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Review

Regulation of glycosaminoglycan structure and atherogenesis

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Abstract. Cardiovascular disease is the major cause of premature death in modern society, and its impact is increasing due to rising rates of obesity and type 2 diabetes. Clinical studies based on targeting metabolic abnormalities and biomarkers demonstrate significant benefits, but always an element of disease remains which is resistant to treatment. Recent evidence has strongly implicated an early interaction of atherogenic lipoproteins with vascular matrix proteoglycans as the initiating step in atherogenesis. Expert commentary has pointed to the need for vascular directed therapies to provide reductions in the residual disease component. We propose that the regulation of synthesis and thus structure of glycosaminoglycans on proteoglycans provides a potential pathway to this reduction. We review existing evidence that the vascular synthesis of glycosaminoglycan chains can be regulated in a manner which reduces lipoprotein binding and the potential application of this strategy to attenuation of the current cardiovascular disease pandemic.

Key words. Proteoglycans; glycosaminoglycans; lipoprotein; atherosclerosis; elongation; sulfation, epimerisation.

Introduction

Expanding, almost exploding, rates of obesity resulting primarily from sedentary work place and lifestyle practices accompanied by excess, often poor quality, energy intake are generating a demographically broadening epidemic of morbidity and mortality from cardiovascular disease. Obesity is associated with rising rates of altered carbohydrate metabolism expressed as insulin resistance leading to impaired fasting glucose and elevated post-challenge levels of glucose and overt type 2 diabetes [1]. The major underlying pathology of cardiovascular disease is inflammatory atherosclerotic vascular disease leading to thrombosis, vessel occlusion, myocardial ischemia, heart failure and death. Clinical trials directed at cardiovascular risk factors such as blood pressure and lipid levels show positive results, but an appreciable element of disease which is resistant to treatment ('residual disease') is always present. Therapies directed at the vessel wall are the suggested pathway to eliminate this residual disease [2]. There are several hypotheses as to the origin of atherosclerosis [3–5] of which the 'response-to-retention' hypothesis is currently prominent. The response-to-retention hypothesis states that the subendothelial retention of atherogenic low-density lipoprotein (LDL) by matrix molecules such as proteoglycans is a critical initiating step in atherogenesis. Recent evidence has strongly implicated the interaction between glycosaminoglycan (GAG) chains on vascular proteoglycans and apolipoprotein B100 on LDL as a critical initiating event in atherogenesis [6].

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Glycosaminoglycan synthesis and structure is therefore a potential target of therapeutic intervention and the subject of this review.

The response-to-retention hypothesis of atherogenesis

The entrapment of lipoproteins in the vessel wall by matrix molecules, most prominently proteoglycans, is continuing to emerge as a critical early step in atherogenesis [7, 8]. This interaction brings together the critical factors of dyslipidemia and the vessel wall as co-contributors to atherogenesis. Unfavourable structural changes in the lipoproteins and/or vascular proteoglycans may occur in diabetes and contribute to the accelerated macrovascular disease in that setting [9].

The understanding of lipoprotein metabolism is well advanced, and the role of apolipoproteins and cofactors such as lipoprotein lipase and sphingomyelinase [10] in promoting binding to proteoglycans has been described [11–13]. Furthermore, it has been demonstrated that fibrate therapy modifies lipoproteins in a manner leading to reduced binding to proteoglycans [14]. In contrast, the biochemical mechanisms determining the biosynthesis of vascular proteoglycans and the role of metabolic and pharmacological agents in regulating proteoglycan metabolism and thus the impact of altered proteoglycan structures on binding to atherogenic lipoproteins has been less well defined [12, 15]. There are three potential avenues of therapeutic attack on the interaction between proteoglycans and lipoproteins as it may relate to the response-to-retention hypothesis and atherogenesis: First, modification of the lipoproteins, second, modification of the structure of the proteoglycans, specifically of the GAG chains on proteoglycans, and third, modification of the interaction between proteoglycans and lipoproteins via GAG mimetics that may bind to LDL [2]. The role of the apolipoproteins and lipid in this model has recently been reviewed [16]; thus, in this article we address the possibilities of GAG regulation and GAG mimetics in attenuating interactions with LDL. We address the changes in proteoglycan structure that may influence the ionic interaction with apolipoprotein B on normal human LDL (referred to as LDL in this review), which is the primary site of binding [17] notwithstanding that the interactions may be different with other apolipoproteins and bridging molecules.

Table 1. Common proteoglycans in the vasculature (adapted from Iozzo [50]).

Proteoglycan	Core protein size (kDa)	GAG chain		Disaccharide composition
		Type	Number	
Perlecan	400–467	HS/CS	3	GlcA + GlcNAc
Versican	265–370	CS	10–30	GlcA + GalNAc
Biglycan	40	CS/DS	2	GlcA + GalNAc or IdoA + GalNAc
Decorin	40	CS/DS	1	GlcA + GalNAc or IdoA + GalNAc
Mimecan	35	KS	2–3	Galactose + GlcNAc

Glucuronic acid (GlcA), *N*-acetyl glucosamine (GlcNAc), *N*-acetyl galactosamine (GalNAc), iduronic acid (IdoA), heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS).

GAG size range 20 kDa[a] to 70 kDa[b] [20].

[a] Human vascular smooth muscle [J. Nigro and P. J. Little, unpublished observations].

[b] Monkey thoracic aorta [20].

Regulation of proteoglycan biosynthesis in vascular smooth muscle

Proteoglycans comprise a core protein with one or more covalently linked GAG chains. The disaccharide components of GAG chains are intrinsically carboxylated and can be further enzymatically sulfated; thus they are negatively charged at physiological pH [18]. The array of proteoglycans produced by vascular smooth muscle cells includes the chondroitin sulfate (CS) proteoglycan, versican, the chondroitin sulfate/dermatan sulfate (DS) proteoglycans, biglycan and decorin, the heparan sulfate (HS) proteoglycan, perlecan and the keratan sulfate (KS) proteoglycan, mimecan (table 1). The expression of the individual core proteins, derived from the respective messenger RNAs (RNAs), is independently regulated [19, 20]. Regulation of individual proteoglycans is apparent in disease states such as osteoarthritis, peridontitis, Alzheimer's, prostate and breast cancer, multiple sclerosis, glomerular disease and Marfan's syndrome, and this independent regulation is assumed to underlie the pathological role of the proteoglycans. The biosynthesis of GAG chains is also regulated [19, 21]. There are diseases of GAG metabolism, including Hurler's, Hunter's and Maroteau-Lamy syndromes, which are enzyme defects in α-l-iduronidase, l-iduronate-2-sulfatase and GalNAc-4-sulfatase activities, respectively.

The structural biochemistry of GAG chains is regulated by drugs and growth factors which have effects on GAG length and sulfation pattern [19]. A multitude of enzymes is involved in the generation and modification of GAG chains. The existence of many of the enzymes has been demonstrated, but their specific role in GAG synthesis in vascular smooth muscle has not been described. The synthesis of GAG chains on proteoglycans commences with the construction of a tetra moiety linking region by the addition of a pentose sugar, xylose, to a serine on the core protein followed by the sequential

addition of two Galactose units and GlcA [22]. The sequential addition of monosaccharides form repeating disaccharide units that create the GAG chain. Synthesis of the main vascular smooth muscle proteoglycans, versican and biglycan/decorin, involves attachment of the initial tetrameric linking sugars as described above. GAG elongation occurs in the cis Golgi through the sequential addition of GalNAc and GlcA residues to the nonreducing end of the growing chain to generate the CS GAG (on all three core proteins). The parallel action of 6-sulfotransferase acting in the cis region forms a partially sulfated GAG chain which proceeds through the Golgi. The partially synthesised proteoglycan passes to the trans Golgi where it is subject to the action of a 4-sulfotransferase and an epimerase. The action of chondroitin C-5 uronosyl epimerase is tightly coupled to the 4-sulfation of the adjacent (GalNAc), producing consecutive repeats of L-IdoA containing disaccharides almost entirely when 4-sulfation has occurred [23]. The 4-sulfotransferase generates a monosulfated disaccharide unit which is the major (>90%) product. 4-Sulfation appears to also make the structure more rigid, and the action of the epimerase generates a stable 4-sulfated DS disaccharide (IdoA-GalNAc). Minor amounts of the disulfated products on the Gal (potentially 4,6-diS) or even an additional 2′-sulfation on the GlcA moiety generating 2′,4,6-tri S are possible – these occur to less than 5–10% of the total disaccharides, but being highly charged may be of biological significance [24]. A very small amount of the GAG chains may escape sulfation. These steps are shown schematically in figure 1a and the positions of the relevant groups on the disaccharide in figure 1b.

The epimerisation mentioned above is a biologically unusual modification that occurs in the trans Golgi. Biglycan and decorin GAG chains, synthesised initially as CS proteoglycans, are subject to the activity of an epimerase which isomerises the carboxylic acid on the 5 position of the GlcA moiety to generate IdoA; such GAGs are then defined as DS. The extent of epimerisation varies markedly, and using chondroitinase AC, ABC and B enzyme digestions we have recently observed very high levels of CS and low levels of DS (but extensive levels of 4-sulfated) GAGs in decorin and biglycan from multiply passaged human internal mammary artery smooth muscle cells [J. Nigro and P. J. Little, unpublished observation] suggesting low levels of epimerase activity in the trans Golgi of these cells. The possibility of the regulation of these steps by drugs and growth factors and the implications for the effect on LDL binding are discussed below.

The applicability and validity of the above model to human vascular smooth muscle proteoglycan biosynthesis can be demonstrated with the use of chlorate, which greatly reduces ^{35}S-sulfate incorporation into proteoglycans and preferentially inhibits sulfation at the 4 position over the 6 position [25]. Chlorate markedly inhibits the incorporation of ^{35}S-sulfate into total proteoglycans produced by human vascular smooth muscle cells even in normal sulfate-containing medium (fig. 2a). Chlorate also greatly inhibits the sulfate incorporation stimulated by treatment of cells with transforming growth factor (TGF)-β (fig. 2a). Fluorophore assisted carbohydrate elec-

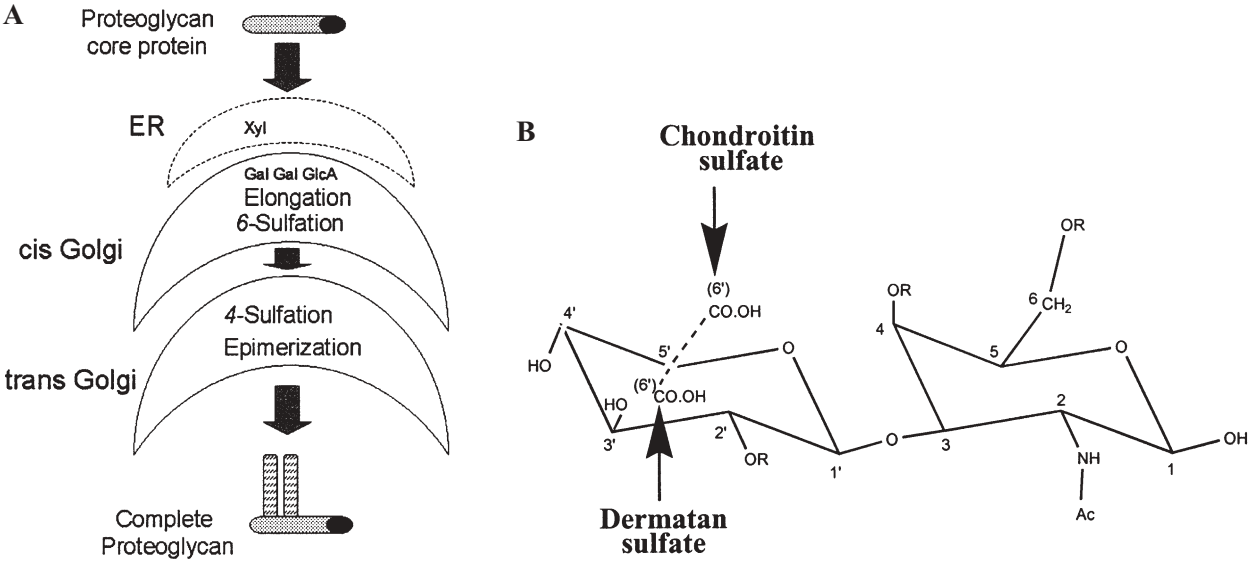

Figure 1. (*A*) Scheme showing the pathways in the synthesis of the glycosaminoglycan chains on proteoglycans to indicate the potential steps of regulation of the pro- and anti-atherogenic aspects of glycosaminoglycan structure. ER, endoplasmic reticulum. (*B*) Structure of chondroitin/dermatan sulfate disaccharides, where R = SO_3^-. The *5′* position is the site of epimerisation of GlcA to IdoA and thus from a chondroitin sulfate (CS) to a dermatan sulfate (DS) GAG chain.

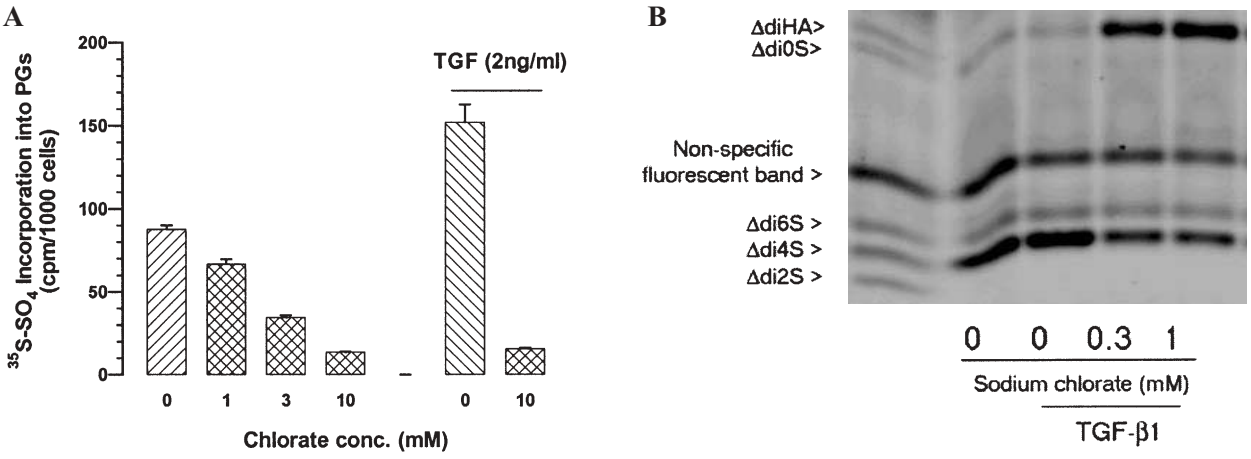

Figure 2. (*A*) Human smooth muscle cells were treated with sodium chlorate (1–10 mM) and TGF-β1 (2 ng/ml) with and without sodium chlorate (10 mM), and vascular proteoglycans were metabolically labelled with ^{35}S-sulfate for 24 h. Proteoglycans were harvested from the culture medium and precipitated using cetylpyridinium chloride, and the radiolabel incorporated into glycosaminoglycans was assessed on a beta counter. Parallel plates were established without radiolabel to count cells. Data are expressed as cpm/1000 cells and are the mean ± SEM of three identical treatments from one experiment. (*B*) Human SMCs were treated with sodium chlorate (0.3-1 mM), and the secreted proteoglycans were harvested from the culture medium. Proteoglycans were purified using DEAE-Sephacel and sequentially digested with proteinase K and chondroitinase ABC. Disaccharides were flurotagged with 2-aminoacridone (AMAC) electrophoresed on a high-percentage acrylamide FACE gel. The gel was imaged using the Glyko FACE imager (SE2000).

trophoresis (FACE) analysis of the resultant products derived from chondroitinase ABC digestion of the GAG chains shows little effect on the level of the 6-sulfated product, but as the inhibition of 4-sulfation occurs in the presence of chlorate, then an increasing amount of the unsulfated disaccharide results, as seen at the top of the gel (fig. 2b). This is consistent with 6-sulfation occurring prior to 4-sulfation as shown in figure 1a and described in detail above.

Theoretical considerations on the structural features of proteoglycans potentially affecting LDL binding

The binding of proteoglycans and GAGs to LDL can be studied by multiple assays, including gel mobility shift assay (GMSA) [7], LDL affinity columns [26, 27], interactions with unlabelled and labelled matrix [28], competition studies of heparin-bound LDL [29] and binding of ^{125}I-LDL to matrix of drug-treated cells [30]. GMSA offers advantages for studies of the regulation of proteoglycans [21], but there are several important considerations for the design of experiments, and the presentation and interpretation of data. An important question would appear to be the choice of radiolabel – the common available choices are ^{35}S-sulfate, ^{3}H-glucosamine or ^{14}C-glucosamine to label the GAG chains, or ^{35}S-methionine/cysteine or other radiolabelled amino acids to label the proteoglycan core proteins. Although we and others have previously used ^{35}S-sulfate GAG-labelled proteoglycans in GMSAs [21, 31], it would appear to be a potentially confounding variable that the label is in the site of variation in binding, i.e. the GAG chains. LDL does not bind to proteoglycan core proteins which have had their GAG chains removed by enzymatic digestion [32]. When demonstrable changes in GAG length and potentially other structural changes are occurring in GAG chains, the chain labelling appears to be less than ideal when other radiolabelling options are available. Thus, we favour the use of core protein labels, ^{35}S-methionine/cysteine and the like, where an identical quantity of proteoglycan core proteins can be applied and the changes in the GAG chains can be allowed to produce changes in LDL binding. The resultant binding curves, after employing a fixed quantity of radiolabelled proteoglycans and a range of LDL concentrations, can be modelled to achieve half-maximal saturation values. Care must be taken in the presentation and interpretation of these data since the basic criteria of binding interactions, particularly of the reversibility and per cent bound for a proper thermodynamic analysis, are not met. Reference to binding affinity in this article should therefore not be taken to mean that the binding isotherm meets classic criteria. We have, however, found that the extent of binding is dependent upon both the amount of LDL and the concentration of proteoglycans. The binding can be demonstrated to be saturable in terms of both proteoglycans and LDL (fig. 3a, b). It appears this experimental protocol produces data which give an insight into the possible interactions that may occur in the vessel wall. The data are best referred to as half-maximal saturation values, with greater numbers representing higher amounts of LDL required

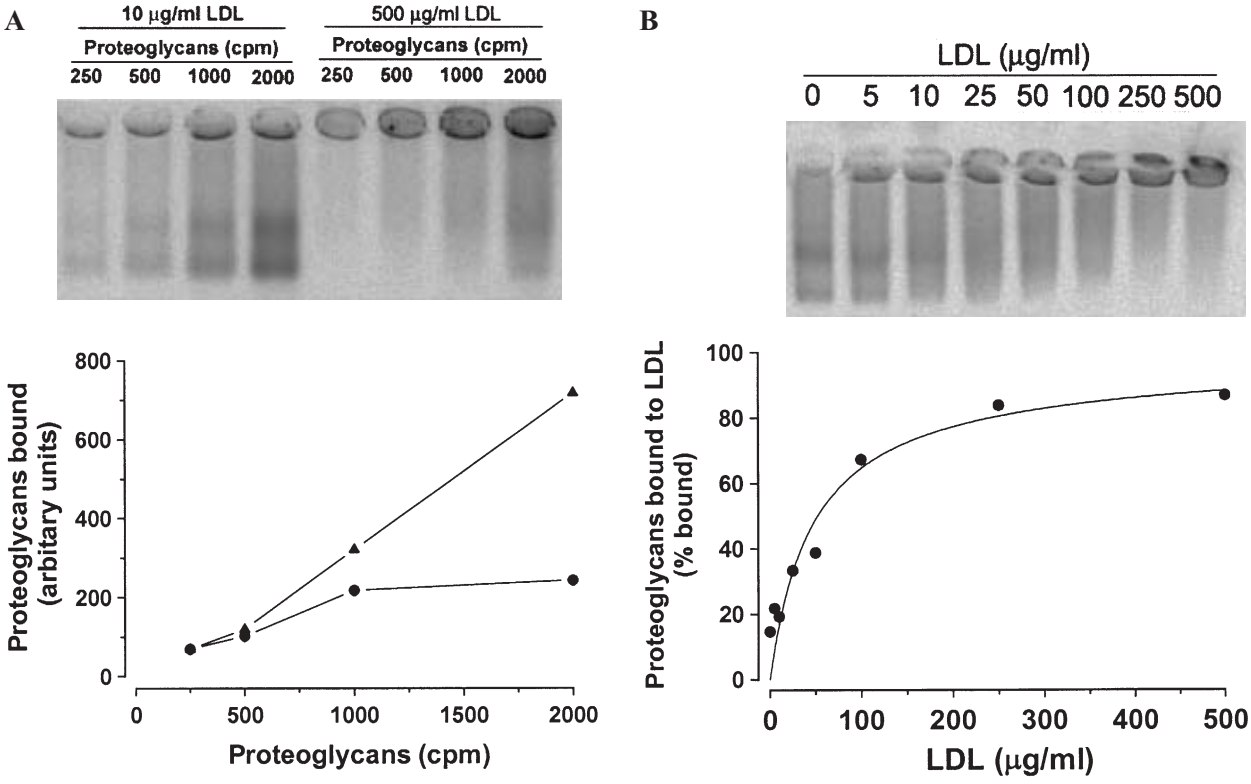

Figure 3. (*A*) Various amounts of proteoglycans (250–2000 cpm) were incubated with LDL at either 10 µg/ml (closed circles) or 500 µg/ml (closed triangles) LDL. Bound and free proteoglycans were separated using the gel mobility shift assay. (*B*) Various amounts of LDL (5–500 µg/ml) were incubated with a fixed amount of proteoglycans (1500 cpm). Bound and free proteoglycans were separated using the gel mobility shift assay.

for saturation representing lower 'affinity' binding and vice versa.

GAG length is clearly regulated and appears to be a determinant of LDL binding. Agents which increase the length of xyloside GAG chains increase the affinity of LDL binding [21]. Thus, the effect of increased length can be seen on fully developed GAGs on mature proteoglycans and shorter GAGs, and therefore occurs over a broad range of size. The simplest interpretation would be that longer GAG chains can accommodate more LDL particles, and hence greater capacity of binding would be observed. However, because gels are usually loaded with equal amounts of radiolabelled proteoglycans and a range of LDL concentrations to saturating values, what is actually observed are changes in apparent 'affinity' of the binding [21]. It is also notable that the binding affinity to intact proteoglycans is an order of magnitude higher than is the binding to the smaller GAGs initiated on xyloside and to free GAG chains. The steric rigidity produced by the core protein produces an enhanced interaction with the apolipoprotein on the LDL and possibly the interaction of the individual LDL particle with more than one GAG. As the change in binding which is related to a change in the size of the GAGs is manifest as an apparent change in the affinity, not simply the extent of binding, then it must be considered that structural changes other than simply GAG elongation are occurring, and this is worthy of considerable further investigation.

A defining step in GAG development and one that is regulated is the sulfation pattern. The disaccharide components of GAGs can be sulfated on the 4 and 6 positions of the hexosamine (GalNAc for CS/DS) or the 2 position of the uronic acid (GlcA for CS and IdoA for DS) (see fig. 1b). The major product is a monosulfated product with the most sulfation occurring on the hexosamine. Disulfated disaccharides occur as minor components, but they are nevertheless more highly charged and thus potentially of importance. The 4-sulfated disaccharide has greater mobility on a FACE gel, suggesting that it has a higher charge density than the 6-sulfated product. Nevertheless, the 6-sulfate is present on an exocyclic carbon and thus would protrude beyond the backbone of the GAG chain more than the 4-sulfated endocyclic product. It is reasonable speculation that the 6-sulfate is more sterically accessible to a positively charged apolipoprotein on LDL and would enhance binding, but this has not been conclusively demonstrated. Sulfation may be regulated by alteration in the level of expression or activity of the relevant enzymes, as suggested above, by alterations in the transit time of the developing macromolecule through the Golgi since the

4 and 6 sulfation enzymes are in different compartments. Sulfation may greatly alter the flexibility of the GAG structure. Flexibility or its converse, rigidity, may contribute to the interaction between the GAG and LDL, with more rigid structures showing higher-affinity binding.

Enzymatic epimerisation of the carboxylic acid group on the 5 position of the GlcA to the IdoA isomer generates, by definition, a GAG change from CS to DS. These molecules show different levels of structural flexibility and rigidity that may be a factor in determining LDL binding. IdoA residues may exist in several isoenergetic conformers as opposed to the single well-defined chair conformation of GlcA [29]. Some evidence that IdoA-containing GAGs may bind more avidly than GlcA-containing GAGs suggests that even though the overall charge densities of the molecules may be similar, IdoA containing GAGs may exhibit local concentrations of charge, 'hot spots', for ionic binding to the apolipoprotein on LDL [17]. There is also an interaction between epimerisation and 4-position sulfation, and again indirect effects on LDL binding are theoretically possible [23]. As GAG chains are synthesised as CS and epimerisation is a late stage event to generate DS, the extent of expression and activity of epimerase will affect the final structure via the extent of conversion of CS to DS. Determining the effect of this on LDL binding and the factors that regulate epimerase activity may be an important question.

A further manner in which growth factors and inflammatory mediators might generate proteoglycans which show enhanced LDL binding would be to alter the proteoglycan profile produced by vascular smooth muscle. Such agents may increase the CS/DS proteoglycans in relation to HS proteoglycans. As CS/DS derivatives show greater binding of LDL [33, 34], this effect may represent a pro-atherogenic action but not one dependent upon changes in the structural features of the individual glycosaminoglycans.

In summary, the multiple enzymes responsible for the elongation, sulfation and epimerisation of GAG chains on proteoglycans in vascular smooth muscle are subject to regulation and may induce structural changes which are theoretically capable of altering the binding properties of the proteoglycans for LDL.

Elongation of GAG chains and enhanced lipoprotein binding

Present data strongly indicates that GAG length is a key determinant of proteoglycan binding affinity, with longer chains exhibiting higher-affinity binding and vice versa. Agents known to increase the size of GAGs on proteoglycan include TGF-β1 and platelet-derived growth factor (PDGF) [19, 21], oxidised LDL [26], free fatty acids [30] and angiotensin II [27, 35, 36]; GAG chains on proteoglycans are also elongated in proliferating cells [31]. There are several assays, including LDL affinity column binding and gel mobility shift assay, for investigating the interaction of proteoglycans with LDL, and in most cases GAG elongation has been shown to increase binding affinity and/or capacity to bind LDL. Following stimulation of vascular smooth muscle cells with growth factors, the binding of both GAG chains chemically released from core proteins and of GAG chains synthesised on exogenous xyloside is increased compared with control, so clearly the GAG chains are the critical site of LDL binding [21].

Angiotensin II, a seven transmembrane G-protein-coupled receptor agonist [27, 35, 36] as well as the tyrosine kinase growth factor, PDGF [19, 37], and serine-threonine kinase growth factor, TGF-β [19, 37], all induce GAG elongation in vascular smooth muscle cells. This indicates the existence of multiple signalling pathways for GAG elongation. With our present knowledge, the biochemical link between receptor activation, intracellular signalling and alterations in GAG synthesis is presently a 'black box', perhaps analogous to our understanding of cell cycle events a decade or so ago. Specifically, whether or not these pathways coalesce through a common intracellular mediator, such as a protein kinase, is unknown.

Pharmacological inhibition of GAG elongation and reduced LDL binding

The therapeutic potential is that shorter GAG chains may lead to lower-affinity binding of lipoproteins, and thus reduced retention of lipoproteins in the vessel wall and less atherosclerosis. Hence, the test of the therapeutic implications of the response to retention hypothesis is to discover agents which specifically reduce the length of GAG chains on proteoglycans either directly, or more likely by preventing the GAG elongation actions of atherogenic growth factors [19]. Such actions may be directed at the growth factor signalling pathways [38] or at the basic underlying processes of proteoglycan biosynthesis [18, 39]. It is likely that the former pathway of antagonising growth factor signalling would be the more successful strategy, since these pathways are activated under the inflammatory conditions existing in a pro-atherosclerotic vessel wall [40]. Furthermore, targeting basic biochemical mechanisms of GAG biosynthesis may interfere with all sites of proteoglycan formation, possibly precipitating unwanted effects such as modification of cartilage.

Little is known about mechanisms for reducing the size of proteoglycans through a shortening of GAG chains. Two calcium antagonists, amlodipine and nifedipine, inhibit proteoglycan biosynthesis, reduce the length of the GAGs and reduce both the capacity of binding to an LDL affinity column and the affinity of binding in a GMSA

[41]. These experiments were conducted in the presence of a low concentration of serum but the absence of atherogenic growth factors, which stimulate GAG elongation. It is further unclear, perhaps even unlikely, that the effect to decrease GAG length results from the inhibition of voltage or receptor-operated calcium channels, the targets of these agents. Furthermore, the effect of intracellular calcium on proteoglycan biosynthesis is presently unknown. Our recent experiments show that other calcium antagonists as well as the calcium channel agonist, Bay K 8644 [J. Nigro and P. J. Little, unpublished observations] inhibit radiosulfate incorporation and reduce the length of GAG chains – intriguingly, the action of Bay K 8633 appears to be more directed towards PDGF than TGF-β-stimulated GAG chain elongation, suggesting some specificity in action. The action of calcium antagonists on proteoglycan biosynthesis becomes another ponderable in the understanding of the anti-atherogenic potential of this very widely used class of anti-hypertensive agents [42].

An emerging area is that of proteoglycan regulation by peroxisome proliferating activating receptor (PPAR) ligands such as those used for hypertriglyceridemia (PPAR-α ligands) and the thiazolidinediones (PPAR-γ ligands), which are the newest class of 'insulin sensitising' agents used for the treatment of hyperglycemia in type 2 diabetes. Olsson and colleagues [30] first showed that darglitazone, a PPAR-γ ligand, inhibits the activation of decorin transcription stimulated by nonesterified free fatty acids. We have recently shown that the PPAR-α ligand, gemfibrozil, inhibits the biosynthesis of proteoglycans in human vascular smooth muscle by reducing the length of GAG chains on proteoglycans [43], and that this effect is maintained in both normal and high glucose environments. We have also shown that the PPAR-γ ligand troglitazone induces small proteoglycans which show reduced LDL binding [44]. The mechanisms of action of these agents remain unknown, so the observed actions would be 'pleiotropic' at this stage.

The only exception to the rule that increased GAG length correlates with increased binding is the recent observation that treatment of monkey aortic smooth muscle cells with the 3-hydroxy-3-methyl glutaryl coenzyme A (HMGCoA) reductase inhibitors simvastatin and cerivastatin results in longer GAG chains which show reduced binding to LDL [45]. The fine structural changes underlying this intriguing result remain to be determined.

Metabolic regulation of GAG elongation

Metabolic regulation of proteoglycan biosynthesis has recently been demonstrated in cells supplemented with glucosamine [32]. Although glucose is a major source of precursors of GAG synthesis, in a series of experiments aimed at demonstrating that elevated glucose mimicking the hyperglycemia of diabetes would have an atherogenic effect on proteoglycan biosynthesis, it was shown that glucose concentration had no effect on proteoglycan metabolism in smooth muscle cells derived from monkey thoracic aorta [32]. However, glucosamine used to activate the hexosamine pathway through which additional carbohydrate flux occurs under high glucose conditions inhibited the biosynthesis of proteoglycans [32]. The reduction in GAG length was dependent on the glucosamine concentration. It might intuitively be expected that GAG chains would be elongated when additional substrate is supplied, but in the case of glucosamine supplementation the reverse was observed. It can be speculated that there is intracellular competition for uptake and utilisation of hexosamines utilised in GAG synthesis, and gross supplementation with one may competitively inhibit the availability of the other, leading to an overall reduction in GAG synthesis and thus chain length. The shorter GAG chains showed reduced affinity binding to LDL, providing a demonstration of the potential for a highly effective agent to reduce GAG chain length and demonstrate anti-atherogenic activity [32]. It is unlikely that glucosamine represents a pathway to a therapeutic agent, but it does provide a tool for further studies and warrants careful monitoring of its effects, as it is widely and increasingly consumed as a nutritional supplement primarily for alleviating osteoarthritis [46].

Sulfation and LDL binding

There has been accumulating evidence that the sulfation pattern of a GAG is altered in both in vivo and in vitro models of atherosclerosis, but whether these changes affect proteoglycan-LDL binding remains controversial. Sulfation of GAGs is essential for LDL binding [47]. For example, the atherosclerosis-susceptible White Carneau pigeon shows an increase in the 6-sulfation of GAGs compared with the atherosclerosis-resistant control [48]. Additionally, it has been shown that there is a two-fold increase in the 6:4-sulfate ratio of GAGs synthesised by monkey aortic smooth muscle cells following stimulation with the atherogenic growth factor PDGF [19]. It has also been suggested that the degree of sulfation, rather than the position of the sulfate groups on the GAGs, determines LDL binding [24]. This work did not directly compare GAGs with 6- versus 4-sulfation in their assay for LDL-proteoglycan interaction, and this question remains unresolved. Gigli and collegues [29] investigated the relationship between GAG size and the degree of sulfation; however they did not specifically compare the difference between proteoglycan-LDL binding with GAGs that were predominantly 6-sulfated with those that were 4-sulfated, of similar sizes [29]. We propose that both 4- and 6-sul-

fation may increase binding to LDL but by different mechanisms. The 6-sulfate is more sterically accessible to LDL particles, and the 4-sulfate produces a rigid molecule of higher charge density (see fig. 2b). The relative atherogenicity of these sulfation patterns and the potential for independent pharmacological and thus therapeutic regulation is an interesting area of future work.

Epimerisation and LDL binding

Various studies show that the binding to LDL is determined by more than the degree of sulfation, and the extent of epimerisation is a further possibility for modification of GAG structure. It appears that activity of the epimerase can be regulated in a manner that will determine the extent of DS in the CS chains of vascular proteoglycans. Gigli and colleagues [29] have demonstrated that glycans containing DS bind with almost one order of magnitude higher affinity to LDL than CS GAGs at equivalent levels of sulfation. Iverius [17] also showed that L-IdoA-containing DS binds more avidly than GlcA containing chondroitin 4-sulfate polysaccharides even at equal charge densities. Vijayagopal and colleagues [47] used two preparations of HS proteoglycans containing almost double the levels of Ido-A (≈20%) compared with CS/DS proteoglycans (≈10%) and found lower binding for the higher IdoA containing HS preparations. These preparations contained similar levels of sulfate (9–10%) but most likely significantly different sulfation patterns as well as differing levels of GlcNAC(HS) and GalNAC(CS/DS). The multitude of structural differences makes it somewhat difficult to independently assess the contribution of the IdoA/GlcA ratio and hence the role of epimerisation to LDL-binding. The use of molecular biology techniques to regulate the level of activity of epimerase, with analysis of GAG structure and LDL-binding analyses will allow for deeper insights into this area.

GAG mimetics as tools to block PG-to-LDL interactions

The interaction between apolipoproteins on lipoproteins and GAG chains in proteoglycans occurs through the ionic interactions between known sites of positive charge on the apolipoproteins and less well defined negatively charged sites on the GAGs on proteoglycans. There is the potential for interactions between large molecules to be interrupted by the introduction of small molecules which mimic one of the binding partners and prevent the critical interaction between the macromolecules. By analogy, the interaction between matrix-bound fibronectin and cell surface integrins can be blocked by short 'RGD' peptides [49]. Staels [2] has recently suggested that vascular directed strategies are required to limit atherosclerosis generated by GAG-to-LDL interactions and has provided a strategy for the interruption of the interaction between GAGs and LDL. Although not strictly a vascular directed strategy, such as modifying GAG chains on proteoglycans produced by vascular smooth muscle cells as proposed in this review, it is an interesting proposition which we have recently addressed experimentally. We chose the simplest model of adding molecules to the LDL and proteoglycan mixture without initial involvement of the potential bridging molecules that may also be participants. We used the large molecules available to us rather than potential oligosaccharides that may be studied later. Thus, we used CS (mol. wt 20–31 kDa, Sigma Chemical Company), HS

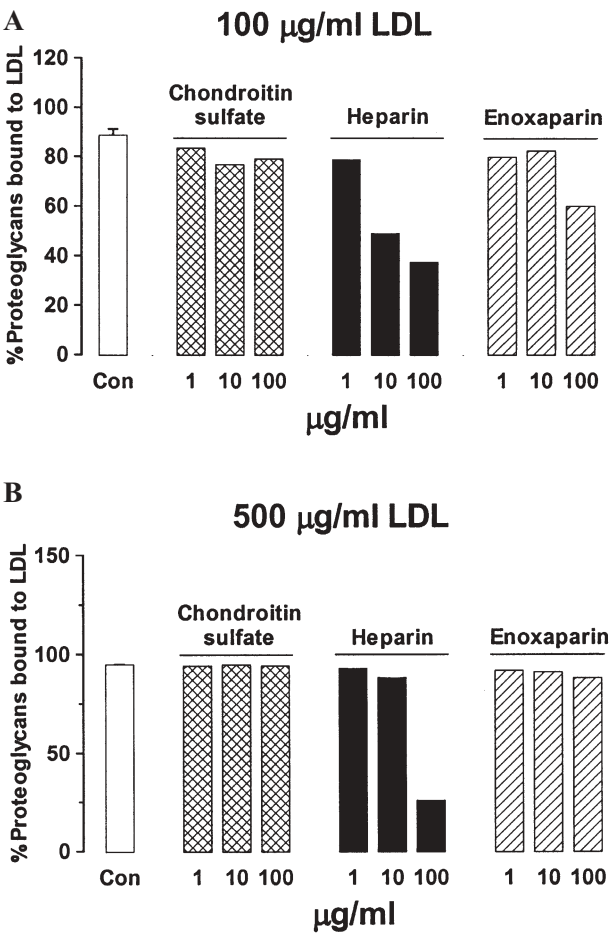

Figure 4. Glycosaminoglycans can interrupt the binding of proteoglycans to LDLs. Chondroitin sulfate, heparin or enoxaparin each at 1, 10 and 100 μg/ml were incubated with either 100 μg/ml (*A*) or 500 μg/ml (*B*) of normal human LDL-cholesterol in the presence of a fixed amount (1500 cpm) of ^{35}S-methionine/cysteine-labelled proteoglycans from smooth muscle cells derived from human internal mammary artery. Bound and free proteoglycans were separated on a flatbed, agarose (0.8%) gel at 4 °C for 2 h. Gels were air-dried, and images were captured using a phosphorimager. Bound and free proteoglycans were quantitated for each condition to express the percentage of proteoglycans bound to LDL. The control was the mean ± SEM of three separate lanes containing LDL and proteoglycans only.

(15.9 kDa) which has been previously shown to bind LDL [29] and the HS-derived enoxaparin (4.45 kDa) and introduced these compounds with human vascular smooth muscle-derived ^{35}S-sulfate-labelled proteoglycans and normal human LDL. We used LDL at 100 µg/ml (fig 4a), which is subsaturating, and 500 µg/ml (fig. 4b), which is a saturating concentration of LDL in this assay. All three molecules showed concentration-dependent inhibition of binding in the presence of subsaturating concentrations of LDL with an order of potency (heparin > enoxaparin > CS). The extent of inhibition of binding was reduced at the higher LDL concentration, and only heparin appreciably inhibited the interaction between proteoglycans and LDL. It is too early to speculate whether it is possible to develop potent oligosaccharides which can perform the same function as these larger molecules, but the data clearly indicate the potential of the Stael's hypothesis to generate agents capable of interfering with the proteoglycan to LDL interaction and thus preventing atherosclerosis.

It would be an interesting speculation that orally active drugs, potentially nutritional supplements or the like [32], might be capable of selectively blocking the interaction between proteoglycans and LDL, but only scant anecdotal evidence is available to support this intriguing proposition.

Conclusion

A challenge to exploit the therapeutic potential of the response to retention hypothesis is to develop mechanistically based agent(s) which modify the elongation, sulfation or isomerisation (epimerisation) of GAG chains on proteoglycans in a manner that reduces atherogenesis as shown in the scheme (fig. 5). Such an agent directed at the blood vessel wall, which modifies vascular proteoglycans, would represent a legitimate example of a therapeutic agent capable of substantially reducing the development and/or progression of atherosclerosis.

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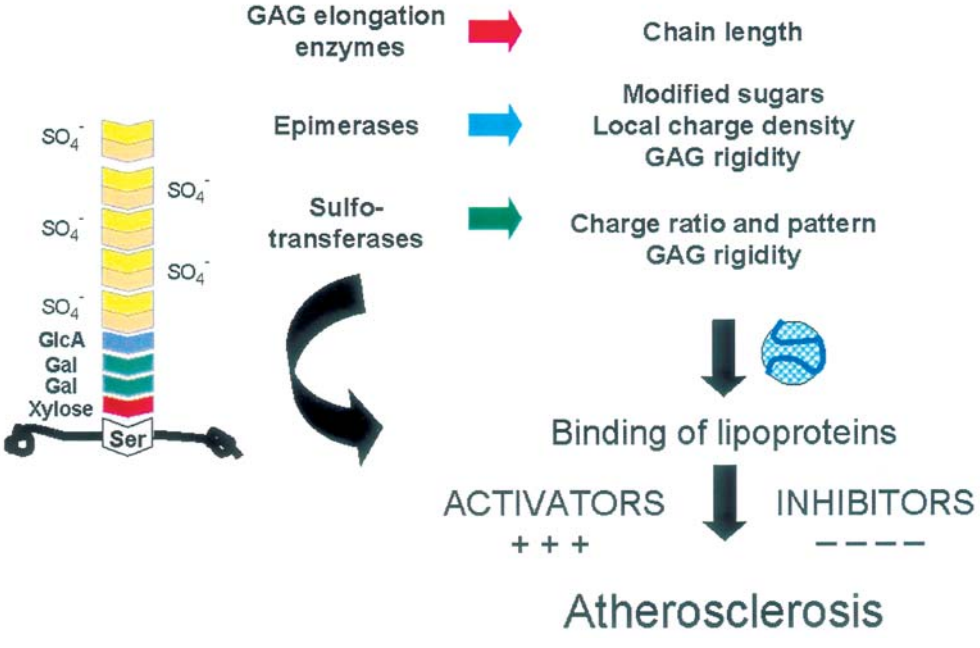

Figure 5. Considerations and implications of GAG synthesis and structure on lipoprotein binding in the development of atherosclerosis and the potential for therapeutic interventions.

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