Commentary

P450 in the Angiogenesis Affair

The Unusual Suspect

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Angiogenesis is a fundamental process of growth and differentiation of new blood vessels in the body. Angiogenesis is new vessel growth from pre-existing vessels by budding, sprouting, and subsequent formation of patent capillaries with lumens, whereas vasculogenesis is new vessel growth from endothelial cell precursors, or stem cells.^{1,2} Both processes occur at different times in different tissues and are likely regulated by complex sets of effectors in physiological and pathological conditions. In recent years, many potent peptide growth factors and cytokines were identified that regulate the angiogenesis process. They include basic fibroblast growth factor (FGF-2), vascular endothelial (VEGF), insulin-like (IGF-I), hepatocyte (HGF), platelet-derived (PDGF), placenta (PIGF), pigment epithelium-derived (PEDF), transforming (TGF- β) growth factors, stromal-derived factor (STF), interleukin (IL)-6, -8, and -10, tumor necrosis factor (TNF)- α , and angiopoietins (for review, see³). Some of these factors are proangiogenic, whereas the others are antiangiogenic. Angiogenic factors act on endothelial cells through cell surface receptors that mediate downstream signaling eliciting mitogenic and motogenic cell responses. In recent years, some signaling pathways of angiogenic growth factor/cytokine receptors have been unraveled. For instance, the importance of protein kinase C β (PKC β) in VEGF signaling has been emphasized and exploited for therapeutic purposes.⁴ At the same time, many aspects of receptor signaling that mediate angiogenic responses remain unclear.³

One of the gaps in our understanding of downstream cellular events leading to angiogenesis seems to be cleared in a publication appearing in the current issue of *American Journal of Pathology* (Chen et al, Am J Pathol, 2005, 165:615-624). In this paper from Scicli's group,⁵ it

is shown that cytochrome P450 4A (CYP4A) plays an important role in angiogenesis. Pharmacological inhibition of CYP4A activity with N-hydroxy-N'-(4-butyl-2-methylphenol) formamidine (HET0016) blocked VEGF-induced endothelial cell proliferation *in vitro*. Furthermore, HET0016 inhibited growth factor-induced angiogenesis in the corneal micropocket model and in the model of corneal angiogenesis induced by glioblastoma implantation. The authors identified 20-hydroxyeicosatetraenoic acid (20-HETE) as a CYP4A metabolite that likely stimulated angiogenesis. They went on to show that a 20-HETE agonist was able to induce endothelial cell mitogenesis *in vitro* and angiogenesis in the rat cornea model. The data clearly implicate CYP4A in angiogenesis and suggest a possible novel way to block this process by pharmacological inhibition of the enzyme. This may be very important for developing drugs aimed at counteracting pathological neovascularization as seen in solid tumors and in various proliferative retinopathies.⁶

The cytochrome P450 (CYP) family of enzymes has been long known to mediate detoxification of steroid hormones, vitamins, xenobiotics, and various drugs.⁷ Many CYPs are expressed in liver, although there are isoforms specific for other tissues, such as heart, vasculature, kidney, lung, and smooth muscle.^{7,8} In addition to the above-mentioned substrates, these microsomal enzymes can also metabolize arachidonic acid. This capacity makes them important players in the regulation of vascular tone, blood flow, and angiogenesis.⁹⁻¹³

Arachidonic acid is the precursor of pro-inflammatory eicosanoids. It is produced from membrane phospholip-

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ids by phospholipase A₂ in response to different stimuli and may be metabolized by several enzymes to yield a variety of important biological mediators. Cyclooxygenases produce prostaglandins and lipoxygenases generate leukotrienes¹⁴ from arachidonic acid. Additionally, arachidonic acid can be metabolized by CYP4A to 20-HETE or by CYP2C and CYP2W to epoxyeicosatrienoic acids (EET). These metabolites have varying effects on blood flow. Whereas EETs are vasorelaxants, 20-HETE is generally a potent vasoconstrictor.¹³ However, in pulmonary arteries 20-HETE may act as vasorelaxant.¹⁵ 20-HETE mediates vascular tone increase through inhibition of K⁺_{Ca} channels, membrane depolarization and further increase in Ca²⁺.^{7,10,16} It is also a vascular oxygen sensor.¹¹ Formation of 20-HETE can be regulated by nitric oxide that inactivates CYP4A.^{17,18} 20-HETE is an important signaling molecule of hormonal systems, such as endothelin-1 and angiotensin II.⁷

What is the evidence for the participation of CYP4A and its product, 20-HETE, in angiogenesis? Sa et al¹⁹ obtained the first data supporting the role of CYP4A and 20-HETE in FGF-2-mediated angiogenesis. They showed that FGF-2 was able to activate phospholipase A₂ in endothelial cells. This could be due to FGF-2-induced increase in the production of arachidonic acid, in turn stimulating CYP4A. Further evidence was obtained by Amaral et al¹⁰ who showed that angiogenesis induced by electrical stimulation in skeletal muscle was dependent on 20-HETE. They made an interesting observation that a neutralizing antibody to a potent angiogenic factor, VEGF, blocked 20-HETE increases induced by stimulation. These results suggested that 20-HETE was important for induction of angiogenesis and that its formation was VEGF-dependent. Presumably, VEGF could increase 20-HETE by stimulating phospholipase A₂²⁰ and arachidonic acid production. Most recently, Jiang et al¹² used adenovirus-mediated transfer to transfect smooth muscle cells and microdissected renal arteries with CYP4A gene. The expression of transfected CYP4A elicited angiogenic activity in the arteries manifested by markedly increased endothelial cell sprouting.

The paper by Chen et al⁵ published in this issue of the *American Journal of Pathology* marks another important step in our understanding of the role of CYP4A and 20-HETE as mediators of growth factor-mediated angiogenesis. The authors have convincingly shown that CYP4A inhibitors abrogated angiogenic response to VEGF, FGF-2, and EGF in an *in vivo* corneal neovascularization model. This effect may of course be explained in different ways. One may postulate a direct role for CYP4A-produced 20-HETE as a signaling molecule downstream of the angiogenic growth factor receptors. As discussed above, there is experimental evidence in support of this mechanism. An alternative possibility is that the growth factors triggered the production of arachidonic acid metabolites that are pro-inflammatory. These products including 20-HETE could mediate recruitment of polymorphonuclear leukocytes and later, macrophages. These cells produce and secrete VEGF, and are thought to be the main mediators of corneal neovascularization.²¹ This would explain why CYP4A in-

hibitors could abrogate the effects of different growth factors with similar efficiencies.⁵ The latter possibility does not exclude a direct effect of 20-HETE, which may be induced by macrophage-produced VEGF. It would be interesting to sort out these issues in future experiments.

The paper by Chen et al⁵ also describes the use of glioblastoma cells implanted in the cornea. Tumor cells elicit a strong angiogenic response that is inhibited by CYP4A blockers. Because glioblastoma cells secrete angiogenic growth factors, there is reason to believe that they may induce corneal angiogenesis through the action of these factors, eg, VEGF. Since the corneal model has some limitations related to its inflammatory nature,²¹ it may be important to further examine the effects of CYP4A inhibitors using tumors grown in other sites, eg, intracranially or subcutaneously.

The development of pathological neovascularization is often associated with hypoxia/ischemia, as observed in malignant tumors and proliferative retinopathies. Hypoxia is known to stimulate important angiogenic mediators including VEGF. This occurs through activation of transcription factor HIF-1 α that increases VEGF expression.^{22,23} The work by Chen et al⁵ now has established a link between CYP4A and VEGF, and it would be interesting to examine the effects of hypoxia/ischemia on this cytochrome and on its angiogenic product, 20-HETE, in conditions promoting angiogenesis. The available data in this area remain controversial. Some members of the CYP family are upregulated by hypoxia. CYP3A6 and CYP4B1, for instance, are directly increased by hypoxia through HIF-1 α mechanism.^{24,25} Moreover, corneal CYP4B1 is increased by hypoxia in parallel with VEGF.²⁶ Interestingly, blockade of phospholipase A₂ during hypoxia in retinal endothelial cells could inhibit stimulation of VEGF production.²⁷ However, in the rat renal artery and vein ischemia-reperfusion injury model, CYP4A and 20-HETE are both decreased.²⁸ These data pertain to 20-HETE measurements not during ischemia but after 3 hours or more of reperfusion, which may have influenced the results. In contrast, recent data in the heart ischemia-reperfusion model show that levels of both CYP4A and 20-HETE increase in the ischemic phase and shortly thereafter. Moreover, exogenous 20-HETE significantly increases infarct size, possibly by exerting its vasoconstricting effect.²⁹ Taken together, there is a need for expanding these studies using other hypoxic/ischemic models with developing neovascularization, for example, models of proliferative retinopathy or experimental tumor growth.

Critical questions to answer in future experiments concern the mechanism by which CYP4A participates in angiogenesis through production of 20-HETE, and the interaction of CYP system with growth factors in the angiogenic process. In other words, what are the downstream signaling events where growth factors, eg, VEGF, converge with the CYP system? Angiogenic growth factors signal through their surface receptors most of which belong to the receptor-type tyrosine kinase class.^{3,4,22} Some growth factors including VEGF have more than one receptor. Signaling pathways of these receptors may be different, which may ensure fine-tuning of the system

depending on the tissue needs.²² Although there is some convergence in signaling pathways of various angiogenic growth factor receptors, they generally use different intermediates, which forms the basis for growth factor synergy.³⁰ Such a synergy makes it difficult to counteract the action of several growth factors together for therapeutic purposes against pathological neovascularization. Therefore, inhibitors of key downstream signaling molecules may prove useful for developing more efficient drugs against unwanted neovascularization.

It was shown recently that contraction of small coronary arteries by 20-HETE depends on the activation of Rho family of small GTPases.³¹ At the same time, Rho is needed for activation of serum response factor, a transcription factor that plays a critical role in VEGF signaling.³² Rho system also mediates induction of endothelial and tumor cell migration by FGF-2, IGF-I, and PDGF.^{33–35} This is the first point of convergence between CYP4A system and angiogenic growth factors. Moreover, both 20-HETE and growth factors are involved in signaling via mitogen-activated protein (MAP) kinase-Ras pathway that is important for growth factor-mediated angiogenesis.^{3,22,36–38} Finally, 20-HETE can activate PKC,³⁹ which is an important signaling mediator of angiogenic growth factors.⁴ In the paper by Chen et al⁵ in this issue of *American Journal of Pathology*, it was shown that CYP4A inhibitors abrogated the mitogenic action of VEGF on the endothelial cells. The analysis of the literature presented above allows one to assume that such inhibitors would also block the action of other angiogenic growth factors. CYP4A and its product, 20-HETE, are, therefore, emerging as novel mediators of angiogenesis that are closely linked to signaling pathways of major angiogenic growth factors. The ability of CYP4A to activate important angiogenic signaling pathways through 20-HETE makes it a very attractive target for future antiangiogenic therapies. An important advantage of CYP4A inhibitors would be their ability to counteract the action of several growth factors together for a more complete blocking of the angiogenic cascade.

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References

1. Bdolah Y, Sukhatme VP, Karumanchi SA: Angiogenic imbalance in the pathophysiology of preeclampsia: newer insights. *Semin Nephrol* 2004, 24:548–556
2. Chan-Ling T, McLeod DS, Hughes S, Baxter L, Chu Y, Hasegawa T, Luty GA: Astrocyte-endothelial cell relationships during human retinal vascular development. *Invest Ophthalmol Vis Sci* 2004, 45:2020–2032
3. Grant MB, Afzal A, Spoerri P, Pan H, Shaw LC, Mames RN: The role of growth factors in the pathogenesis of diabetic retinopathy. *Expert Opin Investig Drugs* 2004, 13:1275–1293
4. He Z, King GL: Protein kinase C β isoform inhibitors: a new treatment for diabetic cardiovascular diseases. *Circulation* 2004, 110:7–9
5. Chen P, Guo M, Wygle D, Edwards P, Falck JR, Roman RJ, Scicli AG: Inhibitors of cytochrome P450 4A suppress angiogenic responses. *Am J Pathol* 2005, 166:615–624
6. Folkman J: Fundamental concepts of the angiogenic process. *Curr Mol Med* 2003, 3:643–651
7. Fleming I: Cytochrome P450 enzymes in vascular homeostasis. *Circ Res* 2001, 89:753–762
8. Zhu D, Zhang C, Medhora M, Jacobs ER: CYP4A mRNA, protein, and product in rat lungs: novel localization in vascular endothelium. *J Appl Physiol* 2002, 93:330–337
9. Harder DR, Roman RJ, Gebremedhin D: Molecular mechanisms controlling nutritive blood flow: role of cytochrome P450 enzymes. *Acta Physiol Scand* 2000, 168:543–549
10. Amaral SL, Maier KG, Schippers DN, Roman RJ, Greene AS: CYP4A metabolites of arachidonic acid and VEGF are mediators of skeletal muscle angiogenesis. *Am J Physiol* 2003, 284:H1528–H1535
11. Sacerdoti D, Gatta A, McGiff JC: Role of cytochrome P450-dependent arachidonic acid metabolites in liver physiology and pathophysiology. *Prostaglandins Other Lipid Mediat* 2003, 72:51–71
12. Jiang M, Mezentsev A, Kemp R, Byun K, Falck JR, Miano JM, Nasjletti A, Abraham NG, Laniado-Schwartzman M: Smooth muscle-specific expression of CYP4A1 induces endothelial sprouting in renal arterial microvessels. *Circ Res* 2004, 94:167–174
13. Pratt PF, Medhora M, Harder DR: Mechanisms regulating cerebral blood flow as therapeutic targets. *Curr Opin Investig Drugs* 2004, 5:952–956
14. Homaidan FR, Chakroun I, Haidar HA, El-Sabban ME: Protein regulators of eicosanoid synthesis: role in inflammation. *Curr Protein Pept Sci* 2002, 3:467–484
15. Yu M, McAndrew RP, Al-Saghir R, Maier KG, Medhora M, Roman RJ, Jacobs ER: Nitric oxide contributes to 20-HETE-induced relaxation of pulmonary arteries. *J Appl Physiol* 2002, 93:1391–1399
16. Sarkis A, Roman RJ: Role of cytochrome P450 metabolites of arachidonic acid in hypertension. *Curr Drug Metab* 2004, 5:245–256
17. Juncos LA, Garvin J, Carretero OA, Ito S: Flow modulates myogenic responses in isolated microperfused rabbit afferent arterioles via endothelium-derived nitric oxide. *J Clin Invest* 1995, 95:2741–2748
18. Lopez B, Moreno C, Salom MG, Roman RJ, Fenoy FJ: Role of guanylyl cyclase and cytochrome P-450 on renal response to nitric oxide. *Am J Physiol* 2001, 281:F420–F427
19. Sa G, Murugesan G, Jaye M, Ivashchenko Y, Fox PL: Activation of phospholipase A₂ by basic fibroblast growth factor via a p42 mitogen-activated protein kinase-dependent phosphorylation pathway in endothelial cells. *J Biol Chem* 1995, 270:2360–2366
20. Wheeler-Jones C, Abu-Ghazaleh R, Cospedal R, Houliston RA, Martin J, Zachary I: Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A₂ in endothelial cells via p42/p44 mitogen-activated protein kinase. *FEBS Lett* 1997, 420:28–32
21. Edelman JL, Castro MR, Wen Y: Correlation of VEGF expression by leukocytes with the growth and regression of blood vessels in the rat cornea. *Invest Ophthalmol Vis Sci* 1999, 40:1112–1123
22. Ferrara N: Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004, 25:581–611
23. Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber HP, Ferrara N, Johnson RS: Loss of HIF-1 α in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell* 2004, 6:485–495
24. Fradette C, du Souich P: Hypoxia-inducible factor-1 and activator protein-1 modulate the upregulation of CYP3A6 induced by hypoxia. *Br J Pharmacol* 2003, 140:1146–1154
25. Mastuygin V, Mezentsev A, Zhang WX, Ashkar S, Dunn MW, Laniado-Schwartzman M: Promoter activity and regulation of the corneal CYP4B1 gene by hypoxia. *J Cell Biochem* 2004, 91:1218–1238
26. Mastuygin V, Mosaed S, Bonazzi A, Dunn MW, Schwartzman ML: Corneal epithelial VEGF and cytochrome P450 4B1 expression in a rabbit model of closed eye contact lens wear. *Curr Eye Res* 2001, 23:1–10
27. Ottino P, Finley J, Rojo E, Ottlecz A, Lambrou GN, Bazan HE, Bazan NG: Hypoxia activates matrix metalloproteinase expression and the VEGF system in monkey choroid-retinal endothelial cells: involvement of cytosolic phospholipase A₂ activity. *Mol Vis* 2004, 10:341–350
28. Hercule H, Oyekan A: Renal cytochrome P450 oxygenases and preglomerular vascular response to arachidonic acid and endothelin-1

- following ischemia/reperfusion. *J Pharmacol Exp Ther* 2002, 302: 717–724
29. Nithipatikom K, Gross ER, Endsley MP, Moore JM, Isbell MA, Falck JR, Campbell WB, Gross GJ: Inhibition of cytochrome P450 ω -hydroxylase: a novel endogenous cardioprotective pathway. *Circ Res* 2004, 95:e65–e71
 30. Castellon R, Hamdi HK, Sacerio I, Aoki AM, Kenney MC, Ljubimov AV: Effects of angiogenic growth factor combinations on retinal endothelial cells. *Exp Eye Res* 2002, 74:523–535
 31. Randriamboavonjy V, Busse R, Fleming I: 20-HETE-induced contraction of small coronary arteries depends on the activation of Rho-kinase. *Hypertension* 2003, 41:801–806
 32. Chai J, Jones MK, Tarnawski AS: Serum response factor is a critical requirement for VEGF signaling in endothelial cells and VEGF-induced angiogenesis. *FASEB J* 2004, 18:1264–1266
 33. Kanda S, Miyata Y, Kanetake H: Role of focal adhesion formation in migration and morphogenesis of endothelial cells. *Cell Signal* 2004, 16:1273–1281
 34. Fukuyama R, Fujita T, Azuma Y, Hirano A, Nakamuta H, Koida M, Komori T: Statins inhibit osteoblast migration by inhibiting Rac-Akt signaling. *Biochem Biophys Res Commun* 2004, 315:636–642
 35. Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y: Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol Cell Biol* 2000, 20:9247–9261
 36. Parmentier JH, Muthalif MM, Nishimoto AT, Malik KU: 20-Hydroxyeicosatetraenoic acid mediates angiotensin II-induced phospholipase D activation in vascular smooth muscle cells. *Hypertension* 2001, 37:623–629
 37. Sun CW, Falck JR, Harder DR, Roman RJ: Role of tyrosine kinase and PKC in the vasoconstrictor response to 20-HETE in renal arterioles. *Hypertension* 1999, 33:414–418
 38. Muthalif MM, Benter IF, Karzoun N, Fatima S, Harper J, Uddin MR, Malik KU: 20-Hydroxyeicosatetraenoic acid mediates calcium/calmodulin-dependent protein kinase II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *Proc Natl Acad Sci USA* 1998, 95:12701–12706
 39. Nowicki S, Chen SL, Aizman O, Cheng XJ, Li D, Nowicki C, Nairn A, Greengard P, Aperia A: 20-Hydroxyeicosatetraenoic acid (20 HETE) activates protein kinase C: role in regulation of rat renal Na⁺,K⁺-ATPase. *J Clin Invest* 1997, 99:1224–1230