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Regulation of endothelial cell functions by basement membrane- and arachidonic acid-derived products

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Abstract

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is required for normal physiological as well as pathological events. The angiogenic process requires endothelial cells to proliferate, migrate, and undergo tubulogenesis. These multistep processes necessitate secretion of pro-angiogenic growth factors, activation of specific intracellular signaling, and interaction of endothelial cells with basement membrane (BM) extracellular matrix components. The generation and release of angiogenic molecules are highly regulated and are influenced by numerous factors, including BM-derived fragments, proteolytic enzymes, as well as metabolites of arachidonic acid (AA). The interactions between these key modulators of angiogenesis is extremely complex, as AA metabolites can regulate the synthesis of soluble angiogenic factors, BM components, as well as enzymes capable of cleaving BM components, which result in the generation of pro- and/or anti-angiogenic products. Furthermore, some BM-derived fragments can alter the expression of AA-converting enzymes and consequently the synthesis of angiogenic factors. In this review we describe the relationship between BM components and AA metabolites with respect to the regulation of endothelial cell functions in health and disease.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is required for both physiological and pathological events, such as embryonic development, wound healing, chronic inflammation, and cancer.¹ In tumor formation, blood vessel recruitment is critical, as tumors can only reach a few cubic millimeters before their mass becomes self-limiting, resulting in hypoxia and necrosis.² For angiogenesis to occur, endothelial cells need to proliferate, migrate, and undergo tubulogenesis. These processes require growth factors, intracellular signaling, as well as interactions with basement membrane (BM) extracellular matrix components and their cleavage products. In addition, it is now accepted that components of the innate immune system, in addition to providing defense mechanisms against many pathogens, are also involved in the control of endothelial cell functions by playing both anti- and pro-angiogenic roles. In this context, the contribution of macrophages, dendritic cells, neutrophils, T cells, as well as mast cells in angiogenesis has been extensively investigated [reviewed in 3–8].

In physiological conditions, endothelial cell functions are tightly regulated by both pro- and anti-angiogenic factors; however in pathological events, such as cancer, there is excess synthesis of pro-angiogenic factors, which leads to increased endothelial cell proliferation,

migration, and tubulogenesis. Generation and release of angiogenic molecules are influenced by numerous factors, including hypoxia, immunologically derived cytokines, proteolytic enzymes, as well as the products of enzymes classically involved in the metabolism of arachidonic acid (AA), such as cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 monooxygenases.^{9–15} In addition to promoting the synthesis of soluble angiogenic factors, AA-converting enzymes regulate the synthesis of BM components^{16–18} as well as enzymes capable of cleaving BM components, thereby generating pro- and/or anti-angiogenic products.^{19–23} Finally, as discussed in detail below, some of these BM-derived factors can alter the expression of endothelial AA-converting enzymes and consequently the synthesis of angiogenic factors.²⁴ In this review we describe how the functions of BM components, COXs, LOXs, and cytochrome P450 monooxygenases, are interlinked with respect to the regulation of endothelial cells in angiogenesis (see also Figure 1). We also discuss how these components might be targeted to inhibit and ideally prevent pathological-associated angiogenesis.

THE ROLE OF BM-DERIVED PRODUCTS IN ANGIOGENESIS

BMs are specialized structures that separate epithelial/endothelial elements from the surrounding stroma. They are composed of many constituents including nonfibrillar collagens (i.e., collagens IV, XV, and XVIII), laminins (i.e., LM-111, LM-332, LM-511, and LM-521), nidogens, agrins, and proteoglycans (i.e., perlecan) [reviewed in 25–29]. Under physiological conditions, the levels of BM components are regulated by a fine equilibrium between synthesis and degradation. BM degradation involves the synthesis and secretion of proteolytic enzymes including cathepsins, the urokinase plasminogen activator system, bone morphogenetic protein/Tolloid-like family of metalloproteases, and matrix metalloproteinases (MMP) (see below for detail). In pathological conditions, excessive degradation of BM components might lead to the generation and release of BM-derived cleavage products capable of altering cell functions. In this context, the observation that vascular BM cleavage products exert anti-angiogenic and/or anti-tumorigenic properties initiated a series of *in vitro* and *in vivo* studies to determine their role as possible anticancer therapies. Table 1 summarizes the BM-derived products described in detail in this article that primarily affect endothelial cell functions.

Perlecan and Angiogenesis

Perlecan, a highly conserved modular heparan sulfate proteoglycan, is one of the most abundant heparan sulfate proteoglycans (HSPGs) of the vertebrate BMs.⁶⁴ The observation that perlecan-null mice show vasculogenesis defects suggested a major role for this HSPG in the control of endothelial cell functions [reviewed in 26]. In addition, the findings that (1) intact perlecan stimulates angiogenesis in a rabbit ear model;⁶⁵ (2) its expression is upregulated in tumor-associated blood vessel;⁶⁶ and (3) downregulation of tumor-derived perlecan results in inhibition of tumor growth and angiogenesis⁶⁷ strongly indicate that this HSPG possesses all the characteristic of a pro-angiogenic factor [also reviewed in 26].

While the intact molecule is a pro-angiogenic factor, endorepellin, the most C-terminus domain of perlecan (also known as domain V), potently inhibits endothelial cell migration and tubulogenesis *in vitro*, as well as angiogenesis in the chorioallantoic membrane and matrigel plug assays.³⁰ Endorepellin is composed of three large globular (LG) domains, named LG1–LG3, with the LG3 motif mediating most of the anti-apoptotic activity of endorepellin.³¹ Endorepellin is able to induce disassembly of the actin cytoskeleton and focal adhesions via interaction with integrin $\alpha_2\beta_1$,³¹ and the endorepellin/integrin $\alpha_2\beta_1$ axis is required for endorepellin-mediated anti-angiogenic activity *in vivo*.³² The finding that exogenously administered endorepellin to tumor-bearing mice not only targets the tumor vasculature but also leads to inhibition of tumor angiogenesis strongly suggests that this

perlecan fragment is an effective anti-angiogenic agent and potentially an anticancer therapeutic.³³

Laminins and Angiogenesis

Laminins are large heterotrimeric glycoproteins composed of one α , one β , and one γ chain.⁶⁸ There are currently five α , four β , and three γ chain genes described in vertebrates and the chains can assemble into at least 15 different heterotrimers [reviewed in 27]. The laminin network is absolutely required for the initial assembly of BMs, and certain laminins (i.e., LM-411, LM-511, and LM-521) are found in various parts of the vasculature. Although mutations in some of these relevant laminin chain genes have no or only modest effects on the integrity of the vasculature [reviewed in 27], cleavage products of some laminin chains and/or expression of laminins not classically associated with the vasculature profoundly impact endothelial cell biology in both physiological and pathological conditions. In this regard, intact LM-111, normally expressed during early embryogenesis,⁶⁹ promotes differentiation of endothelial cells *in vitro*, and angiogenesis in the chicken chorioallantoic membrane assay.⁷⁰ In addition, generation of synthetic overlapping peptides spanning the entire laminin α 1 chain led to the identification of seven peptides capable of promoting endothelial cell adhesion and sprouting *in vitro*, as well as angiogenesis *in vivo*.³⁴ Finally, peptide screening of the laminin α 1 and β 1 chains identified 13 peptides capable of stimulating endothelial sprouting and migration.³⁷ Although the mechanisms of action of these fragments are unknown, it has been shown that at least one of the highly angiogenic α 1-derived peptide binds to integrins α 5 β 1 and α v β 3³⁵ and induces MMP9 synthesis and secretion, thereby promoting matrix degradation.³⁶

Analysis of the laminin α 4 subunit led to the identification of a G domain within this chain capable of supporting endothelial cell adhesion and survival. Antibodies directed against this G domain inhibited endothelial cell proliferation and enhanced apoptosis by preventing α 4 subunit-integrin β 1 interactions.³⁸ Together, these studies indicate that several active domains of LM-111 or α 4-containing laminins may play important roles in controlling different steps in angiogenesis.

Collagen IV and angiogenesis

Collagen IV consists of a family of six homologous α chains (α 1– α 6), each characterized by a short 7 S domain at the N-terminal, a long collagenous domain that occupies the midsection of the molecule, and a noncollagenous (NC) domain positioned at the C-terminal.⁷¹ The NC1 domains of some of the α (IV) chains were originally shown to exert anti-angiogenic activity in the chick cam model.⁴⁰ Since then, numerous studies have been performed to investigate the anti-angiogenic role of these fragments in pathological conditions such as tumor growth and metastasis. The α 1(IV)NC1 domain was demonstrated to prevent endothelial cell proliferation, migration, and tube formation by interacting with integrin α 1 β 1,³⁹ and the α 2(IV)NC1 domain promotes apoptosis and inhibits endothelial cell proliferation, migration, and tube formation^{41–43} by integrin α 1-, α 3-, and α 5-dependent mechanisms.^{40,43,44}

The best studied collagen IV fragment with anti-angiogenic activity is the α 3(IV)NC1 domain. This domain was originally demonstrated to prevent basic fibroblast growth factor-driven angiogenesis in the chick chorioallantoic membrane angiogenesis assay⁴⁰ and later shown to prevent tumor-associated angiogenesis *in vivo* and to inhibit endothelial cell tube formation *in vitro*.⁴⁵ The mechanism whereby this fragment exerts its anti-angiogenic effect is controversial. One group proposes that the peptide sequence encompassing residues 54–132 of the α 3(IV)NC1 domain is responsible for specific anti-angiogenic activity.^{46,72,73} This group claims that the anti-angiogenic activity is exclusively due to interactions between

non-RGD motifs of the $\alpha 3(\text{IV})\text{NC1}$ domain and integrin $\alpha 3$.⁴⁶ Binding of the fragment to integrin $\alpha 3$ inhibits protein synthesis of endothelial cells through a complex transduction pathway involving inhibition of focal adhesion kinase (FAK) and Akt phosphorylation and preventing the dissociation of eukaryotic initiation factor 4E protein from 4E-binding protein 1.⁴⁷ In contrast to these findings, we demonstrated that the $\alpha 3(\text{IV})\text{NC1}$ domain only binds to endothelial cells via integrin $\alpha 3 \beta 1$ and the binding site for this integrin is within residues 185–203.⁴⁸ In addition, we demonstrated that the binding site for $\alpha 3$ -containing integrins is not within the $\alpha 3(\text{IV})\text{NC1}$ domain itself, but rather within an RGD site, which is part of a short collagenous sequence at the N-terminus adjacent to the NC1 domain.⁴⁹ Interestingly, Boosani and colleagues showed that the $\alpha 3(\text{IV})\text{NC1}$ domain binds both integrins $\alpha 3 \beta 1$ and $\alpha v \beta 3$ on endothelial cells²⁴; however, only integrin $\alpha 3 \beta 1$ dependent binding of this fragment is crucial to prevent hypoxia-mediated COX2 expression and COX2-mediated synthesis of angiogenic factors via regulation of I κ B /NF κ B axis.²⁴

In addition to its anti-angiogenic function, the $\alpha 3(\text{IV})\text{NC1}$ domain can also bind tumor cells, where it exerts anti-tumorigenic activity. Melanoma and prostate cells bind the $\alpha 3(\text{IV})\text{NC1}$ domain via CD47/integrin-associated protein, integrin $\alpha 3$ subunit, and integrin $\alpha v \beta 3$ and these interactions are important for inhibition of tumor cell proliferation.^{50,51} Glioma cells can also bind the $\alpha 3(\text{IV})\text{NC1}$ domain via integrin $\alpha v \beta 3$ and are sensitive to $\alpha 3(\text{IV})\text{NC1}$ -mediated growth suppression only if they show intrinsic high levels of the tumor suppressor PTEN and concomitant low levels of activated Akt.⁵² Thus, the $\alpha 3(\text{IV})\text{NC1}$ domain can be viewed as both an anti-angiogenic and anti-tumorigenic factor. An extensive review of how the $\alpha 3(\text{IV})\text{NC1}$ domain might exert its signaling mechanisms and be used as anti-angiogenic agent was recently published.⁷⁴

At present it is unknown whether the $\alpha 4(\text{IV})\text{NC1}$ domain plays any role in endothelial cell functions, and poor anti-angiogenic activity has been attributed to the $\alpha 5(\text{IV})\text{NC1}$ domain.⁴⁰ In contrast, the $\alpha 6(\text{IV})\text{NC1}$ domain prevents angiogenesis and tumor growth, although at present the mechanism remains unexplored.^{40,53}

Collagens XVIII and Angiogenesis

Cleavage of the C-terminal region of the NC domain of collagen XVIII leads to the generation of endostatin, a potent anti-angiogenic peptide.⁷⁵ Endostatin can bind heparin⁷⁶ and it has been postulated that its anti-angiogenic activity resides in its ability to compete with bFGF and vascular endothelial growth factor (VEGF) for heparan sulfate chain binding, thereby preventing the storage of this angiogenic molecules in the matrix milieu.^{55,56} Moreover, this collagen fragment can also bind to the catalytic domain of MMP2, thereby preventing its degrading activity.^{57,58} Studies aimed at determining the identification of endostatin receptors on endothelial cells have identified glypican-1 as a low-affinity binding partner for endostatin.⁵⁹ Glypican-1 is a heparan sulfate proteoglycan bound to the outer surface of the plasma membrane by a glycosyl-phosphatidylinositol anchor that binds endostatin via its glycosaminoglycan residues.⁵⁹ In addition to glypican-1, endostatin has been shown to prevent endothelial cell migration by interacting with $\alpha 1$ -containing as well as $\alpha v \beta 3$ or $\alpha v \beta 5$ integrins^{60,61} (Table 1). The anti-angiogenic activity of human endostatin via binding to integrin $\alpha 5 \beta 1$ is mediated by specific inhibition of the FAK/c-Raf/p38/ERK1 pathway.⁶² Moreover, endostatin interactions with integrin $\alpha 5 \beta 1$ and caveolin-1 at the endothelial cell surface can promote disassembly of actin stress fibers and focal adhesions.⁶³ Interestingly, endostatin can be released by BMs and stored in circulating platelets.⁷⁷ As treatment with COX inhibitors leads to the release of platelet-bound endostatin *in vivo*,⁷⁸ it is possible that exogenous administration of endostatin followed by treatment with COX inhibitors might have potential as an anticancer therapy.

Collagen XV and Angiogenesis

Type XV collagen is structurally homologous to type XVIII collagen, with several interruptions in the triple-helical sequence of the central collagenous domain, a large N-terminus NC globular domain, and a smaller C-terminal NC domain. The C-terminal NC1 domain contains an endostatin-like region, named restin that shows a 60% sequence homology with endostatin.⁷⁹ Despite this high homology, restin differs from endostatin in its binding properties to extracellular matrix macromolecules as well as in its tissue distribution.⁸⁰ Restin does not bind zinc or heparin⁸⁰ and it exhibits a potent antimigratory, but not an antiproliferative, activity on endothelial cells⁸¹ (Table 1).

HOW ARE BIOLOGICALLY ACTIVE BM-DERIVED FRAGMENTS GENERATED *IN VIVO*?

As mentioned above, BM degradation is a process that requires the synthesis and secretion of proteolytic enzymes. Among these enzymes, members of cathepsin and MMP family have been shown to play a key role in the generation of biologically active BM cleavage products. Cathepin L cleaves collagen XVIII, generating endostatin.^{82,83} It can also cleave domain V of perlecan (endorepellin), generating the LG3 domain.⁸⁴ In contrast to this finding, Wang and colleagues have shown that Cathepin S is able to degrade purified 1(IV)NC1 and 2(IV)NC1 domains, suggesting that this enzyme might be involved in the clearance, rather than generation, of anti-angiogenic peptides.⁸⁵ Among the MMP family members, MMP-3, -7, -9, -13, and -20 release endostatin from collagen XVIII,^{54,86} while MMP-9 generates the 3(IV)NC1 domain by direct cleavage of the 3(IV) chain.⁸⁷ In addition to cathepsins and MMPs, elastase has been shown to be involved in the generation of endostatin,^{83,88} and the BMP-1/Tolloid-like proteinases can cleave domain V of perlecan releasing LG3 domain.⁸⁹ To the best of our knowledge, it is at present unclear which enzymes are directly involved in generation of other 1(IV)NC1 domains as well as some of the laminin fragments described above.

THE ROLES OF EICOSANOID LIPIDS IN ANGIOGENESIS

Some of the AA-derived lipids, including prostaglandins, the epoxyeicosatetraenoic acids (EETs), and the hydroxyeicosatetraenoic acids (HETEs) can promote the synthesis of angiogenic factors and/or directly regulate endothelial cell functions.^{19,90–93} In this article we focus on the direct contribution of three enzymes, namely, COXs (generators of prostaglandins), LOXs (generator of 5-, 8-, 12-, and 15-HETEs), and the cytochrome P450-monoxygenases (generators of EETs, 19- and 20-HETEs), in the control of endothelial cell functions in both physiological and pathological angiogenesis. As mentioned above, we will focus on these three enzymes, as the AA metabolites generated by them (1) directly affect endothelial cell functions, (2) can promote the synthesis of BM components, and (3) regulate the synthesis of enzymes involved in the degradation of BM components and (4) their synthesis can be regulated by BM-derived cleavage products.

The COX-derived Products in Angiogenesis

COX1 and COX2 catalyze the rate-limiting steps in the biosynthesis of prostaglandins and thromboxanes from AA. As shown in Figure 2a, the COXs convert AA to prostaglandin H₂ (PGH₂), and the downstream selective isomerases convert PGH₂ to prostacycline, prostaglandins, or thromboxane A₂, all of which are pro-angiogenic.^{94,95}

Prostaglandin E₂ (PGE₂) is the most widely produced prostanoid in the body⁹⁶ and it exerts its cellular effects by binding to four distinct E-prostanoid receptors (EP1–4) that belong to the family of seven transmembrane G protein-coupled rhodopsin-type receptors⁹⁷ (Figure

2b). Despite similar signaling mechanism among these receptors, each receptor has different and often opposing biological effects.⁹⁸ For example, although the EP2 and EP4 receptors upregulate intracellular cAMP levels, they exert different downstream effects on important intracellular mediators, including the PI3K and ERK pathways.^{99,100} Moreover, the EP3 receptor usually counteracts EP2- and EP4-mediated upregulation of cAMP by preferentially coupling to G_i proteins.⁹⁷

Information regarding the role of PGE₂ in angiogenesis has been obtained using cancer models in mice lacking EP receptor expression. EP2-null mice produce significantly fewer and less vascularized tumors than wild-type mice in a two-stage skin carcinogenesis protocol,¹⁰¹ and the EP2 receptor was demonstrated to directly contribute to endothelial cell migration and survival.¹⁰² Similarly, EP3-null mice exhibit decreased tumor-associated angiogenesis following injection of sarcoma or lung carcinoma cells,¹⁰³ and treatment with the selective EP3 agonist ONO-AE-248 promotes angiogenesis in an *in vivo* sponge model assay.¹⁰³ In contrast, the EP1 receptor does not play a role in endothelial cell function or tumor-associated angiogenesis, as the EP1-selective agonists ONO-DI-004 or 17-phenyl - - trinor-PGE₂ failed to promote angiogenesis in a sponge model assay^{103,104} and no differences in the early onset of tumor growth and angiogenesis were observed between tumor-bearing EP1-null and wild-type mice.¹⁰⁵

The contribution of the EP4 receptor to endothelial cell functions is more difficult to evaluate given that 80% of the EP4-null mice die at birth as result of ductus arteriosus.¹⁰⁶ Studies of primary endothelial cells derived from EP4^{flox / flox} mice¹⁰⁷ revealed that this receptor directly controls endothelial cell migration and tubulogenesis, but not proliferation *in vitro*, and that activation of the EP4 receptor by selective agonists promotes angiogenesis *in vivo*.¹⁰⁴ Some of the major effects of the EP receptors on endothelial cell functions are summarized in Figure 2b.

While it is clear that activation of EP receptors in endothelial cells by either selective EP agonists or PGE₂ controls migration, invasion, and tubulogenesis, the role of PGE₂ as an endothelial-specific mitogenic factor is controversial. Mouse microvascular endothelial cell proliferation is not stimulated by PGE₂ and/or EP-selective agonists,¹⁰⁴ and endothelial cells lacking EP4 or EP2 receptors show similar basal proliferation to wild-type cells.^{102,104} However, exogenous PGE₂ inhibits corneal and dermal microvascular endothelial cell growth as well as human umbilical vein endothelial cell (HUVEC) proliferation,^{108,109} whereas low doses of PGE₂ promote HUVEC growth via nitric oxide production.¹¹⁰ Thus, the effects of PGE₂ on endothelial cell proliferation appear to depend on the nature of the endothelial cells themselves.

In addition to directly regulating endothelial cell functions, COX-derived metabolites can also regulate the synthesis of proteinases, thereby contributing to endothelial cell migration/invasion as well as to the generation of biologically active BM-derived products. For example, PGE₂ can promote the clustering and activation of MT1-MMP in endothelial cells¹¹¹ and promote the synthesis of MMP9 in dendritic cells.²⁰ Similarly, treatment of tumor cells with COX2 inhibitors prevents MMP2 and MMP9 synthesis.¹¹² Thus, COX-derived prostanoids can either directly or indirectly promote endothelial cell functions, making COX a potential target for anti-angiogenic therapy (see below for details).

The LOX-derived Products in Angiogenesis

In mammals, LOXs are categorized with respect to their positional specificity of AA oxygenation into 5-, 8-, 12-, and 15-LOXs [reviewed in 113] (Figure 3a). LOXs metabolize AA to the biologically active metabolites hydroperoxy-eicosatetraenoic acids (HPETEs), which are subsequently reduced to corresponding HETEs. Therefore the major AA

metabolites formed by mammalian LOXs are 5-, 8-, 12-, and 15-HETE.¹¹³ The observation that 12-LOX is expressed by endothelial cells¹¹⁴ suggested a role for this enzyme in angiogenesis. In this review we primarily focus on the ability of LOX-derived AA products to control endothelial cell functions. However, it is important to consider that altered expression of LOX isoforms is found in tumor, stromal, or immune cells, strongly suggesting a role for these enzymes in tumor development and growth [reviewed in 113].

Among the four LOX-derived HETEs, 12(S)-HETE promotes both endothelial cell proliferation and migration^{14,115} by stimulating VEGF neosynthesis;¹⁵ by promoting endothelial cell retraction in a protein kinase C (PKC) dependent manner¹¹⁶; and by increasing the surface expression of integrin α_3 ,¹¹⁷ a receptor expressed primarily in angiogenic blood vessels.¹¹⁸

Whereas 12(S)-HETE plays a pro-angiogenic function, the role of 15(S)-HETE is controversial. This LOX-derived product can promote angiogenesis via activation of the PI3K/mammalian target of rapamycin (mTOR) axis,¹¹⁹ and under hypoxia conditions its production supports angiogenesis via MEK1 activation.¹²⁰ In addition, 15(S)-HETE can support angiogenesis by promoting VEGF synthesis in a STAT3-dependent manner.¹²¹ In contrast to this data, mice overexpressing 15-LOX specifically in the endothelium show reduced angiogenesis and tumor formation¹²² most likely by downregulating VEGF, placental growth factor (PLGF), and VEGFR2 expression.¹²³ This data, together with the finding that 15-LOX is upregulated in prostate cancer but downregulated in colon and breast tumors [reviewed in 113], strongly indicates that this enzyme might have both pro- and anti-tumorigenic functions, making it a 'difficult' gene to be targeted for anti-angiogenic and anti-tumorigenic therapy (see also below for details). Some of the major effects of the 12-HETE and 15-HETE on endothelial cell functions are summarized in Figure 3b.

The Cytochrome P450 Monooxygenase-Derived Products in Angiogenesis

Cytochrome P450s (P450) are membrane-bound hemoproteins best known for their roles in the metabolism of toxic chemicals, carcinogens, drugs, as well as endogenous substrates such as steroids, cholesterol, and vitamins.^{124,125} More recently, a functional role for these enzymes in the AA metabolic cascade has been documented.^{126,127} The P450 AA monooxygenase catalyzes the oxidation of AA to four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs; *AA epoxygenase branch = CYP2C isoforms*) and/or 19- and 20-hydroxyeicosatetraenoic acids (19- and 20-HETE; *AA ω -hydroxylase branch = CYP4A isoforms*)¹²⁶ (Figure 4a). The identification of EETs and 20-HETE as products of the *in vivo* metabolism of AA by rodent and human tissues established the epoxygenases and ω -hydroxylases as formal metabolic pathways and suggested a biological role for their metabolites.^{126–128} EETs and HETEs have several important biological activities, including the regulation of angiogenesis [reviewed in 129,130].

The first link between EETs and angiogenesis was obtained in co-cultures of astrocytes and endothelial cells, where EETs from astrocytes promoted endothelial cell growth and formation of capillary-like structures.¹³¹ Moreover, overexpression of CYP2C9 or treatment with exogenous 11,12- or 14,15-EET enhances endothelial cell biological functions.⁹⁰ The 11,12-EET activates PI3K and tyrosine kinases^{90,132} presumably with the participation of COX-2 and the EGF receptor.^{90,133} We identified 5,6- and 8,9-EET as potent pro-angiogenic lipids both *in vitro* and *in vivo*⁹³ and showed that the mitogenic activity of 8,9-EET involved p38 MAPK while that of 5,6-EET involved PI3K.⁹³ EETs also induced angiogenesis in the chick chorioallantoic membrane⁹⁰ as well as in implanted matrigel plugs or sponges.^{93,134} The mechanism whereby EETs exert their pro-angiogenic activities include their ability to (1) activate pro-mitogenic and pro-migratory cell signaling;^{90,93,132} (2) promote the synthesis and activation of MMPs¹⁹ thereby facilitating the release of

heparin bound growth factors (i.e. HB-EGF) from the cell surface;^{90,135} (3) stimulate COX-2 protein expression;¹³³ and (4) recruit monocytes to sites of active angiogenesis by inducing the expression of endothelial VCAM-1¹³⁶ thereby facilitating the release different monocyte-produced angiogenic factors (see also Figure 4b).

Among the HETE products, 20-HETE has been shown to increase endothelial cell proliferation by stimulating VEGF synthesis and VEGFR2 activation,⁹² and inhibitors of CYP4A suppress growth-factor-mediated endothelial cell proliferation *in vitro* and angiogenesis in a cornea assay *in vivo*.¹³⁷

Together, these studies indicate that endogenously produced EETs and HETEs can be considered pro-angiogenic, and targeting either the CYP2C or CYP4A isoforms might be considered a valid tool for preventing unwanted angiogenesis.

ANTI-ANGIOGENIC THERAPY: PRO AND CONTRA

The requirement of a blood supply for tumors to develop, grow, and metastasize has made tumor angiogenesis a potential therapeutic target for the treatment of numerous cancers. However, because of the primary importance of vascular health in the homeostasis of the body, use of anti-angiogenic drugs has both advantages and disadvantages, which we will highlight below.

The Use of ECM Cleavage Products as Anti-Angiogenic Agents

Although extracellular matrix (ECM) cleavage products inhibit tumor-associated angiogenesis, and phase II clinical trials for certain fragments (i.e., endostatin) have been started, there is doubt about the success and safety of these matrix-derived fragments as anti-angiogenic drugs. A recent review by Xu and colleagues¹³⁸ brings to our attention that the largest benefit of these cleavage products occurs when they are administered at the early stages of tumor growth, thus explaining why some of these fragments are ineffective in patients with advanced cancer.¹³⁹ Another major problem with these fragments is their limited half-life. Finally, these anti-angiogenic therapies are almost certainly not sufficient to slow tumor growth and development and can only be used as adjuvant therapies.

Some of these matrix fragments might also act as a 'double edge sword,' as they exert both angio-suppressive and angio-stimulatory effects, as observed for endostatin.¹⁴⁰ Most importantly, it has been recently shown that despite an initial beneficial effect of endostatin and collagen IV-derived fragments, tumors ultimately are able escape angiogenesis inhibition. This effect is due to the ability of tumor cells to upregulate the expression of potent pro-angiogenic factors such as VEGF, PDGF-A, and PDGF-B.¹⁴¹ Finally, given that these fragments bind receptors ubiquitously expressed such as $\alpha 1$ -containing integrins rather than those specifically expressed by endothelial cells, they might damage 'healthy' cells resulting in undesirable side effects. This is exemplified by the fact that in addition to blocking tumor angiogenesis,^{73,87} the $\alpha 3(\text{IV})\text{NC1}$ domain inhibits the function of stimulated neutrophils by raising intracellular levels of cAMP.^{142,143}

Use of LOX inhibitors as Anti-Angiogenic and/or Anti-Tumorigenic Agents

As mentioned above, the observations that (1) 12-LOX is expressed in endothelial cells;¹¹⁴ (2) expression of certain LOX-derived products is upregulated in endothelial cells under hypoxia conditions;¹²⁰ (3) LOX-derived products are pro-angiogenesis (see above for details); and (4) LOX expression and activity are upregulated certain tumor types [reviewed in 113] make LOXs a potential target for anti-angiogenic and anti-tumorigenic therapy. 12-LOX is generally absent in normal epithelia, but overexpressed in various epithelial cancers including colon, esophageal, lung, prostate, and breast cancer.^{144–146} In addition, levels of

12-HETE correlates with progression of various cancers [reviewed in 147]. Studies on human prostate cells indicate that overexpression of 12-LOX results in a significant increase in VEGF expression,¹⁵ and treatment with a pan inhibitor of LOX (nordihydroguaiaretic acid) or with a selective 12-LOX inhibitor (baicalein) prevents VEGF synthesis.¹⁵ In human breast cancer and non-small-cell lung cancer cells, specific inhibition of 12-LOX resulted in significant cell apoptosis via regulation of caspase pathways.¹⁴⁸ Together, this data strongly indicates that 12-LOX can be viewed as a pro-angiogenic and pro-tumorigenic enzyme and its inhibition can provide a valid tool for anti-angiogenic and anti-tumorigenic therapy.

LOX-targeted anti-angiogenic/anti-tumorigenic therapy is more complicated when 15-LOX becomes the target. Pidgeon and colleagues have recently reported that, whereas overexpression of 15-LOX in Du145 prostate cancer cells prevents their growth *in vivo*, overexpression of the same isoforms in PC-3 prostate cells enhances their growth *in vivo*,¹¹³ clearly indicating the difficulty of targeting LOX for anti-tumorigenic activity. Similarly, studies performed on endothelial cells suggest that this isoform can have both pro- and anti-angiogenic activities (see above for details). The picture is further complicated by the fact that LOX-derived eicosanoids in addition to controlling endothelial or tumor cell functions also play a role in regulating the immune response to tumors. Given that the type of infiltrating cell (i.e., T regulatory, T effectors, natural killers, macrophages, neutrophils, granulocytes) highly dictates whether tumor growth is exacerbated or inhibited, the use of lipoxygenase inhibitors as anticancer therapy might be difficult [also reviewed in 113].

Use of COX Inhibitors as Anti-Angiogenic and/or Anti-Tumorigenic Agents

Increased levels of COX-2 and pro-tumorigenic PGE₂ have been observed in various types of tumors including colon, lung, and breast cancer, and its overexpression is associated with poor outcome.¹⁴⁹ In addition, COX-derived prostanoids are pro-angiogenic, which prompted investigators to analyze the ability of COX inhibitors to reduce and ideally prevent the incidence of cancer. Animal studies have clearly shown that nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX inhibitors can reduce incidence and size of tumors. A significant reduction in the number and size of small intestinal polyps in APC^{min/+} mice was observed following treatment with the COX-2 inhibitor celecoxib,¹⁵⁰ and the same inhibitor prevented tumor angiogenesis in an orthotopic model of human colon cancer.¹⁵¹ Furthermore, chronic ingestion of sulindac for 1 year decreased polyp multiplicity and induced regression of polyps in patients with familial adenomatous polyposis or Gardner's syndrome.¹⁵² A large, population-based observational study also demonstrated that low-dose NSAID reduced the relative risk of fatal colon cancer¹⁵³ and chronic ingestion of NSAIDs significantly reduces colon polyp formation and recurrence [154 and reviewed in 155]. Despite these results, in 2005 the U.S. Food and Drug Administration issued a warning concerning the potential cardiovascular side effects of NSAIDs and COX selective inhibitors,¹⁵⁶ as they might lead to prothrombotic risk, increase blood pressure, and heart failure [reviewed in 157]. Finally, the well-described gastrointestinal side effects of traditional NSAIDs make them difficult to consume for long periods. Thus, a better design of COX inhibitors with more restricted side effects and more selective anticancer and anti-angiogenic ability is required, as recently suggested by Reddy and colleagues.¹⁵⁸

Use of P450 inhibitors as Anti-Angiogenic Agents

On the basis of the observation that P450-derived lipids, namely EETs and HETEs, are pro-angiogenic, inhibition of the cytochrome P450 monooxygenases might be a valid tool to reduce and ideally prevent unwanted angiogenesis. *In vitro* studies show that treatment of endothelial cells with the CYP2C inhibitors ketoconazole or MS-PPOH prevents EET formation and consequent cell proliferation, migration, and tubulogenesis.⁹³ Moreover, the CYP4A inhibitor *N*-hydroxy-*N*-(4-butyl-2-methylphenol) formamidine prevented

angiogenesis *in vivo*.¹³⁷ However, these inhibitors are toxic and they can only be used *in vivo* for a very limited time. A more promising P450-anti-angiogenic therapy comes from the observation that the expression of CYP2C and EET production can be inhibited by activators of the peroxisome proliferator-activated receptors (PPAR). Wyeth-14643—a selective PPAR ligand—inhibits endothelial cell proliferation, migration, and tubulogenesis *in vitro* as well as tumor-associated angiogenesis *in vivo*.¹⁵⁹ Interestingly, the anti-angiogenic properties of Wyeth-14643 are associated with a PPAR-mediated downregulation of CYP2C epoxygenase expression and reduced levels of circulating EETs.¹⁵⁹ The observation that Wyeth-14643-mediated inhibition of tumor angiogenesis is independent of the type of tumor cell injected and is absent in PPAR-null mice¹⁵⁹ strongly indicates that Wyeth-14643 can be widely used as anti-angiogenic agent and its effects are PPAR-dependent. Consistent with the observation that PPAR ligands might act as potent anti-angiogenic factors, it has been shown that fenofibrate and Wyeth-14643 suppress VEGF-mediated endothelial cell proliferation by inhibiting Akt phosphorylation^{160,161} and prevent endothelial cell proliferation by inhibiting COX2 expression.¹⁶² Moreover, fenofibrate stimulates tumor cell-derived thrombospondin and endostatin synthesis, thereby indirectly inhibiting angiogenesis.¹⁶⁰ In addition to the beneficial effects of PPAR in inhibiting tumor-associated angiogenesis, loss of host-derived PPAR can be also advantageous by increasing granulocyte infiltration to the tumor site. These immune cells can suppress tumor-associated angiogenesis via excess production of the endogenous angiogenesis inhibitor thrombospondin.¹⁶³ Thus, both activation of PPAR in specific host cells (i.e., endothelial cells) and the concomitant inhibition of PPAR in immune cells (i.e. granulocytes) might lead to the same effect, namely inhibition of tumor-associated angiogenesis [also reviewed in 164].

The effects of PPAR ligands in animal models of tumor angiogenesis should help not only to stimulate further research of their usefulness as anti-angiogenic agents but also to facilitate their evaluation as valid tools for the treatment and/or prevention of tumor development and growth. As several fibric acid derivatives (1) selectively bind to and activate PPAR; (2) are effective hypolipidemic drugs with limited unwanted consequences^{165,166}; and (3) decrease VEGF levels in patients with hyperlipidemia and atherosclerosis,¹⁶⁷ it is conceivable that PPAR ligands might be viewed as safe and tolerable anti-angiogenic molecules to be used in cancer therapy.

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FIGURE 1.

Potential cross-talk between basement membrane components and arachidonic acid metabolizing enzymes. Endothelial cell derived cyclooxygenases, lipoxygenases, and cytochrome p450 monooxygenases stimulate angiogenesis by promoting the synthesis of soluble growth factors (a); basement membrane components (b); and/or matrix degrading enzymes (c). In turn, these degrading enzymes can generate basement membrane-derived fragments capable of regulating the expression of arachidonic acid-metabolizing enzymes (d).

FIGURE 2.

Schematic representation of prostanoid synthesis and functions. (a) Arachidonic acid (AA) is converted by cyclooxygenases (COXs) to PGH_2 . This intermediate is then converted to thromboxane (TxA_2) by the thromboxane synthase (TXS); to prostacyclins (PGI_2) by the prostacyclin synthase (PGIS); to PGD_2 by the prostaglandin D synthases (PGDS); to PGF_2 by the prostaglandin F synthase (PGFS); and to PGE_2 by the prostaglandin E synthase (PGS). (b) PGE_2 can activate four different G protein coupled receptors (EP1–4), thus controlling different endothelial cell (EC) functions.

(a)

<div style="text-align: center;"> <chem>CCCCC/C=C/C(O)/C=C/C/C=C/C(=O)O</chem> 12-HETE </div> <p>Promotes:</p> <ul style="list-style-type: none"> EC proliferation and migration ^{8,109} VEGF synthesis ⁹ EC retraction via activation of PKC ¹¹⁰ Expression of integrin $\alpha v \beta 3$ ¹¹¹ 	<div style="text-align: center;"> <chem>CCCCC/C=C/C/C=C/C(O)/C=C/C(=O)O</chem> 15-HETE </div> <p>Promotes angiogenesis via</p> <ul style="list-style-type: none"> PI3K/mTOR axis ¹¹³ MEK1 activation ¹¹⁴ VEGF synthesis in a STAT3 dependent manner ¹¹⁵ <p>Prevents:</p> <ul style="list-style-type: none"> tumor-associated angiogenesis ¹¹⁶ VEGF, PLGF, and VEGFR2 expression ¹¹⁷
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(b)

FIGURE 3.

Schematic representation of lipoxxygenase-derived hydroxyeicosatetraenoic acid and their functions. (a) Arachidonic acid (AA) is converted by mammalian lipoxxygenases (LOXs) to four major hydroxyeicosatetraenoic acid (5-, 8-, 12-, and 15-HETE). (b) Effect of 12- and 15-HETEs on endothelial cell (EC) functions.

(a)

(b)

FIGURE 4.

Schematic representation of epoxyeicosatrienoic and hydroxyeicosatetraenoic acid synthesis and functions. (a) Arachidonic acid (AA) can be converted by (1) the cytochrome P450 epoxygenases (CYP2C isoforms) to four different epoxyeicosatrienoic acids (5,6 –9–11, 12- and 14,15-EET); or (2) the cytochrome P450 -hydroxylases (CYP4A isoforms) to two different hydroxyeicosatetraenoic acid (19- and 20-HETE). (b) EETs and HETEs can be generated endogenously by endothelial cells and can control different endothelial cell functions.

TABLE 1
Cleavage Products of Basement Membrane (BM) Components and their Effect on Endothelial Cell (EC) Functions

BM components	Products	Biological effect(s)	Surface Receptor(s)	Possible mechanism(s)	References
Perlecan	Endorepellin (domain V)	Inhibition of EC migration <i>in vitro</i> Anti-angiogenic <i>in vivo</i>	Integrin 2 1	Disassembly of the actin cytoskeleton and focal adhesion	30–33
LM-111	1 chain peptides	Stimulation of EC adhesion and sprouting <i>in vitro</i> Pro-angiogenic <i>in vivo</i>	Integrins 5 1 and v 3	Stimulation of MMP9 production	34–36
LM-111	1 and 1 chain peptides	Stimulation of EC sprouting and migration <i>in vitro</i>	Unknown	Unknown	37
LM-411 or LM-421	G domain of the 4 chain	Support of EC adhesion and survival <i>in vitro</i>	1 integrins	Unknown	38
Collagen IV	1 (IV)NC1 domain	Inhibition of EC growth, migration and tube formation <i>in vitro</i> Anti-angiogenic <i>in vivo</i>	Integrin 1 1	Inhibition of FAK, Raf, MEK, ERK, and p38 MAPK activation	39
Collagen IV	2 (IV)NC1 domain	Promotion of EC apoptosis <i>in vitro</i> Inhibition EC proliferation migration and tube formation <i>in vitro</i>	1 integrins as well as integrins v 3 and v 3	Stimulation of procaspase-9 cleavage	40–44
Collagen IV	3 (IV)NC1 domain	Inhibition of EC protein synthesis <i>in vitro</i> Inhibition of EC tube formation <i>in vitro</i> Anti-angiogenic <i>in vitro</i> and <i>in vivo</i>	CD47/IAP Integrins 3 1, v 3, and v 5	Inhibition of protein synthesis. Inhibition of FAK and Akt phosphorylation Inhibition of hypoxia-induced COX2 expression Inhibition of COX2-mediated synthesis of angiogenic factors via the I κ B /NFKB axis	24,40,45–52
Collagen IV	4(IV)NC1 domain	Unknown	Unknown	Unknown	40
Collagen IV	5(IV)NC1 domain	Poor anti-angiogenic <i>in vitro</i>	Unknown	Unknown	40,53
Collagen IV	6(IV)NC1 domain	Inhibition of EC growth <i>in vitro</i> Anti-angiogenic <i>in vivo</i>	Integrin v 3	Unknown	54
Collagen XV	Restin	Inhibition of EC migration <i>in vitro</i>	Unknown	Unknown	55–63
Collagen XVIII	Endostatin	Inhibition of EC migration and growth <i>in vitro</i> Anti-angiogenic <i>in vivo</i>	Glypican-1, 1 integrins, 5 1 v 3 and v 5	Competition with b-FGF and VEGF for heparan sulfate chain binding. Binding to and inhibition of the catalytic domain of MMP2 Inhibition of the FAK/c-Raf/p38/ERK1 pathway Disassembly of actin stress fibers and focal adhesions	