

Effect of Direct Angiogenesis Inhibition in Rheumatoid Arthritis Using a Soluble Vascular Endothelial Growth Factor Receptor 1 Chimeric Protein

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ABSTRACT. Objective. We evaluated the effect of direct angiogenesis inhibition in synovium of patients with rheumatoid arthritis (RA), using a soluble vascular endothelial growth factor receptor 1 (VEGFR1) chimeric protein.

Methods. Dispersed cells from active RA synovial tissues were cocultured on OP9 stromal cells. Control synovial tissues were obtained from patients with injury of the anterior cruciate ligament. Chimeric protein (30 $\mu\text{g/ml}$) of the extracellular domain of VEGFR1 fused to the Fc portion of human IgG1 (VEGFR1-Fc) was added to culture medium. After 10 days, the cells were stained with anti-CD31 antibody and anti-Tie-2 antibody.

Results. Endothelial cells from patients with active RA had high angiogenic growth capacity compared with controls. Proliferation of these endothelial cells was strongly suppressed by VEGFR1-Fc. Quantitative analysis revealed that VEGFR1-Fc inhibited angiogenesis in a dose dependent manner.

Conclusion. VEGFR1-Fc is able to suppress angiogenesis in rheumatoid synovium, suggesting that direct inhibition of angiogenesis activity could serve as a novel therapeutic strategy to prevent progressive synovial hyperplasia and inflammatory reactions in active RA. (J Rheumatol 2002;29:240–5)

Key Indexing Terms:

ANGIOGENESIS INHIBITION ANGIOGENIC GROWTH CAPACITY CHIMERIC PROTEIN
VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 1 RHEUMATOID SYNOVIUM

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown etiology. RA occurs worldwide in all ethnic groups. The characteristic feature of RA is hyperplasia of synovial cells with angiogenesis, although there are a variety of systemic manifestations. The potential of the persistent synovial inflammation to cause cartilage destruc-

tion, bone erosion, and subsequent joint deformities is the hallmark of the disease. No therapeutic management method presently in use is curative. The course of RA is quite variable, and difficult to predict in an individual patient. Some patients experience relentless progressive polyarthritis with marked joint destruction.

An elevated degree of capillary congestion is seen in the proliferation of inflamed synovial tissue in active RA. Recently, it has been reported that angiogenesis precedes all other features in early RA, on the basis of biopsy samples from the synovia of patients with monoarthritis who later developed typical RA¹. Therefore, increased angiogenesis in rheumatoid synovial tissues could serve as the primary target of therapeutic strategy.

Several angiogenic factors are likely to be important in neovascularization. Several positive regulators of angiogenesis have been identified, e.g., the fibroblast growth factor family, the transforming growth factor family, hepatocyte growth factor, tumor necrosis factor (TNF- α), vascular endothelial growth factor (VEGF), angiogenin, interleukin 8 (IL-8), and angiopoietins². Negative regulators include thrombospondin³, the 16 kilodalton fragment of prolactin⁴, the secreted protein that is acidic and rich in cysteine (SPARC)⁵, angiostatin⁶, and endostatin⁷. The interplay between positive and negative regulators of angiogenesis is

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thought to give rise to the complex patterns of vascularization observed in different tissues and organs⁸.

The endothelial cell-specific mitogen VEGF is an important mediator of physiological and pathological angiogenesis⁹. VEGF is essential for embryonic development, and loss of even one VEGF allele results in embryonic lethality^{10,11}. VEGF is also required for growth and survival during early postnatal life¹². However, in the fully developed animal, VEGF is likely to be involved mainly in active angiogenesis processes such as corpus luteum development¹². VEGF binds to its tyrosine kinase receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR), both of which are expressed on endothelial cells¹⁰. Mice homozygous negative for either receptor die between embryonic days 8.5 and 9.5^{13,14}. Strong experimental evidence links VEGFR2 activation to VEGF induced mitogenesis and angiogenesis⁹. Recent studies have unexpectedly shown that VEGFR1 may function in angiogenesis mainly as a ligand-binding molecule, rather than as a signaling tyrosine kinase¹⁵.

Several investigations have shown that concentration of VEGF in synovial fluid is significantly higher in patients with RA than in controls, and that VEGF is strongly expressed in rheumatoid synovial lining cells and subsynovial macrophages¹⁶⁻¹⁸. Further, serum concentration of VEGF is higher in patients with RA than in controls or patients with other arthritides¹⁹. Thus, there is speculation that VEGF is involved in the development of inflammation and tumor-like proliferation of synovial tissue, and may be important in the disease activity of RA.

We examined inhibition of angiogenesis in patients with RA, using a soluble VEGFR1 chimeric protein in which the N-terminal extracellular domain of Flt-1 has been fused to an Fc fragment of human IgG1²⁰. The results suggest that suppression of angiogenesis may be beneficial in the treatment of active RA. To our knowledge, this is the first study to investigate direct blockage of VEGF activity as a means of downregulating angiogenesis in active RA using human tissues.

MATERIALS AND METHODS

Patients and preparation of synovial tissue. Tissue specimens were obtained from 5 patients with RA (stage IV) who satisfied the American Rheumatism Association 1987 diagnostic criteria²¹, and who had a disease duration of 10–25 years. For comparative analysis, tissues from 3 patients with injury of the anterior cruciate ligament (ACL) were also obtained. With informed consent, synovial tissue samples were obtained from patients with active RA during total knee joint arthroplasty, and from patients with injury of the ACL during reconstruction of ruptured ACL.

Immunohistological study. Synovial tissues were fixed in 4% paraformaldehyde [in phosphate buffered saline (PBS, pH 7.4)] for 4 h at 4°C, transferred to 30% sucrose-PBS and left overnight at 4°C, frozen in OCT compound, and stored at –80°C. Frozen synovial tissue was sectioned (5 µm), air dried for 10 min, and stained with immunoperoxidase using an avidin-biotin technique²². All subsequent incubations were performed at room temperature in a moist chamber. Synovial tissues were pretreated with 50 µl of 2% normal horse serum in 3% BSA/PBS for 20 min, incubated with mouse anti-human CD31 (WM-59, mouse anti-human mono-

clonal; Pharmingen, San Diego, CA, USA) or control Mab, and washed in PBS 2 times. They were incubated with a 1/500 dilution of anti-mouse biotinylated antibody in 3% BSA/PBS, washed twice in PBS, incubated with avidin/biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min, and again washed in PBS twice. They were then stained with diaminobenzidine tetrahydrochloride substrate for 5 min, rinsed in tap water for 2 min, and mounted. The sections were photographed under an Olympus AX70 microscope.

Production of recombinant fusion protein. As described²⁰, a recombinant fusion gene containing the extracellular domain of VEGFR1 and the Fc portion of human IgG1 was constructed. VEGFR1-Fc fusion proteins, produced by COS7 cells transfected with the fusion gene in serum-free conditioned medium²³, were purified over protein-A columns (Affi-Gel protein A, Bio-Rad, Hercules, CA, USA). The purity and disulfide linked dimerization of the soluble VEGFR1 chimeric protein were assessed by Coomassie brilliant blue staining of sodium dodecyl sulfate-polyacrylamide gels.

In vitro culture of endothelial cells from synovial tissues. The stromal cell line OP9²⁴ was maintained in α -modified minimum essential medium (α -MEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 20% fetal calf serum (FCS; JRH Bioscience, Lenexa, KS, USA). Synovial tissues were incubated with 0.01% collagenase (Sigma, St. Louis, MO, USA) in α -MEM for 20 min at 37°C. The dispersed cells were drawn through a 23G needle, deposited into a polystyrene tube, and centrifuged at 500 g for 10 min. Debris and aggregated cells were removed with a nylon mesh. The cells in suspension were counted, and viability was tested according to Trypan blue exclusion criteria. These cells (3×10^4 cells/well) were cultured on OP9 stromal cells in RPMI-1640 (Gibco BRL) with 10% FCS and 10^{-5} M 2ME (Sigma) at 37°C in humidified 5% CO₂ air, in the presence or absence of VEGFR1-Fc chimeric protein. After 10 days in culture, endothelial cells were stained using an anti-CD31 antibody (WM-59, mouse anti-human monoclonal), an anti-Tie-2 antibody (C9C3, mouse anti-human monoclonal)²⁵, and a Vector M.O.M. Immunodetection Kit (Vector Laboratories, Burlingame, CA, USA). It has been reported that the experimental agents in this study do not directly damage cells *in vitro*^{26,27}. After CD31 immunohistochemical staining, the number of CD31 positive cells under different culture conditions was counted per well. The average number of 4 wells of the tissue culture from each subject was represented. Cultures were done in replicate.

Statistical analysis. All values were expressed as the mean \pm standard deviation. The Mann-Whitney test was used to analyze experimental data. Results were considered significant at the $p < 0.05$ and $p < 0.01$ level.

RESULTS

Angiogenesis is involved in the proliferation of inflammatory synovial tissue in patients with active RA.

The pathologic hallmark of RA is synovial membrane proliferation and outgrowth associated with erosion of articular cartilage and subchondral bone. To study the extent of angiogenesis in RA, synovial biopsies from 5 patients with RA and 3 patients with injury of the ACL were examined. Histological study on synovial tissues from patients with active RA revealed an elevated degree of capillary congestion, along with mononuclear cell infiltration and proliferation of inflamed rheumatoid synovium into the joint cavity (Figure 1A). The synovia from patients with injury of the ACL were not hyperplastic, and contained very few inflammatory cells (Figure 1B). Immunohistochemical examination of rheumatoid synovial tissue using antibodies to CD31, which is expressed in vascular endothelial cells, showed a large number of vascular endothelial cells that

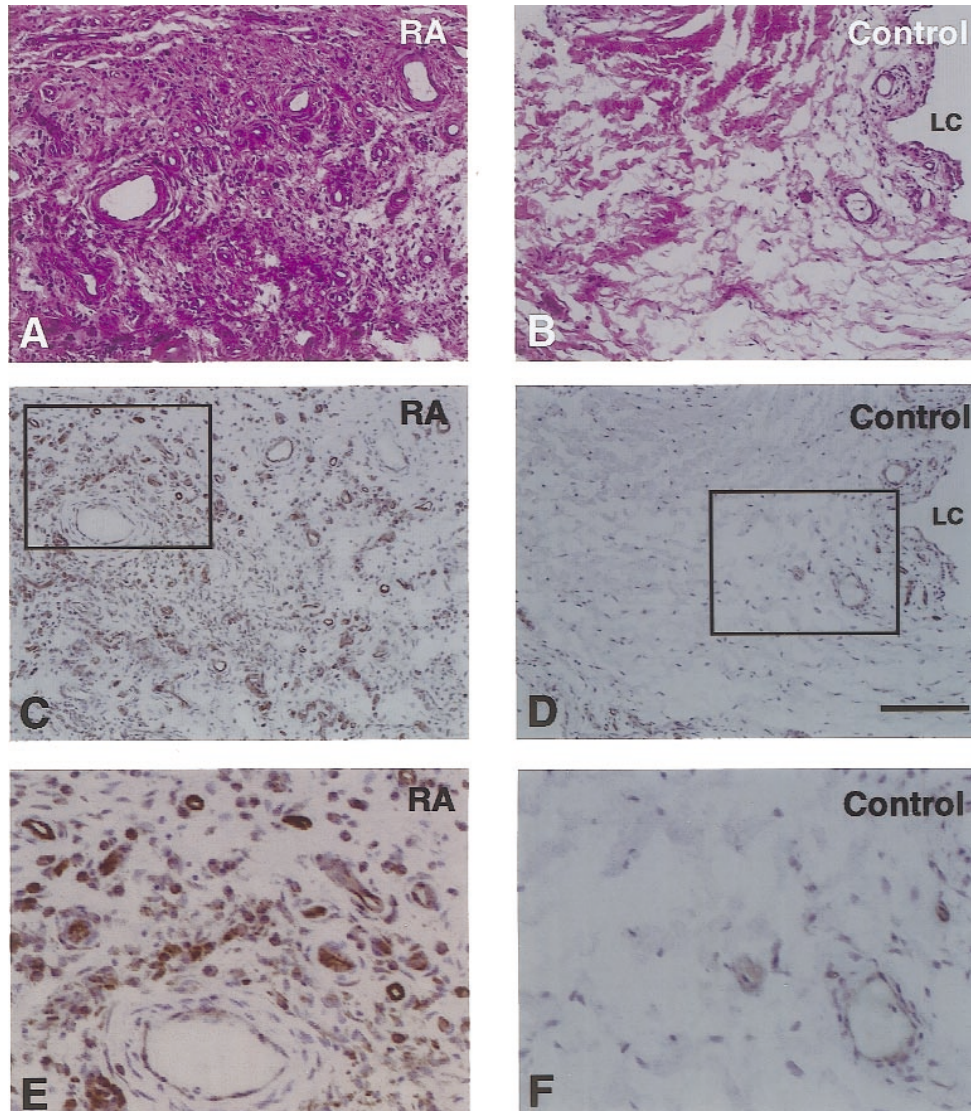


Figure 1. Histological sections of RA or control synovial tissues. H&E staining of synovial tissues (A, B). Histological image of the rheumatoid synovium shows a large number of capillaries, along with extensive infiltration of macrophages, lymphocytes, and plasma cells (A). In control synovium, there is neither an increased number of capillaries nor intrinsic cellular proliferation (B). CD31 immunostaining of synovium (panels C–F). Synovium from a patient with active RA shows a large number of endothelial cells stained with antibodies against CD31 (C). In the control synovial tissue, few endothelial cells are stained with antibodies against CD31 (D). Higher magnification of the boxed area in C is shown in E. Higher magnification of the boxed area in D is shown in F. LC: synovial lining cells. Scale bar indicates 20 μm (A through D).

were similar in morphology to those identified by staining with hematoxylin and eosin (Figures 1C, 1E). In the synovial tissue of controls, there were few endothelial cells that bound antibodies against CD31 (Figures 1D, 1F). Taken together, these findings suggest that angiogenesis in the synovial tissues plays an important role in the pathogenesis of active RA. They also suggest that therapeutic direct suppression of angiogenesis could be effective in treatment of active RA.

VEGFR1-Fc chimeric protein inhibits proliferation of endothelial cells from synovial tissues of active RA.

Although several angiogenic factors are critical for active RA, these molecular mechanisms have not been clarified. Among the involved positive regulators of angiogenesis, VEGF is likely to play a pivotal role in active RA. To examine the proliferation capacity of endothelial cells of rheumatoid synovium, we cocultured dispased cells (3×10^4 cells/well) from synovial tissues of RA on OP9 stromal cells, which can support endothelial proliferation²⁷. After 10 days of culture, the proliferation of endothelial cells was examined by immunohistochemical analysis using antibodies against CD31 and Tie-2, which are known to be

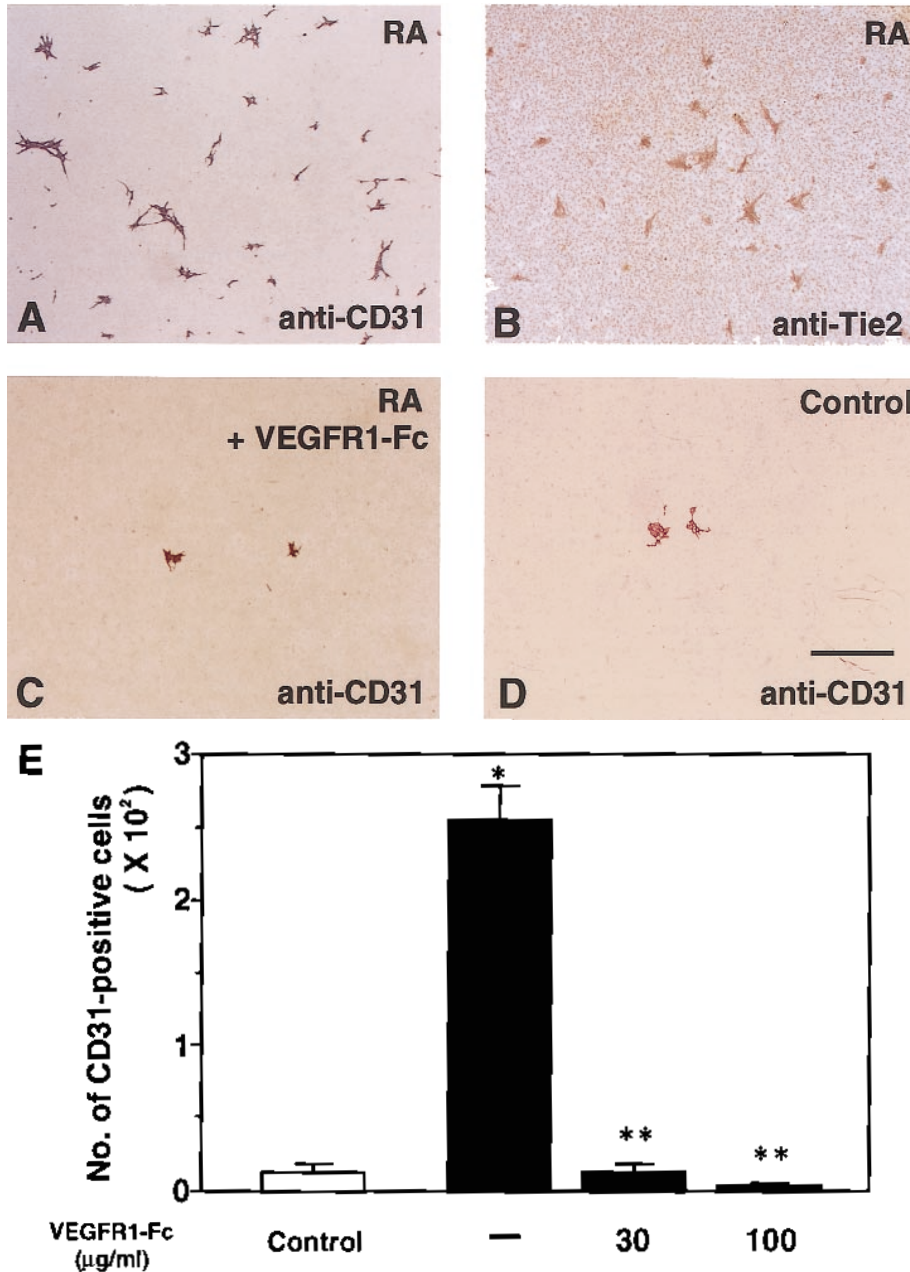


Figure 2. To examine the role of VEGF in endothelial cells, we cocultured dispersed cells (3×10^4 cells/well) from RA synovial tissues on OP9 stromal cells. Culture conditions are described in Materials and Methods. Proliferated endothelial cells (on OP9 stromal cells) stained with the anti-CD31 antibody after 10 days of coculturing (A). These cells were stained with the anti-Tie-2 antibody (B). A chimeric protein (30 $\mu\text{g/ml}$) in which the extracellular domain of VEGFR1 is fused to the Fc of human IgG (VEGFR1-Fc) was added to cultures. After 10 days, these cells were stained with the anti-CD31 antibody. Proliferation of endothelial cells was strongly suppressed by VEGFR1-Fc (C). The number of CD31 positive cells in control synovial tissues was less than the number in RA tissues (D). Scale bar indicates 1 mm (A–D). E: Proliferation of endothelial cells from synovial tissues at different concentrations of VEGFR1-Fc (0, 30, and 100 $\mu\text{g/ml}$). Results represent 5 RA and 3 control samples. Each column represents the mean number of endothelial cells. Error bars indicate SD. * $p < 0.05$, versus endothelial cells from control synovium. ** $p < 0.01$, versus endothelial cells from RA synovium incubated in the absence of VEGFR1-Fc.

specifically expressed in vascular endothelial cells. Proliferated endothelial cells (on OP9 stromal cells) were stained with anti-CD31 antibody and anti-Tie-2 antibody

(Figures 2A, 2B). In RA synovium, the number of CD31 positive cells in culture was 256.8 ± 22.3 , compared to a mean 12.9 ± 1.7 ($p < 0.05$) for endothelial cells from control

synovium (Figure 2D). These results suggest that the endothelial cells from active RA have high angiogenic growth capacity, compared with control donors. To assay whether the function of VEGF is critical to the development of RA, we prepared a chimeric protein in which the extracellular domain of VEGFR1, which contains the ligand binding site, is fused to human IgG-Fc (VEGFR1-Fc). The soluble chimeric protein functions as a competitor of VEGFR1, preventing VEGF from binding to this receptor²⁰. We cultured dispersed cells from rheumatoid synovial tissues on OP9 stromal cells at 37°C with or without VEGFR1-Fc. In the presence of 30 µg/ml VEGFR1-Fc, the number of CD31 positive cells in RA synovium was 12.2 ± 4.7, a decrease of 95.2% from a mean value of 256.8 ± 22.3 for endothelial cells from RA synovial tissues incubated in the absence of VEGFR1-Fc ($p < 0.01$) (Figures 2C, 2E). In the presence of 100 µg/ml VEGFR1-Fc, the number of CD31 positive cells was 2.2 ± 0.7, a 99.1% decrease from the mean for untreated tissues ($p < 0.01$) (Figure 2E). These results show that VEGFR1-Fc drastically inhibits the angiogenic growth capacity of endothelial cells of RA tissues in a dose dependent manner.

DISCUSSION

RA is a chronic inflammatory disorder of unknown cause, typified by invasive synovial proliferation leading to progressive destruction of joints. Proliferating inflammatory synovial tissue (pannus) becomes villous and is vascularized by a great many capillaries (Figure 1). The pannus leads subsequently to destruction of adjacent cartilage and bone, and may result in the joint deformities and dysfunction seen clinically. Angiogenesis (the sprouting of new blood vessels from existing vessels) is one of the earliest histopathologic findings in RA and appears to be required for pannus development²⁸⁻³². Moreover, it has recently been reported that angiogenesis precedes all other features in early RA, on the basis of biopsy samples from the synovia of patients with monoarthritis who later developed typical RA¹. We showed that the endothelial cells in synovia from patients with active RA have higher angiogenic growth capacity than those obtained from control donors (Figure 2). This finding suggests that increased angiogenesis in rheumatoid synovium could serve as the primary target of effective therapy.

Among the implicated positive regulators of angiogenesis, VEGF is a critical mediator of normal and abnormal angiogenesis⁹. In many tumors, VEGF expression is upregulated, and this stimulates tumor angiogenesis and subsequent tumor growth and metastasis³³. Elevated serum levels of VEGF have been reported in patients with a variety of tumor types³⁴. Moreover, monoclonal antibodies to VEGF have been shown to suppress tumor growth in a human-xenograft murine model³⁵. These antibodies are presently under evaluation, in clinical trials, for the treatment of

human cancers. In RA, VEGF is strongly expressed in the rheumatoid synovial lining cells and subsynovial macrophages, and concentration of VEGF in synovial fluid is significantly higher in patients than in controls¹⁶⁻¹⁸. Investigations have revealed that inhibition of angiogenesis with some agents [including AGM-1470³⁶, Mab to TNF- α ³⁷, thalidomide³⁸, $\alpha v\beta 3$ antagonist³⁹, and several disease modifying antirheumatic drugs (DMARD)⁴⁰⁻⁴³] results in amelioration of disease symptoms that is associated with improvements in clinical and laboratory disease activity measures. However, the role of these agents in the regulation of VEGF release has not been fully elucidated, although AGM-1470⁴⁴, Mab to TNF- α ⁴⁵, and bucillamine⁴⁶ have been shown to suppress VEGF levels. It thus seems likely that therapeutic direct suppression of the bioactivity of VEGF could be particularly effective in the treatment of active RA.

We showed that a soluble VEGFR1 chimeric protein can suppress angiogenesis in rheumatoid synovium. Addition of 30 µg/ml VEGFR1-Fc to RA cultures strongly suppressed proliferation of endothelial cells, compared to culturing in the absence of the chimeric protein (Figures 2A, 2B, 2C). This soluble receptor acts by sequestering VEGF and preventing access to its receptors²⁰. Moreover, quantitative analysis revealed that VEGFR1-Fc inhibited angiogenesis in a dose dependent manner (Figure 2E). These results indicate that VEGFR1-Fc drastically inhibits the angiogenic growth capacity of endothelial cells of RA tissues, suggesting that direct inhibition of angiogenesis activity could serve as a novel therapeutic strategy to prevent progressive synovial hyperplasia and inflammatory reactions in active RA.

Nevertheless, anti-VEGF therapy might have undesirable side effects; for example, abnormal organic and functional growth in children. VEGF also has other important physiological functions, in female reproduction, wound healing, and other processes⁹. Thus, there may be certain populations for whom anti-VEGF therapy is not advisable. Finally, we hope these findings will prompt researchers to investigate drug effects, side effects, and dosage schedules of VEGFR1-Fc in treatment of RA in clinical studies.

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