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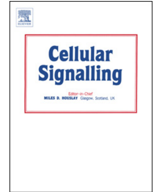

The role of specific Smad linker region phosphorylation in TGF-β mediated expression of glycosaminoglycan synthesizing enzymes in vascular smooth muscle

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ABSTRACT

Hyperelongation of glycosaminoglycan chains on proteoglycans facilitates increased lipoprotein binding in the blood vessel wall and the development of atherosclerosis. Increased mRNA expression of glycosaminoglycan chain synthesizing enzymes in vivo is associated with the development of atherosclerosis. In human vascular smooth muscle, transforming growth factor-β (TGF-β) regulates glycosaminoglycan chain hyperelongation via ERK and p38 as well as Smad2 linker region (Smad2L) phosphorylation. In this study, we identified the involvement of TGF-β receptor, intracellular serine/threonine kinases and specific residues on transcription factor Smad2L that regulate glycosaminoglycan synthesizing enzymes. Of six glycosaminoglycan synthesizing enzymes, xylosyltransferase-1, chondroitin sulfate synthase-1, and chondroitin sulfotransferase-1 were regulated by TGF-β. In addition ERK, p38, PI3K and CDK were found to differentially regulate mRNA expression of each enzyme. Four individual residues in the TGF-β receptor mediator Smad2L can be phosphorylated by these kinases and in turn regulate the synthesis and activity of glycosaminoglycan synthesizing enzymes. Smad2L Thr220 was phosphorylated by CDKs and Smad2L Ser250 by ERK. p38 selectively signalled via Smad2L Ser245. Phosphorylation of Smad2L serine residues induced glycosaminoglycan synthesizing enzymes associated with glycosaminoglycan chain elongation. Phosphorylation of Smad2L Thr220 was associated with XT-1 enzyme regulation, a critical enzyme in chain initiation. These findings provide a deeper understanding of the complex signalling pathways that contribute to glycosaminoglycan chain modification that could be targeted using pharmacological agents to inhibit the development of atherosclerosis.

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1. Introduction

The synthesis of chondroitin sulfate (CS) glycosaminoglycan (GAG) chains on proteoglycans involves the concerted action of a wide range of GAG synthesizing enzymes [1]. After synthesized CS proteoglycan biglycan core protein exits the endoplasmic reticulum, post-translational modifications occur inside the cis/trans Golgi [2]. CS GAG chain synthesis commences with the addition of the linkage region (xylose-galactose-galactose-gluronic acid) by the action of XT-1 [3], GalT-1 [4], GalT-2 [5] and GlcAT-1 [6] enzymes respectively. This linkage region connects the CS GAG chain, composed of alternating monosaccharide units GlcA and GalNAc, to the biglycan core protein [2]. Chondroitin polymerization on the growing CS GAG chain occurs via the action of six glycosyltransferases: chondroitin synthase ChSy-1, ChSy-2, ChSy-3, ChPF, ChGn-1 and ChGn-2 [7–9]. CS GAG chain modifications may include hyperelongation of the repeating disaccharide units and sulfation of hydroxy groups at positions 4 and 6 of the GalNAc residues and position 2 of the GlcA residue by sulfotransferases C4ST-1 and C4ST-2, thereby creating structural diversity of the chain [10]. Transforming

Abbreviations: GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; XT-1, xylosyltransferase-1; GalT-1, β-1,3-galactosyl transferase-1; GlcAT-1, β-1,3-glucuronosyl transferase-1; ChSy-1, chondroitin sulfate synthase-1; ChPF, chondroitin polymerizing factor; ChGn-1, chondroitin *N*-acetylgalactosaminyltransferase-1; C4ST-1, chondroitin sulfotransferase-1.

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growth factor-β (TGF-β) mediated structural modification of the GAG chain either by elongation or sulfation pattern enhances the positive charge of the chain contributing to a higher affinity towards low-density lipoprotein (LDL) binding [11,12]. TGF-β is a pleiotropic growth factor associated with the development of all stages in atherosclerosis [13,14]. Very little is known about the effects of TGF-β on GAG synthesizing enzyme expression in vascular smooth muscle.

Biglycan a proteoglycan secreted by VSMCs in the artery wall in vivo, is present in early atherosclerotic lesions [15] and the GAG chain on biglycan is hyperelongated by TGF-β stimulation in human VSMC in vitro [16]. TGF-β mediated GAG chain hyperelongation on biglycan is dependent upon both transcription and translation [17]. The involvement of transcription factor Smad2 and its binding partners in this pathway has been described in human VSMCs, but the downstream gene targets, presumably the specific GAG synthesizing enzymes are not defined [17]. The canonical TGF-β signalling pathway involves direct carboxy-terminal phosphorylation of Smad2/3 by TGF-β receptor-1 (TGFβR1). Smad2 is directly phosphorylated on two residues Ser465 and 467 [18]. Smad2/3 then complexes with Smad4 for translocation to the nucleus and regulation of gene expression that results in the diverse effects of TGF-β stimulation [19]. In addition to the carboxy-terminal phosphorylation, studies have shown that the linker region of Smad2/3 can also undergo phosphorylation and regulate TGF-β-mediated biological activities [20]. In human Smad2, potential linker region phosphorylation sites are Thr220, Ser245, Ser250 and Ser255. In Smad3, phosphorylation can occur at Thr179, Ser204, Ser208 and Ser213 [21]. According to Matsuzaki et al. [22] Smad linker region phosphorylation can be activated by multiple serine/threonine kinases MAPKs (ERK, JNK and p38), PI3K, CDK, ROCK and GSK3 [16,21]. Previous results from our laboratory have reported that TGF-β stimulates the hyperelongation of GAG chain on biglycan in VSMCs, via Smad2 linker region phosphorylation by ERK and p38 [16]. However, the specific phosphorylation sites in the Smad2 linker region have not been identified. The role and involvement of GAG synthesizing enzyme(s) in the TGF-β-Smad linker region signalling pathway is also unknown. In a mouse model of atherosclerosis, elevated mRNA expression of GAG synthesizing enzymes ChGn-2 and C4ST-1 correlated with increased plaque progression after 8 weeks of high fat Western diet [23]. There is limited information regarding the drivers i.e. growth factors that promote the expression of these GAG synthesizing enzymes in vascular smooth muscle.

In this study we investigated the specific GAG synthesizing enzymes that are stimulated by TGF-β signalling pathways in human VSMCs. Our results show that TGF-β stimulates the mRNA expression of XT-1, ChSy-1, ChPF and C4ST-1 GAG synthesizing enzymes. We also demonstrate that ERK, p38, PI3K and CDK play a role in TGF-β stimulated XT-1, ChSy-1 and C4ST-1 mRNA expression. These serine/threonine kinases differentially phosphorylated four specific Smad2 linker region residues Thr220, Ser245, Ser250 and Ser255 and highlight the selective specificity of the TGF-β signalling pathway in human VSMCs.

2. Materials and methods

2.1. Materials

Foetal bovine serum (FBS) was purchased from In Vitro Technologies Pty. Ltd. (VIC, Australia). Dulbecco's Modified Eagle Medium (DMEM) (0 mM and 25 mM glucose), Trypsin-Versene, penicillin and streptomycin were from GIBCO BRL (NE, USA). Flavopiridol, SB431542, SP600125, SB216763, LY294002, Dulbecco's phosphate-buffered saline (PBS) (10×), sodium dodecyl sulfate (SDS), and 2 mercaptoethanol and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (MO, USA). SB202190 was from Calbiochem (CA, USA). UO126 was from Promega (WI, USA). Quantitative RT-PCR primers (forward and reverse) for GAG synthesizing enzyme genes ChSy-1, C4ST-1 and C4ST-2 were purchased from GeneWorks Pty Ltd (SA, Australia). The primers (forward and reverse) for XT-1, ChPF, GalNacT-2 and 18S, the RNeasy® Mini Kit, the QuantiTect® Reverse Transcription Kit, the QuantiFast™ SYBR® Green PCR kit, and the Rotor-Gene® Q Series software were from Qiagen (VIC, Australia). Human recombinant TGF β, anti-rabbit immunoglobulin-G (IgG) horseradish peroxidase (HRP), anti-mouse IgG HRP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit monoclonal IgG antibody were from Cell Signaling Technology (MA, USA). Amersham ECL Prime chemiluminescent detection reagent is from GE Healthcare (NSW, Australia). C4ST-1 mouse IgG monoclonal antibody was from Abcam (CBG, UK). Bovine serum albumin was from Bovogen Biologicals (VIC, Australia). Bicinchoninic acid protein assay kit, ChSy-1 rabbit IgG polyclonal antibody was from Thermo Scientific (IL, USA). Chemiluminescent molecular weight marker (MagicMark XP) and chromogenic molecular weight marker (BenchMark) were from Invitrogen (AK, New Zealand). TEMED, Tris Base and Glycine were from Amresco (OH, USA). Tween-20, 30% acrylamide/Bis solution, ammonium persulfate, Polyvinylidene fluoride (PVDF) membrane and Image Lab V5.0 imaging software were from BioRad Laboratories (CA, USA). Phospho-Smad2/3L (Thr220) rabbit IgG polyclonal, phospho-Smad2L (Ser245) rabbit IgG polyclonal, phospho-Smad2L (Ser250) rabbit IgG polyclonal and phospho-Smad2L (Ser255) rabbit IgG polyclonal antibodies were a gift from Professor Koichi Matsuzaki (Kansai Medical University, OSA, Japan).

2.2. Tissue culture

Human VSMCs were isolated using the explant technique from discarded segments of saphenous veins from patient donors undergoing surgery at the Alfred Hospital (Melbourne, Australia) under ethics approval from The Alfred Hospital's Ethics Committee. Vascular smooth muscle cells from saphenous veins are known to undergo atherosclerotic changes [24–26]. Cell were seeded into 6-well plates (qRT-PCR experiments) or 60 mm tissue culture dishes (Western blot experiments) at 8×10^5 cells/well in low-glucose (5 mM) DMEM with 10% (v/v) FBS and antibiotics and maintained until confluent. Cells were then serum deprived by culturing in low glucose (5 mM) DMEM with 0.1% (v/v) FBS for 48 h prior to experimentation. Experiments were conducted using cells from passage 11–18.

2.3. Quantitative RT-PCR

Quantification of the mRNA levels of GAG synthesizing enzymes were conducted using quantitative real-time polymerase chain reaction (qRT-PCR). Quiescent cells (90% confluency) were treated with TGF-β (2 ng/ml) for indicated time points. Total RNA was extracted using the Qiagen RNeasy® Mini Kit, and RNA concentration and purity were analyzed using the THERMO Nanodrop 2000. cDNA was synthesized from DNase-treated RNA using the Qiagen QuantiTect® Reverse Transcription kit. Q-RT-PCR was performed using the QuantiFast™ SYBR® Green PCR kit together with primers of the following sequences: XT-1 Forward: 5′-GTGGATCCCGTCAATGTCATC-3′, Reverse: 5′-GTGTGTGAAT TCG GCAGTGG-3′ [27], ChSy-1 Forward 5′-CCCGCCCCAGAAGAAGTC-3′, Reverse 5′-TCTC ATAAACCATTCATACTTGTCCAA-3′ [28], ChPF Forward 5′-AGATCCAGGAGTTACA GTGGGAGAT-3′, Reverse 5′-CCGGGC GGGATGGT-3′ [9], ChGn-2 Forward 5′-GGGC TAATGGCATAGGCTAT CA-3′, Reverse: 5′-CTTTGTCAATTTGGGAATGAAGAA-3′ [29], C4ST-1 Forward: 5′-GGCCCTGCGCAAAG-3′, Reverse: 5′-GGGTGTGTGGGTCGAT GA G-3′ [30], C4ST-2 Forward: 5′-AACGAGGAGTTCTACCGCAAGT-3′, Reverse: 5′-GCTT GTGTGGTTGGCGTACA-3′ [30]. Amplification was performed using the Qiagen Rotor Gene Q thermal cycler. Data was normalized to the ribosomal 18S housekeeping gene. All experiments were performed at least three times and analysis performed in duplicate for each experiment.

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