We have shown previously that vascular endothelial growth factor (VEGF) synthesized by the cellular constituents of small vessels per se, viz. endothelial cells and pericytes, participates in the hypoxia-driven proliferation of both cell types (Nomura, M., Yamagishi, S., Harada, S., Hayashi, Y., Yamashita, T., Yamashita, J., Yamamoto, H. (1995) J. Biol. Chem. 270, 28316–28324; Yamagishi, S., Yonekura, H., Yamamoto, Y., Fujimori, H., Sakurai, S., Tanaka, N., and Yamamoto, H. (1999) Lab. Invest. 79, 501–509). In this study, we examined the expression of the recently isolated VEGF gene family members (placenta growth factor (PIGF), VEGF-B, and VEGF-C) in human dermal microvascular endothelial cells and bovine retinal pericytes cultured under various oxygen tensions. Quantitative reverse transcription-polymerase chain reaction analyses demonstrated that the two cell types possess not only VEGF (VEGF-A) mRNA, but also VEGF-B, VEGF-C, and PIGF mRNAs. Among them, only VEGF-A mRNA was induced under hypoxia. Competitive reverse transcription-polymerase chain reaction showed that, under normoxic conditions, the rank order of mRNA content in endothelial cells was PIGF > VEGF-B > VEGF-C > VEGF-A and that mRNA coding for PIGF was expressed at >100-fold higher levels than VEGF-A mRNA. In pericytes, the rank order was VEGF-C > VEGF-A > VEGF-B > PIGF, and ~7-fold higher levels of VEGF-C mRNA compared with VEGF-A mRNA were noted in this cell type. Furthermore, antisense inhibition of PIGF protein production lowered the endothelial cell synthesis of DNA under hypoxic conditions. The results suggest that these VEGF family members may also take active parts in angiogenesis.

Angiogenesis, the process by which new vascular networks are formed from preexisting capillaries, is physiologically essential for embryogenesis, development, corpus luteum formation, ovulation, and wound repair (1). It is also related to the progression of various pathological conditions such as cancer growth and metastasis, diabetic retinopathy, rheumatoid arthritis, and collateral path formation in occlusive vascular diseases (2). Among the known angiogenic factors, vascular endothelial growth factor (VEGF) has emerged as a central regulator of the angiogenic process under both physiological and pathological conditions (1, 4–6). We have previously shown that the VEGF gene is expressed in vascular endothelial cells and pericytes, the constituents of microvessels wherein angiogenesis takes place, and that the hypoxia-induced proliferation of both cell types is mediated by autocrine VEGF (7, 8). We have also reported that autocrine VEGF-A takes an active part in advanced glycation end product-driven angiogenesis (9) and that VEGF-A can also act on pericytes to stimulate their proliferation and migration (10). Recently, several VEGF-related genes, including placenta growth factor (PIGF) (11), VEGF-B (12), and VEGF-C (13), have been isolated. The VEGF family members have been shown to share common receptors for their actions and have been implicated in the process of angiogenesis (4–6). Coexpression of plural members of the VEGF family has been reported in a variety of normal and tumoral tissues (14–19). However, the expression and significance of these factors in vascular cells per se have not yet been determined. Elucidation of the expression of VEGF family members and their regulation in microvascular cells may provide important insights into the molecular mechanisms underlying blood vessel formation.

In the study, we examined the expression of genes coding for PIGF, VEGF-B, and VEGF-C in human dermal microvascular endothelial cells and bovine retinal pericytes. Because the nucleotide sequences of bovine VEGF-B, VEGF-C, and PIGF mRNAs were not known, we first isolated the bovine cDNA equivalents and determined their primary structures. We then analyzed the expression of these factors in endothelial cells and pericytes cultured under various oxygen tensions and quantified their mRNA content in both cell types by competitive RT-PCR. Furthermore, since PIGF was found to be the most abundantly expressed VEGF family member in endothelial cells, we pursued its role in the hypoxia-driven proliferation of microvascular cells using an antisense approach.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Thymidine (90 Ci/mmol), [γ-32P]ATP (6000 Ci/mmol), [α-32P]CTP (6000 Ci/mmol), and Expre35S–S-protein labeling

1 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PIGF, placenta growth factor; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s).
Cultures under low oxygen tensions were performed as described [1]. Pericytes were isolated from bovine retina and maintained as described [2]. A cluster dish or flask were placed in a controlled atmosphere culture chamber (Bellco, Vineland, NJ), a humidified airtight incubation apparatus was renewed every 24 h, and the O2 and CO2 in the culture chambers was maintained a constant gas composition and kept at 37 °C. The gas phase of CO2/N2 gas mixtures were from Nippon Sanso Corp. (Tokyo, Japan). Hybond-N nylon membrane and protein G-Sepharose 4FF were from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

Cloning of Bovine cDNAs for PIGF, VEGF-B, and VEGF-C—The bovine heart cDNA library was screened as described previously [20] using bovine VEGF-B, VEGF-C, or PIGF cDNA fragments that had been amplified by RT-PCR with bovine heart poly(A)+ RNA as a template. Primers used for the amplification corresponded to nucleotides 443–462 and 631–652 of human PIGF cDNA (11), nucleotides 89–117 and 409–426 of human VEGF-B mRNA (12), and nucleotides 968–987 and 1588–1609 of human VEGF-C cDNA (13). Nucleotide sequences of cloned cDNAs were determined with an Applied Biosystems Model 373 DNA sequencer (Perkin-Elmer).

Cells—Human microvascular endothelial cells isolated from neonatal dermis (Cascade Biologics, Inc., Portland, OR) were maintained in HuMac medium supplemented with 5% fetal bovine serum, 5 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, 10 μg/ml epidermal growth factor, 1 μg/ml hydrocortisone, and 39.5 μg/ml dibutyryl cyclic AMP according to the supplier's instructions (Kurabo Corp., Osaka, Japan). Cells at 5–10 passages were used for the experiments. Pericytes were isolated from bovine retina and maintained as described [21]. Cultures under low oxygen tensions were performed as described [7]. Briefly, cells in the medium supplemented with 5% fetal bovine serum, 5 ng/ml basic fibroblast growth factor, and 10 μg/ml heparin in a cluster dish or flask were placed in a controlled atmosphere culture chamber (Bellco, Vineland, NJ), a humidified airtight incubation apparatus with inflow and outflow valves, into which a gas mixture containing 5% CO2 and 0, 2.5, 5, 10, or 20% O2 balanced with N2 was flushed for 5 min at a flow rate of 10 liter/min. The chamber was sealed to maintain a constant gas composition and kept at 37 °C. The gas phase was renewed every 24 h, and the O2 and CO2 in the culture chambers were immediately equilibrated to the set values by the gas flushing and kept constant for at least 24 h to an accuracy of ±5% (7).

Quantitative RT-PCR—Poly(A)+ RNAs were isolated from cells that had been cultivated under various oxygen tensions, using the QuickPrep mRNA purification kit (Amersham Pharmacia Biotech), and then analyzed by RT-PCR using a GeneAmp RNA PCR kit (Perkin-Elmer) as described previously [7–9]. It is known that alternatively spliced products are generated from VEGF-A, VEGF-B, and PIGF genes (4, 5). The PCR primers were thus designed to discriminate between all the mRNA variants. The oligodeoxyribonucleotide primers and probes employed were as follows. The 5′- and 3′-primers and probe for human PIGF mRNA corresponded to nucleotides 261–283 and 378–398. The resultant PCR products were ligated into a pCR2.1 vector to transform INV + F (Stratagene, La Jolla, CA). Hybridization of the hybridized bands were measured with a BAS1000 BioImage analyzer (Fuji PhotoFilm Co., Ltd., Hamamatsu, Japan). The RT-PCR products were sequence-verified.

Competitive RT-PCR—For competitive RT-PCR (22), each reaction should contain a known amount of a competitor RNA that undergoes primer extension with the same efficiency. The competitor RNA should yield products distinguishable from those derived from endogenous templates on gel electrophoresis. To construct vectors from which such competitor RNAs are generated, composite primers were synthesized and used to amplify cloned bovine cDNA or human endothelial cell poly(A)+ RNA so that the resultant cDNAs would have ~50–200 bp 5′-ends. A composite primer for human and bovine VEGF-A mRNAs was made complementary to nucleotides 44–70 and 234–269 of the human cDNA, but devoid of the internal region 71–243; the primer for human PIGF mRNA corresponded to nucleotides 628–647 and 741–760; that for human VEGF-B mRNA corresponded to nucleotides 196–215 and 301–322; for human VEGF-C mRNA, to nucleotides 340–360 and 513–534; for bovine PIGF mRNA, to nucleotides 429–445 and 561–580; for bovine VEGF-B mRNA, to nucleotides 450–471 and 587–607; and for bovine VEGF-C mRNA to nucleotides 261–283 and 378–398. The resultant PCR products were ligated into a pcCR2.1 vector to transform INV + F (Stratagene, Carlsbad, CA). Competitor RNAs were then synthesized from purified plasmids by in vitro transcription using a riboprobe combination system (Promega, Madison, WI). Concentration was determined by absorbance at 260 nm after denaturation at 65 °C. Two-fold serial dilutions of competitor RNA (ranging between 10 and 10−5 mol) plus 10–60 ng of poly(A)+ RNA underwent a series of RT-PCRs using the same primer set described under "Quantitative RT-PCR" and a GeneAmp RNA PCR kit or a GeneAmp E Z, the RNA PCR kit (Perkin-Elmer). After amplification, aliquots of the reaction mixtures were electrophoresed on a 2.5% agarose gel and stained with SYBR Green (FMC Corp. BioProducts, Rockland, ME). Signal intensities of the bands were measured with an Epilight EP-250 (Aishin Cosmetics Co., Ltd., Toyota, Japan).

Metabolic Labeling and Immunoprecipitation—Subconfluent cultures of endothelial cells were incubated in the presence or absence of PIGF antiserum or sense oligodeoxyribonucleotides for 24 h at 37 °C under 5% O2 and further incubated in methionine/cysteine-free Eagle's minimal essential medium (Sigma) supplemented with 0.5% fetal bovine serum and 200 μCi [35S]methionine/cysteine mixture with or without the antiserum or sense oligodeoxyribonucleotides for 24 or 48 h at 37 °C under 5% O2. After the incubation, the conditioned media were removed, and phenylmethylsulfonyl fluoride was added to a final concentration of 0.5 mm and the samples were then subjected to 4 °C for 10 h. The radioactivities of the supernatants were measured by the trichloroacetic acid precipitation method (23). Aliquots of the saved conditioned media containing the same radioactivities (~1400 μl on average) were precleared with a 30-μl packed volume of protein G-Sepharose precoupled with normal goat IgG (Santa Cruz Biotechnology Inc.) at 4 °C for 2 h with gentle rotation. The precleared conditioned media were then immunoprecipitated with a protein G-Sepharose protein G-400 column (5–15 μl of packed volume) and goat anti-PIGF antibodies or normal goat IgG at 4 °C for 2 h with gentle rotation. We used two types of goat anti-PIGF antibodies for immunoprecipitation; one was a polyclonal antibody raised against recombinant human PIGF protein, and the other was raised against the C-terminal 20-amino acid peptide of human PIGF-1. The former antibody was expected to work with both PIGF-1 and PIGF-2 because the sequence of the bacterially expressed immunogen mostly overlapped between the two isoforms. On the other hand, since the sequence of the C-terminal 20 amino acids employed to raise the latter antibody was perfectly matched with PIGF-1, but only partly identical to PIGF-2 (12 out of 20) due to heterogeneity in that very region, this antibody was expected to scarcely recognize PIGF-2 and thereby to specify PIGF-1. Coupling of antibodies or normal IgG with protein G-Sepharose was performed in buffer A (10 mm Tris-HCl (pH 7.4), 0.15 mM NaCl, 0.01% Triton X-100, and 1 mg/ml bovine serum albumin) at 4 °C for 2 h with gentle rotation. After immunoprecipitation, the gels were washed twice with buffer A and four times with 50 mm Tris-HCl (pH 7.4), 0.15 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. Immunoprecipitated materials were extracted from the Sephadex G-25 columns using 200 μl acetonitrile containing 0.05 mg/ml phosphatidylglycerol and 0.025 μCi [32P] orthophosphate (22). The [32P] phosphatidylglycerol was then electrophoresed on a 12.5% SDS-polyacrylamide gel under reducing conditions. For fluorography, gels were treated with Amplify (Amersham Pharmacia Biotech), dried and exposed to x-ray film at −80 °C.

Antisense Experiments—Phosphorothioate antisense oligodeoxyribo-
nucleotides and their sense counterparts were synthesized and purified by two cycles of reverse-phase high pressure liquid chromatography at TOAGOSEI Co. (Tokyo). PigF antisense and sense oligonucleotides corresponded to nucleotides 322–339 of human PigF mRNA (11), with their sequences then being 5'-CAGCTTCAAGGCGGCT-3' and 5'-ATGCCTGTCATGAGGCT-3', respectively. PigF-A antisense and sense oligonucleotides corresponded to nucleotides 57–78 of human VEGF-A mRNA (3), with their respective sequences being 5'-CCAGCAAGGAAAGTTCATCACT-3' and 5'-ATGAACCTTGTCTGGTTGGG-3' (7). Antisense effects on endothelial cell production of PigF proteins were assessed by immunoprecipitation of 5'-labeled proteins released into culture medium using the Genzyme/Techne anti-recombinant human PigF polyclonal antibody as described above under "Metabolic Labeling and Immunoprecipitation." Antisense effects on endothelial cell DNA synthesis were assessed as described previously (7). Briefly, human microvascular endothelial cells were seeded at a density of 2×10^4 cells/well of a Falcon 24-well plate and placed at 37 °C for 2 h. After cell attachment, media were changed to the HuMedia-EB2 medium supplemented with 5% fetal bovine serum containing antisense or sense oligodeoxyribonucleotides, and cells were incubated for 24 h. Then, [3H]thymidine was added to a final concentration of 5 μCi/ml, and cells were further incubated for 24 h. After incubation, cells were fixed with ice-cold 10% (w/v) trichloroacetic acid for 20 min, followed by neutralization with the same volume of 1N HCl. [3H]radioactivity was measured by liquid scintillation counting.

RESULTS

Isolation and Sequence Determination of the Bovine cDNAs Coding for PigF, VEGF-B, and VEGF-C—The bovine PigF cDNA encodes a 149-amino acid protein with a 20-amino acid signal sequence (Fig. 1A). The nucleotide and deduced amino acid sequences of bovine and human cDNAs shared 74.9 and 75.2% identities, respectively. It has been reported that alternative splicing events give rise to three isoforms of human PigF, namely PigF-1, -2, and -3 (5, 24). In this study, all the bovine cDNAs isolated corresponded to PigF-1, which lacks a heparin-binding domain that endows the ligand with the ability to adsorb the extracellular matrix (24). The bovine VEGF-B cDNA encodes a 188-amino acid protein with a 21-amino acid signal sequence (Fig. 1B). The mature VEGF-B protein of 167 amino acids had three basic amino acid clusters in its carboxy-terminal region that may function as heparin-binding sites (Fig. 1B). Another isoform (VEGF-B186) has been reported to occur in human also by alternative splicing (25). Four out of the five cDNA clones isolated from the heart library corresponded to VEGF-B167 and the nucleotide and deduced amino acid sequences of the bovine and human VEGF-B167 cDNAs shared 93.7 and 93.6% identities, respectively. The remaining one clone encoded VEGF-B186, which lacks putative heparin-binding sites. The bovine VEGF-C cDNA encodes a 240-amino acid protein with a 20-amino acid signal sequence (Fig. 1C). The nucleotide and deduced amino acid sequences of bovine and human cDNAs shared 86.9 and 88.1% identities, respectively.

Expression of the VEGF Family Members in Microvascular Endothelial Cells—As shown in Fig. 2, quantitative RT-PCR analysis demonstrated that microvascular endothelial cells possess not only PigF-A mRNA, but also PigF-B, PigF-C and PigF mRNAs. RT-PCR with PigF-A primers gave signals at 319 and 420 bp, which corresponded to VEGF-A121 and VEGF-A165 mRNAs, respectively. The levels of both VEGF-A121 and VEGF-A165 mRNAs significantly increased as the O2 concentration dropped to 2.5%, 5%, and 10% (Fig. 2A). PigF primers yielded 321- and 384-bp products, which corresponded to PigF-1 and PigF-2 mRNAs, respectively (Fig. 2B). This indicated that microvascular endothelial cells could generate both the soluble and heparin-binding forms of PigF. VEGF-B primers gave signals at 395 and 496 bp, which corresponded to VEGF-B167 and VEGF-B186 mRNAs, respectively; the signal intensity of the latter was consistently stronger than that of the former regardless of the O2 tensions (Fig. 2B), indicating that the soluble form of VEGF-B is predominant in microvascular endothelial cells. With VEGF-C primers, only a single band was noted at 409 bp (Fig. 2C). The levels of mRNAs for VEGF-B, VEGF-C, and PigF were not significantly changed in endothelial cells by exposure to hypoxia or anoxia (Fig. 2, B, D), in contrast with that of VEGF-A mRNA (Fig. 2A).

Expression of the VEGF Family Members in Microvascular Pericytes—As shown in Fig. 3, quantitative RT-PCR analysis demonstrated the presence in bovine retinal pericytes of not only VEGF-A mRNA, but also VEGF-B, VEGF-C, and PigF mRNAs, as in endothelial cells. RT-PCR with VEGF-A primers gave signals at 470 and 590 bp, which corresponded to VEGF-A121 and VEGF-A165 mRNAs, respectively (Fig. 3A). The levels of both mRNA species increased significantly as the atmospheric O2 concentration decreased to 0% (~8-fold) (Fig. 3A). PigF primers yielded 347- and 410-bp products, which corresponded to PigF-1 and PigF-2 mRNAs, respectively (Fig. 3B). VEGF-B primers gave VEGF-B167- and VEGF-B186-corresponding signals at 395 and 496 bp, respectively (Fig. 3C). In contrast with the case in endothelial cells, the former species...
PlGF mRNAs in bovine retinal pericytes. PlGF mRNAs in human microvascular endothelial cells.

hybridized with the respective 32P-end-labeled probes. Autoradiographic exposures were done at −80 °C. Essentially the same results were obtained in several independent experiments.

Fig. 2. RT-PCR analysis of VEGF-A, VEGF-B, VEGF-C, and PlGF mRNAs in human microvascular endothelial cells. Poly(A)＋ RNAs from human microvascular endothelial cells cultured under the indicated oxygen tensions were analyzed by RT-PCR with primers specific to human VEGF-A (A), PlGF (B), VEGF-B (C), VEGF-C (D), and β-actin (E) mRNAs. An aliquot of each RT-PCR mixture was electrophoresed on a 2.5% agarose gel, transferred to a nylon membrane, and hybridized with the respective 32P-end-labeled probes. Autoradiographic exposures were done at −80 °C. Essentially the same results were obtained in several independent experiments.

was more prominent than the latter in pericytes (Fig. 3C). This indicated that the heparin-binding form of VEGF-B predominated in pericytes. With VEGF-C primers, a single band was noted at 348 bp (Fig. 3D), which exactly corresponded to the bovine mRNA. Under hypoxia, the PlGF, VEGF-B, and VEGF-C mRNA levels were essentially unchanged (Fig. 3, B–D).

Quantification of the VEGF Family mRNAs in Endothelial Cells and Pericytes by Competitive RT-PCR—Since the RT-PCR analyses revealed that both endothelial cells and pericytes expressed all the members of the VEGF family tested, we then performed competitive RT-PCR to estimate the relative amounts of VEGF-A, VEGF-B, VEGF-C, and PlGF mRNAs in both cell types cultured under normoxia. After RT-PCR, the products were separated by agarose gel electrophoresis, and the equivalence point, at which the endogenous mRNA template gave the same signal intensity as the competitor RNA included in the reaction, was determined by densitometric scanning. The equivalence point reflects the point at which the initial concentration of the endogenous mRNA concerned is equal to that of the respective competitor, thus providing the quantitative values for the specific mRNA. Figs. 4 and 5 show typical SYBR Green stains together with the data of densitometric analyses in graphic form. The calculated amounts of the VEGF family mRNAs in endothelial cells and pericytes are summarized in Table I. The rank order of their levels in endothelial cells was PlGF > VEGF-B > VEGF-C > VEGF-A. The relative molar ratio of mRNAs for PlGF/VEGF-B/VEGF-C/VEGF-A in endothelial cells was estimated to be −166:20:7:1 when the value for VEGF-A mRNA was given as 1. In pericytes, the levels of the VEGF family mRNAs were ranked as VEGF-C > VEGF-A > VEGF-B > PlGF, and the relative molar ratio of mRNAs for VEGF-C/VEGF-A/VEGF-B/PlGF was −7:1:0.3:0.05 when related to the value for VEGF-A mRNA.

Endothelial Cell Production of PlGF Proteins and Antisense Assay of Their Roles in Hypoxia-induced Endothelial Cell Proliferation—Because mRNA coding for PlGF was present in endothelial cells at an extremely high level above those for the other members including VEGF-A (Table I) and because the physiological significance of this factor is not well understood, we further examined, by immunoprecipitation, whether endothelial cells do produce PlGF proteins and, by an antisense approach, whether they have a role in the proliferation of endothelial cells.

Human microvascular endothelial cells were incubated in the presence of [35S]methionine/cysteine, and 35S-labeled proteins released into culture media were immunoprecipitated with anti-human PlGF polyclonal antibodies. As shown in Fig. 6A, SDS-polyacrylamide gel electrophoresis analysis of the immunoprecipitated materials revealed two major immunoreacted bands at ~25 and ~28 kDa. The ~25- and ~28-kDa bands should represent PlGF-1 and PlGF-2, respectively, because human microvascular endothelial cells contained mRNAs for both PlGF isoforms (Fig. 2B), one having 129 and the other 150 amino acid residues (11, 24), and because the ~25-kDa band was recognized not only by the anti-recombinant PlGF protein antibody, but also by the probably PlGF-1-specific, anti-C-terminal 20-amino acid peptide antibody (Fig. 6A, lanes 2 and 3), whereas the ~28-kDa band was recognized by the former antibody as well (lane 3), but little by the latter antibody (lane 2). The apparently slower migration of PlGF proteins than expected from their primary structures and the broader band patterns could be ascribed to glycosylation and was consistent with results reported by others (11, 18, 24, 26). The data shown in Fig. 6A thus indicate that endothelial cells do translate PlGF mRNAs and release the protein products extracellularly. Furthermore, as shown in Fig. 6B (lanes 2 and 6), the levels of PlGF proteins in culture media increased in a time-dependent manner.

An octadecamer antisense complement of human PlGF mRNA was then synthesized to determine the functional role of PlGF. The region targeted was the 5′-initiator codon region common to both PlGF-1 and PlGF-2 mRNAs. The sense sequence at the same region was used as a control. We checked first whether the antisense oligonucleotides did inhibit the endothelial cell translation of PlGF mRNA by the immunoprecipitation of 35S-labeled proteins secreted into culture media.
using the anti-recombinant PlGF protein antibody. As shown in Fig. 6B, the levels of both PlGF-1 and PlGF-2 were found to be consistently reduced at 24 and 48 h after the addition of the antisense oligonucleotides (2 μM). On the other hand, the sense oligonucleotides gave no change in PlGF production by endothelial cells. The data indicate that, although not completely, the antisense oligonucleotides did inhibit the translation of PlGF mRNA in microvascular endothelial cells.

The effects of PlGF antisense oligonucleotides on hypoxia-induced proliferation of microvascular endothelial cells were examined. When endothelial cells were exposed to 2 or 4 μM antisense oligonucleotides under 5% O₂, endothelial cell synthesis of DNA was found to be markedly inhibited, as when exposed to the same concentrations of VEGF-A antisense oligonucleotides (Fig. 6C). The respective sense controls caused no changes. Furthermore, the inhibitory effects of VEGF-A and PlGF antisense oligonucleotides on endothelial cell growth were found to be additive at lower concentrations (Fig. 6D). Similar results were obtained when the PlGF antisense oligonucleotides were administered to human umbilical cord vein-derived endothelial cells (data not shown).

DISCUSSION

This study has demonstrated for the first time that mRNAs coding for PlGF, VEGF-B, and VEGF-C, the recently identified members of the VEGF family, are present in endothelial cells and pericytes. In both cell types, the levels of mRNAs for VEGF-B, VEGF-C, and PlGF did not increase significantly under hypoxia, by which VEGF-A mRNA was strongly induced (Figs. 2 and 3). This indicated that the expression of the VEGF family members is under controls whose mechanisms are different from those for VEGF-A regulation. In fact, it has been reported that the VEGF-C gene lacks the hypoxia-responsive element in its promoter region (27) and that cytokines and growth factors are the major regulators of VEGF-C expression (14). The structures of the PlGF and VEGF-B promoter regions remain to be elucidated.

The relative abundance of each mRNA species belonging to the VEGF family was also determined in this study (Figs. 4 and 5 and Table I). The fact that these members of the VEGF family are expressed in microvascular endothelial cells and pericytes, the very cell types in which angiogenesis takes place, should deepen our understanding of the molecular basis of the regulation of angiogenesis. VEGF-related factors produced by endothelial cells and pericytes may affect the growth, migration, and tube formation of these cells and gene expression therein in autocrine and/or paracrine manners. In light of the available evidence that 1) microvascular cells express plural types of VEGF receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR), and VEGFR-3 (Flt-4) (7, 8, 28); 2) VEGF-A binds to both VEGFR-1 and VEGFR-2 (4–6); 3) PlGF and VEGF-B bind to VEGFR-1 (4–6); 4) VEGF-C binds to VEGFR-2 and VEGFR-3 (4–6); and 5) PlGF and VEGF-B can heterodimerize with VEGF-A (5, 29), there may be complex interactions among the VEGF family members and their receptor family members.

The finding that microvascular endothelial cells expressed PlGF at >100-fold higher levels than VEGF-A (Table I) appears striking when considering the regulatory mechanisms of angiogenesis. The microvascular endothelial cells did produce PlGF proteins (Fig. 6A), and antisense inhibition of the PlGF production resulted in inhibition of the hypoxia-driven endo-
thelial cell synthesis of DNA (Fig. 6, C and D), suggesting that vascular PlGF may have a physiological role in the regulation of endothelial cell proliferation. The following modes of action should be considered. First, PlGF binds only to VEGFR-1, whereas VEGF-A binds to both VEGFR-1 and VEGFR-2. VEGFR-1 shows at least a 10-fold higher affinity for VEGF-A, but an ~10-fold lower kinase activity than VEGFR-2 (30, 31). Recently, VEGFR-1 has been regarded as a rather negative regulator of endothelial cell proliferation by trapping VEGF-A ligands (31), whereas VEGFR-2 serves as a positive regulator that transduces signals inside to cause the proliferation. Because both PlGF and VEGF-A can share VEGFR-1 as their common receptor molecule (4–6), it is likely that the coexpression of PlGF and VEGF-A may affect the binding of VEGF-A to its receptors and may thus modulate the VEGF-A actions. The presence of such a large amount of PlGF may result in a masking of VEGFR-1 in endothelial cells, thereby inhibiting the VEGF-A binding to this high affinity receptor. This may, in turn, allow access of VEGF-A to the other lower affinity but functioning receptor (VEGFR-2) that is abundantly expressed in endothelial cells. Thus, PlGF can make and keep endothelial cells more sensitive to lower concentrations of VEGF-A that could be supplied by surrounding cells and/or endothelial cells themselves. Second, PlGF is known to heterodimerize with VEGF-A and to exert weak mitogenicity in endothelial cells (29). The low abundance of VEGF-A (Table I) may be supplemented by the heterodimerization with PlGF, thereby contributing to the stimulation of endothelial cell growth. Third, overproduction of the heparin-binding form of PlGF may cause retrieval of sequestered heparin-binding angiogenic factors such as VEGF165 in a soluble form, as has been suggested earlier (32). In support of these speculations is a report by Park et al. (26) that PlGF potentiates the action of VEGF-A at low concentrations to stimulate endothelial cell proliferation.

Since VEGF-B binds to VEGFR-1 (4–6), overproduction of VEGF-B may also lead to the same situation as in the case with PlGF. In contrast, overproduction of VEGF-C could decrease the binding of VEGF-A to VEGFR-2 by competing with VEGF-A since VEGF-C binds to VEGFR-2 (4–6). We (7) and others (28) have shown previously that endothelial cells express VEGFR-3. Although VEGF-C binding to this receptor in the lymphatic endothelium is thought to induce lymphangiogenesis (33), the physiological consequence of VEGF-C binding to VEGFR-3 in vascular endothelial cells is currently unknown.

Table I

| mRNA    | Endothelial cells | Pericyte |
|---------|-------------------|---------|
|         | Content per poly(A) RNA | Relative amount | Content per poly(A) RNA | Relative amount |
| VEGF-A  | 0.7               | 1       | 2               | 1          |
| PlGF    | 116               | 166     | 0.1             | 0.05       |
| VEGF-B  | 14                | 20      | 0.6             | 0.3        |
| VEGF-C  | 5                 | 7       | 13              | 7          |

*Values are calculated from the data presented in Fig. 4.

The value for VEGF-A mRNA is given as 1.

*Values are calculated from the data in Fig. 5.

*These values are related to the value for VEGF-A mRNA.
cells were incubated under hypoxia (5% O\textsubscript{2}) for 24 h and with \[^{3}H\]thy-
were added to the medium at the indicated concentrations (4), and VEGF-E, an Orf virus NZ-T-derived protein with VEGFR-2 binding activity (35). Their expression and roles in vascular cells remain to be determined.

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Placenta Growth Factor and Vascular Endothelial Growth Factor B and C Expression in Microvascular Endothelial Cells and Pericytes: IMPLICATION IN AUTOCRINE AND PARACRINE REGULATION OF ANGIOGENESIS

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