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Vascular Endothelial Growth Factor Expression and Regulation of Murine Collagen-Induced Arthritis

Jing Lu,* Tsuyoshi Kasama,2* Kazuo Kobayashi, † Yoshiyuki Yoda,* Fumitaka Shiozawa,* Michio Hanyuda,* Masao Negishi,* Hirotsugu Ide,* and Mitsuru Adachi*

We have examined the expression and function of the angiogenic factor, vascular endothelial growth factor (VEGF) during the evolution of type II collagen-induced arthritis (CIA). Biologically active VEGF was expressed along a time course that paralleled the expression of two specific VEGF receptors, Flk-1 and Flt-1, and the progression of joint disease. Moreover, levels of VEGF expression correlated with the degree of neovascularization, as defined by vWF levels, and arthritis severity. Macrophage- and fibroblast-like cells, which infiltrated inflamed sites and were then activated by other inflammatory mediators, are probably important sources of VEGF and may thus regulate angiogenesis during the development of CIA. Administration of anti-VEGF antiserum to CIA mice before the onset of arthritis delayed the onset, reduced the severity, and diminished the vWF content of arthritic joints. By contrast, administration of anti-VEGF antiserum after the onset of the disease had no effect on the progression or ultimate severity of the arthritis. These data suggest that VEGF plays a crucial role during an early stage of arthritis development, affecting both neovascularization and the progression of experimentally induced synovitis. *The Journal of Immunology, 2000, 164: 5922–5927.

Type II collagen-induced arthritis (CIA) in the mouse has proven to be a useful model of rheumatoid arthritis (RA), in that it incorporates many of the cell-mediated and humoral immunity characteristics found in human RA. Although the pathogenesis of CIA has not yet been elucidated (1–3), the progression of synovitis in both CIA and RA is characterized by pronounced tumor-like expansion of the synovium. Consequently, neovascularization may play a pivotal step during disease progression.

Neovascularization is a complex process, involving endothelial cell division, selective degradation of vascular basement membranes and surrounding extracellular matrix, and endothelial cell migration. Several polypeptide growth factors have been identified based on their ability to stimulate the proliferation of endothelial cells (4), and a number of angiogenic factors may be important in the neovascularization found in the RA joint. These include TNF-α, acidic and basic fibroblast growth factor, and IL-8 (5). Another important mediator of neovascularization is vascular endothelial growth factor (VEGF), which is a secreted, heparin-binding, homodimeric glycoprotein with several protein variants resulting from alternative mRNA splicing (6, 7). VEGF is an endothelial cell-specific mitogen in vitro and an angiogenic growth factor in vivo. It is known to play an important role in the female reproductive system as well as in such pathological conditions as diabetic retinopathy, certain tumors, and RA (8–11). However, the location and time course of VEGF expression during with the development of CIA have not been delineated, nor has it been determined whether VEGF is directly involved in the induction of the synovitis seen in CIA.

In the present study we show that VEGF is expressed during the development of CIA and that the synovitis can be attenuated by passive immunization with a neutralizing anti-VEGF Ab. Our data support the hypothesis that the expression of specific angiogenic factors during the development of CIA is a key pathologic-component mediating neovascularization in the pannus and the surrounding joint tissue, making VEGF a potentially important factor in the pathogenesis of CIA.

Materials and Methods

Animals and reagents

Male DBA/1J mice (8–10 wk) were purchased from Charles River Japan (Yokohama, Japan), chick type II collagen was obtained from Sigma (St. Louis, MO), recombinant murine VEGF (mVEGF) was purchased from PeproTech (London, U.K.), and goat anti-mVEGF and anti-human von Willebrand factor (vWF) polyclonal Abs (IgG fraction) were obtained from R&D Systems (Minneapolis, MN) and Enzyme Research Laboratories (South Bend, IN), respectively. Monoclonal anti-Mac-1 Ab detects murine macrophages (Cedarlane, Hornby, Ontario, Canada). Monoclonal anti-fibroblast Ab detects murine fibroblasts (Biogenes, Poole, U.K.). All reagents were tested for endotoxin contamination using a Limulus amebocyte lysate test kit (Limulus HS-F test, Wako, Tokyo, Japan). The concentration of endotoxin was consistently below 0.03 EU/ml in all reagents.

Endothelial cell proliferation assays

Endothelial cell proliferation was assayed using HUVECs purchased from Clonetics (Walkersville, MD) and cultured according to the provided protocol. HUVECs were seeded in triplicate in 24-well plates at 5 × 10^4 cells/well. The following day, the culture medium was replaced with serum-free RPMI medium (Dai-Nippon, Tokyo, Japan), and then mVEGF...
was added. VEGF inhibition studies, in which a specific anti-mVEGF neutralizing Ab (10 μg/ml; R & D Systems) was also added to the cultures, were conducted in parallel. After 60 h of culture in the presence of VEGF, [3H]thymidine (1 mM) was added, and its incorporation into DNA was measured by liquid scintillation counting after an additional 12 h.

Preparation of polyclonal anti-mVEGF antiserum

Rabbit polyclonal anti-mVEGF antiserum was prepared according to the methods of Tilton et al. (12). Briefly, the synthesized N-terminal 24 a.a. of mVEGF were coupled to keyhole limpet hemocyanin (KLH) via the maleimide-activated carrier protein that cross-links cysteine to KLH. New Zealand White rabbits were then immunized by intradermal injection of 200 μg of the KLH-linked peptide. After Abs against the carrier protein (KLH) were deleted by passage over a KLH-conjugated column, the antiserum titer was tested for reactivity to mVEGF by ELISA, as described previously (13). Neutralizing activity of anti-mVEGF-peptide antisera was assessed by evaluating inhibition of mVEGF-stimulated HUVEC proliferation; at a dilution of 1/1000, the antiserum inhibited 20 ng of mVEGF in this assay (data not shown).

Induction and evaluation of CIA

Type II CIA was elicited in mice by an immunization with chick type II collagen as previously described (14–16). Paws were individually scored on a scale of 0–3 based on the amount of erythema, swelling, and joint rigidity (arthritis index); the maximum score was thus 12/mouse (16). Inoculations were assigned a value of 100%.

Preparation of aqueous joint extracts

Joint tissues were prepared by first removing the skin and separating the limb below the ankle joint. They were then homogenized on ice in 3 ml of EDTA-containing buffer (19) and then were embedded in OCT compound (Life Technologies). The cultured cells were used from passages 3 to 5, during which time they morphologically resembled fibroblast-like synoviocytes, were positive for anti-murine fibroblast Ab, and were negative for anti-Mac-1 Ab, indicating that they were not macrophage-like synovioocytes (data not shown). [263x673]Anti-VEGF Ab

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control IgG (cpm)</th>
<th>cpm</th>
<th>% suppressiona</th>
</tr>
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<tbody>
<tr>
<td>Medium</td>
<td>568.0 ± 67.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recombinant mVEGF (2 ng/ml)</td>
<td>1829.6 ± 332.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recombinant mVEGF (20 ng/ml)</td>
<td>2434.8 ± 348.2</td>
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</table>

Joint extracts

<table>
<thead>
<tr>
<th>Control</th>
<th>657.4 ± 53.8</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIA</td>
<td>811.8 ± 93.6</td>
<td></td>
</tr>
</tbody>
</table>

Animal histology

VEGF and vWF Ags were visualized immunohistochemically as previously described (16). Joint specimens were initially decalcified for 2 wk in EDTA-containing buffer (19) and then were embedded in OCT compound (Miles, Elkhart, IN). Before staining, 5-μm frozen sections were fixed for 30 min in ice-cold acetone. Endogenous peroxidase activity was quenched by incubating the slides for an additional 30 min in absolute methanol and 3% hydrogen peroxide. The slides were then incubated with polyclonal goat anti-mVEGF Ab (1/500 dilution), goat anti-vWF Ab, or preimmune goat IgG. Biotinylated rabbit anti-goat IgG (BioGenex, San Ramon, CA) and peroxidase-conjugated streptavidin were used as second and third agents, respectively, while the red color was developed using 3-aminon-9-ethylcarbazole in N,N-dimethylformamide. After rinsing with distilled water, the slides were counterstained with Mayer’s hematoxylin. Tissue sections were also examined with Mayer’s hematoxylin. Tissue sections were also examined with Mayer’s hematoxylin. Tissue sections were also examined with Mayer’s hematoxylin.
Statistical analysis

Data were expressed as the mean ± SEM. Differences between VEGF and vWF levels were compared by one-way ANOVA. The incidence and severity of arthritis were evaluated using χ² contingency analysis and Mann-Whitney U test, respectively. Differences were considered significant if p < 0.05.

Results

Expression of VEGF during the evolution of CIA

We initially examined whether arthritic joints contained elements capable of stimulating endothelial cell proliferation. Aqueous joint extracts obtained from mice with severe arthritis (4 days after onset) or from controls without arthritis were tested for their ability to stimulate HUVEC proliferation. In preliminary experiments HUVEC proliferation was elicited by mVEGF (Table I), most likely because cDNAs of human and murine VEGF are highly homologous (20). In addition, the viability of HUVEC at 72 h, as estimated by trypan blue dye exclusion, ranged from 58 to 66% for cultures without serum-free medium. Aqueous extracts from arthritic joints similarly stimulated the proliferation of HUVEC compared with extracts from control joints (Table I). Moreover, addition of neutralizing anti-mVEGF Ab inhibited the activity by 59.3%, confirming that biologically active VEGF was expressed within the arthritic joints.

We characterized the time course to VEGF expression within joints and correlated it with the development of arthritis (Fig. 1). Mice with or without type II collagen immunization were sacrificed every 4 days, and joint tissue was prepared for VEGF analysis. The amount of VEGF Ag present in the joints of arthritic mice reached a maximum on day 4 after the onset of arthritis, which correlated with footpad swelling, and then gradually declined until stabilizing on day 24. Thus, VEGF was expressed primarily during the acute stage of arthritis development, suggesting that VEGF may play a role in the induction and maintenance of CIA. No increase in VEGF was observed in control mice, immunized without type II collagen, at any time (data not shown).

Evaluation of neovascularization by vWF expression

To examine the extent to which VEGF expression correlates with neovascularization in arthritic lesions, the vWF content of affected joints was assessed using an appropriate ELISA. Because vWF is considered to be a marker for endothelial cells (23), we considered vWF levels to be an index of neovascularization. As shown in Fig. 2, vWF levels reached a maximum on day 4 after the onset of arthritis and then gradually declined; neovascularization thus correlated temporally with both VEGF expression and footpad swelling.

Transcription of VEGF and its receptors, Flt-1 and Flk-1, during the development of CIA

VEGF transcription in joint tissue was assessed using RT-PCR and Southern blotting. Several alternative splice variants of VEGF have been described (6), and in fact, the primers used in the present experiments were capable of yielding five spliced forms, 279, 304, 350, 411, and 483 bp in length (24). We found that VEGF transcripts, especially the 279-bp form, were constitutively expressed in joint tissues (Fig. 3A). After the onset of arthritis, however, transcription of the 279- and 304-bp forms was significantly increased until day 24 after onset, which correlates well with the time course of expression of the protein (Fig. 1). Transcription of Flt-1 and Flk-1, the cell surface receptors for mVEGF, was also significantly elevated during the period between days 4 and 12.

FIGURE 1. Time-dependent changes in the VEGF content of aqueous joint extracts and their relation to footpad swelling. CIA was induced by immunization with type II collagen on day 0. Aqueous joint extracts were isolated from joint tissues at the indicated time points and analyzed using an ELISA for VEGF. Data at each time point are the mean ± SEM of at least nine tissue samples. *, p < 0.05.

FIGURE 2. Time-dependent changes in vWF content of aqueous joint extracts plotted together with VEGF content. CIA was induced by immunization with type II collagen on day 0. Aqueous joint extracts were isolated from joint tissues at the indicated time points and analyzed using ELISAs for VEGF and vWF. Note that the data for VEGF are the same as shown in Fig. 1. Data at each time point are the mean ± SEM of at least nine tissue samples. *, p < 0.05.

FIGURE 3. Time course of VEGF, Flt-1, and Flk-1 transcription. Whole RNA was isolated from joint tissues at the indicated time points following primary immunization; transcribed mRNA was amplified by RT-PCR using appropriate primers (A. VEGF; B. Flt-1; C. Flk-1). RNA from mouse aorta served as a positive control (Ao). Ethidium bromide staining; II, Southern blot hybridization with internal probes; D, GAPDH primers were used as an internal control. Data are representative of three independent experiments.
after the onset of arthritis, after which it gradually declined (Fig. 3, B and C).

**Immunolocalization of VEGF and vWF protein during CIA**

Immunolocalization showed VEGF to be mainly associated with infiltrating macrophage- and fibroblast-like cells, identified by morphology and by the reactivity with anti-Mac-1 Ab and anti-fibroblast Ab, respectively, and chondrocytes in chronically inflamed joint tissues (Fig. 4B, arrow); tissue sections stained with preimmune, control IgG showed little or no nonspecific staining (Fig. 4A). Of particular interest to us, VEGF also appeared to be expressed by vascular endothelial cells, but to only a small degree (Fig. 4D, arrow). Identified by their expression of vWF (Fig. 4C, arrowhead), this was in contrast to earlier findings suggesting that vascular endothelial cells are the primary source of VEGF (6, 7).

To further clarify the cellular source of VEGF in the inflamed joint, we isolated fibroblast-like cells from the joints of CIA mice and placed them in culture. When stimulated with TNF-α, the fibroblast-like cells expressed significant amounts of VEGF (Fig. 5, arrowhead), which suggests that the cellular sources of VEGF are probably the macrophage- and fibroblast-like cells that infiltrate the joint during CIA development as well as in the synovium of human RA (10).

**Effects of passive immunization with neutralizing anti-mVEGF antiserum on CIA development**

The results described above show that VEGF is expressed within arthritic joints during the development of CIA and may have a crucial role in the evolution of the ailment. To obtain more direct evidence of VEGF-induced neovascularization, we injected anti-mVEGF antiserum (0.75 ml/time/mouse) i.p. every other day between days 30 and 38 along with type II collagen immunization. The mice were then observed daily, and the incidence and severity of arthritis were assessed. As shown in Fig. 6, we found that administration of anti-mVEGF antiserum attenuated both the incidence and the severity of joint inflammation (arthritis index) in CIA mice. Moreover, histopathologic examination of joints from mice treated with anti-mVEGF antiserum showed less cartilage and bone destruction, less pannus formation, and less fibroblast proliferation than observed in mice treated with control serum (Figs. 7, A and B), and neovascularization (vWF levels) was also significantly reduced (Fig. 7C).

Interestingly, when anti-mVEGF antiserum was administered to CIA mice during the chronic stage (days 50–58), the neutralizing antiserum did not suppress the development or ultimate severity of the arthritis (Fig. 8). This suggests that VEGF acts primarily during the initiation of CIA by regulating neovascularization during that period.

**Discussion**

The data presented in this report demonstrate that VEGF and its receptors, Flt-1 and Flk-1, are expressed within inflamed joints and are very much involved in the development of CIA. Our temporal analyses showed that VEGF expression is coincident with augmented neovascularization, as assessed by the vWF content of inflamed joints, and that passive immunization with anti-VEGF Abs suppresses acute stage synovitis. Thus, VEGF appears to be an...
important regulator of angiogenesis during the development of CIA.

We detected the presence of elements that stimulated endothelial cell proliferation in the extracts of inflamed mouse joints (Table I). Proliferation of vascular endothelial cells is induced not only by VEGF, but also by cytokines such as IL-1, TNF-α, IL-8, and fibroblast growth factor (25, 26). Although we did not assess the specific contributions made by other factors, the fact that inflamed synovitis was markedly (59.3%) inhibited by anti-VEGF Ab strongly suggests that it has a central role in the process.

An unexpected finding was that vascularization reached a maximum during the acute stage of the arthritis and then gradually declined (Fig. 2). Because both vWF and VEGF levels were normalized to total joint protein (percent increased or milligrams per milligrams of protein), one possible explanation is that total joint protein increased relative to vWF and VEGF at later stages (early stage, 0.79 ± 0.09 mg/ml; late stage, 1.92 ± 0.22 mg/ml).

VEGF was originally thought to be an endothelial cell-derived, angiogenic, autocrine factor. However, recent reports have shown that fibroblasts (24, 27, 28), macrophages (10, 29, 30), alveolar epithelial cells (31), vascular smooth muscle cells (32), neutrophils (33), and even chondrocytes (34) also express significant amounts of VEGF. Indeed, most of the cells expressing VEGF in arthritic joints were macrophage- and fibroblast-like cells and chondrocytes, but not endothelial cells (Fig. 4). Furthermore, that TNF-α evoked significant VEGF expression in cultured fibroblast-like cells from arthritic joints (Fig. 5) suggests that inflammatory cells infiltrating inflamed sites are stimulated to express VEGF by other cytokines present there.

Expression of VEGF is known to be regulated by other mediators, including platelet-derived growth factor (35), TGF-β (36), IL-1 (28), oxygen radicals and nitric oxide (30, 37–39), and hypoxia (32, 38, 40). The synovium is chronically hypoxic in RA and probably also in CIA: biochemical evidence of anaerobic metabolism suggests that blood flow is insufficient to meet the high metabolic demands of inflamed synovial tissue (41). In that regard, the cytokines mentioned above are known to be involved in the development and maintenance of CIA (42–44). Thus, hypoxia, various cytokines, oxygen radicals, and nitric oxide may act in concert to stimulate angiogenesis in CIA synovitis through up-regulation of VEGF.

The inhibitory effect of administering neutralizing antiserum clearly showed that VEGF-induced neovascularization is a crucial step in the initiation and early development CIA (Figs. 1, 2, and 6). On the other hand, for reasons that are less clear, arthritis severity was unaffected by administering anti-VEGF antiserum after the onset of the disease (late stage; Fig. 8). One possibility is that newly organized vascular networks may be virtually complete by the chronic stage, making VEGF less important than other immune/inflammatory mediators.

We found that two specific VEGF receptors, Flt-1 and Flk-1, were expressed at the site of synovitis and that the time course of their expression paralleled that for VEGF itself (Fig. 3). This is in agreement with an earlier report showing that Flt-1 and Flk-1 are expressed at low levels in endothelial cells under normal conditions, but are up-regulated at sites where there is a concomitant up-regulation of VEGF (45). Activation of Flt-1 by VEGF does not efficiently induce migration of endothelial cells without simultaneous Flk/KDR activation (46). Nevertheless, a recent report showed that activated monocytes and even dendritic cells, which are APC present at inflamed sites, expressed only Flt-1 and not Flk-1/KDR (47–49). VEGF may thus induce monocyte activation and migration via Flt-1 (47, 50). This idea is supported by the finding that during the development of CIA, infiltration of the synovial tissue by monocytes and neutrophils occurs predominantly during the acute stage (51). Our present findings therefore suggest that infiltrating, activated monocytes contribute to the neovascularization seen during CIA development by acting directly on endothelial cells through secretion of various cytokines, including VEGF, as well as by acting indirectly through recruitment of additional monocytes through the VEGF-Flt-1 pathway.
In conclusion, VEGF appears to be an important endogenous mediator during the development of both RA and CIA synovitis. A more complete understanding of the functions of VEGF and its receptors, Flt-1 and Flk-1, should contribute to our understanding of the pathophysiology of CIA and human RA. More broadly, it should contribute to our understanding of the role of the vascular system in chronic inflammatory responses.

Acknowledgments

I would like to thank T. Takeuchi for her excellent work with the experiments.

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