcR3) [19]. It is a secreted protein of the TNF receptor superfamily. The structure of TNFSF15/DcR3 complex was reported recently [43]. Interestingly, DcR3 is able to neutralize the activities of three different TNFSF members: FasL [44, 45], LIGHT [46], and TNFSF15 [47]. It has been demonstrated that DcR3 interacts with the backbone and side-chain atoms in the membrane-proximal half of TL1A [43]. These results may lead to insights into the structure basis of TNFSF15 signaling.

The Role of TNFSF15 in the Maintenance of Vascular Homeostasis

Expression Patterns

TNFSF15 is detected predominantly in endothelial cells in cell cultures, while the gene transcript is found in many human tissues, including placenta, lung, kidney, skeletal muscle, pancreas, spleen, prostate, small intestine and colon [17, 29]. The distribution profiles of the two TNFSF15 isoforms, VEGI-174 and -251, in human organs and tissues appear to be different [32]. The 7.5 kb transcript encoding VEGI-251 is expressed at high levels in the placenta, kidney, lung and liver, whereas the 2.0 kb transcript corresponding to VEGI-174 is

detected in liver, kidney, skeletal muscle and heart. VEGI-251 is also expressed at high levels in fetal kidney and fetal lung. Overlapping of the two transcripts is detected in prostate, salivary gland and placenta. When examined in cultured cells by using RNase protection assay [32], all three isoforms are found in different types of human endothelial cells, including coronary artery endothelial (HCAE), umbilical-vein endothelial cells (HUVEC), and microvascular endothelial (HMVE) cells. Although the isoforms are present simultaneously, VEGI-251 is by far the most abundant. These expression patterns suggest the possibility of tissue- or developmentally specific functions for the TNFSF15 isoforms. Immunohistochemical analysis indicates that TNFSF15 is mainly associated with established vasculature, with a distribution pattern highly similar to that of endothelial cell markers such as PECOM (CD31) [33, 48].

Regulation of TNFSF15 Expression

TNFSF15 expression has been shown to be stimulated by TNF- α and interleukin-1 [19, 32, 49], whereas TNFSF15 expression is down-regulated by interferon- γ [32], vascular endothelial cell growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) [48]. In a clinical setting, TNFSF15 is found to be prominently present in the vasculature of normal ovary but diminishes in ovarian cancer as the disease progresses [48]. VEGF produced by cancer cells and MCP-1 produced mainly by tumor-infiltrating macrophages and regulatory T cells effectively inhibits TNFSF15 expression in endothelial cells [48]. In another study, TNFSF15 is up-regulated by c.MIF (chicken macrophages migration inhibitory factor) in HD11 chicken macrophages [50]. In bovine uterus, the expression of VEGF and its receptors as well as VEGI during the follicular and luteal phases varied with different cell types, which indicated that ovarian steroid hormones play a significant role in regulating the expression of these cytokines [51]. In a study on prostate cancer, AMPK (5' adenosine monophosphate-activated protein kinase) enhances transcription levels of TNFSF15 and inhibits tumor growth [52]. TNFSF15 expression is also modulated by IL-1 β and chondroitin sulfate in osteoarthritis patients [53], by TLR8 in monocytes [54], or by sodium butyrate in lung vascular cells [55]. A study on the promoter of mouse TNFSF15 [56] shows that NF- κ B subunits p50 and p65 interact with the TNFSF15 promoter, and overexpression of p65 enhances, but that of p50 inhibits TNFSF15 expression. Site-directed mutation studies on the NF- κ B binding site in the TNFSF15 promoter indicate that NF- κ B binding site is critical for TNFSF15 expression. Activation of the NF- κ B signaling pathway by LPS is also reported to participate in TNFSF15 gene expression [57]. These findings indicate that TNFSF15 function is subject to tight controls at gene expression level.

Inhibition of Endothelial Cell Proliferation

Recombinant TNFSF15 (VEGI-174) consisting of the main segment common to all isoforms can induce growth arrest or apoptosis, depending on the stage of the cell in the growth cycle, in HUVEC, adult bovine aortic endothelial (ABAE) cells and bovine pulmonary artery endothelial (BPAE) cells [17, 18, 20]. TNFSF15 exerts two different activities on endothelial cells: early G1 arrest in G0/G1 cells responding to growth stimuli, and programmed death in proliferating cells [20]. These findings allow us to postulate that VEGI may have a role in the maintenance of a quiescent endothelium by enforcing a growth arrest of endothelial cells in an established vasculature and the termination of angiogenesis by inducing apoptosis of proliferating endothelial cells. The growth inhibition effect of TNFSF15 appears to be specific on endothelial cells, as the same protein preparation has little effect on the proliferation of a number of non-endothelial cells such as human coronary artery smooth muscle cells, human breast cancer cells, human T-cell, mouse NIH-3 T3 fibroblast cells, and Lewis lung cancer cells [21, 33]. TNFSF15 must be secreted by endothelial cells in order to induce apoptosis to endothelial cells themselves in an autocrine manner, as non-secreted forms of TNFSF15 when transfected into HUVEC has no effect on the viability of the transfected cells [32]. VEGI-174 recombinant protein is shown to inhibit the growth of human breast cancer MCF7 cells, cervical cancer HeLa cells, and myeloid tumor U-937 and ML-1a cells in culture [58]; however the concentration needed appears to be substantially higher than that for endothelial cells.

Anti-Angiogenesis Effect

The anti-angiogenesis activity of TNFSF15 was mainly assessed in a number of in vitro and in vivo models. In an in vitro angiogenesis model, recombinant VEGI-174 was used to treat ABAE cells grown on a layer of collagen gel [21]. When stimulated with fibroblast growth factor-2 (FGF2) or VEGF, control ABAE cells formed a network of capillary-like tubules in the gel. Addition of the VEGI-174 completely inhibited the capillary formation but did not cause cell death. In another experiment, human vascular endothelial cell line transfected with a TNFSF15-expressing plasmid exhibited a decreased ability to form microtubules in Matrigel [59]. When evaluated in the chick embryo chorioallantoic membrane (CAM) model, recombinant VEGI-174 inhibited about 50 % of the neovascularization induced by either FGF or VEGF [21]. In another study, an adenovirus expressing a fusion protein consisting of endostatin and the C-terminal 151 residues of TNFSF15, termed AdhENDO-VEGI151, were shown to be able to prevent angiogenesis in both the CAM model and a rabbit

corneal neovascularization model [60]. These results indicate that TNFSF15 can inhibit capillary formation regardless of the types of angiogenic stimuli. This is consistent with the view that TNFSF15 stimulates an independent signaling pathway.

Inhibition of Endothelial Progenitor Cell Differentiation in Vitro and in Vivo

VEGI exhibits an inhibitory function on the differentiation of endothelial progenitor cells (EPC) [61]. Treatment of EPC from mouse bone marrow-derived Sca1⁺ mononuclear cells with recombinant VEGI-192 protein results in an inhibition of the expression of a number of endothelial cell markers but not that of stem cell markers compared to vehicle control. Consistently, the VEGI-treated EPCs exhibit decreased ability to adhere, migrate, and form capillary-like structures on Matrigel in vitro. In addition, VEGI induces apoptosis of differentiated EPCs but not early-stage EPCs. These findings indicate that VEGI may participate in the modulation of postnatal vasculogenesis by inhibiting EPC differentiation.

In a study using mouse Lewis lung carcinoma (LLC) tumor model, VEGI shows inhibitory effects on bone marrow (BM)-derived EPC incorporation into the tumors [62]. In that experiment, whole bone marrow from green fluorescent protein transgenic mice was transplanted into C57BL/6 mice, which were then inoculated subcutaneously with LLC cells. Intraperitoneal injection of VEGI significantly inhibited tumor growth and tumor angiogenesis compared to vehicle-treated mice. The number of BM-derived EPC in VEGI-treated tumors was notably less than that in the vehicle-treated group, and most of the apoptotic cells in the VEGI-treated tumors were of bone marrow origin. These findings suggest that VEGI inhibits BM-derived EPC mobilization and prevents their incorporation into LLC tumors by inducing apoptosis specifically of BM-derived EPCs that have differentiated into endothelial cells, resulting in the inhibition of EPC-supported tumor vasculogenesis and tumor growth.

TNFSF15 in Wound Healing

Consistent with an inhibitory role in neovascularization, it was shown [59] that, by immunostaining, TNFSF15 expression levels were low at the wound edge in the acute wound tissue but significantly increased moving away from the wound edge toward normal tissues. In sharp contrast, TNFSF15 is greatest in chronic wound tissue at the wound edge and decreased moving away. At transcript level, TNFSF15 expression is reduced in acute wound tissue compared with chronic wound tissue or normal skin, while in the chronic wound tissue it is comparable to that in the normal

skin. The distribution of TNFSF15 in various wound types is similar to those of hepatocyte growth factor activator inhibitors HAI-1 and HAI-2, which inhibit HGF-induced angiogenesis. These results support the view that TNFSF15 is involved in suppressing the proliferation and differentiation of endothelial cells in a normally quiescent vasculature in adults. Decreased TNFSF15 expression during normal wound healing at the wound edge is consistent with the requirement that new blood vessels form quickly in order to achieve successful healing. An increased expression of TNFSF15 seen at the wound edge of chronic wound tissue, on the other hand, would explain that chronic wounds, which ultimately are slow to heal or remain unhealed, might be the result from inhibition of new blood vessel growth. The increased expression of TNFSF15 in normal skin is consistent with a role in the maintenance of a quiescent vasculature in normal tissue. Furthermore, DR3-TNFSF15 pathway was also reported to take parts in acute kidney injury [63]. The expression of DR3 and TNFSF15 were found to be enhanced in tubular epithelial cells (TECs) during renal injury. A renal injury model induced by cisplatin showed that cisplatin-induced nephrotoxicity diminishes as the DR3-TNFSF15 system is activated, suggesting a beneficial role of TNFSF15 in wound healing.

Anti-Angiogenesis and Anti-Cancer Effect in Tumor Models

Studies on tumor models demonstrate that TNFSF15 treatment can eliminate endothelial cells in the tumor neovasculature. Production of a secreted form of VEGI-174 by engineered MC-38 murine colon cancer cells suppressed colon cancer growth in syngeneic C57BL/6 mice [17]. Histological examination showed a marked reduction of neovascularization in the tumors. The anticancer activity of TNFSF15 was further explored in a Lewis lung cancer murine tumor model [33]. Using recombinant VEGI-192, the study revealed a specific targeting of the endothelial cells. Systemic administration of recombinant VEGI-192 led to marked tumor growth retardation and a nearly 90 % decrease of the number of endothelial cells in the tumor vasculature compared to that in vehicle-treated animals. Interestingly, the study also indicated that, in the absence of the endothelial cells, a residual vascular structure persisted consisting of the basement membrane and the smooth muscle cells. While it is not clear whether the residual vascular structure can support blood circulation in the tumors, it is possible that it may provide a framework or foundation for the recruitment of circulating endothelial progenitor cells to repair the blood vessels in the tumor damaged by anti-angiogenic therapeutic agents.

The anti-cancer effect of TNFSF15 was also demonstrated by using human xenograft tumor models [21, 32]. Chinese hamster ovary (CHO) cells stably transfected with a secreted

form of VEGI-174 were mixed with human breast cancer cells (MDA-MB-231 or MDA-MB-435), and co-inoculated into the mammary fat pads of female nude mice. Marked inhibition of xenograft tumor growth was observed. Since VEGI does not inhibit the growth of either CHO or the breast cancer cells *in vitro*, it is plausible that the antitumor activity of the protein derives from its antiangiogenic function. Similar results were obtained from human prostate cancer PC3 cells co-inoculated with these CHO cells [21]. In another study in which the TNFSF15 gene was transferred to a tumor by subcutaneous injection of the AdhENDO-VEGI-151 adenovirus encoding a secreted form of endostatin-TNFSF15 fusion protein, tumor growth was reduced by 88 % in a liver tumor xenograft model; in contrast, injection of the control viruses encoding either endostatin or TNFSF15 alone did not result in tumor regression [60], possibly because of insufficient inhibitory activity under those experimental conditions.

The anticancer activity of systemically delivered recombinant human TNFSF15 protein was evaluated [33, 52]. C57BL/6 mice bearing LLC tumors were treated with VEGI-192, prepared in *E. coli*, via intraperitoneal or intratumoral routes. The treatment resulted in as much as 50 % inhibition of tumor growth even when the treatment was initiated the tumor volumes had already reached nearly 5 % of the body weight. Consistently, a significantly increased survival time of the treated animals was seen. Moreover, the VEGI-192 treated animals did not exhibit apparent liver or kidney toxicity. In another study, inhibition of tumor formation was also observed when the recombinant protein was given at the time of tumor inoculation [62].

In a recent study, the anti-angiogenesis and anti-tumor potential of VEGI-192 fused with a peptide motif RGD (Arg-Gly-Asp) was assessed [64]. RGD is a well-documented motif that exhibits high affinity toward integrin $\alpha(v)\beta(3)$ abundantly expressed in cancer cells and specifically associated with angiogenesis in tumors [65, 66]. Purified recombinant human RGD-VEGI-192 protein (rhRGD-VEGI-192) inhibited endothelial growth *in vitro* and suppressed neovascularization in CAM assay, to a higher degree as compared with recombinant human VEGI-192 protein (rhVEGI-192). Moreover, rhRGD-VEGI-192, but not rhVEGI-192 protein, apparently targeted MDA-MB-435 breast cancer cells, significantly inhibiting MDA-MB-435 cell growth *in vitro*, triggering apoptosis by activation of caspase-8 as well as caspase-3, and consequently giving rise to significant antitumor effect against human breast cancer xenografts.

Down-Regulation of TNFSF15 Expression in Cancers Under Clinical Conditions

In a survey of clinical specimens, lower levels of TNFSF15 in breast cancer are associated with poorer outcome [67].

TNFSF15 expression profile was assessed at both mRNA and protein levels in human normal and cancer cell lines and in a cohort of 151 mammary tissue samples (33 normal breast tissue and 118 breast cancer tissue) with a 6-year median follow-up period. Patients who had died of breast cancer or had local recurrence of the disease exhibited significantly lower levels of TNFSF15 in comparison to elevated levels in the disease-free patients. High levels of TNFSF15 were also associated with an increased patient survival. Patients with breast tumors expressing reduced levels of TNFSF15 displayed a poorer prognosis than those patients with high levels of TNFSF15. No significant correlations were observed between TNFSF15 expression and tumor grade, TNM classification, or nodal involvement, however. The study indicated that TNFSF15 expression may have a potent influence in breast cancer. TNFSF15 was also reported to be down-regulated in other tumors such as urothelial cancer of the bladder [68].

It was shown recently that TNFSF15 is a critical component of the negative control mechanism that operates in normal ovary but is missing in ovarian cancer [48]. In clinical settings TNFSF15 is present prominently in the vasculature of normal ovary but diminishes in ovarian cancer as the disease progresses. VEGF produced by cancer cells and MCP-1 produced mainly by tumor-infiltrating macrophages and regulatory T cells effectively inhibits TNFSF15 production by endothelial cells *in vitro*. Using a syngeneic mouse tumor model, it is demonstrated that silencing TNFSF15 by topical shRNA treatments prior to and following mouse ovarian cancer ID8 cell inoculation greatly facilitates angiogenesis and tumor growth, whereas systemic application of recombinant TNFSF15 inhibits angiogenesis and tumor growth. These findings indicate that downregulation of TNFSF15 by cancer cells and tumor infiltrating macrophages and T-lymphocytes is a pre-requisite for tumor neovascularization.

The Role of TNFSF15 in the Activation of the Immune System

Functions as a T Cell co-Stimulator

A large collection of evidence indicates that TNFSF15 (TL1A) has an important role in the immune system. It was reported first [19] that TL1A augments the responsiveness of T cells to IL-2, although TL1A alone does not induce proliferation of T cells. Pre-treatment with TL1A enhances T cell proliferation in response to IL-2, and induces secretion of interferon- γ (IFN- γ) and granulocyte-macrophage colony stimulating factor (GM-CSF). Additionally, IL-2 receptor IL-2R α (CD25) and IL-2R β (CD122) expression increase in TL1A treated cells.

TNFSF15 can also synergize with IL-12/IL-18 to augment IFN- γ production in human peripheral blood T cells and NK cells, and enhances IFN- γ production by IL-12/IL-18 stimulated CD56⁺ T cells [23]. The synergistic effect of TNFSF15 on cytokine-induced IFN- γ production is more pronounced on CD4⁺ and CD8⁺ T cells than on CD56⁺ T cells or NK cells. Further study showed that only a small subset of peripheral blood T cells responds to TNFSF15 stimulation with IFN- γ production [24]. TNFSF15 has a marked effect on augmenting IFN- γ production by IL-12/IL-18-primed CCR9⁺ CD4⁺ T cells, which also exhibit a cell surface associated presence of TNFSF15. Another study demonstrated that CD4⁺ CD3⁺CD11c⁺ B220⁺ cells provide survival signals to activated CD4⁺ T cells via their constitutively expressed OX40L (CD252 and TNFSF4) and CD30L (CD153 and TNFSF8) in adult mouse [22]. TL1A has no additional effect on IL-12/IL-18-induced cytotoxicity against an NK-susceptible tumor (K562); however, it promotes cytotoxicity against NK-resistant targets susceptible to lysis only by activated NK cells [69]. Soluble TNFSF15 could promote the proliferation and accumulation of antigen-specific CD8⁺ T cells and their differentiation into CTLs, and enhanced the secondary expansion of endogenous antigen-specific memory CD8⁺ T cells in vivo [50]. Since TRADD is believed to participate in the signaling pathway of TNFSF15-DR3 in T cells, using T cells from TRADD knockout mice, a study showed that the response of both CD4⁺ and CD8⁺ T cells to TL1A is dependent on the presence of TRADD, as TRADD knockout T cells lack the appropriate proliferative response to TL1A [70]. These data indicate TNFSF15 has an important role in T cell stimulation and survival.

Stimulation of Dendritic Cells Maturation

TNFSF15 was reported to function on antigen presenting cells [25, 71]. Recombinant VEGI-192 protein was shown to stimulate maturation of dendritic cells. DCs are professional T-cell activating cells and central component of the innate immune system [27, 72]. Bone marrow-derived immature DCs treated with recombinant VEGI-192 display early activation of maturation signaling molecules NF- κ B, STAT3, p38 and JNK, and cytoskeleton reorganization and dendrite formation [25]. Additionally, these cells reveal a significantly enhanced expression of mature DC specific marker CD83, secondary lymphoid-tissue directing chemokine receptor CCR7, the major histocompatibility complex class-II protein (MHC-II), and co-stimulatory molecules CD40, CD80, and CD86. Functionally, these cells exhibit decreased antigen endocytosis, increased cell-surface distribution of MHC-II, and increased secretion of interleukin-12 and TNF. Moreover, TNFSF15-stimulated DCs are able to facilitate the proliferation and differentiation of CD4⁺ naive

T cells in co-cultures. TNFSF15 was also shown to be strongly up-regulated in human monocytes and dendritic cells when the Fc γ R signaling pathway is activated [71]. The effects of TNFSF15 on antigen presenting cells suggest a role of TNFSF15 in soliciting Th1 responses.

Involvement in Inflammation Modulation

A number of studies demonstrated that TNFSF15 plays a key role in inflammatory bowel disease (IBD) by partially functioning as a Th1-polarizing cytokine. TNFSF15 expression is up-regulated in IBD [57, 73], particularly in Crohn's disease (CD) [43, 50, 57, 74–77] and leprosy [46]. TNFSF15 production is localized to the intestinal lamina propria in macrophages and CD4⁺ and CD8⁺ lymphocytes from CD patients as well as in plasma cells from ulcerative colitis patients. The amount of TNFSF15 protein and the number of TNFSF15-positive cells correlated with the severity of inflammation. An increased number of immunoreactive DR3-positive T lymphocytes are detected in the intestinal lamina propria from IBD patients. Addition of TL1A to cultures of phytohaemagglutinin-stimulated lamina propria mononuclear cells from CD patients significantly augments IFN- γ production. Similarly IFN- γ production by peripheral blood mononuclear cells and lamina propria lymphocytes is, in a dose-dependent manner, augmented by TNFSF15, and this activity is independent of, but in synergy with, IL-12 and IL-18 [23]. In the intestinal mucosa, lamina propria CD4⁺ T cells constitutively express TNFSF15, and the number of those cells is increased in mucosal inflammation. Involvement of TNFSF15 in gut inflammation was further demonstrated with two different models of chronic murine ileitis, namely SAMP1/YitFc and TNF^{ARE} mice [78], considered to be good models for Crohn's disease. TNFSF15 is up-regulated in the inflamed gut mucosa in both models, suggesting its involvement in Th1-mediated diseases such as IBD. In transgenic mice constitutively expressing TNFSF15 in T cells or dendritic cells, the animals develop IL-13-dependent inflammatory small bowel pathology that strikingly resembles the intestinal response to nematode infections [79], and goblet cell hyperplasia in the ileum [80]. Constitutive TNFSF15 expression on lymphoid or myeloid cells could lead to mild intestinal inflammation and fibrosis [81].

TNFSF15 is also involved in atherogenesis, rheumatoid arthritis, chronic skin inflammation, as well as renal tubular inflammation and injury [49, 82–84]. TNFSF15 is involved in atherosclerosis though the induction of pro-inflammatory cytokines or chemokines such as TNF α , MCP-1, and IL-8. Treatment of THP-1 cells with recombinant TL1A in combination with IFN- γ causes enhancement of MMP-9 and IL-8 [49]. In rheumatoid arthritis, marked TNFSF15 expression is seen in either synovial fluids or synovial tissue of

rheumatoid factor positive patients [82]. Monocytes and macrophages of rheumatoid arthritis patients strongly expressed TNFSF15 in response to insoluble immune complexes. The addition of TL1A to organ cultures of human or mouse kidney causes activation of NF- κ B, expression of TNFR2, activation of caspase-3, and induction of apoptosis in human renal tubular epithelial cells [83]. In recent reports, TNFSF15 appears to provide an early signal for Th2 cytokine production in lung, and act as a critical trigger for allergic lung inflammation [85]. TNFSF15 also promotes T helper type 17 cell functions and mediates autoimmune disease [86]. By using a TNFSF15-deficient mouse, it was found that TNFSF15^{-/-} dendritic cells exhibit a reduced capacity in supporting Th17 differentiation and proliferation. Moreover, TNFSF15-null animals display decreased clinical severity in experimental autoimmune encephalomyelitis.

TNFSF15 Initiated Signaling Pathways

Receptor of TNFSF15

DR3 (TNFRSF25) and DcR3 (TNFRSF6B) were identified as receptors for TNFSF15 [19]. Using a 293 F cell line stably expressing full-length TNFSF15 on the cell surface, a Fc-fusion form of the extracellular domain of TNFRSF members including TNFR1, Fas, HveA, DR3, DR4, DR5, DR6, DcR1, DcR2, DcR3, OPG, RANK, AITR, TACI, CD40, and OX40 were evaluated for binding. The results show that only DR3 or DcR3 can bind to TNFSF15 protein on the 293 F cells. In another experiment, a FLAG-tagged soluble form of TNFSF15 was applied to cells transiently transfected with various members of the TNFR family, including TNFR2, LTR, 4-1BB, CD27, CD30, BCMA, DR3, DR4, DR5, DR6, DcR1, DcR2, RANK, HveA, and AITR. The tagged TNFSF15 was found to bind only to DR3-overexpressing cells. In addition, direct interaction between TNFSF15 and DR3 was detected by co-immunoprecipitation. The binding of TNFSF15 to DR3 and DcR3 was also confirmed in a flow cytometry-based assay by other researchers [87].

TNFSF15-Death Receptor-3 Pathway

Death receptor-3 (DR3), also known as TNFRSF25, TRAMP, Apo-3, WSL-1, or LARD, contains a death domain in the cytoplasmic segment that is associated with the induction of apoptosis and NF- κ B activation [88–90]. HEK293 cells overexpressing membrane-bound TNFSF15 were co-cultured with IL-12/IL-18-primed CD4⁺ T cells, could induce a 3-fold increase of IFN- γ in the conditioned media, which demonstrated that TNFSF15 can bind to DR3 through cell-cell contact to induce downstream IFN- γ

secretion enhancement [91]. In a study on DR3-mediated apoptosis [92], TNFSF15 was found to induce the formation of a signaling complex consisting of DR3, TRADD, TRAF2, and RIP and activate the NF- κ B, ERK, JNK, and p38 mitogen-activated protein kinase pathways in human erythroleukemic TF-1 cells; however, apoptosis was not induced under the experimental conditions. Interestingly, it was found in the same experiment that TNFSF15-treatment markedly increase the production of c-IAP2, a known NF- κ B-dependent anti-apoptotic protein. Consistently, inhibition of c-IAP2 production by RNA interference sensitized TF-1 cells to TNFSF15-induced apoptosis. These findings suggest that DR3-mediated NF- κ B may determine whether these cells should live or die. This postulate is supported by a study [83] in which the addition of TNFSF15 to organ cultures of human or mouse kidney was found to cause activation of NF- κ B, expression of TNFR2, activation of caspase-3, and apoptosis in human renal tubular epithelial cells, and that inhibition of NF- κ B activation increased TNFSF15-induced caspase-3 activation and enhanced apoptotic cell death. In another study, in bovine aortic endothelial cells pre-treated with siRNA against NF- κ B, TNFSF15-induced apoptosis is significantly augmented, indicating that a combination therapy using TNFSF15 and NF- κ B inhibitors could be a potent approach for cancer treatment [61].

Mediators Responsible for TNFSF15 Inhibition of Angiogenesis

In proliferating endothelial cells, TNFSF15 (VEGI-174)-induced apoptosis is associated with increased expression of Fas and activation of both SAPK/JNK and p38 MAPK [18]. Inhibition of either pathway attenuates TNFSF15-induced apoptosis, which requires caspase-3 or -7. In another study, TNFSF15 (VEGI-174) activity on endothelial cells was shown to be cell cycle-dependent [20]. TNFSF15 elicits a growth arrest on G0/G1-synchronized ABAE cells by inhibiting cyclin-dependent kinases CDK2, CDK4, and CDK6. These cells exhibit characteristics of early G1 arrest, including the lack of the hyperphosphorylation of the retinoblastoma gene product (pRB) and up-regulation of the c-myc gene. Apoptosis occurs only when the cells have entered the late G1 stage to start the growth cycle at the time of TNFSF15 treatment. These findings suggest a dual role for TNFSF15 in the inhibition of endothelial cell proliferation: the maintenance of growth arrest of quiescent endothelial cells and the induction of apoptosis in proliferating endothelial cells.

TNFSF15 is expressed mainly in endothelial cells [17, 33, 48], however, DR3 is preferentially expressed by lymphocytes and is efficiently induced after T-cell activation [93, 94]. TNFSF15 was shown as a ligand of DR3 in T cells, where the engagement leads to facilitated T-cell proliferation [19]. This is in sharp contrast to the observation that

TNFSF15 treatment causes apoptosis to proliferating endothelial cells [20]. The underlying molecular mechanisms remain unclear. Both TNFSF15 and DR3 were found to be expressed in HUVEC [39]. It was shown that treatment of HUVEC with anti-TNFSF15 or anti-DR3 antibodies leads to increased cell proliferation rate, facilitated motility, and enhanced formation of capillary-like network on extracellular matrix proteins. DcR3 exhibits a similar effect. These studies indicate that DR3 is responsible also for the mediation of the anti-angiogenic activity of TNFSF15.

The Role of Decoy Receptor 3

DcR3 appears to be a naturally existing inhibitor of DR3. DcR3 is overexpressed in malignant tumors arising from esophagus, stomach, glioma, lung, colon, and rectum [95–99], and in certain inflammation diseases such as active ulcerative colitis [100] and rheumatoid arthritis [101]. When a soluble DR3 consisting of only the extracellular domain and DcR3 are added to the culture media in a 2:1 molar ratio, DR3 can no longer bind to TNFSF15-expressing cells [19]. Additionally, TNFSF15 induces significant NF- κ B activation in DR3 transfected cells, but this is completely inhibited by an excess amount of DcR3. DcR3 interferes with the differentiation and maturation of dendritic cells and down-regulates T cell proliferation [38, 102, 103]. Cancer cells engineered to release high levels of DcR3 are protected from FasL-induced apoptotic cell death and chemotaxis, and a decreased immune cell infiltration is seen in tumors formed by these cells [96]. Treatment of HUVEC with DcR3 increases the expression of ICAM-1 and VCAM-1 and secretion of IL-8 [104]. Recently, it was reported that DcR3 binding to TNFSF15 expressed in rheumatoid synovial fibroblasts (RA-FLS) results in decreased cell proliferation induced by inflammatory cytokines [105]. DcR3 also regulates the proliferation of osteoarthritis chondrocytes via ERK signaling and Fas-induced apoptosis [106]. DcR3 is expressed in osteoarthritis and normal chondrocytes. Treatment of chondrocytes with DcR3-Fc protects the cells from Fas-induced apoptosis. DcR3-Fc also enhances chondrocytes proliferation and specifically induces ERK phosphorylation in these cells. It would thus seem that DcR3 is not only a decoy receptor for neutralizing ligands of DR3, but possibly also a cytokine with independent functions in the modulation of apoptosis, immune suppression, angiogenesis, and cancer progression, some of these pathways apparently involving TNFSF15-DR3 interaction.

Conclusions

TNFSF15 is a unique cytokine that plays a critical role in the modulation of neovascularization and inflammation, two

tightly intertwining biological processes. TNFSF15 is a physiologically significant negative regulator of neovascularization. It is highly expressed in a normal vasculature but diminishes in an angiogenic vasculature. The antiangiogenic activity of TNFSF15 is highly specific, since only the proliferating endothelial cells in an angiogenic vasculature will undergo apoptosis in response to TNFSF15 treatment, while the quiescent endothelial cells in established blood vessels do not undergo apoptosis but are unable to enter cell growth cycle in the presence of TNFSF15. It also prevents bone marrow-derived hematopoietic stem cells to differentiate into endothelial progenitor cells. These experimental findings suggest that TNFSF15 function as a gatekeeper of neovascularization whose removal is necessary for the initiation of a new blood vessel growth process. TNFSF15 also takes part in the modulation of immune responses, including stimulation of the Th1 immune responses via augmentation of the activities of antigen presenting cells and T cells, stimulation of the production of a number of essential cytokines, and the acceleration of dendritic cell maturation. These two lines of TNFSF15 activities appear to serve a common goal: to bring inflammatory conditions to a resolution.

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