VEGFR1 Tyrosine Kinase Signaling Promotes Lymphangiogenesis as Well as Angiogenesis Indirectly via Macrophage Recruitment

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Objective—Angiogenesis and lymphangiogenesis are complex phenomena that involve the interplay of several growth factors and receptors. Recently, we have demonstrated that in Keratin-14 (K14) promoter-driven Vegf-A transgenic (Tg) mice, not only angiogenesis but also lymphangiogenesis is stimulated. However, the mechanism by which VEGFR1 is involved in lymphangiogenesis remains unclear.

Methods and Results—To examine how important the tyrosine kinase (TK) of VEGFR1 is in lymphangiogenesis in K14 Vegf-A Tg mice, we crossed the K14 Vegf-A Tg mice with Vegfr1-tk–deficient mice to generate double mutant K14 Vegf-A Tg Vegfr1 tk−/− mice. K14 Vegf-A Tg Vegfr1 tk−/− mice exhibit a remarkable decrease in lymphangiogenesis as well as angiogenesis in subcutaneous tissues. To address the mechanism underlying the decrease in lymphangiogenesis, we investigated the recruitment of monocyte-macrophage-lineage cells into the skin. The recruitment of VEGFR1-expressing macrophages driven by VEGF-A was reduced in K14 Vegf-A Tg Vegfr1 tk−/− mice. Vegf-A Tg mice that received Vegfr1-tk–deficient bone marrow showed a reduction of macrophage recruitment, lymphangiogenesis and angiogenesis compared with those in K14 Vegf-A Tg mice.

Conclusions—VEGFR1 signaling promotes lymphangiogenesis as well as angiogenesis mainly by increasing bone marrow–derived macrophage recruitment. (Arterioscler Thromb Vasc Biol. 2008;28:658-664)

Key Words: VEGF-A ▪ VEGFR1 ▪ lymphangiogenesis
postnatal stage, the abovementioned phenomena suggest that VEGFR1 functions as a negative regulator of vascular development by trapping VEGF-A via its ligand-binding domain in the embryonic stage.

In recent years, lymphangiogenesis has been as much under focus as angiogenesis. Unlike angiogenesis, lymphangiogenesis is believed to be mainly mediated by VEGF-C and VEGF-D and their receptor VEGFR3. VEGF-C and VEGF-D also bind VEGFR2, and VEGF-C could affect the permeability via VEGFR2 signaling. Several studies have suggested VEGFR2 system stimulates lymphangiogenesis as well as angiogenesis.

Lymphatic vessels serve as a key route for drainage of interstitial fluid that accumulates in tissues, because of leakage from blood vessels and lymphocyte circulation. Furthermore, newly developed intra- and peritumoral lymphatic vessels have been reported to increase tumor invasion and metastasis in animal models and human patients. We and others have recently proven that VEGF-A stimulates both angiogenesis and lymphangiogenesis. Chronic transgenic delivery of human VEGF-A to the mouse skin driven by the Keratin 14 (K14) promoter resulted in highly developed blood and lymphatic vessels along with extensive macrophage recruitment. VEGF-A promoted lymphangiogenesis in mouse peritumoral lymphatic vessels, lymphatic metastases, and overexpression of VEGF-A–impregnated pellets in the cornea. However, the possible involvement of VEGFR1 in lymphangiogenesis is yet to be clarified.

To determine the role of VEGFR1 in lymphangiogenesis and angiogenesis, we first examined the degree of angiogenesis and lymphangiogenesis that accompanied macrophage infiltration by using K14 Vegf-A transgenic (Tg) mice and Vegfr1 tk–deficient mice. Next, because VEGF-A is known to recruit VEGFR1-expressing macrophages, we evaluated the effect of blocking the VEGF-A–dependent recruitment of macrophages on both angiogenesis and lymphangiogenesis by using Vegfr1 tk–deficient bone marrow transplantation (BMT). In addition, vascular permeability, which is related to lymph vessel dilation, was examined in Vegfr1 tk−/− mice.

**Methods**

**Mice**

All the experiments using animal models were carried out according to the guidelines set by the Animal Center of the Institute of Medical Science, The University of Tokyo. To obtain Vegfr1 tk−/− mice with the K14 Vegf-A transgene, Balb/c K14 Vegf-A Tg mice were first crossed with Balb/c Vegfr1 tk−/− mice and then the K14 Vegf-A Tg Vegfr1 tk−/− mice were crossed with Balb/c Vegfr1 tk−/− mice. Vegfr1+/LacZ mice were maintained as described previously. Vegfr2+/LacZ mice were purchased from The Jackson laboratory (Bar Harbor, Me). For the experiments, 8-week-old littermates were used.

**Vascular Permeability Assay**

The Miles assay of 8-week-old Balb/c mice (Wild-type [Wt] or Vegfr1 tk−/−) was performed as described previously. Images were photographed using a digital camera (Canon EOS). Results represent the mean±SD of triplicate experiments. Experiments were performed using 3 different groups.

**Bone Marrow Transplantation**

To obtain K14 Vegf-A Tg recipient mice (Balb/c×C57BL/6), we crossed Balb/c K14 Vegf-A Tg male mice and C57BL/6 female mice. Newborn F1 pups were conditioned with intrapartum busulfan (Sigma), 2 intraperitoneal injections of 15 mg/kg of diluted dimethyl sulfoxide (DMSO) on day 18 postcoitus (pc) and day 19 pc, and postnatal total body irradiation (TBI) at a dose of 4.0 Gy; a 2 μL volume of the KSL cell suspension (2×10^6) from the C57BL/6 donor mice was administered via the orbital venous plexus in the pups. Peripheral blood was drawn from recipient animals at 8 weeks posttransplantation to analyze the chimera of donor type HLA class I. The peripheral blood cells of the recipients were stained with fluorescein isothiocyanate (FITC)-conjugated anti H2Kd and PE-conjugated anti H2Kb antibodies. The Analysis was performed on a fluorescence-activated-cell sorter (FACS).

**Statistical Analysis**

An Unpaired Student t test was used for all analyses. Differences were considered to be statistically significant at P<0.05 and P<0.01.

**Results**

**Vegfr1 tk−/− Mice Display a Decrease in Lymphangiogenesis as Well as Angiogenesis**

We first macroscopically observed the ear skin of 8-week-old littermates obtained by mating Vegfr1 tk−/− mice with K14 Vegf-A Tg Vegfr1 tk−/− mice. The redness of the skin was significantly increased in the K14 Vegf-A Tg mice as compared with Wt mice (Supplemental Figure IA versus IC, available online at http://atvb.ahajournals.org). Next, we compared the effect of the VEGFR1 tyrosine kinase and observed that the degree of redness was less in Vegfr1 tk−/− mice than in Vegfr1 Wt mice with K14-driven human VEGF-A (supplemental Figure IB versus IA for Wt ear; Supplemental Figure IE versus C for K14 Vegf-A Tg Vegfr1 tk−/− ear). In addition, the redness of the K14 Vegf-A Tg Vegfr1 tk−/− mice was between that of K14 Vegf-A Tg Vegfr1 tk−/− and K14 Vegf-A Tg Vegfr1 tk−/− mice (Supplemental Figure IC through IE). Next, we examined the ear using whole-mount immunostaining with the blood vessel-specific marker PECAM-1 and lymphatic endothelial specific marker LYVE1. Consistent with the macroscopic observation, the vascular density, length, and joint number in K14 Vegf-A Tg Vegfr1 tk−/− mice were lower than that in K14 Vegf-A Tg Vegfr1 Wt mice (Figure 1 B versus 1A, P<0.05; 1D versus 1C, P<0.05; and graph 1M through 1O).

As compared with the blood vessels, the lymphatic vessels in Wt mice were larger in diameter and possessed a loose lymphatic network and blunt end structures, characteristic features of the lymphatic system. Lymphatic vessels of K14 Vegf-A Tg Vegfr1 tk−/− mice were markedly dilated and increased in number. Interestingly, lymphatic vessel density, length, and joint number were significantly lower in K14 Vegf-A Tg Vegfr1 tk−/− mice than in K14 Vegf-A Tg Vegfr1 tk−/− mice (Figure 1H versus 1G, P<0.05; graph 1P through 1R). Basal levels of lymphatic vessels in Wt and Vegfr1 tk−/− mice without transgene were similar to each other, although blood vessels were slightly lower in Vegfr1 tk−/− mice (Figure 1A to 1R).
VEGFR1 Is Expressed in the Blood Vessels But Not in the Lymphatic Vessels

Lymphangiogenesis was observed to be suppressed in K14 Vegf-A Tg Vegfr1 tk−/− mice. However, it is not clear yet whether this phenomenon was attributable to direct or indirect VEGFR1 signaling. Therefore, we tested VEGFR1 as well as VEGFR2 expression in the vascular and lymphatic vessels by using Vegfr1+/LacZ and Vegfr2+/LacZ heterozygous mice.17,19 The ear skin of 8-week-old Vegfr1 and Vegfr2 heterozygous mice which possess the LacZ gene at each locus was stained by LacZ (Figure 2A and 2F), β-Gal (Figure 2B and 2G), PECAM-1 (Figure 2C and 2H), and LYVE1 (Figure 2D and 2I). VEGFR1 is expressed in the microvascular blood vessels but not in the lymphatic vessels, whereas VEGFR2 is expressed not only in the microvascular blood vessels but also in the lymphatic vessels. These results suggest that VEGFR1 leads to an increase in lymphangiogenesis by the indirect mechanism.

VEGF-A-VEGFR1 Signal Significantly Recruits Macrophages to the Ear

We and others previously demonstrated that VEGFR1 is expressed on monocytes/macrophages,10,11,13 and VEGF-A–dependent migration of macrophages is suppressed in Vegfr1 tk−/− deficient mice.12 In addition, we have recently reported that K14 Vegf-A Tg mice display recruitment of numerous macrophages to the ear.25 Therefore, we examined the local infiltration of monocytes/macrophages in Vegfr1 tk−/− and Vegfr1 tk−/− mice. The infiltration of F4/80-positive macrophages was a little weaker in Vegfr1 tk−/− mice than in Wt mice even in the absence of the Vegf-A transgene, although the difference was not significant (Figure 3B versus 3A in Wt mice, and graph 3E). The number of F4/80-positive macrophages was significantly lower in K14 Vegf-A Tg Vegfr-1 tk−/− mice than in K14 Vegf-A Tg Vegfr1 tk−/− mice (Figure 3D versus 3C, and graph 3E, P<0.05). 

In real-time RT-PCR analysis using whole ear samples, the expression of Il-6 and Mmp-9 mRNA was higher in Vegf-A Tg...
group than in non-Tg group (supplemental Figure III). Therefore, these macrophages recruited by VEGF-A through VEGFR1 appear to be closely related to inflammation in the skin. These results suggest that macrophages are involved in both angiogenesis and lymphangiogenesis.

**VEGF-C Is Secreted From Macrophages**

To explain how VEGFR1 indirectly stimulates lymphangiogenesis, we hypothesized that VEGF-A–recruited macrophages secrete VEGF-C. Previously, VEGF-C secreted from macrophages was noted to be important in inflammatory neovascularization. First, we measured the VEGF-A protein level in the ear-skin protein lysate. As expected, the transgene-derived human VEGF-A protein was detected only in the Tg mice, and its expression was not influenced by the absence or presence of the Vegfr1 tk gene (supplemental Figure IVA). The endogenous protein level of mouse VEGF-A was reduced in Tg mice as compared with that in non-Tg mice (supplemental Figure IVB) suggesting a negative feedback loop. The total VEGF-A level (human and mouse) in Tg mice was approximately twice that in non-Tg mice. Recently, macrophage-lineage cells were reported to secrete VEGF-C.25,27 Because macrophages are an important source of VEGF-C, we next compared the mRNA levels of Vegf-C in whole ear by real-time RT-PCR. The level of Vegf-C mRNA in the whole ear was significantly higher in mice with Vegf-A transgene than in wild-type mice. Furthermore, the Vegf-C mRNA in the whole ear was higher in K14 Vegf-A Tg than in K14 Vegf-A Tg-mice without VEGFR1 TK signal (Vegfr1 tk−/− mice) (Figure 3F, \( P<0.05 \)). These results suggest that secretion of VEGF-C is associated with the infiltrated macrophage.

**Decreased Permeability in Vegfr1 tk−/− Mice**

Stromal exudates from blood vessels result in increased ear thickness, and this may cause dilation of the lymphatic vessels to retract the tissue edema. Next, we performed the Miles assay for vascular permeability, using Vegfr1 tk−/− mice to confirm the involvement of VEGFR1 signaling in the enhancement of vascular permeability induced by VEGF-A. The permeability in the Miles assay was significantly weaker in Vegfr1 tk−/− mice than in Wt mice (Figure 4, \( P<0.05 \)). Vascular permeability is thought to be closely associated with VEGFR2. However, these results reveal that VEGFR1 also promotes vascular permeability along with VEGFR2.

**Transplantation of Vegfr1 tk−/− Bone Marrow Into Vegf-A Tg Mice Decreased Recruitment of Macrophages, and Suppressed Lymphangiogenesis and Angiogenesis**

Macrophages can be recruited to the sites of newly formed blood vessels and lymphatic vessels by VEGF-A via interac-
Vascular permeability is significantly decreased in Vegfr1 tk−/− mice. A, Permeability activity in the Miles assay using TK−/− and Wt mice. Representative images of the skin after the Miles assay demonstrating the vascular permeability in response to the recombinant human VEGF-A and PBS as a control. B, Quantification of the dye leakage. In addition to VEGFR2, VEGFR1 also contributes to vascular permeability (P=0.05).

Our results raise the question of how VEGFR1 is linked to further stimulate angiogenesis through the secretion of cytokines and stromal interactions. Because VEGFR1 is expressed in both macrophages and endothelial cells, we examined which cell type, macrophages or endothelial cells, plays an important role in lymphangiogenesis via VEGFR1. For this experiment, we carried out a bone marrow transplantation (BMT) of Vegfr1 tk−/− cells in K14 Vegf-A Tg mice. Wt or Vegfr1 tk−/− BM KSL (c-Kit+, Sca1+, Lineage-) hematopoietic stem cells were transplanted into newborn transgenic pups, because the development of angiogenesis and lymphangiogenesis usually precedes adulthood. After 8 weeks from the BMT, we examined the chimerism and peripheral leukocyte count in the transplanted mice (supplemental Figure VA). Because the genetic background of recipient mice is Balb/c x C57BL/6 mixture, they have H2Kd and H2Kb HLA class I haplotype (supplemental Figure VB-c). On the other hand, donor mice are C57BL/6 genetic background, thus, they have only H2Kb HLA class I type (supplemental Figure VB-b). Chimerism in K14 Vegf-A Tg mice that underwent BMT changed completely to the donor-derived C57BL/6-type BM cells (supplemental Figure VB-d). Recovery of the peripheral leukocyte count and BM cell density in the Wt and Vegfr1 tk−/− BM cell transplanted groups was almost identical (supplemental Figure IVC, D-G). However, cell densities of the transplanted groups were slightly lower than those of the nontransplanted control groups (supplemental Figure IV, D-G). This might be a result of the brief interval required for the complete recovery of BM cell density.

Next, we asked whether BMT with Vegfr1 tk−/− cells results in fewer macrophages being recruited to the ear or not. K14 Vegf-A Tg mice with Vegfr1 tk−/− BM clearly showed a suppression of macrophage recruitment to the ear (Figure 5A and 5B, graph I). We measured the vascular and lymphatic area as well as these length and joint numbers. The representative vascular and lymphatic pictures are shown in Figure 5C through 5H. Graphs J and K in Figure 5 illustrate that a decrease in macrophages caused by BMT substantially inhibited both angiogenesis and lymphangiogenesis although vascular endothelial cells and the stromal cells were VEGFR1 TK+ (P<0.05 and P<0.01). In addition to vascular and lymphatic areas, those length and joint numbers in Vegf-A Tg mice with Vegfr1 tk−/− BMT were decreased compared with those in Vegf-A Tg mice with Vegfr1 tk+/− BMT (both parameter were P<0.05, data not shown). These results suggest that macrophages recruited via the VEGFR1 signal play an important role in lymphangiogenesis as well as angiogenesis.

Discussion

In this study, we have shown that the VEGFR1 TK signals play a significant role in the promotion of lymphangiogenesis and angiogenesis in a murine model with the K14-promoter-driven human Vegf-A transgene. The signaling of VEGFR1 is considered to result in increased vascular permeability and Vegf-A–dependent migration of macrophages into the skin. Furthermore, the K14 Vegf-A Tg mice transplanted with Vegfr1 tk−/− deficient BM cells did not efficiently recruit macrophages and showed a reduction in angiogenesis and lymphangiogenesis. These results suggest that the signaling of VEGFR1 promotes lymphangiogenesis as well as angiogenesis at least partly via macrophage recruitment and vascular leakage.

Our results raise the question of how VEGFR1 is linked to lymphangiogenesis, directly or indirectly. Lymphangiogenes
thesis is thought to be mainly mediated by the VEGF-C/VEGF-D and VEGFR3 systems.5,9 Also, there is some evidence that VEGF-A has lymphangiogenic properties. Several groups have reported that lymphatic endothelial cells as well as vascular endothelial cells express VEGFR2,8 and the activation of VEGFR2 via VEGF-A promotes lymphangiogenesis.22,31,32 However, our observations indicate that lymphangiogenesis and angiogenesis were reduced in K14 Vegf-A Tg Vegfr1 tk−/− mice compared with Vegfr1 tk wild-type mice although VEGFR2 is intact in both strains (supplemental Figure I and Figure 1). Furthermore, VEGFR1 TK deficiency did not show significant effects on the expression of VEGFR2, VEGFR3, or soluble VEGFR1 (by whole ear real-time RT-PCR analysis, data not shown).12 These results imply that VEGFR1 TK signaling plays a role in lymphangiogenesis. We showed that lymphatic endothelial cells do not express VEGFR1 in Vegfr1+/+LacZ mice by using β-gal staining (Figure 2); thus, we suggest that lymphangiogenesis in mice lacking the VEGFR1 signal is impaired indirectly rather than via direct signaling from VEGFR1 TK in lymphatic endothelial cells.

It is of interest to elucidate in detail the mechanisms which promote lymphangiogenesis. Several studies have indicated that VEGF-A increases vascular permeability in microvessels by activating VEGFR2 in endothelial cells.33,34 However, interestingly, in the Miles assay, we observed that the vascular permeability in a short period in Vegfr1 tk−/− mice was suppressed as compared with that in Wt mice. Together with the impaired vascular leakage in Plgf−/− mice35 and increased vascular leakage in K14 PlGF Tg mice,36,37 these results strongly suggest that VEGFR1 directly contributes to vascular leakage (Figure 4A and 4B). Hence, we consider that the stromal exudates from the blood vessels dependent on VEGFR1 signaling also facilitate lymphangiogenesis to drain the excessive fluid into lymphatic vessels.

We and others have shown that VEGFR1 is well expressed in monocytes/macrophages at both the mRNA and protein levels11,13 and that VEGFR1 is important for VEGF-A–dependent migration of these cells.10,12 Therefore, it is reasonable that the infiltration of monocytes/macrophages into the skin was less extensive in Vegfr1 tk−/−deficient Vegf-A Tg mice than wild-type Vegf-A Tg mice (Figure 3A through 3E). Cursiefen et al and Maruyama et al found that the inhibition of macrophage recruitment suppressed both angiogenesis and lymphangiogenesis in mice with suture-induced inflammatory corneal neovascularization (CNV).27,38 Consistent with these findings, we also demonstrated that the Vegfr-1 tk−/−deficient K14 Vegf-A Tg mice displayed reduced angiogenesis and lymphangiogenesis in parallel with a decrease in the recruitment of VEGFR1-expressing macrophages (Figure 3A through 3E). In addition, it is noteworthy that we observed a decrease in both angiogenesis and lymphangiogenesis in K14 Vegf-A Tg mice after the replacement of VEGFR1-expressing wild-type macrophages with those lacking only VEGFR1 TK signaling.

Recently, Cursiefen et al reported that the recruited macrophages secreted VEGF-C before promoting lymphangiogenesis and angiogenesis.27 In our case, the total amount of VEGF-A including the K14-derived human type and the endogenous mouse type demonstrated no obvious difference between the wild-type and the VEGFR1 signal-deficient condition (supplemental Figure IVA and IVB). The level of Vegf-C mRNA in the whole ear was significantly higher in mice with Vegf-A transgene than in mice without it. Furthermore, the level of Vegf-C mRNA in the whole ear was significantly higher in K14 Vegf-A Tg- than in K14 Vegf-A Tg-mice without VEGFR1 TK signal. These findings also imply that one of the major resources for the VEGF-C secreted into tissues is the recruited macrophages.

The characterization of macrophages infiltrated into Vegf-A Tg mouse skin is an interesting project. Macrophages were recently reported to be divided to 2 types, M1 (inflammatory, potent nitric oxide–producer) and M2 (predomestic, IL12-p40–dependent), however, still these features seem not conclusive yet, and many experiments and discussions are under way to clarify these types. The numbers of macrophages in the skin are significantly increased in Vegf-A Tg condition compared with normal condition, and the total skin tissues in the Tg mice expressed higher levels of interleukin (IL)-6, matrix metalloproteinase (MMP)-9 (supplemental Figure III), and tumor necrosis factor (TNF)α.25 Thus, we suggest that the macrophages recruited into the Vegf-A Tg skin are closely related to inflammation, not to predomestic ones.

These studies indicate the following possibilities for the involvement of VEGFR1 in lymphangiogenesis. First, VEGFR1 contributes to vascular permeability, and stromal exudates from the blood vessels induce lymph vessel dilatation and lymphangiogenesis. Second, VEGF-A recruits BM-derived macrophages in a VEGFR1 signal–dependent manner. Third, monocyte/macrophages recruited in the tissues express VEGF-C, which is the direct activator for VEGFR3. Lymphangiogenesis is thought to occur directly via the VEGF-C/VEGF-D and VEGFR3 systems. However, our observations indicate that the VEGFR1 signals also play an important role in lymphangiogenesis in certain pathological conditions, particularly proinflammatory diseases. The downstream signaling from VEGFR1 toward cell migration is not yet understood. Such a signaling cascade may be an attractive target for the regulation of abnormal lymphangiogenesis.

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Materials and methods

Immunohistological analysis

Samples from 8-week-old mice were fixed in 4% paraformaldehyde (PFA). For histological examination of ear thickness, mouse ear sections embedded in paraffin were stained with hematoxylin/eosin (HE) and then measured using the light microscope Nikon Eclipse TE600 (Nikon, Tokyo, Japan), Axio Vision 3.0 software (Carl Zeiss, Jena, Germany), and a 4 objective lens. For bone marrow (BM) reconstitution examination, mouse femurs were decalcified in EDTA. Next, sagittal paraffin sections were stained with HE and then observed using the light microscope, according to the abovementioned method. For immunofluorescence staining, mouse ear sections were blocked in the TNB buffer (NEN Life Science Products, Wellesley, MA) containing 0.3% Triton X-100. Primary antibodies, rat anti-mouse platelet/endothelial cell adhesion molecule-1 (PECAM-1) (BD Pharmingen, San Diego, CA), rabbit anti-mouse lymphatic vessel endothelial receptor-1 (LYVE1) (Abcam, Cambridge, UK), rabbit anti-β-Gal (Molecular Probes, Eugene, OR), hamster anti-mouse PECAM-1 (Chemicon, Temecula, CA), rat anti-mouse LYVE1 (clone ALY7)\(^1\), and rat anti-mouse F4/80 (Serotec, Oxford, UK), were diluted in the TNB buffer; the ear samples were then incubated overnight in the buffer at 4°C. Subsequently, the samples were washed 3 times, and incubated with secondary antibodies, namely, anti-rat IgG Alexa 546, anti-rabbit IgG Alexa 488 (Molecular Probes), anti-hamster IgG Cy3, and anti-Hamster IgG Cy5 (Jackson ImmunoResearch laboratories, Inc., West grove, PA). Next, the PECAM-1, LYVE1, and β-Gal samples were observed by using the confocal microscope Nikon Eclipse TE600 (Nikon),
Radiance 2000 software (Carl Zeiss), and a 20 /0.50 objective lens; it was then processed with Zeiss LSM Image Browser Ver. 3.5 (Carl Zeiss). In the PECAM-1-positive vascular and LYVE1-positive lymphatic areas, length and joint numbers of vessels were analyzed using KURABO Angiogenesis Image Analyzer Ver. 1.0 (Kurabo, Osaka, Japan). Results are representative of at least 3 independent littermates. The data represent the mean ± S.D. Calculations and analyses for all specimens were performed in 5 different regions. F4/80 samples were observed by fluorescence microscope, using Axio Vision 3.0 software (Carl Zeiss) and A 100 /1.30 objective oil lens, according to the abovementioned method; calculations were subsequently performed. Calculations and analyses for each specimen were performed in 10 different regions.

**LacZ Staining**

Ear samples of 8-week-old Vegfr1+/LacZ and Vegfr2+/LacZ mice were treated as described previously. Then the samples were observed under the Eclipse TE600 microscope (Nikon), using the Axio Vision 3.0 software (Carl Zeiss) and a 20 /0.50 objective lens. Images were processed using Photoshop CS (Adobe Systems, San Jose, CA).

**Enzyme-linked immunosorbent assay (ELISA)**

The ear skin of 8-week-old mice was removed and lysed with the lysis buffer, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and PBS. The concentrations of VEGF-A were determined using human and mouse VEGF-A (R&D systems, Minneapolis, MN) ELISA kits. All the procedures were conducted according to the manufacturer’s instructions. The concentration of each cytokine was presented
as the mean of the total protein in the skin lysates. Experiments were performed using 5 different samples in each case.

**Real-time reverse transcriptase (RT)-PCR**

*Vegf-C, Il-6* and *Mmp-9* mRNA in the 8-week-old Balb/c mice ear was analyzed as described previously. Experiments were performed using 5 different samples in each case. All results are representative of at least 3 independent experiments. The data represent the mean ± S.D.

**Purification of the hematopoietic stem cells**

Hematopoietic stem cells (HSCs), i.e. the KSL (c-Kit+, Sca-1+, Lineage-) cells were purified from BM cells of 10-week-old C57BL/6 Wild-type or *Vegfr1tk/-* mice, and these cells were used for bone marrow transplantation.

**Peripheral blood analysis**

Blood was collected from the mice by retro-orbital bleeding and leukocyte counts were determined using a hemocytometer.
Results

VEGF-A increased ear thickness via VEGFR1 signals

Next, we measured the ear thickness of the mice in each group. The ear was thicker in K14 Vegf-A Tg mice than Wt mice (on average, 389 µm versus 221 µm in Wt mice ear; \( p < 0.001 \)). The thickness of the ear increased in all the layers, i.e., the epidermis, dermis, subdermal adipose, and cartilage layers, of K14 Vegf-A Tg mice. Furthermore, numbers of cells were increased in the ears of K14 Vegf-A Tg mice. This suggests that VEGF-A stimulates the proliferation of these cells. On the other hand, ear thicknesses of Vegfr1 tk-deficient mice with the Vegf-A transgene was lower than that of K14 Vegf-A Tg Vegfr1 tk Wt mice (300 µm versus 389 µm in K14 Vegf-A Tg Vegfr1 tk-/- and tk+/+ mice ear; \( p < 0.001 \)) (Supplemental figure II A-E). We also detected a slight decrease in the ear thickness in Vegfr1 tk-/- mice compared to the Wt mice under the condition without transgene (Supplemental figure II).
References


Supplemental figure I

VEGFR1 tyrosine kinase signal promotes angiogenesis. (A–E) Macroscopic ear redness, a characteristic feature of angiogenesis, increased in a VEGFR1 TK-gene dosage-dependent manner in 8-week-old VEGF-A-transgenic mice. The ears were photographed using a digital camera (Canon EOS, Tokyo, Japan) and the images processed with Photoshop CS (Adobe Systems, San Jose, CA). The scale bar is 5 mm.

Results are representative of at least 3 independent littermates. Wt: wild type, TK-/−: Vegfr1 tk−/−, VA TK+/+: K14 Vegf-A Tg Vegfr1 tk+/+, VA TK+/−: K14 Vegf-A Tg Vegfr1 tk+/−, VA TK−/−: K14 Vegf-A Tg Vegfr1 tk−/−
Supplemental figure II

VEGFR1 increases ear thickness. (A-D) HE staining in the paraffin-embedded ear specimens of 8-week-old mice from each genetic background. The VA TK+/+ mice (C) had thicker ears than the VA TK−/− mice (D). A 4 /0.13 objective lens was used. The scale bar is 200 µm. The ears of Vegfr1 tk−/− mice (B) were thinner than those of Vegfr1 Wt mice (A) regardless of the VEGF-A transgene (B versus A, p < 0.01; D versus C, p < 0.01, and graph E). Results are representative of at least 3 independent littermates. The data represent the mean ± S.D. Calculations and analyses for all specimens were performed in 10 different regions.
Supplemental Figure III

(A-B) The mRNA expression of *IL-6 and Mmp-9* in whole ear was examined by real-time RT-PCR analysis. The expression of *Il-6* and *Mmp-9* mRNA was higher in *Vegf-A* Tg group than in non-Tg group (non-Tg group versus *VA*-Tg group, *p* < 0.05).

Experiments were performed using 5 different samples in each case. All results are representative of at least 3 independent experiments. The data represent the mean ± S.D.
Supplemental figure IV.

(A, B) Amount of human and mouse VEGF-A protein in the ear skin lysate was measured using ELISA. Experiments were performed using 5 different samples in each case. All results are representative of at least 3 independent experiments. The data represent the mean ± S.D.
Supplemental figure V.

Transplantation of Vegfr1 tk+/+ and Vegfr1 tk−/− bone marrow into Vegf-A Tg Vegfr1 tk+/+ mice show recovery of recipient bone marrow. (A) Experimental design for newborn bone marrow transplantation (BMT). (B) Fluorescence-activated cell-sorting (FACS) profiles for analyzing reconstitution. At 8 weeks after transplantation, the peripheral blood cells were analyzed for the contribution of the donor cells. The transplanted recipient mice were completely reconstituted with the C57BL/6 donor cells (a, Balb/c; b, C57BL/6; c, before BMT, Balb/c x C57BL/6; d, after BMT, C57BL/6). (C) Recovery of the peripheral blood count. At 8 weeks after transplantation, the count of the peripheral blood cells was identical to that observed for the non-transplanted mice. No differences were observed in the peripheral blood count between Wt BM-cell-transplanted mice and Vegfr1 tk−/- BM-cell-transplanted
mice. (D-G) HE staining in all the femur specimens. BM cell numbers were not yet completely recovered. However, no difference was observed between Wt BM-cell-transplanted mice and Vegfr1 tk-/- BM-cell-transplanted mice. A 10/0.30 objective lens was used. The scale bar is 200 µm.