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⁺CD34⁺ 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 μ M *N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)formamidinium (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-1 α , 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 as-

sociated 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

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ABBREVIATIONS: EPC, endothelial progenitor cell; 20-HEDE, (6,15) 20-hydroxyeicosadienoic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; AA, arachidonic acid; Ct, threshold cycle; CXCR4, chemokine receptor type 4; CYP4A/F, cytochrome P450 4A/F; EC, endothelial cell; HET0016, *N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)formamidinium; HIF-1 α , hypoxia-inducible factor-1 α ; VE, vascular endothelial; MSC, mesenchymal stem cell; PCR, polymerase chain reaction; KDR, kinase insert domain receptor; RT, reverse transcription; SDF-1 α , stroma-derived factor-1 α ; VEGF, vascular endothelial growth factor; bp, basepairs; DDMS, *N*-methylsulfonyl-12,12-dibromododec-11-enamide; PG, prostaglandin; LC-MS/MS, liquid chromatography/tandem mass spectrometry.

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- α (SDF-1 α), typically released by target tissues (Weidt et al., 2007; Folkens et al., 2009).

Cytochrome P450 4A (CYP4A) is a monooxygenase that metabolizes arachidonic acid (AA) to 20-hydroxyeicosatetraenoic acid (20-HETE). We have shown that 20-HETE stimulates production of both VEGF and hypoxia-inducible factor-1 α (HIF-1 α), 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). We also have shown that 20-HETE induces neovascularization in the rat cornea (Chen et al., 2005). 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 CYP4A/F-20-HETE system regulates neovascularization responses by altering proliferation and migration of EPCs. For this, we extracted human umbilical cord blood and isolated mononuclear 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 and are targeted by the proliferative and angiogenic effects of 20-HETE.

Materials and Methods

Isolation and In Vitro Culture of AC133⁺ EPCs. Progenitor cells positive for the CD133⁺ marker (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 40 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 cell concentration at 5×10^5 to 1×10^6 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 5×10^5 cells/ml.

Flow Cytometry of Endothelial Lineage Cell Surface Markers on AC133⁺ EPCs. Cells expanded in suspension under either

normal growth conditions or conditions that facilitate cell differentiation were harvested, washed in ice-cold $1 \times$ 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 CD31 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 μ g) 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 controls.

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 μ g 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'-AATTTGCCATGAACGAACGAGCTGA-3' and reverse 5'-TGTTCCAAAGGCCACAAGG-3'; CYP4A22 forward 5'-AATTTGCCATGAACGAGCTGA-3' and reverse 5'-GGTCCTTGCTTTCACAAGGG-3'; CYP4F2 forward 5'-CCCGAAACGGAATTGGTTCT-3' and reverse 5'-CCAGCAGCACATATCACCAG-3'; CYP4F3 forward 5'-CCCAAGACGGAAGTGGTTT-3' and reverse 5'-GACCTTAAAGCCCTGGGGGT-3'; CYP2J2 forward 5'-ATGCTCGCGCGATGGGCTC-3' and reverse 5'-TAGTGCTGTCAGAGTGAACC-3'; CYP2C8 forward 5'-TCTTACACGAAGTTACATTA-3' and reverse 5'-TAGATCAGCTACAGTGCCAA-3'; CYP2C9 forward 5'-TGAAGAAGAGCAGATGGCCT-3' and reverse 5'-GAATGTTCACTAGATCTTCA-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 μ l of PCR mix include 2 μ l of reverse transcription product, 2 μ l 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 10 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 $2^{-\Delta\Delta Ct}$ 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

Assessment of 20-HETE Formation. For this, 1×10^6 EPCs were either left untreated or treated with $10 \mu\text{M}$ AA overnight before harvesting the cells. Both cells and media were analyzed for the

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.

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

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 μ M) 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-1 α 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-1 α (Fig. 5). In addition, both HET0016 and 20-HETE prevented SDF-1 α from accelerating EPC migration. We also found that addition of 20-HETE partially reversed the inhibitory effect of HET0016 on SDF-1 α -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

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.

Fig. 3. Comparison of CYP4A11 protein and mRNA expression in EPCs and MSCs. A, total EPC and MSC protein lysates were assayed for human CYP4A11 by Western blot. B, mRNA was extracted using TRIzol and reverse-transcribed. cDNA was amplified by RT-PCR using a CYP4A11 primer.

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-1 α , Endothelial Nitric-Oxide Synthase Phosphorylation, and Dihydroethidium Staining. 20-HETE increased expression of HIF-1 α 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-1 α 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

Fig. 4. LC-MS/MS profile of AA metabolites produced by EPCs. Pooled EPCs (1×10^6) were either left untreated or treated with 10 μ M AA overnight before harvesting the cells. Both the EPC growth media and the EPC pellet were extracted using ethyl acetate, and lipids were separated by high-performance liquid chromatography on a C18 column. LC-MS/MS was performed to assess 20-HETE production as we have described previously. A total of 2 ng of d⁶-20-HETE was added to each sample before ethyl acetate extraction served as the internal positive standard.

Fig. 5. Effects of 20-HETE and CYP4A/F inhibitors on EPC proliferation and migration. A, six-well culture plates containing EPCs were treated with either VEGF (20 ng/ml), 20-HETE (1 μ M), or VEGF plus HET0016 (10 μ M) for 48 h. Changes in cell number are measured, normalized, and expressed as a percentage of the control. Means $\pm$ S.D. of three separate experiments (each in duplicate) are shown. **, $p < 0.05$ versus control; ^, $p < 0.05$ versus VEGF alone. B, EPCs were incubated for 16 h (a) in the presence of either 100 ng/ml SDF-1 α , 20 ng/ml VEGF, or 1 μ M 20-HETE and (b) with either 100 ng/ml SDF-1 or 20 ng/ml VEGF in the presence or absence of 10 μ M HET0016 and/or 10 μ M 20-HEDE in separate experiments. In addition, 10 μ M 20-HETE also was added to one group to determine whether it would reverse the inhibitory effect of HET0016 on SDF-1 α -induced EPC migration. Changes in cell numbers 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. Data represent three separate experiments (each performed in duplicate). **, $p < 0.05$ versus control; #, $p < 0.05$ versus SDF-1 α ; ^, $p < 0.05$ versus SDF-1 α and VEGF.

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

Fig. 6. Effects of EPCs and CYP4A/F-20-HETE inhibitors on tube formation by ECs. A total of 1×10^5 ECs either alone or in the presence of 2×10^4 EPCs washed with phosphate-buffered saline were seeded in wells containing Matrigel without serum or growth factors. In some wells, the ability of EPCs to help ECs form tubes also was tested in the presence of a 20-HETE synthase inhibitor, either HET0016 (10 μ M) or DDMS (10 μ M), as well as 20-HETE antagonist 20-HEDE (10 μ M). EC tube formation was assessed after ~18 h of incubation. *, $p < 0.05$ versus control; #, $p < 0.05$ versus EC+EPC.

expressed. Because these P450s may have distinct patterns of cell distribution, our studies still cannot completely rule out the participation of CYP4F3 in AA ω -hydroxylation by EPCs.

Although EPC pellets produced very little 20-HETE under basal conditions, they released substantial amounts into the medium (~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 ω -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 ω -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 ω -hydroxylase inhibitors such as HET0016 suppress responses to VEGF, it seems unlikely

Fig. 7. Effects of 20-HETE on HIF-1α, VEGF, and SDF-1α expression, endothelial nitric-oxide synthase phosphorylation, and superoxide formation in EPCs. A, EPCs were treated with 1 μM 20-HETE, and protein lysates were subjected to Western blot analysis using anti-HIF-1α, anti-VEGF, anti-SDF-1α, and anti-phosphorylated endothelial nitric-oxide synthase serine 1177 antibodies with actin as a loading control. The blot represents three separate experiments. B, EPCs were treated with dihydroethidium for 30 min, and fluorescence microscopy was used to assess formation of superoxide by 20-HETE. Representative images from three separate experiments are shown.

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-1α 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, permselectivity 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 a 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

Participated in research design: Guo, Janic, Edwards, Arbab, Falck, Roman, and Scicli.

Conducted experiments: Guo, Sheng, Roman, and Arbab.

Contributed new reagents or analytic tools: Janic, Falck, and Arbab.

Performed data analysis: Guo, Roman, and Arbab.

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

References

- Anderson SA, Glod J, Arbab AS, Noel M, Ashari P, Fine HA, and Frank JA (2005) Noninvasive MR imaging of magnetically labeled stem cells to directly identify neovascularization in a glioma model. *Blood* **105**:420–425.
- Arbab AS, Janic B, Knight RA, Anderson SA, Pawelczyk E, Rad AM, Read EJ, Pandit SD, and Frank JA (2008) Detection of migration of locally implanted AC133⁺ stem cells by cellular magnetic resonance imaging with histological findings. *FASEB J* **22**:3234–3246.
- Arbab AS, Pandit SD, Anderson SA, Yocum GT, Bur M, Frenkel V, Khuu HM, Read EJ, and Frank JA (2006) Magnetic resonance imaging and confocal microscopy studies of magnetically labeled endothelial progenitor cells trafficking to sites of tumor angiogenesis. *Stem Cells* **24**:671–678.
- Arnaoutova I, George J, Kleinman HK, and Benton G (2009) The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis* **12**:267–274.
- Au P, Daheron LM, Duda DG, Cohen KS, Tyrrell JA, Lanning RM, Fukumura D, Scadden DT, and Jain RK (2008) Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. *Blood* **111**:1302–1305.
- Bodiga S, Gruenloh SK, Gao Y, Manthathi VL, Dubasi N, Falck JR, Medhora M, and Jacobs ER (2010) 20-HETE-induced nitric oxide production in pulmonary artery endothelial cells is mediated by NADPH oxidase, H₂O₂, and PI3-kinase/Akt. *Am J Physiol Lung Cell Mol Physiol* **298**:L564–L574.
- Butt E, Bernhardt M, Smolenski A, Kotsonis P, Fröhlich LG, Sickmann A, Meyer HE, Lohmann SM, and Schmidt HH (2000) Endothelial nitric-oxide synthase (type III) is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases. *J Biol Chem* **275**:5179–5187.
- Buysschaert I, Carmeliet P, and Dewerchin M (2007) Clinical and fundamental aspects of angiogenesis and anti-angiogenesis. *Acta Clin Belg* **62**:162–169.
- Carmeliet P (2005) Angiogenesis in life, disease and medicine. *Nature* **438**:932–936.
- Chen P, Guo M, Wygle D, Edwards PA, Falck JR, Roman RJ, and Scicli AG (2005) Inhibitors of cytochrome P450 4A suppress angiogenic responses. *Am J Pathol* **166**:615–624.
- Cheng J, Ou JS, Singh H, Falck JR, Narsimhaswamy D, Pritchard KA Jr, and Schwartzman ML (2008) 20-Hydroxyeicosatetraenoic acid causes endothelial dysfunction via eNOS uncoupling. *Am J Physiol Heart Circ Physiol* **294**:H1018–H1026.
- Folkens C, Shaked Y, Man S, Tang T, Lee CR, Zhu Z, Hoffman RM, and Kerbel RS (2009) Glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1. *Cancer Res* **69**:7243–7251.
- Gebremedhin D, Lange AR, Lowry TF, Taheri MR, Birks EK, Hudetz AG, Narayanan J, Falck JR, Okamoto H, Roman RJ, et al. (2000) Production of 20-HETE and its role in autoregulation of cerebral blood flow. *Circ Res* **87**:60–65.
- Guo AM, Arbab AS, Falck JR, Chen P, Edwards PA, Roman RJ, and Scicli AG (2007) Activation of vascular endothelial growth factor through reactive oxygen species mediates 20-hydroxyeicosatetraenoic acid-induced endothelial cell proliferation. *J Pharmacol Exp Ther* **321**:18–27.
- Guo AM, Scicli G, Sheng J, Falck JC, Edwards PA, and Scicli AG (2009) 20-HETE can act as a nonhypoxic regulator of HIF-1α in human microvascular endothelial cells. *Am J Physiol Heart Circ Physiol* **297**:H602–H613.
- Guo AM, Sheng J, Scicli GM, Arbab AS, Lehman NL, Edwards PA, Falck JR, Roman RJ, and Scicli AG (2008) Expression of CYP4A1 in U251 human glioma cell induces hyperproliferative phenotype in vitro and rapidly growing tumors in vivo. *J Pharmacol Exp Ther* **327**:10–19.
- Guo M, Roman RJ, Falck JR, Edwards PA, and Scicli AG (2005) Human U251 glioma cell proliferation is suppressed by HET0016 [N-hydroxy-N'-(4-butyl-2-methylphenyl)formamidin], a selective inhibitor of CYP4A. *J Pharmacol Exp Ther* **315**:526–533.
- Guo M, Roman RJ, Fenstermacher JD, Brown SL, Falck JR, Arbab AS, Edwards PA, and Scicli AG (2006) 9L gliosarcoma cell proliferation and tumor growth in rats are suppressed by N-hydroxy-N'-(4-butyl-2-methylphenol) formamidin (HET0016), a selective inhibitor of CYP4A. *J Pharmacol Exp Ther* **317**:97–108.
- Harris DT and Rogers I (2007) Umbilical cord blood: a unique source of pluripotent stem cells for regenerative medicine. *Curr Stem Cell Res Ther* **2**:301–309.
- Hoening MR, Bianchi C, and Sellke FW (2008) Hypoxia inducible factor-1 α, endothelial progenitor cells, monocytes, cardiovascular risk, wound healing, cobalt and hydralazine: a unifying hypothesis. *Curr Drug Targets* **9**:422–435.
- Janic B and Arbab AS (2010) The role and therapeutic potential of endothelial progenitor cells in tumor neovascularization. *ScientificWorldJournal* **10**:1088–1099.
- Janic B, Guo AM, Iskander AS, Varma NR, Scicli AG, and Arbab AS (2010) Human cord blood-derived AC133⁺ progenitor cells preserve endothelial progenitor characteristics after long term in vitro expansion. *PLoS One* **5**:e9173.
- Jarajapu YP and Grant MB (2010) The promise of cell-based therapies for diabetic complications: challenges and solutions. *Circ Res* **106**:854–869.
- Jiang M, Mezentssev A, Kemp R, Byun K, Falck JR, Miano JM, Nasjletti A, Abraham NG, and Laniado-Schwartzman M (2004) Smooth muscle-specific expression of CYP4A1 induces endothelial sprouting in renal arterial microvessels. *Circ Res* **94**:167–174.
- Kirton JP and Xu Q (2010) Endothelial precursors in vascular repair. *Microvasc Res* **79**:193–199.
- Koga M, Sudo R, Abe Y, Yamamoto K, Ando J, Ikeda M, and Tanishita K (2009) Contribution of rat endothelial progenitor cells on three-dimensional network formation in vitro. *Tissue Eng Part A* **15**:2727–2739.
- Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, and Young PP (2006) VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB J* **20**:1495–1497.
- Miyata N and Roman RJ (2005) Role of 20-hydroxyeicosatetraenoic acid (20-HETE) in vascular system. *J Smooth Muscle Res* **41**:175–193.
- Patenaude A, Parker J, and Karsan A (2010) Involvement of endothelial progenitor cells in tumor vascularization. *Microvasc Res* **79**:217–223.
- Pearson JD (2010) Endothelial progenitor cells—an evolving story. *Microvasc Res* **79**:162–168.
- Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, et al. (2000) Expression of VEGFR-2 and AC133 by circulating human CD34⁺ cells identifies a population of functional endothelial precursors. *Blood* **95**:952–958.
- Penn MS (2010) SDF-1: CXCR4 axis is fundamental for tissue preservation and repair. *Am J Pathol* **177**:2166–2168.
- Roman RJ (2002) P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* **82**:131–185.
- Rosti V, Massa M, Campanelli R, De Amici M, Piccolo G, and Perfetti V (2007) Vascular endothelial growth factor promoted endothelial progenitor cell mobilization into the peripheral blood of a patient with POEMS syndrome. *Haematologica* **92**:1291–1292.
- Sautina L, Sautin Y, Beem E, Zhou Z, Schuler A, Brennan J, Zharikov SI, Diao Y, Bungert J, and Segal MS (2010) Induction of nitric oxide by erythropoietin is mediated by the (β) common receptor and requires interaction with VEGF receptor 2. *Blood* **115**:896–905.
- Senegaglia AC, Barboza LA, Dallagiovanna B, Aita CA, Hansen P, Rebelatto CL, Aguiar AM, Miyagoe NI, Shigunov P, Barchiki F, et al. (2010) Are purified or expanded cord blood-derived CD133⁺ cells better at improving cardiac function? *Exp Biol Med (Maywood)* **235**:119–129.

- Teicher BA (2011) Antiangiogenic agents and targets: a perspective. *Biochem Pharmacol* **81**:6–12.
- Tepper OM, Capla JM, Galiano RD, Ceradini DJ, Callaghan MJ, Kleinman ME, and Gurtner GC (2005) Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow-derived cells. *Blood* **105**:1068–1077.
- Weidt C, Niggemann B, Kasenda B, Drell TL, Zänker KS, and Dittmar T (2007) Stem cell migration: a quintessential stepping stone to successful therapy. *Curr Stem Cell Res Ther* **2**:89–103.
- Zhu D, Zhang C, Medhora M, and Jacobs ER (2002) CYP4A mRNA, protein, and product in rat lungs: novel localization in vascular endothelium. *J Appl Physiol* **93**:330–337.

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