Angiogenesis-Inflammation Cross-Talk: Vascular Endothelial Growth Factor Is Secreted by Activated T Cells and Induces Th1 Polarization

Felix Mor, Francisco J. Quintana and Irun R. Cohen

*J Immunol* 2004; 172:4618-4623; doi: 10.4049/jimmunol.172.7.4618

http://www.jimmunol.org/content/172/7/4618

References

This article cites 50 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/172/7/4618.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Angiogenesis-Inflammation Cross-Talk: Vascular Endothelial Growth Factor Is Secreted by Activated T Cells and Induces Th1 Polarization

Felix Mor,*† Francisco J. Quintana,* and Irun R. Cohen1,2,*

Vascular endothelial growth factor (VEGF) and its receptors are critical in angiogenesis. The main player in the secretion and response to VEGF is the endothelial cell. We initiated this study to test whether T cells can secrete VEGF and are able to respond to it. Here we show that VEGF is secreted by T cells on stimulation by specific Ag or by IL-2 and by hypoxia; thus, activated T cells might enhance angiogenesis. Hypoxia also induced the expression in T cells of VEGFR2, suggesting that T cells might also respond to VEGF. Indeed, VEGF augmented IFN-γ and inhibited IL-10 secretion by T cells responding to mitogen or Ag; thus, VEGF can enhance a Th1 phenotype. Encephalitogenic T cells stimulated in the presence of VEGF caused more severe and prolonged encephalomyelitis. Thus, T cells can play a role in angiogenesis by delivering VEGF to inflammatory sites, and VEGF can augment proinflammatory T cell differentiation. The Journal of Immunology, 2004, 172: 4618–4623.

The study of angiogenesis is a rapidly growing field. New blood vessel formation is critical in the growth and spread of malignant tumors (1) and in the development of microvascular complications of diabetes (2). Therapeutic induction of angiogenesis is being tested in the treatment of myocardial (3) and peripheral ischemic (4) syndromes. Angiogenesis is also important in inflammation. One of the pathological hallmarks of rheumatoid arthritis is the formation of pannus (5), an inflammatory connective tissue mass rich in blood vessels, apparently dependent on angiogenic factors (6, 7).

The endothelial cell has been considered to be the main cellular player in the secretion of angiogenic factors and in the response to them (8). Vascular endothelial growth factor (VEGF)3 is a specific mitogen for vascular endothelial cells (9). VEGF was demonstrated by RT-PCR from various sources (10) including human peripheral blood leukocytes (11), which contain lymphocytes, monocytes, and other cells (12). In contrast, the expression of VEGF receptors has been reported to be limited to endothelial cells (8).

T cells are pivotal in the initiation and progression of inflammation (13). T cells have multiple effects on immune and nonimmune cells through their secretion of a large number of cytokines and chemokines (14). T cells homing to inflammatory sites are in intimate contact with endothelial cells (15) and could influence the angiogenesis associated with inflammation. Because inflammation involves new blood vessel formation (16), we tested whether T cells can deliver and respond to the angiogenic signal VEGF.

Materials and Methods

Animals

Inbred female Lewis rats were supplied by the animal breeding center of the Weizmann Institute of Science (Rehovot, Israel) under the supervision of Harlan Laboratories (Haslett, MI) and an animal welfare committee and were used at 2–3 mo of age.

Primers and Abs

The primers for VEGF were: sense 5′-TGCACCCACAGACAGAA GGGGA-3′ and antisense 5′-TACACCCTTTGCTTGTCA CAT-3′ (product sizes, 497 bp for 120-aa isoform, 629 bp for 164-aa isoform, and 701 bp for 188-aa isoform). Primers for VEGFR1 (Flt-1) were sense 5′- CTTCTCAAGTGCAAGGG-3′ and antisense 5′-AGGATGTTATT GGTCTGCCG-3′ (product size, 543 bp). Primers for VEGFR2 (KDR/Flk-1) were sense 5′-AAGCAAAATGTCAGCAGGT-3′ and antisense 5′-TCTGTCGCTGCTCTGTACGT-3′ (product size, 283 bp). The Abs used were rabbit polyclonal anti-VEGF (sc-507), anti-Flt-1 (sc-316), anti-Flik-1 (sc-504) and anti-rabbit HRP-conjugated Ab, all from Santa Cruz Biotechnology (Santa Cruz, CA). The ELISA kit for murine VEGF was purchased from R&D Systems (Minneapolis, MN).

T cell lines and clones

Ag-specific T cell lines were established from lymph node cells that had been stimulated with Ag (10 μg/ml) for 3 days in stimulation medium, as described (17). The T cell lines used were: anti-p277, reactive to peptide p277 of heat shock protein (HSP) 60 (18); BP10, a Lewis guinea pig myelin basic protein (MBP)-reactive T cell line (17); T cell clones 5, 9, 26, and 22 were derived from a line reactive to β-synuclein peptide p93-111 (19); FISCHER, an MBP-reactive line derived from Fischer 344 rats; A2b, a clone derived from Lewis rats with adjuvant arthritis (20) reactive with peptide p176–190 of mycobacterial HSP65 (21).

RT-PCR assay

Total cellular RNA was isolated by the single-step method using the TRI-REAGENT (Molecular Research Center, Cincinnati, OH). Five micrograms of total RNA were used for the reverse transcriptase reaction. cDNA was prepared, and PCR were performed as described (22).

Western blotting

The protein concentration was determined using the Bio-Rad DC protein assay, and 50 μg of protein were loaded per lane (Bio-Rad, Hercules, CA). After electrophoresis in 12% SDS gel in a minigel apparatus (Bio-Rad), the
ELISA determination of rat VEGF

The levels of VEGF in culture supernatants were measured by a VEGF ELISA kit according to the instructions of the manufacturer (R&D Systems). This assay, which was developed for mouse VEGF, can be used to measure rat VEGF, based on a 70% cross-species reactivity for the detecting Abs.

Hyposxia induction

To induce hypoxia, activated T cells, which are metabolically activated and secreted VEGF, were incubated (5 × 10^6 MBP (10 μg/ml) in the absence or presence of recombinant human VEGF (500 ng/ml; Refs. 25 and 26). T cell blasts were isolated on LymphoPrep gradient (Nycomed, Oslo, Norway). Groups of female Lewis rats (five per group) were injected with the line cells and scored for clinical signs of EAE from day 3 postinjection. Clinical EAE was observed 3–12 days after T cell line injection. Clinical scoring was: +1, paralysis of tail; +1.5, paresis of posterior paws and ataxia; +2, paraplegia; +3, paralysis extending to thoracic spine; +4, a moribund state (17).

Cytokine ELISA

Supernatants were collected after 3 days of stimulation of T cells with Con A or specific Ag. IL-10 and IFN-γ in the culture supernatants were measured by ELISA using the PharMingen OPTEIA kit (PharMingen, San Diego, CA). PharMingen recombinant rat cytokines were used as standards for calibration curves. The results are expressed as picograms per milliliter. The lower limits of sensitivity for the experiments described were 30 pg/ml for both IL-10 and IFN-γ. Recombinant human IL-1α and IL-1β and VEGF were obtained from The National Cancer Institute preclinical repository (Rockville, MD). The recombinant human VEGF was produced by R&D in Sf21 cells, and the protein was purified by sequential chromatography (>97% purity by SDS-PAGE, visualized by silver staining). Recombinant human VEGF is active in the rat (27, 28).

Results

VEGF expression is induced by IL-2 in T cells

To detect the expression in different rat tissues of the various VEGF isoforms by RT-PCR, we used oligonucleotide primers spanning the eight exons of the VEGF gene (29). Similar to previous reports (10), we detected expression in rat tissues of three isoforms, 120 aa, 164 aa, and 188 aa (not shown). To examine the effect of IL-2 on VEGF expression by T cells, we incubated a rat T cell line (anti-p277) with 100 U/ml IL-2 for various times (after 24 h of IL-2 deprivation), in the absence of Ag and APCs, and probed cDNA was prepared from the T cells. Fig. 1 shows that two isoforms of VEGF (encoding 120 aa and 164 aa; Ref. 29) were induced in the T cell line by incubation with IL-2 for 120 min. Incubation of the T cells for 120 min with culture medium containing TCGFs (30) also induced the VEGF isoforms.

To detect VEGF at the protein level, we lysed an activated T cell line (BP10) and various other tissues and ran SDS-PAGE separation with 50 μg of protein per lane. A 21-kDa band representing VEGF164 (the isoform containing 164 aa, analogous to the human VEGF164) was detected in lysates of various rat tissues including T cell line (Fig. 1b). In several tissues (fat and kidney lysates; Fig. 1b), we detected a higher isoform corresponding in size to the 205-aa isoform of VEGF. A band of identical size (21 kDa) was detected by an Ab against VEGF. The 21-kDa band is seen in BP10 (a MBP-reactive T cell line (17), at the end of stimulation) and represents the 164-aa isoform. c, Induction of VEGF by graded concentrations of IL-2. Anti-p277 line cells were incubated for 24 h with 0, 5, 10, 25, 50, or 100 U/ml IL-2. Cells were collected, lysed, and tested by SDS-PAGE. Blotted membranes were immunostained with polyclonal rabbit anti-VEGF. A dose response to IL-2 was seen. With higher concentrations of IL-2, the 21-kDa VEGF band is induced.

VEGF is secreted by T cells

We investigated whether T cells secrete VEGF and whether this secretion can be activated by specific Ag stimulation. We screened for the presence of VEGF in the medium of various T cell clones and lines that had been stimulated with specific Ag for 3 days. Fig. 2a shows that six of seven T cell lines and clones produced VEGF as detected by ELISA. Fig. 2b shows that increasing amounts of VEGF were secreted by two representative T cell lines during 4 days of Ag stimulation; the amount of VEGF in the culture supernatants increased progressively from day 1 to day 4 of Ag-induced stimulation in both the anti-p277 and the FISCHER lines. VEGF was also detected in the supernatant of Con A-activated spleen induced (in a dose-dependent manner) the expression of VEGF164 at the protein level (Fig. 1c). In contrast, incubation with IL-1α or IL-1β did not induce VEGF expression, although these cytokines stimulated proliferation of the T cells as tested by thymidine incorporation (not shown). Thus, T cell proliferation by itself does not appear to induce VEGF. We may conclude that the induction of VEGF by IL-2 is probably unrelated to its mitogenic effect on T cells.

FIGURE 1. Induction of VEGF mRNA and protein by IL-2. a, PCR analysis of cDNA prepared from T cell line anti-p277 (18), which was starved from IL-2 for 24 h (indicated as 0), and incubated with 100 U/ml recombinant human IL-2 for 30 or 120 min (indicated as 30 and 120). FM, Incubation with full medium (containing T cell growth factors (TCGF) and lines that had been stimulated with specific Ag stimulation. We screened for the presence of VEGF in the medium of various T cell clones and lines that had been stimulated with specific Ag for 3 days. Fig. 2a shows that six of seven T cell lines and clones produced VEGF as detected by ELISA. Fig. 2b shows that increasing amounts of VEGF were secreted by two representative T cell lines during 4 days of Ag stimulation; the amount of VEGF in the culture supernatants increased progressively from day 1 to day 4 of Ag-induced stimulation in both the anti-p277 and the FISCHER lines. VEGF was also detected in the supernatant of Con A-activated spleen...
cells (Fig. 2c; TCGF). This finding indicates that the ability to secrete VEGF is present in primary lymphocytes and not only in T cell lines and clones.

**Induction of VEGF and VEGFR2 mRNA and protein by hypoxia**

Hypoxia is a physiological stimulus for VEGF induction in endothelial cells (31). To examine whether hypoxia might also induce VEGF expression in T cells, line T cells 1 day after Ag stimulation (when the cells are highly active and divide in response to IL-2) were incubated in closed Eppendorf tubes for 1–3 h at 37°C, deprived of oxygen (24). The hypoxia resulted in a time-dependent secretion of VEGF by Ag-specific T cell lines during T cell activation. Progressive increases in the amounts of VEGF are detected from day 1 to day 4. p277S6, Anti-p277 line at the sixth stimulation; FISCHERS6, FISCHER line at the sixth stimulation. c, Dose-dependent induction of VEGF secretion. The anti-p277 line was incubated for 3 days with the peptide concentrations indicated, and supernatants were tested by ELISA. TCGF, The second day supernatant of Con A-activated rat spleen cells (30).

**FIGURE 3.** Induction of VEGF and VEGFR2 by hypoxia. Anti-p277 line cells at the beginning (day 1) of the propagation phase were incubated in closed Eppendorf tubes for the times indicated; cDNA was prepared and tested with primers for VEGF and VEGFR2. Both VEGF and VEGFR2 were induced by hypoxia and levels of PCR products peaked at 180 min. G47S3, cDNA from the T cell line reactive to β-synuclein peptide aa 93–111 (19). Control cDNA was amplified with primers for GAPDH. b, Induction of VEGF secretion by hypoxia. T cell line (anti-p277) was incubated at the end of Ag stimulation for 1–4 h in closed Eppendorf tubes and supernatants were tested by ELISA.

**VEGF enhances a Th1 cytokine response profile**

The results presented above show that T cells can synthesize and secrete VEGF and express VEGFR2. Because VEGFR2 is the receptor known to transduce VEGF signaling in endothelial cells (8), we tested whether VEGF could affect T lymphocytes. Incubation of T cells with VEGF did not induce proliferation. Because VEGF prevents apoptosis in endothelial cells (32), we tested the ability of VEGF to prevent dexamethasone-induced apoptosis (18) in both resting and activated T cells. The results of these experiments failed to show any antiapoptotic effect of VEGF on T cells (not shown).

However, we found that T cells responded to VEGF by modifying their cytokine secretion. We activated naïve lymph node cells by various concentrations of the T cell mitogen Con A with or without VEGF (500 ng/ml; Refs. 25 and 26). Fig. 4 shows that the presence of VEGF induced a modest elevation of IFN-γ secretion and a decrease in IL-10 secretion at various concentrations of Con A. An increase in IFN-γ and a decrease in IL-10 is consistent with polarization toward Th1 differentiation (Fig. 4, left). VEGF also influenced the cytokine profile of primed LNC responding to a specific Ag. We obtained draining popliteal lymphocytes from
rats that had recovered from adjuvant arthritis induced 60 days earlier (33), a time point when the rat T cells express also Th2 cytokines in response to target Ags. We stimulated responding T cells with the HSP65 target peptide p176–190 (21). Fig. 4 (right) shows that here too, the presence of VEGF resulted in enhancement of IFN-γ and inhibition of IL-10 secretion by the responding T cells at 0.1, 1, and 50 µg/ml. Fig. 5 (left) shows that BP10, a MBP-specific T cell line, also secreted more IFN-γ and less IL-10 in the presence of VEGF.

To rule out the possibility that the VEGF might have influenced T cell cytokine secretion indirectly by way of APCs or other non-T cells present in the above cultures, we tested the effects of VEGF on a T cell clone (A2b; Ref. 20) that has high expression of MHC class II and proliferates to its specific peptide in the absence of exogenous APCs (21); autopresentation by T cells without APC has been described in human (34) and rat (21) T cells. In this experiment, any effect of VEGF on cytokine production had to be attributed directly to the responding T cells themselves. Here too, the presence of VEGF during stimulation of clone A2b alone resulted in a marked increase in IFN-γ and a decrease in IL-10 secretion (Fig. 5, right).

Enhancement of T cell-mediated EAE by VEGF

EAE is mediated by Th1 T cells reactive to myelin Ags (35). To examine the functional effect of VEGF on T cells mediated inflammation, we stimulated an encephalitogenic T cell line (BP10) with or without VEGF (500 ng/ml; 25 and 26) and injected the activated line cells into naive rats. The VEGF-treated T cells mediated the earlier onset of a more severe and prolonged EAE compared with the T cells stimulated without VEGF (Fig. 6). Thus, VEGF augmented adoptively transferred EAE disease; the enhanced Th1 profile detected in vitro was accompanied by a significant enhancement of the disease in vivo.

Discussion

Previous work has demonstrated the expression of VEGF by peripheral blood lymphocytes (11, 36); but, in addition to T cells, such populations also contain other cells such as monocytes and thrombocytes that might secrete VEGF (12). Other investigators have documented the expression of VEGF by malignant (37) or virus-infected T cells (38, 39). Malignant B cells from patients with chronic lymphatic leukemia (37) and T cells infected with HIV (38) and human T cell leukemia virus type 1 (39) were reported to express VEGF. Basic fibroblast growth factor has been reported to be produced by T cells (40).

The present results demonstrate that both primary T cells stimulated by Con A and primed T cells, T cell lines, and clones responding to specific Ags synthesize and secrete VEGF (Figs. 1 and 2). Secondly, VEGF secretion can be activated by the concentration and duration of stimulation by specific Ag (Fig. 2) or by IL-2 (Fig. 1). Third, both VEGF and VEGFR2 are induced in T cells by hypoxia (Fig. 3). Fourth, VEGF was found to augment secretion of IFN-γ and inhibit secretion of IL-10 in primary T cells and in T cell lines (Figs. 4 and 5). Finally, incubation of a pathogenic T line with VEGF resulted in more severe encephalomyelitis, indicating that the in vitro effect on cytokine secretion was significant in modulation of the in vivo effects of the line (Fig. 6).

The finding that T cells synthesize and secrete VEGF has implications for both the physiology and pathophysiology of inflammation. Aberrant angiogenesis is a characteristic of abnormal inflammation in autoimmune diseases such as rheumatoid arthritis (5), and angiogenesis is a damaging factor in retinitis (41). It is conceivable that the T
cells infiltrating autoimmune lesions (42, 43) contribute to pathogenic angiogenesis by secreting VEGF when they respond to target self-Ags or to the IL-2 produced by other activated T cells.

Physiological angiogenesis too could benefit from VEGF produced by activated T cells. VEGF produced by T cells could activate endothelial cells at sites of T cell activation such as draining lymph nodes. Immune responses are marked by increases in lymph node size and vascularity (44). The present results indicate that activated T cells producing VEGF could contribute to the physiology of lymph node vascularity. Thus, the degree of new blood vessel formation could be fine-tuned by the site and degree of T cell activation.

We and others have shown that activated T cells can serve as vehicles for neurotrophic factors (45), and it is not unlikely that T cell neurotrophins could play a role in the positive contribution of autoimmune T cells to neuroprotection after trauma to the CNS (46). The present findings that activated T cells produce VEGF suggest that activated T cells might also deliver needed angiogenic factors to sites of injury and so promote healing.

Inflammation has been defined as a process induced by damage that normally leads to healing (47). Thus, the role of T cells in inflammation can include molecular signals that can enhance tissue healing, not only tissue destruction (48, 49). The homing of T cells to sites of inflammation provides a mobile source of VEGF that can be controlled by the degree of T cell activation. Moreover, hypoxia associated with tissue damage would lead to the production of angiogenic factors including VEGF at sites of damage; the VEGF would, as we show here, push T cells visiting the site into a proinflammatory Th1 mode and amplify inflammation. VEGF is an important signal in the dialog between the tissues and the immune system (48). It would be of interest to test whether the blood

![FIGURE 5. VEGF augments a Th1 response in T cell lines. ELISA results of cytokine measurements of supernatants of Ag stimulation of line BP10 in the presence of APCs (BP10, left). Cytokine measurements of clone A2b stimulated with peptide p176–190 of HSP65 in the absence of APC (right). Levels of significance in t test are as shown in the legend to Fig. 4.](http://www.jimmunol.org/)

![FIGURE 6. Effects of VEGF on EAE. Administration of BP10 line cells to rats after incubation with VEGF during Ag activation results in earlier and more severe disease. At each time point, disease scores differed significantly between the groups (Mann-Whitney U test; InStat software, p = 0.05 day 5; p = 0.03 day 6; p = 0.008 days 7 and 9; p = 0.01 day 8).](http://www.jimmunol.org/)
vessels at inflammatory sites are indeed newly formed (50, 51) and whether inhibition of VEGF signaling by small molecules (52) or Abs (16) could affect the evolution of the inflammatory process.

References


