Current Topics

Diverse Cell Signaling Events Modulated by Perlecan[†]

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ABSTRACT: Perlecan is a ubiquitous pericellular proteoglycan ideally placed to mediate cell signaling events controlling migration, proliferation, and differentiation. Its control of growth factor signaling usually involves interactions with the heparan sulfate chains covalently coupled to the protein core's N-terminus. However, this modular protein core also binds with relatively high affinity to a number of growth factors and surface receptors, thereby stabilizing cell−matrix links. This review will focus on perlecan−growth factor interactions and describe recent advances in our understanding of this highly conserved proteoglycan during development, cancer growth, and angiogenesis. The pro-angiogenic capacities of perlecan that involve proliferative and migratory signals in response to bound growth factors will be explored, as well as the anti-angiogenic signals resulting from interactions between the C-terminal domain known as endorepellin and integrins that control adhesion of cells to the extracellular matrix. These two somewhat diametrically opposed roles will be discussed in light of new data emerging from various fields which converge on perlecan as a key regulator of cell growth and angiogenesis.

Perlecan was originally isolated in 1980 by Hassell and co-workers from the Engelbreth-Holm-Swarm sarcoma, a basement membrane-secreting tumor, and was soon demonstrated to be expressed also at the cell surface of human colon carcinoma cells (*1*). In spite of their differential expression, the two molecules were shown to have biosynthetic and immunological similarities. Because of its large size (the mRNA encoding perlecan is ~15 kb), it took more than a decade of efforts to complete the cDNA cloning of the full-length mouse perlecan, followed by the complete structure of the human counterpart, its chromosomal mapping, and its genomic organization (*2*). The eponym "perlecan" derives from its ultrastructural appearance of "beads on a string", a feature that can be attributed to the various globular domains interspersed among more linear structures (*1*).

Perlecan is composed of five distinct domains with homology to growth factors and to protein modules involved in lipid metabolism, cell adhesion, and homotypic and heterotypic interactions (*3*). Notably, N-terminal domain I contains three attachment sites for the heparan sulfate side chains with additional attachment sites in carboxyl-terminal domain V (Figure 1). Interestingly, the other two main HSPGs[1] from basement membranes, collagen XVIII and agrin, do not share much structural homology with the exception of agrin domain V. Collagen XVIII is a member of the subfamily of collagens, also known as multiplexins. These collagens, which include collagen XV, harbor a central triple-helical domain that is interrupted and flanked by noncollagenous regions (*4*). The C-terminal, noncollagenous domain of collagen XVIII contains the angiogenesis inhibitor endostatin. Agrin is also a modular HSPG that is best known for its ability to organize postsynaptic differentiation at the neuromuscular junction but is also involved in muscle and renal homeostasis (*5*). The N-terminal region and central region of agrin are quite unique. However, the C-terminal domain has a structural organization similar to that of domain V of perlecan with three laminin-like globular domains interspersed with EGF-like repeats (see below).

Perlecan is a ubiquitous macromolecule that is predominantly a basement membrane/extracellular matrix proteoglycan with an intrinsic ability to self-assemble into dimers and oligomers. It is often secreted into the pericellular space

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[1] Abbreviations: HS, heparan sulfate; HSPG, HS proteoglycan; FGF, fibroblast growth factor; FGFR, FGF receptor; VEGF, vascular endothelial growth factor; VEGFR1 and VEGFR2, VEGF receptors 1 and 2, respectively; PDGF, platelet-derived growth factor.

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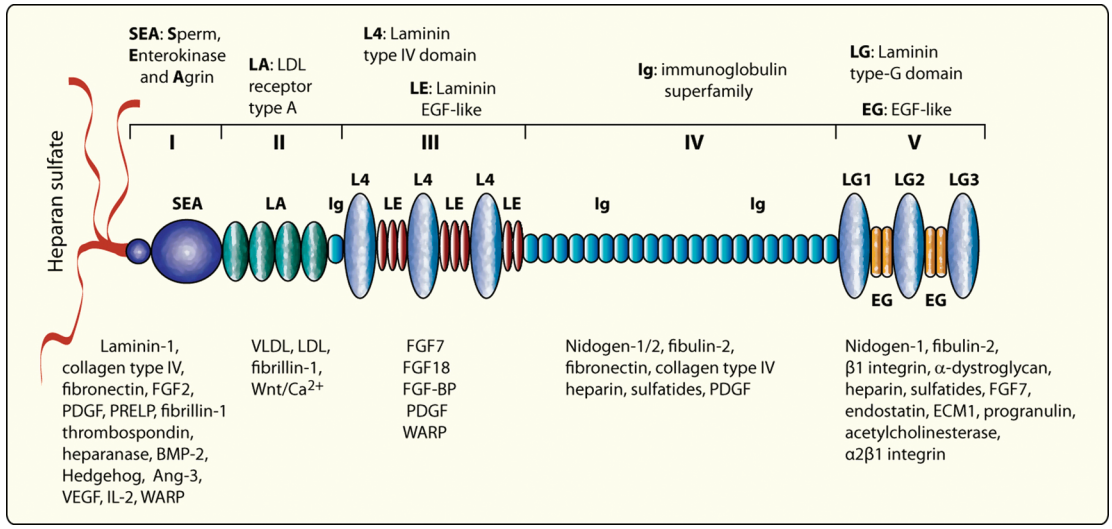

FIGURE 1: Schematic diagram of human perlecan depicting the various domains (Roman numerals) and the abbreviations for each module (top). The bottom panel shows a list of perlecan interactive partners relative to each protein core domain.

where it is ideally situated to mediate the action of signaling molecules that are either secreted by the cells themselves in response to environmental cues or secreted by other cells in a paracrine fashion (*3*). Perlecan's modular protein core interacts with a number of extracellular matrix constituents, receptors, and growth factors (Figure 1 and Table 1). By surrounding the cell, perlecan may act to control the pericellular concentration of mitogens and morphogens. Its widespread expression across species suggests that it may be performing this role for many different types of cells that are responding to different stimuli at the same time. This hypothesis was supported when the effects on embryonic development were studied in perlecan knockout mice. These mice demonstrated a complex series of phenotypes which was not confined to one tissue or organ system (*6, 7*). Most of the mice survived the early stages of embryonic development quite successfully, but then approximately half of them died around embryonic day 11.5 because of either cardiac system failure from intrapericardial hemorrhage due to malformed and transposed major blood vessels or failure of the neural system to develop (*7*). Those mice that progressed to birth died soon after from respiratory failure most probably due to major skeletal abnormalities present in the ribs and diaphragm region (*6*). Histological examination of these mice showed a marked disorganization in the structure and architecture of the developing cartilage tissue (*6*) which may have been caused by disturbed signaling gradients. Other skeletal changes included shortened long bones and a dwarflike phenotype similar to that seen in human Schwartz-Jampel syndrome, a condition shown to be due to a mutation in the perlecan gene (*1*). A complication with these types of studies is the possibility of an upregulation of other heparan sulfate proteoglycans (HSPGs) in the basement membranes and extracellular matrix that may perform similar functions leading to compensation of the phenotype in some animals. This is particularly relevant because the growth signaling molecules bind to the HS chains which may be very similar among HSPGs. This may have been the case in some of the perlecan-deficient mice where an increase in the level of type XVIII collagen and/or agrin could have provided enough HS with the appropriate structure to replace the roles of perlecan (*8*). The presence of HS is absolutely required for successful embryonic development because zygotes totally lacking the ability to synthesize any did not proceed past the early gastrulation phase of development. One could hypothesize that a total lack of HS would lead to a loss of all mitogen/morphogen gradients, and while the cells could grow to the multicellular blastula stage, the diffusion of cytokines away from the cells would cause a failure in the formation of a tube critical to gastrulation (*9*). Mice that specifically lack type XVIII collagen have abnormalities in eye development and some effects on angiogenesis (*4*), whereas animals lacking agrin have defective neuromuscular junctions due to the inability of the synapses to localize the acetylcholine receptors correctly (*5*). Although it is tempting to suggest that agrin is specific for neural tissue, it has been shown to be produced by chondrocytes and to be localized to basement membranes in the kidney in a manner similar to that of collagen XVIII (*5*).

The important role of HS and the fact that type XVIII collagen can compensate for the lack of perlecan were also demonstrated when mice that produced HS-deficient perlecan were bred with mice deficient in collagen type XVIII. This resulted in mice that displayed an ocular phenotype that was more severe than that in those animals expressing the HS-deficient perlecan (*8*).

Mutations of the *Caenorhabditis elegans* perlecan ortholog, UNC-52, cause defects in the formation and maintenance of the muscle myofilament lattice. Notably, perlecan/UNC-52 affects gonadal leader cell migration by modulating the bioactivity of several growth factors, including FGF, TGFβ, and Wnt (*10*). In *Drosophila*, perlecan/*Trol* stimulates neuroblast proliferation (*11*) and modulates FGF and Hedgehog signaling, and this interaction is mitogenic for neural stem cells (*12*). Perlecan also potentiates cell cycle progression and neuronal differentiation in the murine cerebral hemispheres and regulates Sonic Hedgehog availability in the floor plate (*13*). Thus, it is likely that perlecan may play multiple developmental roles by concentrating growth factors and morphogens near the cell surface and by restricting their subsequent diffusion (*10*).

Table 1: Activation of Multiple Signaling Pathways via Interactions of Perlecan with Growth Factors and Morphogens

growth factor	interacting perlecan moiety	effect or activity
FGF2	heparan sulfate chains domain I	mostly activation of FGF receptor function; required for receptor activation; enhanced growth, angiogenesis and chondrogenesis
FGF7	domain III and domain V/endorepellin	required for receptor activation and mitogenic activity in colon carcinoma cells and keratinocytes
FGF10	heparan sulfate chains, specific microdomains	stimulates FGFR2b activity and branching morphogenesis in the submandibular salivary gland
FGF18	domain III	regulation of growth plate chondrocyte proliferation, hypertrophy, and vascularization
VEGF	heparan sulfate chains; colocalization with perlecan in tumor angiogenic vessels	enhanced tumor angiogenesis and survival
PDGF	HS chains and domains III and IV	storage of PDGF and modulation of PDGF receptor activity
Progranulin	domain V/endorepellin	promotes cell migration and invasion of bladder cancer cells
Hedgehog	domain V/endorepellin of *Trol* in *Drosophila*	activation of neural stem cell division in *Drosophila*; required for receptor activity; control of neurogenesis in the developing telencephalon
TGFβ/Wnt	protein core/UNC-52 *Trol* in *Drosophila*	migration of gonadal leader cells in *Caenorhabditis elegans*
IL-2	heparan sulfate chains	neuroblast proliferation in *Drosophila*; enhanced proliferation of IL-2-dependent cells

PERLECAN SIGNALING AND FGFs

Perlecan binds to many growth factors, particularly those from the fibroblast growth factor family, known regulators of neovascularization. It has been shown that the HS chains are responsible for the binding to FGF1, -2, -7, -9, -10, and -18 (*14*), and such interactions may lead to enhanced angiogenesis and chondrogenesis. Most of the research performed on the binding of perlecan to growth factors has concentrated on FGFs because of the availability of good research tools, including recombinant growth factors and their receptors, antibodies, and the Baf32 cell line that has been transfected with the genes for the various FGFR isoforms (*14*). The other point about FGFs is that they are produced by many cell types and in most cases signal cells to proliferate. These results suggest that FGF bioactivity is strongly influenced by the contextual environment in which the growth factor is presented and expand the repertoire of subtle variations and sometimes paradoxical effects perlecan can display in particular pericellular environments.

Perlecan around the chondrocyte has been shown to localize FGF2 and act as a mechanotransducer by causing proliferation via activation of MAP kinases Erk1 and Erk2 (*15*), both major signaling molecules involved in the response to FGFs. In addition to FGF2, smooth muscle cells also express FGFR1, which has been shown to be important in proliferation and, in particular, during neointimal formation that causes restenosis following angioplasty of atherosclerotic lesions within blood vessels (*16*). The role of HS in smooth muscle cell proliferation has been shown to be important; when it is removed by a combination of bacterial heparinase digestions, the cells at a site of injury no longer respond to introduced FGF2 (*17*).

Perlecan is also involved in the binding and bioactivity of FGF7, also known as keratinocyte growth factor. Colon carcinoma cells, in which the perlecan gene is disrupted by targeted homologous recombination, grow slowly, fail to respond to exogenous FGF7 with or without heparin, and are less tumorigenic when injected into immunocompromised mice (*18*). In an engineered human skin model, perlecan-deficient keratinocytes form a poorly organized epidermis which is partially restored by exogenous FGF7 (*19*). Finally, the protein core of perlecan binds to FGF18, a key factor for chondrogenesis, and alters the mitogenic effect of FGF18 on growth plate chondrocytes (*20*). This finding is also supported by the similarity in cartilage phenotype between perlecan null and the FGF18 null mice which both exhibit a defect in endochondral ossification (*1*).

Perlecan HS is also involved in branching morphogenesis of the submandibular salivary gland via specific interactions with FGF10 (*21*). In this ex vivo model, heparanase colocalizes with perlecan in the glandular basement membrane and liberates FGF10 bound to the heparan sulfate chains. This leads to a signaling cascade which activates MAPK, stimulates the formation of epithelial clefts, and ultimately enhances branching morphogenesis. The specificity of this interaction was demonstrated by surface plasmon resonance studies showing that FGF10 and FGF10−FGFR2b complexes bound to HS chains on perlecan and that these complexes could be liberated by heparanase (*21*).

The role of HS and heparin has been modeled and indicated that at some concentrations and in solution they are capable of signaling through their cognate receptors, whereas at higher concentrations, they can inhibit growth. Perlecan's HS has been shown to signal through FGF2 and FGFR1 in solution, but these results have been obtained using perlecan derived from cultured endothelial cells and the genetically manipulated Baf32 cell line (*22*).

Perlecan is a key component of a basement membrane-like structure surrounding chondrocytes (*23*) and, together with dystroglycan, promotes basement membrane differentiation and maintenance of cell polarity in *Drosophila* follicle cell epithelium (*24*). Perlecan can be substituted not only with HS but also with chondroitin sulfate. Interestingly, chondroitin sulfate perlecan enhances collagen fibrillogenesis in cartilage (*25*), thereby providing a plausible explanation for the chondrodysplasia observed in the perlecan-null mice. Moreover, the chondroitin sulfate moiety in perlecan inhibits delivery of FGF2 to its cognate receptor, FGFR3, in the cartilage growth plate (*26*).

All of these results need to be confirmed in vivo but support the hypothesis that perlecan is an inactive sink for FGFs; this would partly explain why cells that are surrounded by perlecan and produce FGF do not proliferate out of control. Instead, they remain in a quiescent state unresponsive to many mitogenic signals. Whereas HS chains favor FGF−FGFR interaction, chondroitin sulfate chains in perlecan could act as "negative" regulators of FGF−FGFR interaction, primarily by physically constraining the FGFs from contacting their cognate receptors. It would be of interest to determine the structure of the HS attached to the different perlecan species to determine the specific microdomain structures that are responsible for mediating these various signals.

PERLECAN AND OTHER GROWTH FACTOR SIGNALING

Some families of growth factors have been shown to demonstrate differential binding to perlecan HS with one such example being the VEGFs. One of the longer isoforms, $VEGF_{189}$, which contains exon 6 that encodes a basic stretch of amino acids and which has been shown to be responsible for matrix localization, binds to perlecan HS derived from endothelial cells, whereas the shorter and more strongly expressed $VEGF_{165}$ does not (J. M. Whitelock and S. E. Stringer, unpublished observations). Interestingly, a fraction that included both the secreted and cell surface HSPGs from fibroblasts was shown to bind $VEGF_{165}$ (*27*). This would support the idea that perlecan localizes the larger forms of VEGF to the matrix but does not sequester the shorter forms, enabling them to diffuse through the pericellular matrix and bind to the cell surface HSPGs where they can signal the cell through either neuropilin or the VEGF tyrosine kinase receptors displayed on the cell surface (*28*). This hypothesis is supported by an elegant study in zebrafish where the localization of VEGF in the matrix was disturbed by knocking down the expression of the enzyme 6-*O*-sulfotransferase which affects the levels of sulfation present within HSPGs (*29*). Cell proliferation occurs satisfactorily, but the process of branching morphogenesis is severely retarded. One would speculate that the perlecan produced by endothelial cells undergoing angiogenesis would have small amounts of 6-*O*-sulfate, and if it produced a perlecan that had a high proportion of these sulfate groups, it would prevent angiogenesis by hindering the diffusion of VEGF. This may be a process that cells use to modulate the response of the endothelial cells to VEGFs produced in the pericellular environment. Interestingly, this may also be a factor in PDGF signaling. PDGF has been shown to contain an alternatively spliced exon that contains "heparin-binding" or matrix localization sequences. Both PDGF homodimers bind to perlecan HS derived from endothelial cells (*30*), and the inhibition of smooth muscle cell growth by perlecan may involve the inhibition of PDGF signaling which has downstream effects on FGF2 signaling. Finally, the LDL repeats in perlecan domain II, a module predicted to interact with lipids (*31*), are involved in uptake of LDL and VLDL (*32*).

Thus, perlecan might be indirectly involved in the complex interplay among these signaling pathways during cartilage development and differentiation.

PRO-ANGIOGENIC ACTIVITY

Perlecan is strongly expressed in the stroma of various types of solid tumors. It is often associated with the microvasculature which provides nutrients and oxygen to the growing neoplastic cells, and its expression correlates with a more aggressive phenotype. In 1994, we reported the first evidence that perlecan could be involved in angiogenesis. We found that in tumor xenografts composed of human-derived prostate carcinoma cells and mouse-derived stromal elements, perlecan secreted by the human prostate cancer cells was deposited along the newly formed (angiogenic) vessels of the tumor xenografts. Thus, we hypothesized that perlecan might directly contribute to the scaffolding of angiogenic blood vessels (*3*). Nearly concurrently, it was demonstrated that perlecan is the major cofactor for the activity of FGF2, a powerful angiogenic factor, and for the specific interaction with its cognate receptor leading to enhanced mitogenesis and angiogenesis. Notably, antisense targeting of endogenous perlecan in a variety of transformed cells, including colon carcinoma and melanoma cells, causes a significant inhibition of tumor growth and angiogenesis (*3*). Seemingly, colon carcinoma cells with a somatic cell mutation leading to a perlecan null phenotype show growth retardation and minimal angiogenesis in tumor xenografts (*18*). The central role of perlecan in angiogenesis is further confirmed by genetic manipulation leading to complete ablation of the perlecan gene (*6, 7*). A significant proportion of perlecan-null mice develop numerous vascular anomalies, including transposition of the great arteries and abnormal coronary arteries (*1*). In an animal model expressing a mutated form of perlecan lacking the canonical glycosaminoglycan attachment site, and thus lacking HS side chains, there is impaired angiogenesis and retarded tumor growth (*33*), whereas perlecan is required to inhibit thrombosis in an animal model of deep vascular injury (*16*). A recent study adds a new dimension to these results because it demonstrates that regulation of perlecan gene expression is regulated by a mechano-transduction pathway in endothelial cells and that this is a key mechanism through which endothelial cells inhibit vascular smooth muscle cell proliferation in response to changes in mechanical environment (*34*).

A central function of perlecan in cardiovascular development and angiogenesis has recently been demonstrated in the zebrafish *Danio rerio*. Morpholino-mediated knockdown targeting three separate regions of the perlecan mRNA showed relatively normal development of axial vessels, dorsal aorta, and posterior cardinal vein, but a blunted and anomalous development of the angiogenic intersegmental and dorsal longitudinal anastomotic vessels [Figure 2A,B (*35*)]. Notably, the perlecan morphant phenotype could be rescued by microinjecting human perlecan into single-cell embryos. The overall phenotype of the perlecan morphants is similar to that evoked by null mutations or knockdown of VEGFR2, phospholipase Cγ-1, a major downstream target of VEGF–VEGFR angiogenic signaling, VEGFR2 receptor blockade by the small molecule SU5416, or by antisense knockdown of VEGFA. Thus, it is possible that perlecan is required for the proper targeting of VEGF to its cognate receptor during developmental angiogenesis.

In hepatoblastoma xenografts, VEGF is deposited in the same perivascular pattern as tumor-derived perlecan (*36*)

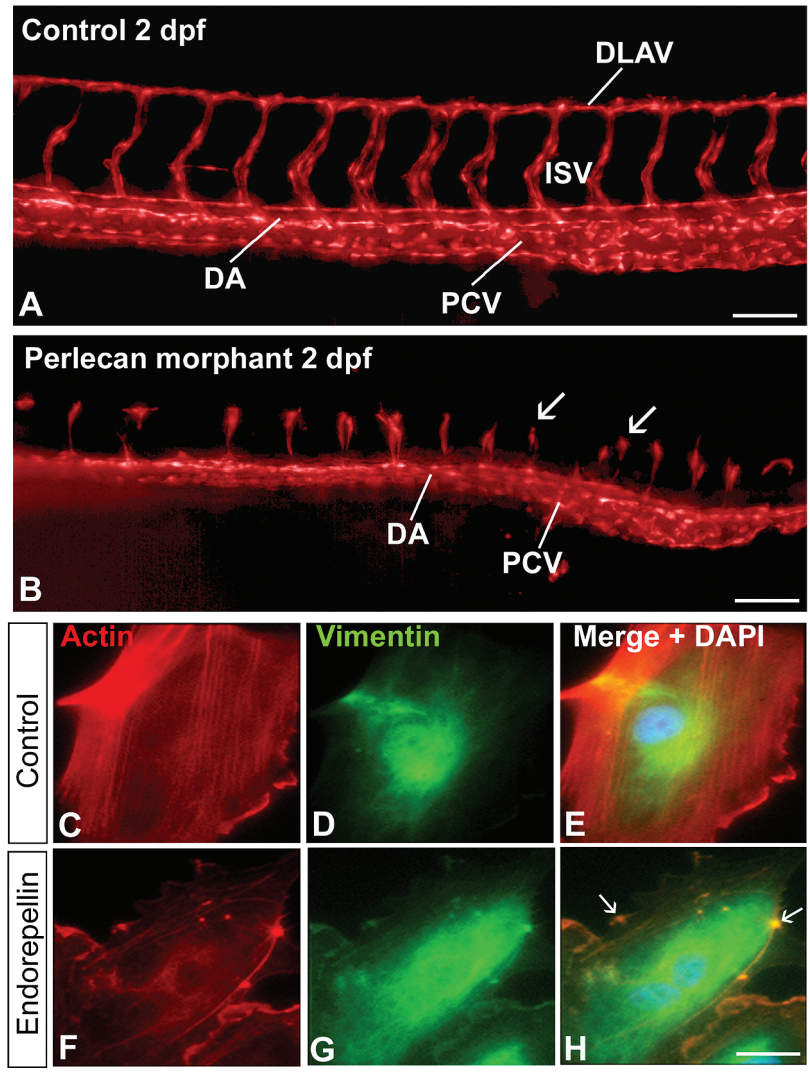

FIGURE 2: Pro- and anti-angiogenic activity of perlecan and endorepellin, respectively. (A and B) Effects of perlecan knockdown on the development of zebrafish embryonic vasculature. The vasculature is visualized in red (pseudocolor) and derives from the transgenic expression of GFP driven by the *fli*1 promoter specific for endothelial cells. (A) Epifluorescence microscopy with three-dimensional deconvolution of the trunk vessels from a zebrafish control embryo at 2 days postfertilization (dpf). Note the correct formation of the dorsal aorta (DA), posterior cardinal vein (PCV), intersegmental vessels (ISV), and dorsal longitudinal anastomotic vessels (DLAV). (B) In contrast, the perlecan morphants exhibit relatively normal axial vessels but significantly blunted ISVs and no DLAVs (arrows). The bar is 100 μm. (C−H) Double immunofluorescent micrographs of human endothelial cells following a 30 min exposure to endorepellin using rhodamine-phalloidin to label actin (red) or antibodies against vimentin (green). Notice the endorepellin-evoked disruption of the actin cytoskeleton (F−H) in contrast to the preservation of the actin microfilaments in untreated endothelial cells (C−E). The arrows in panel H show bundles of actin filaments collapsed onto the plasmalemma. The bar is 5 μm.

and the vascular recovery following VEGF blockade by systemic delivery of soluble VEGFR1 and VEGFR2 is mediated by enhanced expression of perlecan at such locations. Concurrently, there is an increase in the level of heparanase in the perivascular zones. Perlecan-bound VEGF might be dynamically regulated by heparanase-mediated release from the HS chains of perlecan and/or by proteolytic processing of the perlecan protein core with ultimate release of domain I-associated HS−VEGF complexes in a manner similar to that shown previously for domain I-associated FGF complexes (*37*). Thus, sequestration and release of perlecan-bound VEGF in the tumor microenvironment represent steps of a mechanism for continuous vessel growth and tumor progression. The net result is a protracted activation of VEGFR2 which caused a sustained activation of the Akt pathway promoting survival and angiogenesis (*36*).

Interestingly, HSPGs can also act across cells or "in trans" (*9*) and, specifically, can potentiate in trans VEGFR-mediated angiogenesis (*38*). Arteries and arterioles are surrounded by mural cells, either vascular smooth muscle cells for large arteries and veins or pericytes for capillaries. Mural cell HSPGs, most likely including perlecan which is a major product of smooth muscle cells and pericytes, can transactivate VEGFR2 on endothelial cells by enhancing signal transduction and by facilitating the formation of receptor−ligand complexes on endothelial cells (*38*). Thus, perlecan occupies a central role in angiogenesis because it can potentially mediate not only the VEGF and VEGFR axis but also the transactivation of smooth muscle cells and pericytes during angiogenesis.

While the overwhelming majority of the reports support a pro-angiogenic activity of the parent perlecan proteoglycan, other studies suggest the possibility that perlecan might

inhibit tumor growth and angiogenesis (*39*). These apparently contradicting data could be reconciled by considering the fact that perlecan acts in a cell context-specific manner. In the vast majority of epithelial tumors (i.e., cancers), perlecan may be required for presenting FGF2 and VEGF to the expanding tumor vasculature, whereas in sarcomas, perlecan might be inhibitory via the liberation of cryptic anti-angiogenic fragments (see the next section).

ANTI-ANGIOGENIC PROPERTIES: CRYPTIC C-TERMINAL FRAGMENTS

During a search for perlecan binding partners using the yeast two-hybrid system and domain V of perlecan as the bait, we isolated a highly interactive cDNA clone which encoded the NC1 domain of collagen type XVIII (*40*) comprising the powerful anti-angiogenic fragment named endostatin. We soon realized that domain V of the perlecan protein core harbored a powerful angiostatic activity as demonstrated by various in vitro and in vivo angiogenic assays; this region was renamed endorepellin to designate its intrinsic anti-endothelial activity (*40*). Endorepellin is composed of three laminin-like globular domains (LG1−LG3) interspersed with four EGF-like modules (Figures 1 and 3) and interacts specifically with the α2β1 integrin, an established receptor for collagen I, in platelets (*41*) and endothelial cells (*42*). In the latter, endorepellin triggers a signaling cascade that leads to disruption of the endothelial actin cytoskeleton (Figure 2C−H) and thus to cytostasis (*3, 42−44*). Using a proteomic approach, several key proteins involved in angiogenesis, including β-actin, were significantly downregulated via exposure of endothelial cells to recombinant endorepellin (*45*). Importantly, systemic delivery of human recombinant endorepellin to tumor xenograft-bearing mice causes a marked suppression of tumor growth and metabolic rate mediated by a sustained downregulation of the tumor angiogenic network (*46*). Genetic analysis using siRNA-mediated block of endogenous α2β1 integrin or animals lacking the α2β1 integrin receptor has definitively shown that this is a key receptor for endorepellin, and thus for the perlecan protein core, and has further demonstrated that endorepellin targets the tumor xenograft vasculature in an α2β1 integrin-dependent manner (*47*). Endorepellin might represent a member of the family of cryptic domains residing within larger parent molecules of the extracellular microenvironment that acts in a dominant negative manner.

Notably, the last laminin-like globular domain, LG3 (Figure 3), possesses most of the biological activity (*42*) and can be released from the parent molecule by BMP-1/Tolloid-like metalloproteinases (*48*) which recognize an ND dipeptide that is highly conserved across species, including human, mouse, *Drosophila*, and zebrafish (*35*). This highly conserved region within the perlecan protein core together with the high degree of conservation of BMP-1/Tolloid-like metalloproteinases suggests that liberation of LG3 might be physiologically important. Mutations in LG3 molecules displaying lower or no affinity for calcium (*48*) disrupt LG3 angiostatic activity (Figure 3). It is noteworthy that the two proximal globular domains of endorepellin, LG1 and LG2, might be occupied by a number of high-affinity ligands such as dystroglycan and endostatin within basement membranes and on cell surfaces. In contrast, LG3 might be relatively

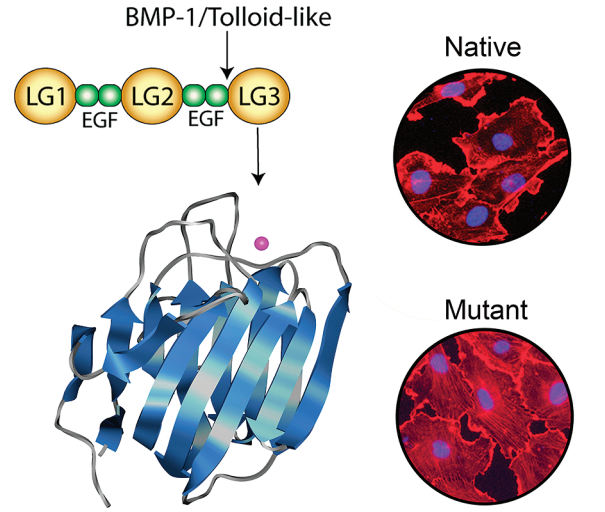

FIGURE 3: Endorepellin is cleaved by the BMP-1/Tolloid proteases, liberating the anti-angiogenic LG3 domain. The illustration depicts a graphic illustration of endorepellin and a comparative model of LG3. Highlighted in magenta is a calcium ion shown to be vital for function. To the right are immunofluorescence images of endothelial cells stained for actin (red) and depicting the effects of LG3 (notice the disruption of the actin cytoskeleton) and of an LG3 mutant which exhibits a reduced affinity for calcium. Nuclei are stained blue with DAPI.

accessible and thus likely to be released by partial proteolysis, a process that is common to most LG domains of laminin. Indeed, LG3 fragments with identical N-terminal residues (i.e., cleaved by BMP-1/Tolloid-like metalloproteinases) have been found in the urine of patients with end-stage renal failure (*49*) and chronic allograft nephropathy (*50*), and in the amniotic fluid of pregnant women with a marked increase in women with symptoms of premature rupture of fetal membranes (*51*) and those carrying trisomy 21, Down syndrome, fetuses (*52*). In addition, endorepellin fragments have been found in the media conditioned by apoptotic endothelial cells (*53*). In this case, secreted LG3 interacts with the α2β1 integrin receptor of fibroblasts and triggers a signaling cascade that leads to activation of an anti-apoptotic pathway and potentially to a fibrogenic response (*53*).

We hypothesize that endorepellin and LG3 are liberated via partial proteolysis during tissue remodeling and cancer growth, thereby representing an additional layer of control for angiogenesis, which also depends on the cellular context and specific integrin expression. In line with this fine-tuning, circulating LG3 levels have been shown to be reduced in patients with breast cancer (*54*), suggesting that reduced titers might be a useful biomarker for cancer progression and invasion.

A COMMON THEME: RELEASE OF BIOACTIVE FRAGMENTS AND THEIR FINE BALANCE

A common theme is emerging from a growing body of literature. The main postulate is that processing of extracellular matrix proteins is not a random event but is a guided and focused biological process that can affect either positively or negatively the growth of cells and, in particular, angiogenesis. For example, cathepsin L, a cysteine protease of the papain superfamily, cleaves collagen XVIII in the hinge region of the NC1 domain, thereby liberating endostatin, a strong anti-angiogenic factor (*4*). Efficient endostatin

generation requires a moderately acidic pH, a typical feature of the tumor microenvironment. Interestingly, apoptotic endothelial cells secrete cathepsin L which, in turn, cleaves endorepellin near its C-terminal region, thereby liberating endorepellin's angiostatic LG3 domain (*55*). Thus, cathepsin L and BMP1/Tolloid-like proteases acting in concert could liberate LG3 from the perlecan associated with the cell surface or embedded within the basement membrane. Finally, cathepsin L has recently been shown to be a key enzyme required for the conversion of proheparanase into an active heparanase by specifically cleaving multiple sites within the linker region (*56*). Thus, differential expression of cathepsin L may have opposite effects on angiogenesis by generating either anti-angiogenic factors (endostatin and endorepellin's LG3) or pro-angiogenic factors (FGF, VEGF, PDGF, etc.) via heparanase-mediated cleavage of the HS chains of perlecan and collagen XVIII. The molecular understanding of this fine balance between pro- and anti-angiogenic activities will undoubtedly lead to a better treatment of cancer and other diseases where angiogenesis is prevalent.

AN ENDOREPELLIN-LIKE STRUCTURE IN AGRIN

Agrin, another basement membrane and synaptic HSPG, has an endorepellin-like domain at its C-terminus. This domain comprises three LG modules interspersed with three EGF-like repeats (*5*). Notably, endorepellin-like and LG3 fragments are generated from agrin by a specific serine protease, neurotrypsin (*57*). Neurotrypsin cleaves agrin at two homologous sites, liberating a 90 kDa fragment and the C-terminal globular domain, LG3 (*57*). The release of cryptic fragments within agrin could promote interactions with other proteins and receptors that were inaccessible to full-length agrin. While there is no evidence that any of these modules affects angiogenesis, there is ample evidence that they play important biological roles and can also mediate signaling events propagated from surface receptors. For example, the endorepellin-like region of agrin is involved in binding to dystroglycan and integrins (*5*). In addition, the LG3 module of agrin signals through a synaptic receptor that has been recently identified as the Na^+,K^+-ATPase (*58*). Agrin LG3 inhibitory activity evokes membrane depolarization and increases action potential in neurons by interacting with the α3 subunit of the Na^+,K^+-ATPase, a member of the family of ion pumps (*58*). Elegant work by Rüegg and collaborators (*59*) has shown that a mini-agrin gene composed of the N-terminus (which binds to laminin γ1) and the C-terminal endorepellin-like structure (which binds to α-dystroglycan) can function as a biological linker and can ameliorate muscular dystrophy caused by mutation in the laminin α2 gene (*59*). Interestingly, chimeric proteins composed of the N-terminus of agrin and the mouse endorepellin can also have similar linker and stabilizing activity and rescue the dystrophic phenotype (*59*). Thus, it is conceivable that modules containing LG domains interspersed with EGF-like repeats, such as those present in perlecan and agrin, might have developed unique cell- and tissue-specific functions. Not surprisingly, these modules have been conserved for more than 500 million years of evolution. Moreover, utilization of endorepellin-like structures which bind with high affinity to surface receptors such as α-dystroglycan and α2β1 integrin might be utilized in the future for the treatment of diseases in which these two receptors are involved.

ADDITIONAL BIOLOGICAL PROPERTIES OF ENDOREPELLIN AND LG3

In a different pathological setting, LG3 is released by apoptotic endothelial cells induced by serum starvation which leads to enhanced proteolytic processing of extracellular matrix constituents, including perlecan (*53*). Released LG3 causes an α2β1 integrin-dependent anti-apoptotic pathway in fibroblasts, which express high levels of this receptor, and this could have a potential effect on abnormal fibrogenic healing (*53*). This is another example of a cell-specific context in which cryptic perlecan fragments might show a diverse effect. Indeed, in various fibrotic diseases, apoptosis of endothelial cells precedes the recruitment of fibroblasts, and thus, release of LG3 could affect not only angiogenesis but also the production of collagen and the overall fibrotic response.

Quantitative proteomic analysis of the pancreatic cancer secretome has shown that a fragment of perlecan encompassing endorepellin is increased in size approximately 5-fold relative to the secretome of normal pancreatic ductal cells (*60*), suggesting that endorepellin and LG3 might serve as a potential biomarker for pancreatic cancer. We have found LG3 abundantly secreted by human colon carcinoma cells (*48*) and a variety of transformed cells (unpublished observation). Thus, endorepellin and LG3 could be used as a biomarker for pancreatic cancer and other forms of cancer since LG3 is soluble, circulating in the blood, and can be secreted into several biological fluids, including urine and amniotic fluid. This makes detection of LG3 a feasible procedure with potential diagnostic and prognostic value.

A WORKING MODEL OF PERLECAN'S ROLE IN TUMOR ANGIOGENESIS

Extracellular matrix-derived signals control vascular morphogenesis and remodeling. Growth factors such as FGF and VEGF require not only their receptors for full biological activity but also essential cofactors such as HS and, indirectly, the protein core of perlecan and other extracellular HSPGs. Thus, perlecan functions in targeting, storage, and delivery of growth factors to their functional receptors. It has been recently proposed that complex molecules such as perlecan, which reaches 100−200 nm in length, could serve to cluster various ectodomains of transmembrane proteins, stabilize their interactions, and thus create a stable signaling complex. During tumor progression, the vascular basement membrane undergoes constant remodeling, and when heparanase is preponderant, it could release growth factors from the HS chains of perlecan (Figure 4). These increased levels of growth factors together with the cofactor HS would activate their respective cognate receptors which, in turn, would activate the pro-survival activity of Akt and ultimately promote angiogenesis and tumor progression. Obviously, marked proteolysis would also generate a large number of growth factors and cytokines that are bound to the protein core, including PDGF, FGF7, and FGF2. When proteolysis is somewhat "limited", endorepellin and LG3 could be liberated in the tumor microenvironment to counteract the FGF−FGFR and VEGF−VEGFR2 axes: endorepellin in-

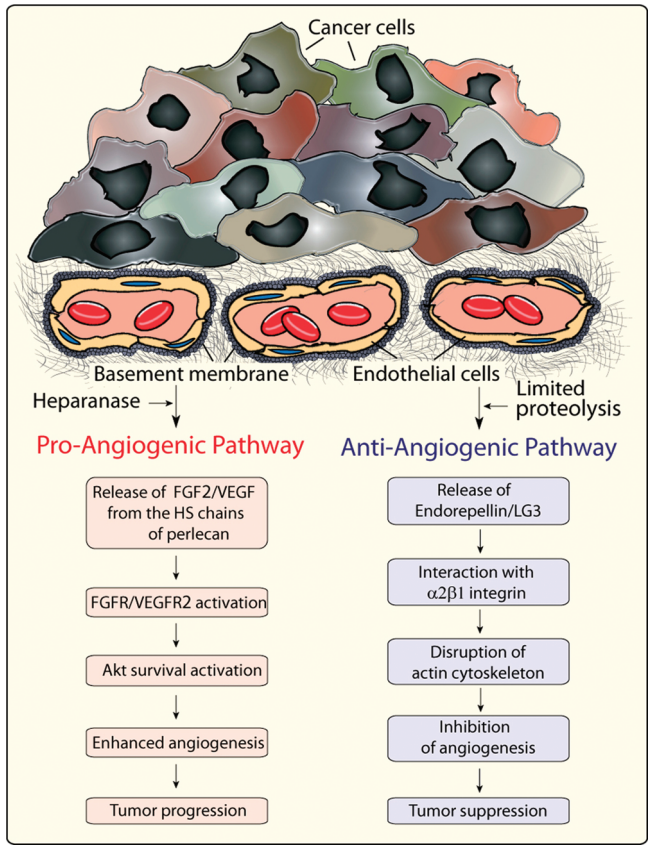

FIGURE 4: Working model showing the potential dual role of perlecan in tumor angiogenesis. Heparanase-mediated release of growth factors, primarily FGF2 and VEGF, bound to the heparan sulfate chains of perlecan would lead to sustained activation of FGF−FGFR and VEGFR2−Akt pathways, with consequent enhanced survival of endothelial cells and increased levels of tumor angiogenesis and tumor progression. On the other hand, limited proteolysis of the perlecan protein core could liberate endorepellin and LG3; this evokes an $\alpha 2\beta 1$ integrin-dependent disruption of the endothelial cell actin cytoskeleton, inhibition of endothelial cell migration, a reduced level of angiogenesis, and a reduced level of tumor growth.

teracts with the $\alpha 2\beta 1$ integrin receptor and triggers a signaling cascade that leads to disruption of the endothelial cell actin cytoskeleton, inhibition of cell motility, and ultimately inhibition of angiogenesis and concurrent tumor suppression (Figure 4). This conceptual framework could be easily applied to other endogenous inhibitors of angiogenesis associated with the basement membrane, such as those derived from various basement membrane collagens.

Understanding the balance between pro- and anti-angiogenic cues will be of great therapeutic potential in the future. Would blocking heparanase, for instance, be a suitable treatment for certain forms of highly vascularized cancers? What protease inhibitors would be most beneficial for tilting the balance toward a less vascularized or avascular condition? Should heparin mimetics be used in tumor therapy to cause the diffusion of growth factors away from the tumor cells? Would combination therapy work? These important questions can conceivably be answered in the near future after we elucidate the role each component plays in the complex processes of vascular generation, regression, and remodeling that occur during cancer evolution.

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