REVIEW ARTICLE

The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices

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INTRODUCTION

In the pre-molecular era of biochemistry, scientists assigned rather unprepossessing physiological roles to proteoglycans: they merely provided an inert scaffolding upon which cellular and organismal development could take place. The preceding decade, however, has witnessed an explosion of innovative technology which has created an interest in, and brought to the fore, a wealth of information in the field of proteoglycan research. It has originated a whole new perspective in our thinking of proteoglycan gene structure, function and evolution. We are entering the golden age of proteoglycan research and are on the brink of discovering human genetic diseases linked to specific proteoglycan gene families.

Perlecan is a multifaceted, modular proteoglycan that is present in virtually all vascularized tissues with a distribution that is primarily, but not exclusively, confined to basement membranes. The complex pleiotropy of perlecan suggests that this gene product is involved in several developmental processes, at both the early and late stages of embryogenesis, as well as in pervasive human diseases such as cancer and diabetes.

This review provides a propitious opportunity to comment on some new horizons and research lines opened up principally by the recent molecular data on perlecan gene structure, expression and topology.

HISTORICAL AND GENERAL CONSIDERATIONS

The neologism perlecan, a mnemonic from *perl* (bead or gem-like) and *can* (glycosaminoglycan), was coined by Hassell and co-workers [1] to symbolize the 'beads-on-a-string' appearance of isolated perlecan molecules as visualized by rotary shadowing. This term is now widely accepted and designates a gene family encoding modular, relatively large and ubiquitous glycoproteins which, as we discuss below, may or may not carry covalently linked glycosaminoglycan side chains.

The story of perlecan began over two decades ago with three key observations: a role for proteoglycans in branching morphogenesis [2], the demonstration of spatially ordered polyanionic sites in the basal lamina of breast ducts [3], and the discovery of heparan sulphate in the glomerular basement membrane [4]. Perhaps the work that has had the greatest impact on our understanding of perlecan is that of Hassell and collaborators [5]. These investigators first purified in chemically useful amounts a large, low-density heparan sulphate proteoglycan from a mouse sarcoma that was known to synthesize excessive

quantities of basement membrane constituents [6–8]. The subsequent availability of immunological reagents recognizing specific domains in the protein core, rather than in the heparan sulphate side chains, has stimulated research in other cellular systems. These landmark studies have undoubtedly stimulated the work on perlecan and have set the stage for more recent discoveries, including the molecular cloning of the cDNA, the elucidation of the protein-core modules, and the characterization and chromosomal mapping of the gene.

One of the truisms of recent research is the perception that the size of the perlecan protein core has gradually increased over time. From earlier two-digit estimates, it has now reached the three-digit range for the protein core (400–470 kDa) and over four-digit size for the fully glycosylated proteoglycan in certain cellular systems [9]. Molecular cloning and peptide sequence analyses have now definitively proven that perlecan is not only synthesized by Engelbreth–Holm–Swarm (EHS) tumour cells, but also by cells as disparate as melanoma [10], colon carcinoma [11], lung fibroblastic [12], endothelial [13], and mammary epithelial [14] cells. Perlecan is an archetypal molecule from which multiple species of proteoglycan could be derived either via a well-refined proteolytic processing pathway or via a pattern of differential exon usage. The fact that antibodies to perlecan consistently identify a single precursor product of 400–450 kDa [9,15–17], strongly favours the existence of a single gene for this proteoglycan family. The multidomain nature of perlecan is reminiscent of other modular proteins which are often constituents of the extracellular matrix and may result from a mosaic blend of exons with diverse origins [18].

MOLECULAR ORGANIZATION: THE GENE, THE PROTEIN AND THE FIVE MODULES OF PERLECAN

Elucidation of the complete cDNA sequences of the mouse [1], human [19,20] and nematode [21] perlecan, together with the complete structural characterization of the human perlecan gene [22], have provided us with the detailed structural picture of the molecule which we possess today, and have clearly shown that the primary design of perlecan encompasses discrete molecular domains (Figure 1). There is a remarkable preservation of individual domains, each largely composed of a subset of exons flanked on either side by introns in the same phase, i.e. interrupting the coding sequence between codon triplets or after the first or second nucleotide. This homogeneity in codon phasing constitutes a powerful mechanism for the coupling of functional units via intronic recombination, thereby preserving a continuous open reading frame. The most striking feature of perlecan, in

Abbreviations used: LDL, low-density lipoprotein; LRP, LDL-receptor-related protein; N-CAM, neural cell adhesion molecule; EGF, epidermal growth factor; EHS, Engelbreth–Holm–Swarm; RFLP, restriction-fragment length polymorphisms; RT-PCR, reverse transcriptase-polymerase chain reaction; bFGF, basic fibroblast growth factor.

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Figure 1 Schematic representation of human chromosome 1, the exonic organization of human perlecan gene and the deduced protein structure from human, mouse and nematode *C. elegans*

The distinct domains of the human perlecan gene are represented by the differently coloured rectangles containing the number of exons. The corresponding protein modules are designated by the Roman numerals underneath, and their colour-coded symbols are shown at the bottom of the figure.

addition to its colossal dimensions, is the presence of numerous internal repeats and the montage of modular protein domains. These modules harbour protein motifs with domain similarities to known polypeptides involved (i) in the binding and delivery of nutrients and lipids, (ii) in cellular growth and (iii) in intercellular signalling pathways.

The human perlecan gene (*HSPG2*) resides on the telomeric region of chromosome 1

Previous studies have provided evidence that a single gene is present in the haploid human [22–25] and mouse [26] genomes. The first evidence for the presence of perlecan on the telomeric region of human chromosome 1 was obtained by cross-species *in situ* hybridization [23] using as the probe a mouse cDNA encompassing the neural cell adhesion molecule (N-CAM) domain of perlecan [10]. This localization was subsequently confirmed by two independent studies using human probes under highly stringent conditions and employing human/rodent somatic cell hybrids containing partial deletions of chromosome 1 [24] and by *in situ* hybridization analysis [25]. Both studies clearly showed that the perlecan gene, whose genomic designation is *HSPG2*, is located within a narrow region of the telomeric

portion of chromosome 1 (Figure 1), with the most likely primary site being the 1p36.1 band. It is noteworthy that the mouse perlecan gene has been localized to chromosome 4 adjacent to the alkaline phosphatase-2 gene [26]. Because the same gene is also present in human 1p36.1-p34 [27], there is strong evidence for the preservation of a syntenic region between human chromosome 1 and mouse chromosome 4. Although no disease has been assigned to an abnormality in the perlecan gene locus, there are numerous reports involving either deletions or loss of heterozygosity of the short arm of chromosome 1 in familial cutaneous melanoma [28,29], neuroblastoma [30,31], multiple endocrine neoplasia type 2 [32], breast carcinoma [33] and colorectal cancer [34]. In addition, it has been proposed that the distal portion of chromosome 1 may contain one, or several, tumour suppressor genes [33]. Since several restriction-fragment length polymorphisms (RFLP), generated with either *TaqI*/*EcoRI* [24] or *BamHI* [25], have been identified in the human perlecan gene, these RFLPs could be useful in linkage of this locus with diseases connected to the perlecan gene.

The promoter of perlecan

Transcription of the perlecan gene is driven by a promoter which is highly enriched in GC content, with over 80% GC in the 500 bp immediately upstream of the leader exon (Figure 2). It has a CpG/GpC ratio of approx. 0.9, thus indicating the absence of CpG suppression [35]. The promoter contains four GC boxes and three GGGCGG hexanucleotide sequences which are known binding sites for the zinc-finger transcription factor SP1 [36]. Moreover, five GGGCGG hexanucleotide sequences and a GC-box were found in the first exon and first intron. This region also contained 21 CCGG tetranucleotide sequences, which is the recognition site for the methylation-sensitive *HpaII* enzyme. The more distal part of the perlecan promoter contained two viral-enhancer AP2 motifs and three short palindromic direct and indirect repeats, which by forming a secondary structure could influence the regulation of perlecan gene expression. These features, together with the lack of canonical TATA or CAAT boxes, are often associated with multiple transcription start sites. Indeed, both primer extension and S1 nuclease mapping have demonstrated at least five distinct transcription start sites [22]. In addition to these characteristics, the 5' end of the perlecan gene and its promoter are found in a CpG island, also referred to as a *HpaII* tiny-fragment island [35]. Such DNA regions contain clusters of unmethylated CpG dinucleotides, a microenvironment in which the number of CpG approximately equals the number of GpC [35]. These islands have been correlated with transcriptional control regions and are typically observed in genes that encode oncoproteins, growth factors, transcription factors and housekeeping proteins. This is in agreement with the ubiquitous nature and the constitutive expression of perlecan in most tissues. Similar promoter features are observed in several extracellular matrix genes and cell-adhesion-molecule genes, including those encoding human laminin B1 [37] and B2 [38] chains, and N-CAM [39]. The promoter regions of these genes also exhibit a high GC content and contain SP1-binding sites as putative regulators of transcription (Figure 2). The similarities among these molecules, from promoter to gene arrangement and protein structure, suggest common regulatory mechanisms and therefore functional significance.

Domain I: a module unique to perlecan

Following the signal peptide, there is a small domain of 172 amino acids that contains three consecutive Ser-Gly-Asp (SGD)

Figure 2 Comparison between the relative G + C composition in the promoter region of the human perlecan gene and that of the related proteins laminin B1, laminin B2 and N-CAM

Each panel shows the percentage of G + C content in the first 600 bp of promoter and approx. 250 bp of the 5' end of the designated genes. The G + C content was determined from a window of 50 bp. The symbols of the various *cis*-acting elements are summarized in the diagram at the bottom of the figure.

tripeptides, the predicted site for attachment of the heparan sulphate side chains [1]. Whether this is the only domain capable of being substituted with glycosaminoglycans or just one of the possible glycosylation sites needs to be established by future experiments. It should be pointed out, however, that the perlecan protein core harbours over 50 Ser-Gly (SG) dipeptides, some of which are flanked by acidic amino acids and would comply with a proposed consensus sequence for a glycosaminoglycan attachment site [40,41]. The SGD-sequence region shares features with proposed glycanation sites in other heparan sulphate proteoglycans (Figure 3) and is most similar to sequences found in some of the syndecan family of cell-surface proteoglycans [42–45]. In contrast with the other domains, domain I does not harbour any internal repeats, lacks cysteine residues and is quite enriched in acidic amino acids. These physicochemical attributes render this domain significantly different from the others. Indeed, neither its amino acid nor its nucleotide sequence is related to

Gene	Sequence	Reference
Human perlecan	<u>DS</u> <u>IS</u> <u>SGDDL</u> <u>SG</u> <u>DL</u> <u>SG</u> <u>DF</u> <u>Q</u> <u>M</u>	[19, 20]
Mouse perlecan	<u>DDA</u> <u>SG</u> <u>DL</u> <u>SG</u> <u>DV</u> <u>SG</u> <u>DF</u> <u>Q</u> <u>M</u>	[1]
Syndecan-1	<u>EDQ</u> <u>E</u> <u>SG</u> <u>DD</u> <u>S</u> PAA <u>E</u> <u>SG</u> <u>ED</u> FT <u>F</u> <u>T</u> <u>SG</u> <u>ENT</u>	[43]
Syndecan-3	PVSGGP <u>SG</u> <u>DF</u> <u>E</u>	[44]
Syndecan-4	<u>DF</u> <u>EL</u> <u>SG</u> <u>SL</u> <u>D</u>	[142]
Betaglycan	GY <u>ED</u> <u>LE</u> <u>SG</u> <u>D</u> <u>NG</u>	[143]
Glypican	PQA <u>E</u> <u>I</u> <u>SG</u> <u>EH</u> <u>L</u> <u>D</u> <u>SG</u> <u>SS</u> <u>SG</u> <u>D</u> <u>G</u> <u>C</u>	[45]

Figure 3 Potential heparan sulphate attachment sites in perlecan and other heparan sulphate proteoglycans

Comparison of the putative heparan sulphate attachment sites in domain I of human and mouse perlecan and similar potential GAG attachment sites in human syndecan-1, rat syndecan-3 (N-syndecan), human syndecan-4 (amphiglycan), rat betaglycan (transforming growth factor- β type-III receptor) and human glypican. Most of the sequences comply at least in part with the proposed GAG attachment consensus sequence (D/E)GSG(D/E). Ser-Gly sequences are underlined and shown in dark red, while flanking acidic residues are shown in light red. Some gaps have been introduced into the sequences in order to optimize alignments.

any previously characterized entry in the available databases: it seems that this is the only domain unique to perlecan. Of note, is the exonic organization of this domain, inasmuch as the first SGD triplet is split between exons 2 and 3 [22]. Because these exons are separated by phase I introns at either end, it is plausible that exon 3 may be spliced out; a consequence of this would be the synthesis of a polypeptide without heparan sulphate side chains. That this event may occur is supported by the observation that colon carcinoma cells, under routine culture conditions, can release perlecan protein core into the medium without heparan sulphate side chains [9].

Collectively, these observations suggest that perlecan can also be a non-glycanated protein, a notion that has been corroborated by the recent detection of perlecan protein core in cartilage [46], and confirmed by our immunohistochemical data using a monoclonal antibody specific for domain III (cf. Figure 8). One fundamental question that needs to be addressed in future studies is to what extent this gene product is secreted as a glycoprotein rather than as a proteoglycan and what functional roles it may have during ontogeny and neoplastic growth.

Domain II: identity with the low-density lipoprotein (LDL) receptor

The second discrete domain of perlecan is homologous to the ligand-binding portion of the LDL receptor [47]. This region is encoded by three distinct exons, as is the LDL receptor gene, and contains four cysteine-rich motifs that are also shared by a number of binding molecules (Figure 4). The overall genomic organization of this domain is remarkably similar to that of the LDL receptor. For example, the first two cysteine-rich repeats of perlecan and the corresponding repeats in the LDL receptor are encoded by one exon of nearly identical size. The distal two repeats are also encoded by one highly conserved exon. More startlingly, all three exons are interrupted by phase I introns, thus allowing for the possibility of alternative splicing. It has been postulated that via alternative exon usage, the LDL receptor gene can generate multiple forms of the receptor with varying degrees of affinity for LDL [47]. The consensus sequence of the LDL-receptor repeat has six conserved cysteine residues spaced over an approx. 40-amino-acid region (Figure 4). It is iterated

Gene	Sequence										Copy number	Reference	
Human perlecan	C	GPQEAA	C	R-----NGH	C	IPRDYL	C	DGQED	C	E <u>DGSDE</u> LD--	C	4	[19, 20]
Mouse perlecan	C	GPQEAS	C	H-----SGH	C	IPRDYL	C	DGQED	C	R <u>DGSDE</u> LG--	C	4	[1]
Nematode <i>unc-52</i> /perlecan	C	MADEKA	C	G-----NNE	C	VKNDYV	C	DGEPD	C	RDRSDEAN--	C	3	[21]
Complement C9	C	GND-FQ	C	S-----TGR	C	IKMLRL	C	NGDND	C	GDFSDEDD--	C	1	[48]
Heyman nephritis antigen	C	QPTQFR	C	P-----DHR	C	ISPLYV	C	DGDKD	C	A <u>DGSDE</u> G---	C	13	[49]
α_2 -Macroglobulin receptor	C	GVDEFR	C	K-----DSGR	C	IPARWK	C	DGEDD	C	G <u>DGSDE</u> PKKE	C	31	[50]
Nematode LRP	C	TPDQFK	C	V---SSGL	C	IPASWK	C	DGQQD	C	D <u>DGSDE</u> PKFG	C	35	[51]
Quail receptor for RS virus	C	PPGQFR	C	SEPPGAHGE	C	YPQDWL	C	DGHPD	C	DDGRDEWG--	C	1	[52]
Chick receptor for RS virus	C	SPGQFH	C	SEPRDPQTD	C	YPLEWL	C	DGHPD	C	DDGRDEWG--	C	1	[52]
LDL receptor consensus	C	F	C	G	C	I	C	DG	D	<u>DGSDE</u>	C	7	[47]

Figure 4 Amino acid sequence similarity between perlecan domain II and the ligand motif of the LDL receptor and related proteins

The strong similarity in the second repeat of domain II of the human [19,20], mouse [1] and nematode [21] perlecan are compared with regions of similarity in complement C9 [48], the fourth repeat of the antigen glycoprotein, also known as GP330, which causes the Heymann nephritis [49], the fifth repeat of the α_2 -macroglobulin receptor [50] also known as LRP, the twelfth repeat of the nematode LRP [51] and the avian receptors for the Rous sarcoma (RS) virus [52]. The consensus sequence of the human LDL receptor [47] is presented at the bottom. Alignment was obtained using the FASTP and ALIGN programs in the GCG and PCGENE packages respectively. The pentapeptide DGSDE, a proposed binding site for LDL, is underlined and coloured in dark red. The six conserved cysteine residues are shown in light red. Gaps, introduced to optimize alignment, are shown as dashes.

seven times and contains an additional 11 amino acids that define this LDL-receptor repeat [47]. Interestingly, mammalian perlecan contains the pentapeptide DGSDE, a sequence that mediates ligand binding by the LDL receptor [47]. One to several copies of this repeat are found in a number of molecules including mouse [1] and nematode [21] perlecan, complement C9 [48], the auto-immune target glycoprotein (also known as GP330) involved in Heymann nephritis [49], the α_2 -macroglobulin receptor [50], the LDL-receptor-related protein (LRP) protein in *C. elegans*, a protein which appears to be the orthologue of the mammalian α_2 -macroglobulin receptor [51], and the receptors for subgroup A of the Rous sarcoma virus in the quail and chicken [52]. Expression of these viral genes renders mammalian cells susceptible to Rous sarcoma virus infection [52], thus opening the possibility that viruses may exploit similar protein motifs to gain entry into host cells.

Perhaps one of the most interesting, yet speculative, functions of perlecan is the possible role of this pericellular proteoglycan in the metabolism of LDL. Because perlecan is expressed at high levels along the sinusoidal spaces of the liver (cf. Figure 7), spleen and in the subendothelial region of all blood vessels, it seems logical to speculate that LDL would be in contact with perlecan during its normal circulatory life as well as during endothelial denudation or in hyperlipidaemic states. Binding of lipids to the basement membranes and extracellular matrices of blood vessels is an established observation, and this property has been generally attributed to the glycosaminoglycan chains [53]; but no direct link between LDL binding and protein cores has been established. A plausible scenario would include the formation of a supra-molecular complex between perlecan, either associated with the basement membrane or the cell surface, and LDL. This complex could conceivably be stabilized by the interaction of the closely clustered heparan sulphate chains that are known to bind apolipoproteins [54]. Interestingly, the extracellular concentration of LDL can in turn modulate the expression of perlecan in cultured endothelial cells [55]. An alternate view would be to consider domain II simply as providing a sorting mechanism for the polarized secretion of perlecan to the basolateral region as proposed for mammary epithelial cells [14]; however, perlecan can be localized around tumour cells without any apparent

vectorial secretion but still in close association with the plasma membrane [56].

Domain III: homology with the short arm of laminin A chain

Domain III exhibits extensive similarity with the internal segments of laminin [57,58], particularly with the region of the A chain that comprises one of the short arms of the laminin multimer [58]. This region consists of three distinct globular subdomains, characteristically devoid of cysteine residues, which are flanked on either side by cysteine-rich extended domains. The globular regions have an overall amino acid configuration which resembles that of two subregions of the laminin A chain, the IVa and IVb subdomains [58], and to a lesser extent the laminin B2 subdomain IV (Figure 5). The rod-like segments are composed of a series of repeats, each typically harbouring eight highly conserved cysteine and two glycine residues arranged in essentially the same pattern as that of laminin domains III or V. The entire domain is encoded by 27 exons ranging in size from 50 to 231 bp. Interestingly, there is no apparent correlation between the exon arrangement and protein subdomains, a phenomenon that occurs in all the related genes [22]. This considerable divergence suggests that the laminin portion of the perlecan gene might have evolved from an ancestral gene enduring significant exon shuffling. We have also found [22] a duplication of the distal portion of exon 30 in a genomic region adjacent to exon 23. This duplicated exon, although not yet formally discovered in any mammalian mRNA, could conceivably be spliced out, thereby forming a truncated perlecan species. Another feature of this human perlecan domain is that it lacks the RGD triplet, a sequence that is present in the mouse species and may be involved in cell-surface binding (see below).

Domain IV: homology with the immunoglobulin superfamily and N-CAM

Domain IV has two distinguishing features: (i) it is the largest domain of human perlecan, encoding a polypeptide of over 200 kDa; and (ii) it is the most iterative domain, containing 21 consecutive repeats typically observed in the members of the

Figure 5 (a) Schematic representation of human laminin and perlecan and (b) homology plot analysis of the respective homologous domains

(a) The three chains of laminin are represented with their distinct subdomains. Subdomain IV and the G-domain of laminin are colour-coded in dark red and light red respectively, to indicate the homologies with the perlecan protein core shown on the right. Of note, a recent report [141] has proposed a new nomenclature for the heterotrimeric forms of laminin. Accordingly, the laminin isoform depicted in (a) would be called laminin-1 and the three chains A, B1 and B2 would be designated as $\alpha 1$, $\beta 1$ and $\gamma 1$ respectively. (b) The 1286 amino acids of subdomains V to IIIa of the A chain of laminin were compared with the 1172 residues of perlecan domain III (upper panel), and the 950 amino acids of the G-domain of laminin were compared with the 1005 residues of perlecan domain V (lower panel). Homology-plot analyses were done using the Dot-Plot program (GCG package) with a window setting of 30 and a stringency of 15. A single dot indicates that > 15 residues match in a searching area of 30 amino acids.

immunoglobulin gene superfamily [59]. In contrast with the human, the mouse and nematode perlecan species contain only 14 repeats (Figure 1). This domain is encoded by 40 exons and harbours the largest collection of Ig repeats so far described. Similar domains are shared by all members of the Ig gene superfamily, which includes molecules involved in immune recognition as well as molecules with no immunological function. This structural unit is characterized by a primary amino acid sequence of 70–100 residues with an essentially invariant disulphide bridge spanning 50–70 residues, and numerous con-

served glycine and tryptophan residues [19]. Specifically, the 21 consecutive Ig repeats resemble the organization of the N-CAM [59], which is an established member of the Ig superfamily. With the exception of repeats 17–21, each Ig repeat is encoded by two exons, thus supporting the proposal that the Ig superfamily was founded on an ancestral block of two exons [59]. The sequences that exhibit the strongest internal sequence similarity follow a strict pattern; specifically, the first half of the repeat is always interrupted by a phase 0 intron after the dipeptide His-Glu, whereas the other half of the repeat is invariably interrupted by

a phase I intron. This arrangement makes this region an optimal candidate for alternative exon usage or skipping (see below). It is noteworthy that in the mouse [1], the first and the twelfth Ig repeats contain an extra cysteine which may be involved in disulphide bonding and would thus generate a more compact, globular N-CAM region [1,60]. The same possibility could occur for the human species inasmuch as these two cysteine residues, although located much further apart because of the intervening Ig-like repeats (Figure 1), are still fully preserved [19]. These data are consistent with the multiple globular domains observed in rotary-shadowed pictures of isolated perlecan molecules from the EHS tumour matrix [8,61]. The main function of this module is still conjectural. By analogy with other molecules containing the same motifs [62], this module could: (i) stabilize homophilic protein-protein interactions; (ii) promote dimerization of perlecan molecules within the basement membrane, thus contributing to the stabilization of this matrix; or (iii) enhance the adhesion of neighbouring cells carrying perlecan at their surfaces.

Domain V: homology with the C-terminus of laminin A chain

This protein module comprises the C-terminal end of the mammalian species of perlecan, but surprisingly is missing in the nematode orthologue (Figure 1). Domain V is encoded by 16 exons and composed of three globular and four epidermal growth factor (EGF)-like motifs, exhibiting structural similarity with the C-terminal, globular region of the laminin A chain [58] (Figure 5). As in the case of domain III, the other region with sequence similarity to the laminin molecule, domain V, also lacks correlation between subdomain boundaries and exon structure, indicating that the two laminin-like regions of perlecan have diverged from the ancestral gene quite significantly during evolution. As previously proposed [63], the relationship of exons to protein structure probably represents a degenerate state of ancestral correspondence between exons and structure/function modules of proteins. The four EGF-like repeats of perlecan domain V are composed of about 40 amino acids each, with a consensus sequence typically found in EGF type-I repeats [64] and several conserved glycine residues that may be involved in the proper folding of this region. It is noteworthy that the presence of alternating globular and EGF-like domains has now been recognized in a number of molecules that are involved in cell adhesion and growth. For example, significant structural similarity exists between the C-termini of perlecan and neurexins; the latter comprise a large family of polymorphic cell-surface proteins, as well as secreted variants [65,66]. The repeats of the three distinct species of neurexin (I α , II α , and III α) show a similar degree of homology to agrin, a component of the basement membrane that causes the aggregation of acetylcholine receptors [67], as well as to laminin [57,58] and perlecan [1,19,20]. Interestingly, in the case of neurexin III α [66], the carboxyl end can undergo extensive alternative splicing events with the potential of generating a membrane-bound and a truncated form, suggesting that some forms of neurexin III α may be secreted. The terminal domain of perlecan could be involved in both homotypic and heterotypic interactions, as in the case of the mouse EHS proteoglycan [61,68]. It could also be involved in the modulation of cellular growth via the EGF repeats, inasmuch as comparable EGF-like repeats of mouse laminin can induce cellular growth in cultured cells possessing EGF receptors but not in cells lacking the receptor [69]. In line with this view is the fact that synthetic peptides from the globular G domain of laminin can promote cell adhesion and neurite outgrowth, and can interact with heparin and the β 1 integrin subunit [70]. Domain V contains two SGxG

tetrapeptides which may be substituted with glycosaminoglycan side chains other than heparan sulphate. It is noteworthy that hybrid forms of perlecan containing both chondroitin and heparan sulphate side chains have been described recently in a cell line derived from the EHS tumour [71] and in human corneal fibroblasts [72]. In addition, a hybrid dermatan/heparan sulphate proteoglycan from human placenta [73] has been shown to be immunologically related to human perlecan [19].

Finally, domain V contains two conserved Leu-Arg-Glu (LRE) tripeptides that have been invoked as the primary sites for the binding of motor neurons to s-laminin in the synaptic basement membrane [74]. Neurons bind to immobilized LRE-containing peptides, and soluble LRE sequences block attachment of neurons to s-laminin fragments [74]. The presence of two LRE motifs in human perlecan, in contrast with the mouse form where only one is conserved [1], suggests that these motifs may play important roles in perlecan-dependent neurite outgrowth.

WIDESPREAD EXPRESSION OF PERLECAN: FUNCTIONAL IMPLICATIONS

Perlecan is one of the earliest gene products encoding basement membrane constituents to be expressed in embryonic life: it can be detected at the 2–4-cell stage of the mouse embryo, and is later deposited in all embryonic basement membranes [75]. Interestingly, perlecan is found at the exterior surface of the trophectoderm during the attachment phase of blastocyst implantation *in vivo*, an expression that correlates with the acquisition of attachment competence by mouse embryos *in vitro* [76].

The cell- and tissue-specific expression of perlecan is now being re-evaluated inasmuch as a number of epithelial and mesenchymal cells are found to express the perlecan message. For example, both colon carcinoma cells and fibroblasts express a single approx. 14 kb transcript (Figure 6, lanes 1 and 2 respectively), and several perlecan domains, including I, II and IV, can be detected by reverse-transcriptase polymerase chain reaction (RT-PCR) in colon carcinoma, osteosarcoma, fetal lung and skin fibroblastic, and prostate carcinoma cells (Figure 6, lanes 3–10). Similarly, *in situ* hybridization analyses have demonstrated perlecan message in a variety of cells independent of whether they assemble a classical basement membrane [19,77]. A number of studies have localized perlecan to various basement membranes and pericellular regions [5,19,71,75,77–83]. We have recently developed a monoclonal antibody that recognizes a recombinant fusion protein containing domain III of human perlecan and have investigated systematically its expression in human tissues [77]. Perlecan is present not only in the basement membrane zone of every vascularized organ, but also in the tumour stroma of several human cancers (Figure 7). Of interest is an abundant deposition of perlecan in the perisinusoidal space of the liver (Figure 7b), as well as in the sinusoidal spaces of all the lymphoreticular and endocrine organs investigated [77]. Because of the large blood flow through these tissues, perlecan would be continuously exposed to circulating blood elements and immunocompetent cells.

It is now becoming apparent that perlecan is diffusely present in the pericellular matrices of a number of organs, and that the fibroblast, a cell type that does not assemble a traditional basement membrane, is a main producer of the proteoglycan. For example, *in situ* hybridization studies of human skin [77] have demonstrated perlecan message in the fibroblasts of the upper dermis but not in keratinocytes. Perlecan has been localized to the dermo-epidermal basement membrane of fetal skin during the first trimester of life, and as early as 54 days of intra-uterine development [80]. The presence of perlecan at the earliest time at

Figure 6 Expression of perlecan in normal and neoplastic human cells

A schematic representation of the perlecan protein structure and the various domains (roman numerals) is shown in (a). The arrows indicate the sense and antisense primers used in RT-PCR to amplify a 500 bp fragment in domain I–II, and a 750 bp fragment in domain IV, a region contained between Ig repeats 13 and 16 respectively. (b) and (c) show Northern blotting (lanes 1 and 2) and Southern blotting of RT-PCR products amplified from domain I–II (lanes 3–5) or domain IV (lanes 6–10). The following cellular RNAs were analysed: WiDr colon carcinoma cells (lanes 1 and 6), neonatal skin fibroblasts (lane 2), MG-63 osteosarcoma cells (lanes 3 and 8), CRL-1262 embryonic lung fibroblasts (lanes 4 and 9), PC-3 prostate carcinoma cells (lanes 5 and 10), and IMR-90 embryonic lung fibroblasts (lane 7). For Northern blotting, approx. 20 µg of total RNA was separated in a denaturing formaldehyde gel, transferred to a nylon membrane and hybridized under high stringency with a PCR-generated perlecan probe encompassing part of domain IV. For Southern blotting, the cDNAs amplified by RT-PCR were separated by analytical agarose gel electrophoresis, transferred to a nylon membrane and hybridized with PCR-amplified probes corresponding to the same regions of approx. 500 and 750 bp respectively. The Northern blotting was exposed for 48 h, while the Southern blotting was exposed for approx. 30 min.

which basement membrane is formed, supports the hypothesis that this large proteoglycan contributes to the early structural integrity of the epidermal–connective tissue interface [80]. In the developing intestine, by contrast, perlecan is manufactured by epithelial cells and is subsequently assembled into a basement membrane which includes the mesenchyme-derived collagen type IV [84]. Together, these findings suggest that basement membranes are the product of both ectoderm and mesoderm and that co-operation between different cells is necessary for correct orientation and assembly into a functionally complete basement membrane.

There has long been a controversy regarding the intrabasement membrane topology of perlecan. Earlier immunoelectron microscopic studies [85] have shown that the major heparan sulphate proteoglycan of renal glomeruli is restricted to the lamina rara interna; this corresponds to the concentration of polyanionic sites [86]. It is now felt, however, that this antigen probably represents a proteolytically processed form of perlecan, inasmuch

as other studies have shown a diffuse presence of perlecan throughout the basement membrane [82,87]. Further support for this view is offered by the observation that monoclonal antibodies directed toward an epitope of perlecan [88] stain only the lamina densa on the stromal side of the basement membrane, thus indicating a vectorial insertion of perlecan. This view has the N-terminus with the heparan sulphate chains near the plasma membrane, whereas globular domain V would reach across the basement membrane and could interact with other extracellular matrix molecules. This orientation is reminiscent of that of laminin, where the short arms are considered to be buried in the basement membrane, whereas the long arm can traverse the full thickness [68]. Double tracks of approx. 4.5 nm width, visualized in the glomerular basement membrane and Reichert's membrane, have been interpreted as representing submicroscopical images of perlecan [89,90]. In human colon carcinoma cells, perlecan is localized to the cell surface [11] and can be acylated with both myristate and palmitate, two long-chain fatty acids [91]. This post-translational modification may contribute to the binding and vectorial secretion of perlecan under the appropriate circumstances.

In the liver, perlecan is expressed primarily by the sinusoidal endothelial cells but not by the hepatocytes. However, when hepatocytes are cultured *in vitro* they gain the ability to express perlecan [81], implying a central role of perlecan for cellular survival and growth. A traditional dogma needs also to be revised insofar as immunoreactive perlecan is found in normal articular cartilage (Figure 8a) and abundant deposits are seen in human chondrosarcomas (Figures 8c and 8d). This observation is provocative because cartilage is an archetypal tissue that does not contain blood vessels and does not assemble a basement membrane. The question is why a gene product as complex and heterogeneous as perlecan is expressed in these tissues. The answer probably lies in the fact that a significant proportion of perlecan can escape post-translational modifications and can be secreted as a glycoprotein, i.e. lacking the heparan sulphate side chains [9]. This view has now been confirmed experimentally in rat chondrosarcoma cells by the finding of a perlecan protein core that, instead of being converted into a fully glycanated proteoglycan, is released without attachment of the glycosaminoglycan side chains [46]. This product appears to be localized in the pericellular environment of the chondrocyte and to be involved in the attachment of these cells to their own substratum [46].

In summary, several generalizations can be drawn from the studies of perlecan expression discussed above: (i) perlecan appears to be more ubiquitous than previously considered, with a distribution that is not rigorously limited to epithelial/mesenchymal interfaces; (ii) epithelial/mesenchymal cell co-operation is required for full perlecan expression; (iii) qualitative and quantitative expression of the mature proteoglycan is tissue-specific; and (iv) post-translational modifications of perlecan, and especially the attachment of heparan sulphate side chains, can be bypassed under certain tissue-specific conditions.

BINDING PROPERTIES OF PERLECAN

Binding to extracellular matrix proteins: homotypic and heterotypic interactions

The discovery that this large, multimeric macromolecule can self-assemble into a dynamic structure implies that perlecan is directly involved in the formation of basement membranes in living organisms [61,68]. This realization surfaced from key experiments [61] which showed that perlecan can self-aggregate into dimeric or multimeric forms when incubated under neutral isotonic

conditions. This homotypic interaction is apparently mediated by the carboxyl end of the perlecan protein core, or at least the end opposite to the glycosaminoglycan-binding region, the heparan sulphate chains appearing peripherally oriented [61]. Intriguingly, a recent report suggests that in EHS tumour perlecan, the heparan sulphate chains may be located at the carboxyl end [92]. The evidence for this is indirect and inferred from peptide sequencing and size estimation of proteolytic fragments obtained from purified EHS perlecan; however, no peptide sequence could be obtained from the fragment shown to be carrying the heparan sulphate chains [92]. Direct biochemical evidence is, therefore, needed to establish the precise site of glycosaminoglycan binding and clarify these seemingly contradictory results. In addition to these homotypic interactions, a number of proteoglycans, which have now been recognized as perlecan, have been involved in heterotypic interactions with numerous extracellular macromolecules including laminin [68,93], nidogen [93,94] and fibronectin [73,95]. Earlier workers have observed that, in order to purify and extract perlecan from the EHS tumour, denaturing solvents and chaotropic agents such as guanidinium chloride were required [5,6,96]. This indicates that perlecan is tightly bound to other matrix macromolecules and that both the protein core and the polyanionic chains might be involved in these interactions. For example, during EHS tumour purification, a significant proportion of laminin remains associated with perlecan and is removed only after CsCl gradient centrifugation [68]. Another basement membrane molecule that can interact with perlecan is nidogen [93]. Nidogen binds the protein core of perlecan via amino acid sequences mapped to the G2 domain [94], a region that also harbours binding sites for collagen type IV. It has been speculated that nidogen, via its laminin-binding region in the globular G3 domain together with the G2-binding region, may modulate the formation of a supramolecular complex among perlecan, laminin and collagen type IV in a heparan sulphate chain-independent fashion [97].

A large heparan sulphate proteoglycan from human fibroblasts binds fibronectin, thereby functioning as mediator of fibronectin-cell surface interaction in fibroblasts [98] and perhaps other cell types [73], and this interaction may anchor the ubiquitous fibronectin to certain basement membranes. This proteoglycan has been subsequently shown to be identical with human perlecan by comparison of its peptide sequence with the amino acid sequence deduced from cDNA cloning [19]. Intriguingly, a basement membrane heparan/dermatan sulphate hybrid proteoglycan, which was isolated from human placenta [73], also appears to be identical with perlecan [19]. This proteoglycan also binds fibronectin, apparently via covalent interactions, and may contribute to some of the fundamental functions of fibronectin in cell adhesion and spreading [73].

Binding to cell surfaces

About a decade ago, we reported that human colon carcinoma cells synthesize a surface proteoglycan which cross-reacted with anti-perlecan antibodies [11]. Later, it was unequivocally shown by cDNA cloning to be perlecan [24]. This colon carcinoma perlecan is closely associated with the plasma membrane: it is localized to the microvillar surface [56], the side opposite to the basement membrane; it cannot be displaced by high concentrations of heparin; and it requires detergent or mild trypsinization for its efficient removal from the cell surface [11]. We interpreted these data as being indicative of an intercalated proteoglycan sharing immunological and structural features with the basement membrane proteoglycan purified from the mouse

EHS tumour [5]. These observations can now be partly reconciled with the notion that perlecan may be closely linked to the cell surface via receptor proteins recognizing the protein core and possibly stabilized by the heparan sulphate chains. Earlier studies, in fact, have proposed that perlecan can bind cell-surface receptors, originally shown to be of a size smaller than usual integrins [99]. More recent investigations, however, have clearly shown that the attachment and spreading of several tumour cells on a perlecan substratum is mediated by $\beta 1$ integrins, but not by the $\alpha 6 \beta 1$ receptor typically observed for laminin [92]. Aortic endothelial cells, which synthesize and deposit perlecan [13], interact with the protein core of perlecan via $\beta 1$ and $\beta 3$ integrins, and this binding is partially RGD-dependent [100]. Apparently, this interaction is sensitive to a cell-mediated effect of heparin and heparan sulphate which, therefore, may act as regulatory ligands [100]. The cell-adhesive properties of perlecan were assigned to an approx. 160-kDa fragment which was localized to the C-terminus of the proteoglycan, a region containing part of the N-CAM repeats and domain V [100]. Note, however, that human perlecan does not contain an RGD sequence and therefore these findings may not be relevant to the human species.

Binding to growth factors

Recent discoveries in the field of growth factor and cytokine research have provided novel information regarding the role of pericellular heparan sulphate proteoglycans in the storage, binding and delivery of such compounds [95,101,102]. A cluster of important papers have reported a close association between basic fibroblast growth factor (bFGF), which is a heparin-binding angiogenic protein [95], and basement membrane constituents including mouse perlecan [103–107]. The bFGF appears to bind heparan sulphate chains and is released from the bovine cornea subendothelial matrix by heparin or heparinase, but not by chondroitin sulphate or chondroitinase [103]. It has been proposed that the sequestration of angiogenic endothelial cell mitogens in the basement membrane may be a general mechanism for regulating accessibility of these substances to the vascular endothelium [103]. Abnormal release of bFGF during wound healing or cancer invasion may contribute to local tissue neovascularization [108]. Interestingly, both heparinases I and III inhibit neovascularization *in vivo* and proliferation of capillary endothelial cells mediated by bFGF *in vitro* [109]. Because approx. 70% of bFGF added to capillary endothelial cells binds to the extracellular matrix, while only 7% binds to the cells [110], it is likely that specific sequences in the heparan sulphate chains covalently attached to perlecan protein core are directly implicated in the control of angiogenesis. In agreement with this view, is the discovery of a high affinity ($K_d \approx 30$ nM) between iodinated bFGF and EHS tumour perlecan [104]. This binding, at least in endothelial cells, seems to protect bFGF from proteolytic degradation [106], and bFGF-heparan sulphate complexes can be released from these cells via a plasminogen activator-mediated proteolysis [107]. High-affinity ($K_d \approx 1.5$ nM) binding of interferon- γ to heparan sulphate chains of an EHS tumour-derived extract has also been reported [111]. Finally, a recent study has shown an interaction between transforming growth factor- β and perlecan in the basement membrane of mouse endodermal cells [112], an intriguing observation inasmuch as this growth factor can in turn modulate the expression of perlecan in colon carcinoma cells [113].

The mechanism of action of bFGF at the cell surface has been partly elucidated by studies involving mutant cell strains lacking the enzymes necessary for the biosynthesis of heparan sulphate chains. Upon transfection of the FGF receptor, the defective

Figure 7 Immunolocalization of perlecan in human tissues using a monoclonal antibody against a recombinant fusion protein containing domain III of human perlecan protein core

The monoclonal antibody 7B5 was shown previously to react by Western blotting exclusively and specifically with the laminin-like domain III, a fusion protein generated in a prokaryotic expression system, and not to cross-react with the mouse perlecan [77]. The human perlecan epitope (red staining) is present in the basement membrane of oral mucosa (a, arrowheads), in the perisinusoidal space of the liver (b), in the basement membranes of endothelial cells (c, small arrowhead) and prostate epithelium (c, larger arrowhead), but can also be observed as diffuse deposits within the tumour stroma of breast (d) and colon (e) cancers. (f) shows a tumour xenograft 2 weeks after subcutaneous injection of approx. 1×10^6 PC-3 prostate carcinoma cells in a nude SCID mouse. Notice the positive reaction around small blood vessels (arrowheads), which are of mouse origin, and around the human tumour cells. In contrast, the basement membrane zone of the mouse skin (asterisks) is totally unreactive in agreement with the lack of cross-reactivity of our monoclonal antibody. (g) is a higher magnification showing intense reactivity around newly formed tumour microvessels (arrowheads). [Original magnification: (a) and (b), $\times 350$; (c–e), $\times 450$; (f), $\times 250$; (g), $\times 600$.]

cells could not bind bFGF unless supplemented with exogenous heparin or heparan sulphate [114]. In contrast, the wild-type cells were capable of binding bFGF without the need of exogenous glycosaminoglycan.

Subsequently, heparin-dependence was shown both for the interaction of bFGF with a recombinant FGF receptor species and for bFGF-induced mitogenesis [115]. An intuitive explanation for this phenomenon is that binding of the growth factor to the glycosaminoglycan may alter the conformation of bFGF,

thereby facilitating its interactions with the receptor. The specific binding site for bFGF has now been identified as a unique carbohydrate sequence within the heparan sulphate chains purified from the [^3H]glucosamine-labelled total proteoglycan pool synthesized by skin fibroblasts [116]. Using bFGF-affinity chromatography, a heptad-saccharide containing both N-sulphate and iduronic acid 2-sulphate residues was shown to be essential for binding activity to bFGF [116]. Although it is not known whether extracellular matrix heparan sulphate proteo-

Figure 8 Immunolocalization of perlecan in human cartilage and chondrosarcoma tissues using a monoclonal antibody directed against domain III of human perlecan protein core

Notice the presence of immunoreactive perlecan in close association with normal human articular cartilage chondrocytes (**a**, arrowheads), while the entire cartilage matrix is unreactive. Panel (**b**) is a control section lacking the monoclonal antibody. Panels (**c**) and (**d**) represent two separate cases of human chondrosarcoma tissue showing intense perlecan staining around atypical chondrocytes (**c**, arrowheads) or as large deposits within the tumour (**d**, asterisk). (Original magnification: **a** and **b**, $\times 650$; **c** and **d**, $\times 450$).

glycans such as perlecan bind bFGF through the same oligosaccharide, the fact that the bFGF-specific oligosaccharide was isolated from the heparan sulphate released into the medium by human skin fibroblasts strongly implicates perlecan as a major source of this material.

In summary, the current picture of perlecan–growth factor interactions is quite complex and in a state of constant flux. As we gain more information about protein and carbohydrate isoforms and specific functional subsequences, we will be able to define more precisely the roles these interactions may play during organogenesis and development. The central theme of this research line is that the multiple autocrine or paracrine effector molecules might be influenced by the selective temporal and spatial expression of pericellular heparan sulphate proteoglycans during morphogenesis and development. A more subtle pathway would involve processing via mild degradation with proteases or endoglycosidases, thereby releasing the factors in a heparan sulphate-bound, active form.

NEW CLUES ABOUT FUNCTION: PERLECAN ISOFORMS

Alternatively-spliced variants in the mouse and nematode species

The perlecan story is complicated by the existence of variant forms in the mouse [1] and *Caenorhabditis elegans* [21] species. Comparison between domain IV of the mouse and human molecules shows that the mouse species lacks seven Ig repeats, from the middle of the fifth to the middle of the twelfth repeat (Figure 1). This generates a perlecan protein core of smaller size (approx. 396 versus approx. 467 kDa). Recently, however, three additional Ig-like repeats, aligning quite well with the human repeats, have been found in the mouse species and larger mRNA transcripts have been identified [60]. Because chromosomal *in*

situ hybridization and somatic cell hybridization studies have all supported the existence of only a single perlecan gene, it is likely that alternate exon usage is the principal mechanism responsible for the generation of these transcripts. An extensive search by our laboratory for smaller perlecan variants in humans using RT-PCR and primers encompassing critical regions of domains I, II and IV, has so far failed to reveal any such species. By Northern blotting, we have constantly detected a single transcript of approx. 14 kb (cf. Figure 6). We can cautiously conclude from these data that the larger form of perlecan is the predominant species, and if smaller variants exist in human, they probably represent minor components or tissue-specific isoforms. This is in agreement with the fact that the complete human perlecan cDNA sequence is based on clones isolated from seven different cDNA libraries [19,20,24,25]. In addition, numerous RNAs and cDNA libraries have been screened in our laboratory and the amplified bands were all of the predicted size and sequence. It would be very interesting to determine whether a specific perlecan isoform might be linked to the transformed phenotype. Nevertheless, the high degree of conservation of the various perlecan domains between the human and the mouse species indicates that these modular units are important for perlecan function. This concept is further supported by the discovery of the *unc-52*, a miniaturized form of perlecan gene, in the nematode *C. elegans* [21]. This protein harbours three consecutive domains that resemble the LDL receptor, the laminin short chain and the N-CAM respectively, but is devoid of both the N- and C-terminal domains of perlecan (Figure 1). Domain I in the nematode, a stretch of 28 amino acids including a signal peptide, has little similarity to its mammalian counterpart, with the exception that both are hydrophilic and do not contain cysteine residues or internal repetitive sequences. In addition, the *C. elegans* domain I lacks the triplet SGD and thus it is not known whether this

species is synthesized as a proteoglycan or as a protein. Domains II and III are similar to the mammalian species with the exception of one less globular repeat in each domain. As in the mouse species, domain IV of *C. elegans* contains 14 Ig-like repeats with two additional repeats flanking domain II. The presence of a signal peptide and the immunological localization of *unc-52* protein in the nematode basement membrane strongly indicate that this gene product is an extracellular macromolecule. The nematode perlecan-like protein undergoes extensive alternative splicing with several isoforms varying in their C-termini [21]. The longest transcript consists of 26 exons that span approx. 15 kb of genomic DNA. The additional two smaller transcripts are generated by differential utilization of polyadenylation signals located approx. 8.5 kb apart and by differential splicing of the exons at the 3' end of the *unc-52* gene [21]. All the exons encoding domain IV are located between the two polyadenylation signals, and thus the two larger transcripts encode proteins containing all or part of domain IV, whereas the smaller transcript encodes a truncated polypeptide that is completely devoid of domain IV repeats.

Implication of perlecan-like molecule in muscle cell biology

An unsuspected role of perlecan-like molecules derives from research on a commonly vilified organism, the worm, and opens the possibility that genetic mutations at the perlecan locus may contribute to skeletal muscle pathology in humans. Interestingly, mutations of the *unc-52* gene induce disruption of the skeletal muscle architecture of *C. elegans* [21]. Moerman and co-workers [21,117] have used suppression analysis to identify a new gene involved in maintaining the attachment of myofilaments to the cell surface and basement membrane of the body-wall muscle cells. Based on their complex pattern of complementation, two major types of mutations occur in this animal: a lethal form associated with a complete loss of function for at least one of the *unc-52* alleles and a viable, but paralysed, variant caused by the insertion of a Tc1 transposon into the eighth Ig-like repeat of domain IV [21]. The disorganized body-wall muscle phenotypes in both mutant forms suggest that perlecan-like molecules are involved in maintaining the structural integrity of the skeletal muscle. The findings in *C. elegans* indicate that the abnormal but viable phenotype is caused by the synthesis of a truncated *unc-52* protein, while the lethal phenotype is caused by the complete absence of the protein. At least in the nematode, the integrity of the N-CAM region is essential for full attachment of myofilaments to the basement membrane. Because of the widespread expression of mammalian perlecan in skeletal and smooth muscle, it is possible that some forms of human congenital myopathy may also be due to abnormally truncated forms of perlecan. Is there any genetic disease, pertinent to the muscular dystrophy *sensu lato*, that is linked to abnormal perlecan? This is an important question that may be addressed in the future by targeted disruption of the gene in transgenic animals.

PERLECAN IN HUMAN PATHOLOGY

Given the current concept that basement membranes provide directional anchors for cells and a scaffolding that fosters tissue morphogenesis, and given the recent realization that they originate through a process of cell assembly driven by 'mass-action' [68], it is not surprising that the deregulated expression of even a single vital constituent could lead to serious complications, and ultimately to a pathological phenotype. Below, we discuss contemporary knowledge regarding two examples of human pathological processes in which perlecan may play an active role.

Diabetic microangiopathy

In long-term diabetes mellitus a number of complications are inflicted on the diabetic individual. Among the most clinically significant are the microangiopathies. These are disruption of the normal function of vascular capillary beds. A hallmark of these disease processes is a significant thickening, or sometimes duplication, of the microvascular basement membrane (Figure 9), with chronic progression as a function of time. Paradoxically, this thickening of the basement membrane is accompanied by a loss of function, allowing charged serum molecules which are normally retained within the circulation to pass across the matrix. This produces systemic effects in skin and muscle, but the most marked clinical consequences are observed in the renal glomerulus in diabetic nephropathy and in the retina with hard exudate formation, microaneurysms, haemorrhaging and neo-vascularization and blindness in the proliferative end stage of diabetic retinopathy [118]. The physicochemical changes in the properties of the basement membrane were assumed to be linked to some change in matrix composition, and with much of the charge barrier present attributable to the heparan sulphate chains of proteoglycans [86], these macromolecules have become the subject of considerable interest in the aetiology of microangiopathies. A number of approaches have been developed in the investigation of these complications, many involving experimental models of diabetes which are either induced by chemical destruction of pancreatic islet cells or by the use of strains of animals bred to be genetically susceptible to the disease. Problems facing investigators were ones familiar to matrix researchers: the affected structures in most vascular beds were too diffusely distributed and too insoluble to yield adequate material for biochemical analysis. Most studies therefore focused on the kidney as the organ of choice; the glomerulus providing the most concentrated and abundant amounts of relatively pure capillary basement membrane. Incubation of isolated glomeruli from streptozotocin-induced diabetic and non-diabetic rats with radiolabelled sulphate has shown that the former glomeruli incorporate less sulphate into the basement membrane, while the total uronic acid content is unchanged [119,120], thereby indicating possible undersulphation of the glycosaminoglycan chains. As an indication that these effects might be somewhat more widespread, it has also been shown that liver heparan

Figure 9 Electron micrograph of a small capillary from a patient with long-standing diabetes mellitus

Notice the duplication of the basement membrane (multiple arrowheads) surrounding the endothelial cells (EC). The lumen of the blood vessel is indicated by an asterisk.

sulphate proteoglycans from streptozotocin-induced diabetic rats were also undersulphated, possibly through inhibition of N-deacetylase, one of the enzymes required for N-sulphation of glycosaminoglycans [121]. However, Klein et al. [122] found no decrease in sulphate-labelled proteoglycans in glomeruli from streptozotocin-treated rats, and suggested that alterations in the interactions of heparan sulphate proteoglycans with other basement membrane components were responsible for the decrease in heparan sulphate proteoglycan content.

In an attempt to circumvent problems related to quantity of material, some workers have examined the synthesis of proteoglycans in EHS tumours grown in diabetic host animals. Interestingly, EHS tumours from streptozotocin-treated diabetic mice incubated with high glucose showed a marked increase in radiolabelled sulphate incorporation into proteoglycans [123]. In contrast, other studies showed that in EHS tumours grown in genetically (non-insulin-dependent) diabetic [124] or streptozotocin-treated diabetic mice [125], a reduction in sulphate incorporation correlated with a reduction in the level of heparan sulphate proteoglycan protein core. However, using steady-state mRNA levels to examine protein-core synthesis in a mouse model of non-insulin-dependent diabetes, no absolute changes in kidney cortex levels of perlecan mRNA were observed [126], although there was proportionally less when compared with the elevated levels of type-IV collagen in diabetic animals with demonstrated glomerular basement membrane thickening.

In the few studies performed with human material, a similarly confusing picture emerges. In an early study [127], the amount of heparan sulphate in whole kidney cortex and medulla was found to be increased in some diabetic patients. However, other investigators [128] found a significantly reduced heparan sulphate content in the glomerular basement membrane of diabetics, with no change in the degree of sulphation.

To summarize, there is evidence for major alterations in heparan sulphate proteoglycan in diabetic nephropathy. These abnormalities can take place via: (i) an increase in sulphate incorporation; (ii) a decrease in charge via a decrease in protein-core biosynthesis or enhanced degradation; (iii) a decrease in sulphation alone; (iv) a dilution effect brought on by unbalanced synthesis of other basement membrane constituents; and (v) a loss of heparan sulphate proteoglycan following perturbations in the forces anchoring this macromolecule in the basement membrane matrix.

Taken at face value, these results are confusing and conflicting. It can be difficult, even in the light of more recent knowledge, to re-interpret the data presented in these studies given the differences in experimental approach and measurements made in some studies, which were omitted from others. A key question is to what extent the numerous sulphate incorporation experiments could be interpreted as reflecting perlecan biosynthesis/turnover. While clearly not the only proteoglycan present in all basement membranes, current evidence suggests that perlecan is the major heparan sulphate proteoglycan of the matrices affected by diabetic microangiopathy, including the glomerular basement membrane, and thus it is probably safe to assume that alterations to the perlecan molecule could contribute to the consequent pathology.

Tumour progression and invasion: perlecan in the metastatic cascade

It is becoming apparent that heparan sulphate proteoglycans are key players in many interactions between the cells and their environment. During tumour cell dissemination, these relationships become modulated, further enhancing the disorganization

of the basement membrane. Given its polyhedral nature, perlecan holds a key position in the basement membrane to influence the processes leading to tumour invasion. Previous work on basement membrane heparan sulphate proteoglycans indicated that sulphated glycosaminoglycan chains are degraded at a faster rate by melanoma cells with high metastatic potential [129] by an endoglucuronidase or heparanase [130], reinforcing the functional importance of heparan sulphate chains in invasion. Recently, differences in the sulphation pattern of basement membrane proteoglycans were identified in Lewis lung carcinoma cells with high metastatic potential [131]. Evidence of a major role for heparan sulphate chains in tumour formation is further provided by the work of Esko and co-workers [132]. These authors generated mutant cell lines deficient in heparan sulphate chains and demonstrated that none of the mutants grew abnormally *in vitro*, thus suggesting that these glycosaminoglycans are not essential for growth. However, surprising results were obtained when the deficient cells were challenged to grow as tumours in athymic *nu/nu* mice. Whereas mutants grossly deficient in heparan sulphate synthesis were not tumorigenic, mutants with partially reduced levels were tumorigenic and required approx. 10% of the wild-type levels of heparan sulphate biosynthesis to express the malignant phenotype [132]. In general, any significant change in the sulphation pattern or enzymic degradation of the heparan sulphate may result in defects in assembly and stability of the basement membrane, especially in the surrounding endothelial cells, and may therefore facilitate the invasion/extravasation of tumour cells. There is no doubt that any alteration in this equilibrium will have an influence on the process of local invasion.

The protein core of perlecan is also capable of interacting with components of the matrix to affect cell adhesive behaviour. An emerging picture is that perlecan is directly involved in supporting the growth and invasion of tumour cells *in vivo* via its ability to capture and store angiogenic factors, such as bFGF [104]. Studies of cell-surface proteoglycans on human melanomas have shown that a predominance of heparan sulphate proteoglycan is a marker for a more aggressive phenotype [133]. Also, perlecan isolated from the EHS tumour stimulates invasiveness of melanoma cells in an *in vitro* assay [134]. The growth of transformed endothelial cells, but not of normal cells, on reconstituted basement membranes is severely suppressed when perlecan is degraded [135]. Furthermore, we and others have observed abundant deposits of perlecan in the stroma of a number of malignant tissues including breast and colon cancer (cf. Figures 7d and 7e respectively). Pronounced stromal reaction was observed in human colon carcinoma with perlecan and type-IV collagen forming fibrillar deposits, a reaction not observed in colonic polyps [136]. In a series of experiments using the prostate carcinoma cell line PC-3 to induce tumour xenografts in immunodeficient severe combined immunodeficient ('SCID') mice, we found that the newly formed microvessels in the tumour stroma were intensely reactive with a monoclonal antibody against perlecan (Figures 7f and 7g). Because our monoclonal antibody does not recognize mouse perlecan, as shown by the lack of staining of the mouse skin basement membrane (Figure 7f, asterisks), we conclude that the perlecan that is deposited around the tumour microvessels derives from the human PC-3 carcinoma cells. In contrast, the endothelial cells lining the tumour microvessels were of mouse origin as they failed to react with a monoclonal antibody against human factor VIII (results not shown). In summary, these data suggest that the prostate carcinoma cells synthesize perlecan, as also proven by the RT-PCR data (cf. Figure 6), and that this molecule induces or favours neovascularization of the transplanted tumour. A gra-

dient of immunoreactivity was also visible, with the most reactive microvessels localized at or around the growing edge of the tumour xenograft (Figure 7f). If this occurs in human tumours, then neoplastic clones with an ability to over-express perlecan would have a growth advantage over clones that lack this phenotypic trait. The sequestration of bFGF by perlecan and its release by tumour-cell-derived enzymes [103,104] could constitute a plausible autocrine or paracrine loop influencing local tumour growth or invasion. Accordingly, bFGF has been localized to various human tumours in the digestive [137] and nervous [138] systems. The abnormal deposition of bFGF in the extracellular matrix of gastric cancers was related primarily to areas of increased vascular permeability and active angiogenesis [137]. The close association between tumour cells and the vascular basement membrane observed in gastric cancer with liver metastases has been proposed to be specific for a more aggressive behaviour [139], whereas lung carcinoma cells with higher metastatic propensity adhere to a subendothelial matrix more efficiently than cells with lower metastatic potential [140]. These studies are in agreement with our observation in tumour xenografts discussed above and suggest that the ability of tumour cells to form a well-defined basement membrane is positively correlated with their adhesiveness to the vascular basement membrane *in vivo*.

CONCLUDING REMARKS

The recent advances in the chemistry and biology of heparan sulphate proteoglycans and their ligand interactions are having a considerable scientific and clinical impact. Contemporary evidence indicates that (i) perlecan is a highly conserved gene product with isoforms present in the nematode and mouse, (ii) that this molecule can exist as a glycoprotein and (iii) that its expression is not restricted to basement membranes. The composite, multidomain perlecan is made up of a montage of protein modules that are evolutionarily related to molecules involved in nutrient metabolism, mitogenesis and cell adhesion. The emerging portrait of perlecan biology is based principally on perlecan's ability to interact with cells and their products. This active participation occurs via permissive structural hierarchies in which the level of interaction, whether homotypic or heterotypic, is dictated by the structural motifs and their constitutive binding characteristics. Characterization of the genomic structure, chromosomal mapping of the gene, discovery of several RFLPs and of alternatively spliced variants are important conceptual advances that will undoubtedly facilitate a more comprehensive understanding of this molecule. The stage is now set to unravel the role of perlecan in fundamental biological processes wherein tissue remodelling occurs, inasmuch as this chimeric molecule is utilized by cells during embryonic development, wound repair and cancer growth. Systematic studies are needed to learn about the function of the individual protein modules and to develop transgenic animals with deregulated or abrogated perlecan expression. Collectively these studies should provide novel avenues of research and perhaps discover unforeseeable functions for this important molecule. One of the future challenges is to define the signal transducing pathways through which perlecan can activate specific sets of genes in different cell types.

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