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Chondroitin Sulfate Prevents Platelet Derived Growth Factor-Mediated Phosphorylation of PDGF-Rβ in Normal Human Fibroblasts Severely Impairing Mitogenic Responses

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Abstract Platelet-derived growth factor (PDGF) is a major polypeptide mitogen for cells of mesenchymal origin such as fibroblasts. Chondroitin sulfate chains (CS), which are abundant in the extracellular matrix have been shown to physically interact with PDGF-BB modulating its biological function. The aim of the present study was to examine the involvement of CS on PDGF-BB induced proliferative responses and receptor activation in human lung fibroblasts. The addition of exogenous free CS chains caused a significant downregulation of the PDGF-BB mediated mitogenic and chemotactic responses. Similar results were obtained by the increase of endogenous CS biosynthesis after β-D-xyloside treatment. Furthermore, removal of the membrane-bound CS chains by selective enzymatic treatment significantly increased the proliferative capacity of human fibroblasts. Analysis of PDGF-R phosphorylation in the presence of CS or β-D-xyloside, revealed a reduction of PDGF-Rβ phosphorylation in the tyrosine residue 1021. These results demonstrate, for the first time, that CS either soluble or surface bound downregulates the mitogenic responses of PDGF-BB in normal human lung fibroblasts through the reduction of PDGF-Rβ phosphorylation. J. Cell. Biochem. 103: 1866–1876, 2008.

Key words: chondroitin sulfate; fibroblasts; PDGF-BB; PDGFR

Platelet-derived growth factor (PDGF) is the major polypeptide mitogen for cells of mesenchymal origin. PDGF delivers a survival signal, by inhibiting cell apoptosis and promoting cell proliferation [Kelly et al., 1985]. PDGF is formed by heterodimerization or homodimerization of two distinct but highly homologous polypeptides, A and B. Both A and B chains can be produced as long and short isoforms through the alternative splicing of exon 6. The short isoforms are the most prevalent in the extracellular matrix and are shown to be more stable and active than the long isoforms which are secreted in small quantities. It is suggested that the long isoforms are immobilized on the cell surface through a stretch of positively charged amino acids called the retention motif and are destined to serve autocrine purposes [Pollock and Richardson, 1992; Andersson et al., 1994]. The retention motif is a carboxyterminal stretch of highly basic amino acids which can interact with negatively charged biomolecules such as the glycosaminoglycans (GAGs).

PDGF signaling is mediated through binding to the high affinity receptors α and β, which possess tyrosine kinase properties. Previous

Abbreviations used: PDGF, platelet derived growth factor; CS, chondroitin sulfate chains; GAGs, glycosaminoglycans; b-FGF, basic fibroblast growth factor; TGFβ2, transforming growth factor β2; DS, dermatan sulfate; SMCs, human smooth muscle cells; FBS, fetal bovine serum; DLF, human lung fibroblasts; PBS, phosphate buffer saline; HS, heparan sulfate.

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studies have demonstrated that growth factor signaling is significantly assisted by the interaction with proteoglycans. A well known example is the interaction of basic FGF with heparan sulfate (HS) proteoglycans through the GAG chains, which stabilize its binding with the FGF receptor and thus enhance its signal delivery [Rapraeger et al., 1991]. Likewise, PDGF interacts with proteoglycans mainly through the GAG moiety. The physical interaction of the PDGF isoforms with matrix and cell associated GAGs has been studied in detail. Binding to GAGs is mediated by electrostatic interaction of the negatively charged GAGs with the positively charged amino-acid stretches in the PDGF sequence as well as with the retention motif of the long PDGF isoforms [Fager et al., 1995]. Both the long and the short isoforms of PDGF can bind GAGs, the former with 10- to 100-fold greater affinity [Lustig et al., 1999]. Specifically, PDGF-BB has been shown to bind HS, chondroitin sulfate A (CS mainly sulphated at C-4 of galactosamine), dermatan sulfate (DS) and heparin while the short PDGF-BB isoform may be stored on the cell surface by binding on heparan sulfate GAG chains [Garcia-Olivas et al., 2003].

The interplay of PDGF with matrix and cell associated GAGs can regulate its mitogenic function. Specifically, it has been shown that heparin [Lustig et al., 1996], HS and DS [Fager et al., 1995] prevent the proliferation of human smooth muscle cells (SMCs) induced by PDGF. Furthermore, it has recently been shown that hyaluronan by binding to CD44 induces recruitment of phosphatases, which in turn inhibit PDGF-BB induced phosphorylation of PDGF-Rβ in human fibroblasts [Li et al., 2006]. CS containing decorin has been shown to significantly affect PDGF signaling in SMCs as regards to cell proliferation, migration, and collagen synthesis [Nili et al., 2003]. On the other hand, studies of our group on fibrosarcoma cell lines suggested that secreted CS, but not DS or heparin, can enhance PDGF-BB mediated signaling through tyrosine kinase dependent pathways [Fthenou et al., 2006]. The aim of the present study was, therefore, to examine the involvement of exogenous or surface bound CS on PDGF-BB induced receptor activation and proliferative responses in human lung fibroblasts. Our results demonstrate that both soluble and surface bound CS can downregulate mitogenic responses of PDGF-BB by reducing PDGF-Rβ phosphorylation.

MATERIALS AND METHODS

Materials

PDGF-BB, TGFβ2, and bFGF were obtained from R&D diagnostics. Chondroitin sulphate type A (CS), dermatan sulphate (DS), heparin, sodium chlorate, β-D-xyloside, and genistein were obtained from Sigma Chemical Co. (St. Louis, MO). Chondroitinase ABC and Heparitinase were purchased from Seikagaku Kogyo Co (Tokyo, Japan). Cell culture reagents were obtained from Biochrom KG (Berlin, Germany). [Methyl]-^{3}H thymidine was purchased from (Moravek Biochemicals).

Cell Culture

Human lung fibroblasts (DLF), passage 6–10, were kindly provided by Dr Kletsas (Demokritos, Athens, Greece). The cells were grown in DMEM with 10% fetal bovine serum (FBS). Prior to stimulation with growth factors the cells were incubated in 2% FBS medium for 24 h. PDGF-BB, TGFβ2, and bFGF (final concentration 10 ng/ml which was selected after pilot dose response experiments) were added in 2% FBS medium. Prior to RNA extraction the cells were treated with each growth factor for 24 h.

RNA Isolation and Real-Time PCR

Total ribonucleic acid was isolated with the TRIzol method (GibcoBRL) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis using the ThermoScript™ RT-PCR System (Invitrogen). The primers were mRNA specific to avoid misleading results from traces of DNA contamination. PDGF-Ra primer forward 5′-CCTGGCTGAAAAACAATCTGACT-3′, PDGF-Ra primer reverse 5′-CAGCTTTAATTTGCTTCGATACCTT-3′, PDGF-Rβ forward 5′-GCTCAC-CATCATCTCCCTTATCA-3′, PDGF-Rβ reverse 5′-CCTCATCATGCTTTGGC-AGAAGAAGCC-3′, GAPDH forward 5′-GGAAGGTGAAGGTCGGAGTCA-3′ and GAPDH reverse 5′-GTCATTGATGGCAACAATATCCACT-3′. For the real-time PCR reaction we utilized the QuantiTech SYBR Green master mix (Qiagen) in a total volume of 20 μl. Standard curves were run in each optimized assay which produced a linear plot of threshold cycle (Ct) against log (dilution). The specificity of the PCR reaction

was verified by melting point analysis. The real-time PCR reactions were carried out in an ABI 7000 cycler. The amount of each target was quantified based on the concentration of the standard curve and was presented as arbitrary units. The quantity of each target was normalized against the quantity of GAPDH.

Proliferation Assay

Growing cells from non-confluent cultures were harvested and seeded in 24 well plates (Costar) at a density of 20×10^3 cells per well in 1 ml of DMEM (10% FBS). The cells were allowed to rest overnight. Prior to stimulation the cells were incubated in medium with 2% FBS for 24 h. The medium was replaced with fresh medium supplemented with heparin, CS or DS at concentrations of 1, 10, 30, and 100 μg/ml, PDGF-BB (10 ng/ml), β-D-xyloside (50 μg/ml) chondroitinase ABC (0.1 u/ml), and heparitinase (0.001 u/ml). In the case of β-D-xyloside, chondroitinase ABC and heparitinase, PDGF-BB (10 ng/ml) was added after 1 h of treatment, in fresh medium with 2% FBS. After 32 h of incubation [methyl]-^{3}H thymidine (Moravek Biochemicals) was added up to the final concentration of 0.2 μCi/ml and the cells were incubated for further 16 h (total assay treatment time 48 h). Radioactivity was measured in a liquid scintillation spectrometer (Beckman Instruments).

Wound Healing

Human lung fibroblasts were seeded in 24-well culture plates and cultured in DMEM supplemented with 10% FBS until confluent. The cells were starved for 24 h in DMEM with 2% FBS. The cell layer was wounded by scratching with a sterile 10 μl pipette tip. Detached cells were removed by washing two times with 2% FBS media. The cells were treated with PDGF-BB (10 ng/ml), CS (30 μg/ml) and co-treated with PDGF-BB (10 ng/ml) and CS (30 μg/ml). The wound closure was monitored using a digital image processor connected to a microscope, at six different positions across the wound. The cells motility was quantified by image analysis (ImageJ 1.4.3.67 Launcher Symmetry Software).

Chemotaxis Assay

DLF cell chemotaxis was assessed using 24 transwell plates (Cat. 3422, Corning, Inc.). 5.000 DLF cells were loaded into the upper well of the chamber with 2% FBS DMEM. Chemoatractants [PDGF-BB (10 ng/ml) and CS (30 μg/ml)] were placed in the bottom chamber. After 24 h incubation time the migrated cells under the bottom of the insert were dislodged using Cell Detachment Solution (Cat. 90131, Chemicon), lysed using Cell Lysis Buffer (Cat. 90130, Chemicon) and cell number measured using CyQuant GR Dye (Cat. 90132) on a fluorometer (Biotek) using the 480/520 nm filter set.

Western Blot

Growing cells from non-confluent cultures were harvested and seeded in 25 cm^2 flasks. The cells were allowed to rest overnight in 10% FBS. Cells were incubated in 2% FBS supplemented medium 24 h prior to stimulation with PDGF-BB (10 ng/ml), CS (30 μg/ml), and β-D-xyloside (50 μg/ml). The incubation time with PDGF-BB, CS, and β-D-xyloside was 30 min in order to study the phosphorylation of PDGF receptors. After that, the cells were lysed with Tris/HCL 50 mM, EDTA 0.5 M, Triton X-100 1%, NaCl 0.1%, and protease/phosphatase inhibitors (PMSF 1 mM, NEM 5 mM, benzamidin 5 mM, sodium cholate 1%, orthovanadate 1 mM). The cell lysates were electrophoresed on 8% polyacrylamide Tris/Glycine gels and transferred to nitrocellulose membranes in 10 mM CAPS, pH 11, and containing 10% methanol. The membranes were blocked for 12 h with PBS containing 0.1% Tween-20 (PBS-T) and 5% (w/v) low-fat milk powder. The membranes were incubated for 1 h at room temperature with primary antibodies in PBS containing 0.1% Tween-20 (PBS-T) and 1% (w/v) low-fat milk powder. The immune complexes were detected after incubation with peroxidase-conjugated anti-rabbit or anti-goat antibody (Santa Cruz Biotechnology), diluted (1:4,000) in PBS-T, 1% low-fat milk, with the SuperSignal West Pico Chemiluminescent substrate (Pierce), according to the manufacturer' instructions. The antibodies used in the present study were: rabbit anti-PDGF-Rβ, goat anti-p-PDGF-Rβ (tyr 1021), and goat anti-Actin (all from Santa Cruz Biotechnology).

For the dot blot analysis, lung fibroblasts were seeded in 12 well plates. They were serum starved for 24 h and then treated with β-D-xyloside (50 μg/ml) for 48 h in 2% FBS medium. Ten microliter of the culture supernatant was spotted on a positively charged PVDF membrane (Biotrace HP 0.45 μm, Gelman

Sciences) using a dot blot device (Bio-Dot apparatus, BIO-RAD). In addition, cells after 1 h chondroitinase ABC (0.1 u/ml) treatment were lysed as described in the previous section and 10 μl of lysate was spotted on the membrane. On the same membrane a standard curve was included using dilutions of purified CSA chains (0.31 up to 5 mg/ml) diluted in 2%FBS medium. The amount of standard curve solution used for spotting was 10 μl. The membranes were blocked for 1 h with PBS containing 0.1% Tween-20 (PBS-T) and 5% (w/v) low-fat milk powder, at room temperature. The membrane was incubated for 1 h at room temperature with monoclonal anti-mouse CSA (Sigma) primary antibody diluted 1:200 in PBS containing 0.1% Tween-20 (PBS-T) and 1% (w/v) low-fat milk powder. The immune complexes were detected after incubation with anti-mouse (Santa Cruz Biotechnology) diluted 1:2,000 in PBS containing 0.1% Tween-20 (PBS-T) and 1% (w/v) low-fat milk powder, with the SuperSignal West Pico Chemiluminescent substrate (Pierce), according to the manufacturer' instructions.

Statistical Analysis

The statistical significance was evaluated using the *t*-test and the one way completely randomized variance analysis (ANOVA) using the Microcal Origin (version 5.0) software.

RESULTS

Normal lung fibroblasts (DLF) (passage 6–10) were utilized in this study (Fig. 1A). PDGFR α and β were analyzed at the level of mRNA by real time PCR and at protein level by Western blotting, under serum starvation (2% FBS) culture conditions. The fibroblasts expressed highly both receptors (Fig. 1B). DLF cells demonstrated high proliferative responses after PDGF-BB (10 ng/ml) stimulation (Fig. 1C). bFGF and TGFβ2 were used as a positive and negative control for fibroblast proliferation, respectively.

To examine the effects of endogenously synthesised GAGs on PDGF-BB function, we utilized β-D-xyloside, which is used as an artificial acceptor of CS/DS and HS chains during biosynthesis enhancing the formation of secreted GAG chains. As shown in Figure 2B, β-D-xyloside alone inhibited marginally the proliferation of normal lung fibroblasts. However, the inhibitory effect of β-D-xyloside was more pronounced on the PDGF-BB-induced stimulation of DLF proliferation (Fig. 2B). These results indicate that the synthesis of CS/DS and/or HS GAG chains can somehow interfere with PDGF-BB mitogenic signaling. However, they cannot completely inhibit the PDGF-BB signaling.

In order to examine the possible participation of different cell-associated GAGs to the PDGF-BB

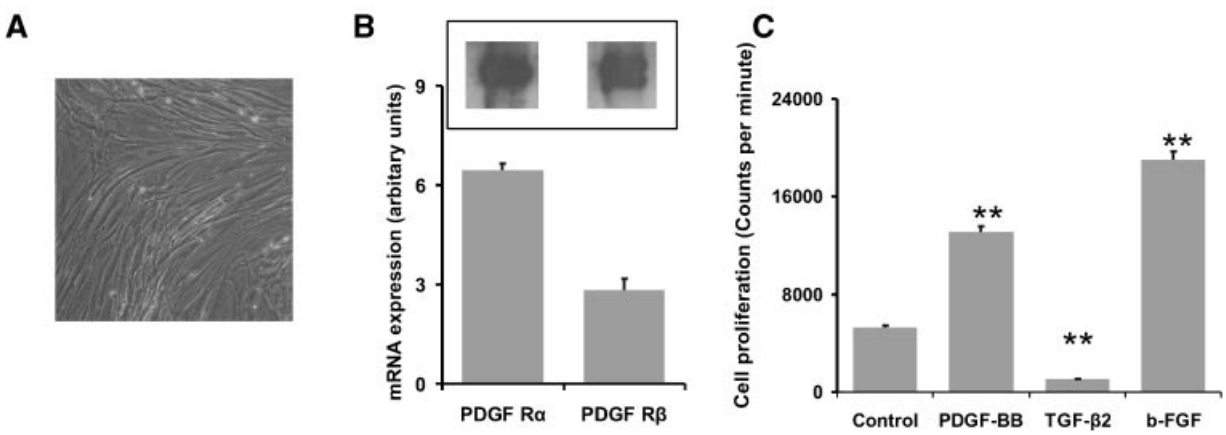

Fig. 1. Analysis of DLF fibroblast morphology, PDGF receptor expression status and the effect of PDGF-BB, TGFβ2, and b-FGF on their proliferation. **A**: Microscopic image of cultured (1×10^6 cells per 75 cm^2 flask) DLF lung fibroblasts. **B**: Western blot analysis of PDGF receptors in serum-starved (2% FBS) DLF cell extracts (1×10^6 cells per 75cm^2 flask). The cells were lysed and immunobloted with PDGF-Rβ and -Rα antibodies (diluted in 1/800 and 1/200, respectively). The diagram represents real-time PCR quantification of PDGF-Rβ and -Rα mRNA in DLF cells after 24 h serum starvation (2% FBS). DLF cells were cultured (5×10^5 cells per 25 cm^2 flask) in 2% FBS for 24 h whereupon the RNA was extracted and cDNA synthesized. **C**: DLF cells were starved (2% FBS) for 24 h and treated with 10 ng/ml PDGF-BB, TGFβ2 and b-FGF for 48 h and cell proliferation measured using [methyl]-^{3}[H]thymidine incorporation. Means ± SEM plotted; n = 5. Statistical significance: $^*P < 0.01$, $^{**}P < 0.001$; ns: non-significant.

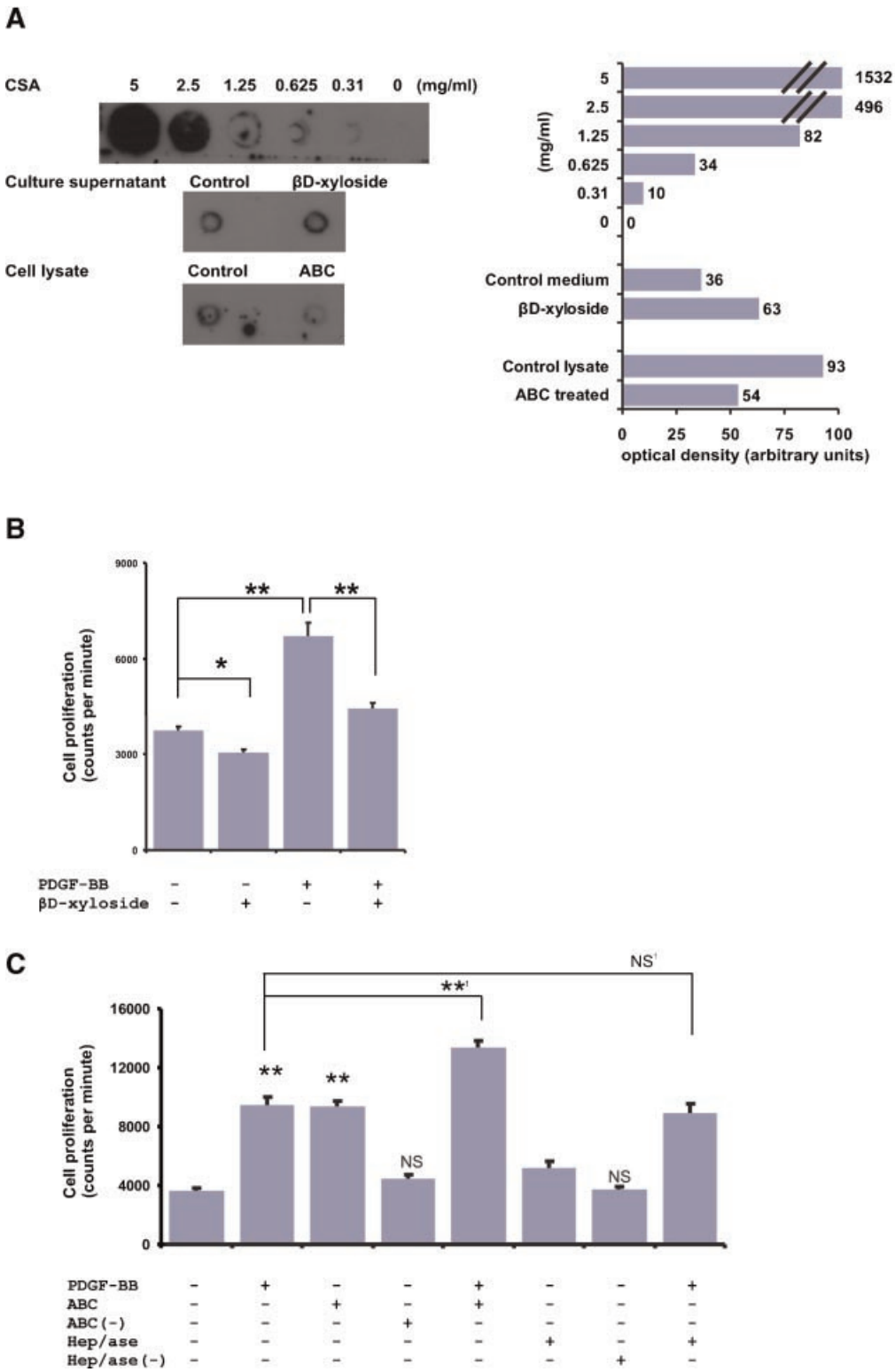

Fig. 2. Effects of PDGF-BB, β-D-xyloside (βDx), chondroitinase ABC and heparitinase on human lung fibroblast cell proliferation. **A**: Detection of chondoitin sulphate A chains by a dot Western blot analysis. Increasing concentrations of CSA (0.31 up to 5 mg/ml) were spotted on a PVDF membrane. β-D-xyloside: cultured medium from lung fibroblasts treated with β-D-xyloside (50 µg/ml) for 48 h. ABC: cell lysate derived from lung fibroblasts treated with chondoitinase ABC (0.1 u/ml) for 1 h. To the right, the bar chart depicts the spot optical density (sample values are expressed in arbitrary units). **B**,**C**: DLF cells were starved for 24 h and treated for 48 h with combinations of PDGF-BB (B) (10 ng/ml) or with β-D xyloside (50 µg/ml); chondroitinase ABC (0.1 u/ml) (C), heat inactivated chondroitinase ABC [ABC(−)] (0.1 u/ml), heparitinase (0.001 u/ml) (C) and heat inactivated [Hep/ase(−)] (0.001 u/ml), whereupon the cell proliferation was measured using [methyl]-^{3}[H] thymidine incorporation. Means ± SEM plotted; n = 5. Statistical significance: $^{*}P < 0.01$, $^{**}P < 0.001$; ns, non-significant. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

function, DLF cells were treated with chondroitinase ABC or heparitinase. The enzymic treatment of the cells with chondroitinase ABC aimed at the preferential removal of CS and DS chains (Fig. 2A), while that with heparitinase targeted at the removal of HS chains. The removal of surface GAGs is a transient event since the cells will very quickly start to restore surface GAG content. As shown in Figure 2C, chondroitinase ABC treatment demonstrated a significant stimulatory effect on the basal levels of proliferation. Furthermore, combined treatment with chondroitinase ABC and PDGF-BB resulted in a costimulatory effect on the level of proliferation. Removal of cell associated HS by heparitinase treatment had a minor stimulatory effect on the basal proliferation, whereas it had no significant effect on the PDGF-BB-induced proliferation of the DLF cells. Heat-inactivated chondroitinase ABC, indicated in Figure 2C as ABC (−), and heparitinase, indicated in Figure 2C as hep/ase (−), had no effect on the cell proliferation indicating that the observed effects were dependent on their enzymic activity. The obtained data indicated that the removal of cell associated CS/DS and not HS chains seemed to enhance PDGF-BB mitogenic function. Nevertheless, we cannot exclude the possibility that the GAG effector pathway is acting independently.

In order to evaluate the specific role of CS and DS on PDGF mitogenic function, purified CS, DS, and heparin were exogenously added in the presence or absence of PDGF-BB and the effects on cell proliferation examined. The CS, DS and heparin used in this study have been previously subjected to an extensive biochemical characterization [Syrokou et al., 1999a; Nikitovic et al., 2005]. In pilot dose response experiments, CS and DS had no statistically significant effects, whereas heparin demonstrated a pronounced dose dependent inhibition. None of the GAGs had either cytotoxic effects or caused significant changes on the cell morphology. Combined treatment of DLF cells with CS (30 μg/ml), DS (30 μg/ml) or heparin (10 μg/ml) with PDGF-BB (10 ng/ml) resulted in a significant inhibitory effect for CS and heparin on the PDGF-BB-induced proliferation (Fig. 3A). The inhibitory effect of PDGF in combination with heparin may be attributed to the highly inhibitory action of heparin on the basal proliferation of these cells.

To examine whether the observed inhibition of PDGF-BB signaling by CS affects also its ability to promote migration and chemotaxis, wound healing and chemotaxis assays were performed. It was established that combined treatment of PDGF-BB and CS severely reduced both the cell motility (Fig. 3B) as well as the chemotactic response of the fibroblasts (Fig. 3C). Furthermore, CS autonomously affected the migration and chemotaxis of the DLF cells. The obtained results indicate that CS, and not DS, is responsible for the inhibition of the PDGF-BB-induced proliferation in normal lung fibroblasts shown above. Furthermore, CS appears to affect both the PDGF-dependent and independent motility and chemotactic responses of the lung fibroblasts.

To determine whether the CS inhibitory effects on the PDGF-BB-induced cell proliferation was mediated by modulation of PDGF receptor levels, CS, DS, heparin, and PDGF-BB were exogenously added and the gene expression level of PDGF-Rα and -Rβ were monitored by real-time PCR. Both PDGF-Rα and -Rβ transcripts were negatively affected by PDGF-BB, in agreement with the negative feedback pathway which has been previously reported for this growth factor [Tingstrom et al., 1992]. PDGF-Rα was not significantly affected by CS and up-regulated by DS (53%) and heparin (61%) (Fig. 4). PDGF-Rβ levels were not significantly affected by any of the added GAGs. Furthermore, the combined treatment of CS and PDGF-BB did not significantly affect PDGF-Rα and -Rβ receptors at the protein level as was found by Western blot analysis (data not shown). These data demonstrated that the inhibitory effect of CS on the mitogenic function of PDGF-BB is not caused by reduced signaling due to either transcriptional or translational downregulation of the corresponding receptors .

To assess the PDGF receptor activation, the phospho-specific antibody anti-p-PDGF-Rβ (tyr 1021), which specifically recognizes the phosphorylation of the tyrosine 1021 residue was used (Fig. 5). PDGF-BB efficiently stimulates phosphorylation of tyrosine 1021 residue, on PDGF-Rβ. Following combined treatment of PDGF-BB and exogenous CS, a significant reduction of PDGF-Rβ phosphorylation was found. Combination of β-D-xyloside with PDGF-BB resulted in an almost complete inhibition of PDGF-Rβ phosphorylation. These results demonstrated that the exogenous addition of CS and/or the enhancement of endogenous CS chain

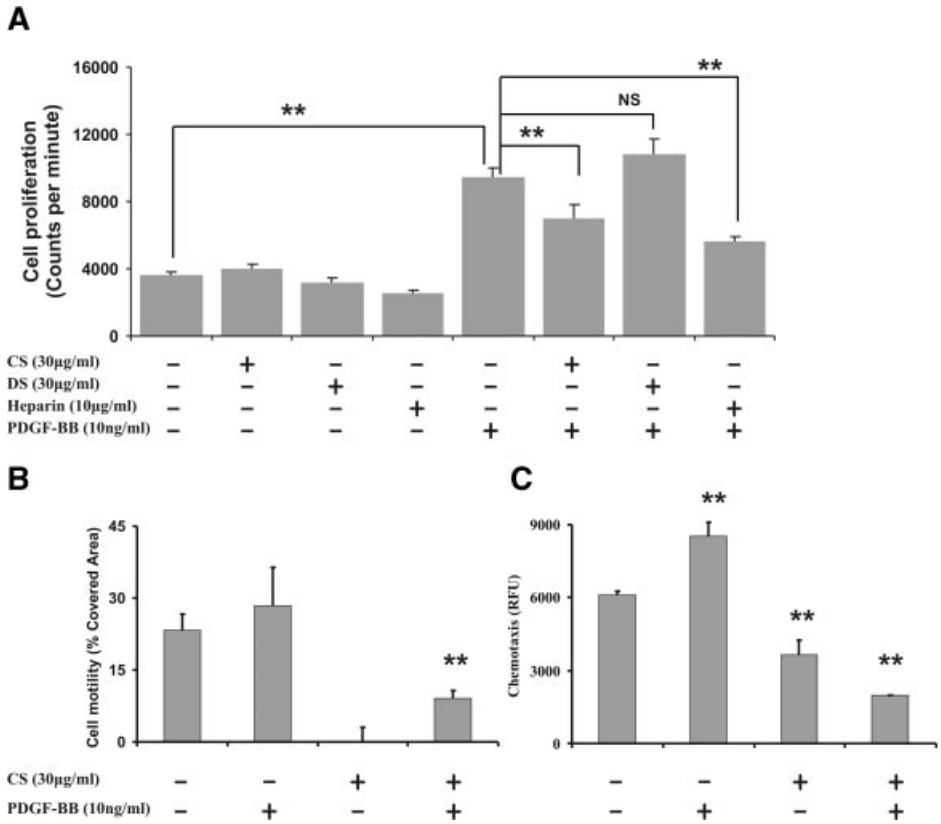

Fig. 3. Effect of exogenous CS, DS (CSB), and heparin on DLF mitogenic response. **A**: Serum starved (2% FBS) DLF cells were treated with a combination of CS (30 μg/ml), DS (30 μg/ml), or heparin (10 μg/ml) and PDGF-BB (10 ng/ml). Means ± SEM plotted; n = 3. Statistical significance: $*P < 0.01$, $**P < 0.001$; ns: non-significant. **B**: Wound healing assay. The cells were treated with PDGF-BB (10 ng/ml), CS (30 μg/ml), and co-treated with PDGF-BB (10 ng/ml) and CS (30 μg/ml). The cell layer was wounded by scratching with a sterile 10 μl tip. The wound closure was monitored at six different positions across the wound. The cell motility was quantified by image analysis. Means ± SEM plotted; n = 6. Statistical significance: $*P < 0.01$, $**P < 0.001$; ns, non-significant. **C**: Chemotaxis Assay. 5.000 DLF cells were loaded into the upper chamber of the 24-transwell plate with 2% FBS DMEM. Chemoatractants [PDGF-BB (10 ng/ml) and CS (30 μg/ml)] were placed in the bottom chamber. After 24 h incubation time the migrated cells under the bottom of the insert were dislodged using Cell Detachment Solution (Invitrogen) and cell number measured on a fluorometer. Means ± SEM plotted; n = 3. Statistical significance: $*P < 0.01$, $**P < 0.001$; ns, non-significant.

synthesis can abolish almost completely the PDGF-Rβ phosphorylation. It can be therefore concluded that secreted CS chains may account for the severely impaired PDGF-BB-related mitogenic responses.

DISCUSSION

This study demonstrated that PDGF-BB receptor signaling and mitogenic responses can be significantly affected by both endogenous and exogenously added CS. The CS chains were found to downregulate the proliferative responses of fibroblasts by reducing the phosphorylation of PDGF-Rβ. PDGF is a strong mitogen and chemoattractant in mesenchymal cells, including fibroblasts, myofibroblasts and smooth muscle cells. The phosphorylation of PDGF receptor triggers downstream a number of signaling enzymes [Tallquist and Kazlauskas, 2004]. The normal human lung fibroblasts that were used in the present study expressed both PDGF receptors at high levels and their growth was found to be responsive to PDGF-BB stimulation. The encounter/interaction of PDGF and fibroblasts usually takes place in a microenvironment rich in extracellular matrix components. GAGs have been shown to physically interact with PDGF-BB through the positive amino-acid stretches found in its sequence [Fager et al., 1995]. This interaction affects the bioavailability of PDGF through storage and/or controlled release and has the potential to affect its biological functions.

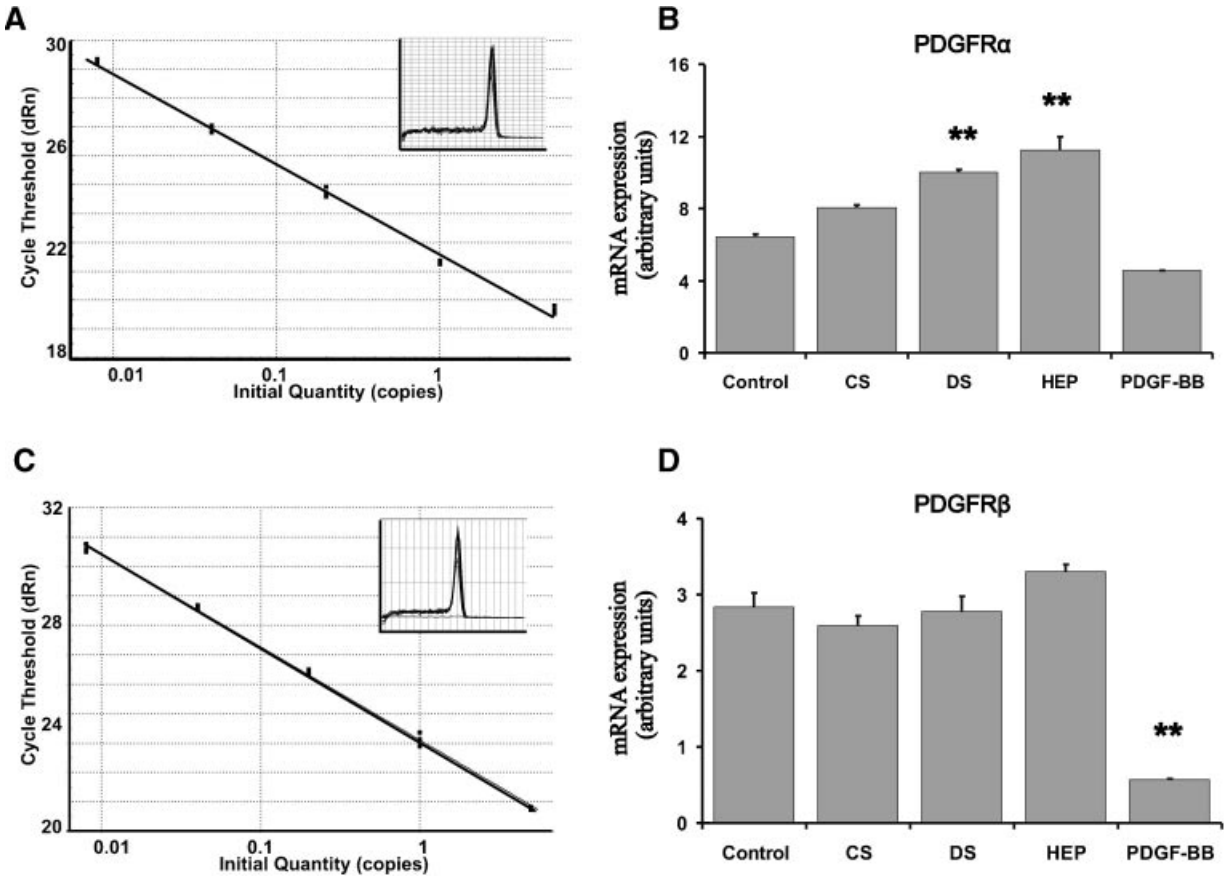

Fig. 4. Effects of exogenous CS, DS, and heparin on the expression of PDGF-Rα (**B**) and -Rβ (**D**). After 24 h serum starvation (2% FBS), DLF fibroblasts were treated for 24 h with CS, DS, heparin, and PDGF-BB. RNA was extracted and cDNA was synthesized. The real-time PCR reaction was performed with the QuantiTech SYBR Green master mix in a total volume of 20 µl. Standard curves were run in each optimized assay producing a linear plot of threshold cycle (Ct) against log dilution (**A**,**C**). The specificity of the PCR reaction was verified by melting point analysis (small window in A, C). The mRNA level of PDGF Rα and Rβ were quantified by utilizing the standard curves and presented as arbitrary units (B,D). The quantity of each target was normalized against the quantity of GAPDH. Means ± SEM plotted; n = 3. Statistical significance: $*P < 0.01$, $**P < 0.001$; ns, non-significant.

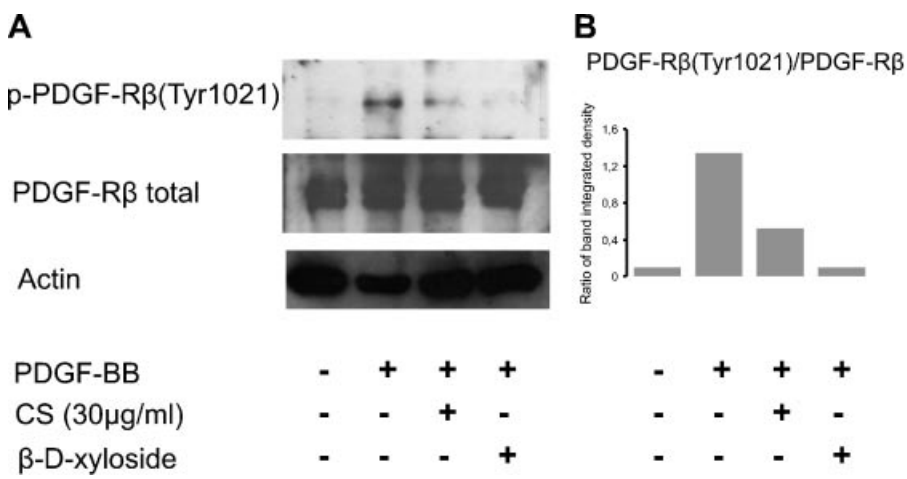

Fig. 5. Effect of CS and xyloside on the phosphorylation of tyrosine 1021 PDGF Rβ. Cells were incubated in 2% FBS supplemented medium, for 24 h prior to stimulation with PDGF-BB for 30 min were then lysed as described in Materials and Methods Section. Cell extracts were analyzed using Western blot analysis. **A**: The Western blot analysis of p-PDGF-Rβ (Tyr1021) and PDGF-Rβ total, (**B**) the intensities of the bands were measured and the ratio between p-PDGF-Rβ (Tyr1021)/PDGF-Rβ total are indicated on the graph.

The endogenous production of free GAG chains can affect both cell apoptosis [Cartel and Post, 2005] and proliferation, as it has been previously shown in several normal and cancerous cell lines treated with β-D-xyloside [Miao et al., 1995]. The aglycone derivative of β-D-xyloside used in the present study was *p*-nitrophenyl, which is known to induce mainly chondroitin/DS synthesis [Lugemwa and Esko, 1991]. Combined treatment of lung fibroblasts with β-D-xyloside and PDGF-BB resulted in an inhibition of the PDGF-BB-induced cell proliferation, demonstrating a potential inhibitory effect of the endogenous GAGs on PDGF-BB function. Specific GAG-degrading lyases on live cells have been routinely used for examining the activities of chondroitin sulfate and heparan sulphate proteoglycans of the cell membrane [Rapraeger and Yeaman, 1989; Maeda et al., 1996; Lyon et al., 1997; Milev et al., 1998; Denholm et al., 2000]. Combined chondroitinase ABC and PDGF-BB treatment in the present study demonstrated an increased level of proliferation. These results confirm the data from the β-D-xyloside treatment where it was shown that the increase of the endogenous CS/DS biosynthesis inhibited PDGF-BB-induced growth response.

In order to establish which GAG type exerted the observed negative effect, we exogenously applied CS, DS, and heparin preparations which we had previously biochemically characterized [Nikitovic et al., 2005]. Heparin resulted in a highly inhibitory effect on the basal level of proliferation, in agreement with our previously reported data on fibrosarcoma, osteosarcoma [Nikitovic et al., 2005; Fthenou et al., 2006] and mesothelioma cells [Syrokou et al., 1999b]. Similarly, DS, heparin, and heparan sulfate have been found to inhibit the proliferation of normal fibroblasts [Ferrao and Mason, 1993; Westergren-Thorsson et al., 1993], although in our study DS was mildly inhibitory only in the highest concentration. CS has been previously shown to mildly promote the proliferation of cancer cell lines [Fthenou et al., 2006], its effect on the growth of normal lung fibroblasts of the present study was marginal inhibition. Combined treatment of DLF cells with CS, DS, or heparin with PDGF-BB resulted in a significant inhibitory effect for CS and heparin on PDGF-dependent signaling. The inhibitory effect of heparin is probably due to its highly inhibitory action on the basal proliferation, whereas, CS exerted significant inhibitory effects on PDGF-BB function in agreement with our previous findings. Therefore, CS chains specifically and not DS, are responsible for the inhibition of the PDGF-BB-induced proliferation. Apart from its mitogenic properties, PDGF-BB has been shown to promote cell motility and to function as a chemotactic signal for cells of mesenchymal origin [Schneller et al., 1997; Woodard et al., 1998; Ronnstrand and Heldin, 2001]. Our study demonstrated that both these functions are severely affected by the presence of free CS chains indicating that CS causes a generalized blockade of the PDGF-BB signaling pathway. This blockade could not be attributed to reduced signaling due to transcriptional or translational downregulation of the corresponding receptors. Similar inhibition of the migratory responses has been demonstrated in dermal fibroblasts after treatment with hyaluronan [Selbi et al., 2006].

In a previous study it has been shown that chondroitinase ABC treatment of CHO cells did not increase the actual binding of PDGF-BB on the membrane [Garcia-Olivas et al., 2003], therefore the reported increased PDGF-BB mitogenic activity after chondroitinase ABC treatment might be due to enhanced signaling. On the other hand CS although it has been shown to bind with high affinity directly to PDGF-BB [Fager et al., 1995] cannot block the binding of PDGF-BB to the surface of CHO cells, [Garcia-Olivas et al., 2003] indicating that the observed negative effects of the free CS chains cannot be attributed to a direct inhibition of the interaction with the PDGF receptor. Upon interaction with PDGF, the PDGF receptor dimerizes and becomes phosphorylated on a number of tyrosine residues of its intacellular domain. Our study focused on the phosphorylation of tyrosine residue 1021 on PDGF-Rβ known to be specific for receptor activation. It was demonstrated that tyrosine 1021 on PDGF-Rβ becomes phosphorylated in the presence of PDGF-BB and it is therefore considered responsible for the observed PDGF-BB-induced mitogenic effects. Furthermore, treating the cells with free CS chains or β-D-xyloside prior to PDGF-BB stimulation resulted in a significantly reduced phosphorylation of tyrosine 1021 on PDGF-Rβ. In agreement with our results, it has previously been shown that CS-bearing decorin can prevent PDGF-Rβ phosphorylation resulting in significant

reduction of SMC proliferation, migration and collagen synthesis [Nili et al., 2003].

The results of this study suggest the possible involvement of an unknown chondroitin sulfate proteoglycan which has the ability to inhibit the phosphorylation of PDGFRs. Free or membrane bound chondroitin sulfate chains could potentially recruit this inhibitor and direct it on PDGF-Rβ, reducing its phosphorylation. A very promising candidate is the transmembrane receptor CD44 which has recently been shown by co-immunoprecipitation to be in close proximity to the PDGF-Rβ [Li et al., 2006]. CD44 contains a single domain which can bind both CS and hyaluronan GAG chains. The interaction of CD44 with hyaluronan has been studied in detail in the last years [de La Motte et al., 1999; Lesley et al., 2000; Selbi et al., 2006] and has been found to be extremely important for the formation of the pericellular matrix. Recently, it has been shown that upon hyluronan binding CD44 can recruit phosphatases that reduce the phosphorylation of PDGF-Rβ [Li et al., 2006]. Further investigation of the involvement of CD44 in the CS-dependent regulation of PDGF signaling is under way in our laboratory.

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