To understand the role of eicosanoids in angiogenesis, we have studied the effect of lipoxygenase metabolites of arachidonic acid on human microvascular endothelial cell (HMVEC) DNA synthesis. Among the various lipoxygenase metabolites of arachidonic acid tested, 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE) induced DNA synthesis in HMVEC. 5(S)-HETE also stimulated Jak-2, STAT-1, and STAT-3 tyrosine phosphorylation and STAT-3-DNA binding activity. Tyrphostin AG490, a specific inhibitor of Jak-2, significantly reduced tyrosine phosphorylation and DNA binding activity of STAT-3 and DNA synthesis induced by 5(S)-HETE. In addition, 5(S)-HETE stimulated phosphatidylinositol 3-kinase (PI3-kinase) activity and phosphorylation of its downstream targets Akt, p70S6K, and 4E-BP1 and their effectors ribosomal protein S6 and eIF4E. LY294002 and rapamycin, potent inhibitors of PI3-kinase and mTOR, respectively, also blocked the DNA synthesis induced by 5(S)-HETE. Interestingly, AG490 attenuated 5(S)-HETE-induced PI3-kinase activity and phosphorylation of Akt, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E. 5(S)-HETE induced the expression of basic fibroblast growth factor 2 (bFGF-2) in a Jak-2- and PI3-kinase-dependent manner. In addition, a neutralizing anti-bFGF-2 antibody completely blocked 5(S)-HETE-induced DNA synthesis in HMVEC. Together these results suggest that 5(S)-HETE stimulates HMVEC growth via Jak-2- and PI3-kinase-dependent induction of expression of bFGF-2. These findings also reveal a cross-talk between Jak-2 and PI3-kinase in response to 5(S)-HETE in HMVEC.

Arachidonic acid, a polyunsaturated fatty acid, is an important component of membrane phospholipids and is acutely released in response to a variety of agonists, including growth factors, cytokines, and hormones (1–4). Upon release, it is either metabolized via the cyclooxygenase, lipoxygenase (LOX),1 or cytochrome P450 monooxygenase pathways producing prostaglandins, hydroperoxyeicosatetraenoic acids, and epoxyeicosatrienioic acids, respectively, or reincorporated into membrane phospholipids via esterification involving arachidonoyl-CoA synthetase and arachidonoyl-lyso phospholipid transferase (1, 5). Arachidonic acid and its metabolites, known as eicosanoids, are involved in the regulation of a variety of biological processes including vascular tone (5, 6). In addition, these lipid molecules have been reported to mediate intracellular signaling events in response to a number of stimuli (7–12). A large body of pharmacological data suggests that arachidonic acid and its eicosanoid metabolites stimulate mitogenesis in various cell types, including cancer cells (13–20).

Formation of new capillaries, a process known as angiogenesis, plays an important role in embryonic development and wound healing (21, 22). Angiogenesis also plays a role in the progression of various proliferative diseases such as atherosclerosis, cancer, and diabetic retinopathy (21–25). Proliferation and motility of endothelial cells are essential events of angiogenesis (23). Factors such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) that influence endothelial cell proliferation and motility are, therefore, likely involved in embryonic development and disease processes (21–25). In recent years, some studies using pharmacological approaches have reported that eicosanoids play a role in angiogenesis, although the underlying mechanisms are yet to be investigated (26).

Janus kinases are a group of non-receptor tyrosine kinases that via phosphorylating modulate the activities of a group of transcriptional factors, namely signal transducers and activators of transcription (STATs) (27, 28). STATs have been reported to be involved in the regulation of cell growth and differentiation (29–31). Similarly, phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway has been reported to play a major role in agonist-induced cell survival and growth (32–35). To understand the role of eicosanoids in angiogenesis, we have studied the effect of the LOX metabolites of arachidonic acid on HMVEC DNA synthesis. Here, we report for the first time that 5(S)-HETE, the 5-LOX metabolite of arachidonic acid, stimulates DNA synthesis in HMVEC, and this event is mediated via Jak-2- and PI3-kinase-dependent induction of expression of bFGF-2.

**MATERIALS AND METHODS**

*Reagents—Aprotinin, dithiothreitol, phenylmethylsulfonyl fluoride, sodium orthovanadate, sodium deoxycholate, leupeptin, HEPES, and acetic acid; HMVEC, human microvascular endothelial cells; PI3-kinase, phosphatidylinositol 3-kinase; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor.*

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1 The abbreviations used are: LOX, lipoxygenase; bFGF-2, basic fibroblast growth factor 2; 5(S)-HETE, 5(S)-hydroxyeicosatetraenoic acid; HMVEC, human microvascular endothelial cells; PI3-kinase, phosphatidylinositol 3-kinase; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor.

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phosphatidylinositol were purchased from Sigma. LY294002 and rapamycin were obtained from Calbiochem. 5(S)-HETE, 15(S)-HETE, leukotriene B₄, leukotriene C₄, and leukotriene D₄ were bought from Cayman Chemicals (Ann Arbor, MI). Phospho-specific anti-Akt (9271), anti-eIF4E (9741), anti-p70S6K (9205), and anti-4E-BP1 (9451) were obtained from Cell Signaling Technology (Beverly, MA). Phospho-specific anti-Jak-2 (9131S) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Phospho-specific anti-PI3-kinase (SC-2571) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PI3-kinase (66–195), anti-bFGF-2, and anti-VEGF antibodies and neutralizing antibodies of bFGF-2 and VEGF were from Upstate Biotechnology, Inc. (Lake Placid, NY). T4 polynucleotide kinase nucleotides for STAT-3 (5′-GATCTTCTGGAATATCGAT-3′) was procured from Invitrogen. [γ-32P]ATP (3000 Ci/mmol) and [H]thymidine (20 Ci/mmol) were obtained from PerkinElmer Life Sciences.

**Cell Culture**—HMVEC were bought from Clonetics (Walkersville, MD). Cells were grown in endothelial basal medium 2 (CC-3156) containing EGM-2 MV SingleQuots (CC-4147). Cultures were maintained at 37 °C in a humidified 95% air, 5% CO₂ atmosphere. Cells were growth-arrested by incubating in endothelial basal medium 2 for 24 h and used to perform the experiments.

**DNA Synthesis**—HMVEC with and without appropriate treatments were pulse-labeled with 1 μCi/ml [3H]thymidine for the indicated times. After lysis, cells were washed with cold phosphate-buffered saline, trypsinned, and collected by centrifugation. The cell pellet was suspended in cold 10% (v/v) trichloroacetic acid and vortexed vigorously to lyse cells. After standing on ice for 20 min, the cell lysis mixture was passed through a glass fiber filter (GFC, Whatman). The filter was washed once with cold 5% trichloroacetic acid and once with cold 70% (v/v) ethanol. The filter was dried, placed in a liquid scintillation vial containing the scintillant fluid, and the radioactivity was measured in a liquid scintillation counter (LS 5000TA, Beckman Instruments).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared from treated or untreated HMVEC as described previously (36). The protein content of the nuclear extracts was determined using the Micro BCA protein assay reagent kit (Pierce). Protein-DNA complexes were formed by incubating 5 μg of nuclear protein in a total volume of 20 μl consisting of 15 mM HEPES, pH 7.9, 3 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 4.5 μg of bovine serum albumin, 2 μg of poly(dI-dC), 15% glycerol, and 100,000 cpm of 32P-labeled oligonucleotide probe for 30 min on ice. The protein-DNA complexes were resolved by electrophoresis on a 4% polyacrylamide gel using 1× Tris-glycine-EDETA buffer (25 mM Tris-HCl, pH 8.5, 200 mM glycine, 0.1 mM EDTA). Double-stranded oligonucleotides were labeled with [γ-32P]ATP using the T4 polynucleotide kinase kit (Invitrogen) following the supplier’s protocol.

**PI3-kinase Assay**—PI3-kinase activity was measured as described in a previously published protocol (37). After appropriate treatments, cells were lysed in 1 ml of lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 2 μl leupeptin, 10 units/ml aprotinin, and 400 μM phenylmethylsulfonyl fluoride) for 20 min on ice. The cell lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4 °C. The protein content of the supernatants was determined as described above. Five hundred micromolars of protein from control and each treatment were incubated in 30 μl of TnE buffer and incubated with 10 μl of 2 mg/ml phosphatidylinositol, 10 μl of 100 mM MgCl₂, 2 μl of 100 mM ATP, and 20 μl of [γ-32P]ATP for 10 min at 22 °C. The reaction was terminated by the addition of 20 μl of 5× HCL and 10 μl of 100 mM chloroform-methanol (1:1) mix. The aqueous and organic phases were separated by centrifugation at 2000 rpm for 10 min. The organic phase containing the phosphoinositide phosphates was spotted onto silica gel 60A TLC plate coated with 1% potassium oxalate and separated in a solvent system consisting of chloroform:methanol:water: ammonium hydroxide (90:70:14.6:5.4). The TLC plate was exposed to X-Omat AR x-ray film for 4–6 h at −80 °C and developed.

**Western Blot Analysis**—After appropriate treatments, HMVEC were rinsed with cold phosphate-buffered saline and frozen immediately in liquid nitrogen. Cells were lysed by thawing in 250 μl of lysis buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate) and scraped into 1.5-ml Eppendorf tubes. After standing on ice for 20 min the cell lysates were cleared by centrifugation at 12,000 rpm for 20 min at 4 °C. Cell lysates containing equal amounts of protein were resolved by electrophoresis on 0.1% SDS and 10% polyacrylamide gels. The proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Biosciences). After blocking in 10 mM Tris-HCl, pH 7.4, 10% nonfat dry milk, the membrane was treated with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected using chemiluminescence reagent kit (Amersham Biosciences).

**Statistics**—All the experiments were repeated three times with similar results. Data on DNA synthesis are presented as the mean ± S.D. The treatment effects were analyzed by Student’s t test, p values < 0.05 were considered to be statistically significant. In the case of PI3-kinase activity, electrophoretic mobility shift assay, and Western blot analysis, one representative set of data is shown.

**RESULTS AND DISCUSSION**

To understand the role of eicosanoids in angiogenesis, we have studied the effect of various LOX metabolites of arachidonic acid on HMVEC DNA synthesis. Quiescent HMVEC were treated with and without 1 μM of indicated eicosanoids for 24 h,
and DNA synthesis was measured by pulse-labeling cells with 1 μCi/ml [3H]thymidine for the last 2 h of the 24-h incubation period. Among the LOX metabolites of arachidonic acid tested, only 5(S)-HETE induced DNA synthesis by about 80% over control (Fig. 1). To learn the signaling events of 5(S)-HETE-induced DNA synthesis, the role of the Jak/STAT pathway was studied. Quiescent HMVEC were treated with and without 5(S)-HETE (1 μM) for the indicated times, and cell extracts were prepared. Equal amounts of protein (40 μg) from control and each treatment were analyzed by Western blotting for pJak-2 and pSTAT-3 using their phospho-specific antibodies. As a loading control, the same blot was reprobed with anti-Jak-2 antibodies.

AG490 significantly inhibited 5(S)-HETE-induced STAT-3-DNA binding activity. To find the role of Jak/STAT pathway in 5(S)-HETE-induced HMVEC growth, the effect of AG490 on 5(S)-HETE-induced DNA synthesis was studied. As shown in Fig. 5, AG490 significantly inhibited 5(S)-HETE-induced DNA synthesis.

The PI3-kinase/Akt pathway plays an important role in cell

**Fig. 3.** Effects of 5(S)-, 12(S)-, and 15(S)-HETE on tyrosine phosphorylation of Jak-2 and STAT-3 in HMVEC. Growth-arrested HMVEC were treated with and without 1 μM of 5(S)-, 12(S)-, or 15(S)-HETE for 30 min, and cell extracts were prepared. Equal amounts of protein (40 μg) from control and each treatment were analyzed by Western blotting for pJak-2 and pSTAT-3 using their phospho-specific antibodies. As a loading control, the same blot was reprobed with anti-Jak-2 antibodies.

**Fig. 4.** AG490, a potent inhibitor of Jak-2, reduces 5(S)-HETE-induced tyrosine phosphorylation and DNA binding activity of STAT-3 in HMVEC. A, growth-arrested HMVEC were treated with and without 5(S)-HETE (1 μM) in the presence and absence of AG490 (25 μM) for 5 min, and cell extracts were prepared. Equal amounts of protein (40 μg) from control and each treatment were analyzed by Western blotting for pSTAT-3 using its phospho-specific antibodies. As a loading control, the same blot was reprobed with anti-STAT-3 antibodies. B, growth-arrested HMVEC were treated with and without 5(S)-HETE (1 μM) in the presence and absence of AG490 (25 μM) for 2 h, and nuclear extracts were prepared. Five micrograms of nuclear protein from control and each treatment were incubated with 100,000 cpm of 32P-labeled STAT-3 consensus oligonucleotide probe, and the DNA-protein complexes were separated by electrophoretic mobility shift assay and subjected to autoradiography. For supershift analysis, the DNA-protein complexes were incubated with 1 μg of anti-STAT-3 antibodies for 2 h before subjecting the complexes to electrophoretic mobility shift assay. For competitive analysis, nuclear protein was first incubated with excessive cold oligos followed by incubation with labeled oligos. For clarity of the appearance of supershifts, longer exposure autoradiographic signals of the respective lanes are shown in right panel. Ab, antibody.
survival (32–35). In recent years, it was reported that the LOX and cytochrome P450 monooxygenase metabolites of arachidonic acid, particularly, 12(S)-HETE and 14, 15-epoxyeicosatrienoic acid, play a role in cell survival (39–41).

Because cell survival activity is required for cell growth, we wanted to study the effect of 5(S)-HETE on activation of PI3-kinase/Akt signaling. Quiescent HMVEC were treated with and without 5(S)-HETE in the presence and absence of LY294002 (25 μM), a potent and specific inhibitor of PI3-kinase, and cell extracts were prepared. Equal amounts of protein from control and each treatment were assayed for PI3-kinase activity. 5(S)-HETE stimulated PI3-kinase activity 2-fold, and this response was completely inhibited by LY294002 (Fig. 6).

We next tested the effect of 5(S)-HETE on the phosphorylation of PI3-kinase downstream targets, Akt (32–34), p70S6K (42, 43), ribosomal protein S6, 4E-BP1, and eIF4E in a PI3-kinase-dependent manner. Growth-arrested HMVEC were treated with and without 5(S)-HETE (1 μM) in the presence and absence of LY294002 (25 μM) for the indicated times, and cell extracts were prepared. Equal amounts of protein (40 μg) from control and each treatment were analyzed by Western blotting for Akt, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E phosphorylation using their phospho-specific antibodies. For loading control, the blot was reprobed only with anti-Akt antibodies because the same blot was used for probing with all the indicated phospho-specific antibodies. pRPS6, phosphorylated ribosomal protein S6.
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(44, 45), and eIF4E (46). 5(S)-HETE stimulated serine phosphorylation of Akt, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E exhibit a requirement for Jak-2 in HMVEC. Growth-arrested HMVEC were treated with and without 5(S)-HETE (1 μM) in the presence and absence of AG490 (25 μM) for the indicated times, and cell extracts were prepared. A, equal amounts of protein (400 μg) from control and each treatment were immunoprecipitated with 3 mg of anti-PI3-kinase antibodies, and the kinase activity was measured in the immunocomplexes as described in legend to Fig. 6. PIP3, inositol 1,4,5-trisphosphate. B, equal amounts of protein (40 μg) from control and each treatment were analyzed by Western blotting for Akt, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E phosphorylation using their phospho-specific antibodies. For loading control, the blot was reprobed only with anti-Akt antibodies because the same blot was used for probing with all the indicated phospho-specific antibodies.

Fig. 10. 5(S)-HETE induces bFGF-2 expression in HMVEC. Growth-arrested HMVEC were treated with and without 5(S)-HETE (1 μM) in the presence and absence of AG490 (25 μM) or LY294002 (25 μM) for the indicated times, and cell extracts were prepared. Equal amounts of protein (40 μg) from control and each treatment were analyzed by Western blotting for bFGF-2 or VEGF using their specific antibodies.

Fig. 11. Neutralizing anti-bFGF-2 but not anti-VEGF antibodies inhibit 5(S)-HETE-induced DNA synthesis in HMVEC. Growth-arrested HMVEC were treated with and without 5(S)-HETE (1 μM) in the presence and absence of neutralizing anti-bFGF-2 or anti-VEGF antibodies for 24 h and DNA synthesis was measured by pulse-labeling cells with 1 μCi/ml [3H]thymidine for the last 12 h of the 24-h treatment period. *, p < 0.01 versus control; **, p < 0.01 versus 5(S)-HETE treatment alone.
the activation of PI3-kinase and its downstream targets by 5(S)-HETE in HMVEC.

Among a variety of factors, bFGF-2 and VEGF are potent mitogens for endothelial cells and play an important role in angiogenesis (22, 23). To test whether 5(S)-HETE stimulates HMVEC growth via induction of expression of angiogenic factors, we studied its effect on the expression of bFGF-2 and VEGF. Although having a modest effect on VEGF expression (1.7-fold), 5(S)-HETE induced the expression of bFGF-2 by 3-fold compared with control (Fig. 10). In addition, 5(S)-HETE-induced expression of bFGF-2 but not VEGF was attenuated by AG490 and LY294002, the inhibitors of Jak-2 and PI3-kinase, respectively (Fig. 10). To test the role of 5(S)-HETE in 5(S)-HETE-induced HMVEC DNA synthesis, quiescent cells were treated with and without 5(S)-HETE (1 μM) in the presence and absence of neutralizing anti-bFGF-2 (3 μg/ml) or anti-VEGF (3 μg/ml) antibodies for 24 h, and DNA synthesis was measured by [3H]thymidine incorporation. 5(S)-HETE-induced DNA synthesis was completely inhibited by neutralizing anti-bFGF-2 but not anti-VEGF antibodies (Fig. 11).

The important findings of the present study are as follows. 1) Among the LOX products of arachidonic acid examined, 5(S)-HETE significantly induced DNA synthesis in HMVEC; 2) 5(S)-HETE stimulated the tyrosine phosphorylation of Jak-2, STAT-1, and STAT-3 in a time-dependent manner; 3) AG490, a potent and specific inhibitor of Jak-2, substantially reduced tyrosine phosphorylation and DNA binding activity of STAT-3 and DNA synthesis induced by 5(S)-HETE; 4) 5(S)-HETE stimulated PI3-kinase activity and phosphorylation of its downstream molecules, Akt, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E induced by 5(S)-HETE; 5) 5(S)-HETE-induced DNA synthesis was completely inhibited by neutralizing anti-bFGF-2 but not anti-VEGF antibodies (Fig. 11).

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