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THE REGULATORY EFFECTS OF CYTOKINES ON LYMPHATIC ANGIOGENESIS

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ABSTRACT

The effects of fibroblast growth factor basic (bFGF), transforming growth factor α (TGFα), recombinant human epidermal growth factor (EGF), recombinant human tumor necrosis factor α (TNFα), and recombinant interleukin 1α (IL-1α) on lymphatic angiogenesis were assessed in cultured newborn bovine lymphatic endothelial cells (NBLEC). bFGF, TGFα, and EGF stimulated the proliferation of NBLEC in a dose-dependent manner, but the combination of either two growth factors did not show synergistic effects on NBLEC DNA synthesis. TNFα and IL-1α suppressed the multiplication of NBLEC. Treatment with bFGF markedly increased the migration of NBLEC. The tissue plasminogen activator (t-PA) activity was enhanced by bFGF. TNFα also promoted NBLEC t-PA activity.

These results suggest that bFGF is a major multifunctional lymphatic endothelial cell targeted cytokine, and both growth and pro-inflammatory cytokines exert differential regulatory effects on lymphatic endothelial cell proliferation, migration and t-PA activity.

The lymphatic system has important functions in maintaining the homeostasis of the whole body. Lymphatic obstruction and aplasia are major causes of chronic lymphedema. Therefore, the effective restoration of lymph flow after operations, trauma, and inflammation is an important clinical issue.

Previously we found that the regeneration of lymphatics is conditional. Thus, a transplanted lymph node loses its architecture due to failure of regrowth of afferent lymphatics (1). Although after tissue injury newly formed small lymphatics develop, as granulation tissue matures into scar tissue the number of new lymphatics decreases or disappears (2). Most likely there is a balance in the tissues between stimuli promoting the proliferation of lymphatic endothelium and the progression of fibrosis; the latter tending to hinder the continued generation of new lymphatic vessels (2).

During the last two decades, an increasing body of information has accumulated about the effects of cytokines on wound healing and on angiogenesis (3). However, until now little is known about the influence of these factors on lymphatic regeneration. To investigate whether cytokines also regulate lymphatic angiogenesis, we examined the effects of recombinant human epidermal growth factor (EGF), transforming growth factor α (TGFα), fibroblast growth factor basic (bFGF), recombinant human interleukin 1 alpha (IL-1α), and recombinant human tumor necrosis factor alpha (TNFα) on the angiogenic response of newborn bovine lymphatic endothelial cells (NBLEC).

EGF and TGFα are structurally related proteins, promote angiogenesis, bind to blood

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vascular endothelial cells and promote their growth. TGFα produced by tumor cells also contributes significantly to angiogenesis (4).

bFGF is a potent mitogen for several cell types including blood vascular and blood capillary endothelial cells and induces an angiogenic response *in vivo* (5-7).

Interleukin 1, formerly described as lymphocyte-activating factor, exerts pleiotropic effects on a variety of cells including thymocytes, keratinocytes and fibroblasts. IL-1 also stimulates the proliferation of human dermal microvascular endothelial cells (HDMEC) but not human umbilical vein endothelial cells (HUVEC) (8).

TNFα is a macrophage-derived cytokine (9) that inhibits the proliferation of various cell lines *in vitro* including HUVEC (10), but not HDMEC (8).

MATERIAL AND METHODS

Fresh bovine thoracic duct and mesenteric lymphatics were obtained at the local abattoir. After dissecting away the adjacent fat tissue, the lymphatic vessel was cannulated at the distal end and perfused with phosphate buffered saline (PBS) to remove residual lymph, then filled with a 0.2% collagenase solution (Sigma, type II) and incubated at 37°C. After 30 min, the collagenase solution was collected and the lymphatic vessel reflushed with PBS. The total cell suspension was centrifuged and the cells resuspended in RPMI1640 (Gibco) supplemented with 20% fetal calf serum (FCS), 15mM/l Hepes buffer, 100u/ml penicillin, 100u/ml streptomycin and seeded into a 6-multiwell plate (Nunclon). The cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO_2. Subcultures were obtained by digestion of primary cultures with 0.1% trypsin and replating of harvested cells. At confluence, the cells formed a monolayer which demonstrated contact inhibition, displayed the cobblestone morphology characteristic of normal endothelial cells and showed a positive immunoperoxidase staining with anti-human factor VIII antibody (Dako).

For proliferation assays, the second passage of lymphatic endothelial cells were seeded in a 96-multiwell plate with a density of 2000 cells/well. One day later, cells were incubated with fresh medium containing cytokines or with medium alone for 4 days. The medium was renewed after 2 days. Four wells for each concentration were treated identically. At the given time points, the cells were harvested after trypsin digestion and the cell number was counted.

For determination of the cytokine effects on DNA synthesis, NBLEC were seeded in 96-multiwell tissue culture plates with a seeding density of 2000 cell/well and treated as described above. Quadruplicate dishes were incubated for 10 h at 37°C with 0.5µCi/ml 3H-thymidine (22Ci/mM, Chinese Academy of Sciences, Shanghai Nuclear Technique Co.). After completion of incubation, the cells were washed twice with PBS and harvested onto glass fiber paper, and then counted in a liquid scintillation counter (Beckman). Three separate studies were performed. In further experiments, different combinations of growth factors were then tested for the effect on NBLEC DNA synthesis for 4 days.

To test the effect of cytokines on cell motility, primary passage of NBLEC were grown in a 24-well plate. The nearly confluent monolayers were then slightly damaged by removing a portion (3 mm in width) of the cells using an angled scraper (rubber policeman) at the central part of the well. The remaining cells were further incubated for 24 h in the presence of different cytokines. The cells were then fixed and stained with crystal violet and 4% formalin solution. Cell migration was determined as the distance measured under a microscope at ten random locations along the injury line to the cells farthest from the injury site (11,12).

For tissue plasminogen activator (t-PA) activity assay, confluence cultures of NBLEC grown in 24-well plates were incubated for 24

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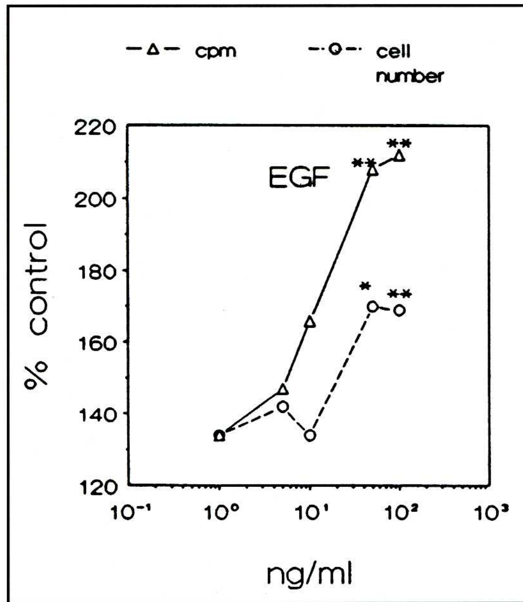

*Fig. 1. Second-passage of NBLEC. Cell growth as determined by cell number and 3H-thymidine incorporation 4 d after treatment with EGF. The concentrations were 1, 5, 10, 50, 100 ng/ml, respectively. The changes are expressed as percent variation from control. Dunnett' test was used for statistical analysis. *P<0.05, **P<0.01.*

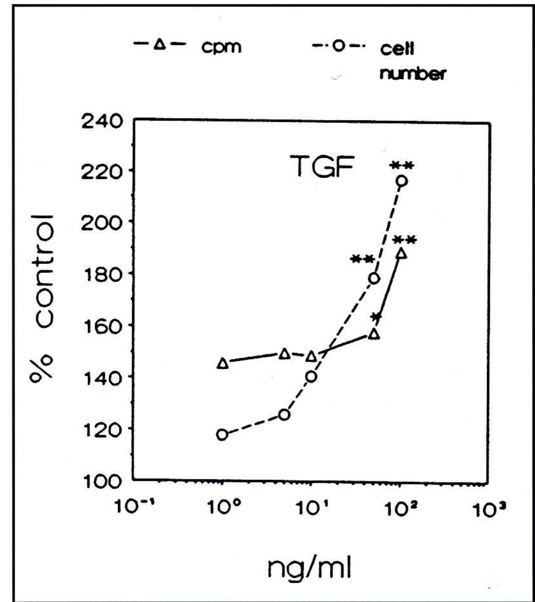

Fig. 2. Second-passage of NBLEC. Cell growth was determined by cell number and 3H-thymidine incorporation after treatment with TGF. The concentrations were 1, 5, 10, 50, 100 ng/ml, respectively. All changes are expressed as percent variation from control.

h under various conditions (see the Figure Legends). After incubation, these cells were washed twice with cold PBS and extracted in 0.5% Triton X-100, in 0.1 M Tris-HCl, pH 8.1. Aliquots of cell extracts of 100 µl were incubated in a microtiter plate with plasminogen and plasmin chromogenic substrate S2390 Dval-Phe-Lys-PNA (Shanghai Medical University). After incubation at 37°C, the plate was read at 405 nm with an automatic microplate reader. At the same time, the protein concentration was measured using the Lowry method (13). The t-PA activity was expressed as IU per µg protein. Four separate studies were performed for each set of results.

Cytokine Treatments

EGF, bFGF and TGFα were obtained from Sigma Co. IL-1α (2x10^7 u/mg) and TNFα (2x10^6 u/mg) were kindly provided by Dainippon Pharmaceutical Co., Ltd. (Japan). For proliferation assays, NBLEC were treated with 0.1 to 1000 u/ml of TNFα and IL-1α and 1 ng to 100 ng/ml of EGF, bFGF, and TGFα for up to 4 days. For migration and t-PA activity assays, NBLEC were treated with 100 u/ml of TNFα and IL-1α and 20 ng/ml of EGF, bFGF, TGFα, respectively for 24 h.

Statistical Analysis

All data were obtained by three independent experiments as described above, and the results are shown as mean ± standard deviation. Statistical significances were calculated using the Dunnett test (for cell growth) and student t test (for other comparisons).

RESULTS

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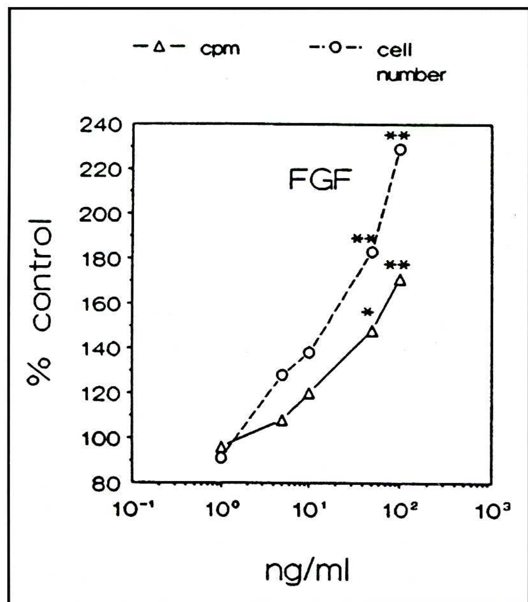

Fig. 3. Second-passage of NBLEC. Cell growth as determined by cell number and 3H-thymidine incorporation after treatment with bFGF. The concentrations were 1, 5, 10, 50, 100 ng/ml, respectively. The changes are expressed as percent variation from control.

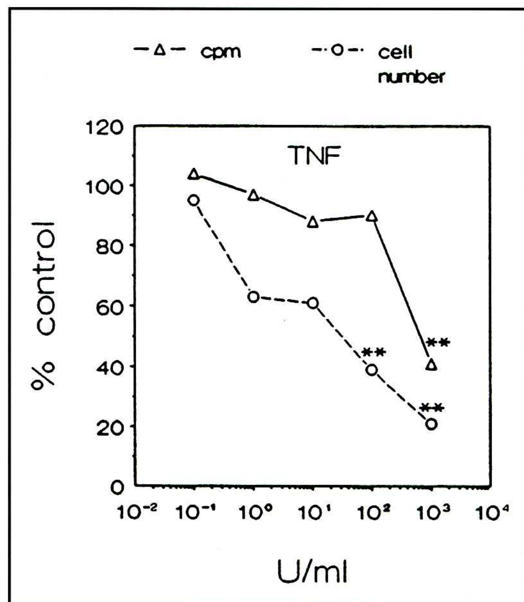

Fig. 4. Second-passage of NBLEC. Cell growth as determined by cell number and 3H-thymidine incorporation 4 days after treatment with TNF. The concentrations were 0.1, 1, 10, 100, 1000 U/ml, respectively. The changes are expressed as percent variation from control.

Cytokine Effects on NBLEC Proliferation

Treatment with EGF induced a dose-dependent stimulation of NBLEC proliferation as measured by direct cell counting and 3H-thymidine incorporation *(Fig. 1).* The minimal effective concentration was 1 ng/ml, and a significant stimulation of cell proliferation was observed above 50 ng/ml. TGFα was also found to be a potent stimulator of NBLEC multiplication with a pronounced effect at 50 to 100 ng/ml *(Fig. 2).* As shown in *Fig. 3*, bFGF also increased both cell multiplication and 3H-TdR incorporation. A significant stimulation began at concentrations of 50-100 ng/ml (similar to EGF and TGFα) but the minimal effective concentration started at 10 ng/ml. In testing for the cooperative effects of growth factors on lymphatic endothelial cell DNA synthesis, none of the cytokines showed a synergistic effect with one another expressed as 3H-thymidine incorporation when compared with the results of a single cytokine treatment.

When NBLEC were exposed to the inflammatory cytokines, both the cell number and the capacity to take up 3H-thymidine were reduced. In TNFα group, the inhibitory effect was seen at a concentration of 100 u/ml *(Fig. 4)*. IL-1α inhibited cell proliferation and 3H-TdR incorporation at a concentration of 0.1 u/ml. Higher concentrations did not enhance the effect *(Fig. 5).*

Cytokine Effects on Cell Migration

After treatment with the growth factors, only bFGF showed a significant effect on promoting the motility of NBLEC as compared by the distance the cell migrated

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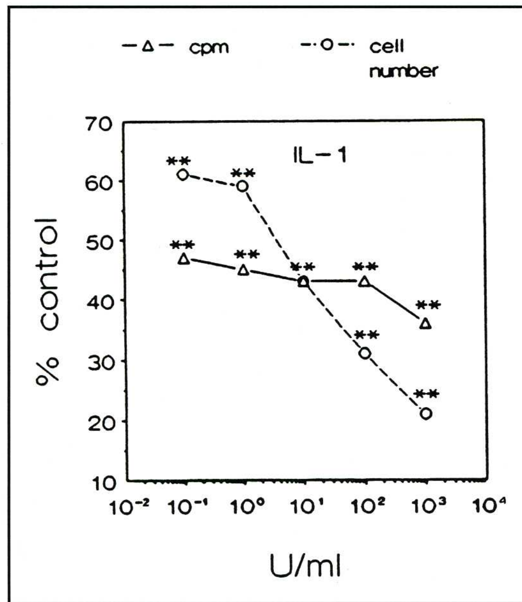

Fig. 5. Second-passage of NBLEC. Cell growth was determined by cell number and thymidine incorporation after treatment with IL-1. The concentrations were 0.1, 1, 10, 100, 1000 U/ml. The changes are expressed as percent variation from control.

TABLE 1
Effect of Cytokines on NBLEC Motility

Cytokines	Migration Distance (um)
EGF	8.8 ± 2.6
TGFα	8.9 ± 2.7
bFGF	18.7 ± 2.7**
IL-1α	5.7 ± 2.1
TNFα	3.1 ± 1.7*
control	7.7 ± 1.1

*P<0.05; **P<0.01

from the injury line *(Table 1 and Fig. 6)*. TNFα in the culture inhibited cell motility *(Fig. 6 and Table 1)*. EGF, TGFα, and IL-1α showed no effect on NBLEC migration in the culture plate.

Cytokine Effect on the Cell-Associated t-PA Activity

The cell associated t-PA activity increased significantly with bFGF *(Fig. 7)*, but was unaffected by EGF or TGFα. Addition of TNFα, however, increased NBLEC t-PA activity *(Fig. 7)*.

DISCUSSION

Angiogenesis involves dynamic functions of endothelia (14). The response of blood endothelial cells to angiogenic factors *in vivo* can be divided into four components: dissolution of basement membrane, migration from the vascular wall through perivascular connective tissue, proliferation and formation of loops (4,11). These components, in turn, may correlate with cultured endothelial cells exposed to angiogenic stimuli, production of proteases, increased motility, and increased rate of multiplication (15).

Our study suggests that cytokines which stimulate blood vascular angiogenesis *in vivo* also stimulate the proliferation, protease activity and migration of cultured lymphatic endothelial cells. However, the growth and pro-inflammatory cytokines exert different regulatory effects on the angiogenic functions of NBLEC.

EGF, TGFα and bFGF, the growth factors tested in this study, each showed a remarkable effect on NBLEC proliferation. The minimal effective concentration was 1 ng/ml for EGF and TGFα and 10 ng/ml for bFGF. These concentration doses conform to those previously shown for stimulating the proliferation of blood microvascular endothelial cells (3). However, the responses of blood vascular and lymphatic endothelial cells to mitogens have some differences. In a

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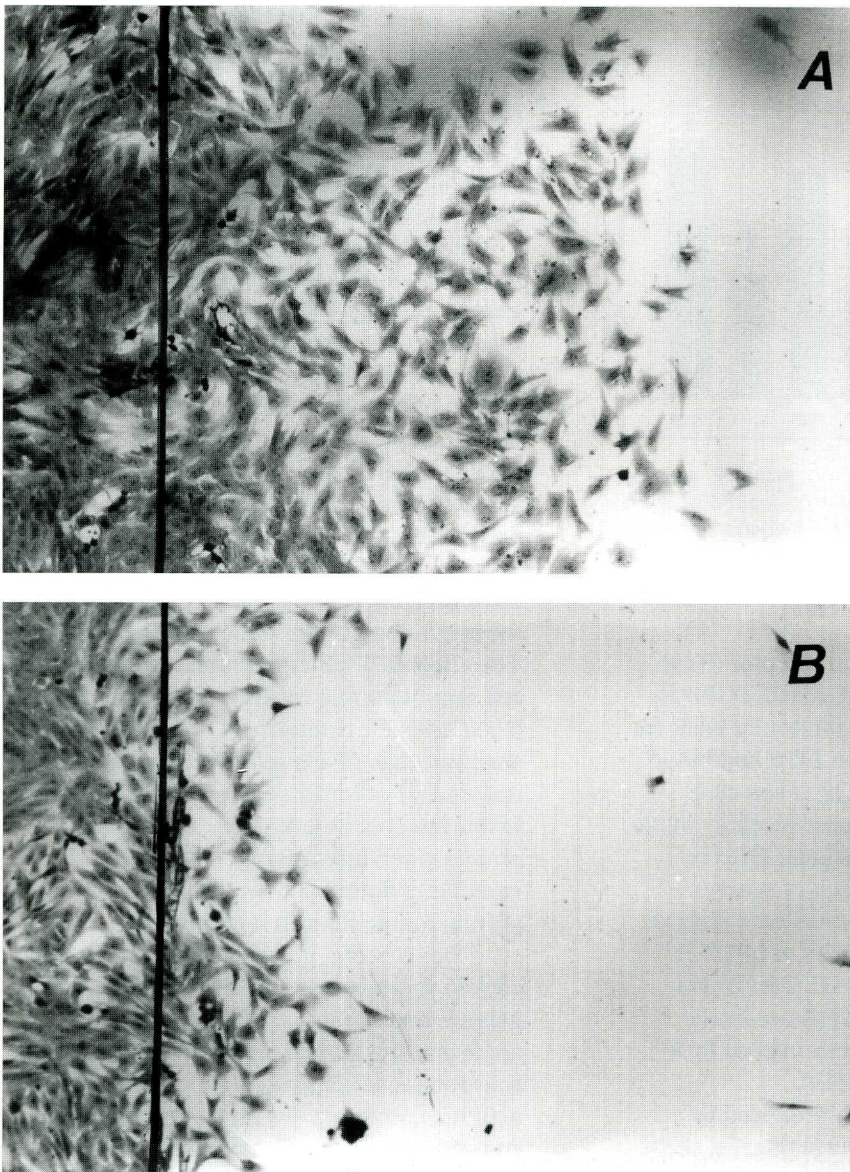

Fig. 6. Pictures of a wound in confluent NBLEC. Cytokines were added at the time of injury. The wound site is indicated by the vertical line. The cultures were exposed to cytokines for 24 h and cell migration was observed. (A) after treatment with bFGF; (B) after treatment with TNFα (x72).

previous report (16), bovine blood vascular endothelial cells failed to respond to EGF at a concentration of 10 ng/ml and no receptor for EGF was detected on these cells. In our study, bovine lymphatic endothelial cells responded to EGF at a concentration of 1 ng/ml with a significant proliferative response at 15 ng/ml and 100 ng/ml. The reasons for this discrepancy of the mitogenic response between blood vascular and lymphatic endothelial cells to EGF is unclear. Whether there are functional EGF receptors on NBLEC awaits further investigation.

To further elucidate the effects of growth factors on NBLEC, we treated the cultured cells with a combination of these factors. However, there was no synergistic promotion effect on cell DNA synthesis. Because EGF

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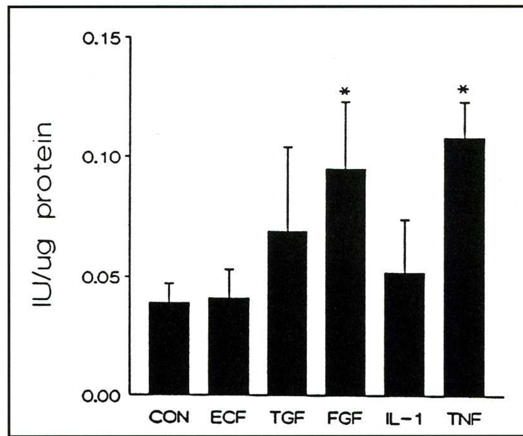

Fig. 7. Effect of cytokines on primary passage NBLEC t-PA activity. Cell were incubated with cytokines for 24 h. For statistical analysis student t-test was used. (CON=control)

and TGFα share a common receptor (3), a lack of synergy between EGF and TGFα is not overly surprising. The molecular basis for the lack of synergy between EGF and bFGF or between TGFα and bFGF is less clear. Perhaps the difference of post-receptor signal transduction between bFGF and EGF or TGFα contributes to the lack of synergy between these cytokines. It has been suggested that the mitogenic activity of bFGF is mediated by protein kinase C (PKC) (17), whereas the activity of EGF is not likely mediated by PKC (18,19) but instead by a calcium mediated process (19).

IL-1α and TNFα, both produced by cells of monocyte/macrophage lineage, share a number of biological actions on blood vascular endothelial cells, including the stimulation of procoagulant activity, expression of certain surface antigens, induction of morphologic changes, and stimulation of neutrophil and lymphocyte adherence. With regard to EC proliferation, however, results have been at variance. Some have found that IL-1 increased EC proliferation (8,20), but others did not (21,22). On the other hand, TNFα consistently inhibits *in vitro* blood vascular EC proliferation (22-24). We observed that both IL-1α and TNFα suppressed thymidine incorporation and proliferation of NBLEC at low concentrations. Moreover, the inhibitory effect of TNFα on cultured NBLEC growth was dose-dependent, but the suppressive effect of IL-1α did not increase with higher concentration.

Migration of EC is an early angiogenic response which is a major step in angiogenesis and possibly under the regulation of cytokines (14). Among the growth factors tested, bFGF strongly promoted the locomotion of lymphatic endothelial cells in a wound assay. This result is similar to the effect of bFGF on blood vascular endothelium and coincides with its effect on NBLEC proliferation. In contrast to EGF's mitogenic effect on NBLEC multiplication, neither EGF nor TNFα showed a demonstrable effect on the migration of lymphatic endothelial cells. The effect of TNFα on lymphatic endothelial cell migration was contrary to that on its blood vascular counterpart. Whereas we observed that TNFα *inhibited* lymphatic endothelial cell migration on a culture plate, Leibovien et al observed (25) that TNFα *promoted* the migration of bovine microvascular endothelial cells through a polycarbonate filter in a microwell.

The secretion of enzymes by endothelial cells to degrade extracellular matrix components is another important event during angiogenesis. An increase in t-PA, as well as collagenase levels may provide the proteolytic activities necessary for the penetration of endothelial cells into surrounding tissues. t-PA converts plasminogen into plasmin, which is a broad spectrum protease able to digest most tissue proteins. In addition, plasmin converts latent collagenase to active collagenase which can degrade plasmin-resistant tissue protein. It has been presumed that an increase in PA and collagenase activities may derive from an increased rate of endothelial cell multiplication as these cells respond to the angiogenic stimulus (15). The results concerning the effect of mitogenic factors on endothelial cell t-PA production

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have been discordant (15,26-29). In our study, the cell-associated t-PA activity was tested by using chromogenic subtracts assay (17) using the supposition that cell-associated PA levels correlate with the amount of PA secreted into the medium (15). We found that bFGF significantly increased the t-PA activity of NBLEC, an effect similar to that on bovine blood capillary endothelial cells (28,29). Nevertheless, bFGF and TGFα did not concomitantly stimulate NBLEC t-PA activity although they showed strong mitogenic effect on cell replication. These results do not support the hypothesis that increased t-PA activity derives from enhanced cell proliferation. bFGF was also the most potent angiogenesis mediator for the cultured NBLEC. It promoted cell proliferation, migration, and t-PA activity, processes that are characteristic of angiogenesis *in vivo*. In the course of biological processes such as embryonic development, wound healing, perhaps inflammation, growth factors such as EGF and bFGF may have pivotal effects on lymphatic development and regeneration.

Treatment with TNFα significantly induced the cell-associated t-PA activity of NBLEC, a result at variance with Laschinger et al (30), wherein TNFα promoted a decrease in free PA in conditioned medium (CM) and cell layer, an increase in plasminogen activator inhibitor (PAI), and a concomitant increase in complexes of t-PA-PAI-1 in CM. This discrepancy may in part relate to differences in methodology. Thus, the concentration of TNFα in the study of Laschinger et al was 200 u/ml. This dosage was much higher than what we used and the effect of TNFα varies with dose (25,31). At lower concentration, TNFα shows a promoting effect on tissue granulation *in vivo* and bovine microvascular cell growth *in vitro*. On the other hand, at higher doses TNFα inhibits tissue repair and cell growth. Alternatively, PAI may be inactivated by acidification (32). At this stage, accordingly, it is unclear whether increased t-PA activity is due to an increase in pure t-PA or an increase of a t-PA-PAI complex. Because t-PA and PAI are confined to different subcellular compartments (32), their individual activity may in future studies be separated.

It remains to be determined whether cytokine-modulated angiogenesis of lymphatic and blood vascular endothelium is similar. TNF is an indirect angiogenic agonist *in vivo* (33) although it inhibits growth of blood vascular endothelial cells *in vitro*. Perhaps TNF acts through its pro-inflammatory actions by recruiting lymphocytes and macrophages that could be the source of more direct-acting factors (14,33). As is well known, recurrent infection (cellulitis and lymphangitis) are common complications of longstanding peripheral lymphedema. Infiltration of monocytes and lymphocytes around microvessels in limb lymphedema is common (34) and mononuclear cells are potent producers of EC growth factor (35,36), including epidermal growth factor (37) and fibroblast growth factors (38). Such factors may stimulate EC proliferation at the site of inflammation. In this manner, IL-1α and TNFα may have an important role in the modulation of EC proliferation. Many more small blood vessels exist in chronic lymphedema (34) and blood flow is notably higher in the lymphedematous limb compared with the intact contralateral limb (39). Nonetheless, it remains unclear whether and how these cytokines influence lymphatic neovascularization during a pathologic process. Casley-Smith et al (40) claim that qualitatively there are more microvessels (both lymphatic and blood vessels) in lymphedematous tissue, and, if so, may account for the intense edema and fibrosis seen with inflammation/infection in such limbs.

In conclusion, cytokines were demonstrated as important regulators of angiogenic function of cultured lymphatic endothelial cells. bFGF was a major multifunctional lymphatic endothelial cell targeted cytokine which promoted NBLEC proliferation, locomotion, and proteinase production, three

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key elements needed for neovascularization. Whereas EGF and TGFα stimulated proliferation of NBLEC, they had no effect on EC t-PA activity and locomotion. The pro-inflammatory cytokines IL-1α and TNFα inhibited NBLEC growth and locomotion, but TNFα enhanced cell t-PA activity. Together these data suggest that endothelial cell proliferation, locomotion and proteinase production are independent events in angiogenesis, a highly complex process seemingly regulated by a variety of cytokines. Further studies along these lines are needed to better define the role of various cytokines on lymphangiogenesis *in vivo*.

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