Vascular endothelial growth factor (VEGF) is a prime regulator of normal and pathological angiogenesis. Three related endothelial cell growth factors, VEGF-B, VEGF-C, and VEGF-D were recently cloned. We have here studied the regulation of VEGF-C, a lymphatic endothelial growth factor, by angiogenic proinflammatory cytokines. Interleukin (IL)-1β induced a concentration- and a time-dependent increase in VEGF-C, but not in VEGF-B, mRNA steady-state levels in human lung fibroblasts. The increase in VEGF-C mRNA levels was mainly due to increased transcription rather than elevated mRNA stability as detected by the nuclear run-on method and by following mRNA decay in the presence of an inhibitor of transcription, respectively. In contrast, angiopoietin-1 mRNA, encoding the ligand for the endothelial-specific Tek/Tie-2 receptor, was down-regulated by IL-1β. Tumor necrosis factor-α and IL-1α also elevated VEGF-C mRNA steady-state levels, whereas the IL-1 receptor antagonist and dexamethasone inhibited the effect of IL-1β. Experiments with cycloheximide indicated that the effect of IL-1β was independent of protein synthesis. Hypoxia, which is an important inducer of VEGF expression, had no effect on VEGF-B or VEGF-C mRNA levels. IL-1β and tumor necrosis factor-α also stimulated the production of VEGF-C protein by the fibroblasts. Cytokines and growth factors have previously been shown to down-regulate VEGF receptors in vascular endothelial cells. We found that the mRNA for the VEGF- and VEGF-C-binding VEGFR-2 (KDR/Fk-1) was stimulated by IL-1β in human umbilical vein endothelial cells, whereas the mRNA levels of VEGFR-1 (Flt-1) and VEGFR-3 (Flt-4) were not altered. Our data suggest that in addition to VEGF, VEGF-C may also serve as an endothelial stimulus at sites of cytokine activation. In particular, these results raise the possibility that certain proinflammatory cytokines regulate the lymphatic vessels indirectly via VEGF-C.

Angiogenesis, most commonly involving sprouting of capillaries from preexisting blood vessels, starts with proteolysis of the subendothelial extracellular matrix followed by migration and proliferation of endothelial cells (1). Thereafter, the newly grown endothelium differentiates into tubelike structures, which are stabilized by mesenchymal components, preventing leakage and fused to allow blood circulation. The significance of angiogenesis is now widely accepted in many physiological and pathological conditions (2). Angiogenesis is needed for embryonic development, for several female reproductive functions, and for wound healing and other repair processes, including collateral blood vessel formation in ischemic limb and heart diseases (1–3). Angiogenesis also occurs in nonmalignant diseases, such as diabetic retinopathy, atherosclerosis, psoriasis, and rheumatoid arthritis, and its importance in solid tumor growth has been demonstrated in multiple experimental models. Several growth factors and cytokines promote angiogenesis in animal models. Most of them, including tumor necrosis factor (TNF)α and interleukin (IL)-1, do not stimulate endothelial cell growth in culture and are thus called indirect angiogenic factors (4). Once tubes composed of endothelial cells are formed, angiogenesis is apparently completed by stabilization of these vessels through migration of pericytes and production of basement membrane components. Both of these processes have been suggested to be facilitated via endothelial cell specific Tek/Tie-2 receptor ligand angiopoietin-1 (Ang-1) (5–8). In addition to angiogenic inducers, several endogenous inhibitors of angiogenesis have been recently characterized (2, 9). The switch to an angiogenic phenotype in tumors may thus be triggered by overproduction of factors in favor of angiogenesis or reduction of inhibitor release, both of which can depend on the activation of onecogenes and inactivation of antionecogenes (1).

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor or vasculotropin, is a glycoprotein with homology to platelet-derived growth factor (PDGF), and it is translated into multiple protein forms due to alternative exon splicing (reviewed in Refs. 10–14). VEGF is a prototype of directly acting angiogenic factors, because it is secreted in a biologically active form, its receptors are found at sites of angiogenesis, and it is the most specific endothelial cell growth factor known thus far. VEGF also induces vascular permeability, regulates production of proteases and their inhibitors, and promotes endothelial cell differentiation, movement, and survival, which are related to its angiogenic properties.

Up-regulation of VEGF synthesis by hypoxia and by several indirectly acting angiogenic factors also supports its role in angiogenesis. VEGF transduces its signals through Flt-1 and Flt-4, also known as VEGF receptor (VEGFR), VEGFR-1, and VEGFR-2, respectively.
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(VEGFR-1) and KDR/Flik-1 (VEGFR-2) tyrosine kinase receptors. However, mitogenesis of endothelial cells may be signaled exclusively via VEGFR-2. Genetic disruption of VEGF or its receptors has indicated that they are necessary for vasculogenesis (differentiation of endothelial cells from their precursors) and angiogenesis (15–18). Further, injection of VEGF can induce blood vessel formation, whereas antibodies against VEGF, soluble extracellular domain of VEGF-1 or overexpression of a dominant negative VEGF-2 inhibit angiogenesis and subsequent tumor growth in animal models (13, 14, 19, 20).

VEGF-B (also known as VEGF-related factor) (21–25), VEGF-C (also known as VEGF-related protein and VEGF-2) (26–30), and c-fos-induced growth factor (also known as VEGF-D) (31, 32) are recently discovered members of the VEGF family. Thus, this family is currently composed of five members, including also the placenta growth factor (33).

VEGF-B is an endothelial cell mitogen, which is expressed in most tissues, but especially highly in the heart and skeletal muscle (23). VEGF-C induces endothelial cell migration and vascular permeability (26, 34), but, unlike VEGF, it is a relatively weak mitogen for blood vascular endothelial cells (28, 30). VEGF-C mRNA is expressed at low levels in many tissues, most prominently in the heart, placenta, skeletal muscle, ovary, and the small intestine, and it is also present in certain tumor cell lines (26, 35). VEGF-C is the ligand for VEGFR-2 and for the third member of the VEGF receptor family, VEGFR-3 (also known as Flt-4), which is expressed mainly on venous endothelium in early embryos and lymphatic endothelium in adult tissues (27, 36, 37). Indeed, mice overexpressing VEGF-C in basal keratinocytes developed hyperplastic lymphatic vessels in the skin (38). Although VEGF induces growth of vascular and lymphatic endothelial cells in vitro, it seems to be specific for blood vessel growth in vivo (39).

The existence of a gene family consisting of several related growth factors suggests that these family members have overlapping but distinct functions and that they may be differentially regulated. The lymphatic endothelial growth factor VEGF-C (38, 39) in particular could be important in inflammatory conditions by controlling the composition and pressure of interstitial fluid and by facilitating lymphocyte trafficking. We have therefore studied the regulation of the expression of VEGF-C in comparison with VEGF and VEGF-B by the angiogenic proinflammatory cytokines and anti-inflammatory agents.

**MATERIALS AND METHODS**

**Cell Culture and Cytokine and Inhibitor Treatments**—Diploid human lung fibroblasts (IMR-90, American Type Culture Collection CCL-186) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), glutamine, and antibiotics (Life Technologies, Inc.) containing 10% FCS, endothelial cell growth supplement (50 μg/ml; Upstate Biotechnology Inc., Lake Placid, NY), heparin (5 units/ml, Sigma), antibiotics, and glutamine, and the experiments were performed at passage 4. Before initiation of the experiment, HUVECs were grown confluent, then starved in 5% FCS in medium 199 for 24 h, after which either IL-1β (10 ng/ml) or TNF-α (50 ng/ml) were added to the starvation media for 6 h.

**Hypoxia Treatment**—Confluent cultures of IMR-90 cells were grown on 10-cm diameter tissue culture plates containing 10 ml of DMEM and 5% FCS. These cultures were exposed to hypoxia by incubating the cells for 6 h in the Anaerocult A anaerobic culture jar supplied by Merck (Darmstadt, Germany).

**Isolation and Analysis of RNA**—Total RNA was isolated using the Trizol reagent (Life Technologies) and quantitated by absorbance at 260 nm. The poly(A)+ mRNA fraction was isolated with the PolyATrac mRNA isolation system (Promega, Madison, WI). For Northern blots, 20 μg total RNA or 1 μg of poly(A)+ mRNA were denatured in 1× MAGE buffer, 50 mM dimethyl sulfide, and 10 mM phosphate buffer at 50°C for 60 min, electrophoresed, and transferred to nylon membranes (Micron Separations Inc., Westborough, MA). Note that for whole organ or tissue RNA preparation, the lungs were minced and digested with proteinase K. In all cases, washing steps were followed by DNAase I treatment (W. Kaelin, Jr., personal communication). The nylon filters were baked at 80°C for 2 h at about 0.1% SDS. The blots were hybridized in 2× SSC, 0.1% SDS, 1× SSC, and 0.1% SDS for 4 h at 65°C. The nylon membranes were washed twice for 5 min in 6× SSC, 0.1% SDS and exposed to autoradiographic film at −70°C. The drug treatments included actinomycin D (10 ng/ml), cycloheximide (10 μg/ml), and cyclooxygenase-2 as a positive control for a cytokine-induced gene (39). The hurn probe was a 1.4-kb reverse phase-polymerase chain reaction fragment containing the open reading frame, a kind gift from Dr. Yuji Gunji. The receptor probes were the following: VEGFR-1 (nucleotides 706–2310; GenBank™ accession no. X51602), VEGFR-2 (nucleotides 6–715; GenBank™ accession no. X61656), VEGF-C (nucleotides 1–595; GenBank™ accession no. X68203), Tie-1 (nucleotides 1–2190; GenBank™ accession no. X60507), and Tek/Tie-2 (nucleotides 1550–2285; GenBank™ accession no. L06149, a kind gift from Eola Kukk). Probes were purified by gel filtration through nick columns (Pharmacia, Uppsala, Sweden) and used at 1×10⁶ cpm/ml. Hybridizations were done at 42°C for 7 h in solution containing 50% formamide, 6× SSC, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 100 μg/ml herring sperm DNA, 100 μg/ml yeast RNA, and 0.5% SDS. Membranes were washed three times at 50°C for 15 min in 0.1× SSC and 0.1% SDS. The probes were then hybridized to Fuj film x-ray film after quantitation of the signals with the Fujifilm IP reader Bio-Image analyzing system BAS1500 (Tokyo, Japan) and the MacBas software supplied by the manufacturer and visualized by autoradiography.

**Analysis of Transcription**—For nuclear run-on analysis IMR-90 cells were first starved for 48 h then incubated for 3 h with or without IL-1β (10 ng/ml) in DMEM supplemented with 0.5% FCS (10 cm dishes each). Total RNA was extracted from one control dish and one IL-1β-treated dish and used for Northern blotting. The nuclei were isolated from nine control and nine IL-1β-treated dishes as described by Greenberg and Ziff (42). Extraction of the RNA and nuclear transcription were performed as described by Celano et al. (43). [32P]UTP (3000 Ci/mmol) labeled nuclear RNA samples (1×10⁶ cpm/sample) were then hybridized to linearized plasmid DNA (pDNA) containing inserts of VEGF-C and GAPDH, or no insert. The probes were quantitated with the Bio-Image Analyser using the MacBas software, after which background was subtracted from VEGF-C and GAPDH signals.

**Analysis of mRNA Half-life**—Degradation of the mRNAs were studied in human IMR-90 lung fibroblasts. The cells were starved in DMEM supplemented with 0.5% FCS for 48 h and shifted to medium containing 5% FCS for 4 h prior to mRNA stability measurements. After two washes with DMEM, medium containing 0.5% FCS and actinomycin D (10 μg/ml, Sigma) as an inhibitor of transcription, with or without IL-1β (10 ng/ml), was added for 1, 2, or 4 h.

**Detection of VEGF-C Protein**—Confluent IMR-90 cell cultures were first starved for 48 h in DMEM supplemented with 0.5% FCS and then preincubated with or without IL-1β (10 ng/ml) or TNF-α (50 ng/ml) for 4 h. After two washes with phosphate-buffered saline, metabolic labeling was carried out by addition of 100 μCi/ml of Pro-mix 1–3S in vitro cell labeling mix (Amersham, Buckinghamshire, UK) to methionine- and cysteine-free Eagle’s minimal essential medium, with or without the cytokines. After 6 h the medium were collected and centrifuged to remove the cellular debris. Aliquots of the culture media were precipitated with trichloroacetic acid and measured for incorporated radioactivity. Equal counts/min of the media were immunoprecipitated with anti-VEGF antiserum as described earlier (34). The immunoprecipitated proteins were separated in SDS-polyacrylamide gel electrophoresis and visualized and quantitated by the Bio-Image analyzer system.

**Statistical Analysis**—Statistical significance was calculated by Stu-
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RESULTS

Kinetics and Concentration Dependence of IL-1β-induced VEGF-C mRNA Expression—To study the kinetics and concentration-dependence of VEGF-C mRNA regulation, IMR-90 cells were stimulated with 10 ng/ml IL-1β for 2–48 h. Total RNA was isolated and analyzed by Northern blot hybridization using mixture of VEGF, VEGF-B, and VEGF-C probes. GAPDH served as a loading control. Mobilities of the 28 and 18 S ribosomal RNA bands are indicated. B, the cells were incubated with IL-1β (0.001–10 ng/ml) for 6 h and analyzed for the respective RNAs. Results in the graphs are shown as fold induction of the respective controls after normalization for the GAPDH signals.

Effect of Dexamethasone (Dex) and Cycloheximide (Chx) on IL-1β-induced VEGF-C mRNA expression—A, IMR-90 cells were incubated with or without IL-1β (10 ng/ml), dexamethasone (10 μM), cycloheximide (10 μM), or a combination of IL-1β and either dexamethasone or cycloheximide for 6 h. B, IMR-90 cells were incubated with or without IL-1β (1 ng/ml), dexamethasone (1 μM), or a combination of both for 6 h. The cells treated with dexamethasome or cycloheximide and IL-1β were first preincubated with dexamethasone (1 μM) for 12 h. Cycloheximide treatment was performed as in A. Values represent arbitrary densitometric units normalized for the GAPDH signals (means ± S.E. from six (Dex) and three (Chx) different experiments). Asterisks indicate a significant (p < 0.05) difference as compared with the controls or IL-1β + IL-1β + Dex.

Effect of Hypoxia on IL-1β-induced VEGF-C mRNA Expression—Hypoxia is an important stimulus for angiogenesis and an inducer of VEGF expression (10–14). IMR-90 cells, hypoxia induced VEGF mRNA but not VEGF-B or VEGF-C mRNAs (Fig. 5). Further, when hypoxia was combined with IL-1β treatment, no additional VEGF stimulation was found in comparison to that of hypoxia alone. This is in contrast with the stimulation obtained with PDGF, which was potentiated by the elevation of VEGF-C mRNA steady-state levels. In contrast, indomethacin, which is a nonsteroidal anti-inflammatory drug and an inhibitor of prostanoid production, did not inhibit the effects of IL-1β (not shown).

To investigate whether ongoing protein synthesis is necessary for IL-1β-induced VEGF-C expression, cycloheximide, an inhibitor of protein synthesis, was administered to the cells. Fig. 3 shows that cycloheximide did not inhibit the effect of IL-1β, indicating a protein synthesis-independent mechanism of action. IL-1ra was used to investigate the specificity of IL-1β effects in IMR-90 cells. This protein binds to the signal transducing IL-1 receptor type I and blocks the effects of IL-1β and IL-1α (44). Fig. 4 shows that IL-1ra inhibited IL-1β-induced VEGF-C and VEGF mRNA expression, confirming the receptor-dependent action of IL-1β.
hypoxia (45) (Fig. 5). Moreover, cyclooxygenase-2 expression was recently reported to be induced by hypoxia in human vascular endothelial cells (46), but this did not seem to be the case in the IMR-90 cells (Fig. 5).

Effect of IL-1β on VEGF-C mRNA Transcription and Stability—To investigate a possible mechanism contributing to IL-1β-induced VEGF-C mRNA expression, we performed nuclear run-on experiments, which indicated that transcription of VEGF-C is induced to similar extent as are the steady state levels of VEGF-C mRNA (Fig. 6A). We also measured the half-life of VEGF-C mRNA in the presence of an inhibitor of transcription, actinomycin D, with or without IL-1β for 1–4 h. As shown in Fig. 6B, the half-life of VEGF-C in the absence of IL-1β was approximately 3.5 h and it was somewhat prolonged in the presence of IL-1β (t1/2 > 4 h). VEGF mRNA was degraded considerably faster with a half-life of approximately 1 h, whereas the IL-1β-stabilized mRNA had a half-life of approximately 2 h, which is consistent with the results obtained in rat aortic smooth muscle cells (47). The level of VEGF-B mRNA was not reduced during the 4 h experiment. These data suggest that the mechanism of the IL-1β-induced VEGF-C mRNA expression operates mostly at the transcriptional level.

IL-1β and TNF-α Stimulate the Production of the VEGF-C Protein—IL-1β and TNF-α stimulated the production of metabolically labeled VEGF-C protein (6- and 9-fold, respectively), as detected by immunoprecipitation of the metabolically labeled proteins secreted into the culture media using VEGF-C-specific polyclonal antibodies (Fig. 7). The major protein precipitated from the culture medium corresponds to the partially processed VEGF-C isoform, which is biologically active (34).
mRNAs, but VEGF-B expression was not affected by these cytokines. In contrast, IL-1β decreased Ang-1 mRNA levels. This is consistent with the hypothesis that the Tek/Tie-2 signaling pathway may be important in the destabilization of blood vessels during angiogenesis and play a role later during the stabilization of the newly formed vessels (5–8). We also found that IL-1β and TNF-α stimulate the production of a partially processed form of the VEGF-C protein, which is an active ligand of the lymphatic endothelial cell receptor VEGFR-3 (34). Since the mature form of VEGF-C protein is released from its precursor as a result of proteolytic cleavages, production or activation of such proteases could be also mediated at sites of cytokine activation in vivo. It remains to be studied whether cytokines regulate these post-translational processes.

VEGF expression has previously been shown to be stimulated by IL-1α in human synovial fibroblasts (51), by IL-1β in rat aortic smooth muscle cells (47), and by IL-6 in tumor cell lines (52). In addition to our fibroblast model, TNF-α was found to stimulate VEGF expression in keratinocytes (53) and in tumor cell lines (52), but not in vascular smooth muscle cells (47). Hypoxia induces VEGF expression by transcriptional activation via hypoxia-inducible factor-1 and by post-transcriptional stabilization of the mRNA (54–59). The mechanism of action of IL-1β was also suggested to depend both on transcriptional activation and on post-transcriptional mRNA stabilization (47). In the case of VEGF, the rapid mRNA decay depends on AU-rich protein binding instability elements found in the 3′-untranslated region (55, 58), which are not present in the VEGF-C mRNA (60). This is consistent with our data, which show that VEGF-C mRNA has a relatively long half-life and that only a small stabilizing effect can be obtained with IL-1β. All this suggests that mRNA stabilization is not as important for VEGF-C regulation as it is for VEGF. Indeed, our nuclear run-on data suggest that IL-1β induces expression of VEGF-C at the transcriptional level. Li et al. (47) hypothesised that IL-1β may facilitate transcriptional activation of the VEGF gene via AP-1 sites found in its 5′-flanking region (61). Data published by Chilov et al. (60) indicate that the 5′-flanking region of the VEGF-C gene contains a putative binding site for NF-κB, which is an important transcription factor for signal transduction of proinflammatory cytokines (62). Interestingly, NF-κB sites have also been identified in the mouse, but not the human, VEGF gene (59, 61).

Glucocorticoids are important anti-inflammatory agents that also inhibit angiogenesis and reduce brain tumor-associated vascular permeability (63, 64). Heiss et al. (65) recently suggested that the inhibition of vascular permeability by dexamethasone is delivered via inhibition of VEGF production. Further, serum- and PDGF-induced VEGF expression was inhibited by dexamethasone in glioma cell lines (65). We found that IL-1β-induced expression of VEGF and VEGF-C was inhibited by dexamethasone in IMR-90 fibroblasts. Whether the effect of dexamethasone is due to direct inhibition of VEGF-C transcription or due to interference with the IL-1β signaling pathway remains to be studied. Proinflammatory cytokines stimulate the production of prostanoids and dexamethasone inhibits it via the inducible cyclooxygenase-2 pathway (41, 66). Since prostanoids are considered to be indirect angiogenic agents acting by inducing synthesis of VEGF (51, 67), we investigated, whether the induction of VEGF-C by cytokines is prostanoid-dependent. Indomethacin, a cyclooxygenase-inhibiting nonsteroidal anti-inflammatory drug, did not inhibit the IL-1β-induced VEGF-C mRNA expression confirming the mechanism to be independent of prostanoids.

Namiki et al. (68) recently reported that HUVECs and human dermal microvascular endothelial cells express VEGF mRNA, but only after induction with PMA or hypoxia. In HUVECs we detected VEGF-B and VEGF-C mRNAs but no VEGF mRNA. Furthermore, VEGF-C mRNA level was increased by treatment with IL-1β and TNF-α. Since neither VEGF nor VEGF-C expression has been detected in endothelial cells in vivo, the significance of these findings remains to be determined. In fact, VEGF-C was detected in mesenchymal cells around the vessels expressing VEGFR-3, suggesting a paracrine mode of action (27). It is, however, tempting to speculate that, in defined conditions, such as during cytokine activation, VEGF-C could modulate endothelial cell responses in an autocrine manner.

VEGFRs are specifically expressed on endothelial cells in vivo (14). Their expression levels are high during embryogenesis, low in most adult tissues, and again elevated in many tumors. However, only a few examples of VEGFRs mRNA regulation exist. TNF-α was recently shown to down-regulate VEGFR-1 and VEGFR-2 mRNAs in HUVECs and in human aortic endothelial cells (48), whereas PMA induced all three VEGFR mRNAs in HUVECs (49). We found that IL-1β elevated the level of VEGFR-2 mRNA in HUVECs. These data support the hypothesis that cytokines could also induce responsiveness of endothelial cells to VEGF and VEGF-C by increasing the expression of VEGFR-2.

We have here shown that proinflammatory cytokines up-regulate the expression of VEGF-C. Our data suggest that, in addition to VEGF, VEGF-C may serve as an endothelial stimulus at sites of cytokine activation. Since VEGF-C primarily targets endothelium of lymphatic vessels, these results raise the possibility that certain proinflammatory cytokines affect the lymphatic vessels indirectly via VEGF-C. This may be important in controlling the composition and pressure of interstitial fluid and in facilitating lymphocyte trafficking and thus have an important role in inflammatory processes.

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Proinflammatory Cytokines Regulate Expression of the Lymphatic Endothelial Mitogen Vascular Endothelial Growth Factor-C
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