Review Article

Involvement of vascular endothelial growth factor receptor-1 in rheumatoid arthritis

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Vascular endothelial growth factor (VEGF) and its receptor family including VEGFR-1 (Flt-1) were recently shown to be involved in pathological angiogenesis including tumor angiogenesis, tumor metastases, inflammatory disease such as rheumatoid arthritis, psoriasis, and atherosclerosis. Rheumatoid arthritis (RA) is a chronic systemic disease characterized by an inflammatory erosive synovicitis and a pannus of inflammatory vascular tissue, leading to irreversible cartilage and bone destruction. A few cytokines such as TNF-α, IL-1 and IL-6 are known to be involved in RA. VEGF-A was reported to be highly expressed in synovial fluid in human RA, suggesting a role in RA progression. We have recently shown that VEGFR-1 is expressed not only in vascular endothelial cells but also in inflammatory cells, especially in monocyte/macrophage. However, the molecular basis of their actions on RA is not fully understood. Here we report that in a murine model of RA, deletion of the tyrosine kinase (TK) domain of VEGFR-1 (*Vegfr-1 tk-/-*) decreased the incidence and clinical symptoms of RA. Pathological symptoms, such as synovial hyperplasia, inflammatory infiltrates, pannus formation and cartilage/bone destruction became milder in *Vegfr-1 tk-/-* mice compared with *Wild-type* (*Wt*) mice in the *Human T-cell Leukemia Virus-1 pX* (*HTLV-1 pX*) induced chronic models. VEGFR-1 TK-deficient bone marrow cells showed a suppression of multi-lineage colony formation. Furthermore, macrophages induced to differentiate *in vitro* showed a decrease in phagocytosis and the secretion of Interleukin-6 (IL-6) and VEGF-A. Treatment of this RA model with a small molecule inhibitor for VEGFR TK, KRN951, also attenuated the arthritis. These results indicate that the VEGFR-1 TK signaling modulates the proliferation of bone marrow hematopoietic cells and immunity of monocyte/macrophages, and promotes chronic inflammation, which may be a new target in the treatment of RA.

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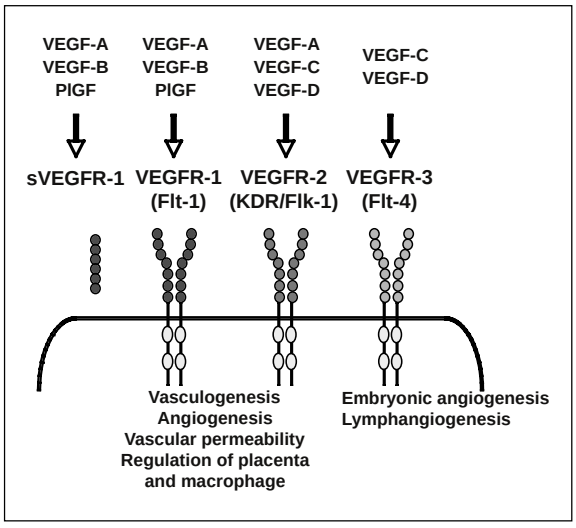

Fig.1 VEGF and VEGF receptors

Schematic outline of the VEGF family (VEGF-A, -B, -C, -D, PlGF) and their receptors, the receptor tyrosine kinases VEGFR-1 VEGFR-2 and VEGFR-3. VEGFR-1 occurs as a soluble form denoted sVEGFR-1. VEGF-A, a major contributor to angiogenesis, binds and activates VEGFR-1 as well as VEGFR-2, and regulates vasculogenesis, angiogenesis, vascular permeability, inflammatory response, and carcinogenesis. The soluble form of VEGFR-1 appers to be an important modulator of the placental vasculature. VEGF-C and VEGF-D mainly activates VEGFR-3 and regulates embryonic angiogenesis and lymphangiogenesis.

Structure and signaling of VEGFR-1

Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) including VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4) form a regulatory system crucial for normal development and pathological angiogenesis. VEGF-A binds and activates two tyrosine kinase receptors, VEGFR-1 and VEGFR-2[1-4]. VEGFRs are structurally related to the Fms/Kit/PDGFR family, and contain an extracellular domain, transmembrane domain, tyrosine kinase (TK) domain, and carboxy terminal region[5]. The extra cellular domain carries seven immunoglobulin (Ig)-like repeats, and the ability to bind ligands is localized to the 2nd and 3rd domains[5,6]. The affinity for VEGF-A of VEGFR-1 is very high, with a Kd of about 2-10 pM, which is at least one order of magnitude higher than that of VEGFR-2. On the other hand, the tyrosine kinase activity of VEGFR-1 is relatively weak compared to that of VEGFR-2[7]. VEGF-A does not stimulate the proliferation of NIH3T3 cells overexpressing VEGFR-1, and Placental growth factor (PlGF) and VEGF-B, VEGFR-1 specific ligands, do not stimulate significantly the proliferation of VEGFR-1- and VEGFR-2-expressing primary endothelial cells in the culture[8,9]. All VEGF receptors are expressed to different levels, in vascular and lymphatic endothelial cells. VEGFR-1 and VEGFR-2 are highly expressed on vascular endothelial cells[10-13]. VEGFR-1 is expressed not only in vascular endothelial cells but also in monocyte/macrophages[14,15], and its signaling is involved in the migration of macrophages toward VEGF-A[16]. Furthermore, VEGFR-1 expression has been observed in dendritic cells, osteoclast, pericytes and trophoblasts in the placenta[12,17,18]. The significance of VEGFR-1 expression in these non-endothelial cells is still not clear.

VEGFR-1 as a negative regulator of angiogenesis in embryogenesis

Mice lacking *Vegfr-2* die in the embryonic stage due to a severe deficiency of vascular development[19]. In contrast, mice lacking *Vegfr-1* die due to over-growth and disorganization of the vascular system, not due to a poor vascularization[20]. Interestingly, however, mouse embryos lacking the TK domain of VEGFR-1 (*Vegfr-1 tk-/-*) survive without significant defects[21]. These mice showed disruption of the migration of macrophages toward VEGF-A[21]. Since VEGFR-1 has high affinity for VEGF-A, but only weak tyrosine kinase activity, these results strongly suggest that VEGFR-1 functions as a negative regulator of vascular development by trapping VEGF-A via its ligand-binding domain at embryogenesis[22].

VEGFR-1 tyrosine kinase as positive regulator in adult stages

Recently, various studies indicated that the expression of VEGFs and VEGFRs is upregulated in various diseases including tumor angiogenesis, tumor-dependent ascites formation, metastases, inflammatory disease such as rheumatoid arthritis, psoriasis, and atherosclerosis[2,4,23-25]. VEGFR-1-mediated signaling was shown to play a significant role in a variety of pathological vascularization directly by stimulating endothelial cell function and indirectly by mediating recruitment of bone marrow-derived cells[26,27]. VEGFR-1 is expressed on macrophage-lineage cells and also promotes disease progression by stimulating migration of inflammatory cells into the lesion of arthritis[24,28] and atherosclerosis[29].

We studied growth of Lewis lung carcinoma growth in primary subcutaneous injection and following metastatic sites by using *Vegfr-1 tk-/-* mice. We found that VEGFR-1 significantly enhances tumor metastases in the lung via induction of matrix metalloproteinase 9 (MMP9) in this tissue[30,31]. Recently, several

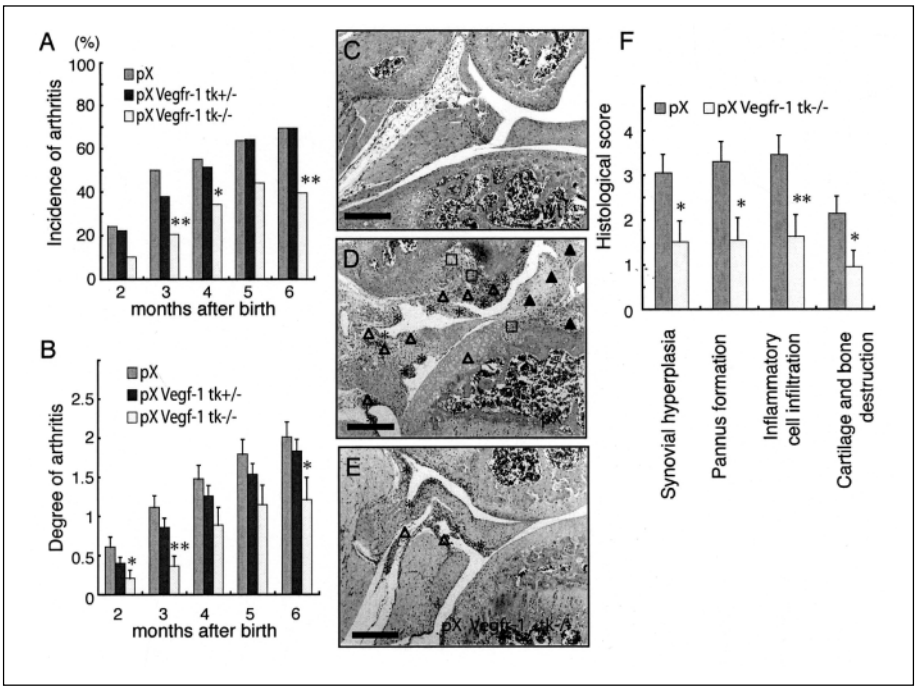

Fig.2 Signals from VEGFR-1 tyrosine kinase contribute to the onset and progression of arthritis

(A) The incidence of arthritis was significantly lower in *pX Vegfr-1 tk-/-* mice than *pX* mice. In addition, the incidence of arthritis in *pX Vegfr-1 tk+/-* heterozygotes was slightly lower in the early stages. (B) Clinical grades of arthritis were reduced depending on the deficiency of the VEGFR-1 TK domain from 2 to 6 months after birth. *pX Vegfr-1 tk+/-* mice showed mild clinical scores in between those of *pX* and *pX Vegfr-1 tk-/-* mice. (C-E) Cross sections of ankle joints in control and RA mice. Joints of wild-type mice (C) show no remarkable change. Joints of *pX* mice (D) show synovial hyperplasia(＊), inflammatory cell infiltration (△), pannus formation(▲), and loss of cartilage and bone(□). These findings are milder in *pX Vegfr-1 tk-/-* mice (E). (F) Histological scores of the degree of synovial hyperplasia, etc. in paws and ankles. Scores of *pX Vegfr-1 tk-/-* mice were about half those of the *pX* mice.

papers described the involvement of abnormal angiogenesis in the progression of atherosclerosis[28,29]. Soluble VEGFR-1 (sVEGFR-1) treatment in mouse models of atherosclerosis significantly suppressed the degree of disease[29]. Recruitment of macrophages was decreased in these conditions, strongly suggesting VEGF-VEGFR-1 signaling is involved in atherosclerosis. Hattori et al. showed that VEGFR-1 is important for the reconstitution of hematopoiesis in bone marrow (BM) recovery after irradiation[27] and Niida et al. showed that VEGFR-1 TK plays pivoital role in osteoclast functions for BM recnstruction in M-CSF-deficinet (op/op) mice[32].

Rheumatoid arthritis and angiogenesis

Rheumatoid arthritis (RA)[33] is a chronic systemic disease characterized by an inflammatory erosive synovicitis, which shows marked neovascularization, inflammatory cell infiltration, and synovial hyperplasia. These pathological reactions gradually induce a pannus with inflammatory vascular tissue, leading to an irreversible loss of cartilage and bone[34,35]. VEGF-A is highly expressed in synovial fluid in RA[36]. Immunohistochemical and in situ hybridization studies on the synovial tissues have shown that VEGF-A is strongly expressed in synovial macrophages, fibroblasts surrounding microvessels, and vascular smooth muscle cells[36,37]. In inflamed joints, many cytokines including VEGF and the pro-inflammatory interleukine (IL)-1, IL-6 and tumor necrosis factor-α (TNFα) play important roles in the pathogenesis of RA[38]. A recent study indicated that the artificial blocking of TNFα and IL-6-receptor by neutralizing antibodies significantly suppressed clinical as well as histological scores in patients[39].

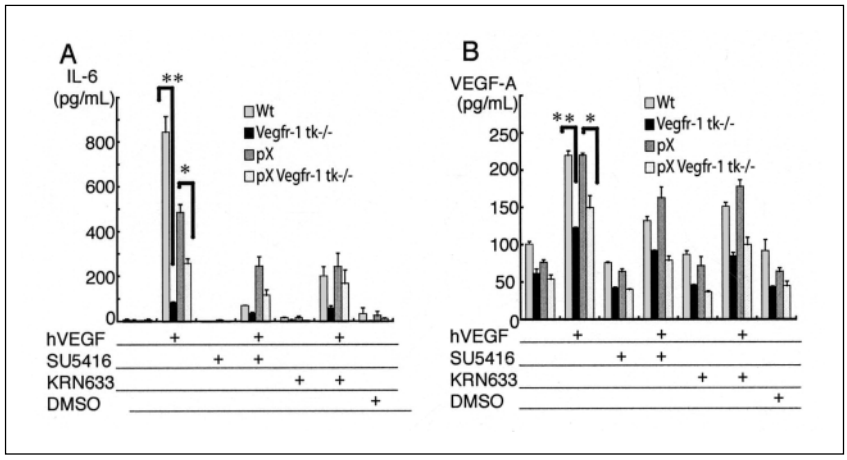

Fig.3 Cytokine-secretion and phagocytosis of macrophages in *Vegfr-1 tk*-/- mice

(A-B) IL-6 and VEGF-A were secreted from macrophage upon stimulated with VEGF-A (100ng/ml). (A) IL-6 was secreted in response to VEGF-A. Secretion of IL-6 from *Vegfr-1 tk-/-* macrophages was low compared to that from the *Wild-type* macrophages. (B) Mouse VEGF-A was secreted from macrophages in the absence of VEGF-A, and the secretion increased on stimulation with exogenous VEGF-A. The secretion from *Vegfr-1 tk-/-* macrophages was about half that from wild-type macrophages.

VEGFR-1 tyrosine kinase contributes to the onset and the progression of arthritis

We and others found that VEGFR-1 is involved in the progression of arthritis. Luttun et al. found that, using blocking antibodies against mouse VEGFR-1 or VEGFR-2, treatment with anti-VEGFR-1 Ab more efficiently suppressed an adjuvant-induced inflammatory arthritis, than did treatment with anti-VEGFR-2 Ab[28]. DeBandt et al. reported that VEGFR-1 is involved in a model of chronic arthritis in which KRN (the $\alpha v \beta 6$ TCR-transgene)/NOD(non-obese diabetic) mice were used for the induction of inflammation[40]. Their model system is different from ours, therefore, results obtained with several independent animal models of arthritis clearly support the importance of VEGFR-1 in this disease. Furthermore, we proved the involvement of VEGFR-1 signaling in rheumatoid arthritis by using *Vegfr-1 tk-/-* mouse and *Human T-cell Leukemia Virus-1* (*HTLV-1*) *pX* transgenic mouse[41]. This *pX* mice model is more similar to human RA than other models, such as collagen-antibody-induced arthritis, in terms of chronic progression, the production of rheumatoid factor, and pathological findings[42]. We measured the incidence and clinical grade of arthritis in the presence or absence of VEGFR-1 TK signals. The incidence of arthritis, such as paw swelling, erythema, and ankylosis, and the clinical scores, such as redness and swelling of the ankle or wrist were significantly lower in *pX Vegfr-1 tk-/-* mice than *Vegfr-1 wild-type pX* transgenic mice (Fig.2AB). Furthermore, *pX Vegfr-1 tk+/-* mice showed mild clinical scores in between those of *pX* mice and *pX Vegfr-1 tk-/-* mice (Fig.2B). The histological difference determined as synovial hyperplasia, inflammatory infiltrates, pannus formation, and loss of cartilage/bone were reduced by about half in *pX Vegfr-1 tk-/-* mice compared to *pX wild-type* mice (Fig.2C-F). However, we did not find the difference of capillary formation in the pannus of synovial tissue. These results suggest that VEGFR-1 tyrosine kinase-dependent signals contribute to the symptoms of arthritis including pathological findings in a gene-dosage-dependent manner[41].

Secretion of cytokines and phagocytosis of macrophages in arthritis

We examined local infiltration and the functions of monocyte/macrophages in these mice. The infiltration of inflammatory cells into arthritic joints was significantly less extensive without VEGFR-1 signals (Fig.2D-F). Cytokines and growth factors and its receptors are the chatracterized system in the rheumatoid joints. RA is a chronic disease of late onset, and multiple pathways of inflammation and immune systems appear to be involved. The neutralizing antibodies of TNFα and IL-6-receptor decreased clinical symptom in patients[39]. Therefore, we focused on the function of macrophages. Secretion of IL-6 and VEGF-A was measured in the presence or absence of hVEGF-A. IL-6 and VEGF-A was secreted in response to hVEGF-A, however, much less IL-6 and VEGF-A were secreted from *Vegfr-1 tk-/-* macrophages than *Vegfr-1 wild-type* cells. (Fig.3AB). Macrophages are multi-functional cells involved in immunological reactions and phagocytosis. Therefore, we examined the extent of phagocytosis using macrophages. *Wild-type* BM-derived and Macrophage colony-stimulating factor (M-CSF)-stimulated macrophages efficiently phagocytized both fluorescent dextran and LPS.

Surprisingly, macrophages from *Vegfr-1 tk*-deficient mice showed significantly less phagocytotic activity. Therefore, in addition to the suppression of VEGF-A-dependent migration, *Vegfr-1 tk*-deficient macrophages were dysfunctional in the secretion of IL-6 and VEGF-A as well as phagocytosis under these experimental conditions[41].

VEGFR tyrosine kinase inhibition as therapeutic approaches

It has become evident that VEGF receptors as well as VEGF-A are critical targets of developing new drugs to suppress a range of disease, particularly malignancies. There are various approaches against VEGF/VEGFR signals such as neutralizing antibody against ligands and receptors, as well as inhibitors of VEGFRs. To confirm the therapeutic effect of VEGFR kinase-inhibitors on arthritis, we administered such a small molecule tyrosine kinase inhibitor for VEGFR, KRN951[43] (kindly provided from Kirin Brewery, Gumma, Japan), to mice with *pX*-induced chronic arthritis and collagen-antibody-induced acute arthritis. We treated the mice with KRN951 for 5 straight days (oral, 20mg/kg/day) a week from 8 to 26 weeks of age. Administration of KRN951 reduced the progression of arthritis compared with the control. Histological abnormalities in the treated group decreased 11 to 25% compared with the control. The administration of KRN951 in the acute model also reduced the progression of the symptoms of arthritis in a dose-dependent manner[41]. The suppressive effect of *Vegfr-1 tk*-deficiency on the RA model and that of the treatment with the pan-VEGFR-kinase inhibitor KRN951 were similar, suggesting that VEGFR-1 signaling is more strongly related to the progression of RA than VEGFR-2 signaling.

VEGFR-1 signals in the proliferation/differentiation of hematopoietic cells

Another hallmark of VEGFR-1 signals is important mediator of stem-cell recruitment and mobilization. Hattori et al. investigated the role of VEGFR-1 signals during the hemetopoietic recovery after irradiation. Treatment with blocking monoclonal antibody to VEGFR-1 inhibited the hematopoietic recovery. In contrast delivery of PlGF to irradiated mice improved it[27].

A hematopoietic activity is capacity for colonies to form bone marrow mononuclear cells (BMMNCs) *in vitro*[44]. We showed the colony-forming ability of *Vegfr-1 tk-/-* BMMNCs was reduced to about 70% of that of *Wild-type* BMMNCs, and all the progenitors including erythroid-colonies, myeloid-colonies, and more immature mixed-colonies were equally affected (Fig.4).

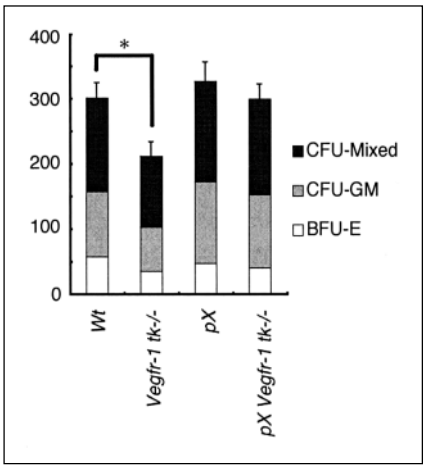

Fig.4 VEGFR-1 is associated with the proliferation of hematopoietic cells

The number of colony forming units (CFU) in *Vegfr-1 tk-/-* was decreased in all progenitor cells (BFU-E, CFU-GM, CFU-Mixed) compared to that in *Wild-type* mice. Each progenitor cell also showed a decrease in the number of colonies.

However, the number of Sca-1 and CD34-positive cells corresponding to hemetopoietic stem cells (HSCs) among BMMNCs were almost the same in these mice[41]. These results suggest that a deficiency of VEGFR-1 signaling may reduce the proliferation of HSCs, but not the number of these cells in BM.

Conclusion

In our study, we have shown that VEGFR-1 TK signals play a significant role in the progression of RA in murine models of both chronic and acute arthritis. Furthermore, the involvement of VEGFR-1 signals is considered to be gene-dosage-dependent since the clinical and histological scores of RA in *Vegfr-1 tk+/-* heterozygous *pX* mice were in between those of *wild-type pX* and *Vegfr-1 tk-/-* mice. Several functions of macrophages such as the secretion of IL-6 and VEGF-A, phagocytosis, and VEGF-A-dependent migration were clearly suppressed in *Vegfr-1 tk-/-* mice. In addition, the proliferation of HSCs decreased about 30% in the *in vitro* assay in these mice. These results suggest that the kinase activity of VEGFR-1 is important in a variety of steps during the progression of pathological inflammatory diseases such as RA (Fig.5). This study may support the idea that VEGFR-1 TK activity is a good pharmaceutical target for control of chronic RA.

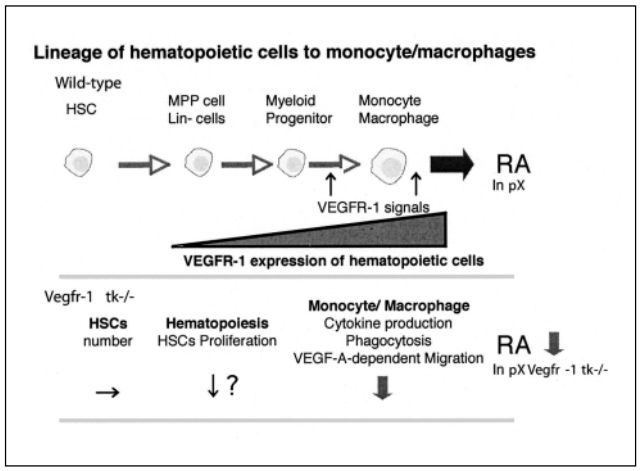

Fig.5 VEGFR-1 tyrosine kinase signaling is involved in arthritis by modulating hematopoiesis and promoting the differentiation of monocyte/macrophages

A schematic model of the VEGFR-1 TK signals associated with arthritis. (Upper panel) Immature monocyte/macrophages derived from BM hematopoietic cells, differentiate and migrate into the circulation. VEGFR-1 is expressed in monocyte/macrophage lineages. VEGFR-1 signals mobilize inflammatory cells to the peripheral tissues and RA-joints, and stimulate secretion of inflammatory cytokines to promote RA. (Lower panel) VEGFR-1 signal-deficient macrophages show suppressed cytokine secretion, phagocytosis, and VEGF-dependent migration, resulting in a decrease in rheumatoid arthritis.

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