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LIPOPROTEIN-MATRIX INTERACTIONS IN MACROVASCULAR DISEASE IN DIABETES

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1. ABSTRACT

The retention of atherogenic lipoproteins in the artery wall through their interactions with the arterial extracellular matrix is a critical step in the development of atherosclerosis, as outlined in the 'response to retention' hypothesis. Lipoprotein retention by vascular proteoglycans is thought to be the principle means of lipoprotein retention, although lipoprotein binding to other components of the extracellular matrix has been reported. The interactions of lipoproteins and proteoglycans can be direct through ionic interactions between the negatively charged glycosaminoglycan chains of proteoglycans and positively charged residues of apolipoproteins B and E, or can be mediated through bridging molecules such as lipoprotein lipase. Retention of atherogenic lipoproteins within the artery wall environment leads to pathophysiologically important modifications of the lipoproteins, including oxidation. Oxidation of lipoproteins leads to increased uptake by macrophages, leading to the formation of foam cells. This article reviews the scientific evidence in support of the response to retention hypothesis, with a specific focus on the effects of diabetes to modify lipoprotein retention.

2. INTRODUCTION

The importance of lipoprotein retention by extracellular matrix molecules in the development of atherosclerosis has been outlined in the 'response to retention' hypothesis (1). This article will review the scientific evidence that supports this hypothesis, and specifically will address the question of whether lipoprotein retention by matrix molecules is increased in diabetes. Since the major matrix molecules that have been implicated in the retention of lipoproteins are the proteoglycans, this article will focus on the interactions of lipoproteins with extracellular vascular proteoglycans, but also will evaluate the role of non-proteoglycan components of matrix in atherogenesis in diabetes.

2.1. Response to retention hypothesis

The response to retention hypothesis of early atherogenesis was described in 1995 as a comprehensive hypothesis for the early stages of atherosclerosis (1). The hypothesis states that the retention of atherogenic lipoproteins in the artery wall is a key initial step in the pathogenesis of the early atherosclerotic lesion. The basis of this hypothesis is that

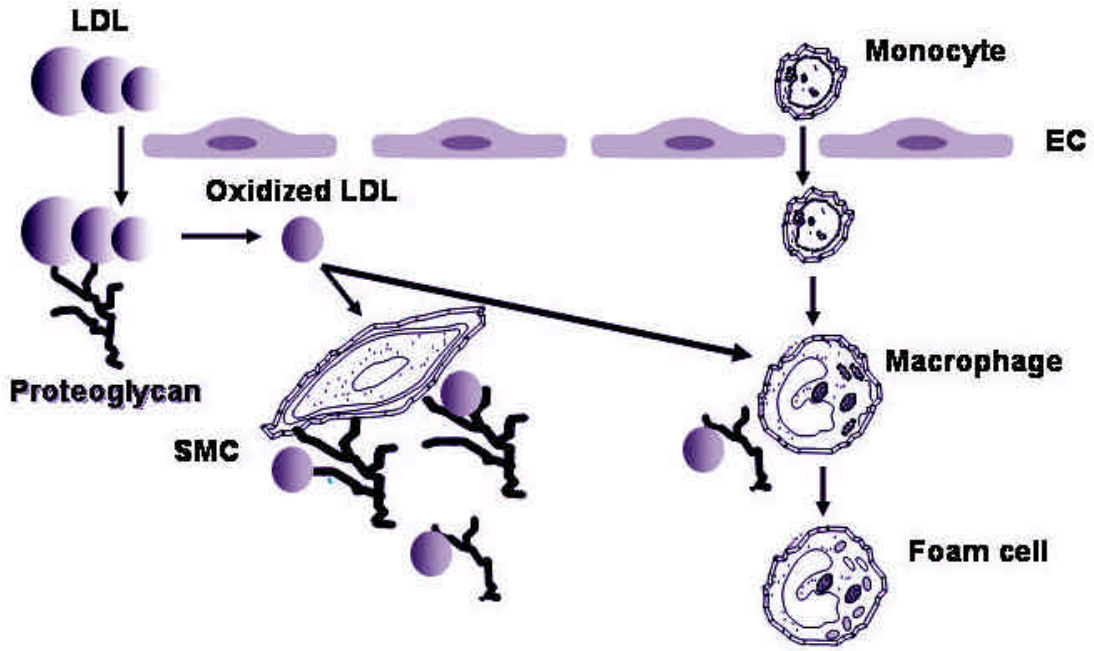

Figure 1. Interactions between lipoproteins and artery wall proteoglycans in atherosclerosis. LDL that crosses the endothelial barrier and enters the artery wall is bound by extracellular matrix proteoglycans, leading to its retention. LDL that is bound to proteoglycans is more susceptible to modification, such as oxidation. Oxidized LDL binds to other components of the extracellular matrix, and can stimulate modifications of the extracellular matrix. Oxidation of LDL also triggers expression of moncyte adhesion molecules and chemotactic factors leading to the recruitment of circulating monocytes into the artery wall. The monocytes then become activated and differentiate, and take up oxidized LDL leading to the formation of foam cells. Diabetes can affect this process at multiple levels.

atherogenic lipoproteins which enter the artery wall bind to, and are retained by, their interactions with vascular proteoglycans secreted by smooth muscle cells and other cell types. The interactions between lipoproteins and proteoglycans are ionic in nature, occurring between the negatively charged sulfate and carboxyl residues on the glycosaminoglycan side chains of proteoglycans and the positively charged residues of apolipoproteins B and E. The interactions can be direct, or mediated through bridging molecules such as lipoprotein lipase (2, 3). A key point of the response to retention hypothesis is that retention of lipoproteins within the microenvironment of the artery wall leads to pathophysiologically important lipoprotein modifications, such as oxidation. Oxidative modification of lipoproteins leads to increased uptake by macrophages, leading to the formation of foam cells (figure 1) (4). Lipoprotein retention in the artery wall matrix is characteristic of all stages of atherogenesis.

The critical role of lipoprotein retention by proteoglycans was recently demonstrated by Skalen *et al*, who generated transgenic mice expressing proteoglycan-binding-defective apo B, LDL receptor binding-defective apo B, or wild type control apo B. Apo B with defective binding to the LDL receptor was generated by inducing a point mutation resulting in a conformational change that disrupts LDL-receptor binding, but not proteoglycan binding. Proteoglycan binding-defective apo B was created by changing lysine 3363 to glutamic acid, which impairs the interaction of the molecule with proteoglycans but not with the LDL receptor. This group also generated two mouse strains carrying apo B defective in binding to both proteoglycans and to the LDL receptor. The transgenic mice were then fed an atherogenic diet (1.2% cholesterol, 0.5% cholic acid, 18% fat) for 20 weeks. The mice with proteoglycan-binding-defective LDL (regardless of whether LDL receptor binding was intact or defective) developed significantly less atherosclerosis than the other groups, which had LDL with intact proteoglycan binding properties (5). Thus, the critical role played by proteoglycans in mediating the retention of lipoproteins within the artery wall was clearly illustrated.

2.2. Composition of vascular extracellular matrix

The vascular extracellular matrix is a heterogeneous mixture of molecules that are capable of interacting with lipoproteins, leading to their retention in the artery wall. Extracellular matrix components include elastin, fibronectin, laminin, collagen, hyaluronan and various glycoproteins including proteoglycans. Proteoglycans are thought to be the component most responsible for lipoprotein retention. Proteoglycans are a heterogeneous group of molecules composed of a core protein to which one or more glycosaminoglycan side chains are attached. Individual proteoglycans are identified by their specific core protein and by the disaccharide composition of their glycosaminoglycan chains. Proteoglycan composition and distribution vary by tissue,

and correspondingly they have a variety of functions in different tissues. The major vascular smooth muscle cell derived proteoglycans are the large chondroitin sulfate proteoglycan versican, the small dermatan sulfate proteoglycans biglycan and decorin, and the heparan sulfate proteoglycan perlecan (6). Human monocyte-derived macrophages synthesize two forms of proteoglycans that bind LDL with high affinity: a large form with chondroitin sulfate/dermatan sulfate glycosaminoglycans, and a smaller form which contains chondroitin sulfate, dermatan sulfate, and heparan sulfate glycosaminoglycans. Both these secreted proteoglycans were identified as proteoglycan forms of the growth factor, macrophage colony-stimulating factor (PG-MCSF) (7). Proteoglycans also are synthesized by endothelial cells. The primary role of endothelial heparan sulfate proteoglycans is to anchor the dimeric (active) form of lipoprotein lipase to the cell surface (see section 6; reviewed in (8)).

3. LIPOPROTEIN DETERMINANTS OF RETENTION

3.1. Proteoglycan binding site of apo B100

Apo B100 is essentially the only protein component of LDL, and there is one molecule of apo B100 per LDL particle. Apo B100 is a very large monomeric protein (molecular weight approximately 550 kDa) that is not transferred between particles, and is highly insoluble in aqueous solution. Thus, apo B100 remains with a lipoprotein particle during its evolution from triglyceride (TG)-rich lipoprotein to LDL. The structure of apo B100 in LDL has been difficult to determine, but it is thought that the first 89% of the molecule wraps once around the LDL like a thick ribbon, meeting its origin at approximately residue 4050. The carboxy-terminal 11% is thought to stretch back into one hemisphere and cross the ribbon between residues 3000 and 3500 (9, 10). The principal proteoglycan-binding region of apo B-100 was identified as site B, a cluster of basic amino acids located at residues 3359-3369 (11). This is also the region that is critical for binding to the LDL receptor. Substitution of neutral amino acids in this site abolishes both the LDL receptor binding and proteoglycan binding activities (11). However, Boren *et al* were able to separately affect the LDL receptor and proteoglycan binding activities by introducing single point mutations. Insertion of a glutamic acid into the basic cluster RKR in site B (K3363E mutation) of apo B100 led to defective proteoglycan binding, but normal LDL receptor binding. In contrast, substitution of glutamine for arginine (R3500Q) disrupted LDL receptor binding but these LDL interacted normally with proteoglycans. Although this residue (3500) is outside the receptor-binding site (site B) it disrupts receptor binding by inducing a conformational change (12).

3.2. Proteoglycan binding site of apo B48

Apo B48 is the major form of apolipoprotein B in apo E deficient mice, a mouse model of severe atherosclerosis. Apo B48 is truncated at residue 2153, and thus contains the amino-terminal 48% of the apo B100 molecule. Thus apo B48 does not include site B, the major proteoglycan binding site of apo B100. However, in gene-targeted mice expressing only apo B48, or only apo B100, atherosclerosis developed to a similar extent given similarly high cholesterol levels (13). Further studies by Boren's group have identified site B-Ib (comprising residues 84-94) as the principal proteoglycan binding site of apo B48 (14). To determine the role of site B-Ib in the proteoglycan binding of apo B100, they also evaluated the proteoglycan binding affinity of an artificial particle, apo B80 (containing the amino-terminal 80% of apo B100), which contains both sites B and B-Ib. The proteoglycan binding affinity was greatest for apo B80, least for apo B48, and in between for apo B100. Thus, they propose that when both sites B and B-Ib are exposed (apo B80) the proteoglycan binding affinity of the LDL is increased. Apo B100 has a lower proteoglycan binding affinity due to obstruction or masking of site B-Ib by the carboxyl-terminal 11% of the apo B100 molecule, thereby only site B is available for proteoglycan interactions. The binding affinity of apo B100 is greater than that of apo B48. However, the binding of apo E-containing apo B48 particles is greater than apo E depleted apo B48 particles, suggesting that apo E on apo B48 particles can act cooperatively to increase the binding of apo B48 to proteoglycans (14). The presence of a proteoglycan binding site in apo B48 accounts for the equal atherosclerosis seen in apo B48-only and apo B100-only expressing mice.

3.3. Proteoglycan binding site of apo E

Apo E is a 34 kDa apolipoprotein which is a component of several classes of lipoproteins including triglyceride (TG)-rich lipoproteins and their remnants, and HDL. Apo E has several variants due to a genetically determined polymorphism at a single gene locus, resulting in the expression of 6 phenotypes: E4/E4, E3/E3, E2/E2, E4/E3, E4/E2, E3/E2. The E3 form is the most common. The structures of the different variants have been determined. Analysis of delipidated apo E demonstrates two potential proteoglycan binding sites, located at residues 142-147 and between residues 243-272. The lipoprotein receptor binding domain of apo E is in the vicinity of residues 140-160. Thus, in apo E, as with apo B100, the LDL receptor binding and proteoglycan binding regions overlap. Biochemical analysis of apo E complexed to phospholipids or on the surface of a lipoprotein particle demonstrates that only the first binding site, residues 142-147 are available to interact with proteoglycans (15). Thus the conformation of apo E complexed to lipids is such that the second potential proteoglycan binding site is masked. The conformation of apo E may also depend on the particle size, as studies have indicated that TG-rich lipoproteins from hypertriglyceridemic subjects can bind to the LDL receptor via apo E whereas TG-rich lipoproteins from normotriglyceridemic subjects cannot (16). Thus, the size of the lipoprotein particle associated with the apo E might affect the conformation of the apo E molecule, which subsequently could alter its ability to interact with the LDL receptor or proteoglycans.

Apo E also was shown to mediate the binding of HDL to proteoglycans, in that HDL containing apo E, but not apo E-free HDL, was able to bind to the purified proteoglycan, biglycan (17). This binding was reduced by

reducing the positive charges on lysine and arginine residues of apo E, confirming the ionic nature of the interactions between apo E and proteoglycans. TG-rich lipoproteins contain both apo B and apo E. Apo E enrichment of TG-rich particles could lead to increased proteoglycan binding affinity if the apo E molecules acted cooperatively with the apo B molecule to increase proteoglycan binding affinity. Several groups have reported increased apo E content of TG-rich lipoproteins in diabetes (18-20), although we and others have not confirmed this finding (21-23). When we examined TG-rich lipoproteins isolated from individuals with type 2 diabetes or age-matched control subjects we did not detect any differences in binding affinities for biglycan, or for the LDL receptor (23). Thus the role played by apo E in mediating binding of apo B-containing lipoproteins to proteoglycans is unclear. Apo E may be anti-atherogenic by leading to vascular wall retention of HDL, thereby blocking a binding site that could otherwise be occupied by the more atherogenic LDL. Alternately, apo E-mediated retention of HDL, or of TG-rich lipoproteins, could be pro-atherogenic, by contributing to lipid accumulation in the artery wall. The conformation of apo E in its association with different lipid particles appears to affect its ability to interact with proteoglycans, or with the LDL-receptor. Thus, whether the interaction of apo E-containing particles with artery wall proteoglycans is pro- atherogenic or anti-atherogenic may depend on the lipoprotein particle associated with the apo E, and on the level and conformation of apo E.

3.4. Post-binding structural modifications can further increase binding to proteoglycans

Although eight separate glycosaminoglycan binding sites have been identified in apo B100, it appears that only site B is functional when the apo B is incorporated into a lipoprotein particle (14). However, the initial interaction of LDL with proteoglycans may induce structural conformations that can unmask other binding sites and contribute to the retention of the particle in the extracellular matrix. For example, proteolysis of apo B100 increases the binding of LDL to proteoglycans, presumably by unveiling other binding sites in the fragmented apo B100 molecule. However, this was only true if proteolysis of apo B100 followed proteoglycan binding. Studies subjecting LDL particles to proteolysis in solution first, then evaluating proteoglycan interactions did not show increased binding (24). Thus, the pattern of proteolytic cleavage of apo B100 must depend on whether the LDL particles are proteoglycan bound or free. Fusion of proteolysed LDL particles further increased their binding (24). This is likely mediated by the increased number of apo B100 fragments available for proteoglycan interactions in the fused LDL particles (25). Fusion of LDL particles in the absence of proteolysis also increases the strength of proteoglycan binding (24). Similarly, binding of LDL to extracellular matrix was increased in the presence of lipoprotein lipase (LPL) and sphingomyelinase, which led to aggregation of the LDL particles (26). *In vivo*, proteolysis and fusion of LDL particles likely occur simultaneously, thus increasing the strength of the association of the lipid retention in the artery wall, even after the initial binding step.

3.5. Lipoprotein-proteoglycan interactions are charge mediated

Several groups have demonstrated that lipoprotein modifications that decrease the positive charges on apo B or apo E lead to decreased proteoglycan binding. For example, oxidation of LDL, which decreases the positive charges on the apo B100, leads to decreased proteoglycan binding. Extensively oxidized LDL does not demonstrate any proteoglycan binding (3, 27). Similarly, LDL treated with malondialdehyde, which mimics modification of lysine residues during LDL oxidation, also prevents proteoglycan binding (27). Glycation of lipoproteins similarly leads to decreased positive charges due to formation of lysine adducts. All classes of lipoproteins have increased amounts of glycation in diabetes compared to controls (28). Studies examining the binding of apo E to heparin demonstrate decreased binding for glycated apo E compared to unmodified apo E, and this was true for apo E glycation *in vivo* or *in vitro* (29). However, one study reports that LDL isolated from streptozotocin-induced diabetic monkeys had greater binding affinity for proteoglycans isolated from thoracic aortas than did LDL isolated from nondiabetic monkeys (30), which is counter to what is expected based on the above considerations. Glycation has been shown to decrease the interactions of LDL with the LDL receptor (31), and to decrease the affinity of apo E for proteoglycans, so the mechanism leading to increased proteoglycan binding for the LDL isolated from diabetic monkeys is unclear. It is possible that this increased binding was mediated through conformational modification of glycated apo B, or possibly the binding interactions were mediated via LPL.

3.6. Retention of oxidized LDL

Oxidation of LDL has been shown to decrease its retention by purified proteoglycans (3, 27). However, oxidized LDL or oxidation specific epitopes have been identified in atherosclerotic plaques (32-34). Oxidized LDL has been shown to bind to complex extracellular matrices synthesized by a variety of cell types including vascular smooth muscle cells (35), macrophages (36), and mesangial cells (37, 38). These extracellular matrices are a heterogeneous mixture of components including collagen, elastin, fibronectin, laminin, proteoglycans, hyaluronan, and glycoproteins. Oxidized LDL binds matrices to a greater extent than does native LDL, and this binding does not appear to be mediated via proteoglycan components of the matrices (35). However, oxidized LDL is known to bind to proteoglycans via a bridging molecule, such as lipoprotein lipase (LPL) (3, 36, 39). Binding of oxidized LDL to extracellular matrices via LPL is at least partially dependent on proteoglycans, in that digestion of glycosaminoglycan chains decreases the binding of oxidized LDL (36). The nonproteoglycan components of the extracellular matrix that mediate the binding of oxidized LDL are not known, and may be mediated by a variety of matrix components. For example, digestion of the matrix with pronase or with trypsin markedly reduced the binding of both native and oxidized LDL (35).

3.7. Retention of Lipoprotein (a)

Lipoprotein (a) [Lp(a)] is a lipoprotein very similar to LDL in terms of its cholesterol and phospholipid content. Lp(a) also contains apo B-100, but in addition contains apolipoprotein (a), a glycoprotein that resembles

plasminogen. Elevated levels of Lp(a) are linked to increased risk of heart disease by epidemiological evidence (40, 41). The mechanism by which Lp(a) leads to cardiovascular disease is not clear, and Lp(a) has been identified as an emerging risk factor by the National Cholesterol Education Panel guidelines (42). One mechanism by which Lp(a) may lead to increased cardiovascular disease is by inhibiting plasmin formation and clot lysis by competing with plasminogen for binding at sites of fibrin deposition. However, Lp(a) does bind to components of the extracellular matrix, and thus accumulation of Lp(a) may function similarly to LDL by leading to cholesterol deposition and foam cell formation. The binding interactions between Lp(a) and extracellular matrix molecules appear to be mediated via ionic interactions with glycosaminoglycan chains on proteoglycans (43, 44). However, hydrophobic interactions between apo (a) and the decorin core protein also have been reported (43).

3.8. Lipoprotein abnormalities in diabetes

Type 2 diabetes is characterized by a dyslipidemia with increased triglyceride and low HDL cholesterol levels. The lipoprotein profile in type 2 diabetes thus consists of elevated levels of very low density lipoprotein (VLDL) and chylomicrons and their remnants. The absolute concentrations of LDL cholesterol are similar, or perhaps even lower than non-diabetic subjects (45). However, the LDL in subjects with diabetes is more likely to be of the more atherogenic small dense phenotype. Type 2 diabetes is associated with increased production and decreased clearance of triglycerides and apo B, resulting in excess numbers of VLDL particles. Similarly, there is increased production of LDL particles, and these LDL are more likely to be glycated (28) and/or oxidized. Several studies have demonstrated an association between small dense LDL with increased atherosclerosis (46-50), and small dense LDL has been hypothesized to have greater binding affinity for proteoglycans compared to large buoyant LDL (51, 52). Our laboratory evaluated the effect of lipoprotein size and density on binding to biglycan in vitro, and demonstrated that within an individual, the small dense LDL had greater binding affinity for biglycan than the large buoyant LDL (53). In comparisons of different lipoprotein particles, LDL had the greatest binding to biglycan, followed by intermediate density lipoprotein (IDL) and VLDL (53). Thus, although glycation and/or oxidation of LDL appear to decrease the proteoglycan binding affinity, the increased atherosclerosis in diabetes may be more attributable to increased binding affinity conferred by excess small dense LDL. Alternately, increased oxidation of lipoproteins in diabetes may lead to increased binding to non-proteoglycan extracellular matrix components (see below).

4 PROTEOGLYCAN DETERMINANTS OF LIPOPROTEIN RETENTION

4.1. Lipoprotein-proteoglycan interactions are mediated via glycosaminoglycan chains

The binding of lipoproteins to proteoglycans is affected by modifications of either component. The binding affinity of proteoglycans for lipoproteins appears to relate to the length and extent of sulfation of the glycosaminoglycan chains of the various proteoglycans. For example, glycosaminoglycans extracted from atherosclerosis-susceptible human arteries are longer than and demonstrate greater LDL retention properties compared to glycosaminoglycans isolated from atherosclerosis-resistant arteries (54). Similarly, Camejo *et al* have reported that proteoglycans synthesized by proliferating cells have longer glycosaminoglycan chains and increased LDL binding affinity compared to proteoglycans synthesized by quiescent cells (55). Our group has shown that proteoglycans synthesized by vascular smooth muscle cells exposed to oxidized LDL (56), or to transforming growth factor beta (TGF-beta) (57), have longer glycosaminoglycan chains and increased LDL binding affinity compared to proteoglycans synthesized by control cells. The binding affinity of proteoglycans synthesized by vascular smooth muscle cells in culture is approximately 5.5×10^{-8} M LDL(58). In addition, it has been reported that the sulfate content of glycosaminoglycans affects lipoprotein retention, with increased sulfation (and thus increased negative charge) of glycosaminoglycans conferring increased LDL retention (59). Conversely, proteoglycans synthesized under conditions leading to shorter glycosaminoglycan chains have reduced LDL binding affinity compared to control proteoglycans (58, 60). For example, vascular smooth muscle cells exposed to glucosamine synthesize proteoglycans that are smaller, with less sulfate incorporation than control proteoglycans, and had lower binding affinity than control proteoglycans (58). Vascular smooth muscle cells exposed to calcium channel blockers amlodipine or nifedipine, had decreased synthesis of proteoglycans, and the proteoglycans synthesized were smaller, with less sulfate incorporation, and had less LDL binding than proteoglycans synthesized by unstimulated cells (60). Similarly, vascular smooth muscle cells exposed to troglitazone (a thiazolidinedione compound formerly used in the treatment of diabetes and insulin resistance) had decreased LDL binding affinity (Tannock and Chait, unpublished observations). Thus, the current understanding of proteoglycan-lipoprotein interactions is that the binding affinity of glycosaminoglycans for LDL is related in large part to their length and degree of sulfation.

4.2. Proteoglycan core protein-mediated lipoprotein binding

Although most studies indicate that the binding interactions between lipoproteins and proteoglycans are mediated through the glycosaminoglycan side chains of proteoglycans, it has been suggested that the binding of lipoproteins to proteoglycans may be mediated, in part, via the core proteins of proteoglycans. Evaluation of the interactions between apo E and biglycan suggested that binding is mediated by a charge interaction between a region in the C-terminal domain of apo E and the core protein of biglycan, independent of glycosaminoglycans (61). The same group also reported that apo (a), an apolipoprotein molecule associated with Lp(a), also binds to the core protein of decorin. However, the binding of intact Lp(a) to decorin involved both apo B100-glycosaminoglycan and apo (a)-decorin core protein interactions (43). In a series of studies our group has demonstrated that native human LDL has high binding affinity for purified proteoglycans, but a lower binding affinity to glycosaminoglycan chains cleaved from proteoglycans, or to

artificial glycosaminoglycans (glycosaminoglycans synthesized on artificial acceptor molecules and thus never associated with core proteins) (57, 58). However, we were unable to demonstrate binding of LDL to the core proteins of proteoglycans cleaved of glycosaminoglycan chains (57). Thus, the interactions of lipoproteins with proteoglycans are mediated through ionic interactions between apolipoproteins B and/or E with glycosaminoglycan chains of proteoglycans, but interactions of lipoproteins with the core proteins of proteoglycans may also contribute to lipoprotein retention.

4.3. Proteoglycan distribution in atherosclerosis

Several studies have demonstrated an altered distribution of proteoglycans in atherosclerosis-susceptible arteries. In general, atherosclerotic lesions demonstrate a relative increase in chondroitin sulfate (eg. versican) and dermatan sulfate proteoglycans (eg. biglycan and decorin), with a relative decrease in amount of heparan sulfate proteoglycans (eg. perlecan) (62-65). This relative increase in chondroitin sulfate and dermatan sulfate proteoglycans and relative decrease in heparan sulfate proteoglycans is exaggerated in atherosclerotic arteries obtained from individuals with diabetes (66, 67). The relative distribution of proteoglycans in atherosclerosis appears to be dynamic: examination of aorta proteoglycans from monkeys with diet-induced atherosclerosis demonstrated decreased aortic heparan sulfate proteoglycans with increased severity of lesions, yet aortic heparan sulfate content increased with regression of atherosclerotic lesions. Aortic chondroitin sulfate proteoglycans followed an opposite trend, that is increased aortic chondroitin sulfate with increased lesion severity, and decreased aortic chondroitin sulfate with lesion regression (68). The distribution of proteoglycans also differs within the atherosclerotic lesion suggesting that different proteoglycans could have different roles in atherogenesis (69, 70). For example, biglycan, a small dermatan sulfate proteoglycan, demonstrated striking colocalization with apolipoproteins B and E within atherosclerotic lesions, whereas no such colocalization was found with versican, a large chondroitin sulfate proteoglycan (71). Furthermore, Cardoso and Mourao compared the LDL binding affinity of proteoglycans isolated from normal arteries of young adults who died from accidental causes, and found that LDL binding affinity of the isolated proteoglycans reflected the relative susceptibility of the respective artery to developing atherosclerosis. The total concentration of proteoglycans and collagen was similar between the different arteries, but the LDL binding affinity was greatest for proteoglycans extracted from the abdominal aorta, and least for proteoglycans extracted from the pulmonary artery, reflective of the relative susceptibility to atherosclerosis (54). Thus, alterations in the relative synthesis and distribution of proteoglycan species within atherosclerotic susceptible areas can affect the retention of lipoproteins.

4.4. Proteoglycan synthesis

Proteoglycans are synthesized by a variety of cell types; however, vascular proteoglycans are mainly made by arterial smooth muscle cells. Proteoglycan synthesis is differentially regulated at the transcriptional, posttranscriptional and posttranslational levels (72). Glycosaminoglycans are linear polysaccharides of N-acetylglucosamine, N-sulfonylglucosamine or N-acetylgalactosamine residues alternating with glucuronic acid, iduronic acid or galactose residues in glycosidic linkage. The proteoglycan classes are determined by the specific disaccharide repeat patterns of the glycosaminoglycan chains. The glycosaminoglycan chains are covalently attached to a core protein at the amino acid serine via an O-glycosidic linkage. In general, a particular core protein has predominantly one type of glycosaminoglycan chain (72). Proteoglycan core protein expression and glycosaminoglycan structure are subject to different, and sometimes independent regulation. For example, vascular smooth muscle cells exposed to TGF-beta increase their steady state mRNA levels for biglycan and versican core proteins, but do not change their mRNA levels for decorin. However, all three types of proteoglycans are larger due to the synthesis of longer glycosaminoglycan chains (73, 74). Oxidized LDL preferentially increases the synthesis of the biglycan core protein, but causes non-specific glycosaminoglycan chain elongation for versican, biglycan and decorin (56). Vascular smooth muscle cells exposed to platelet derived growth factor (PDGF) specifically increase versican mRNA and core protein synthesis, but also synthesize longer glycosaminoglycan chains on all proteoglycans (73). Protein kinase C activation using 12-O-tetradeconoylphorbol-13-acetate (TPA) can mimic the effect of PDGF on glycosaminoglycan chains, but not the effect on versican expression (75). However, the signaling pathways involved in regulation of core protein expression, or of glycosaminoglycan synthesis have not been identified.

4.5. Effect of diabetes on proteoglycan synthesis and LDL binding affinity

Metabolic abnormalities in diabetes include high glucose and insulin levels, high levels of free fatty acids, and increased oxidative stress. The effect of high glucose levels on proteoglycan synthesis is not clear. We were not able to demonstrate any effect of high glucose levels on the synthesis of proteoglycans by vascular smooth muscle cells, either in the quiescent state (58) or after TGF-beta stimulation (57). Mesangial cells synthesize proteoglycans that are very similar to those synthesized by vascular smooth muscle cells (6, 76). Several groups have reported that high glucose levels lead to increased proteoglycan synthesis by mesangial cells (77-79), an effect mediated through the hexosamine pathway leading to increased autocrine induction of TGF-beta (80-82). However, other groups have found decreased mesangial cell proteoglycan synthesis in response to high glucose levels (83, 84). Potential differences in the response of vascular smooth muscle cells and mesangial cells to high glucose levels include different rates of glucose uptake and utilization, and different signaling pathways. *In vivo*, studies have demonstrated an exaggerated increase in chondroitin sulfate and dermatan sulfate proteoglycans, and an exaggerated decrease in heparan sulfate proteoglycans in atherosclerotic lesions from subjects with diabetes compared to lesions from nondiabetic subjects (66, 67). Our laboratory previously has demonstrated that versican has greater LDL binding affinity than biglycan (3), and that both versican and biglycan have greater LDL binding affinity than heparan sulfate proteoglycans (Lewis and Chait,

unpublished observations). Thus, the exaggerated increase in chondroitin and dermatan sulfate proteoglycans in diabetes may lead to greater LDL retention in the artery wall, and contribute to the increased atherosclerosis in diabetes. The factors leading to this altered distribution have not been identified.

High insulin levels are common in diabetes. Insulin stimulation of pig (85), or human (86) vascular smooth muscle cells was shown to stimulate the synthesis of both secreted and cell-associated proteoglycans. However, the glycosaminoglycan chains synthesized by pig cells exposed to insulin were shorter than those synthesized by unexposed cells (85). LDL binding affinity was not assessed in either of these studies, but would be expected to be decreased compared to control proteoglycans given the smaller glycosaminoglycan chains. However, the increased synthesis of proteoglycans induced by insulin may be proatherogenic by simple increased mass of artery wall proteoglycans available to interact with lipoproteins.

Diabetes is associated with high levels of free fatty acids. Stimulation of human aortic smooth muscle cells *in vitro* with moderate to high levels of linoleic acid, a polyunsaturated fatty acid, caused increased mRNA levels for the core proteins of the proteoglycans versican, decorin, and syndecan 4 but not fibronectin or perlecan, compared with control cells. Linoleic acid stimulation also resulted in increased glycosaminoglycan chain length for decorin (87).

However, treatment of diabetes with thiazolidinediones may protect against atherosclerosis, by decreasing the LDL binding affinity of proteoglycans. Thiazolidinediones are a class of medications used in the treatment of diabetes due to their ability to increase insulin sensitivity. Their mechanism of action is not fully understood, but they are ligands for peroxisome proliferator-activated receptors (PPAR)-gamma. Olsson *et al* demonstrated that the induction of the decorin gene by linoleic acid was inhibited by co-stimulation of the cells with darglitazone, a thiazolidinedione member (87). Our group has demonstrated that vascular proteoglycans synthesized in the presence of troglitazone have decreased LDL binding affinity, related to the effect of troglitazone to decrease the size and sulfate incorporation of nascent proteoglycans (Tannock and Chait, unpublished observations). Animal models of atherosclerosis fed troglitazone, or rosiglitazone, have demonstrated decreased early atherosclerosis, possibly due to decreased lipoprotein retention by modified artery wall proteoglycans (88-90). In renal studies, troglitazone was shown to block mesangial expansion and inhibit development of diabetic nephropathy (91). Further studies are required to determine if thiazolidinedione therapy of diabetes will confer protection against development and/or progression of atherosclerosis.

5. NONPROTEOGLYCAN DETERMINANTS OF LIPOPROTEIN RETENTION

5.1. Lipoprotein lipase

Lipoprotein lipase (LPL) is a key enzyme involved in hydrolyzing triglycerides and liberating free fatty acids in the plasma. LPL also plays a role as a "bridging" molecule as it has domains that can interact with lipoproteins and proteoglycans. For example, LPL is involved in binding TG-rich lipoproteins to hepatic heparan sulfate proteoglycans aiding non-receptor-dependent removal of lipoproteins by hepatocytes (92, 93). Immunohistochemisty demonstrates LPL staining in the arterial intima in association with intimal cells and with extracellular matrix (94, 95). Oxidized LDL binds very poorly to proteoglycans in the absence of LPL (35, 56), yet oxidized LDL is present in the extracellular matrix of atherosclerotic lesions (96). Several groups have identified the importance of LPL in mediating the binding of both native and oxidized LDL to extracellular matrix (3, 26, 36, 39). Thus LPL may have a pro-atherogenic role by enabling the binding of oxidized LDL to vascular proteoglycans, leading to the accumulation of oxidized LDL in the artery wall (2, 97).

5.2. Collagen

Collagens, a family of highly characteristic fibrous proteins, are the major proteins of the extracellular matrix (98). Collagens are characterized by a stiff triple-stranded helical structure, and can aggregate into larger bundles termed collagen fibers which measure several micrometers in diameter. The normal artery wall contains various types of collagen, with types I and III predominating. The amount of collagen is increased in atherosclerotic lesions compared to normal artery wall (98). Several studies have suggested an association between LDL and collagen in the arterial intima (99-101). Although LDL does appear to have weak binding to collagen *in vitro* (102-105), the binding of LDL is increased significantly in the presence of bridging molecules such as LPL (95, 106, 107). Modification of either LDL or of collagen can affect their interactions. For example, collagen has greater binding to oxLDL than to native LDL (108). Advanced nonenzymatic glycosylation of collagen led to increased LDL binding, and the authors suggest that the increased atherosclerosis in diabetes may in part be related to increased LDL trapping by advanced glycosylation endproducts caused by hyperglycemia in diabetes (109).

5.3. Elastin

The artery wall undergoes repeated cycles of extension and recoil with each cardiac contraction. Elastic fibers are important components of the artery wall due to their ability to extend and recoil without damage. The combination of tensile strength from collagen, and elastic function from elastin allows the artery wall to function. However, loss of elasticity is a component of atherosclerosis, and is due to degradation of artery wall elastin. Elastin is a highly hydrophobic, nonglycosylated protein rich in proline and glycine. Elastin fibers in the extracellular space form extensive cross links via lysine residues. Elastic fibers also contain a microfibril glycoprotein on their surface. Lipid deposition adjacent to, and within, elastin fibers has been documented (110, 111). Several groups have reported that elastin is capable of interactions with lipoproteins (112, 113). Podet *et al* reported high affinity interactions between human LDL and human aortic elastin with a binding constant of 3.6×10^{-8} M (114). This value is very similar to binding constants

obtained by our laboratory for the interactions of LDL with proteoglycans (3, 23, 58). Elastin obtained from atherosclerotic lesions was reported to have increased binding capacity, but decreased binding affinity, for LDL (114, 115). Although it is not clear if LDL would have access to elastin fibers in the healthy artery wall, degradation of elastin occurs during atherogenesis. Furthermore, vascular smooth muscle cells exposed to LDL have increased synthesis of elastase (116), which may increase the exposure of elastin Thus, retention of LDL by interactions with artery wall elastin is feasible; however, further studies to elicit the mechanism and physiological sequelae of binding are required.

5.4. Fibronectin and Laminin

Fibronectin and laminin are important constituents of the extracellular matrix that play key roles in mediating the attachment of cells to the underlying matrix. Fibronectin exists in three forms of differing solubility, ranging from highly soluble plasma fibronectin, to highly insoluble matrix fibronectin. Fibronectin is a dimer that is folded into a series of globular domains with different functions. For example, domains can bind collagen, heparin, and cell surface receptors. Laminin is a large complex of 3 long polypeptide chains arranged into a number of functional domains. Like fibronectin, laminin is capable of binding collagen, heparan sulfate proteoglycans, and cell surface receptors. *In vitro* studies have revealed very poor binding of LDL or oxidized LDL to either laminin or fibronectin (105). However, Lp(a) has been reported to bind to fibronectin via bridging peptide molecules, defensins, which are released by activated neutrophils (117). Another role for fibronectin in the extracellular matrix is to bind glycosaminoglycan complexes, leading to increased macrophage uptake of LDL-heparin-collagen complexes (118). Thus, interactions with proteoglycan and/or nonproteoglycan components of matrix can lead to the retention of atherogenic lipoproteins within the artery wall. There are many reasons to speculate that these interactions are increased in diabetes, contributing to the increased atherosclerosis seen in individuals with diabetes.

5.5. Effect of diabetes on the synthesis of nonproteoglycan components of matrix

Several studies have demonstrated structural differences in extracellular matrix between arteries from diabetic and nondiabetic subjects. For example, the concentration of type IV collagen was increased in both plaque and non-plaque areas from aortas from diabetic subjects compared to controls (119). In tissue culture studies media containing diabetic serum stimulated the synthesis of procollagen type I and of fibronectin; however, these effects were not mimicked by increasing the glucose and/or insulin content of normal serum, thus the stimulating factor is not known (120). Similarly, in studies of other cell types in culture, high glucose was shown to lead to increased synthesis of fibronectin and collagen type III by renal fibroblasts (121), and to increased laminin synthesis by rat endothelial cells (122). In addition, extracellular matrix components are susceptible to modifications such as glycation. In studies of diabetic rats it was found that aortic collagen had much higher content of advanced glycation end products than did tendon collagen (123). Glycation of collagen was shown to increase LDL binding (109). Glycation of other matrix molecules may similarly increase their retention of LDL, although this has not yet been proven. However, increased synthesis of extracellular matrix components in diabetes, or increased modifications such as glycation, likely affect the relative interactions of lipoproteins with matrix components.

6. SEQUELAE OF LIPOPROTEIN RETENTION

Lipoproteins that have been bound by the extracellular matrix have increased retention time within the intima. LDL that is bound to proteoglycans are more susceptible to modification, including oxidation, proteolysis, or fusion; all of which may further increase the strength of the binding (as reviewed in section 2.4). Modification of the bound lipoproteins, for example by oxidation, or digestion by enzymes such as secretory phospholipase A2 and sphingomyelinase, can release biologically active lipids such as oxidized phospholipids, oxysterols, free fatty acids, and ceramide. These biologically active lipids can be ingested by cells, and may induce further, proatherogenic changes in the extracellular matrix. For example, our laboratory recently identified that lysophosphatidylcholine, lysophosphatidic acid, and 7-ketocholesterol all are capable of stimulating the vascular smooth muscle cells to synthesize proteoglycans with longer glycosaminoglycan chains and enhanced lipoprotein binding properties (124). Thus, release of these biologically active lipids can perpetuate the development of lipoprotein binding-avid matrix. Furthermore, these biologically active lipids may trigger inflammatory reactions, or be cytotoxic (as reviewed in (8)).

Atherosclerotic plaques are characterized by foam cell deposits. Both macrophages and arterial smooth muscle cells have been shown to bind and internalize lipoproteins, leading to the formation of foam cells. *In vitro* studies have demonstrated that macrophages bind and internalize proteoglycan bound-LDL more efficiently than native LDL (4). As described earlier, LDL that has bound to proteoglycans or matrix often aggregates. Sakr *et al* demonstrate that the binding and uptake of aggregated lipoproteins requires cytoskeletal-mediated processes. These authors postulate a series of sequential events; first a prolonged cell-surface contact between the macrophage cell surface and the retained lipoproteins, leading to partial selective cholesteryl ester uptake and hydrolysis, internalization and degradation of lipoproteins, stimulation of acyl-CoA:cholesterol acyltransferase (ACAT) leading to accumulation of intracellular cholesteryl ester and foam cell formation (125). Kaplan and Aviram report that macrophages had higher uptake of extracellular matrix bound oxidized LDL than of extracellular matrix bound native LDL (126). Lipoprotein lipase was required for this cellular uptake. Additionally, they demonstrate that extracellular matrix glycosaminoglycans bound to the lipoproteins are internalized along with the lipoproteins. Macrophages that have taken up lipoproteins then release more LPL (127, 128), and can stimulate the smooth muscle

cells to migrate and transform from the contractile state to the proliferative state (129). Macrophages then secrete other biologically active molecules such as cytokines, growth factors and pro-oxidants, which can amplify early events in the developing atherosclerotic lesion, leading to progression of atherogenesis, plaque instability and acute clinical events (as reviewed in (130)).

7. PERSPECTIVE

The retention of atherogenic lipoproteins by artery wall proteoglycans and extracellular matrix leads to a series of events that initiate the development of the atherosclerotic plaque. Lipoproteins that have been bound in the artery wall are more susceptible to oxidative modification and aggregation. Modified lipoproteins are avidly taken up by macrophages and smooth muscle cells leading to the formation of foam cells, a hallmark of the early atherosclerotic lesion. Diabetes is characterized by premature and extensive atherosclerosis that is multifactorial in etiology. Diabetes can lead to increased atherosclerosis at several steps in the response to retention hypothesis: in diabetes there is an exaggeration of the altered proteoglycan distribution between atherosclerotic and lesion-free areas; there is altered synthesis of both proteoglycan and non-proteoglycan components of the extracellular matrix, there is increased amounts of atherogenic lipoprotein particles to interact with arterial proteoglycans and matrix, and there is increased oxidative stress that can modify lipoproteins affecting both their interactions with the extracellular matix and their susceptibility to cellular uptake. The mechanisms and pathways by which diabetes alters the extracellular matrix composition and structure have not been fully identified, but likely injurious factors include hyperglycemia, dyslipidemia, increased oxidative stress, excess free fatty acid flux, and downstream pathways induced by these factors. Thus, the metabolic milieu of diabetes can contribute via multiple pathways to the excess of cardiovascular disease observed in this population.

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