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Transforming growth factor- β regulation of proteoglycan synthesis in
vascular smooth muscle:
contribution to lipid binding and accelerated atherosclerosis in diabetes.

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Abstract

Atherosclerosis is accelerated in the setting of diabetes but the factors driving this phenomenon remain elusive. Hyperglycemia leads to elevated levels of transforming growth factor- β (TGF- β) and TGF- β has been implicated as a factor in atherosclerosis. Given the established association between hyperglycemia and elevated TGF- β it is plausible that elevated TGF- β levels in diabetes play a pathogenic role in the development of accelerated atherosclerosis in diabetes. TGF- β is a potent regulator of extracellular matrix synthesis, including many actions on proteoglycan synthesis which lead to increased binding to LDL and therefore potentially increased lipid retention in the vessel wall and accelerated atherosclerosis. TGF- β signals through the canonical TGF- β receptor I (T β RI/Alk V) mediated phosphorylation of Smad transcription factors and is also known to involve, positively and negatively, interactions with the Mitogen Activated Protein (MAP) kinase pathways. This review will focus on the effects of TGF- β on proteoglycan synthesis in vascular smooth muscle and particularly the signaling pathways through which TGF- β exerts its effects because those pathways may be therapeutic targets for the prevention of pathological modifications in the proteoglycan component of the vessel wall in the vascular diseases of diabetes.

Introduction

Cardiovascular disease is the largest single cause of premature mortality in developed nations ¹. World-wide increases in obesity, metabolic syndrome and diabetes are driving higher rates of cardiovascular disease ^{2,3}. Individuals with diabetes have numerous acute issues such as hypoglycemic events however the main impact of diabetes on health is through the development of its microvascular (retinopathy, nephropathy, neuropathy and impotency) and macrovascular (myocardial infarction, stroke) complications ^{4,5}. The major manifestation of cardiovascular disease or coronary artery disease is an acute myocardial infarction leading to death or subsequently heart failure. Atherosclerosis, the underlying pathology of macrovascular disease, is accelerated by the plasma milieu and other factors associated with diabetes resulting in higher levels of cardiovascular disease in this group ^{6,7}. Acute ischemic events have a more profound effect in people with diabetes because of the more pervasive vascular disease and the higher susceptibility of the myocardium to the effects of ischemia ^{8,9}. The underlying pathology is atherosclerosis, the slow accumulation of cholesterol in the sub-endothelium of the artery wall and the development over several decades of complex lesions termed plaques ^{7,10-14}. Some plaques are labile and the rupture of such so-called vulnerable plaques leads to the adverse clinical event ^{15,16}. The extended risk factors include cigarette smoking, hypertension, hyperlipidemia and hyperglycemia and non-modifiable factors such as age and genetic predisposition ¹⁷. Medical therapies are based on lowering risk factors and anti-hypertensives and anti-hyperlipidemia agents are quite efficacious ^{18,19}. The role of glucose and hyperglycemia in driving accelerated atherosclerosis and increased rates of CVD are well established both epidemiologically ²⁰ and investigationally such as the recent study of Intravascular

Ultrasound (IVUS) of plaque progression in people with diabetes ²¹. However, in contrast to the established efficacy of agents which lower plasma cholesterol and blood pressure the role of anti-hyperglycemic agents in preventing cardiovascular disease is highly controversial ^{22,23}. The reasons for this are most perplexing ²⁴. Thus, in spite of multiple therapies, rates of cardiovascular events are still high and a better understanding of the drivers of atherosclerosis in the setting of diabetes is required and this will hopefully lead to the identification of new targets and ultimately new treatment modalities.

There are several theories in relation to the origins of atherosclerosis. These include the Response to Injury hypothesis of Ross and Glomset ²⁵, Response to Retention Hypothesis of Tabas, Williams and later Boren ^{13,26}, the oxidation hypothesis ²⁷ and the role of hyperglycemia and Advanced Glycation End (AGE) products ^{28,29}. The major processes initiating atherosclerosis are the initial deposition and accumulation of lipid in the vessel wall ³⁰ and the subsequent inflammatory response which ultimately generates the complex and sometimes labile plaques ^{11,12,15,16}. Nakashima *et al.* ³¹ demonstrated that in human coronary arteries the deposition of lipid occurs before the inflammatory response and the deposited apolipoproteins associated with the lipid co-localize with proteoglycans specifically biglycan and decorin ³¹. Thus the extracellular matrix and in particular the lipid-binding proteoglycan component is altered in the neointima of atherosclerosis susceptible arteries and it forms the permissive environment for the initiation of atherosclerosis ^{14,32,33}. Whether or not the initial lipid retention by proteoglycans is the forerunner of the inflammatory response or the inflammatory response is independent is unknown. The former alternative seems more likely and if so then proteoglycan to

lipid interaction is integral to the initiation of atherosclerosis and potentially represents a therapeutic target for the prevention of the disease process^{34,35}.

The impact of the factors associated with diabetes on the production of proteoglycans by VSMCs has been investigated in detail by Tannock, Little and colleagues³⁶⁻³⁹. Surprisingly, many of the obvious factors, including hyperglycemia, have no effect on proteoglycan synthesis and structure under the conditions so far investigated^{36,37}. Glucosamine, investigated as a component of the hexosamine pathway, has the potential to act as a precursor for glycosaminoglycan (GAG) synthesis and thus increase proteoglycan and GAG chain synthesis⁴⁰. Our studies actually found that it has the opposite effect and exogenous glucosamine causes concentration dependent inhibition of GAG elongation³⁶ which we later showed to be due to competition with glucose uptake leading to cellular ATP depletion³⁸. Elevated free fatty acids are a component of the early dysmetabolic status of diabetes⁴¹ and free fatty acids also have a modest effect on proteoglycan synthesis in VSMC⁴².

The major effect of the diabetic milieu appears to be indirect due to the action of high glucose concentrations to stimulate the secretion of TGF- β ^{37,43}. The hyperglycemia in patients with Type 2 diabetes leads to increased renal production of TGF- β ⁴³. TGF- β is a potent regulator of extracellular matrix synthesis, including many actions on proteoglycan synthesis^{39,44-47}. TGF- β induces changes in the proteoglycans secreted by VSMCs leading to increased binding to LDL³⁹ and therefore potentially increased lipid retention in the vessel wall and accelerated atherosclerosis¹⁴. TGF- β has been implicated in many diseases including tumor metastasis and renal and cardiac fibrosis⁴⁸⁻⁵⁰. Hyperglycemia has also been linked to activation of TGF- β signaling pathways regulating processes associated with the complications of diabetes⁵¹. TGF- β is strongly implicated in atherosclerosis and it is

highly expressed in atherosclerotic plaques (see Fig. 1). TGF- β and its receptors are present in atherosclerotic lesions⁵² and play an important role in regulating vessel proteoglycan synthesis and lipoprotein retention^{52,53}. Thus, given the established association between hyperglycemia and elevated TGF- β ⁴³, it is plausible that elevated TGF- β levels in diabetes plays a pathogenic role in the development of accelerated atherosclerosis in diabetes. On this basis we have reviewed the actions of TGF- β on proteoglycan synthesis in VSMC as a surrogate for the effects of the diabetic milieu on the vascular extracellular matrix. This review will focus on the effects of TGF- β on proteoglycan synthesis in vascular smooth muscle and particularly the signaling pathways through which TGF- β exerts its effects because those pathways may be therapeutic targets for the prevention of pathological modifications in the proteoglycan component of the vessel wall in diabetic vascular disease.

TGF- β and TGF- β receptors

TGF- β is a multifunctional growth factor that controls cellular processes involved in proliferation, differentiation, apoptosis, extracellular matrix accumulation and the immune system^{49,50,54}. The TGF- β superfamily of ligands include: Bone morphogenetic proteins (BMPs), Growth and differentiation factors (GDFs), Anti-müllerian hormone (AMH), Activin, Nodal and TGF- β s⁵⁵. TGF- β is a secreted protein that exists in three isoforms called TGF- β 1, TGF- β 2 and TGF- β 3. Three isoforms are highly conserved in mammals, and deletion of the isoforms in mice leads to distinct phenotypes⁵⁶⁻⁵⁸. All three TGF- β s are synthesized as precursor molecules containing an N-terminal propeptide region in addition to the TGF- β homodimer⁵⁹. The N-terminal signal peptide (propeptide region) consists of a 20-30 amino acid domain called latency associated peptide (LAP) required for secretion from the cell

and a 112-114 amino acid C-terminal region that becomes the mature TGF- β molecule following its release from the propeptide region by proteolytic cleavage⁶⁰. TGF- β homodimer interacts with a LAP forming a complex called Small Latent Complex (SLC). This complex remains in the cell until it is bound by a protein called Latent TGF- β -Binding Protein (LTBP), forming a larger complex called Large Latent Complex (LLC). It is LLC that get secreted to the extracellular matrix⁶¹. In most cases, before the LLC is secreted, the TGF- β precursor is cleaved from the propeptide but remains attached to it by noncovalent bonds⁶². After its secretion, it remains in the extracellular matrix as an inactivated complex containing both the LTBP and the LAP which need to be further processed in order to release active TGF- β ⁶³.

TGF- β receptors are the major family of cell surface receptors which are serine/threonine kinases but they also possess weak tyrosine kinase activity⁶⁴. They exist in several different isoforms that can be homo- or heterodimeric⁶⁵. Receptor types I (T β RI) and II (T β RII) are signaling receptors consisting of an N-terminal extracellular ligand binding domain, a transmembrane region and a C-terminal serine/threonine kinase domain. T β RI, but not T β RII receptors contain a GS domain (cytosolic and immediately N-terminal to the kinase domain) which consists of a series of about thirty glycine-serine repeats⁵⁴. TGF- β receptor type III is the most abundant and a non-signaling receptor which plays a role in transferring TGF- β to its signaling receptors and has a high affinity for both TGF- β 1 and TGF- β 2⁶⁶. Type III receptors are membrane proteoglycans that carry heparan and chondroitin sulfate GAG chains. The binding site for TGF- β resides in the 100-120-kDa core polypeptide of this receptor. The type III receptor is highly sensitive to cleavage by trypsin. Trypsin action releases the GAG-containing domain of the receptor leaving a 60-kDa membrane-associated domain that contains the cross-linked ligand⁶⁶.

In mammals there are seven known type I receptors and five type II receptors⁶⁷. Signaling begins with a TGF- β ligand binding to a constitutively active type II receptor dimer, which recruits a type I receptor dimer forming a hetero-tetrameric complex with the ligand on the cell surface. The binding of a TGF- β family ligand causes the rotation of the receptors so that their cytoplasmic kinase domains are arranged in a catalytically favorable orientation. This enables the type II receptor to phosphorylate multiple serine and threonine residues in the GS region of the type I receptor.

TGF- β signals via the T β RI/Alk V receptor and the canonical Smad pathway involving extreme C-terminal phosphorylation of receptor-regulated (R)-Smad transcription factors and translocation of Smad complexes to the nucleus where they regulate transcription^{50,54}. T β RI can also activate Mitogen Activated Protein (MAP) kinases leading to the phosphorylation of R-Smads in their linker region, a response that can inhibit or promote TGF- β signaling⁶⁸. The latter pathway is known somewhat incongruously as non-Smad or Smad independent signaling notwithstanding that it involves ultimately Smad phosphorylation.

The Smad family and TGF- β signaling via phosphorylation of Smad carboxyl terminal

The activated type I receptor phosphorylates cytoplasmic proteins of the C-terminal regions of Smad family. Smads are a class of proteins that modulate the activity of TGF- β ligands and consist of three domains: (1) an N-terminal Mad-homology 1 (MH1) domain which carries nuclear localization signals (NLS) and a DNA-binding domain; (2) a linker region of variable sequence and length that interact with prolyl-isomerases and ubiquitin ligases; (3) a C-terminal MH2 domain which

binds to type I receptor and mediates Smad homo- and hetero-oligomerization as well as mediates the transactivation of nuclear Smad complexes ⁶⁹. There are three classes of Smad: (1) the receptor-regulated Smads (R-Smad) which include Smad1, Smad2, Smad3, Smad5 and Smad9 (sometimes referred to as Smad8) ⁷⁰; (2) the common-mediator Smad (co-Smad) which includes only Smad4, which interacts with R-Smads to participate in signaling ⁷¹; (3) The antagonistic or inhibitory Smads (I-Smad) which include Smad6 and Smad7, which block the activation of R-Smads and Co-Smads and can also signal-terminating initiate degradation ⁷². There are five receptor regulated Smads. TGF- β s, Activins, Nodals and some GDFs are mediated by Smad2 and Smad3, while BMPs, AMH and a few GDFs are mediated by Smad1, Smad5 and Smad9.

The C-terminal phosphorylation of receptor activated R-Smads allows them to bind the co-Smad, Smad 4. This resulting R-Smad/co-Smad complex which consist of trimers of two R-Smads and one Smad4, is then imported into the nucleus where they act as transcription factors and participate in the regulation of target gene expression ⁵⁰.

TGF- β signaling via phosphorylation of the Smad2 linker region

TGF- β signaling is also known to involve positively and negatively in interaction with Mitogen Activated Protein (MAP)/ Extracellular signal-Regulated Kinases (ERK) pathway ^{50,54,73}. TGF- β stimulates the phosphorylation of ERK1/2 ⁷³; the activated ERK then phosphorylates Smad2 on threonine residues and possibly serine residues in the linker region which can induce the expression of the inhibitory I-Smads, Smad 6 and Smad7, that can hinder the translocation of the Smad2 into the nucleus and resistance to TGF- β signaling ⁷³.

Proteoglycans

Proteoglycans are glycosylated proteins which have covalently attached highly anionic GAGs ⁷⁴. Many forms of proteoglycans are present in all extracellular matrices of connective tissues including the blood vessel wall, where they are a component of the extracellular matrix as well as a cell-surface component of vascular cells. The major biological function of proteoglycans derives from the physicochemical characteristics of the core protein and carbohydrate components of the molecule. Core proteins determine the cellular location and serve as scaffolds for the GAG chains and the highly ionic chains determine physical properties of tissues and bind factors including growth factors and apolipoproteins. The GAG chains are long, linear carbohydrate polymers that are highly negatively charged under physiological conditions, due to the occurrence of sulfate and uronic acid groups. The complementary roles of core protein and GAG are exemplified by the observation that binding of low-density lipoprotein (LDL) to GAG chains of intact proteoglycans occurs with an order of magnitude higher affinity than the binding to free GAG chains ⁷⁵.

Proteoglycans can be categorized depending upon the nature of their GAG chains. These chains may be: chondroitin sulfate, decorin sulfate, heparan sulfate and keratan sulfate. Proteoglycans have distinct biological functions and their involvement in many aspects of cell and tissue activities has been demonstrated. For example, aggrecan, forms a substantial structural component of cartilage and is composed of both decorin sulfate and keratan sulfate GAG chains. Large chondroitin sulfate proteoglycan, versican, is prominent in blood vessels ⁷⁶. The smaller leucine-rich proteoglycans includes biglycan and decorin, which are widely distributed in many connective tissues, may have functions in regulating collagen fibril formation and in

modifying the activity of TGF- β ⁷⁷. Perlecan, the major heparan sulfate proteoglycan in the glomerular basement membrane, may play an important role as the major anionic site responsible for the charge selectivity in glomerular filtration ⁷⁸.

Vascular smooth muscle cells isolated and passaged from human vessels, secrete predominantly the small chondroitin/decorin sulfate proteoglycans: biglycan, decorin, lesser amounts of versican and heparan sulfate proteoglycan, perlecan ⁷⁹. TGF- β ⁷⁵ and oxidized LDL ⁸⁰ regulate the GAG chains elongation and the proportion of the chondroitin/decorin sulfate proteoglycans in the component mixture of secreted proteoglycans. These effects lead to enhanced binding of proteoglycans to atherogenic apolipoproteins ⁸¹. In the initiation of atherosclerosis, the growth factor-modified biglycan with elongated GAG chains has been termed by us as “hyperelongated biglycan” ²⁵. The modifications to GAGs on chondroitin sulfate and decorin sulfate proteoglycans increases the binding of GAGs to apolipoproteins ^{75,81,82}; the hydrolytic and proteolytic modifications of apolipoproteins increase the binding strength of GAGs to LDL ^{80,83}.

Actions of TGF- β on proteoglycan synthesis in vascular smooth muscle

Actions of TGF- β on proteoglycan synthesis

TGF- β stimulates proteoglycan synthesis, specifically the expression of biglycan and an elongation of its decorin sulfate (DS) GAG chains and this results in an increased binding of the proteoglycan to LDL ^{39,44,46,47}. In very early studies, Wight and colleagues ^{44,45} found that TGF- β causes a 3.3-fold increase in radiolabeled sulfate incorporation into biglycan in monkey arterial smooth muscle cells, but as expected it

had no effect on core protein size. Northern blotting analysis also revealed a more than two fold increase in biglycan mRNA expression⁴⁵. TGF- β had no appreciable effect on decorin core protein synthesis or GAG elongation on decorin⁴⁵. We have confirmed and extended these findings in human VSMCs^{46,47}. TGF- β increases radiolabeled incorporation into biglycan, associated with a marked increase in proteoglycan size which is specifically associated with an increase in the size of the GAG chains^{39,47}. TGF- β also increases [³⁵S]-methionine/cysteine incorporation indicating an increase in core protein synthesis⁴⁶ and consistent with the induction of biglycan mRNA first described by Schonherr *et al.*⁴⁵. All responses to TGF- β are concentration-dependently inhibited by the T β RI/Alk V inhibitor SB431542 indicating that they arise from T β RI/Alk V⁴⁶.

TGF- β receptor signaling pathway regulating biglycan synthesis

TGF- β can also activate MAP kinases leading to downstream signaling completely independent of Smads but also causing phosphorylation of Smads in the linker region⁵⁰ a response originally associated with the inhibition of Smad translocation to the nucleus and this is antagonistic to TGF- β signaling⁷³. We recently investigated the ability of TGF- β to stimulate the phosphorylation of p38, Extracellular signal-Regulated Kinases (ERK) and Jun N-terminal Kinase (JNK) MAP kinases as well as Smad2 phosphorylation at both its C-terminal and in the linker region⁴⁷. TGF- β stimulated the phosphorylation of all three MAP kinases⁴⁷. Pharmacological inhibition of ERK and p38 MAP kinase blocked TGF- β mediated GAG elongation and expression of biglycan whereas inhibition of JNK had no effect. Inhibition of ERK and p38 but not JNK MAP kinases attenuated the effect of TGF- β to increase phosphorylation of Smad2 linker region⁴⁷. High levels of phosphorylation

of Smad2 linker region were detected in a nuclear fraction of TGF- β treated cells implying that this response was not antagonistic to TGF- β signaling. Thus, TGF- β signaling via ERK and p38 MAP kinases and leading to the phosphorylation of the linker region of Smad2 mediates its effects of TGF- β on biglycan synthesis and GAG elongation in human vascular smooth muscle cells⁴⁷. We have also demonstrated that the size of the effect mediated by the signaling pathways is sufficient to alter binding to LDL because the binding of radiolabelled proteoglycans to normal human LDL is reduced in proteoglycan harvested from cells treated with inhibitors of the TGF- β receptor (T β RI) or p38 MAP kinase⁴⁶.

Consequences of TGF- β stimulated proteoglycan synthesis: increased lipid binding

TGF- β has two actions on proteoglycan synthesis which can enhance the atherogenicity of the extracellular matrix. TGF- β stimulates the expression of biglycan which is the proteoglycan most closely associated with lipid binding in early human atherosclerosis³⁰ and TGF- β also stimulates the elongation of GAG chains leading to hyperelongated chains which show enhanced binding to LDL^{14,39,84}. Highly anionic (sulfated and carboxylated) GAG chains on proteoglycans bind to a multitude of molecules but practically and of interest to the development of atherosclerosis they bind to cationic residues on the apolipoproteins (B100) on human LDL^{85,86}. Lipids bind to the GAGs in the vessel wall⁸⁷. Due to the complex nature of the interacting species it is difficult to describe the interaction in classical receptor-binding terms but when an equal quantity of radiolabelled proteoglycan core proteins from control and TGF- β treated cells is added to an *in vitro* gel mobility shift

assay then the latter show higher affinity binding as evidenced by a shift to the left of the binding isotherm³⁹. The rate limiting step for passages of lipoproteins through the vessel wall is the release from the wall to the lymph so an increase in binding affinity will delay the transition of the lipid and ultimately induce the inflammatory response that generates the complex plaques⁸⁸.

As described above, TGF- β signaling involves Smad transcription factors acting through the canonical C terminal phosphorylation pathways but also as shown recently by us potentially via the TGF- β -stimulated MAP kinase (p38 and Erk) mediated phosphorylation of the linker region of Smad2⁴⁷. Multiple other hormones and growth factors activate MAP kinases in VSMCs and these have the potential to cause Smad linker phosphorylation. Such a response might synergize with the actions of TGF- β in diabetes and indeed we have reported that the actions of TGF- β and PDGF are additive in causing GAG elongation on biglycan³⁴ and by extrapolation causing increased binding to LDL. Thus, hyperglycemia driven increased level of TGF- β can synergize with other factors known to be elevated in diabetes to drive hyperelongation of GAG chains on lipid binding proteoglycans (see schema in Fig. 2) and thus has the potential to be the driving factor and the link between hyperglycemia and accelerated development of atherosclerosis in diabetes⁸⁹

Conclusions

Proteoglycans are complex molecules being comprised of both protein and carbohydrate moieties. These entities have completely different synthetic processes consistent with normal cell biology of the synthesis of proteins and GAGs^{40,90}. All of these features can be involved in pathology because growth factors can alter the

proteoglycan composition of the extracellular matrix by altering the relative expression of proteoglycan core proteins or independently by causing the elongation of the GAG chains, known as hyperelongation¹⁴. Each of these processes regulating core proteins and GAG synthesis is activated by signaling pathways which are sometimes similar but can also be completely distinct⁹¹. Considerable progress has been made in revealing the signaling pathways that control the synthesis and structure of proteoglycans in vascular smooth muscle from the hormone and growth factors^{39,92-94}, metabolic factors³⁶ through to the involvement of transcription factors⁹⁵ that regulate the genes that control growth factor stimulated proteoglycan synthesis. Amongst these factors TGF- β is one of the most prominent activators of proteoglycan syntheses and it leads to increased binding to LDL. Given the established association between hyperglycemia and elevated TGF- β we have speculated that elevated TGF- β levels in diabetes play a pathogenic role in the development of accelerated atherosclerosis in diabetes. Blocking growth factor actions on proteoglycan synthesis with many known cardiovascular and diabetes drugs attenuates lipid binding and potential provides pleiotropic actions which assist in the prevention of atherosclerosis^{96,97}. These signaling pathways have the potential to be therapeutic targets for vascular diseases such as atherosclerosis especially when targeted by highly specific agents^{34,35}. Further studies will reveal the potential for these pathways to represent therapeutic targets that may be successfully exploited without interfering with the normal physiological functions of TGF- β .

Figures

Figure 1. Atherosclerotic plaques show expression of TGF- β . Sections of abdominal aorta from a hypercholesterolemia non-human primate, *Macaca nemestrina*, and containing advanced fibrous plaques were immunostained with antibodies specific for control IgG (left panel) and with TGF- β 1 (right panel). Positive staining for TGF- β 1 was seen in macrophages in the core of the lesion as well as macrophages scattered in the fibrous cap and near the luminal surface. Magnification, X60.

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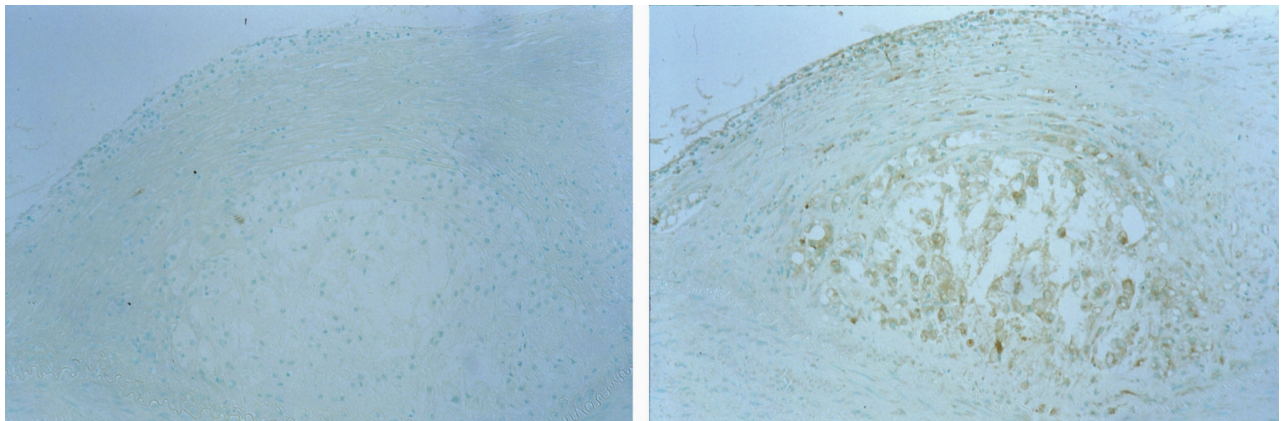
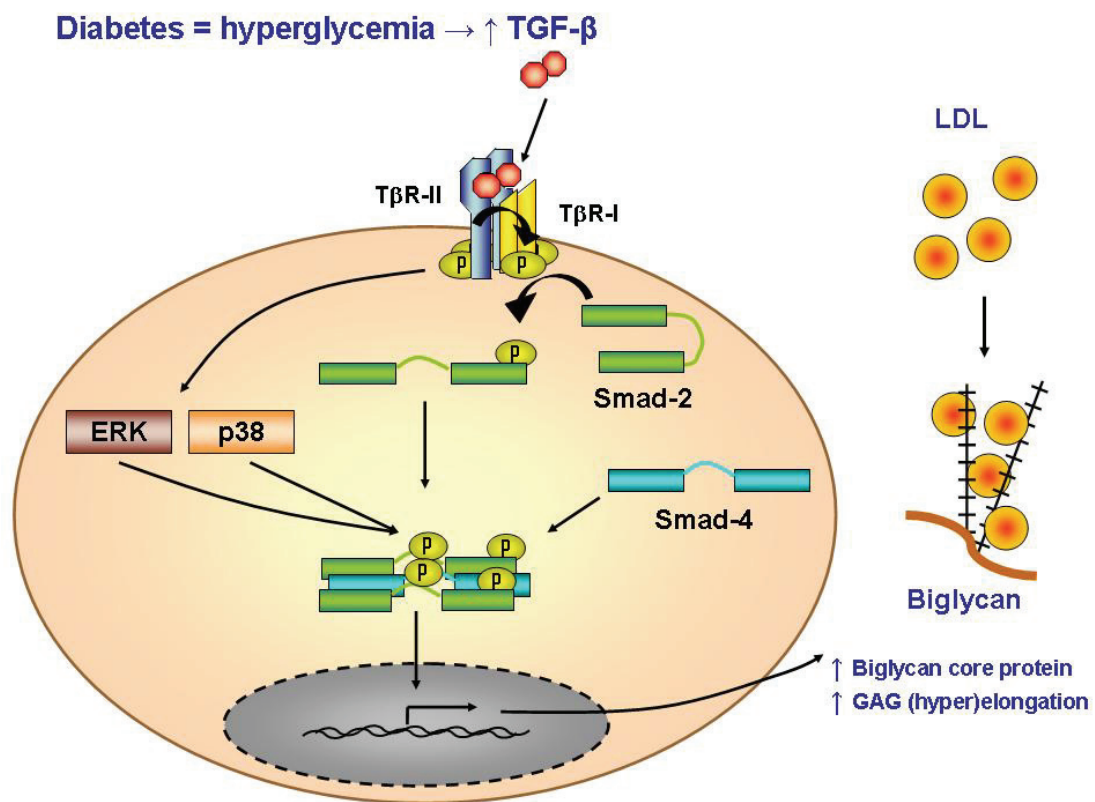


Figure 2. Schema showing our current knowledge of the signaling pathway through which Transforming Growth Factor (TGF)- β stimulates changes in the synthesis and structure of proteoglycans secreted by vascular smooth muscle cells. These changes lead to increased in vitro binding of proteoglycan to LDL and given the established relationship between hyperglycemia and elevated TGF- β levels, these effects potentially underlie the increased atherosclerosis observed in people with diabetes. The pathway leading to the stimulation of biglycan synthesis involves the canonical as well as the MAP kinase linker region phosphorylation pathway (see text for details and references). Erk: Extracellular regulated kinase; p38: p38 Mitogen Activated Protein (MAP) kinases; T β R-I and T β R-II are the Type I and II TGF- β receptors, respectively. Smad is the Smad transcription factors as part of the T β R-I signaling pathways.



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