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 synovitis 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.
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\(^1\). 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\(^5\). 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 (PIGF) and VEGF-B, VEGFR-1 specific ligands, do not stimulate significantly the proliferation of VEGF-A- and VEGF-B-expressing primary endothelial cells in the culture\(^9\). 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\(^10\). 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\(^2,23\). VEGF-1-mediated signaling was shown to play a significant role in a variety of pathologi cal vascularization directly by stimulating endothelial cell function and indirectly by mediating recruitment of bone marrow-derived cells\(^26,27\). VEGF-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
papers described the involvement of abnormal angiogenesis in the progression of atherosclerosis\textsuperscript{28,29}. Soluble VEGFR-1 (sVEGFR-1) treatment in mouse models of atherosclerosis significantly suppressed the degree of disease\textsuperscript{39}. 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\textsuperscript{27} and Niida et al. showed that VEGFR-1 TK plays pivotal role in osteoclast functions for BM reconstruction in M-CSF-deficient (op/op) mice\textsuperscript{30}.

**Rheumatoid arthritis and angiogenesis**

Rheumatoid arthritis (RA)\textsuperscript{30} 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\textsuperscript{14,15}. VEGF-A is highly expressed in synovial fluid in RA\textsuperscript{30}. 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\textsuperscript{16,37}. In inflamed joints, many cytokines including VEGF and the pro-inflammatory interleukine (IL)-1, IL-6 and tumor necrosis factor-\textalpha (TNF\textalpha) play important roles in the pathogenesis of RA\textsuperscript{30}. A recent study indicated that the artificial blocking of TNF\textalpha and IL-6-receptor by neutralizing antibodies significantly suppressed clinical as well as histological scores in patients\textsuperscript{30}.

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\textsuperscript{-/-} mice than pX mice. In addition, the incidence of arthritis in pX Vegfr-1 tk\textsuperscript{+/-} 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\textsuperscript{+/-} mice showed mild clinical scores in between those of pX and pX Vegfr-1 tk\textsuperscript{-/-} 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 (\textDelta), pannus formation (▲), and loss of cartilage and bone (□). These findings are milder in pX Vegfr-1 tk\textsuperscript{-/-} mice (E). (F) Histological scores of the degree of synovial hyperplasia, etc. in paws and ankles. Scores of pX Vegfr-1 tk\textsuperscript{-/-} mice were about half those of the pX mice.
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 Ab59. DeBandt et al. reported that VEGFR-1 is involved in a model of chronic arthritis in which KRN (the αβ TCR-transgene)/NOD (non-obese diabetic) mice were used for the induction of inflammation60. 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 mouse61. 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 findings62. 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 manner63.

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 characterized 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 patients64. 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 phagocytic 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\(^{43}\).

**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\(^{42}\) (kindly provided from Kirin Brewery, Gumma, Japan), to mice with \(\rho\)X-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 hematopoietic recovery after irradiation. Treatment with blocking monoclonal antibody to VEGFR-1 inhibited the hematopoietic recovery. In contrast delivery of PIGF to irradiated mice improved it\(^{45}\).

A hematopoietic activity is capacity for colonies to form bone marrow mononuclear cells (BMMNCs) in vitro\(^{46}\). 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).

However, the number of Sca-1 and CD34-positive cells corresponding to hematopoietic stem cells (HSCs) among BMMNCs were almost the same in these mice\(^{47}\). 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 \(\rho\)X mice were in between those of wild-type \(\rho\)X 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 showed suppressed cytokine secretion, phagocytosis, and VEGF-dependent migration, resulting in a decrease in rheumatoid arthritis.

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