The Cytochrome P450 4A/F-20-Hydroxyeicosatetraenoic Acid System: A Regulator of Endothelial Precursor Cells Derived from Human Umbilical Cord Blood


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ABSTRACT

Endothelial progenitor cells (EPCs) contribute to physiological and pathological neovascularization. Previous data have suggested that the cytochrome P450 4A/F (CYP4A/F)-20-hydroxyeicosatetraenoic acid (20-HETE) system regulates neovascularization. Therefore, we studied whether the angiogenic effects of the CYP4A/F-20-HETE system involve regulation of EPC function. We extracted human umbilical cord blood and isolated EPCs, which express AC133 and kinase insert domain receptor (KDR) surface markers and contain mRNA and protein for CYP4A11 and CYP4A22 enzymes, as opposed to mesenchymal stem cells, which only express negligible amounts of CYP4A11/22. When EPCs were incubated with arachidonic acid, they produced 20-HETE, which stimulated the cells to proliferate and migrate, as did vascular endothelial growth factor. Incubation with 1 μM N-hydroxy-N’-(4-butyl-2-methylphenyl)formamidine (HET0016), a selective inhibitor of 20-HETE synthesis, reduced the proliferative and migratory effects of vascular endothelial growth factor and also significantly abolished EPC migration mediated by stroma-derived factor-1α, as did (6,15) 20-hydroxyeicosadienoic acid. Coculturing EPCs and endothelial cells on a Matrigel matrix led to tube formation, which in turn was inhibited by both HET0016 and 20-hydroxyeicosadienoic acid. We concluded that the CYP4A/F-20-HETE system is expressed in EPCs and can act as both an autocrine and a paracrine regulatory factor.

Introduction

Angiogenesis, the growth of new blood vessels from existing ones, and vasculogenesis, the de novo formation of blood vessels, are essential for organ development. They also play a critical role in tissue repair and regeneration and in the response to ischemia and cardiovascular repair/regeneration (Carmeliet, 2005). Unchecked neovascularization also is associated with human diseases, including cancer and age-related macular degeneration. Thus, the ability to regulate angiogenic responses is critical. Therapeutic manipulation of blood vessel formation (either excessive or impaired) requires a precise understanding of the numerous factors capable of regulating neovascularization (Pearson, 2010).

Circulating endothelial progenitor cells (EPCs) contribute to postnatal vasculogenesis and angiogenesis, and a growing body of evidence suggests that they play an important role in neovascularization and hence repair of damaged tissue (Janic and Arbab, 2010; Jarajapu and Grant, 2010; Kirton and Xu, 2010). Neovascularization may involve sprouting from neighboring vessels in response to increases in locally formed vascular endothelial growth factor (VEGF) and also may be supported by mobilization and functional incorporation of bone marrow-derived EPCs. Therefore, EPCs derived from human umbilical cord blood and isolated EPCs, which express AC133 and kinase insert domain receptor (KDR) surface markers and contain mRNA and protein for CYP4A11 and CYP4A22 enzymes, as opposed to mesenchymal stem cells, which only express negligible amounts of CYP4A11/22. When EPCs were incubated with arachidonic acid, they produced 20-HETE, which stimulated the cells to proliferate and migrate, as did vascular endothelial growth factor. Incubation with 1 μM N-hydroxy-N’-(4-butyl-2-methylphenyl)formamidine (HET0016), a selective inhibitor of 20-HETE synthesis, reduced the proliferative and migratory effects of vascular endothelial growth factor and also significantly abolished EPC migration mediated by stroma-derived factor-1α, as did (6,15) 20-hydroxyeicosadienoic acid. Coculturing EPCs and endothelial cells on a Matrigel matrix led to tube formation, which in turn was inhibited by both HET0016 and 20-hydroxyeicosadienoic acid. We concluded that the CYP4A/F-20-HETE system is expressed in EPCs and can act as both an autocrine and a paracrine regulatory factor.

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from the bone marrow may provide a novel therapeutic target in patients with vascular disease. Neovascularization also plays a critical role in tumor formation, where it involves recruitment of circulating EPCs from bone marrow as well as sprouting of vessels from pre-existing endothelial cells (ECs) (Janic and Arbab, 2010; Kirton and Xu, 2010; Patenaude et al., 2010; Pearson, 2010). Although EPCs are considered to originate in bone marrow, they are also present in other tissues, including cord blood.

VEGF plays a critical role in the regulation of EPC function by increasing mobilization of EPCs from the bone marrow and mediating their migration into the circulation (Li et al., 2006; Rosti et al., 2007). In turn, EPCs promote vascular growth and repair by increasing production of VEGF and other factors within ischemic tissue (Tepper et al., 2005). EPCs express chemokine receptor type 4 (CXCR4), which allows migration to sites of neovascularization in response to its ligand, stroma-derived factor-1-alpha (SDF-1α), typically released by target tissues (Weidt et al., 2007; Folkins et al., 2009).

Cytochrome P450 4A (CYP4A) is a monooxygenase that metabolizes arachidonic acid (AA) to 20-hydroxyicosatetraenoic acid (20-HETE). We have shown that 20-HETE stimulates production of both VEGF and hypoxia-inducible factor-1α (HIF-1α), essential regulators of angiogenic responses in ECs. In addition, 20-HETE increases EC proliferation and migration, both essential steps in the angiogenic cascade (Guo et al., 2007, 2009). Thus, 20-HETE activates both the release of angiogenic factors and the growth responses of vascular cells in vivo. We examined the hypothesis that the CYP4A4F-20-HETE system regulates neovascularization responses by altering proliferation and migration of EPCs. For this, we extracted human umbilical cord blood and isolated monocellular cells positive for AC133 and CD34 (Peichev et al., 2000; Janic and Arbab, 2010; Janic et al., 2010). We have found that these cells express CYP4A11 and synthesize 20-HETE.

**Materials and Methods**

**Isolation and In Vitro Culture of AC133**

Progenitor cells positive for the CD133** super antigen AC133** were isolated from umbilical cord blood obtained from subjects who volunteered for Institutional Review Board-approved protocols. Mononuclear cells were separated out by Ficoll gradient centrifugation, and AC133** cells were isolated by immunomagnetic positive selection using the MidiMACS system (Miltenyi Biotec, Auburn, CA). Freshly isolated AC133** cells were suspended in Stemline II medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20 ng/ml stem cell factor, 40 ng/ml FLT3, and 10 ng/ml thrombopoietin (all from CellGenix, Antioch, IL). They were maintained at 37°C in a humidified incubator containing 5% CO2 as a suspension culture for 30 days, keeping the cell concentration at 5×10^6 cells/ml. Throughout this expansion period, the cells were cultured in Falcon six-well dishes (BD Biosciences, San Jose, CA) and monitored daily using an inverted phase contrast microscope. After mitosis, viable cells were counted by trypan blue exclusion assay, and a growth curve was generated. Then, mitosis was induced by adding freshly prepared medium so as to adjust the concentration to 5×10^6 cells/ml.

**Flow Cytometry of Endothelial Lineage Cell Surface Markers on AC133**

Cells expanded in suspension under either normal growth conditions or conditions that facilitate cell differentiation were harvested, washed in ice-cold 1× phosphate-buffered saline, and incubated for 30 min on ice in the dark with a fluorescently labeled antibody. Flow cytometry was performed with an LSR II flow cytometer (BD Biosciences), and 10,000 or more events were analyzed for each sample. Live cells were gated based on their forward and side angle light scatter and analyzed using CellQuest Pro software (BD Biosciences). The antibodies used for flow cytometry were as follows: mouse anti-human CD133 IgG1 (Miltenyi Biotec), mouse anti-human CD34 IgG1 (BioLegend, San Diego, CA), mouse anti-human CD117 IgG1 (BioLegend), mouse anti-human CD29 IgG1 (BioLegend), mouse anti-human CD54 IgG2a (BioLegend), mouse anti-human kinase insert domain receptor (KDR) (VEGFR2) IgG1 (R&D Systems, Minneapolis, MN), mouse anti-human vascular endothelial (VE)-cadherin IgG2b (R&D Systems), mouse anti-human CD62E IgG2a (BioLegend), mouse anti-human CD184 IgG2a (BioLegend), and mouse anti-human CD105 IgG2a (BioLegend).

**Western Blot Analysis**

Homogenates were prepared from EPCs and mesenchymal stem cells (MSCs) using radioimmunoprecipitation assay buffer as described previously (Guo et al., 2005). Equal amounts of protein (20–40 μg) were separated on a 14% Tris-glycine gel, transferred to a polyvinylidene difluoride membrane, and incubated with a CYP4A11 primary antibody (RDI Division of Fitzgerald Industries, Acton, MA) at a dilution of 1:500. The membranes were incubated with an anti-rabbit horseradish peroxidase antibody and developed by enhanced chemiluminescence. We then treated the blots with protein stripping buffer, reblocked with blocking agent, and then immunostained with an antibody against human β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using the same development system to serve as loading control.

**Reverse Transcription Polymerase Chain Reaction.** RNA was extracted from EPCs or MSCs using TRIzol reagent and treated with DNase, and its concentration was measured by absorbance at 260 nm. Then, 1 μg of RNA was reverse-transcribed using a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). Primers used for polymerase chain reaction (PCR) were CYP4A11 forward 5′-AATTTTGCATAGCAGGAGAGCTGA-3′ and reverse 5′-TGGTCTACAAGGCACAAAGG-3′; CYP4A22 forward 5′-AATTTCGCTAGATGACCAGGTA-3′ and reverse 5′-GATTCCTTGTCCACAAAGG-3′; CYP4F2 forward 5′-CCGAAACAGGATGTGCTCT-3′ and reverse 5′-CCAGACGCATACATACGGA-3′; CYP4F3 forward 5′-CCCCAGAGCGAACTGTTT-3′ and reverse 5′-GACCTTAAAGCCCTGCTG-3′; CYP2J2 forward 5′-ATGTTCTCGGTTGGATGGCT-3′ and reverse 5′-TGGTTGCTCGAGTGAACC-3′; CYP2C8 forward 5′-TCTTACAGAAGTCTTCATAT-3′ and reverse 5′-GAGTCTTTGAGACCTGATTC-3′; CYP2C9 forward 5′-TGGTCCAGTTGGTTGGCT-3′ and reverse 5′-ATAATACGTTGCTGATTC-3′. Amplification was performed at 95°C for 3 min, followed by 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR was extended at 72°C for 10 min, and the products were separated by electrophoresis on 1% agarose gels. Expected product sizes were as follows: CYP4A11, 500 basepairs (bp); CYP4A22, 172 bp; CYP4F2, 110 bp; CYP4F3, 160 bp; CYP2J2, 435 bp; CYP2C8, 169 bp; CYP2C9, 147 bp.

**Real-Time PCR.** Real-time PCR was performed using a LightCycler 3 (Roche, Indianapolis, IN). The 20 μl of PCR mix include 2 μl of reverse transcription product, 2 μl of LightCycler FastStart DNA Master SYBR mix, 0.5 unit of LightCycler uracil-DNA glycosylase, and 10 pmol of various primers. The reactions were incubated at 95°C for 10 min, followed by 55 cycles at 95°C for 5 s, 55°C for 5 s, and 72°C for 15 s. The threshold cycle (Ct) data were determined using default threshold settings. The Ct was defined as the fractional cycle number at which the fluorescence passes a fixed threshold. The 2^-ΔCt method was used to calculate the relative changes in the gene expression. The products were collected, and molecular weights of the products were confirmed by electrophoresis on 1% agarose gels.

**Cell Proliferation Assay.** Proliferation studies were performed as described previously (Guo et al., 2005, 2006). EPCs were incubated with...
VEGF (20 ng/ml) or 20-HETE (1 μM) for 48 h. In some experiments, the cultures were exposed to VEGF in the presence of 10 μM N-hydroxy-N’-(4-butyl-2-methylphenyl)formamidine (HET0016), a selective inhibitor of 20-HETE synthesis. The effects of VEGF and 20-HETE on proliferation were assessed by cell counting, using EPCs treated with solvent (0.01% ethanol) as a control. Cell counts were normalized to ethanol and expressed as a percentage of the control.

**Cell Migration Assay.** A total of 1×10⁵ EPCs in medium lacking both serum and growth factors were placed in the upper chamber of a cell migration transwell (QCM Chemotaxis 5-μm 24-well cell migration assay; Millipore, Billerica, MA), and VEGF (20 ng/ml), SDF-1 (100 ng/ml), or 20-HETE (1 μM) was added to the bottom well for 16 h to study its effects on EPC migration. In separate experiments, EPCs were incubated with either 100 ng/ml SDF-1 or 20 ng/ml VEGF in the presence or absence of 10 μM HET0016 and/or 10 μM (6,15) 20-hydroxyeicosadienoic acid (20-HED). In addition, 10 μM 20-HETE was added to one group to determine whether it would reverse the inhibitory effects of HET0016 on SDF-1-mediated EPC migration. Changes in the numbers of cells both attached to the membrane and contained in the lower chamber were quantified using a fluorescence-based migration assay kit following the manufacturer’s recommended protocol.

**EC Tube Formation Assay.** The ability of EPCs or ECs to form tubes resembling capillaries was assessed by seeding the cells in wells containing 35-mm BioCoat Matrigel (BD Biosciences). A total of 1×10⁵ EPCs either alone or in the presence of 2×10⁵ ECs washed with phosphate-buffered saline were seeded in wells containing Matrigel without serum or growth factors, and tube formation was assessed after ~18 h of incubation. To establish whether the CYP4A/F-20-HETE system contributes to the effects of EPC-assisted EC tube formation, a 20-HETE synthesis inhibitor, either 10 μM HET0016, 10 μM N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS), or 10 μM 20-HED, was added. Images were taken from random fields of Matrigel wells, and the tube lengths were tracked using Sigma-plot and normalized to pixel counts.

**Assessment of 20-HETE Formation.** For this, 1×10⁵ EPCs were either left untreated or treated with 10 μM AA overnight before harvesting the cells. Both cells and media were analyzed for the presence of 20-HETE and other lipid metabolites as described previously (Guo et al., 2008). 1d-20-HETE (2 ng) added to each sample before lipid extraction with ethyl acetate served as the internal positive standard. The organic phases were collected and dried under nitrogen.

Samples were reconstituted with 50% methanol and water, and the metabolites were separated by high-performance liquid chromatography on a BetaBasic C18 column (150×2.1 mm, 3 μm; Thermo Fisher Scientific, Waltham, MA) at a flow rate of 0.2 ml/min using an isocratic elution starting from a 51:49:0.01 mixture of acetone/tritritile/methanol/water/acetic acid for 30 min followed by a step change to 68:13:19:0.01 acetone/tritritile/methanol/water/acetic acid for 15 min. The effluent was ionized using a negative ion electro spray (450°C, 4500 V) with the collision-activated dissociation gas set at 7 l/min. All of the transitions had a scan time of 0.2 s and a unit resolution in both Q1 and Q3 set at 0.7 ± 0.1 full width at half-maximum. Peaks eluting with a mass/charge ratio (m/z) of 319 to 301 (HETEs and epoxyeicosatrienoic acids), 337 to 319 (DiHETEs), 319 to 245 (20-HETEs), 325 to 251 (d6-20-HETE), 351 to 271 (prostaglandins (PG)D2 and E2), 353 to 309 (PGF2), 369 to 245 (PGF1α), and 369 to 195 (thromboxane A2) were monitored as multiple reactions using a triple quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA).

**Statistical Analysis.** Data were analyzed using analysis of variance followed by Tukey’s test or Student’s t test when only two groups were studied, taking p < 0.05 as significant.

**Results**

**Isolation and Characterization of EPCs.** Mononuclear cells were isolated from human umbilical cord blood. AC133⁺ and CD34⁺ cells were separated out by immunomagnetic-positive selection, and expression of AC133 and CD34 cell surface markers was confirmed in vitro by flow cytometry (Fig. 1A). When these cells were plated and cultured on a fibronectin-coated surface in the presence of 2% fetal bovine serum and 2 ng/ml VEGF for 10 days, they became differentiated, exhibiting a cobblestone pattern and expression of cell surface markers on EPCs. A, expression of AC133 and CD34 progenitor markers on EPCs was analyzed by flow cytometry. B, expression of CD31, CD34, and CD117 (progenitor markers) and CD31, CD29, CD184, CD62, KDR, VE-cadherin, and CD105 (endothelial lineage cell surface markers) on EPCs. Before differentiation, 4-day-old EPC cultures were used. After differentiation, EPCs were induced to differentiate into mature ECs for 2 weeks. We used AC133⁺ EPCs (before differentiation) for all of the studies.

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**Fig. 1.** Flow cytometry of cell surface markers on EPCs. A, expression of AC133 and CD34 progenitor markers on EPCs was analyzed by flow cytometry. B, expression of CD31, CD34, and CD117 (progenitor markers) and CD31, CD29, CD184, CD62, KDR, VE-cadherin, and CD105 (endothelial lineage cell surface markers) on EPCs. Before differentiation, 4-day-old EPC cultures were used. After differentiation, EPCs were induced to differentiate into mature ECs for 2 weeks. We used AC133⁺ EPCs (before differentiation) for all of the studies.
mature EC-specific markers. Comparison of CD133, CD34, and CD117 (progenitor markers) and CD31, CD29, CD184, CD62, KDR, VE-cadherin, and CD105 (endothelial lineage cell surface markers) on EPCs before and after differentiation confirmed that the AC133+ and CD34+ EPCs that we isolated matured into ECs (Fig. 1B).

**CYP4A/F-20-HETE System in EPCs.** EPCs expressed mRNA for CYP4A11, CYP4A22, and CYP4F2—all of which are known to produce 20-HETE—along with the epoxide-synthesizing enzymes CYP2C8 and CYP2C9 (Fig. 2A). CYP4F3 was not detectable in EPCs by reverse transcription (RT)-PCR (data not shown). To determine the relative mRNA expression of these genes, real-time PCR also was performed in EPCs. We found that CYP4A11 and CYP4A22 have relatively high expression in EPCs, whereas CYP4F2, CYP2C8, and CYP2C9 are minimally expressed (Fig. 2B).

Western blots confirmed that EPCs also express CYP4A11/22 protein (Fig. 3). Due to the relatively high expression of CYP4A11 in EPCs, we examined whether this 20-HETE synthase is differentially expressed in another different type of stem cells, MSCs. It is interesting to note that RT-PCR of cDNA from MSCs isolated from umbilical cord blood showed negligible expression of CYP4A11 (Fig. 3B). A similar observation also was recorded for CYP4A11 protein expression.

When these cells were incubated with medium containing AA (10 μM) for 60 min and 20-HETE was analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS), they were shown to produce 20-HETE along with 5-, 12-, and 15-HETE and several epoxyeicosatrienoic acids (Fig. 4). The majority of 20-HETE in EPCs was detected in the culture media not in the cell pellets.

**Effects of 20-HETE on EPC Proliferation and Migration.** Additional studies were performed to determine the effects of 20-HETE on EPC proliferation and migration, with VEGF and SDF-1α serving as a corresponding positive control. Although both 20-HETE and VEGF increased EPC proliferation and migration, on a molar basis VEGF was more potent than 20-HETE. Blocking 20-HETE synthesis with HET0016 inhibited both proliferation and migration responses to VEGF and SDF-1α (Fig. 5). In addition, both HET0016 and 20-HEDE prevented SDF-1α from accelerating EPC migration. We also found that addition of 20-HETE partially reversed the inhibitory effect of HET0016 on SDF-1α-mediated EPC migration.

**Effects of 20-HETE Synthesis Inhibitors on EPC-Induced EC Tube Formation.** Coculture of EPCs with ECs markedly increased tube formation as seen on a Matrigel assay, and this effect was significantly inhibited by HET0016 (Fig. 6). DDMS, another inhibitor of 20-HETE synthesis, had a similar effect on EPC-mediated EC tube formation even

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**Fig. 2.** mRNA analysis of P450s enzymes in EPCs. A, RNA was extracted from cord blood EPCs using TRIzol and reverse-transcribed. cDNA was amplified by RT-PCR using CYP4A11, CYP4A22, CYP4F2, CYP2C8, and CYP2C9 primers. Glyceraldehyde-3-phosphate dehydrogenase served as a loading control. Representative gels from three separate experiments are shown. B, real-time PCR analysis of CYP4A11, CYP4A22, CYP4F2, CYP2C8, and CYP2C9 relative mRNA expression also was performed in EPCs. Glyceraldehyde-3-phosphate dehydrogenase again was used as a control. Data represent at least three individual experiments in triplicate.
though it is chemically dissimilar to HET0016, and so did 20-HEDE (Fig. 6).

Effects of 20-HETE on Expression of the Angiogenic Regulators VEGF and HIF-1α, Endothelial Nitric-Oxide Synthase Phosphorylation, and Dihydroethidium Staining. 20-HETE increased expression of HIF-1α and VEGF as well as phosphorylation of serine 1177 in endothelial nitric-oxide synthase (Fig. 7A). It also increased dihydroethidium staining in EPCs, most likely by stimulating superoxide formation (Fig. 7B).

Discussion

Over the past decade there has been considerable interest in the role of EPCs in angiogenesis. EPCs are considered to be bone marrow-derived cells that have the capacity to proliferate and migrate to sites of neovascularization, where they differentiate into mature ECs. However, EPCs are also present in other tissues. Many groups have used peripheral and placental umbilical cord blood as a source of EPCs (Harris and Rogers, 2007). We isolated cells expressing CD34, AC133, and VEGFR2 from placental cord blood. These cells are thought to represent an immature EPC population that when plated on a matrix can give rise to AC133+CD34+VEGFR2+ cells, which are more mature and have limited proliferative capacity (Janic and Arbab, 2010; Janic et al., 2010). Such cells behave like EPCs, migrating to sites of neovascularization and becoming integrated into vascular networks (Janic and Arbab, 2010; Janic et al., 2010). Cord blood AC133+ cells represent a pluripotent adult progenitor cell population that could be valuable in vascular repair; moreover, cord blood generates more EPCs than any other source of EPCs (Harris and Rogers, 2007).

We and others (Jiang et al., 2004; Chen et al., 2005; Buyschaert et al., 2007; Guo et al., 2007) have shown that the CYP4A–20-HETE system plays an important role in angiogenesis. 20-HETE is angiogenic both in vitro and in vivo (Jiang et al., 2004; Guo et al., 2007, 2009) and activates VEGF and HIF-1α in ECs (Guo et al., 2007, 2009). In the vasculature, the main source of 20-HETE is the smooth muscle layer (Gebremedhin et al., 2000), although in the lung it also is produced by ECs (Zhu et al., 2002). We have tested a
total of six batches of EPCs randomly isolated from more than 20 donors. CYP4A11 and CYP4A22 mRNA (RT-PCR) and protein (Western blot) were expressed in each of the six batches. Three additional batches from different donors also were subjected to real-time PCR analysis and showed mRNA expression of both isozymes. The ratio of one isoform to another tended to vary. This simply may reflect the different donors, because cord blood was obtained from volunteers without regard for age, race, or health status. Although we failed to detect CYP4F3 RNA in EPCs by RT-PCR, we did not specifically analyze whether CYP4F3a or CYP4F3b RNA was expressed. Because these P450s may have distinct patterns of cell distribution, our studies still cannot completely rule out the participation of CYP4F3 in AA \( \Delta^5 \)-hydroxylation by EPCs.

Although EPC pellets produced very little 20-HETE under basal conditions, they released substantial amounts into the medium (\( \sim 200 \) pg/million cells) when incubated with AA, showing that they contained enzymatically active 20-HETE synthases. EPCs responded to exogenous 20-HETE by proliferating and migrating, showing that 20-HETE stimulates basic functions of EPCs. It is worth noting that 20-HETE is highly unstable in the cell culturing system based on our previous experiences working with ECs. Thus, it is most likely that the increased EPC proliferation observed at 48 h after 20-HETE addition resulted from its immediate mitogenic effects. 20-HETE may reach EPCs via either a paracrine or an autocrine pathway (or both), because \( \Delta^5 \)-hydroxylases are microsomal enzymes (Roman, 2002). EPC supernatants also contained small amounts of various epoxides, a result consistent with low levels of epoxide-producing enzymes being present.

We previously reported that 20-HETE synthesis inhibitors blocked the proliferative and migratory responses of ECs to VEGF; indeed, the selective AA \( \Delta^5 \)-hydroxylase inhibitor HET0016 not only blunted EC growth and migration responses to VEGF in vitro but also angiogenic responses to VEGF and other growth factors in vivo (Chen et al., 2005; Guo et al., 2007). To determine whether blocking the CYP4A/F-20-HETE system would have a similar action on the proliferative and migratory responses of EPCs to VEGF, we coincubated VEGF with HET0016 and found that both responses were eliminated entirely. Although we do not know exactly how \( \Delta^5 \)-hydroxylase inhibitors such as HET0016 suppress responses to VEGF, it seems unlikely...
that HET0016 acts on sites near EPCs, because blocking 20-HETE synthesis with a structurally and mechanistically different inhibitor, DDMS, had the same effect. HET0016 lessened the migratory effect of SDF-1 on EPCs, suggesting that some metabolite produced by ω-hydroxylase, from either AA or some other lipid substrate, is necessary for VEGF or SDF-1α to activate its receptor signaling cascade. Adding 20-HETE partially reversed the inhibitory effects of HET0016. The fact that HET0016 inhibited two separate factors (VEGF and SDF-1α) that act via different receptors suggests that 20-HETE synthesis is an essential component of their common signaling pathway. Further studies are required to elucidate the exact mechanism involved. When we used 20-HEDE, a competitive antagonist of the vasoconstrictor actions of 20-HETE, we found that it markedly reduced EPC migratory responses to VEGF and SDF-1α.

ECs plated on Matrigel formed a network of tube-like structures resembling capillaries, indirectly demonstrating that differentiation of ECs is necessarily the initial step in the angiogenic cascade (Arnaoutova et al., 2009). When human umbilical vascular endothelial cells or EPCs were plated on Matrigel in the absence of growth factors, they did not form tubes; however, when EPCs and ECs were plated together, a network of EC tubes was formed, suggesting that EPCs secrete some factor(s) that induce ECs to differentiate and form tubes that contribute to angiogenesis (Koga et al., 2009). The fact that the tube formation induced by EPCs was inhibited by HET0016, DDMS, and 20-HEDE is consistent with 20-HETE being an important mediator of EPC-induced EC differentiation.

In human microvascular ECs, 20-HETE increases phosphorylation of VEGF and nitric-oxide synthase and also enhances superoxide formation, which in turn increases HIF-1α (Guo et al., 2007, 2009). We found that 20-HETE increased both superoxide and HIF-1α in EPCs. Production of both SDF-1α and VEGF is regulated by HIF-1α (Hoenig et al., 2008), and both were increased by 20-HETE.

Phosphorylation of serine 1177 has been associated with increased synthesis of nitric oxide (Butt et al., 2000), and we found that it was markedly increased in EPCs treated with 20-HETE just as others reported in ECs (Cheng et al., 2008; Bodiga et al., 2010), pointing out the close similarities between these progenitor cells and mature ECs. Both the proliferative and the migratory effects of 20-HETE could be directly due to stimulation of some unknown receptor (Miyata and Roman, 2005), or they might be secondary to increases in nitric oxide and VEGF (Sautina et al., 2010).

The increases in SDF-1α induced by 20-HETE are worth noting. It might be that 20-HETE caused EPCs to attract CXCR4+ cells such as stem cells and leukocytes. CXCL12 (SDF-1)/CXCR4 is believed to be involved in angiogenesis (Teicher, 2011) and has been identified as a key factor in the recruitment of stem cells to injured tissue. Increased expression of SDF-1 secondary to 20-HETE stimulation could have multiple effects, including recruitment of bone marrow-derived stem cells and local inhibition of apoptosis of cells expressing CXCR4 (Penn, 2010).

Our research raises the question of whether EPCs derived from umbilical cords behave like EPCs derived from bone marrow. Functionally, both cell lines are very similar. Ves-
sels derived from the umbilical cord exhibit normal blood flow, permissiveness to macromolecules, and induction of leukocyte–EC interactions in response to cytokine activation just like normal vessels (Au et al., 2008). CD133+ cells derived from cord blood and EPCs derived from mouse bone marrow showed no differences in migration and accumulation (Anderson et al., 2005; Arbab et al., 2006, 2008). Patients at high risk of cardiovascular disease have fewer EPCs, and those they do have exhibit greater senescence in vitro. Treatment with EPCs derived from human umbilical cord blood could help the restore stem cell function and enhance neovascularization (Senegaglia et al., 2010) particularly in the sick and elderly. Future studies should examine whether increasing 20-HETE stimulation of EPCs by overexpression of 20-HETE synthase would heighten production of SDF-1, VEGF, and nitric oxide in these patients, which could increase angiogenic transduction pathways and help in tissue repair.

In summary, AC133+ and CD34+ cells isolated from human umbilical cord blood express mRNA and protein for CYP4A11 and CYP4A22 and produce 20-HETE in the presence of AA. EPCs respond to 20-HETE with increased migration and proliferation and increases in genes known to regulate angiogenesis. Selective inhibitors of 20-HETE synthesis attenuate VEGF-induced migration and proliferation of EPCs and hence EPC-induced EC tube formation. These data suggest that the CYP4A/F-20-HETE system plays an important role in the regulation of the EPC functions associated with angiogenic responses.

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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Guo, Janic, Roman, Edwards, Arbab, and Scicli.

References


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