# Vascular wall proteoglycan synthesis and structure as a target for the prevention of atherosclerosis



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**Abstract:** Atherosclerosis is the underlying pathology of most cardiovascular disease and it represents the major cause of premature death in modern societies. Current therapies target risk factors being hypertension, hypercholesterolemia, hypertriglyceridemia and hyperglycemia when diabetes is present however the maximum efficacy of these strategies is often 30% or less. Areas of vascular biology that may lead to the development of a complementary vascular wall directed therapy are: inflammation, oxidation, endothelial dysfunction, diabetes-specific factors —hyperglycemia and advanced glycation endproducts and lipid retention by vascular matrix specifically proteoglycans. The major structural features of proteoglycans that determine low-density lipoprotein (LDL) binding are the length and sulfation pattern on the glycosaminoglycan (GAG) chains. Emerging data discussed in this review indicates that these structural properties are subject to considerable regulation by vasoactive substances possibly using novel signaling pathways. For example, GAG elongation stimulated by platelet-derived growth factor is not blocked by the receptor tyrosine kinase antagonist, genistein suggesting that there may be a previously unknown signaling pathway involved in this response. Thus, modifying proteoglycan synthesis and structure may represent a prime target to prevent LDL binding and entrapment in the vessel wall and thus prevent the development and progression of atherosclerosis.

**Keywords:** proteoglycans, signaling, lipoproteins, atherosclerosis

## Introduction

The World Health Organization estimates that cardiovascular diseases are responsible for 30 percent of all deaths worldwide each year, with atherosclerotic vascular disease the principal cardiovascular disorder responsible for the global rise in mortality (Bonow et al 2002). Combined with the explosion in the rates of obesity and type 2 diabetes, an unprecedented increase in the incidence of cardiovascular disease is predicted for the future. Atherosclerotic vascular disease is potentially preventable by lifestyle changes, therapeutics or a combination of both strategies.

Atherosclerosis is the result of a complex interaction between lipoproteins, extracellular matrix and cells of the vessel wall leading to the formation of a lesion known as an atherosclerotic plaque. A significant and potentially fatal outcome is acute plaque rupture leading to the formation of a thrombus—which may occlude the blood vessel at the rupture point or downstream causing ischemia and necrosis of affected tissues (Glass and Witztum 2001; Libby 2003). Current therapies target "risk factors" that contribute to increased incidence of cardiovascular disease, such as hypertension, hypercholesterolemia, hypertriglyceridemia, and hyperglycemia when diabetes is present. The maximum efficacy of these strategies as determined from multiple clinical trials is limited to around 30% (Pignone et al 2000). Notably, although atherosclerosis occurs within the vessel wall there is no pharmaceutical treatment that directly targets the blood vessel wall and is capable of preventing

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the atherosclerotic process. Appropriate new target molecules are urgently needed to provide complementary anti-atherosclerotic therapies that may add to the success of risk factor directed strategies. This issue is well recognized by the pharmaceutical industry as expressed by Novartis Institutes for BioMedical Research: "Despite significant improvement in the prognosis of atherosclerosis patients treated with statins, the disease process continues to progress in a significant proportion of patients. Thus, alternative (or adjunctive) novel therapies that inhibit other triggers for this pathological state are needed…" (www.novartisinstitutes.com/DiseaseAreas/Cardiovascular/)

Thus, there is widespread agreement on the successful role of statins and other cardiovascular risk factor directed strategies but importantly there is also acknowledgement of a large amount of resistant or residual disease (atherosclerosis). With two decades of vascular biology research and considerably increased knowledge of the molecular and cellular mechanisms of atherosclerosis an opportunity for the identification of new therapeutic targets and the development of targeted drugs is available.

The major areas of research that may potentially provide a vascular directed agent for the prevention of atherosclerosis are: inflammation (Ross 1999; Libby 2006), oxidation (Steinberg et al 1989; Witztum 1994; Stocker and Keaney 2004), endothelial dysfunction (Davignon and Ganz 2004), diabetes specific factors—hyperglycemia and advanced glycation end products (AGEs) (Brownlee 2001; Forbes et al 2004), and lipid retention by vascular matrix specifically proteoglycans (Skalen et al 2002).

Special attention is given to the area of lipid retention as favored by our laboratory for the following reasons: firstly lipid retention is an essential and very early step in the atherosclerotic cascade indeed without this event atherosclerosis cannot be induced in animal models of the disease (reviewed in Olofsson and Boren 2005); secondly, proteoglycans are the major lipid-binding component present in the arterial wall able to interact with lipids in the early stages of atherosclerotic plaque development (Camejo 1982; Skalen et al 2002); thirdly, signaling pathways of proteoglycan and glycosaminoglycan (GAG) structural changes appear to be highly novel and provide tissue and pathological specificity and fourthly, the turnover of proteoglycans is sufficiently high to provide an opportunity for therapeutic modification of the extracellular matrix.

## Low density lipoprotein binding to proteoglycans—the "response to retention" hypothesis

The "response to retention hypothesis proposes that sub-endothelial retention of lipoproteins by matrix proteoglycans, most prominently chondroitin sulfate (CS)/dermatan sulfate (DS) proteoglycans is a critical initiating step in atherogenesis (Williams and Tabas 1995). The binding of lipoproteins to these molecules leads to their entrapment and eventual phagocytosis by macrophages and other cells. Therefore understanding the precise mechanisms by which lipid is trapped and retained within blood vessels is critical to understanding atherogenesis and may provide targets for new therapies aimed at reducing plaque formation by directly targeting the vascular wall.

A study reported in *Nature* by the Boren Laboratory in Gothenburg in 2002 supported not only the "response to retention hypothesis" but also specifically the role of proteoglycans in lipoprotein binding (Skalen et al 2002). Transgenic mice expressing human wild-type apoB100 containing low-density lipoprotein (LDL) with normal proteoglycan binding or genetically altered LDL such that LDL-proteoglycan binding was defective were generated. After 20 weeks of feeding on a Western diet, mice with mutations of the apoB100 gene developed significantly less atherosclerosis due to the decreased ability of apoB100-modified LDL to bind with artery wall proteoglycans. This study presents direct evidence that the binding of LDL to proteoglycans in the blood vessel wall is a key step in atherogenesis. The authors concluded "thus, atherosclerosis is initiated by sub-endothelial retention of atherogenic lipoproteins". In an associated commentary it was stated "… therapies that act directly on the arterial wall are needed" (Staels 2002).

LDL normally passes through the blood vessel wall by a process of endothelial transcytosis and efflux through the vessel wall (see Nigro et al 2006). Retention of LDL within the vessel wall and efflux to the lymph are rate-limiting steps (Proctor 2002). GAG chains on proteoglycans are highly negatively charged with over 90% of the disaccharide moieties containing an ionized sulfate group and all contain an ionized carboxylic acid group. The only manner in which the contribution of apo B100 on the LDL can be altered is by lowering the concentration in the plasma. It is not a viable therapeutic option to alter the protein sequence by the genetic manipulation employed by Boren and colleagues in their mouse study (Skalen et al 2002). Therefore, an alternative approach is to modify the synthesis and structure of the proteoglycans in the vessel wall.

Camejo and colleagues (1993) described an in vitro assay, somewhat equivalent to a receptor-binding assay, in which the binding of radiolabelled proteoglycans to LDL can be studied rapidly. Many vasoactive agonists and hormones have been shown to increase the size of the GAG chains on proteoglycans and in many cases (using the Camejo methodology) it has been demonstrated that this leads to greater binding to LDL. There have been only a few studies on the possibility of inhibiting this GAG elongation.

Our laboratory is addressing the question of inhibiting atherogenic changes in GAG synthesis and structure as a strategy to prevent atherosclerosis. In the late 1990s we embarked on a major program to study the hormonal and metabolic factors and their signaling pathways controlling GAG synthesis and structure on vascular smooth muscle proteoglycans. The most notable finding in the field at that time was the work of Schonherr et al (1997) in the Wight Laboratory in Seattle which showed that platelet derived growth factor (PDGF)-mediated elongation of GAG chains on proteoglycans synthesized by primate vascular smooth muscle cells (VSMCs) was *not* blocked by genistein. On the basis that genistein was a broad-spectrum tyrosine kinase inhibitor it was concluded that genistein-sensitive (ie, most if not all) tyrosine kinases were not involved in GAG elongation. This is a very important statement because it implies signaling via the PDGF receptor that is independent of the receptor tyrosine autophosphorylation which is the "classic" signaling pathway for PDGF receptor signaling, for example in cell proliferation (Little et al 2003). Thus regulation of proteoglycan (PG) synthesis and structure, specifically GAG structure may be through novel signaling pathways and investigations of such pathways may lead to important information on the regulation of PG synthesis but also new information on signaling in vascular smooth muscle.

## GAG elongation

The biochemical mechanisms by which the regulation of elongation of CS/DS chains of PGs occurs and specifically the factors that control final GAG chain length are unknown. Possible mechanisms include the activity of the multitude of chain elongation enzymes, the activity of the transporters that provide the requisite substrates and the transition time of the growing GAG chain through the Golgi synthetic process. As the importance of GAG chain length in PG function continues to increase it will become very important to understand the processes controlling GAG elongation. Many factors have been found to elongate GAG chains and these include growth factors such as PDGF (Schonherr et al 1993) and transforming growth factor-β (TGFβ) (Little et al 2002), vasoactive hormones such as angiotensin II (Figueroa and Vijayagopal 2002), and metabolic factors such as oxidized LDL (Chang et al 2000) and free fatty acids (Olsson et al 1999). As the activated vessel wall contains multiple growth factors and vasoactive substances, we recently considered if the interaction of these agents might have an additive effect on GAG elongation—both PDGF and TGFβ (at maximally effective doses) increase GAG length and these effects are clearly additive (Figure 1). Matrix can control the length of GAG chains (Figueroa et al 2004) but this is most likely secondary to a role in controlling proliferation since increased proliferation is associated with elongation of GAG chains of proteoglycans. One drug family—the HMGCoA reductase inhibitors (statin drugs) —also elongate GAG chains on vSMCs PGs (Meyers et al 2003). In most of these cases it has been demonstrated that the elongated GAG chains are associated with an increased proteoglycan/LDL binding. The only exception is the PGs secreted by statin treated cells in which the GAG elongation effect is overwhelmed by an inhibitory effect on sulfation as discussed below.

The process of GAG elongation is controlled independently from the presence of the PG core protein. Cells can be provided with exogenous "xyloside"—the first sugar attached to the serine of the core protein in the tetrasaccharide linker regions preceding the GAG chain—and short GAGs will be synthesized on that xyloside. The resultant molecules are about 10–15 kDa and thus a quarter of the size of the GAG chains on normal PGs. The size of the xyloside GAGs is regulated in parallel with the effects on GAG chains of PGs—for example TGFβ stimulates elongation of GAG chains on PGs and also elongation of xyloside GAGs in human VSMCs. Interestingly, although the affinity of binding LDL is almost one order of magnitude lower for binding to xyloside GAGs compared with free chains, the enhanced LDL binding is also observed in xyloside GAGs from TGFβ-treated cells (Little et al 2002).

The signaling pathways through which agents stimulate GAG elongation are poorly understood but potentially of immense importance as drug targets. It is most likely that the signaling pathways will provide the tissue and disease specificity required for the development of a therapeutic agent in this area. The most intriguing finding is that the action of PDGF to elongate GAG chains on VSMC PGs is not blocked by genistein (Schonherr et al 1997). In PG synthesis by primate VSMCs, genistein inhibits the expression

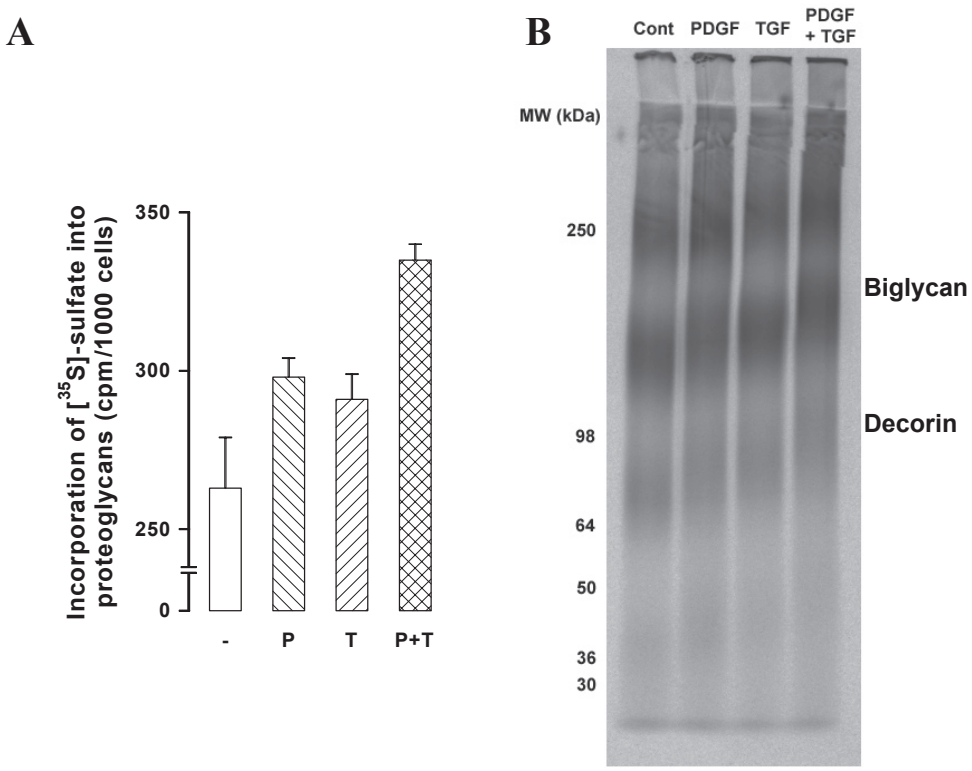


**Figure 1** Stimulation of vascular smooth muscle cells with both TGF-β1 and PDGF has an additive effect to increase [$^{35}$S]-sulfate incorporation into proteoglycans. Human VSMC treated with DMEM (–), PDGF (P, 50 ng/ml), TGF-β1 (T; 1 ng/mL) or PDGF plus TGF-β1 (P + T; 50 ng/mL and 1 ng/mL respectively) for 24 h were metabolically labelled with $^{35}$S-$SO_4$ (**A**) $^{35}SO_4$-labeled proteoglycans secreted into the medium were analyzed by CPC precipitation. (**B**) SDS-PAGE analysis of secreted PGs 20 000 cpm were loaded per lane. Gels were visualized by phospho-imaging.
**Abbreviations:** PDGF: platelet-derived growth factor; TGF: transforming growth factor; VSMC: vascular smooth muscle cell.

of the core protein for versican so this appears to be via the classic signaling pathway (Figure 2). There are only a very small number of responses to PDGF which are not sensitive to genistein (Mundschau et al 1994; Pelletier and Boynton 1994; Cartel et al 2001). The possibility that there is a completely novel PDGF signaling pathway controlling GAG elongation is intriguing. TGFβ also signals GAG elongation. The biochemically similar signaling pathway to the tyrosine kinase activity of the PDGF receptor would be the serine/threonine kinase activity of the TGFβ Type 1 receptor (ALK-5) which phosphorylates Smad proteins (Agrotis et al 2005). An inhibitor of ALK-5 has recently become available (Inman et al 2002). This compound will be able to be used to assess if the GAG elongation mediated by TGFβ is through the classic serine/threonine kinase pathway associated with collagen synthesis and fibrosis and thus whether the signaling pathway for PDGF is itself unique or indicative of a new set of growth factor signaling pathways associated with CS/DS GAG synthesis.

The process of GAG elongation is susceptible to pharmacological inhibition. Receptor mediated elongation such as for Ang II can be blocked by receptor antagonists (Figueroa and Vijayagopal 2002). Signaling pathway antagonists such as the MAP kinase inhibitor PD98059 (Shimizu-Hirota et al 2001) block the action of Ang II and epidermal growth factor receptor (EGFR) transactivation by Ang II is blocked by EGFR inhibitor AG1478 (Shimizu-Hirota et al 2001). A multitude of cardiovascular agents block GAG elongation and represent pleiotropic actions of these drugs. The peroxisome proliferator-activated receptor-α (PPARα) ligands, the lipid-lowering fibrates gemfibrozil and fenofibrate (Wiklund et al 1996; Nigro et al 2002, 2004), block GAG elongation and reduce lipid LDL binding. The PPARγ ligands, the insulin-sensitizing thiazolidinediones, rosiglitazone, and pioglitazone (Tannock et al 2004; Rodriguez-Lee et al 2006) as well as the experimental agent darglitazone (Olsson et al 1999), block GAG elongation and reduce LDL binding to PGs. Calcium channel blockers inhibit GAG elongation (Vijayagopal and Subramaniam 2001) but the mechanism is not dependent upon inhibition of voltage-sensitive calcium channels (Survase et al 2005).

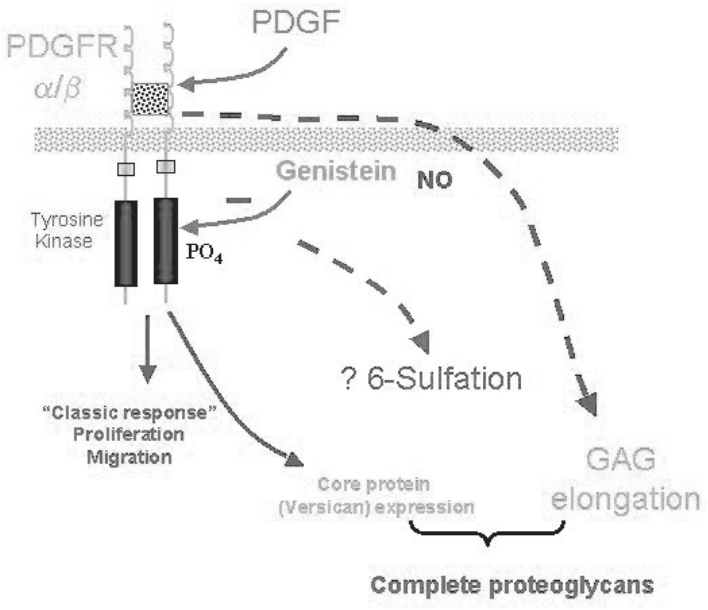


**Figure 2** PDGF-mediated signaling pathways for proteoglycan synthesis have classic and novel components. The PDGF stimulated signaling pathway for proteoglycan core protein expression is of the classic type as associated with proliferation and is sensitive to the PDGF receptor tyrosine kinase inhibitor, genistein. GAG elongation is not sensitive to genistein and the effect of genistein on the PDGF effect to increase the 6:4 sulfation ratio is unknown. The implication is that GAG elongation may be mediated by a completely novel PDGF signaling pathway which would provide an opportunity for the development of a specific therapeutic agent targeting GAG elongation but not other PDGF responses.
**Abbreviations:** GAG: glycosaminoglycan; PDGF: platelet-derived growth factor.

Many of these cardiovascular drugs reduce atherosclerosis in animal models of the disease. For example rosiglitazone inhibits the development of atherosclerosis in ApoE KO mice rendered diabetic with streptozotocin (Calkin et al 2005). Troglitazone also blocks the development of atherosclerosis in mice (Collins et al 2001). A multitude of anti-atherogenic processes are considered as the mechanisms resulting in a reduction in atherosclerosis, but it is also likely that an inhibition of GAG elongation leading to reduced LDL retention in the vessel wall may also be a positive contributing factor.

It will be very interesting to determine if a highly specific and potent inhibitor of GAG elongation can be developed. Such a compound could be used in animal models to test the potential for a therapeutic agent based on inhibition of GAG to be developed for human therapeutic use. Currently to elucidate the potential side effects of PG and GAG modifications it is necessary to examine the consequences of mutant PG genes in human patients and gene manipulation in mouse models. The extensive involvement of PGs in extracellular matrix and tissue stability as well as their roles in growth factor signaling indicate the potential areas of impact PG and GAG alterations may influence. Regulation of PG and GAG chain biosynthesis and metabolism is implicated in numerous human disease states (Ballinger et al 2004). To date at least 11 genes encoding PGs have been ablated in mice including biglycan, decorin, versican and perlecan (Aszodi et al 2006) with some gene knockouts resulting in embryonic lethality, eg, versican and perlecan, while others show mild to severe phenotypes (Aszodi et al 2006). Perlecan-null mice ($Hspg2^{-/-}$) are either embryonic lethal or die at birth and have a severe cartilage defect which presents as a disorganized growth plate with impaired chondrocyte proliferation and differentiation. These abnormalities arise from perlecan no longer being available to bind and regulate matrix metalloproteinases and thereby block the degradation of the collagen fibrils in cartilage (Costell et al 1999) as well as resulting in impaired FGF-2 signaling (Arikawa-Hirasawa et al 1999). Mice expressing perlecan that lack attachment sites for three heparan sulfate chains ($Hspg2^{\Delta 3/\Delta 3}$) survive but have defective eye lenses (Rossi et al 2003) demonstrating the capacity for altered PG molecules to result in altered phenotypes with variable severity. Undoubtedly it will be necessary to employ mouse knock-in models with specific PG and/or GAG mutations to provide a clearer identification of the consequences of specific PG and GAG modifications.

## GAG sulfation

A major structural feature of CS/DS GAG chains is the sulfation pattern. Disaccharide units can be sulfated on the

4 and 6 positions of the N-Acetylgalactosamine (GalNAc) moiety or the 2' position on the uronic acid (GlcA) moiety (see Figure 3). As is the case for GAG elongation very little is currently known of the actual enzymes mediating CS/DS GAG sulfation in VSMC. The temporal aspects of sulfation are known, with 6 position sulfation occurring before the sulfation at the 4 position of the disaccharide. In VSMC the GAG product contains about 80%–90% monosulfation at the 4 position and 5% at the 6 position with negligible amounts of 2' position sulfation observed (Ballinger et al 2004). The temporal aspects can be demonstrated using sodium chlorate, which is a specific inhibitor of 4 position sulfation—in the presence of increasing concentrations of chlorate there is a decreasing amount of the sulfation at the 4 position (Ballinger et al 2004).

Growth factors can regulate the sulfation pattern on GAGs. PDGF stimulates an increase in 6:4 position sulfation ratio of monosulfated disaccharides (Schonherr et al 1993). In contrast TGFβ does not alter the 6:4 position sulfation ratio in monkey arterial SMCs (Schonherr et al 1993), although as noted above both growth factors cause elongation of GAG chains on VSMC PGs.

The most prominent sulfation positions on GAGs have different impacts on the biochemistry in relation to LDL binding. Sulfation at the 6 position is present on an exocyclic carbon and is thus more sterically accessible to an anionic binding site on the ApoB100 on LDL (Boren et al 1998). Sulfation at the 4 position produces an entity of apparently higher peak charge density compared with the 6-sulfated disaccharide as suggested by the increased migration on the charge-based FACE separation system (Ballinger et al 2004). There has been little work on the impact of GAG sulfation on LDL binding. Disaccharides derived from PGs from the atherosclerosis susceptible White Carneau pigeon have a higher 6:4 position sulfation ratio than the atherosclerosis-resistant Show Pigeon (Edwards and Wagner 1988) and display higher binding affinity for plasma LDL (Steele and Wagner 1987). This sulfation pattern of GAGs is typical of PGs at aortic atherosclerotic lesion prone-regions (Curwen and Smith 1977).

Nothing is known of the signaling pathways that control sulfation of GAGs. As PDGF stimulates an increase in the 6:4 position sulfation ratio it will be very interesting to determine if this response is sensitive or resistant to genistein —this will indicate whether or not the signaling pathway for two properties of GAG structure—elongation and sulfation —are both mediated via the novel genistein-resistant

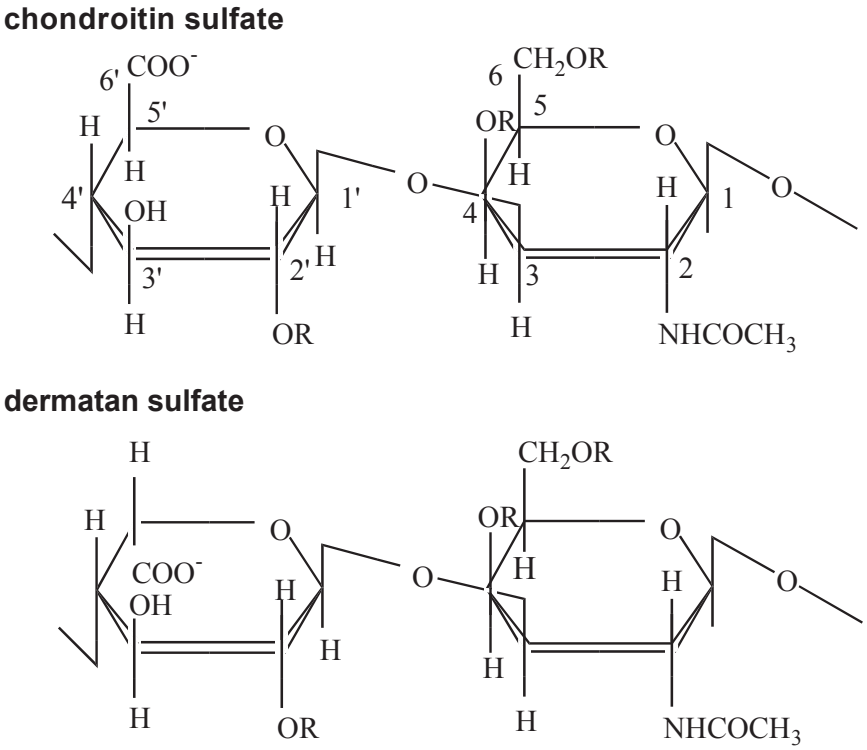


**Figure 3** A chondroitin sulfate disaccharide unit and a dermatan sulfate disaccharide unit showing theoretical sulfation positions R = $SO_3^-$.

PDGF pathway or that only the elongation pathway is novel (Figure 2). It will be interesting to determine if the action of PDGF, and any other factors which may affect sulfation, leads to enhanced LDL binding specifically due to a change in sulfation pattern. If both 6 position sulfation and GAG elongation turn out to enhance LDL binding and thus be potentially pro-atherogenic then developing an agent that inhibits both of these responses will be critical. If it evolves that both these responses are regulated through the same pathway then the potential therapeutic agent will be effective in blocking both atherogenic responses.

Few agents have been studied for their effects on sulfation pattern. The lipid-lowering fibrate, fenofibrate, does not affect the sulfation pattern of proteoglycans secreted by control or TGFβ-treated VSMCs (Nigro et al 2004) although this study did not examine the effect on PDGF, which in contrast to TGFβ actually increases the 6:4 position sulfation ratio. Several statin drugs inhibit radiosulfate incorporation into proteoglycans in primate vSMCs (Meyers et al 2003). Although the statin effect was observed in the presence of proliferating VSMCs and statins inhibit proliferation, the effect was observed when corrected for radiosulfate incorporation on a per cell basis (Meyers et al 2003). As noted above statins somewhat anomalously cause GAG chain elongation. PGs from statin-treated cells show reduced binding to LDL (Meyers et al 2003). In this situation, hyposulfation of the GAG chains appears to greatly contribute to the net outcome of decreased proteoglycan/LDL binding observed, over-riding the effect of GAG chain elongation. The effect of statins to inhibit GAG sulfation is intriguing and warrants further investigation.

## Conclusions

There is a great need and a large therapeutic window for the development of a vascular-directed agent that acts in concert with existing cardiovascular risk factor directed strategies to prevent atherosclerosis. Numerous potential mechanisms of action have emerged from the last several decades of research in vascular biology and pathology and these are being actively investigated in cell and animal systems. None has yet reached the clinic. Of the possible mechanisms one pathway is based on the role of vascular proteoglycans in the very early step of retaining lipid in the vessel wall. This retention occurs on the CS/DS GAG chains of proteoglycans and it has been demonstrated that changes in the GAG chains increase lipid binding in vitro. Modification of GAG chain synthesis and biochemical structure provides a pathway that may be drug targeted. The investigation and development of agents which modify GAG chain synthesis needs to be actively pursued in order to ascertain the extent of the potential to reduce atherosclerosis and cardiovascular disease.

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