Tissue distribution of perlecan domains III and V during embryonic and fetal human development

Matthias Roediger, Jenny Kruegel, Nicolai Miosge and Nikolaus Gersdorff
Department of Prosthodontics, Tissue Regeneration Work Group, Georg-August-University Goettingen, Germany

Summary. A major component of basement membranes (BMs) is perlecan, a five-domain heparan sulphate proteoglycan. During murine embryogenesis, nearly all BMs of mesenchymal origin express perlecan, and it is believed to participate in the supramolecular assembly of BMs. However, the distribution of perlecan in human embryonic and fetal tissues is widely unknown, except for cartilage anlagen of developing extremities and the fetal spine. Clinical syndromes, caused by perlecan-associated mutations or gene-defects, suggest its multifunctional involvement during human development. Here we reveal the immunohistochemistry of perlecan domains III and V during human development from gestational weeks (gw) 6 to 12 in basement membrane zones (BMZs) of the developing brain, nervous system, blood vessels, skin, lung, heart, kidney, liver, intestine and skeletal system. Interestingly, a difference in the distribution of the two perlecan domains was found in the endoneurium of ganglia. Domain III is strongly present from gw 6 onwards, while domain V shows attenuated expression at this stage and has been detected abundantly only from gw 8 onwards, possibly indicating vascularization of the endoneurium during this early stage. We found perlecan to be present particularly at those stages of human development where epithelial-mesenchymal interactions occur.

Key words: Perlecan, Basement membrane, Human embryogenesis, Immunohistochemistry

Introduction

Basement membranes (BMs) are specialized structures of the extracellular matrix closely apposed to epithelia, endothelia, peripheral nerve axons, fat cells and muscle cells. They are known to fulfil various functions during development and in adulthood. BMs divide tissues into compartments, separating the epithelium and stroma, and regulate many cellular activities, including growth, differentiation and cell migration (Timpl and Aumailley, 1989).

Laminin-111 (Timpl et al., 1979), nidogen-1 (Carlin et al., 1981), nidogen-2 (Kohfeld et al., 1998), collagen type IV (Kühn, 1995) and perlecan (Noonan and Hassel, 1993) have all been identified as major components of BMs.

Perlecan is a heparan sulphate proteoglycan found in many adult connective tissues, and was first isolated from a mouse tumor (Engelbreth-Holm-Swarm, EHS) (Hassel et al., 1980). Perlecan is expressed in cartilage and several other mesenchymal tissues (French et al., 1999; Hassell et al., 2002; Melrose et al., 2006), and is present in nearly all BMs, as well as in all mesenchymal organs, such as kidney, lung, liver and the gastrointestinal tract. Furthermore, it is found in numerous connective tissues during vasculogenesis, as well as in heart, pericardium, blood vessels and cartilage during murine embryogenesis (E11-17.5), suggesting a crucial role in maturation and maintenance of differentiated tissues (Handler et al., 1997). In addition, it has been demonstrated for early-stage murine embryogenesis (E12-17.5) that perlecan controls the response of the neuroepithelium to growth factors, and that perlecan-null brains show atrophy and decreased numbers of cells in mitosis (Girós et al., 2007). In chick embryos perlecan was detected very early from stage X (morula) to Hamburger-Hamilton stage (HH) 17 (29 somites) with varying intensity in neuroepithelium, lung rudiments, aortic arches, myocardium, tubule rudiments and gut. The authors suggest a crucial role for perlecan in maintaining BM integrity, mediation of epithelization, adhesive separation and maintenance of neuroepithelium in brain and tissue architecture in heart, aorta and gut (Soulintzi and Zagris, 2007).

Many studies suggest functions for perlecan during cell growth, differentiation and tissue organization, as it has been shown to exhibit binding sites for various BM components. Perlecan may also play a structural role in
Perlecan in human organogenesis

addition to its involvement in cell-matrix interactions (Hopf et al., 1999; Miosge et al., 2003).

Perlecan is involved in angiogenesis, regulation of wound healing and bone formation (Knox and Whitelock, 2006). Additionally, perlecan is postulated to mediate the binding of proteins to the protein core and/or its glycosaminoglycan (GAG) chains, and may play a role in the pathogenesis of human late-stage osteoarthritis (Tesche and Miosge, 2004).

Mutations and gene defects, associated with perlecan, cause various syndromes, suggesting perlecan’s functional involvement in human development, for example, Schwartz-Jampel Syndrome, a myotonic myopathy associated with chondrodysplasia (Nicole et al., 2000; Arikawa-Hirasawa et al., 2002), as well as dyssegmental dysplasia of Silverman-Handmaker type, a human disorder identified to be a functional null mutation of perlecan (Arikawa-Hirasawa et al., 2001).

The human perlecan gene (HSPG2) consists of 97 exons and is located in the telomeric region of chromosome 1. HSPG2 encodes a large 467 kDa protein consisting of five distinct domains, I-V (Noonan et al., 1991), with homologies to laminin, low-density lipoprotein (LDL) receptor, epithelial growth factor (EGF) and neural cell adhesion molecule (N-CAM).

The main region of GAG substitution in human perlecan is domain I, which contains a cluster of three potential attachment sites (Konyesi and Silbert, 1995) and promotes binding to laminin-111 and collagen type IV when substituted with heparan sulfate (HS). There are a variety of additional interactions, including nidogen-2, fibulin-2, fibronectin, PRELP (proline/arginine-rich and leucine-rich repeat protein)/prolargin, and types XIII and XVIII collagen (Hopf et al., 2001; Bengtsson et al., 2002; Miosge et al., 2003).

The N-terminal domain III of perlecan has been detected in BMs of several mouse organs by immunohistochemistry (Couchman et al., 1995) and in human adult tissues, including the pituitary gland, skin, breast, thymus, prostate, colon, lung, pancreas, spleen, heart, lung, vascular BMs and in the subendothelial regions of sinusoidal vessels of liver, spleen, lymph node and pituitary gland (Murdoch et al., 1994).

The C-terminal domain V of perlecan promotes beta 1 integrin-mediated cell adhesion and binds heparin, nidogens and fibulin-2 (Brown et al., 1997) and is also involved in GAG attachment, substitution and the binding of several carbohydrate and protein ligands (Friedrich et al., 1999). The localization of perlecan domain V is not as well documented as that of domain III.

BM components like perlecan are well known to participate in numerous cell biological processes during embryogenesis. Other than its localization in hyaline cartilage anlagen in extremities (Melrose et al., 2006) and spine of the fetal human (Melrose et al., 2003), little is known about perlecan localization during early human development.

In this study, we systematically examined the localization of perlecan in human embryonic and fetal tissues with the aid of immunohistochemistry. We found perlecan to be present particularly at those developmental stages where epithelial-mesenchymal interactions occur.

Materials and methods

Tissue sources

Aborted human embryos and fetuses were obtained according to the regulations of the Ethics Committee of the Medical Faculty of the University of Goettingen. The cut-off point for the differentiation between embryos and fetuses has been defined to range from gestational week (gw) 8 to 9 (Moore, 1982). We investigated three embryos of gw 6 to 7, two of gw 8, two fetuses of gw 9, two of gw 10 and two fetuses of gw 12. The stages were determined according to O’Rahilly and Mueller (O’Rahilly and Mueller, 1987). No malformations or abnormalities were observed in these specimens. The abortion procedure leads to destruction of much of the embryonic tissues, limiting the tissues we could investigate.

Tissue processing

The specimens were transported in histidine-tryptophan-ketoglutarate solution at 4°C to ensure tissue preservation (Bretschneider, 1980) and were fixed with 3.7% paraformaldehyde in phosphate buffer, pH 7.2, at 4°C for 24 h, and then serially dehydrated in ethanol from 30% to 100% and embedded in paraffin according to standard protocols (Gersdorff et al., 2005). Serial sections of 5 µm thickness were cut with a Reichert’s microtome. Every fifth section was stained with hematoxylin and eosin (HE) for topological orientation within the anatomical regions examined.

Antibodies

Rabbit polyclonal antibodies against perlecan domain III-3 (1030, Schulze et al., 1996) and perlecan domain V (1056, Brown et al., 1997) were kindly provided by Dr. Takako Sasaki (Shriners Hospital, Portland, Oregon).

Secondary antibodies used for immunostaining were purchased from Dako (Hamburg, Germany).

Immunohistochemistry

Immunoperoxidase staining was performed on paraffin-embedded tissue sections as follows: Tissues were deparaffinised, rehydrated and rinsed for 10 min in phosphate buffered saline (PBS), pH 7.2. Endogenous peroxidase was inhibited with a solution of 3% \( \text{H}_2\text{O}_2 \) in
methanol for 45 min in the dark. The sections were pretreated for 5 min with 10 µg/mL protease XXIV (Sigma Deisenhofen, Germany). The antibodies 1030 (perlecan domain III) and 1056 (perlecan domain V) were used at a 1:100 dilution in PBS/BSA and incubated for 1 h at room temperature. Each step was followed by a 10-min rinse in PBS. The secondary antibody was applied at a 1:50 dilution in PBS/BSA, followed by incubation with the PAP complex (diluted in 1:150 in PBS/BSA) for 30 min at room temperature. The sections were counterstained with hematoxylin. Negative controls omitted the primary antibody.

Results

A complete list of our results is given in Table 1 with the exception of those from the skeletal system, which are described in the text below.

Neuronal system

The chorda dorsalis induces the morphogenesis of the neuroectoderm to form the neural tube at the beginning of gw 4 (Sadler, 2005). Staining for perlecan was found in the endoneurium of peripheral ganglia and the basement membrane zones (BMZs) of capillaries. Perlecan domain III was localized in all embryonic and fetal specimens from gw 6 to 12 (Fig. 1A), whereas perlecan domain V showed only weak staining at gw 6 and was abundantly found from gw 8 onwards (Fig. 1B,E). Neuronal cells were negative for staining of perlecan domain III at all stages examined and showed only attenuated distribution of domain V (Fig. 1A,B).

Fig. 1. Localization of perlecan in the neuronal system during human embryogenesis. A. Immunostaining for perlecan domain III was positive in the endoneurium of a nerve (N) and ganglion (G) at gw 6 (arrows). B. Endoneurium (arrows) of a nerve (N) and neuronal cells (asterisk) at gw 6 showed weak staining for perlecan domain V. C. Localization of perlecan domain V in BMZs of neuroectoderm (open arrows) at gw 6. D. Immunostaining for perlecan domain V in BMZs of neuroectoderm (open arrows) and capillaries (arrowheads) at gw 6. E. Localization of perlecan domain V in the endoneurium of spinal ganglia (arrows), condensed mesenchyme (triangle) and cartilage anlagen (asterisk) at gw 9. Bars: A-D, 15 µm; E, 60 µm.
BMZs of the neuroectoderm and of capillaries stained positive for both perlecan domains III and V between gw 6 to 12 (Fig. 1C,D), while no staining was detected for either perlecan domain in neuroectodermal cells.

**Blood vessels**

Already at gw 3, in the visceral mesoderm, angioblasts differentiate into endothelial cells (O’Rahilly

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BMZ: basement membrane zone; ++: positive reaction; +: intermediate reaction; -: negative reaction; Ø: tissues not available.
**Liver**

Development starts with the budding of entodermal cells from the distal end of the foregut (O’Rahilly and Müller, 1996). From gw 6 onwards to week 12, in the developing liver, both perlecan domains were present in BMZs of sinusoids and capillaries (Fig. 2F). Perlecan was also detected in the early mesenchyme at gw 6 and 7 (Fig. 2F). However, hepatocytes and hematopoietic cells were negative at all stages examined.

**Lung**

Lung buds emerge between gw 4 and 5 also as a diverticulum of the entoderm. Branching morphogenesis results in development of bronchioli and alveoli at gw 6 to 8, while cartilage and smooth muscles originate from visceral mesoderm (Burri, 1984; O’Rahilly and Müller, 1996). In the embryonic lung, perlecan domains III and V were specifically detected in BMZs of the bronchioli and of the bronchial capillaries from gw 6 to 12 (Fig. 2A,B). Low levels of perlecan were shown for mesenchymal cells (Fig. 2B).

**Kidney**

From cranial to caudal, three renal systems evolve (pronephros, mesonephros and metanephros). At gw 6, urinary secretion starts in the mesonephros lasting up to gw 12 (Saxen and Sariola, 1987). At all stages of renal development examined, perlecan domains III and V were expressed in the BMZs of glomeruli, tubules and capillaries. The surrounding mesenchymal cells, however, were negative for perlecan (Fig. 2C-E).

**Skin**

Skin development starts from two compartments. The epidermis derives from the ectoderm and the dermis from the mesoderm (Smith and Holbrook, 1986). The BMZs of dermal-epidermal junctions and of capillaries stained positive for perlecan domains III and V, while keratinocytes of the epidermis, dermis and the loose mesenchyme were negative (Fig. 2G).

**Pancreas**

BMZs of the developing exocrine gland were positive for perlecan at all developmental stages examined. Furthermore, perlecan was localized to the BMZs of glandular capillaries, while epithelial cells revealed no staining for perlecan (Table 1).

**Intestine**

Already at gw 4, the early foregut forms. Connective tissues and the musculature of the gastro-intestinal tract derive from the surrounding mesenchyme (O’Rahilly and Müller, 1996). The developing intestine stained positive for perlecan in the epithelial BMZ, as did the BMZs of the capillaries in the tunica submucosa and adventitia. Perlecan was absent in epithelial cells, mesenchymal cells and the developing lamina muscularis prior to gw 8. From gw 8 onward, perlecan domains III and V were expressed in the lamina muscularis and submucosa (Fig. 2K,L).

**Heart**

In embryonic week 4, the heart anlage consists of the endocardial tube and the developing myocardial layer separated from each other by the cell-free, homogeneous cardiac jelly (CJ), which also surrounds individual myocytes (Wenink, 1976). Perlecan was detected at gw 6 in the BMZs of the endo- and pericardium, as well as of capillaries, and was present until gw 12.

Perlecan was not detected between gw 6 and 7 in developing cardiomyocytes. At gw 8, the mesenchyme of the endocardial cushion tissues stained positive for perlecan domain V (Fig. 2H). Perlecan was also detected intracellularly and in BMZs of cardiomyocytes from gw 8 to 12 (Fig. 2I,J).

**Skeletal system**

The condensed mesenchyme at gw 6 and 7 represents the first developmental step towards cartilage anlagen, and exhibits weak staining for perlecan domains III and V. However, perlecan was localized to the BMZs of capillaries (Fig. 3A). At gw 8, late condensed mesenchyme and early vertebral cartilage anlagen were positive for perlecan domain III (Fig. 3B), and perlecan domain V (Fig. 3C). Both perlecan domains were expressed in capillaries. At gw 9, chondrocytes and the pericellular matrix of rib cartilage anlagen were perlecan-negative, whereas the interterritorial matrix and BMZs of capillaries stained positive (Fig. 3D,E). Ossification of the long bones starts around gw 8 and localization of perlecan domains III and V was also observed in zones of chondral and desmal ossification. Hypertrophic chondrocyte staining decreased with increasing levels of perlecan domain III toward the metaphysis (Fig. 3F,G). Proliferating chondrocytes of the epiphyses of long bones at gw 12 exhibited staining for perlecan domain III in the interterritorial matrix, and in contrast to cartilage anlagen at gw 9, perlecan was additionally expressed in the pericellular matrix (Fig 3H,I). Furthermore, BMZs of capillaries exhibited staining for perlecan domain III, while the perichondrium was negative at all stages examined.
Fig. 2. Localization of perlecan during human embryogenesis. A. Perlecan domain V in the BMZs of bronchioli (open arrows) and of blood vessels (arrow head) at gw 8. B. Perlecan domain III in the BMZs of bronchioli (open arrows), of blood vessels (arrow head) and in the mesenchyme (M) at gw 12. C, D. Perlecan domain III localization in the BMZs of tubuli (open arrow), glomeruli (arrows) and capillaries (arrowhead) in human embryonic kidney at gw 7. E. Perlecan domain III in the BMZs of kidney tubules at gw 9 (open arrows). F. Sinusoid (S) of developing liver at gw 7. Perlecan domain III was detected in BMZs of sinusoids (open arrows) and in early mesenchyme (M). G. Perlecan domain III in dermal-epidermal BMZ (open arrows) of the skin at gw 12. Epidermis (arrows) and loose mesenchyme (LM) were negative. H. Perlecan domain V (open arrow) in endocardial cushion tissue (ECT) at gw 8. I, J. Perlecan domain III in cardiomyocytes (CM) and in BMZs of cardiomyocytes (open arrow) at gw 9. K, L. Perlecan domain III in intestinal mesenchyme (M) and in BMZs of capillaries (arrowhead) and epithelium (open arrows) at gw 10. Bars: A, D, F-H, J, L, 15 µm; B, C, E, I, K, 60 µm.
Fig. 3. Localization of perlecan in the skeletal system during human embryogenesis. A. At gw 6, condensed mesenchyme (triangle), the first developmental step toward cartilage anlagen revealed weak staining for perlecan domain III (asterisk), while BMZs of blood vessels (arrowhead) exhibited distinct staining. B. At gw 8, perlecan domain III was detected in BMZs of capillaries (arrowhead), in late condensed mesenchyme (triangle), as well as in vertebral cartilage anlagen (asterisk). C. At gw 8, positive staining for perlecan domain V was seen in vertebral cartilage anlagen (asterisk) and in BMZs of capillaries (arrowhead). D, E. Staining for perlecan domain III in the interterritorial matrix (open arrows) of rib cartilage anlagen (asterisk) and BMZs of blood vessels (arrowheads) at gw 9. Zones of the pericellular matrix were negative. F, G. Zones of chondral ossification (CO), desmal ossification (DO) and BMZs of capillaries (arrowhead) in developing long bones displayed positive staining for perlecan domain III at gw 9. Hypertrophic chondrocyte (asterisk) staining is reduced with increasing levels of perlecan domain III toward the metaphysis. H, I. Perlecan domain III in BMZs of capillaries (arrowhead) and in cartilage anlagen of the epiphysis of long bones (asterisk), where proliferating chondrocytes (open arrows) and pericellular matrix (arrows) displayed positive staining (gw 12). Bars: A-D, F, H, 60 µm; E, G, I, 15 µm.
Embryogenesis.

Epithelial-mesenchymal interactions are necessary for the development of several organs (Grobstein, 1956). The early presence of perlecan in human embryonic kidney in BMZs of glomeruli and tubules may indicate its involvement in tubulo- and glomerulogenesis. Also, during human gut development, beginning at gw 8, intestinal connective tissues showed staining for perlecan, which is consistent with results for murine embryonic development (Handler et al., 1997). Perlecan was also detected in murine lung development (Handler et al., 1997). We found only low levels of perlecan between gw 6 to 12 in mesenchymal cells of the lung of human embryos. Both domains were detected distinctively in the BMZs of bronchioli of the developing lung from gw 6 onwards. Taken together, these results indicate the involvement of perlecan in epithelial-mesenchymal interactions during early human embryogenesis.

Another aspect to substantiate perlecan’s significance for development, inflammation and wound repair might be the high affinity to growth factors. For example, the interaction between fibroblast growth factor 2 (FGF-2) and perlecan enhances affinity to tyrosine kinase receptors and is essential for its phosphorylation. Furthermore, for FGF binding and activation of the tyrosine kinase receptors the formation of FGF-2-heparin complexes are essential (Segev et al., 2004).

During human neuronal development, perlecan domain III is expressed in the endoneurium of ganglia and in BMZs of capillaries in human embryos from gw 6. However, only weak staining for perlecan domain V was detected in the endoneurium prior to gw 9. The low level of domain V may indicate vascularization of the endoneurium during this early stage. Perlecan domain V (endorepellin) inhibits angiogenesis (Gonzalez et al., 2005). Processed perlecan lacking domain V might have been detected by the anti-domain III antibody. Starting at gw 9, the endoneurium and BMZs of capillaries revealed distinct staining for perlecan domain V, possibly indicating that vascularization has been completed already at this developmental stage.

Furthermore, our results lead us to hypothesize that perlecan might also be involved in the regulation of angiogenesis in embryonic, fetal and adult human tissues. Perlecan domains III and V were detected in the BMZs of capillaries and blood vessels in all organs and at all stages examined. Also, high levels of perlecan were detected in human primary liver tumors around newly formed blood vessels (Roskams et al., 1998). Perlecan is postulated to play a prominent role in both the activation and inhibition of angiogenesis, depending on its cellular context, consistent with the expression of perlecan in both, vascularized and hypovascularized tissues (Knox and Whitelock, 2006; Bix and Iozzo, 2008).

In the heart, in adult human tissues, perlecan domain III was localized to BMZs of the endocardium, epicardial and subendothelial BMZs of capillaries, but was absent in cardiac myocytes (Murdoch et al., 1994). We found perlecan domains III and V in the developing human heart in BMZs of endo- and pericardium at gw 6. Perlecan was not detected in the cytoplasm and BMZs of cardiomyocytes prior to gw 8. A lack of perlecan expression leads to the absence of normal BM structures around myocardial cells, indicating the importance of perlecan in cardiac development (Olson, 1999). Knockdown of perlecan in zebrafish resulted in abnormal actin filament orientation and disorganized sarcomeres, suggesting the involvement of perlecan in myopathies (Zoeller et al., 2008). This phenotype was partially rescued by injection of human perlecan and endorepellin, extending the survival time up to the larval stage. BM structures may need additional support starting from gw 8, while other heparansulfate proteoglycans, such as agrin, may fulfill this function in earlier stages. Perlecan-null mouse embryos displayed many abnormalities, including discontinuities in the myocardial wall, which result in hemorrhage or malpositioning of the larger arteries, associated with hyperplasia of mesenchymal cells in endocardial cushions (Costell et al., 1999, 2002). We also found the endocardial cushion tissues positive for perlecan domain V at gw 8. Perlecan domain V may have different functions in mice than in humans, for example, there are structural distinctions such as alternative GAG attachment sites in murine domain V compared to the human (Tapanadechopone et al., 1999).

Perlecan has been localized in cartilaginous fetal human tissues after gw 12 and strongly associated with venules and arterioles in the synovial lining (Melrose et al., 2006). We found perlecan domains III and V strongly expressed in cartilage anlagen beginning at gw 8. Condensed mesenchyme showed low levels of perlecan at earlier stages of development. Developing bones stained positive for perlecan domains III and V, which appeared in zones of chondral and desmal ossification at gw 8 and 9. Perlecan is involved in the pathogenesis in late stages of osteoarthritis, as protein and mRNA levels are upregulated adjacent to the main cartilage defect as an attempt to stabilize the extracellular matrix (Tesche and Miosge, 2004). Perlecan-deficient mouse embryos develop severe skeletal dysplasias (Arikawa-Hirasawa et al. 1999; Costell et al., 1999). Growth plates of long bones deficient in perlecan are severely disrupted in the proliferative and hypertrophic zones (Knox and Whitelock, 2006), consistent with our results of perlecan localization during chondral ossification in long bones. Schwartz-Jampel-Syndrome results from missense and splicing mutations of the gene encoding perlecan generating truncated versions of perlecan that lacked domain V or significantly reduced levels of wild-type perlecan (Nicole et al., 2000; Arikawa-Hirasawa et al., 2002). This could imply a strong association of perlecan domains III and especially V with chondrogenesis.
Perlecan knockdown in zebrafish revealed abnormal phenotypes with a pronounced curvature of the tail and trunk caused by severe defects in the musculoskeletal system (Zoeller et al., 2008).

In summary, our results indicate an important role of perlecan during human embryogenesis. We found perlecan to be present particularly at those stages of development where epithelial-mesenchymal interactions occur. Perlecan is a component of the BMZs that is ubiquitously expressed between gw 6 to gw 12 in all tissues investigated.

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