

Flavopiridol Inhibits TGF- β -Stimulated Biglycan Synthesis by Blocking Linker Region Phosphorylation and Nuclear Translocation of Smad2

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Received August 8, 2017; accepted January 3, 2018

ABSTRACT

Transforming growth factor- β (TGF- β) is a pleiotropic growth factor implicated in the development of atherosclerosis for its role in mediating glycosaminoglycan (GAG) chain hyperelongation on the proteoglycan biglycan, a phenomenon that increases the binding of atherogenic lipoproteins in the vessel wall. Phosphorylation of the transcription factor Smad has emerged as a critical step in the signaling pathways that control the synthesis of biglycan, both the core protein and the GAG chains. We have used flavopiridol, a well-known cyclin-dependent kinase inhibitor, to study the role of linker region phosphorylation in the TGF- β -stimulated synthesis of biglycan. We used radiolabeled incorporation and SDS-PAGE to assess proteoglycan synthesis, real-time polymerase chain reaction to assess gene expression, and chromatin immunoprecipitation to assess the binding of Smads to the promoter region of GAG

synthesizing genes. Flavopiridol blocked TGF- β -stimulated synthesis of mRNA for the GAG synthesizing enzymes, and chondroitin 4-sulfotransferase (C4ST-1), chondroitin sulfate synthase-1 (ChSy-1) and TGF- β -mediated proteoglycans synthesis as well as GAG hyperelongation. Flavopiridol blocked TGF- β -stimulated Smad2 phosphorylation at both the serine triplet and the isolated threonine residue in the linker region. The binding of Smad to the promoter region of the C4ST-1 and ChSy-1 genes was stimulated by TGF- β , and this response was blocked by flavopiridol, demonstrating that linker region phosphorylated Smad can pass to the nucleus and positively regulate transcription. These results demonstrate the validity of the kinases, which phosphorylate the Smad linker region as potential therapeutic target(s) for the development of an agent to prevent atherosclerosis.

Introduction

Cardiovascular disease is the leading cause of death among adults worldwide and atherosclerosis is the major underlying pathology (Nigro et al., 2006; Deaton et al., 2011). The early stages of the development of atherosclerotic lesions in human pathology occur due to the accumulation of atherogenic lipids on proteoglycans, mostly the chondroitin sulfate (CS)/

dermatan sulfate (DS) proteoglycan, biglycan (Nakashima et al., 2007, 2008). Modification of the synthesis and structure of proteoglycans, predominantly glycosaminoglycan (GAG) chain hyperelongation, results in increased binding to apolipoproteins on lipids *in vitro* (Little et al., 2002, 2008; Ballinger et al., 2004) leading to the trapping of atherogenic low-density lipoproteins (LDL) in the blood vessel wall. GAG chain hyperelongation occurs by growth factor stimulation of the expression of the GAG chain synthesizing enzymes in vascular smooth muscle cells (VSMCs) (Little et al., 2002; Ivey and Little, 2008; Yang et al., 2009, 2010; Burch et al., 2010; Cardoso et al., 2010; Osman et al., 2014). Prevention of this change in proteoglycan structure by targeting the hormone and growth factor signaling pathways has been proposed and demonstrated as a therapeutic target to prevent atherosclerosis (Ballinger et al., 2004; Little et al., 2007, 2011; Osman

The authors declare that they have no conflicts of interest with the contents of this article.

This study was supported by a National Heart Foundation of Australia Grant-in-aid (to P.J.L. and N.O.). M.A.R. was supported by the Academic Trainee Scholarship from the International Islamic University of Malaysia. Support was provided by The University of Queensland Head of School support package (to P.J.L.) and the University of Queensland Faculty of Health and Behavioural Sciences via the School of Pharmacy (to P.M.).

<https://doi.org/10.1124/jpet.117.244483>.

ABBREVIATIONS: Alk-5, activin-like kinase; C4ST-1, chondroitin 4-sulfotransferase; CDK, cyclin-dependent kinase; ChSy-1, chondroitin sulfate synthase-1; CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; HRP, horseradish peroxidase; LDL, low-density lipoproteins; RT-PCR, real-time polymerase chain reaction; SB431542, 4-(5-benzo[1,3]dioxol-5-yl-4-pyridin-2-yl-1*H*-imidazol-2-yl)-benzamide; Ser, serine; TGF- β , transforming growth factor β ; TGF β RI, transforming growth factor- β receptor; Thr, threonine; VSMC, vascular smooth muscle cell; XT-1, xylosyltransferase-1.

et al., 2008); the signaling pathways are the preferred target because these pathways are specific for VSMCs, which is not the case for the action of the elongation enzymes themselves that are ubiquitously expressed and functional in most tissues of the body. Vasoactive growth factors mediate proteoglycan core protein expression and independently modify the structure of the GAG chains on proteoglycans by stimulating the expression of the genes for the GAG chain elongation enzymes (Osman et al., 2011; Kamato et al., 2016; Rostam et al., 2016).

Transforming growth factor- β (TGF- β) is a pleiotropic growth factor linked to vascular disease (Bobik et al., 1999), which acts via serine (Ser)/threonine (Thr) kinase cell surface receptors (Derynck and Zhang, 2003; Massagué et al., 2005). TGF- β stimulates the expression of biglycan in VSMCs and also stimulates the elongation of its GAG chains, which results hyperelongated GAG chains that show increased binding to LDL (Little et al., 2002; Burch et al., 2010; Rostam et al., 2016). TGF- β signaling involves the regulation of gene expression by Smad transcription factors (Massagué et al., 2005). This signaling pathway is responsible for the transcription and translation of enzymes, which can regulate GAG chain synthesis and structure (Yang et al., 2009). Anggraeni et al. (2011) showed a correlation between increased mRNA expression of the synthesizing enzymes chondroitin 4-sulfotransferase (C4ST-1) and chondroitin *N*-acetylgalactosaminyltransferase-2 and GAG elongation with lipid deposition and the development of atherosclerosis in a mouse model (Anggraeni et al., 2011).

To date, most studies on TGF- β signaling pathways have focused on the response of TGF- β receptors (TGF β RI), also known as activin-like kinase (Alk-5), which directly activate Smad transcription factors (Smad2 or Smad3) in the carboxy terminus (Derynck and Zhang, 2003; Massagué et al., 2005). However, specific residues of the Smad linker region phosphorylation can regulate a wide range of cellular events (Kamato et al., 2013; Yumoto et al., 2013). The Smad linker region pathway signals through activation of Ser/Thr kinases including mitogen-activated protein kinase, extracellular-signal regulated kinase, Jun N-terminal kinase and p38 kinase, Akt, cyclin-dependent kinase (CDK), rho-associated protein kinase, calcium calmodulin-dependent kinase, and glycogen synthase kinase-3, as well as via activation of the tyrosine kinase Src and phosphatidylinositol 3'-kinase (Kamato et al., 2013). Mitogen-activated protein kinase and CDK show a preference for specific Ser/Thr residues in the linker region essential for the regulation, stability, activity, and nuclear transport of R-Smads (Matsuzaki et al., 2009; Burch et al., 2011).

In the original characterization of the cell biology of linker region phosphorylation of Smad transcription factors, Ras-dependent linker region phosphorylation inhibited the nuclear translocation, and hence the gene regulatory action of phosphorylated Smad (Kretschmar et al., 1997). However, in contrast, our data have demonstrated that Smad linker region phosphorylation is essential to the process of GAG chain elongation and hyperelongation on biglycan (Burch et al., 2010). Thus, questions about Smad linker region phosphorylation and the role of linker region phosphorylation on the nuclear translocation of these transcription factors have not been answered.

In a previous study on the role of various kinase inhibitors in blocking Smad phosphorylation and GAG hyperelongation, we identified flavopiridol as a potent inhibitor of GAG

elongation (Rostam et al., 2016). Flavopiridol (Alvocidib) was the first CDK inhibitor to be tested in human clinical trials (Senderowicz and Sausville, 2000). It is a flavonoid alkaloid and CDK9 kinase inhibitor previously under clinical development for the treatment of acute myeloid leukemia (Mariaule and Belmont, 2014). Flavopiridol thus presents as a useful tool to study the role of linker region phosphorylation in TGF- β -stimulated biglycan synthesis. In a study on kinase inhibitors, Smad linker phosphorylation and TGF- β stimulation of GAG gene expression, GAG elongation and core protein (biglycan) expression, flavopiridol had very potent and substantial inhibitory effects (Rostam et al., 2016). We have reported on the role of Smad linker region phosphorylation on driving the expression of genes for enzymes that are rate limiting for the elongation of GAG chains on biglycan and also on the resultant size of the biglycan molecules related to changes in the size of the CS/DS GAG chains (Rostam et al., 2016). Therefore, based on the potency and efficacy of flavopiridol in this *in vitro* model of atherogenesis, we have used this compound to further explore the role of Smad linker region phosphorylation in driving the expression of GAG synthesizing genes.

We report that flavopiridol inhibits GAG elongation on biglycan, Smad linker region phosphorylation and Smad binding to consensus sites on the promoter regions of C4ST-1 and chondroitin sulfate synthase-1 (ChSy-1), demonstrating a pathway that convincingly shows the involvement of Smads in GAG hyperelongation in VSMCs. The data point to very specific pathways, which may represent therapeutic targets for the prevention of the changes in biglycan structure that mediate lipid binding in the vessel wall as the earliest stage of human atherosclerosis.

Materials and Methods

Materials. Human recombinant TGF- β was purchased from R&D systems (Minneapolis, MN). Fetal bovine serum was purchased from CSL (Parkville, VIC, Australia). Cell culture materials were purchased from GIBCO BRL (Grand Island, NY). Trypsin-versene, antibiotics (penicillin, streptomycin), flavopiridol and 4-(5-benzo[1,3]-dioxol-5-yl-4-pyridin-2-yl-1*H*-imidazol-2-yl)-benzamide (SB431542), Dulbecco's phosphate-buffered saline (10 \times), SDS, and 2-mercaptoethanol and dimethylsulfoxide were purchased from Sigma-Aldrich (St. Louis, MO). The 18S primer, RNeasy Mini Kit, QuantiTect Reverse Transcription Kit, QuantiFast SYBR Green PCR Kit, and the Rotor-Gene Q Series software were purchased from Qiagen (Chadstone, VIC, Australia). Anti-rabbit IgG horseradish peroxidase (HRP), anti-mouse IgG HRP, anti-phospho-Smad2 (Ser245/255/250), anti- α tubulin, and anti-glyceraldehyde-3-phosphate dehydrogenase antibody were purchased from Cell Signaling Technology (Danvers, MA). Primers for GAG synthesizing genes and biglycan (Table 1) were purchased from GeneWorks Pty. Ltd. (Thebarton, SA, Australia). Amersham ECL Prime chemiluminescent detection reagent was from GE Healthcare (Paramatta, NSW, Australia). Bovine serum albumin was purchased from Bovogen Biologicals Pty. Ltd. (Keilor, VIC, Australia). Bicinchoninic acid protein assay kit was purchased from Thermo Scientific (Rockford, IL). Chemiluminescent molecular weight marker (MagicMark XP) and chromogenic molecular weight marker (BenchMark) were purchased from Invitrogen (Auckland, New Zealand). Tetramethylethylenediamine (TEMED), Tris base, and glycine were purchased from Amresco (Solon, OH). Tween-20, 30% acrylamide/Bis solution, ammonium persulfate, polyvinylidene fluoride membrane, and Image Laboratory version 5.0 imaging software were purchased from BioRad Laboratories (Hercules, CA). Phospho-Smad2/3L (Thr220/Thr179) rabbit IgG

TABLE 1
Target gene and primer sequence

Target Gene	RT-PCR Primer Sequence (5'-3')
C4ST-1	Forward 5' AGG GTC GTG CAG TTT CCT TA 3' Reverse 5' AGG GTT AGG GGG TTT CTG GT 3'
ChSy-1	Forward 5'-GAA GAG GAC GGG GAC AGC-3' Reverse 5'-CCT TTA AGA GGG GAC CAT GC-3'
XT-1	Forward 5'-AAG GTC CGC ATA CCA AAC AC-3' Reverse 5'-TAT CCT CAT GGG GTG GT-3'
Biglycan	Forward 5'-CTC AAC TAC CTG CGC ATC TCA G-3' Reverse 5'-GAT GGC CTG GAT TTT GTT GTG-3'

polyclonal antibody was a gift from Koichi Matsuzaki (Kansai Medical University, Osaka, Japan). Carrier-free [³⁵S]-SO₄ was purchased from ICN Biomedicals (Irvine, CA). Cetylpyridinium chloride was purchased from Unilab Chemicals & Pharmaceuticals Pvt. Ltd. (Mumbai, India).

Tissue Culture. Human VSMCs were isolated using the explant technique from discarded segments of the saphenous veins from patient donors undergoing surgery at Alfred Hospital (Melbourne, VIC, Australia) under ethics approval from The Alfred Hospital Ethics Committee. Cells were seeded into six-well plates at 8×10^5 cells/well in low-glucose (5 mM) Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum and antibiotics and maintained until confluent. Cells were then serum deprived by culturing in low glucose (5 mM) Dulbecco's modified Eagle's medium with 0.1% (v/v) fetal bovine serum for 48 hours prior to experimentation. Experiments were conducted using cells from passages 14–19.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis. Measurement of the mRNA levels of GAG synthesizing enzymes was conducted using quantitative RT-PCR. Total RNA was extracted using the Qiagen RNeasy Mini Kit. cDNA (1000 ng/ μ l) was synthesized using the Qiagen QuantiTect Reverse Transcription Kit. Quantitative RT-PCR was performed using the QuantiFast SYBR Green PCR Kit. Data were normalized to the ribosomal 18S house-keeping gene. All experiments were performed at least three times.

Western Blotting. Whole cell lysates were resolved on 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% (w/v) bovine serum albumin and incubated with primary antibody (1:1000) as indicated in figure legends 4 and 5, followed by species-specific secondary antibody (anti-rabbit IgG HRP and anti-mouse IgG HRP). Enhanced chemiluminescence was used to detect protein of interest. Blots were imaged using the BioRad gel documentation system and densitometry analysis was performed with the Image Lab version 5.0 imaging software (BioRad Laboratories). Each experiment was conducted at least three times.

Quantification of Radiolabel Incorporation into Proteoglycans. Quiescent cells in 24-well plates were changed to fresh medium containing [³⁵S]-sulfate (50 μ Ci/ml) in the presence or absence of TGF- β (2 ng/ml) and inhibitors for 24 hours. Secreted proteoglycans were harvested from the media with added protease inhibitors (5 mM benzamide in 0.1 M 6-aminocaproic acid). Incorporation of the radiolabel into proteoglycans was measured by the cetylpyridinium chloride precipitation assay described previously (Nigro et al., 2002; Tannock et al., 2002).

Determination of Proteoglycan Size. Proteoglycans, labeled with [³⁵S]-sulfate, were isolated through DEAE-Sephacel anionic exchange mini columns (GE Lifesciences, Paramatta, Australia). Samples were washed with low-salt buffer (8 M urea, 0.25 M NaCl, 2 mM disodium EDTA, and 0.5% Triton X-100). Proteoglycans were eluted using high-salt buffer (8 M urea, 3 M NaCl, 2 mM disodium EDTA, and 0.5% Triton X-100). Aliquots (25,000 cpm) were precipitated (1.3% potassium acetate, 95% ethanol) and chondroitin sulfate was added as a cold carrier. Samples were resuspended in buffer (8 M urea, 2 mM disodium EDTA, pH 7.5), to which an equal volume of sample buffer was added. Radiolabeled proteoglycans were

separated on 4%–13% acrylamide gels with a 3% stacking gel. Processed and dried gels were scanned on a Cyclone Plus Phosphor Imager (Perkin Elmer, Melbourne, AUS).

Chromatin Immunoprecipitation Assay. Approximately, 6×10^6 cells were cultured in a serum-free medium for 48 hours before being treated with TGF- β (2 ng/ml) for 6 hours. For both flavopiridol and TGF- β treatment, cells were preincubated for 30 minutes with 500 nM flavopiridol before being treated with TGF- β for 6 hours. After TGF- β incubation, the cells were treated with 1.0% formaldehyde for 10 minutes at room temperature before harvesting, washed twice with cold phosphate-buffered saline, and resuspended in 1X lysis buffer (50 mM Tris-HCl, pH8.1, 10 mM EDTA, and 1.0% SDS) supplemented with protease inhibitors (Roche, Sydney, AUS). Cell suspensions were sonicated for 10 cycles (10 seconds for each cycle followed by 1 minute interval) using sonicator with half of its maximum capacity and centrifuged at 20,000g for 10 minutes. Extracts were diluted at 1:10 ratio with immunoprecipitation dilution buffer (50 mM Tris-HCl, pH8.1, 150 mM NaCl, 2 mM EDTA, and 1.0% TritonX-100) and incubated for 2 hours with 25 μ l of protein A/G beads (Santa Cruz Biotech, Shanghai, China) at 40°C. Extracts were centrifuged and the supernatants were reincubated overnight with anti-Smad antibody (Cell Signaling Technology) or corresponding control IgG at 4°C. DNA bound protein-antibody complexes were captured after further incubation with 50 μ l of protein A/G beads for 60 minutes. Complexes were washed once with TSE-I (20 mM Tris-HCl, pH8.1, 150 mM NaCl, 2 mM EDTA, 1.0% TritonX-100, and 0.1% SDS), four times with TSE-II (20 mM Tris-HCl, pH8.1, 500 mM NaCl, 2 mM EDTA, 1.0% TritonX-100, and 0.1% SDS), once with buffer-III (10 mM Tris-HCl, pH8.1, 1 mM EDTA, 1.0% Deoxycholate, 1.0% NP-40, and 0.25 M LiCl), and finally three times with TE (10 mM Tris-HCl and 1 mM EDTA). DNA-protein complexes were extracted after incubating twice (5 minutes each time) with freshly prepared extraction buffer (1.0% SDS and 100 mM NaHCO₃), and then incubated another 6 hours at 65°C to uncouple the protein-DNA complex. The DNA fragments were precipitated by adding twice the volume of 100% ethanol, 150 mM of sodium acetate, and 10 μ g of glycogen, and finally fragments were purified through a polymerase chain reaction purification kit (Invitrogen, Scoresby, AUS). Elutes were analyzed through RT-PCR to determine the enrichments. The polymerase chain reaction condition for ChSy-1 was 95°C for 2 minutes/95°C for 10 seconds, 56°C for 15 seconds, and 72°C for 30 seconds/40 cycles, while the polymerase chain reaction condition for C4ST-1 and xylosyltransferase-1 (XT-1) was 95°C for 2 minutes/95°C 10 seconds, 55°C for 15 seconds, and 72°C for 30 seconds/40 cycles.

Images. The experiments in this paper were conducted with multiple CDK inhibitors; however, the lack of specificity of these inhibitors makes interpretation of the data uncertain. Therefore, with the known actions of flavopiridol from our previous paper (Rostam et al., 2016) on Smad phosphorylation, we have presented the data only for flavopiridol, and thus the gel images have been prepared to only show the flavopiridol and relevant other control agents being the agonist (TGF- β) and control antagonist (SB431542).

Statistical Analysis. Data are presented as mean \pm S.E.M. and analyzed for statistical significance using one-way analysis of variance, followed by the least significant difference post hoc analysis. Results were considered statistically significant at $P < 0.05$ or $P < 0.01$, as indicated.

Results

Flavopiridol Concentration Dependently Inhibits the TGF- β -Stimulated mRNA Expression of GAG Synthesizing Enzymes in Human VSMCs. C4ST-1 and ChSy-1 are leading candidates to be rate limiting enzymes for the elongation of GAG chains, while XT-1 catalyzes the addition of a xylose residue to the serine in the biglycan core protein as the initial step in the formation of a GAG chain (Götting et al., 2000; Izumikawa et al., 2011). We investigated the effects of

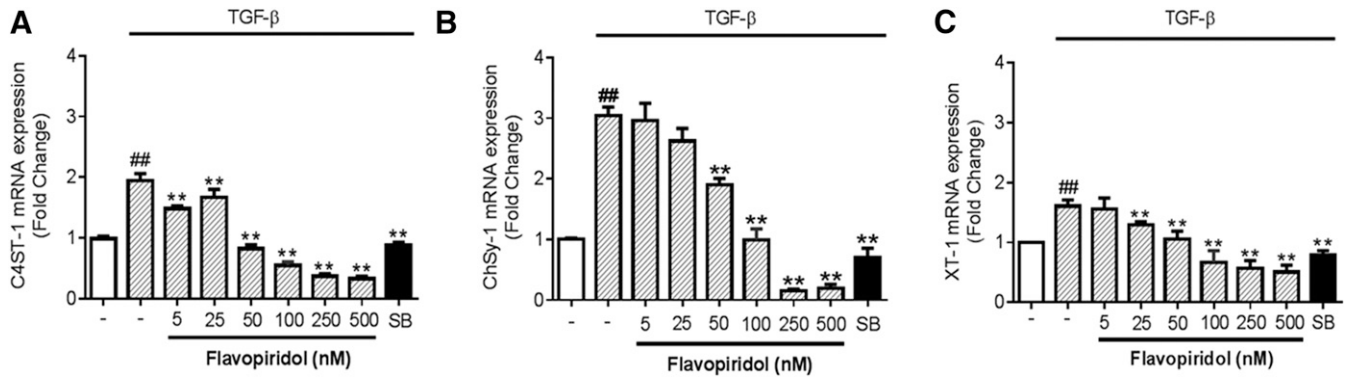


Fig. 1. Effect of flavopiridol on TGF- β -stimulated mRNA expression of GAG synthesizing enzymes (C4ST-1, ChSy-1, and XT-1). VSMCs were preincubated with flavopiridol (5–500 nM) or SB431542 (3 μ M) for 30 minutes before being treated with TGF- β (2 ng/ml) for 6 hours. The expression of (A) C4ST-1, (B) ChSy-1, and (C) XT-1 was assessed by real-time polymerase chain reaction. Histograms represent the mean \pm S.E.M. of three individual experiments, which indicate the fold change of mRNA expression compared with untreated control. ## P < 0.01 basal vs. TGF- β , ** P < 0.01 inhibitor vs. TGF- β . Experiments were analyzed using one-way analysis of variance.

flavopiridol on TGF- β -stimulated expression of these three GAG synthesizing enzymes (Fig. 1). VSMCs were treated with TGF- β (2 ng/ml) for 6 hours and the mRNA expression of C4ST-1, ChSy-1, and XT-1 was upregulated 2.0-fold, 3.0-fold, and 1.5-fold, respectively (P < 0.01) (Fig. 1) compared with untreated controls. Treatment of VSMCs with flavopiridol (5–500 nM) caused a concentration-dependent decrease in TGF- β -stimulated mRNA expression of C4ST-1, ChSy-1, and XT-1 (Fig. 1). The TGF β RI/Alk-5 inhibitor, SB431542 (Burch et al., 2010) abolished TGF- β -mediated C4ST-1, ChSy-1, and XT-1 mRNA expression (Fig. 1, lane 9).

Effect of Flavopiridol on TGF β RI/Alk-5-Stimulated Biglycan mRNA Expression in VSMCs. Biglycan is one of the major lipid binding CS/DS proteoglycans produced by VSMCs. Biglycan consists of a core protein to which two CS/DS GAG chains are covalently attached. Growth factors including TGF- β regulate the core protein and GAG chain synthesis (Ballinger et al., 2004; Osman et al., 2011). As a prelude to studies on the expression of GAG initiation and elongation genes, we also investigated the effects of flavopiridol on the TGF- β -stimulated synthesis of biglycan mRNA (Fig. 2) (Ballinger et al., 2004). TGF- β increases the expression of biglycan in VSMCs via the Akt pathway, but this pathway is not involved in GAG hyperelongation (Burch et al., 2010). TGF- β treatment increased biglycan mRNA expression 1.4-fold (P < 0.05) (Fig. 2). Both flavopiridol (1 μ M) and SB431542 (3 μ M) treatment totally inhibited TGF- β -stimulated biglycan mRNA expression (Fig. 2).

Flavopiridol Effects on TGF- β -Stimulated Biglycan Synthesis and GAG Hyperelongation. Although the elongation enzymes, C4ST-1 and ChSy-1, are purported or teleologically considered to be rate limiting in the synthesis and elongation of GAG chains, this has not been definitively demonstrated. Therefore, to confirm that the action of flavopiridol on the expression of these genes (C4ST-1 and ChSy-1) is functionally relevant, we assessed its effects at two concentrations on the size of biglycan molecules synthesized and secreted by TGF- β -stimulated human VSMCs. Thus, as an assessment of total proteoglycan synthesis being a combination of core protein expression and incorporation of sulfate into GAG chains (Ballinger et al., 2004), we used a cetylpyridinium chloride precipitation assay and measured the incorporation of radioactive sulfate [35 S]-SO $_4$ into secreted

proteoglycans (mostly biglycan) over 24 hours. Treatment with TGF- β increased [35 S]-SO $_4$ incorporation into secreted proteoglycans by 2-fold compared with untreated controls (Fig. 3, A and C). When VSMCs were treated with flavopiridol (1 μ M) for 24 hours, there was complete inhibition of [35 S]-SO $_4$ incorporation into proteoglycans (Fig. 3A). Flavopiridol (10 μ M) also elicited a similar effect (Fig. 3C). The TGF β RI/Alk-5 inhibitor SB431542 completely inhibited TGF- β -stimulated [35 S]-SO $_4$ incorporation into proteoglycans in these cells (Fig. 3, A and C).

Assessment of the apparent size of biglycan molecules provides evidence of changes in the size of the GAG chains because the size of the core protein is fixed and only the CS/DS chain size can vary (Little et al., 2002; Ballinger et al., 2004). Biglycan synthesized and secreted by TGF- β -treated cells showed a marked decrease in electrophoretic mobility (corresponding to an increase in the apparent size of the molecules) compared with biglycan synthesized and secreted by untreated cells (Fig. 3, B and D). In the presence of flavopiridol

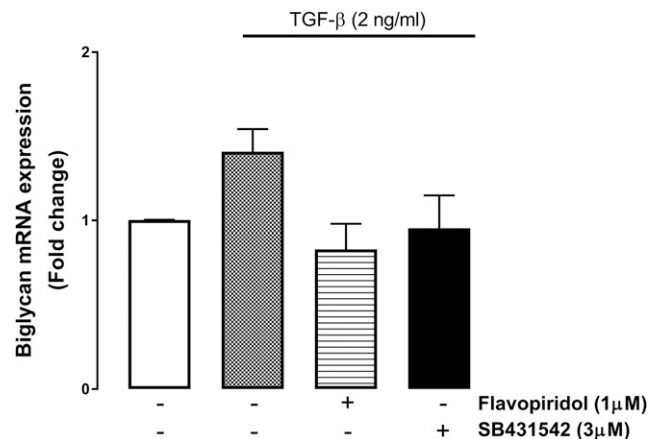


Fig. 2. Flavopiridol and biglycan mRNA. VSMCs were preincubated in the presence and absence of flavopiridol (1 μ M), and an Alk-5 inhibitor SB431542 (3 μ M) for 30 minutes, followed by treatment with TGF- β (2 ng/ml) for 4 hours. The histogram shows the mean fold change \pm S.E.M. from three individual experiments compared with untreated control. ## P < 0.01 basal vs. TGF- β , ** P < 0.01; * P < 0.05 inhibitor vs. TGF- β , using one-way analysis of variance.

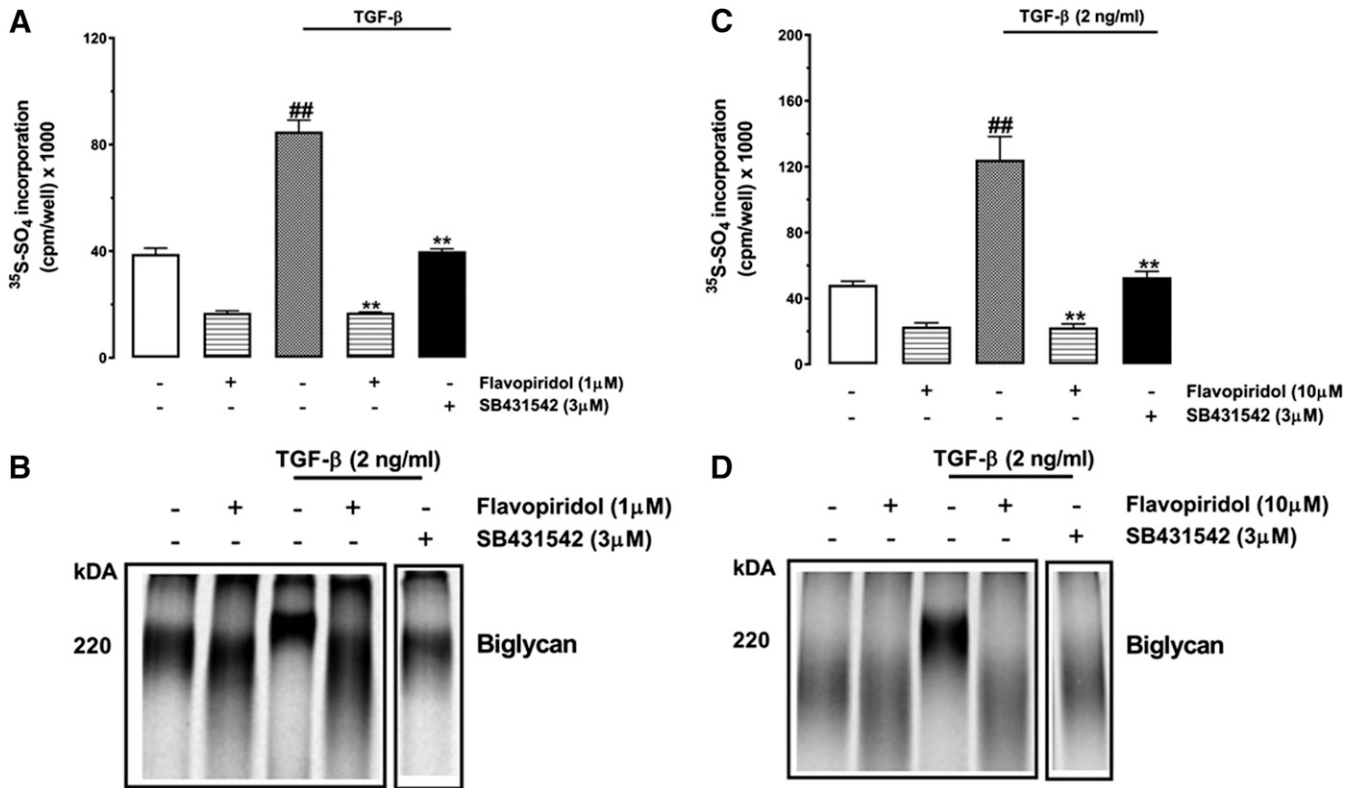


Fig. 3. Effect of flavopiridol on TGF- β -stimulated radiolabeled incorporation into proteoglycans. (A) VSMCs were treated with or without flavopiridol (1 μ M) and SB431542 (3 μ M) for 24 hours in presence of TGF- β (2 ng/ml) and [35 S]-SO $_4$ (50 μ Ci/ml). Medium containing secreted proteoglycans was harvested, spotted onto 3MM paper, and quantitated by cetylpyridinium chloride precipitation to measure radiolabel incorporation. (B) Secreted proteoglycans were isolated using ion exchange chromatography (DEAE-Sephacel), concentrated by ethanol/potassium acetate precipitation, and electrophoresed on 4%–13% SDS-PAGE. The histogram shows the mean \pm S.E.M. of the fold change compared with untreated control. $^{##}P < 0.01$ basal vs. TGF- β , $^{**}P < 0.01$; $^{*}P < 0.05$ inhibitor vs. TGF- β , $n = 3$ experiments using one-way analysis of variance. (C) Similar treatment as indicated in (A) with flavopiridol 10 μ M. (D) Similar method as detailed in (B) with flavopiridol at 10 μ M.

(1 and 10 μ M), the effect of TGF- β treatment was blocked such that there was an increase in biglycan electrophoretic mobility (Fig. 3, B and D). VSMCs treated with TGF- β in the presence of SB431542 had a similar biglycan size to control cells (Fig. 3, B and D). These results demonstrate that the increase in the size of biglycan molecules in TGF- β -stimulated VSMCs is blocked by flavopiridol. It can also be noted in these experiments that TGF- β stimulation increases the expression of biglycan core proteins consistent with the mRNA expression data shown (Fig. 2). In the SDS-PAGE shown in Fig. 3, lane 3 versus lane 1, strong darkening of the band consistent with higher levels of biglycan can be seen, and this effect is blocked by flavopiridol (Fig. 3, lane 4 vs. lane 3). The data show that the effects of flavopiridol on the expression of C4ST-1 and ChSy-1 correlate with the effects on the size of biglycan molecules, which is consistent with the proposition that the activity of these two enzymes is rate limiting for the synthesis and elongation of GAG chains.

Effect of Flavopiridol on TGF- β -Stimulated Smad Linker Region Phosphorylation of Specific Serine and Threonine Sites. As a starting point for the investigation of the role of Smad linker region phosphorylation in TGF- β -stimulated GAG gene expression and GAG hyperelongation, we investigated its effect on the phosphorylation of the cluster of serine residues (Ser245, 250, and 255) as well as the threonine site (Thr220) in the linker region of Smad2. Western

blotting was used to determine the time course of TGF β RI-mediated Smad2 linker region phosphorylation. The level of phospho-Smad2L (Ser245, 250, and 255) reached a peak 1 hour post-TGF- β treatment (Fig. 4A). Treatment with TGF- β for 1 hour increased the phosphorylation of Smad2L (Ser245/Ser250/Ser255) by 2.7-fold (Fig. 4B), while flavopiridol (1 μ M) treatment inhibited this TGF- β -mediated phosphorylation. SB431542 (3 μ M) inhibited the TGF- β -mediated phosphorylation of Smad2L (Fig. 4B).

Site-specific antibody for phospho-Thr220 was used to investigate the involvement of the Thr220 residue (Matsuzaki et al., 2009; Kamato et al., 2014). We have previously reported that TGF- β treatment stimulates the phosphorylation of Thr220 with a peak response at 1 hour (Rostam et al., 2016). In the current experiments, TGF- β (2 ng/ml) stimulation of VSMCs resulted in a 2.3-fold increase in phospho-Smad2L (Thr220) (Fig. 5). Flavopiridol completely prevented the TGF- β -induced phosphorylation of Smad2L (Thr220). These results indicate the phosphorylation of multiple Smad linker region phosphorylation sites that can be blocked by flavopiridol are most likely responsible for the effects of TGF- β on the expression of GAG synthesizing genes and GAG hyperelongation. The temporal aspects of the phosphorylation of the Ser and Thr sites were sufficiently different to suggest different signaling pathways; however, the total inhibition of both pathways by flavopiridol highlights its broad inhibitory effects.

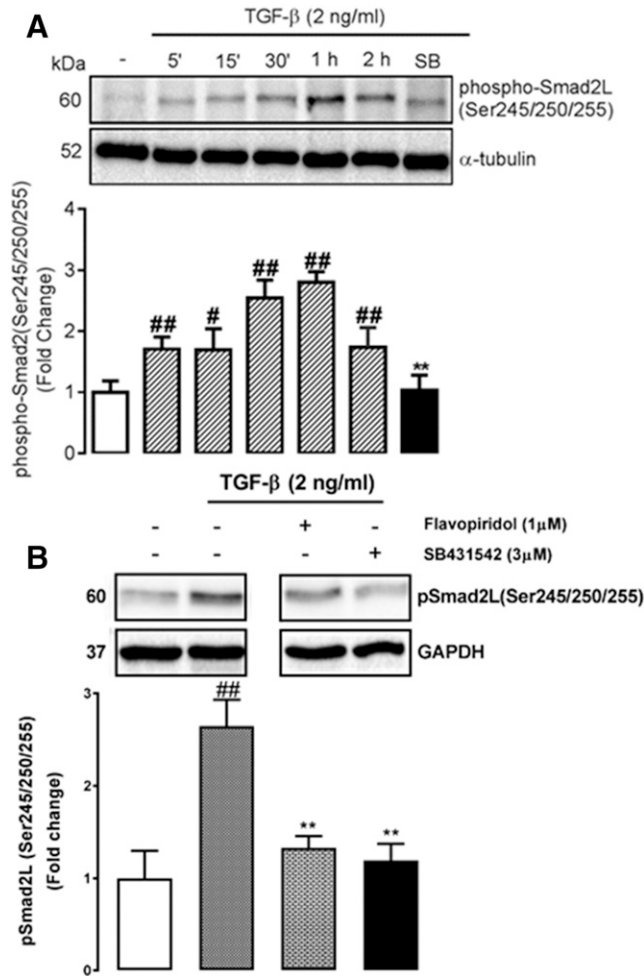


Fig. 4. TGF- β -mediated phosphorylation of Smad2 linker region (Ser245/250/255) in human VSMCs. (A) Time course (0–2 hours) of TGF- β (2 ng/ml) on phospho-Smad2L levels in VSMCs. Western blot of cell lysates were probed with primary antibody phospho-Smad2L (Ser245/250/255) rabbit polyclonal or α -tubulin rabbit polyclonal antibody followed by peroxidase-labeled Rabbit IgG secondary antibody. (B) Effects of flavopiridol on TGF- β /Alk-5-mediated Smad2L phosphorylation in VSMCs. Cells were treated with TGF- β (2 ng/ml) for 1 hour in the presence and absence of flavopiridol (1 μ M) as well as the Alk-5 inhibitor SB431542 (3 μ M). The membrane was probed with phospho-Smad2L (Ser245/250/255) as detailed in (A). The Western blot is representative of three independent experiments. The histogram is a densitometric quantitation of the three independent experiments and indicates the mean \pm S.E.M. of the fold change over that observed in the untreated control. ## P < 0.01 basal vs. TGF- β , ** P < 0.01; * P < 0.05 inhibitor vs. TGF- β , using one-way analysis of variance.

Smads Bind to Promoter Regions of GAG Synthesizing Genes in a Flavopiridol-Dependent Manner. The role of linker region phosphorylation of Smads in determining phosphor-Smad distribution, and specifically in modulating Smad nuclear translocation, is a major unresolved question in TGF- β signaling and cell biology. We previously observed dose-dependent inhibition of TGF- β -stimulated C4ST-1, ChSy-1, and XT-1 mRNA expression in the presence of flavopiridol (Fig. 1). Considering the Smad linker region phosphorylation results presented in Figs. 4 and 5, these data suggest that flavopiridol-dependent linker region Smad phosphorylation is critical for the TGF- β -driven expression of GAG synthesizing genes. Analysis of the promoter region sequences of GAG genes investigated in this study revealed single

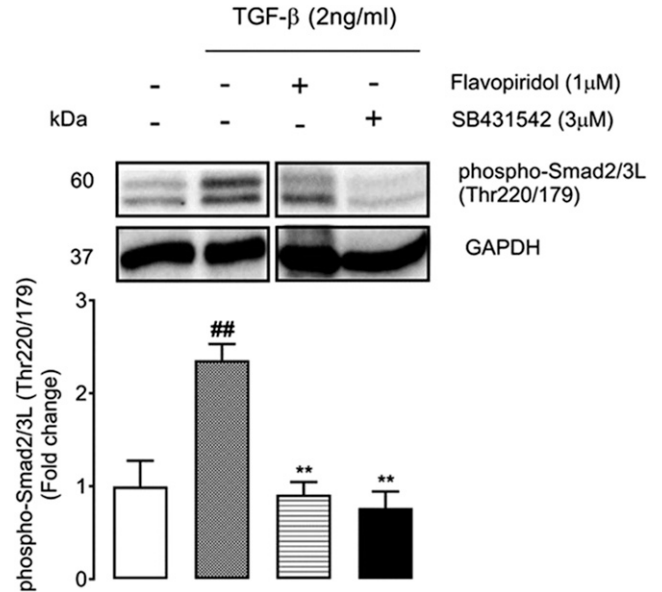


Fig. 5. The effects of flavopiridol on TGF- β /Alk-5 mediated Smad2L (Thr220) phosphorylation in VSMCs. VSMCs were preincubated for 30 minutes in the presence of flavopiridol (1 μ M) or the Alk-5 inhibitor SB431542 (3 μ M) with TGF- β (2 ng/ml) stimulation for 1 hour. Western blot is representative of cell lysates probed with a phospho-Smad2L (Thr220) rabbit polyclonal primary antibody, followed by peroxidase-labeled Rabbit IgG secondary antibody together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) HRP-conjugated monoclonal antibody for equal loading. Histograms are a densitometric quantitation of three independent experiments and show the mean \pm S.E.M. of the fold change of expression relative to untreated controls. ## P < 0.01 basal vs. TGF- β , ** P < 0.01; * P < 0.05 inhibitor vs. TGF- β , using one-way analysis of variance.

consensus Smad binding sites were present 600 base pairs upstream of the C4ST-1 and ChSy-1 initiation sites, but no equivalent Smad binding site was present in the promoter region of the XT-1 gene. XT-1 expression could thus serve as a negative control for studies of Smad binding to C4ST-1 and ChSy-1 genes. We performed chromatin immunoprecipitation assays to determine the enrichment of Smad binding in that region after TGF- β treatment. We observed a marked increase of Smad binding in the promoter of C4ST-1 (>20-fold) and ChSy-1 (>5-fold) genes and the increased binding was absent for the XT-1 gene (Fig. 6). In VSMCs pretreated with flavopiridol (500 nM), TGF- β -stimulated Smad enrichment was markedly reduced for both the C4ST-1 and ChSy-1 genes. In relation to XT-1 expression, since there was no stimulation with TGF- β there was no inhibition by flavopiridol. These data demonstrate that TGF- β -stimulated GAG gene expression is mediated directly by Smad transcription factors that are linker region polyphosphorylated by a flavopiridol-sensitive kinase, most likely a CDK.

Discussion

Proteoglycans with structural modifications are implicated in the early stages of the atherosclerotic process and the pathways regulating their synthesis and properties are potential targets for the development of therapeutic agents. Such a therapeutic agent would block GAG hyperelongation and reduce the binding of the proteoglycan to LDL and such a therapeutic approach would be used in tandem with a

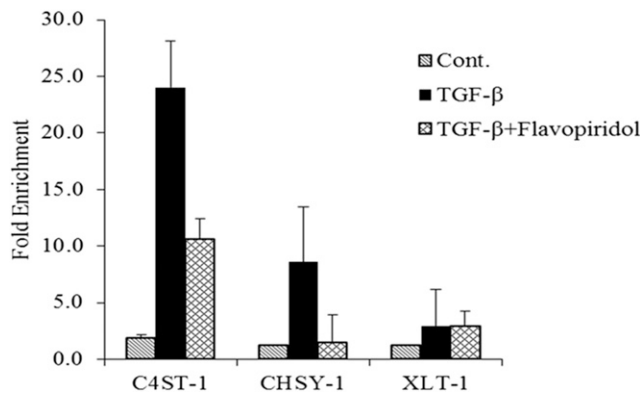


Fig. 6. TGF- β -induced GAG gene expression is associated with increased Smad binding in the promoter region of C4ST-1 and ChSy-1 but not in XT-1. VSMCs were treated with TGF- β (2 ng/ml) for 6 hours to stimulate gene activation. To determine the effect of flavopiridol on Smad binding, cells were preincubated with flavopiridol before they were treated with TGF- β for 6 hours. Extracts were used to perform chromatin immunoprecipitation assays by using anti-Smad antibody at 1:50 dilution or normal rabbit IgG (1.0 μ g) as control. The amount of Smad binding was estimated by RT-PCR by using gene-specific primers described in the *Materials and Methods*. For each gene, Smad enrichment was represented as fold change in comparison with the corresponding control IgG. Data presented here are the mean \pm S.D. of two independent experiments and in each case RT-PCR was performed at least twice to confirm the reproducibility of the results.

3-hydroxy-3-methyl-glutaryl-coenzymeA (HMG-CoA) reductase inhibitor (statin), which reduces blood cholesterol levels (Little et al., 2007, 2008, 2011). TGF- β plays a role in atherosclerosis and in this context it is a potent and highly effective agent to mediate hyperelongation of GAG chains on the proteoglycan, biglycan. TGF- β signals via its type I and II cell surface Ser/Thr kinase receptors and on to regulation of the phosphorylation of regulatory Smads (Smad2/3), which pass to the nucleus and regulate gene transcription (Derynck and Zhang, 2003; Massagué et al., 2005). There are multiple pathways for the regulation of Smads (Burch et al., 2011). Phosphorylation of Smad2 in the linker region has emerged as a pathway regulating the expression of genes for enzymes that mediate GAG elongation. In this study, we investigated the role of Smad linker region phosphorylation in mediating the expression of GAG elongation genes in human VSMCs using flavopiridol, a well-known CDK inhibitor that inhibited the increased expression of these genes (Rostam et al., 2016).

We show that flavopiridol inhibits 1) TGF- β stimulation of the expression of genes that are considered to be rate limiting for the elongation of GAG chains on biglycan, and 2) TGF- β stimulated increase in the size of the biglycan molecules, which reflects the increased size of the CS/DS GAG chains (Little et al., 2002, 2010, 2013). TGF- β treatment increased the phosphorylation of the serine triplet as well as the isolated threonine residue in the linker region of Smad2 (Rostam et al., 2016). We also found that the linker region and the carboxy-terminal phosphorylated Smad bound to the critical regions of the genes for C4ST-1 and ChSy-1 but not XT-1, exactly as predicted from the structural analysis of these regions. The increased binding of the linker region of phosphorylated Smad2 to the promoter region of C4ST-1 and ChSy-1 shows that this transcription factor can pass to the nucleus and regulate—in our case upregulate—gene transcription. Our data thus strongly support the notion that the Smad linker

region is a site of integration of TGF- β signaling as well as mediating transcriptional regulation of GAG synthesizing genes in the cell nucleus.

Linker region phosphorylation was originally demonstrated to be inhibitory for TGF- β signaling by blocking the translocation of this phosphorylated entity to the cell nucleus (Kretzschmar et al., 1999). Our data clearly show that for the TGF- β -mediated regulation of GAG gene expression the Smad linker region facilitates and does not inhibit this response. These discrepancies in gene expression and cellular responses require further analysis of TGF- β signaling and the role of Smad linker region phosphorylation as a master regulator and integrator. Indeed, in view of the clear role of linker region phosphorylation in mediating the upregulation of gene expression, we also suggest that it is worth readdressing the role of carboxy terminal phosphorylation in TGF- β signaling.

In VSMCs, GAG hyperelongation by TGF- β stimulation is dependent on both transcription and translation (Yang et al., 2009). GAG synthesizing enzymes (C4ST-1, ChSy-1, and XT-1) are responsible for sulfation and GAG chain (hyper) elongation; multiple other hormones and growth factors stimulate GAG elongation (Ballinger et al., 2009; Getachew et al., 2010). In many studies, the vascular endothelial growth factor stimulates GAG elongation, but not in retinal endothelial cells (Al Gwairi et al., 2016). In relation to the in vivo relevance of these findings, Anggraeni et al. (2011) have shown that the mRNA expression of some GAG synthesizing genes increases over 8 weeks in an atherosclerotic mouse model and this increase correlates with increased atherosclerosis in mice.

TGF- β -mediated proteoglycan synthesis in VSMCs involves Smad2 linker region phosphorylation (Burch et al., 2010). The Smad3 linker region is a target for CDK2 and CDK4 phosphorylation (Matsuura et al., 2004). In epithelial cells, TGF β RI and Ras-associated kinases, including Erk, c-Jun NH₂-terminal kinase, and CDK4 (Matsuura et al., 2004) differentially phosphorylate Smad2/3 in the carboxy-terminus, linker region, or both (Matsuzaki et al., 2009). Smad2/3 phospho-isoforms can differentially interact with Smad4 to either translocate or be blocked from entering the nucleus to initiate gene transcription (Derynck and Zhang, 2003). Kinase-specific phosphorylation of Smad2/3 isoforms creates a complex signaling cascade that regulates the switching of TGF- β -mediated tumor suppressive effects in early stages of cancer to advanced carcinomas (Matsuzaki, 2011). In VSMCs, the specificity of these signaling pathways is not well understood; however, it is important and further analyses will facilitate better understanding of the cell biology of TGF- β signaling and the identification of new therapeutic targets—perhaps the kinases that are in the cascades leading to Smad linker region phosphorylation—for the treatment of atherosclerosis (Little et al., 2007).

In vascular endothelial cells, Smad2 linker region residues are phosphorylated by different Ser/Thr kinases that alter plasminogen-activator inhibitor 1 mRNA expression (Kamato et al., 2014). Here, we used two antibodies, with one detecting the cluster of serine residues (Ser245/250/255) and the second detecting the phosphorylated threonine residue (Thr220). Flavopiridol blocked all of these phosphorylations; therefore, we are not able to describe a relationship between individual residues that are phosphorylated and the expression of

individual GAG synthesizing enzymes, but this work is currently under way in our laboratory.

Although there have been many studies on the role played by Smads in the expression of genes (Matsuzaki, 2013; Morikawa et al., 2013), there are none for GAG genes showing the definitive involvement of these transcription factors. Indeed, the current favored hypothesis is that linker region phosphorylation of Smad2/3 prevents the translocation of the Smad entity to the nucleus, whereas our data indicate a correlation between the phosphorylation status of the linker region and increased expression of GAG elongation genes. This is an important point in the understanding of the cell biology of TGF- β signaling. To investigate this point we used chromatin immunoprecipitation assays to assess the direct binding of Smads to promoter regions of three GAG synthesizing genes. We determined bioinformatically that the two GAG genes, C4ST-1 and ChSy-1, possessed Smad binding sites, whereas the GAG chain initiation enzyme XT-1 did not possess this Smad binding site. TGF- β treatment of VSMCs led to a marked increase in Smad enrichment on the C4ST-1 and ChSy-1 genes but not on the XT-1 gene. Both of these responses were attenuated in cells treated with flavopiridol. These data strongly suggest that there is direct activation of Smad-mediated GAG gene expression by TGF- β and that this is dependent on Smad linker region phosphorylation (as described previously). It is noted that studies of XT-1 expression after TGF- β treatment showed small but statistically significant increases (see Fig. 6). This indicates that there are Smad independent pathways of TGF- β -stimulated GAG gene expression, which is not inconsistent with our previous findings of the existence of multiple pathways mediating the effects of TGF- β in VSMCs.

The therapeutic rationale for these studies is that there is the potential for a specific kinase inhibitor or an inhibitor of multiple kinases (Bernard et al., 2016) to be a therapeutic agent for the reduction in atherosclerosis and the prevention of cardiovascular disease (Little et al., 2007). It can also be mentioned that there is an alternative approach of targeting GAG chain biosynthesis, which is to use GAG-directed antibodies that block the ionic interaction between GAG chains and ApoB100; the veracity of this approach has recently been demonstrated in a mouse model of intimal hyperplasia, where the accelerated atherosclerosis was shown to be due to lipoprotein binding to modified proteoglycans and the interaction—and hence the atherosclerosis—could be blocked by GAG-directed antibodies (Kijani et al., 2017). It follows in this area that an anti-proteoglycan, or more specifically an anti-GAG agent, would work in therapeutic tandem with a HMG-CoA reductase inhibitor such as a statin. Here, the statin would reduce the blood cholesterol level (Gotto, 2002), and a proteoglycan synthesis inhibitor capable blocking the hyperelongation of GAG chains on biglycan or the interaction between GAG chains and LDL in the vessel wall would render vessel wall less sticky for atherogenic lipoproteins (Ballinger et al., 2010). The impact of a CDK inhibitor on normal physiology needs to be considered. In clinical practice CDK inhibitors are associated with a range of adverse effects that include secretory diarrhea and a proinflammatory syndrome (Senderowicz and Sausville, 2000). Thus, the potential exists for CDK inhibitors, such as flavopiridol and related agents, to have a variety of effects beyond the identified therapeutic target, which in this case is the role of

hyperelongated GAG chains in the etiology of the early stages of atherosclerosis. The need will be for studies in animal models and later in translational studies to establish if a highly specific inhibitor of the signaling pathways mediating GAG hyperelongation can successfully target proteoglycan changes in the vessel wall associated with the early stages of atherosclerosis at doses that do not generate unwanted side effects. We have already provided proof-of-concept data for this hypothesis (Ballinger et al., 2010; Getachew et al., 2010) and now we need to determine the optimum target in the signaling pathways that mediate growth factor effects of GAG synthesizing enzyme gene expression and GAG hyperelongation in human VSMCs.

Authorship Contributions

Participated in research design: Little, Osman, Cao, Piva, Zheng.
Conducted experiments: Rostam, Shajimoon, Getachew, Mitra.
Performed data analysis: Rostam, Getachew, Mitra, Kamato.
Wrote or contributed to the writing of the manuscript: Rostam, Little, Osman, Cao, Zheng, Kamato, Piva.

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