

# Smad and p38 MAP Kinase-mediated Signaling of Proteoglycan Synthesis in Vascular Smooth Muscle\*

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Atherosclerosis is the underlying pathological process of most cardiovascular disease. A critical component of the "response to retention" hypothesis of atherogenesis is proteoglycan/low density lipoprotein (LDL) binding. Transforming growth factor  $\beta$  (TGF- $\beta$ ) is present in atherosclerotic lesions, regulates vascular smooth muscle cell (VSMC) proteoglycan synthesis via an unknown signaling pathway, and increases proteoglycan/LDL binding. This pathway was investigated using the activin receptor-like kinase 5 (ALK5) inhibitor SB431542 and inhibitors of p38 MAP kinase as a possible downstream or alternative mediator. TGF- $\beta$  stimulated and SB431542 inhibited the phosphorylation of Smad2/3. In human VSMC, TGF- $\beta$  increased [<sup>35</sup>S]sulfate incorporation into proteoglycans associated with a 19% increase in glycosaminoglycan (GAG) chain size by size exclusion chromatography. SB431542 caused a concentration-dependent decrease in TGF- $\beta$ -mediated [<sup>35</sup>S]sulfate incorporation with 92% inhibition at 3  $\mu$ M. Two different p38 MAP kinase inhibitors, SB203580 and SB202190, but not the inactive analogue SB202474, concentration-dependently blocked TGF- $\beta$ -mediated [<sup>35</sup>S]sulfate incorporation. TGF- $\beta$  increased [<sup>3</sup>H]glucosamine incorporation into glycosaminoglycans by 180% and [<sup>35</sup>S]Met/Cys incorporation into proteoglycan core proteins by 35% with both effects completely inhibited by SB431542. Blocking both Smad2/3 and p38 MAP kinase pathways prevented the effect of TGF- $\beta$  to increase proteoglycan to LDL binding. TGF- $\beta$  mediates its effects on proteoglycan synthesis in VSMCs via the ALK5/Smad2/3 phosphorylation pathway as well as via the p38 MAP kinase signaling cascade. Further studies of downstream pathways controlling proteoglycan synthesis may identify potential therapeutic targets for the prevention of atherosclerosis and cardiovascular disease.

Cardiovascular disease is currently the leading cause of death and illness globally (1, 2). Atherosclerosis is the underlying cause of this growing burden of cardiovascular disease (3), which results in conditions such as heart attack and stroke (4). Current therapies are based mostly on reducing risk factors

such as hypercholesterolemia, but none achieves greater than 30% efficacy in clinical trials (5). No therapy directed at the vessel currently exists, but it is postulated that a vessel wall-directed therapy may act synergistically with a risk factor-directed strategy to reduce the progression of atherosclerosis.

Nakashima *et al.* (6) have recently provided definitive evidence that human atherosclerosis commences with the deposition of lipid in the outer area of diffuse intimal thickenings; furthermore, they show that this retention and deposition are associated with expression of the proteoglycan, biglycan. Lipoproteins bind to GAG<sup>2</sup> and not core proteins (7), and thus changes in GAG structure are most likely responsible for lipid binding and retention. TGF- $\beta$  is strongly implicated in atherosclerosis (8). We have demonstrated that TGF- $\beta$  elongates GAG chains on (VSM)-derived proteoglycans; these GAG chains, as intact proteoglycans or free GAG chains, show enhanced binding to human LDL (7).

Inhibition of GAG elongation represents a potential new class of agents to prevent lipid deposition and atherosclerosis. Hence a detailed knowledge of the agonists and the signaling pathways that control GAG synthesis and structure is required to provide information to allow for the rational development of an agent that may be specific for inhibiting GAG elongation in VSM.

TGF- $\beta$  transduces its effects from the cell membrane to the nucleus through distantly related but structurally similar transmembrane Ser/Thr kinase receptors known as type I receptors (T $\beta$ R-I/ALK5), constitutively active type II receptors (T $\beta$ R-II) (9, 10), and their downstream effectors, known as Smad proteins (11–14). In arterial smooth muscle cells TGF- $\beta$  up-regulates biglycan mRNA (15), and in the pancreatic cell line PANC-1, TGF- $\beta$ -induced biglycan up-regulation has been demonstrated to be mediated by p38 MAP kinase signaling (16, 17). Recent progress in discovering the TGF- $\beta$  signaling cascade makes it possible to attempt therapeutic strategies by disrupting the signaling cascade (18). Nothing is known of the TGF- $\beta$  signaling pathway that controls GAG synthesis in VSM. We addressed the question of whether the TGF- $\beta$  receptor sig-

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<sup>2</sup> The abbreviations used are: GAG, glycosaminoglycan; TGF- $\beta$ , transforming growth factor  $\beta$ ; VSM, vascular smooth muscle; VSMC, vascular smooth muscle cell; ALK, activin receptor-like kinase; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; ANOVA, analysis of variance; MAP, mitogen-activated protein; LDL, low density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CPC, cetyl pyridinium chloride; MOPS, 4-morpholinopropanesulfonic acid.

naling pathway involving Smad 2/3 phosphorylation is involved in the regulation of GAG chain synthesis in VSM and whether p38 MAP kinase signaling is involved. We further evaluated whether the TGF- $\beta$ -mediated enhancement of LDL binding is prevented by inhibiting the TGF- $\beta$  signaling pathway.

## EXPERIMENTAL PROCEDURES

**Materials**—Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen Corp., fetal bovine serum (FBS) and penicillin-streptomycin-fungizone solution from CSL Ltd., and trypsin-versene from JRH Biosciences Inc. 90-mm and 60-mm plates, 75-cm<sup>3</sup> flasks, and 24-well plates were from BD Biosciences. Human TGF- $\beta$  and anti-human TGF- $\beta$ 1/2/3 monoclonal antibody were from R&D Systems, Minneapolis, MN. PDGF, SB431542, 6-amino caproic acid, benzamidine hydrochloride, methyl- $\beta$ -D-xylopyranoside (xyloside), DEAE-Sepharcel, Sepharose CL-6B, proteinase K, sodium orthovanadate, and sodium borohydride were from Sigma. Imatinib was purchased as Glivec capsules from Novartis. The specific p38 inhibitors SB203580 and SB202190 and the negative control inhibitor SB202474 were purchased from Calbiochem. Sulfur-35-Na<sub>2</sub>SO<sub>4</sub> ([<sup>35</sup>S]sulfate), Tran<sup>35</sup>S-label ([<sup>35</sup>S]Met/Cys), D-glucosamine-HCL, [6-<sup>3</sup>H] ([<sup>3</sup>H]glucosamine), and [<sup>3</sup>H]thymidine were from MP Biomedicals. Cetyl pyridinium chloride (CPC) was from Unilab Chemicals and Pharmaceuticals. Whatman No. 3MM chromatography paper was from Biolab. Instagel-plus scintillation fluid was from PerkinElmer Life Sciences, Poly-Prep columns from Bio-Rad, and BCA protein assay from Pierce-Perbio. Rainbow <sup>14</sup>C-methylated protein molecular weight marker was from Amersham Biosciences. Smooth muscle  $\alpha$ -actin antibody was from Dako and phospho-Smad2/3 (Ser<sup>465/467</sup>) rabbit polyclonal antibody from chemicon. The ECL Western blotting analysis system, Hyperfilm<sup>TM</sup>ECL, anti-rabbit IgG, and anti-mouse IgG were from Amersham Biosciences.

**Cell Culture**—Human internal mammary artery and saphenous vein VSMCs were derived from discarded vessels at the Alfred Hospital approved by Alfred Hospital Ethics Committee. Cells were maintained in DMEM supplemented with low glucose (5 mM), 10% FBS, and antibiotics. For experimentation, cells between passages 6 and 15 were cultured. Confluent cultures were deprived of serum by incubation in DMEM low glucose with 0.1% FBS for 48 h prior to treatment.

**Quantitation of Radiolabel Incorporation into Proteoglycans**—Quiescent cells were treated in 5 mM glucose-DMEM, 0.1% FBS, 0.1% Me<sub>2</sub>SO with SB431542 (3  $\mu$ M), SB203580 (1–10  $\mu$ M), SB202190 (1–10  $\mu$ M), or SB202474 (1–10  $\mu$ M) and exposed to [<sup>35</sup>S]sulfate (50–100  $\mu$ Ci/ml) or [<sup>3</sup>H]glucosamine (10  $\mu$ Ci/ml) under basal conditions or in the presence of TGF- $\beta$  (2 ng/ml) for 24 h unless stated otherwise. Secreted proteoglycans were harvested, and radiolabel incorporation into proteoglycans was quantitated using the CPC precipitation assay (19).

**Chemical Cleavage of GAG Chains**—Human VSMCs were rendered quiescent and treated in 0.5 ml DMEM, 0.1% FBS with SB431542 (3  $\mu$ M) under basal conditions and in the presence of TGF- $\beta$  (2 ng/ml) for 4 h prior to the addition of [<sup>35</sup>S]sulfate (50  $\mu$ Ci/ml) for a further 24 h. Secreted proteoglycans were harvested, isolated, and concentrated as described previously (20).

To cleave the GAG chains chemically through a  $\beta$ -elimination reaction, pelleted proteoglycans were treated with sodium borohydride (1 M) in NaOH (50 mM) for 24 h at 45 °C. The reaction was terminated with glacial acetic acid.

**Synthesis of Xyloside-initiated GAG Chains**—Quiescent human VSMCs were treated in 0.5 ml DMEM, 0.1% FBS supplemented with methyl  $\beta$ -D-xylopyranoside (xyloside) (0.5 mM) with SB431542 (3  $\mu$ M) under basal conditions in the presence of TGF- $\beta$  (2 ng/ml) for 4 h prior to the addition of [<sup>35</sup>S]sulfate (50  $\mu$ Ci/ml) for a further 24 h. Secreted proteoglycans were harvested, isolated, and concentrated as described previously (20).

**Size Analysis of Proteoglycan/GAG Length by SDS-PAGE and Size Exclusion Chromatography**—Proteoglycans, cleaved GAG chains, and xyloside-associated GAG chains were sized by SDS-PAGE as described previously (19). Cleaved GAG chains and xyloside-associated GAG chains were sized on Sepharose CL-6B columns eluted in guanidine buffer as described previously (21). Data were standardized by calculating  $K_{av}$  values.

**Thymidine Incorporation into DNA**—VSMCs were treated with PDGF (50 ng/ml) and other agents for 16 h, after which time [<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml) was added for 3 h at 37 °C. Cells were washed with ice-cold Dulbecco's phosphate-buffered saline (with Ca<sup>2+</sup> and Mg<sup>2+</sup>), incubated on ice for 30 min with 0.2 M HClO<sub>4</sub> to precipitate DNA and then washed with HClO<sub>4</sub>. Cells were digested by the addition of 0.2 M NaOH for 1 h at 37 °C, and the reaction was neutralized using 6% acetic acid. Radioactive incorporation was assessed on a liquid scintillation counter.

**Neutralization of TGF- $\beta$  with anti-TGF- $\beta$ 1/2/3 Antibody**—VSMCs were incubated with anti-human-TGF- $\beta$ 1/2/3 monoclonal antibody (1–50-fold molar excess) for 40 min prior to treatment with growth factor and radiolabeling to determine the concentration of antibody required to neutralize TGF- $\beta$ . Mouse IgG was used as an isotype control. In subsequent experiments, a 20-fold molar excess of anti-TGF- $\beta$ 1/2/3 antibody was used.

**Western Blotting**—Total cell lysates were resolved on 10% SDS-PAGE and transferred onto polyvinylidene difluoride. Membranes were blocked with 5% skim milk powder and incubated with anti-phosphorylated Smad2/3 (pSmad2/3) rabbit polyclonal antibody followed by horseradish peroxidase-anti-rabbit IgG and ECL detection. Blots from three experiments were quantified by densitometry using NIH ImageJ software.

**Proteoglycan/LDL Binding by Gel Mobility Shift Assay**—Human VSMCs were cultured, rendered quiescent as described, and treated with SB431542 (3  $\mu$ M) or SB202190 (10  $\mu$ M) in the presence of TGF- $\beta$  (2 ng/ml) for 12 h, at which time fresh treatments were added with [<sup>35</sup>S]Met/Cys (50  $\mu$ Ci/ml) to radiolabel proteoglycan core proteins (37 °C, 24 h). The secreted proteoglycans were harvested and isolated as described and dialyzed into a physiological buffer (20 mM MOPS, 140 mM NaCl, 5 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, pH 7.4). Increasing concentrations of LDL (0–0.5 mg/ml) purified from human blood (22) were incubated with equal counts (1250 cpm) of proteoglycans for 1 h at 37 °C. Samples were run on flat-bed agarose gels (0.7%) as described previously (23).

**Statistical Analysis**—Normalized data are presented as the mean  $\pm$  S.E. of three independent experiments conducted in

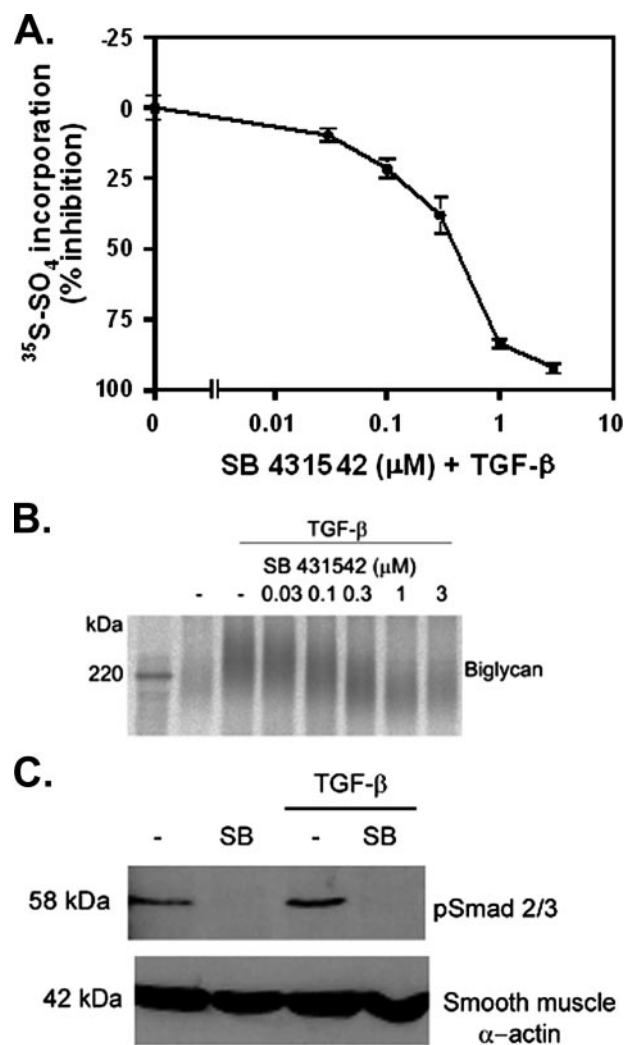
## Smad and p38 Signaling of Proteoglycan Synthesis

triplicate unless stated otherwise. Data were analyzed for statistical significance using a one-way analysis of variance (ANOVA), a two-way ANOVA, or a Student's paired *t* test as stated.

### RESULTS

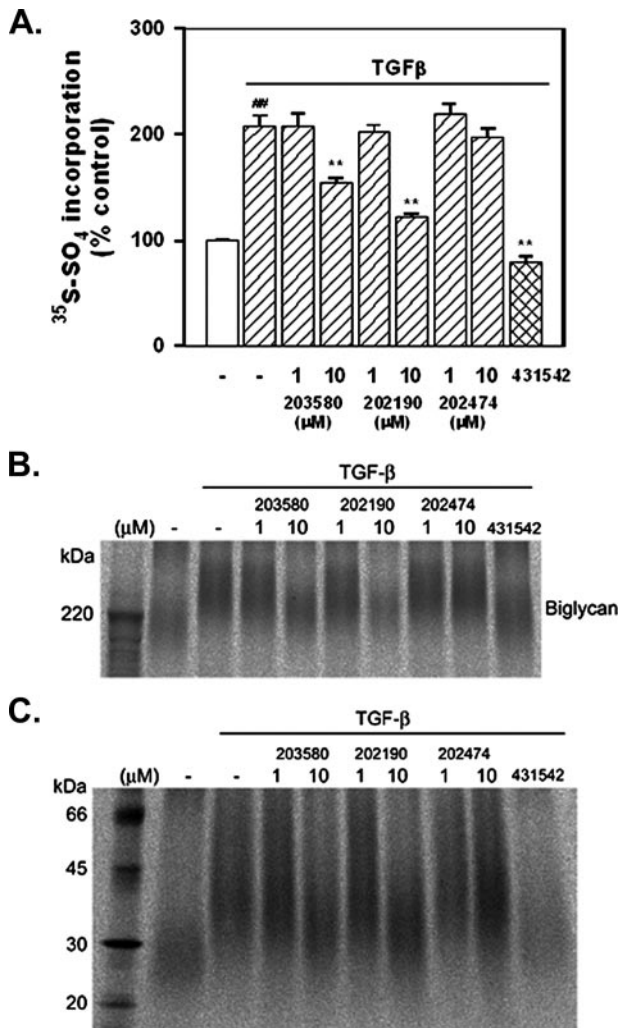
**Smad and p38 MAP Kinase-mediated Proteoglycan Synthesis in VSMC**—To determine the extent of TGF- $\beta$ /ALK5-mediated proteoglycan synthesis in human VSMC, we used the ALK5 inhibitor SB431542 (24). Cells were treated with TGF- $\beta$  (2 ng/ml) in the presence of SB431542 (0–3  $\mu$ M). SB431542 showed a concentration-dependent decrease of TGF- $\beta$ -mediated [ $^{35}$ S]sulfate incorporation into secreted proteoglycans (Fig. 1A). [ $^{35}$ S]Sulfate incorporation in the presence of TGF- $\beta$  was normalized to 100% for its effect at 10  $\mu$ M and SB431542 at 3  $\mu$ M decreased TGF- $\beta$ -mediated [ $^{35}$ S]sulfate incorporation by 92% (Fig. 1A). The IC<sub>50</sub> value was determined to be 0.78  $\mu$ M (comparable to kinase assay IC<sub>50</sub> data from the supplier). SB431542 at 3  $\mu$ M showed near maximum inhibition with no decrease in cell numbers and was used for subsequent experiments. Separation of proteoglycans on SDS-PAGE showed a concentration-dependent increase in electrophoretic mobility in the presence of SB431542 relative to TGF- $\beta$  treatment alone (Fig. 1B), indicating a concentration-dependent decrease in proteoglycan size upon ALK5 inhibition with SB431542. In the classic TGF- $\beta$  signaling pathway, the effects of TGF- $\beta$  are mediated via phosphorylation of Smad2/3 by ALK5 (11). We determined the level of expression of pSmad2/3 protein in whole cell lysates of human VSMC by Western blot. An ~58-kDa band corresponding to pSmad2/3 was observed in control cells (Fig. 1C, lane 1) indicating the presence of pSmad2/3 under basal conditions. The pSmad2/3 band intensity in TGF- $\beta$  (2 ng/ml)-treated cells (Fig. 1C, lane 3) was increased significantly *versus* control (24  $\pm$  5%, *p* < 0.01), indicating stimulation of Smad2/3 phosphorylation. Treatment with the ALK5 inhibitor SB431542 (3  $\mu$ M) abolished the pSmad2/3 band in both control cells and TGF- $\beta$ -treated cells. These results demonstrate that in human VSMC SB431542 inhibits phosphorylation of Smad2/3 by ALK5 in the presence of TGF- $\beta$ , confirming that SB431542 is able to effectively inhibit the classic TGF- $\beta$  Ser/Thr kinase signaling pathway.

Recently p38 MAP kinase was demonstrated to regulate biglycan expression (16). To determine whether p38 MAP kinase is a mediator of ALK5 signaling in GAG elongation, in our cells we used two inhibitors, SB203580 and SB202190, that specifically block activity of p38 MAP kinase but not ALK5 at concentrations  $\leq$  10  $\mu$ M (25). Both inhibitors concentration-dependently blocked TGF- $\beta$ -mediated [ $^{35}$ S]sulfate incorporation into secreted proteoglycans (Fig. 2A) with a maximum decrease of 50% at 10  $\mu$ M SB203580 (*p* < 0.01) and 80% at 10  $\mu$ M SB202190. There was no significant inhibition observed with the negative control inhibitor SB202474 (Fig. 2A). Separation of proteoglycans on an SDS-polyacrylamide gel showed an increase in electrophoretic mobility of biglycan in the presence of either 10  $\mu$ M SB203580 or SB202190 relative to TGF- $\beta$  treatment alone (Fig. 2B, lane 3 *versus* lanes 4/5 and 6/7), indicating that inhibition of p38 MAP kinase activity by either inhibitor is sufficient to interfere with GAG synthesis in VSMC.



**FIGURE 1. Determination of the role of ALK5 activity in TGF- $\beta$ -mediated proteoglycan synthesis in human VSMCs using the ALK5 inhibitor SB431542.** A, VSMCs were treated with TGF- $\beta$  (2 ng/ml) in the presence of SB431542 (0–3  $\mu$ M) and radiolabeled with [ $^{35}$ S]sulfate (50  $\mu\text{Ci/ml}$ ) for 24 h. Medium containing secreted proteoglycans was spotted onto chromatography paper, and CPC was precipitated to assess radiolabel incorporation into proteoglycans. Normalized data are shown as the mean  $\pm$  S.E. from two experiments in triplicate. B, secreted proteoglycans were isolated using ion-exchange chromatography (DEAE-Sephacel) and concentrated by ethanol precipitation. Electrophoretic mobility of proteoglycans was assessed by SDS-PAGE on a 4–13% acrylamide gradient gel. A representative gel is shown. C, inhibitory effects of SB431542 on TGF- $\beta$ /ALK5-mediated Smad2/3 phosphorylation in human VSMCs. VSMCs were treated with TGF- $\beta$  (2 ng/ml) and SB431542 (3  $\mu$ M) for 24 h. Cell lysates were collected, and proteins were resolved by SDS-PAGE on a 10% acrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with anti-phospho-Smad2/3 rabbit polyclonal antibody (1:500 dilution) overnight at 4  $^{\circ}\text{C}$  followed by incubation with anti-rabbit IgG (1:5000 dilution) for 1 h at room temperature. The membrane was then reprobed with anti-smooth muscle  $\alpha$ -actin mouse monoclonal antibody (1:1000 dilution) for 1 h at room temperature followed by incubation with anti-mouse IgG (1:5000 dilution) for 1 h.

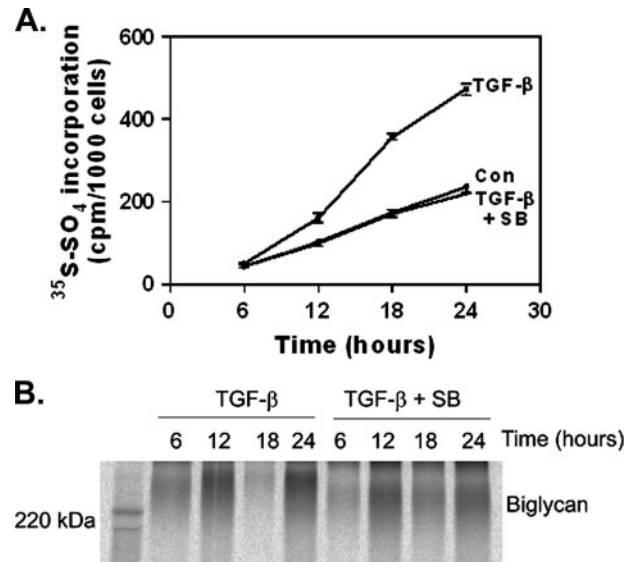
To assess whether the change in biglycan size we observed was because of alterations in GAG length, we chemically cleaved the GAG chains from proteoglycan core proteins. In the presence of TGF- $\beta$  the cleaved GAG chains showed a decrease in electrophoretic mobility compared with control, indicating an increased size (Fig. 2C, lane 2 *versus* 3). Electrophoretic mobility of GAG chains in TGF- $\beta$ -treated cells



**FIGURE 2. p38 MAP kinase inhibitors block TGF- $\beta$ -mediated GAG elongation in human VSMCs.** VSMCs were treated with SB203580 (1–10  $\mu\text{M}$ ), SB202190 (1–10  $\mu\text{M}$ ), SB202474 (1–10  $\mu\text{M}$ ), and SB431542 (3  $\mu\text{M}$ ) in the presence of TGF- $\beta$  (2 ng/ml) and [ $^{35}\text{S}$ ]sulfate (50  $\mu\text{Ci/ml}$ ) for 24 h. *A*, medium containing secreted proteoglycans was spotted onto chromatography paper and CPC precipitated to assess radiolabel incorporation. Results are the mean  $\pm$  S.E. of data normalized to control from three separate experiments in triplicate. #,  $p < 0.01$  versus control, and \*\*,  $p < 0.01$  versus TGF- $\beta$  alone, using a one-way ANOVA. *B*, secreted proteoglycans were isolated using ion-exchange chromatography (DEAE-Sephacel) and concentrated by ethanol precipitation. Electrophoretic mobility of complete proteoglycans was assessed by SDS-PAGE on a 4–13% acrylamide gradient gel. The gel is representative of three identical experiments. *C*, GAG chains were chemically cleaved as described under “Experimental Procedures” and separated by SDS-PAGE on a 4–20% acrylamide gel. The gel is representative of three analyses from three separate experiments.

was inhibited to control levels in the presence of 10  $\mu\text{M}$  SB203580 or 10  $\mu\text{M}$  SB202190 (Fig. 2C). SB202474 had no detectable effect on TGF- $\beta$ -mediated GAG elongation. The results with the p38 inhibitors clearly demonstrate that TGF- $\beta$ -induced GAG elongation in VSMC is dependent on p38 MAP kinase signaling. Taken together with our earlier findings, this indicates that TGF- $\beta$  signals via ALK5/Smad2/3 and p38 MAP kinase to induce GAG elongation in human VSMC.

**Temporal Effects of SB431542 on TGF- $\beta$ /ALK5-mediated Proteoglycan Synthesis in Human VSMCs**—Subsequent experiments focused specifically on ALK5/Smads to determine the

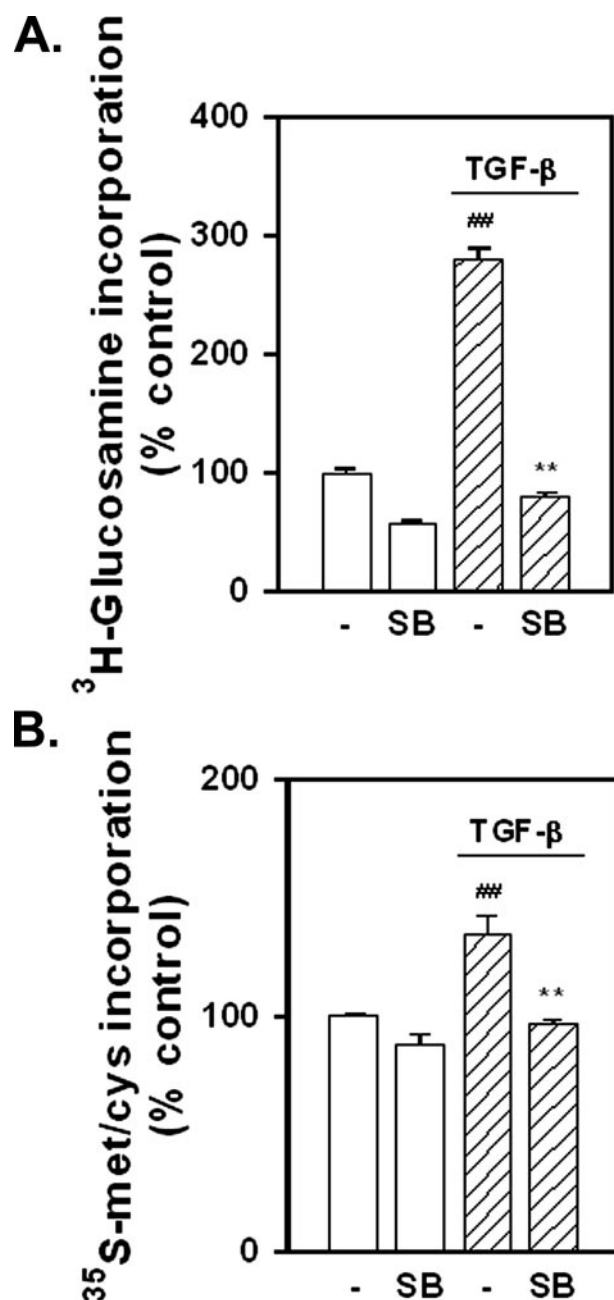


**FIGURE 3. Temporal effects of SB431542 on TGF- $\beta$ /ALK5-mediated proteoglycan synthesis in human VSMCs.** *A*, VSMCs were treated with TGF- $\beta$  (2 ng/ml) and SB431542 (SB) (3  $\mu\text{M}$ ) and radiolabeled with [ $^{35}\text{S}$ ]sulfate (100  $\mu\text{Ci/ml}$ ) for 6, 12, 18, and 24 h. Medium containing secreted proteoglycans was spotted onto chromatography paper and CPC precipitated to assess radiolabel incorporation into proteoglycans. The experiment was performed twice, and a representative is shown. *B*, secreted proteoglycans were isolated using ion-exchange chromatography (DEAE-Sephacel) and concentrated by ethanol precipitation. Electrophoretic mobility of proteoglycans was assessed by SDS-PAGE on a 4–13% acrylamide gradient gel. The gel is representative of two identical experiments.

extent of changes that signaling via these molecules produces in VSMC proteoglycans. We undertook a time course study of ALK5 inhibition of TGF- $\beta$ -mediated sulfate incorporation. SB431542 (3  $\mu\text{M}$ ) decreased TGF- $\beta$  (2 ng/ml)-mediated [ $^{35}\text{S}$ ]sulfate incorporation to control levels (Fig. 3A). TGF- $\beta$  caused a decrease in electrophoretic mobility, indicating an increase in proteoglycan size, within 12 h of stimulation (Fig. 3B). No change in the electrophoretic mobility of proteoglycans from TGF- $\beta$ -treated cells was observed in the presence of SB431542 (Fig. 3B, lanes 6–9), suggesting that the action of SB431542 occurs prior to the action of TGF- $\beta$  on proteoglycan synthesis and therefore earlier than 12 h.

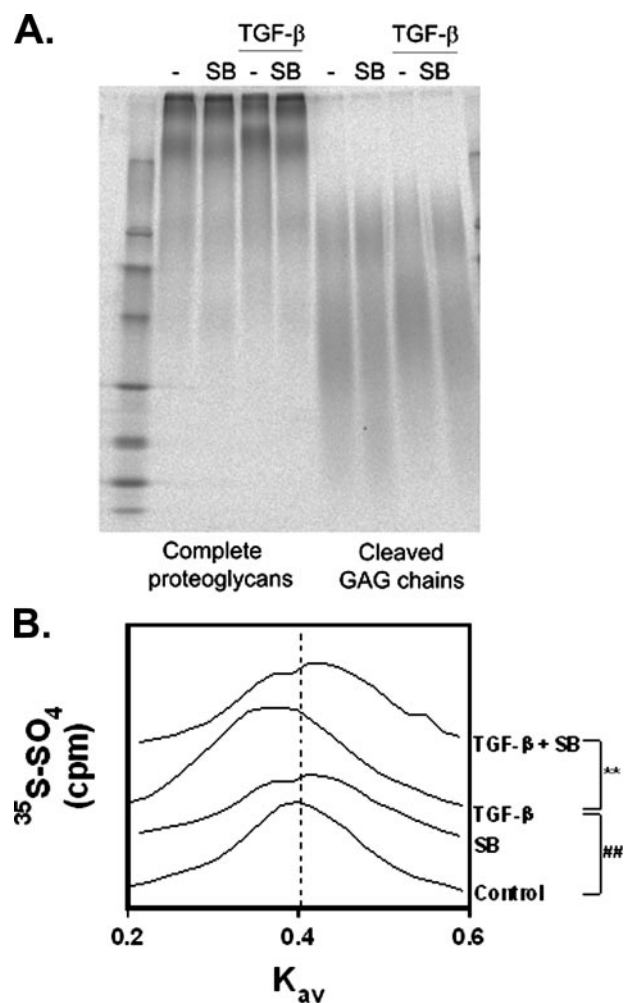
**Effects of TGF- $\beta$  on GAG Synthesis in Human VSMCs Using the ALK5 Inhibitor SB431542 and Glucosamine as an Alternative Radiolabel**—Using the radiolabeled monosaccharide [ $^3\text{H}$ ]glucosamine we confirmed the inhibitory effects of SB431542 on VSMC proteoglycans. TGF- $\beta$  (2 ng/ml) caused a 180% increase in [ $^3\text{H}$ ]glucosamine incorporation compared with control ( $p < 0.01$ ) indicating an increase in GAG synthesis. SB431542 (3  $\mu\text{M}$ ) decreased basal [ $^3\text{H}$ ]glucosamine incorporation by 43% and TGF- $\beta$ -mediated [ $^3\text{H}$ ]glucosamine incorporation by 72% ( $p < 0.01$ ) (Fig. 4A) indicating inhibition of ALK5-mediated GAG synthesis of VSMC proteoglycans.

**TGF- $\beta$  Increases Proteoglycan Core Protein Synthesis via the ALK/Smad2/3 Pathway**—[ $^{35}\text{S}$ ]Met/Cys incorporation was assessed to investigate changes in total proteoglycan core protein synthesis. TGF- $\beta$  increased [ $^{35}\text{S}$ ]Met/Cys incorporation by 35% compared with control ( $p < 0.01$ ) indicating an increase in core protein synthesis. SB431542 (3  $\mu\text{M}$ ) decreased basal



**FIGURE 4. ALK5 inhibition blocks the effects of TGF- $\beta$  on glucosamine incorporation into proteoglycans and total proteoglycan core protein synthesis in human VSMCs.** *A*, VSMCs were treated with TGF- $\beta$  (2 ng/ml) and SB431542 (SB) (3  $\mu\text{M}$ ) and radiolabeled with [ $^3\text{H}$ ]glucosamine (10  $\mu\text{Ci/ml}$ ) for 24 h. Medium containing secreted proteoglycans was spotted onto chromatography paper and CPC precipitated to assess radiolabel incorporation into GAGs. Results shown are the mean  $\pm$  S.E. of normalized data from three separate experiments in triplicate. <sup>##</sup>,  $p < 0.01$  versus control, and <sup>\*\*</sup>,  $p < 0.01$  versus TGF- $\beta$ , using a one-way ANOVA. *B*, VSMCs were treated with TGF- $\beta$  (2 ng/ml) and SB431542 (3  $\mu\text{M}$ ) and radiolabeled with [ $^{35}\text{S}$ ]Met/Cys (50  $\mu\text{Ci/ml}$ ) for 24 h to assess total proteoglycan core protein synthesis. Medium containing secreted proteoglycans was spotted onto chromatography paper and CPC precipitated to assess radiolabel incorporation into core proteins. Results shown are the mean  $\pm$  S.E. of normalized data from three separate experiments in triplicate. <sup>##</sup>,  $p < 0.01$  versus control, and <sup>\*\*</sup>,  $p < 0.01$  versus TGF- $\beta$  using a one-way ANOVA.

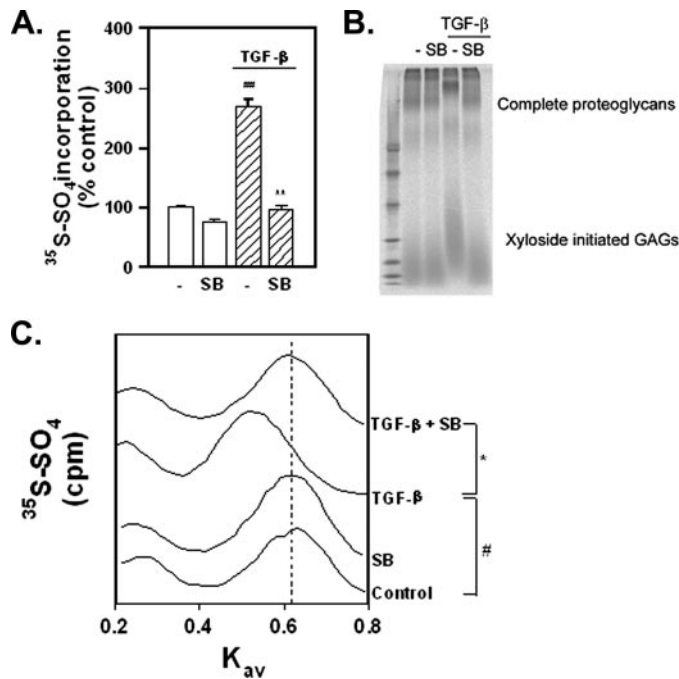
[ $^{35}\text{S}$ ]Met/Cys incorporation by 12% and TGF- $\beta$ -mediated [ $^{35}\text{S}$ ]Met/Cys incorporation by 27% ( $p < 0.01$ ) (Fig. 4B) indicating inhibition of ALK5-mediated proteoglycan core protein synthesis.



**FIGURE 5. Inhibition of ALK5 signaling prevents TGF- $\beta$ -induced GAG chain synthesis and elongation in human VSMCs.** VSMCs were treated with TGF- $\beta$  (2 ng/ml) and SB431542 (3  $\mu\text{M}$ ). [ $^{35}\text{S}$ ]Sulfate (50  $\mu\text{Ci/ml}$ ) was added 4 h post-treatment and proteoglycans radiolabeled for a further 24 h. Secreted proteoglycans were isolated using ion-exchange chromatography (DEAE-Sephacel) and concentrated by ethanol precipitation. GAG chains were chemically cleaved by incubation in 1 M NaBH $_4$ , 0.05 M NaOH at 45  $^\circ\text{C}$  for 24 h. *A*, electrophoretic mobility of intact proteoglycans and chemically cleaved GAG chains assessed by SDS-PAGE on a 4–20% acrylamide gradient gel. The gel is representative of three identical experiments. *B*, size exclusion chromatography of cleaved GAG chains was performed using a Sepharose CL-6B column. The dotted vertical line indicates  $K_{av}$  of cleaved GAG chains from control cells. Three measures were performed from three separate experiments, and a representative is shown. <sup>##</sup>,  $p < 0.01$  versus control, and <sup>\*\*</sup>,  $p < 0.02$  versus TGF- $\beta$  using a paired Student's  $t$  test. SB, SB431542.

#### *Inhibition of ALK5 Blocks TGF- $\beta$ -induced GAG Elongation Dependently and Independently of Proteoglycan Core Proteins—*

To assess whether the changes in proteoglycan size that we observed were specifically because of alterations in GAG length, we chemically cleaved the GAG chains from proteoglycan core proteins. The electrophoretic mobility of cleaved GAG chains was greater compared with complete proteoglycans (Fig. 5A). In the presence of TGF- $\beta$  the cleaved GAG chains showed a decrease in electrophoretic mobility compared with control, indicating an increased size. Electrophoretic mobility of GAG chains in TGF- $\beta$ -treated cells was inhibited to control levels in the presence of 3  $\mu\text{M}$  SB431542 (Fig. 5A). These changes were definitively evaluated by size exclusion chromatography. A representative size exclusion analysis experiment is shown where



**FIGURE 6. ALK5 inhibitor SB431542 blocks the effect of TGF- $\beta$  on xyloside-initiated GAG chain synthesis in human VSMCs.** VSMCs were treated with SB431542 (SB) (3  $\mu\text{M}$ ) and TGF- $\beta$  (2 ng/ml). [ $^{35}\text{S}$ ]Sulfate (50  $\mu\text{Ci/ml}$ ) was added 4 h post-treatment, and proteoglycans were radiolabeled for 24 h. For analysis of GAG synthesis independent of proteoglycan core protein, treatment medium was supplemented with a "false acceptor," 0.5 mM xyloside. **A**, medium containing secreted proteoglycans was spotted onto chromatography paper and CPC precipitated to assess radiolabel incorporation. Results shown are the mean  $\pm$  S.E. of normalized data from three separate experiments in triplicate. ##,  $p < 0.01$  versus control, and \*\*,  $p < 0.01$  versus TGF- $\beta$ , using a one-way ANOVA. **B**, xyloside-initiated and -secreted GAGs were isolated using ion-exchange chromatography (DEAE-Sephacel) and concentrated by ethanol precipitation. Electrophoretic mobility of xyloside-initiated GAG chains was assessed by SDS-PAGE on a 4–16% acrylamide gradient gel. The gel is representative of three identical experiments. **C**, size exclusion chromatography of cleaved GAG chains was performed using a Sepharose CL-6B column. Data are plotted as  $K_{av}$  against [ $^{35}\text{S}$ ]sulfate (cpm). The dotted vertical line indicates the  $K_{av}$  of xyloside-initiated GAG chains from control cells. Three measures were performed from three separate experiments, and a representative is shown. #,  $p < 0.05$  versus control, and \*,  $p < 0.05$  versus TGF- $\beta$ , using a paired Student's  $t$  test.

the vertical line represents the mean  $K_{av}$  of GAG chains from control cells (Fig. 5B). GAG chains from TGF- $\beta$ -treated cells have a  $K_{av}$  value of  $0.39 \pm 0.01$  compared with control ( $p < 0.01$ ), where  $K_{av}$  is  $0.42 \pm 0.01$ , indicating TGF- $\beta$ -mediated GAG elongation. Inhibition of ALK5 with SB431542 resulted in a  $K_{av}$  of  $0.44 \pm 0.02$  compared with TGF- $\beta$  treatment alone ( $p < 0.02$ ), indicating that SB431542 inhibited TGF- $\beta$  stimulation of GAG elongation.

We used  $\beta$ -D-xyloside (an initiator of GAG synthesis) to examine GAG synthesis independently of core protein synthesis. TGF- $\beta$  (2 ng/ml) increased [ $^{35}\text{S}$ ]sulfate incorporation by 169% compared with control ( $p < 0.01$ ) (Fig. 6A). SB431542 (3  $\mu\text{M}$ ) decreased basal [ $^{35}\text{S}$ ]sulfate incorporation by 24% and TGF- $\beta$ -mediated [ $^{35}\text{S}$ ]sulfate incorporation by 64% ( $p < 0.01$ ) (Fig. 6A). Xyloside-initiated GAG chains were separated by SDS-PAGE. Electrophoretic mobility of xyloside-initiated proteoglycans in the presence of TGF- $\beta$  was reduced compared with control (Fig. 6B, lanes 2 and 4). Inhibition of ALK5 by SB431542 prevented the effect of TGF- $\beta$  on proteoglycan synthesis and had the same electrophoretic mobility when com-

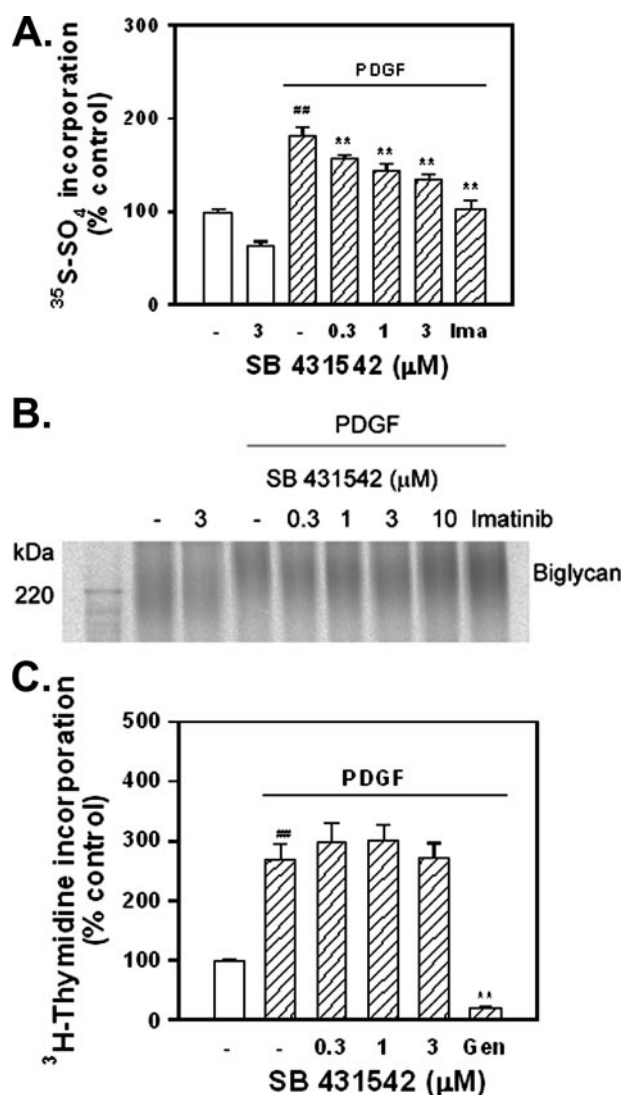
pared with control (Fig. 6B, lane 5). Complete proteoglycans also may be seen at the top of the gel. As determined by size exclusion chromatography, xyloside-initiated GAG chains from TGF- $\beta$ -treated cells have a  $K_{av}$  value of  $0.52 \pm 0.02$  compared with control cells ( $p < 0.05$ ) which have a  $K_{av}$  of  $0.62 \pm 0.01$  ( $p < 0.05$ ), demonstrating an increase in GAG length in the presence of TGF- $\beta$ . Treatment with SB431542 resulted in a  $K_{av}$  of 0.60 compared with TGF- $\beta$  treatment alone ( $p < 0.05$ ), verifying that SB431542 inhibited TGF- $\beta$ -mediated stimulation of GAG synthesis.

**SB431542 Inhibits PDGF-mediated GAG Synthesis but Not VSMC Proliferation**—To examine the specificity of SB431542, we evaluated its action on the tyrosine kinase growth factor, PDGF. It was expected that at the concentration specific for ALK5 inhibition (3  $\mu\text{M}$ ), there would be no effect on PDGF. PDGF (50 ng/ml) stimulated [ $^{35}\text{S}$ ]sulfate incorporation by 82% relative to control ( $p < 0.01$ ) (Fig. 7). Treatment of cells with SB431542 (0–3  $\mu\text{M}$ ) showed a concentration-dependent decrease of PDGF-mediated [ $^{35}\text{S}$ ]sulfate incorporation into secreted proteoglycans (Fig. 7A) with a maximum decrease of 60% at 3  $\mu\text{M}$  ( $p < 0.01$ ). Imatinib, a protein-tyrosine kinase inhibitor (26) that inhibits PDGF-mediated GAG elongation,<sup>3</sup> was used as a positive control and inhibited PDGF-mediated [ $^{35}\text{S}$ ]sulfate incorporation by 97% ( $p < 0.01$ ) (Fig. 7A). Separation of proteoglycans on SDS-PAGE showed a concentration-dependent increase in electrophoretic mobility in the presence of SB431542 relative to PDGF treatment alone (Fig. 7B), indicating a concentration-dependent decrease in proteoglycan size upon treatment with SB431542.

The effect on VSMC mitogenic response as a classic PDGF cellular action was assessed to investigate whether SB431542 would inhibit PDGFR-mediated responses (Fig. 7C). PDGF caused a 170% increase in [ $^3\text{H}$ ]thymidine incorporation compared with control ( $p < 0.01$ ). No change in [ $^3\text{H}$ ]thymidine incorporation was observed in the presence of SB431542, indicating that SB431542 does not block the PDGFR and is not a PDGFR tyrosine kinase inhibitor. Genistein, used here as a positive control, inhibited PDGF-mediated [ $^3\text{H}$ ]thymidine incorporation by 92% ( $p < 0.01$ ).

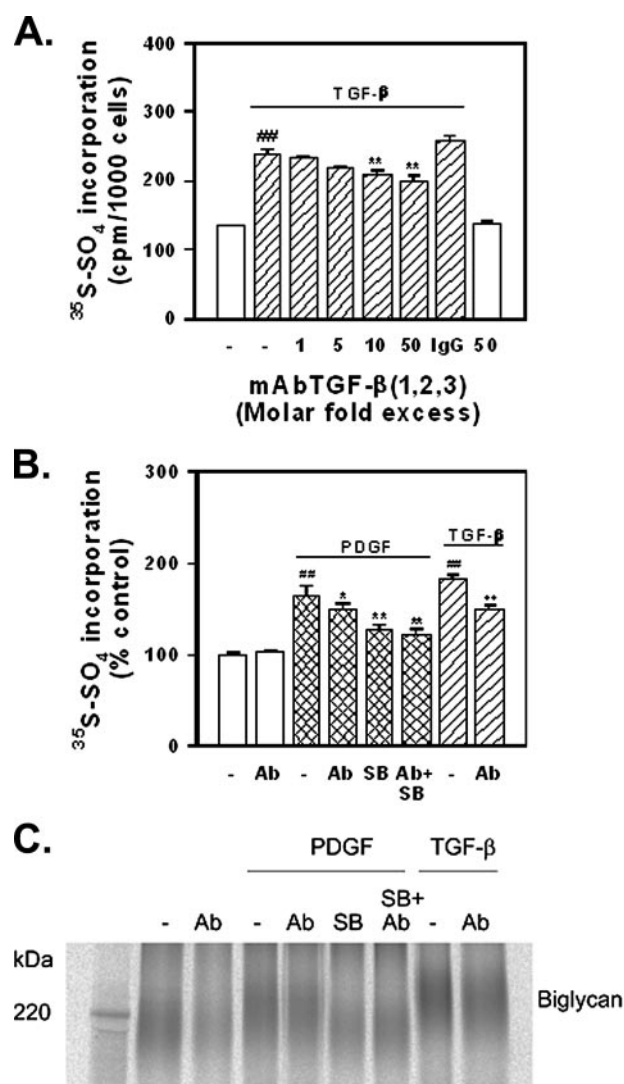
PDGF is known to mediate TGF- $\beta$  release from VSMCs (27). To investigate whether PDGF mediates the release of a functional amount of TGF- $\beta$ , we used a neutralizing antibody to block the effects of any TGF- $\beta$ . Cells were incubated with anti-TGF- $\beta$ 1/2/3 antibody (1–50-fold molar excess) in the presence of TGF- $\beta$  (2 ng/ml) to generate a concentration inhibition curve (Fig. 8A). At both 10- and 50-fold molar excess, the anti-TGF- $\beta$  antibody inhibited TGF- $\beta$ -mediated [ $^{35}\text{S}$ ]sulfate incorporation into proteoglycans ( $p < 0.01$ ) (Fig. 8A). Mouse IgG, used as an isotype control, had no effect on TGF- $\beta$ -mediated [ $^{35}\text{S}$ ]sulfate incorporation as expected. The anti-TGF- $\beta$  antibody (50-fold molar excess) showed no effect under basal conditions (Fig. 8A). The effect of the antibody at 20-fold molar excess was assessed against PDGF to investigate potential PDGF-mediated TGF- $\beta$  release from VSMCs. The antibody showed no effect under basal conditions. The antibody

<sup>3</sup> M. L. Ballinger and P. J. Little, unpublished observations.



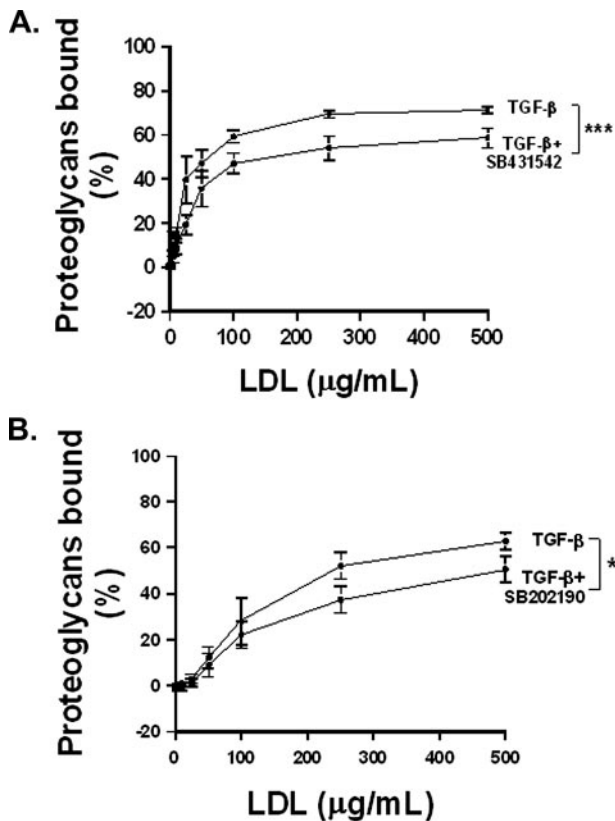
**FIGURE 7. Determination of the specificity of action of SB431542 by examining its effects on tyrosine kinase growth factor (PDGF)-activated processes in human VSMCs.** A, VSMCs were treated with PDGF (50 ng/ml) and SB431542 (0–3  $\mu\text{M}$ ) and radiolabeled with [ $^{35}\text{S}$ ]sulfate (50  $\mu\text{Ci/ml}$ ) for 24 h. Imatinib (1  $\mu\text{M}$ ) was used as a positive control. Medium containing secreted proteoglycans was spotted onto chromatography paper and CPC precipitated to assess radiolabel incorporation. Results are the mean  $\pm$  S.E. of data normalized to control from three experiments in triplicate. ##,  $p < 0.01$  versus control, and \*\*,  $p < 0.01$  versus PDGF, using a one-way ANOVA. B, secreted proteoglycans were isolated using ion-exchange chromatography (DEAE-Sephacel) and concentrated by ethanol precipitation. Electrophoretic mobility of proteoglycans was assessed by SDS-PAGE on a 4–13% acrylamide gradient gel. The gel is representative of three identical experiments. C, human VSMCs were treated with PDGF (50 ng/ml) and SB431542 (0–3  $\mu\text{M}$ ) for 16 h followed by the addition of [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci/ml}$ ) for a further 3 h. Genistein (100  $\mu\text{M}$ ) was used as a positive control. Cells were washed with ice-cold phosphate-buffered saline, incubated with  $\text{HClO}_4$  (0.2 M), and digested with NaOH (0.2 M). Results are the mean  $\pm$  S.E. of data normalized to control from three experiments in triplicate. ##,  $p < 0.01$  versus control, and \*\*,  $p < 0.01$  versus PDGF, using a one-way ANOVA.

decreased PDGF (50 ng/ml)-mediated [ $^{35}\text{S}$ ]sulfate incorporation by 24% ( $p < 0.05$ ) (Fig. 8B), suggesting that such a TGF- $\beta$  release may contribute to the action of PDGF on proteoglycan synthesis in VSMCs. The antibody was used with TGF- $\beta$  as a positive control and was found to decrease TGF- $\beta$ -mediated [ $^{35}\text{S}$ ]sulfate incorporation by 40% (Fig. 8B). Effects of the antibody on [ $^{35}\text{S}$ ]sulfate incorporation are mirrored on an SDS-



**FIGURE 8. Effects of an anti-TGF- $\beta$ 1/2/3 antibody on PDGF-mediated proteoglycan synthesis in human VSMCs.** A, VSMCs were incubated with anti-human-TGF- $\beta$ 1/2/3 monoclonal antibody (mAb; 1–50-fold molar excess) for 40 min prior to treatment with TGF- $\beta$  (2 ng/ml) and radiolabeling with [ $^{35}\text{S}$ ]sulfate (50  $\mu\text{Ci/ml}$ ). Mouse IgG was used as an isotype control. Medium containing secreted proteoglycans was spotted onto chromatography paper and CPC precipitated to assess radiolabel incorporation. Results are the mean  $\pm$  S.E. of data normalized to control from three experiments in triplicate. ##,  $p < 0.01$  versus control, and \*\*,  $p < 0.01$  versus TGF- $\beta$  alone, using a one-way ANOVA. B, VSMCs were incubated with anti-human-TGF- $\beta$ 1/2/3 monoclonal antibody (Ab; 20-fold molar excess) prior to treatment with PDGF (50 ng/ml) and SB431542 (3  $\mu\text{M}$ ) in the presence of [ $^{35}\text{S}$ ]sulfate (50  $\mu\text{Ci/ml}$ ). TGF- $\beta$  was used as a positive control. Radiolabel incorporation was assessed as described under “Experimental Procedures.” Results are the mean  $\pm$  S.E. of data normalized to control from three experiments in triplicate. ##,  $p < 0.01$  versus control, \*,  $p < 0.05$  versus PDGF, \*\*,  $p < 0.01$  versus PDGF, and +,  $p < 0.01$  versus TGF- $\beta$ , using a one-way ANOVA. C, secreted proteoglycans were isolated using ion-exchange chromatography (DEAE-Sephacel) and concentrated by ethanol precipitation. Electrophoretic mobility of proteoglycans was assessed by SDS-PAGE on a 4–13% acrylamide gradient gel. The gel is representative of three identical experiments. SB, SB431542.

PAGE analysis (Fig. 8C). SB431542 (3  $\mu\text{M}$ ) decreased PDGF-mediated [ $^{35}\text{S}$ ]sulfate incorporation by 57% (Fig. 8B) and increased electrophoretic mobility (Fig. 8C). The presence of SB431542 and the antibody together in PDGF-treated cells did not decrease [ $^{35}\text{S}$ ]sulfate incorporation any further (Fig. 8B). The data indicate that SB431542 inhibits PDGF-mediated pro-



**FIGURE 9. ALK5 and p38 MAP kinase inhibition reduces LDL binding to proteoglycans derived from TGF- $\beta$ -treated human VSMCs.** VSMCs were treated with TGF- $\beta$  (2 ng/ml) and the ALK5 inhibitor SB431542 (3  $\mu$ M) (A) or the p38 MAP kinase inhibitor SB202190 (10  $\mu$ M) (B), and core proteins were radiolabeled with [ $^{35}$ S]Met/Cys (50  $\mu$ Ci/ml). Core protein-radiolabeled proteoglycans were isolated and equal counts combined with increasing concentrations of LDL. Flat-bed agarose gels were utilized for separation of bound and free proteoglycans in the presence of TGF- $\beta$  and SB431542 or SB202190. Calculation of bound proteoglycans was performed using image analysis software with the results shown as the mean  $\pm$  S.E. from two experiments analyzed in duplicate. \*\*\*,  $p < 0.001$ , and \*,  $p < 0.05$ , using a two-way interactive ANOVA.

teoglycan synthesis in VSMCs and may inhibit an autocrine effect of TGF- $\beta$  released following PDGF stimulation.

**Inhibition of ALK5 and p38 MAP Kinase Reduces LDL Binding to Proteoglycans from TGF- $\beta$ -treated Human VSMCs**—Gel mobility shift assays were performed to assess proteoglycan-LDL binding. Proteoglycans derived from cells treated with SB431542 (3  $\mu$ M) under basal conditions had similar LDL binding to control proteoglycans (data not shown). However, proteoglycans derived from SB431542-treated cells in the presence of TGF- $\beta$  had a decreased binding to LDL compared with TGF- $\beta$  treatment alone ( $p < 0.001$ ) with half maximal saturation values of 50 and 25  $\mu$ g/ml LDL, respectively (Fig. 9A). Similarly, proteoglycans from SB202190-treated cells in the presence of TGF- $\beta$  exhibited decreased LDL binding compared with TGF- $\beta$  treatment ( $p < 0.05$ ) (Fig. 9B), indicating that inhibition of TGF- $\beta$ -mediated signaling via ALK5/Smad2/3 or p38 MAP kinase results in proteoglycans with a decreased propensity to bind LDL.

## DISCUSSION

A TGF- $\beta$ -activated Smad2/3 phosphorylation pathway is present in human internal mammary artery-derived VSMCs

and, along with p38 MAP kinase signaling, is responsible for the TGF- $\beta$ -mediated elongation of GAG chains on proteoglycans. Inhibiting Smad2/3 phosphorylation with SB431542 antagonized the GAG elongation action of TGF- $\beta$ , and inhibition of p38 kinase also blocked TGF- $\beta$ -mediated GAG elongation. Further investigation revealed that SB431542 partially inhibited the GAG elongation action of another atherogenic growth factor, PDGF. However, we demonstrated that this latter action was likely due to the PDGF-stimulated autocrine secretion of functional levels of TGF- $\beta$  and that SB431542 has no direct action to inhibit a classic PDGFR-mediated response such as VSMC proliferation (28). Finally, inhibition of phosphorylation of Smad2/3 and p38 MAP kinase with SB431542 and SB202190, respectively, reversed TGF- $\beta$ -mediated increases in proteoglycan/LDL binding.

TGF- $\beta$  is a secreted cytokine that controls a plethora of cellular processes, including cell proliferation, differentiation, apoptosis, extracellular matrix production, and immune functions (9, 10, 14, 29). TGF- $\beta$  signals through Ser/Thr kinase receptors type I (T $\beta$ RI/ALK 5) and type II (T $\beta$ RII) (9, 10) and subsequently the Smad proteins (11–14). TGF- $\beta$  stimulates the synthesis of elongated GAG chains on VSM proteoglycans (7, 30). Elongation of GAG chains on VSM proteoglycans is of particular interest in cardiovascular disease because it leads to enhanced binding of proteoglycans to lipoproteins on LDL (7, 21).

It has been shown definitively that, in the early stages of human atherosclerosis, the pathological process begins with lipid deposition in the basal layers of diffuse intimal thickenings (6). Interestingly, the lipid deposition described in early atherosclerotic lesions occurs in association with enrichment of the proteoglycan biglycan (6). As such, proteoglycan synthesis, and specifically GAG synthesis, represents a potential therapeutic target (6, 21). Recent advances in elucidation of TGF- $\beta$  signaling pathways may provide insights into possible therapeutic strategies for the future (18).

The use of small specific inhibitors developed for therapeutics can be a powerful tool in dissecting complex signal transduction pathways (31). This study focused on TGF- $\beta$  signaling by the use of a potent ALK5 inhibitor, SB431542 (31). SB431542 also inhibits the ALK4 and ALK7 receptors, which are highly related to ALK5 in their kinase domains, but it has no effect on any of the other ALKs (24). SB431542 acts by competitively inhibiting ATP binding. It inhibits TGF- $\beta$ - and activin-induced phosphorylation of Smad2 and Smad3, which is mediated by ALK4/5/7 (24). SB431542 has previously been shown to prevent TGF- $\beta$ -induced gene expression of collagen, plasminogen activator inhibitor-1, and connective tissue growth factor (32). Future studies will enable us to obtain a clearer picture of the effects of this inhibitor on gene transcriptional events related to proteoglycans in VSM. Our findings show SB431542 to be useful as a novel experimental tool for gaining understanding of TGF- $\beta$  signaling leading to proteoglycan synthesis in VSMCs. Further studies using antibodies to individual Smads or a commercially available inhibitor of ALK5-mediated Smad3 phosphorylation will further define and delineate these signaling pathways.

Very little is known about the signaling biochemistry operating between cell surface receptors and the ultimate changes in



## Smad and p38 Signaling of Proteoglycan Synthesis

GAG synthesis and structure. PDGF stimulates GAG elongation and the expression of mRNA for proteoglycan core proteins, but only the increased expression of the core protein versican is blocked by the broad spectrum protein-tyrosine kinase inhibitor genistein (30, 33). Genistein inhibits PDGF-activated VSMC proliferation (28). The fact that GAG elongation stimulated by PDGF is not blocked by genistein suggests that a novel signaling pathway dependent upon the PDGFR but independent of its tyrosine kinase activity is responsible for mediating GAG elongation in VSMCs. This observation generated our interest in the signaling pathway by which TGF- $\beta$  stimulated GAG elongation in VSMCs, which revealed that TGF- $\beta$ -mediated GAG elongation proceeds via a known ALK5 and Smad2/3 pathway.

In VSMC TGF- $\beta$ -mediated activation of p38 MAP kinase has been shown to induce expression of thrombospondin (34), vascular endothelial growth factor (35) and is involved in induction of smooth muscle marker genes (36). Although TGF- $\beta$  has been demonstrated to regulate biglycan expression via p38 MAP kinase in the pancreatic adenocarcinoma cell line PANC-1 (16, 17), in the present study we have described an involvement of p38 MAP kinase in TGF- $\beta$ -mediated proteoglycan synthesis in VSMC and demonstrated that inhibition of this pathway results in proteoglycans with decreased LDL binding. Using two p38 MAP kinase inhibitors at concentrations that enable highly specific inhibition, the results clearly demonstrate a role for p38 MAP kinase in TGF- $\beta$  signaling in ALK5-mediated GAG synthesis. We observed inhibition of GAG chain elongation by both the Smad inhibitor and the p38 inhibitors. It remains an interesting question as to the relationship between Smad2/3 and p38 MAP kinase, particularly whether or not p38 MAP kinase is downstream and therefore Smad-dependent or is otherwise a Smad-independent pathway. A recent report in human cardiac fibroblasts has shown that TGF- $\beta$  induction of GAG synthesis occurs through both ALK5 and p38 signaling (37). Further studies will facilitate the identification of the signaling intermediates that dictate TGF- $\beta$ -mediated effects on proteoglycans in VSMCs, including the possible downstream association of p38 MAP kinase to upstream Smad2/3 phosphorylation.

Although molecular manipulations that interfere with TGF- $\beta$  signaling lead to deleterious actions on the immune system in animal models, there are very little data available on the impact of small molecule TGF- $\beta$  inhibitors (38). Compared with the molecular genetics approach, these agents may either be more specific in their action or indeed favorably less efficacious in inhibiting TGF- $\beta$  signaling. Small molecule inhibitors provide an opportunity to evaluate the potential of inhibiting growth factor signaling pathways for the prevention of changes in the synthesis and structure of VSMC proteoglycans. The data provided in this paper indicate that a suitable TGF- $\beta$  signaling inhibitor should be evaluated for its potential to prevent lipid deposition and atherosclerosis.

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## **Smad and p38 MAP Kinase-mediated Signaling of Proteoglycan Synthesis in Vascular Smooth Muscle**

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