Possible Participation of Autocrine and Paracrine Vascular Endothelial Growth Factors in Hypoxia-induced Proliferation of Endothelial Cells and Pericytes*

(Received for publication, October 24, 1994, and in revised form, August 31, 1995)

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Hypoxia is the principal factor that causes angiogenesis. These experiments were conducted to explore how it induces the proliferation of vascular cells, a key step in angiogenesis. Human umbilical vein endothelial cells and bovine retinal pericytes were grown in controlled atmosphere culture chambers containing various concentrations of oxygen. The numbers of both endothelial cells and pericytes increased significantly under hypoxic conditions: the $O_2$ concentrations that achieved maximal growth promotion were 10% for endothelial cells and 2.5% for pericytes. Quantitative reverse transcription-polymerase chain reaction analysis revealed that mRNAs coding for the secretory forms of vascular endothelial growth factor (VEGF), a mitogen specific to endothelial cells, were present in both endothelial cells and pericytes and that their levels increased significantly in the two cell types as the atmospheric $O_2$ concentration decreased. The two genes for VEGF receptors, kinase insert domain-containing receptor (kdr) and fms-like tyrosine kinase 1 (flt1), were found to be constitutively expressed in endothelial cells, and their relative mRNA levels were ranked in that order. On the other hand, only flt1 mRNA was detected in pericytes under hypoxic conditions. Furthermore, most anti-sense oligodeoxyribonucleotides complementary to VEGF mRNA efficiently inhibited DNA synthesis in endothelial cells. These results indicate that autocrine and paracrine VEGFs may take part in the hypoxia-induced proliferation of endothelial cells.

Angiogenesis is a process by which new vascular networks are formed from pre-existing capillaries (1). Physiologically, it is essential for embryogenesis, development, ovulation, corpus luteum formation, and wound repair. In addition, it occurs during the progression of various pathological conditions such as cancers, diabetic retinopathy, rheumatoid arthritis, and occlusive vascular diseases, e.g. neovascularization is needed by solid tumors to access sufficient nutrients and oxygen for growth (1, 2). To determine how angiogenesis is induced under these circumstances is therefore important for clarifying the pathogenesis, prevention, and treatment of such diseases as well as for understanding the basis of the physiological processes involved.

A decrease in tissue oxygen concentrations has been considered as the leading cause of angiogenesis (3). However, the mechanisms underlying the induction of angiogenesis by hypoxia are still poorly understood. Using primary cultured vascular cells, we have been investigating the biochemical basis underlying various vascular functions and disturbances (4–7). In this study, we employed a hypoxic culture system and examined how low oxygen tensions affect the proliferation of endothelial cells and pericytes, a key step in angiogenesis; the latter cell type is the microvascular constituent encircling the endothelium, which we showed plays important roles in the growth, function, and damage of endothelial cells (4, 5). The first part of this paper describes the accelerated growth of both endothelial cells and pericytes caused by hypoxia.

The following parts of this paper deal with the molecular mechanism underlying the hypoxia-induced proliferation of the vascular cells. Recently, there has been an explosive growth in knowledge regarding angiogenic growth factors (8–10), including endothelial cell-specific mitogen. This factor was initially identified in the conditioned medium of bovine pituitary follicular stalk cells (11, 12), and its expression was subsequently shown to increase in human gliomas under hypoxic conditions (13, 14). The presence of VEGF in vascular cells, including endothelial cells (15), smooth muscle cells (16), and mesangial cells (17), has also been noted. Here we show that endothelial cells and pericytes per se can produce secretory forms of VEGF in response to hypoxia. We also determined the VEGF receptor subtypes expressed in these two cell types. Furthermore, a functional relationship between the vascular VEGF system and hypoxia-driven endothelial cell growth was tested by manipulating VEGF gene expression with anti-sense DNA.

**EXPERIMENTAL PROCEDURES**

Materials—RPMI 1640 and Medium 199 containing Hanks’ salts were purchased from Life Technologies, Inc. Dulbecco’s modified Eagle’s medium was from Nissui Pharmaceutical Co. Ltd. (Tokyo). Fetal bovine serum (FBS) was from ICN Biomedicals Inc. (Costa Mesa, CA). Endothelial cell growth supplement was from Collaborative Research (Bedford, MA). Eheparin was from Sigma. $O_2/CO_2/N_2$ gas mixtures were from Nippon Sanso Corp. (Tokyo). Reagents for DNA synthesis and oligodeoxynucleotide purification cartridge columns were from

*This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan (to H. Y.), the J. Y. the J. Diabetes Foundation (to H. Y.), the Mochida Memorial Foundation for Medical and Pharmaceutical Research (to H. Y.), and the Hokkoku Foundation for Cancer Research (to H. Y., S. Y., and M. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article therefore must here be marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; nt, nucleotide(s); bp, base pair(s); bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; PDGF-B, platelet-derived growth factor B.
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Applied Biosystems, Inc. (Foster City, CA). [3H]Thymidine, [35S]methionine were from DuPont NEN. Reverse transcriptase and T4 polynucleotide kinase were from Takara (Koyo, J apan). AmpliTaq DNA polymerase was from Perkin-Elmer. Hybrid-n+ nyl non membranes were from Amersham (Buckinghamshire, United Kingdom). Monoclonal antibody A4.6.1 was raised against human VEGF (18) was kind gift by Dr. K. Yamauchi, Tokyo, Japan.

Cells—Vascular endothelial cells were primary-cultured from human umbilical cord veins as described previously (4) and maintained in a gelatin-coated Falcon 3110 tissue culture flask in RPMI 1640 Medium 199 (1:1) supplemented with 15% FBS, 100 μg/ml endothelial cell growth supplement, and 25 μg/ml heparin at 37 °C under 5% CO2, 95% air. Cells at 25–30 passages were used for experiments; at 30–35 passages, cells were identified as endothelial cells by uptake of acetylated low density lipoprotein (4). Pericytes were isolated from bovine retina as described (4) and maintained in a Falcon 3110 culture flask in Dulbecco’s modified Eagle’s medium supplemented with 20% FBS under 5% CO2, 95% air. Cells at 5–10 passages were used for experiments; 95% of the cells were identified as pericytes by their characteristic (Bacharach, Inc., Pittsburgh, PA) prior to and after gas and medium were changed.

Primers and Probes—Oligodeoxyribonucleotide primers and probes for reverse transcription-polymerase chain reactions (RT-PCRs) were synthesized with an Applied Biosystems Model 392 DNA synthesizer and purified with an oligonucleotide purification cartridge. Primer sequences were 5′-GAGAAATGTGGCCTCAGAACATGAAACTTTCCTGGTGCACATC-3′ (nucleotides (nt) 44–70 plus EcoRI adapter) and 5′-GAGGACCAATCTGGAGAGTACAGTATGATGTC-3′ (nt 3232–3258 plus EcoRI adapter) and 5′-GAGCATCCGTTAACACTATAATACATGTCGTTTCATGCTATC-3′ (complement of nt 4289–4313 plus Splh adapter) (20) for fms-like tyrosine kinase (fkt) mRNA, 5′-TATAGATGGTGTAACCCAGGG-3′ (nt 873–892) and 5′-TTGTCCTACGAGACGCTCGG-3′ (nt 3232–3257) (21) for E-isoform of smooth muscle actin. Sequences of antisense and sense oligodeoxyribonucleotides were synthesized with the Model 392 DNA synthesizer by the phosphorothioate approach using tetraethylthiuram disulfide (22). 

RESULTS

Hypoxia Stimulates the Growth of Both Endothelial Cells and Pericytes—Human umbilical vein endothelial cells and bovine retinal pericytes were cultured in controlled atmosphere culture chambers containing 2.5, 5, and 10% O2. Fig. 1A shows the growth curves of endothelial cells. Under the standard oxygen tension (20%), the viable cell number began to gradually increase on day 2 and doubled on day 7. When exposed to 10% O2, endothelial cells started to grow within 24 h without noticeable lag and increased in an almost linear fashion, reaching on day 7 a level –3.5-fold higher than at the beginning of the hypoxic culture at every time point examined. The viable cell number under 10% O2 was significantly larger than that under 20% O2. Five percent O2 also supershifted the curve, but to a lesser extent than did 10% O2. When the 02 tension was further reduced to 2.5%, the viable cell number instead decreased, and almost all the endothelial cells were dead within 3 days. The growth of pericytes was also stimulated under hypoxic conditions (Fig. 1B).

Both Endothelial Cells and Pericytes Express mRNA for Secretory Forms of VEGF—In response to hypoxia, mRNA for VEGF was isolated from cells that had been exposed to low oxygen tensions and analyzed by an RT-PCR technique to determine the expression of the VEGF gene. It has been known that four alternatively spliced products can be generated from
the single VEGF gene, yielding different protein products composed of 121, 165, 189, and 206 amino acids, designated as VEGF_{121}, VEGF_{165}, VEGF_{189}, and VEGF_{206}, respectively. Among them, only VEGF_{121} and VEGF_{165} are secreted and induce mitogenesis of endothelial cells; VEGF_{189} and VEGF_{206} are membrane-anchored and act as vascular permeability factors (31, 33). Since Northern blot analysis could not clearly discriminate the four mRNA species, we employed a more sensitive RT-PCR technique to determine which VEGF forms are expressed in the vascular cells. For this, we designed primer and an internal probe against the regions common to the four alternatively spliced products (Fig. 2A); with them, 486-, 618-, 690- and 741-bp-long cDNA fragments would be amplified from respective VEGF_{121}, VEGF_{165}, VEGF_{189}, and VEGF_{206} mRNA templates (19).

Fig. 2 (B and C) shows titration curves of RT-PCR products for determining the quantitative range in which the reactions proceeded exponentially. Poly(A)^+ RNA from U251 cells, a human glioma cell line, was used as a standard template; this cell line has been known to produce high amounts of VEGF (13, 14). Signal intensities of the products obtained with U251 poly(A)^+ RNA were plotted as functions of template amount and cycle number. The products increased linearly up to 50 ng (Fig. 2B) and up to 30 cycles (Fig. 2C); hence, we chose 30-ng templates and 25 cycles as the conditions for VEGF mRNA analysis.

As shown in Fig. 2D, poly(A)^+ RNAs from endothelial cells and pericytes gave signals at 486 and 618 bp, which corresponded to mRNAs for VEGF_{121} and VEGF_{165}, respectively, as did U251 poly(A)^+ RNA. The levels of the two mRNA species increased significantly in these cells as the atmospheric O_2 concentration decreased from 20% to 0%. The sum of the 486- and 618-bp band intensities was strongest in anoxic cultures; in endothelial cells and pericytes, it was 8- and 9-fold higher than respective normoxic cultures when standardized with the signal intensities of ß-actin mRNA as an internal control. On the other hand, signals for VEGF_{189} and VEGF_{206} mRNAs were not detected in either the vascular cells or the glioma cells throughout these experiments.

Determination of VEGF Receptor Subtypes Expressed in Endothelial Cells and Pericytes—To exert its action, locally produced VEGF requires locally expressed receptors. We then examined VEGF receptor gene expression in endothelial cells and pericytes. So far, two kinds of VEGF receptors, flt1 (20, 34) and kdr (22, 23), and their homologue, flt4 (21, 35), have been identified. Although flt4-encoded protein has recently been shown not to act as a receptor for VEGF (36), the three have a quite similar structure, each containing seven extracellular immunoglobulin-like domains, one transmembrane domain, and two cytoplasmic tyrosine kinase domains; their mRNAs exhibit high homologies in both nucleotide and deduced amino acid sequences (35). Accordingly, the RT-PCR method rather than Northern blotting seemed suitable for differential mRNA detection as in the case with VEGF mRNAs. Primers and probes were designed so that they would correspond to the regions where sequence homologies among the three are relatively low and would generate products of differing lengths (Fig. 3A). Data in Fig. 3B show that each set of primers and a probe worked well in specifying their corresponding mRNA; 1098-bp DNA fragments amplified with flt1 primers hybridized to the flt1 probe, but not the kdr probe, and 555-bp RT-PCR products with kdr primers hybridized only to the kdr probe. Based on the titration of the products (Fig. 3, C and D), RT-PCR was performed with 30 ng of template poly(A)^+ RNA for 25 cycles for the quantitative detection of each receptor mRNA.

As shown in Fig. 3E, endothelial cells were found to contain mRNAs for flt1 and kdr. Between the two, the intensities of the hybridization signals were much stronger for kdr mRNA than for flt1 mRNA. In contrast with VEGF mRNAs, the levels of kdr and flt1 mRNAs were essentially unchanged when atmospheric O_2 tensions were lowered.

On the other hand, in pericytes, neither of the two mRNA species was detected under normoxic conditions. However, as shown in Fig. 3F, the 1098-bp band corresponding to flt1 mRNA was visible in pericytes grown at 2.5% O_2 and became...
clearly marked at 0% kdr mRNA remained undetected at 2.5 and 0% O₂ even when the template amount and the cycle number were raised to 100 ng and 35 cycles, respectively (data not shown).

Antisense Oligodeoxyribonucleotides against VEGF mRNA Inhibit DNA Synthesis in Hypoxic Endothelial Cell Culture—We next tested whether vascular VEGF could be functionally related to the hypoxia-induced proliferation of endothelial cells. For this, a 22-mer antisense oligodeoxyribonucleotide complement of the 5′-region of human VEGF mRNAs and the corresponding sense oligodeoxyribonucleotide were synthesized and administered to the culture medium in which endothelial cells were grown. After attachment to the dish, cells were incubated with various concentrations of oligodeoxyribonucleotides under 10% O₂ for 24 h, and then [³H]thymidine incorporation was measured. As shown in Fig. 4A, the antisense oligodeoxyribonucleotide was found to inhibit [³H]thymidine incorporation into the endothelial cells in a dose-dependent manner; at 1, 2, 5, and 10 μM, 6.9, 55.1, 71.6, and 86.5% inhibitions were achieved, respectively. Control sense oligodeoxyribonucleotides showed no significant change.

Fig. 4B shows the time course of endothelial cell synthesis of DNA in response to the antisense oligodeoxyribonucleotide under 10% O₂. DNA synthesis began to decrease as early as 4 h after the oligodeoxyribonucleotide administration. The inhibition of DNA synthesis with similar kinetics was also noted under 20% O₂ (Fig. 4C).

Evidence That the Antisense Oligodeoxyribonucleotides Block VEGF Expression—To confirm whether the antisense oligodeoxyribonucleotides did block the expression of VEGF mRNA, we examined by immunoprecipitation denovo VEGF synthesis in endothelial cells that had been treated with or without the antisense or sense oligodeoxyribonucleotides. As shown in Fig. 5, the anti-VEGF monoclonal antibody specifically recognized 35S-labeled proteins that migrated to the positions of 22 and 18 kDa, and the amounts of the 22- and 18-kDa proteins were found to be consistently lowered by the antisense oligodeoxyribonucleotides in a dose-dependent manner. At 2 and 10 μM, 52 and 78% inhibitions were achieved, respectively. On the other hand, the sense oligodeoxyribonucleotides did not inhibit VEGF synthesis in endothelial cells.

Antisense Oligodeoxyribonucleotides against Different Re-
Fig. 3. Quantitative RT-PCR analysis of VEGF receptor mRNAs. A, schematic representation of human VEGF receptor mRNAs. Boxes indicate open reading frames. Arrows and bars indicate primers and probes, respectively, for flt1, kdr, and flt4 mRNA detections. Expected sizes of RT-PCR products are indicated on the right. B, specific detection of flt1, kdr, and flt4 mRNAs. RT-PCR products yielded with each set of primers were specifically recognized by the corresponding probe. Numbers on the left indicate lengths of the products in base pairs. C and D, titration curves of RT-PCR products. Poly(A)$^+\,$RNA from endothelial cells was used as the template. Signal intensities of RT-PCR products are expressed as the arbitrary logarithm values of radioactivities and plotted against template amounts (C) and against cycle numbers (D). Radioactivities of hybridization bands were measured with a Fujix BA100 BioImage analyzer. ●, flt1; □, kdr; ○, β-actin.

E, RT-PCR analysis of VEGF receptor mRNAs in endothelial cells. Thirty nanograms of poly(A)$^+\,$RNA from endothelial cells incubated under the indicated O$_2$ concentrations was amplified by RT-PCR and hybridized with $^{32}$P-end-labeled probes specific to flt1 and kdr (upper panels) and β-actin (lower panels) mRNAs. PCR amplification for the latter was performed for 15 cycles. The flt1 blot was exposed for 32 h, and the kdr blot for 1 h. RT($-$), the reaction without reverse transcriptase. F, RT-PCR analysis of VEGF receptor mRNAs in pericytes. Thirty nanograms of poly(A)$^+\,$RNA from pericytes incubated under the indicated O$_2$ concentrations was amplified by RT-PCR and hybridized with $^{32}$P-end-labeled probes specific to flt1 (upper panel) and β-actin (lower panel) mRNAs. PCR amplification for the latter was performed for 15 cycles. The film was exposed for 7 days.
regions of VEGF Also Inhibit Endothelial Cell Synthesis of DNA—As additional controls, we prepared 10 independent antisense oligodeoxyribonucleotide species against different regions of VEGF mRNA, including the 5’- and 3’-untranslated regions and the VEGF open reading frame. As shown in Fig. 6, six antisense species out of the 10 could significantly inhibit endothelial cell synthesis of DNA under 10% O2.

FIG. 4. Effect of antisense oligodeoxyribonucleotides on DNA synthesis in endothelial cells. A, dose dependence. After endothelial cells had been cultured for 24 h in the presence of the indicated concentrations of antisense (hatched columns) or sense (black columns) oligodeoxyribonucleotides, [3H]thymidine was added to a final concentration of 1 μCi/ml, and the cells were further cultured for 4 h. The culture was carried out under 10% O2 in RPMI 1640 Medium 199 (1:1) supplemented with 15% FBS, but without endothelial cell growth supplement or heparin. 3H radioactivity incorporated is expressed as disintegrations/minute. Columns represent the means of triplicate experiments. Bars indicate standard deviations. **, p < 0.01 compared with the value for the culture without oligodeoxyribonucleotides. The control without oligodeoxyribonucleotides is also shown (open column). B and C, time courses under 10 and 20% O2, respectively. Four hours after the addition of [3H]thymidine, 10 μM oligodeoxyribonucleotide was administered, and 3H radioactivity incorporation into endothelial cell DNA was assayed at the indicated time points. Each point represents the mean of triplicate experiments. Vertical bars show standard deviation when larger than the symbol. **, p < 0.01 compared with the value without oligodeoxyribonucleotides (Student’s t test). ● and ○, cumulative DNA synthesis without oligodeoxyribonucleotides; ■ and □, with antisense oligodeoxyribonucleotides; and ■ and □, with sense oligodeoxyribonucleotides.

FIG. 5. Effect of antisense oligodeoxyribonucleotides on VEGF synthesis in endothelial cells. Endothelial cells were incubated with [35S]methionine in the presence or absence of the indicated concentrations of antisense or sense oligodeoxyribonucleotides, lysed, and immunoprecipitated as described under “Experimental Procedures.” A, fluorogram of total labeled proteins. Proteins were electrophoresed on a 12% SDS-polyacrylamide gel under reducing conditions. Ten-microliter aliquots of cell lysate were loaded per lane. Bars on the left indicate molecular mass markers in kilodaltons. B, immunoprecipitates. Immuno-reacted materials that had been prepared from 8.0 × 106 dpm of lysate each except for pericytes (4.0 × 106 dpm) were electrophoresed under the same conditions as described for A. Specific immunoprecipitates were marked at 22 and 18 kDa. Note that pericytes synthesized VEGF, as did U251 cells.

DISCUSSION

The process of angiogenesis is thought to consist of the following four steps: 1) proteolytic degradation of the basement membrane, 2) migration of endothelial cells, 3) proliferation of endothelial cells, and 4) tube formation. The final step is completed when new capillaries are covered with pericytes (1, 2). In this study, we have focused on how the proliferation of endothelial cells and pericytes is affected by hypoxia, the leading cause of angiogenesis.

This study has confirmed that low atmospheric O2 tensions can result in the stimulation of the proliferation of both human umbilical vein endothelial cells and bovine retinal pericytes. The O2 concentrations that induced maximal growth promotion were 10% for endothelial cells and 2.5% for pericytes. This is comparable with earlier reports that human umbilical vein endothelial cells grew at the greatest rate under 7.5% O2 (37) and bovine brain microvascular pericytes under 3% O2 (38).

This study has also demonstrated for the first time that mRNAs coding for VEGF, a potent endothelial cell mitogen, are present not only in endothelial cells but also in pericytes, and that their level is significantly elevated in both cell types as atmospheric O2 concentrations decrease. Moreover, it is VEGF121 and VEGF165, the secretory forms of VEGF, that are coded for by the mRNAs expressed in the vascular cells. VEGF is a growth factor known to be present in a variety of tissues, including ovary (39, 40) and malignant tumors (41–44), where angiogenesis takes place. Its expression has been shown to be localized around necrotic foci in brain tumors (45) and to be enhanced by O2 depletion in glioblastoma cell cultures (13, 14). Iizuka et al. (46), using an RT-PCR analysis similar to that conducted in this study, recently showed that VEGF mRNA levels are elevated during hypoxia in human osteosarcoma
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cells. From these observations, VEGF has been regarded as the principal angiogenic factor under ischemic and hypoxic conditions. Our finding that vascular cells per se can express the VEGF gene in response to hypoxia would seem, therefore, to have significant implications in angiogenesis. Vascular VEGF may participate in the process of angiogenesis in autocrine and paracrine manners.

In general, the actions of growth factors are mediated by receptors on their target cells (47, 48). In this study, we have also identified VEGF receptor subtypes expressed in endothelial cells and pericytes. Endothelial cells were found to constitutively express the two genes for flt1 and kdr, regardless of the atmospheric O2 tensions. The rank order of mRNA abundance was kdr >> flt1, suggesting that Kdr is the major VEGF receptor species expressed in human umbilical vein endothelial cells. On the other hand, only flt1 mRNA was detected in pericytes under hypoxic conditions. The difference in the mode of Kdr gene expression between the two cell types is of interest in understanding the regulation of VEGF receptor gene expression. Transcripts of the kdr gene were not detected in pericytes; given the relatively low level of the transcript as determined by Northern blot analysis with human kdr cDNA (data not shown) and the lack of bovine sequence, it was unfortunate that RT-PCR could not be used to evaluate the level of the kdr transcript.

The results obtained indicate that vascular endothelial cells per se possess a system for triggering their own growth. In endothelial cells, VEGF expression was inducible by low O2 concentrations, whereas receptor expressions were constitutive. This may be an indication that ligand expression is a rate-limiting step in the putative autocrine actions of VEGF, as in the case of the keratinocyte growth factor system in dermal wound healing (49). We therefore manipulated VEGF expression with antisense oligodeoxyribonucleotides to test the functional role of the VEGF system. Antisense oligodeoxyribonucleotides complementary to the 5'-region of VEGF mRNA, encompassing the initiator codon, were found to cause a dose- and time-dependent inhibition of DNA synthesis in endothelial cells cultured under hypoxic conditions (Fig. 4), probably through a blockade of the translation of VEGF mRNA (Fig. 5). We also conducted control experiments with additional antisense oligodeoxyribonucleotide species against different regions of VEGF mRNA, including the 5'- and 3'-untranslated regions and the VEGF open reading frame, the majority of which (6 of 10) could also efficiently inhibit endothelial cell synthesis of DNA (Fig. 6); arrests of the ribosome transition (50) and an RNase H-like activity-driven degradation of target mRNA (51) would account for the antisense DNA action. The results could be regarded as evidence that vascular VEGF is causally related to the hypoxia-induced proliferation of endothelial cells.

In light of these findings, we propose a model for the mechanism of the hypoxia-induced proliferation of vascular cells (Fig. 7). When the local oxygen concentration is lowered, VEGF gene expression would be induced in endothelial cells and in pericytes to produce secretory forms of VEGF. VEGF in turn may act on Kdr and Flt1 receptors on endothelial cells in autocrine and paracrine manners, thereby causing the proliferation of endothelial cells, which may lead to angiogenesis. Basal amounts of vascular VEGF synthesized in normoxic states may promote the maintenance of microvascular homeostasis, as suggested by our observation that the antisense VEGF oligodeoxyribonucleotide could modify endothelial cell DNA synthesis under 20% O2 (Fig. 4C). According to this model, we can also suggest a possible approach for the prevention of angiogenesis. Interruptions of the series of biochemical events at certain steps might halt the process of angiogenesis, e.g. antisense DNA/RNA against VEGF mRNA may have therapeutic potential in the treatment of proliferative angiopathies or tumors. In support of the above-mentioned model, Aiello et

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2 Human umbilical vein endothelial cells responded to a recombinant VEGF added to the medium. Based on a standard curve obtained by plotting radioactivities of incorporated [3H]thymidine as a function of incorporated [3H]thymidine as a function of the VEGF concentration, 10% O2 was deduced to be equivalent to 0.15 ng/ml VEGF in the induction of DNA synthesis (M. Nomura and H. Yamamoto, unpublished data). The mitogenic activities of VEGF and endothelial cell growth supplement were not affected by a heparin wash (52) under the present conditions.
al. (53) recently reported that ocular VEGF levels were abnormally high in a large population of patients with actively proliferative diabetic retinopathy, but dropped following successful treatment.

The model proposed was based on in vitro observations withcultivated endothelial cells and pericytes. Although it can partially explain how hypoxia causes endothelial proliferation, the involvement of other growth factors must also be taken into account when considering the processes that would occur in vivo. In addition to VEGF, acidic fibroblast growth factor, basic fibroblast growth factor (bFGF), epidermal growth factor, transforming growth factor-α, transforming growth factor-β (TGF-β), and platelet-derived growth factor B (PDGF-B) have been implicated in angiogenesis (54–57). Among them, bFGF, TGF-β, and PDGF-B seem particularly important because they can be produced by endothelial cells themselves, thus possessing potential autocrine/paracrine activities. bFGF is a mitogen for a wide variety of cell types, playing diverse roles in vascular and nervous systems as well as in connective tissues (58).

Concerning vascular functions, bFGF has been reported to stimulate not only endothelial cell mitosis and chemotaxis, but also tube formation. Furthermore, it can induce collagenases and plasminogen activator, which would promote degradation of the basement membrane of the parental vessels (59). These observations indicate that bFGF may be related to all the steps of angiogenesis. However, the expression of this growth factor has been shown not to be influenced by hypoxia (60). TGF-β inhibits the proliferation of endothelial cells in culture (61), but is known to induce new capillary formation in vivo (56). This apparently paradoxical effect of TGF-β may be explained by the fact that this factor is chemotactic for macrophages and causes them to release angiogenic factors (56). However, TGF-β expression has also been shown not to be affected by hypoxia (60). PDGF-B is a major serum mitogen for mesenchymally derived cells. Since PDGF-B is not only released by platelets, but also secreted by cells involved in inflammatory responses, it has been suggested to play a role in wound healing (10). Although PDGF-B was previously thought to be devoid of mitogenic activity on endothelial cells, Funa et al. (62) have recently demonstrated that functional PDGF-B receptors are expressed on hyperplastic capillary endothelial cells in malignant glioma, suggesting that autocrine PDGF-B has a role in the proliferation of endothelial cells. In contrast to bFGF and TGF-β, hypoxia-induced up-regulation of the PDGF-B gene has also been reported (60). Available evidence thus suggests that the major autocrine/paracrine growth factors involved in the control of endothelial cell growth under normoxic conditions would be bFGF, VEGF, and PDGF-B. Under hypoxic conditions, induced VEGF and PDGF-B would mainly account for the endothelial proliferation.

We have shown previously that pericytes can restrict the replication of co-cultured endothelial cells and suggested TGF-β and heparan sulfate as the candidate regulatory molecules (4). We also have shown that endothelium-dependent stimulation of pericyte growth is mediated mainly by endothelin-1 (5), a hypoxia-inducible mitogen (63). In the present study, pericytes were demonstrated to express the VEGF gene in response to hypoxia, as do endothelial cells. Therefore, pericytes may be regarded as cells that can exert both negative and positive growth effects on their microvascular counterpart. Although the role of pericytes during angiogenesis is poorly understood, it is likely that hypoxia would turn them predominantly mitogenic, thereby promoting the growth of endothelial cells together with that of pericytes through synergistic actions of VEGF, PDGF-B, and endothelin-1. Whether VEGF in fact stimulates pericyte replication, however, remains to be established because of the following observations. Midy and Plouët (64) reported that VEGF stimulated the migration, but not mitosis, of bovine osteoblasts, a cell type of the same mesenchymal origin as pericytes. Vascular smooth muscle cells, the equivalent of pericytes in larger vessels, have also been reported not to respond to VEGF (16). Seetharam et al. (65) showed that transfection of fibroblasts failed to confer responsiveness to VEGF on NIH3T3 cells.

Acknowledgments—We thank Shinichi Matsudaira and Reiko Kitamura for assistance, Shinichi Tsubamoto for monitoring O₂, and Brent Bell for reading the manuscript.

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J. Biol. Chem. 1995, 270:28316-28324.
doi: 10.1074/jbc.270.47.28316

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