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Inhibition of human dermal fibroblast proliferation by removal of dermatan sulfate

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Abstract

In the current study, a glycosaminoglycan lyase, chondroitinase B, was used to study the role of dermatan sulfate proteoglycans on human dermal fibroblast proliferation. Pretreatment with chondroitinase B significantly decreased fibroblast proliferative responses to serum (20% to 55%). In contrast, heparinase III and chondroitinase AC were less effective in inhibiting fibroblast proliferation to serum. Analysis of glycosaminoglycans on chondroitinase B-treated fibroblasts confirmed that dermatan sulfate was removed from fibroblasts by this enzyme. Chondroitinase B treatment also decreased proliferation to basic fibroblast growth factor (bFGF) by 20% and reduced receptor binding by 25%. Heparinase III inhibited bFGF binding by 73%, but decreased proliferation to bFGF by only 21%. Chondroitinase AC had no effect on bFGF proliferation or binding. These data suggest that dermatan sulfate proteoglycans play a significant role in the control of human dermal fibroblast proliferation.

Keywords: Dermatan; Chondroitin sulfate; Fibroblast; Proliferation; Chondroitinase

1. Introduction

Proteoglycans on the cell surface and in the extracellular matrix contain variable glycosaminoglycan chains, which include heparan sulfate, chondroitin sulfate and dermatan sulfate. While some proteoglycans contain only one type of glycosaminoglycan, others contain a mixture of heparan and chondroitin sulfate or dermatan sulfate (Jackson et al., 1991; Rapraeger, 1989). The functions of proteoglycans and their component parts have been extensively studied, with much of the emphasis on the roles of heparin and heparan sulfate on cell metabolism (Kjellen and Lindahl, 1991; Vlodavsky et al., 1995; Yayon et al., 1991). Much less is known about the biological activities of chondroitin sulfate and dermatan sulfate containing proteoglycans, particularly as relates to their effects on cell proliferation.

In much of the work done to examine the biological roles of different proteoglycans, there has been no clear means of assigning activities specifically to either chondroitin sulfate or dermatan sulfate, and thus chondroitin sulfate most often refers to chondroitin sulfate A, chondroitin sulfate C as well as dermatan sulfate (also called chondroitin sulfate B). Evidence for a role of chondroitin sulfate proteoglycans in cell proliferation includes studies, which have examined the effects of the addition of exogenous glycosaminoglycans; those, which have utilized inhibitors of glycosaminoglycan synthesis or sulfation; and those, which have utilized different glycosaminoglycan lyases.

Conflicting data exist, which indicate that the addition of exogenous chondroitin sulfate or dermatan sulfate increased (Penc and Gallo, 1998; Tao et al., 1997; Volpi et al., 1994), decreased (Westergren-Thorsson et al., 1993), or had no effect (Ferrao and Mason, 1993; Schmidt et al., 1992) on cell proliferation. However, the effects of soluble glycosaminoglycans on cell metabolism may be quite different from that of glycosaminoglycans incorporated into proteoglycans on the plasma membrane. In this respect, β-xyloside, which increases soluble chondroitin sulfate and decreases cell-associated chondroitin sulfate, has been

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shown to reduce smooth muscle cell proliferation (Hamati et al., 1989). Similar experiments, with chlorate, which inhibits chondroitin sulfate and heparan sulfate proteoglycan sulfation, have demonstrated a decrease in fibroblast responsiveness to growth factors (Fannon and Nugent, 1996). While chlorate and β-xylosides have been used to examine the relative contributions of heparan sulfate and chondroitin sulfate proteoglycans to control of the cell cycle (Keller et al., 1989; Miao et al., 1995; Schwartz, 1977), there are currently no inhibitors, which can selectively block dermatan sulfate proteoglycan synthesis. There are however, specific glycosaminoglycan lyases, which can remove heparan sulfate, chondroitin sulfate or dermatan sulfate from cells.

Many studies examining the activities of chondroitin sulfate proteoglycans (Lyon et al., 1998; Maeda et al., 1996; Milev et al., 1998; Rapraeger, 1989) have utilized one such enzyme, chondroitinase ABC, which is capable of degrading chondroitin sulfate A, chondroitin sulfate C and chondroitin sulfate B (dermatan sulfate). Other recent work has used chondroitinase B, a glycosaminoglycan lyase, which acts only on dermatan sulfate (Gu et al., 1995), to examine the role of dermatan sulfate in wound healing (Penc and Gallo, 1998) and cell activation (Penc et al., 1999).

In the present experiments, chondroitinase B, was used to selectively remove dermatan sulfate from human skin fibroblasts, and to determine the influence of dermatan sulfate proteoglycans on fibroblast proliferation. Results of these experiments suggest that dermatan sulfate proteoglycans are important components of the complex extracellular matrix control system, which regulate cellular responses to growth factors.

2. Materials and methods

2.1. Materials

Dulbecco's minimal essential medium, Fischer's medium, phosphate buffered saline and fetal bovine serum were from GIBCO, Grand Island, NY. Dermatan sulfate, chondroitin sulfate A and C, and heparan sulfate were purchased form Celsus Laboratories, Cincinnati, OH. Basic fibroblast growth factor (bFGF) was purchased from Peprotech, Rocky Hill, NJ. Calcein acetoxymethyl (AM) ester and CyQuant proliferation kits were from Molecular Probes, Eugene, OR.

2.2. Enzymes

Chondroitinase B (no EC number), chondroitinase AC (EC 4.2.2.5) and heparinase III (EC 4.2.2.8) are recombinant proteins expressed in *Flavobacterium heparinum* (Gu et al., 1995; Su et al., 1996). Enzymes were purified from *F. heparinum*, after which specific activity and substrate specificity were determined for each enzyme, using a kinetic spectrophotometric assay, performed essentially as described by (Gu et al., 1995). The specific activities of the enzymes were: 100 IU/mg for chondroitinase B, 160 IU/mg for chondroitinase AC, and 60 IU/mg for heparinase III. Each enzyme had a purity of at least 98% as assessed by high-pressure liquid chromatography. Heat-inactivated chondroitinase B was prepared by heating the enzyme at 100°C for 10 min prior to dilution in growth medium. Enzymatic analysis showed that activity was reduced to $< 0.01\%$ of the original activity by this procedure.

2.3. Proliferation assay

Human dermal fibroblasts were obtained from Clonetics, San Diego, CA. Cells were cultured in Dulbeccos' minimal essential medium containing 1% antibiotics and 10% fetal bovine serum. The proliferation assay was performed as previously described (Denholm and Phan, 1989). Briefly, cells were plated in medium w/10% serum; 24 h later this medium was replaced with serum-free medium, and incubation continued for an additional 24 h. Cells were then treated with either serum-free medium alone, or medium containing the indicated concentration of enzyme for 1 h at 37°C. Following enzyme treatment, cells were rinsed $1\times$ with serum-free medium, then given medium w/10% serum and incubated for 48 h. In experiments using bFGF, medium containing 2 mg/ml bovine serum albumin was used, with or without 1.0 ng/ml bFGF. Controls for each experiment were: (negative) untreated cells incubated in serum-free medium, and (positive) untreated cells incubated in medium with 10% serum. The number of cells per well was quantitated using the CyQuant assay method from Molecular Probes, Eugene, OR. Fluorescence/well was determined using a CytoFluor Series 4000 fluorescent plate reader (PerSeptive Biosystems) and cell numbers calculated from a standard curve. The average number of cells/well in negative controls was $3.0 \pm 0.3 \times 10^4$, and for positive controls was $9.0 \pm 0.8 \times 10^4$ (mean ± S.E.M.; $n = 10$). Based on controls for each experiment, data is represented as % Inhibition, where:

$$\%\text{Inhibition} = 1 - \left[(\#\,\text{cells/well enzyme-treated}) / (\#\,\text{cells/well untreated})\right] \times 100\%.$$

2.4. Analysis of cell-associated glycosaminoglycans

The relative amount of cell-associated glycosaminoglycans on fibroblasts, were analyzed using a dot blot assay, as previously described (Rapraeger and Yeaman, 1980). Briefly, glycosaminoglycans were labeled by incubating fibroblasts with [^{35}S]sodium sulfate for 3 days in Fischer's

medium with 10% serum. Following labeling, cells were washed 3× in serum-free medium, then treated for 1 h with either medium alone, or medium containing 1.0 IU/ml of chondroitinase B. After treatment and washing to remove enzyme, medium was replaced with fresh Fischer's medium containing 10% serum, and incubation continued for 6 to 72 h. Cells were washed, then lysed with 10 mM Tris–HCl pH 8.0, 8 M urea, 0.1% Triton X-100. Cell lysates were heated to100°C for 5 min. Cell number/well was estimated using the CyQuant assay (see Section 2.3). Lysate equivalent to 10^4 cells was added to each slot of a the dot blot containing a Zeta-Probe nylon filter (Bio-Rad). Blots were cut into segments and treated with either phosphate-buffered saline with 1.0 mM$CaCl_2$, 0.5 mM $MgCl_2$ (PBS), or PBS containing 1.0 IU/ml of chondroitinase B for 1 h at 37°C. Blots were washed and counted as described previously. The relative amount of dermatan sulfate was calculated as:

$$\%\,\text{dermatan sulfate} = \frac{(\text{cpm PBS treated lysate}) - (\text{cpm enzyme treated lysate})}{(\text{cpm PBS treated lysate})}.$$

2.5. Western blot analysis of cell-associated glycosaminoglycans

Fibroblasts were grown to confluence in 75 cm^2 flasks. Cells were treated with either medium alone, 1.0 or 10 IU/ml chondroitinase B, or 1.0 IU/ml of heparinase III for 1 h at 37°C. After enzyme treatment, cells were washed and lysed with extraction buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM sodium orthovanadate, 1% Triton X-100, 5 mM EDTA, 2 mM phenylmethylsulfonylfluoride, 10 mM sodium fluoride, 10 μg/ml leupeptin). Protein concentrations in lysates were determined using the Micro-BCA assay kit from Pierce (Rockford, IL). Lysate equivalent to 10 μg of cell protein for each dot was applied to Immobilon filters using a dot blot apparatus. The primary antibody was a monoclonal antibody (3G10, Seikagaku America, Falmouth, MA) which recognizes a heparan sulfate epitope generated by digesting heparan sulfate. An Amersham ECL detection kit with peroxidase conjugated goat anti-mouse immunoglobulin (IgG_2) was used to develop the blot.

2.6. Agarose gel electrophoresis of glycosaminoglycans

Glycosaminoglycans were labeled by incubation of fibroblasts with [^{35}S]sodium sulfate for 48 h at 37°C, as described under analysis of glycosaminoglycans. Cells were treated with either medium alone, or medium containing 1.0 IU/ml of chondroitinase B for 1 h. Glycosaminoglycans released into the medium were precipitated with Cetavalon, resuspended in sample buffer and applied to 0.5% agarose gels in 0.04 M barium acetate, pH 5.8, as described by Volpi (1993). Standards were unlabeled dermatan sulfate, chondroitin sulfate A and heparan sulfate at a concentration of 125 μg in 5 μl of sample buffer, and disaccharide fragments of dermatan sulfate prepared by pre-incubation of 20 mg/ml dermatan sulfate with 10 IU/ml of chondroitinase B for 1 h. Following electrophoresis and drying with a Gel Air-drying system (BioRad), gels were stained with 0.2% toluidine blue to visualize standards. Film was then applied to gels for detection of [^{35}S]-labeled glycosaminoglycans.

2.7. Receptor binding

Iodinated bFGF was obtained from Dupont NEN, specific activity > 1200 Ci/mmol. Fibroblasts were plated in 48 well dishes and grown to confluence. Prior to binding assays, cells were treated with medium or enzyme as indicated for proliferation assays. Following enzyme treatments, cells were chilled and binding assays carried out at 4°C. Cells were incubated for 1 h with 25 ng/ml of [^{125}I]bFGF alone, or with the addition of 25 μg/ml of unlabeled bFGF in binding buffer (Dulbecco's minimal essential medium, 25 mM HEPES, 0.05% gelatin). Following incubation with bFGF, cells were washed 2× with ice cold binding buffer. Glycosaminoglycan-bound [^{125}I]bFGF was removed with two rinses with wash buffer(2 M NaCl in 20 mM HEPES, pH 7.4). Receptor bound [^{125}I]bFGF was removed by washing 2× with wash buffer (pH 4.0) (Fannon and Nugent, 1996).

2.8. Adherence and viability

Fibroblasts were plated in 96 well plates as described for proliferation assays. On Day 2, prior to enzyme treatment, cells were labeled with 1 μM calcein for 20 min at 37°C. Following labeling, cells were washed once in serum-free medium to remove unincorporated dye. Cells were then treated with phenol red-free medium alone or with 1.0 or 10 IU/ml chondroitinase B for 1 h, rinsed 1× with medium after which fresh medium was added to wells. Fluorescence was determined at an excitation of 485 nm and emission of 530 nm. For determination of lactate dehydrogenase (LDH) release, unlabeled cells were treated exactly as described above. One hundred microliters was removed from each well immediately (0 h) and 18 h after enzyme treatment. LDH in the medium was determined using the CytoTox 96 LDH assay kit from Promega, Madison, WI.

2.9. Statistical analysis

Differences between control and enzyme-treated cells were analyzed using analysis of variance. When applicable, the Tukey method of multiple comparisons was used to determine whether there were statistically significant

differences ($P < 0.05$) between groups (Neter and Wasserman, 1974).

3. Results

3.1. Effects on proliferation

Initial experiments were performed to determine if removal of dermatan sulfate from fibroblasts with chondroitinase B would have any effect on proliferation of these cells. Proliferation of fibroblasts in response to 10% fetal bovine serum was inhibited in a dose-dependent manner when cells were pretreated for 1 h with 0.001 to 3.0 IU/ml of chondroitinase B (Fig. 1, top panel).

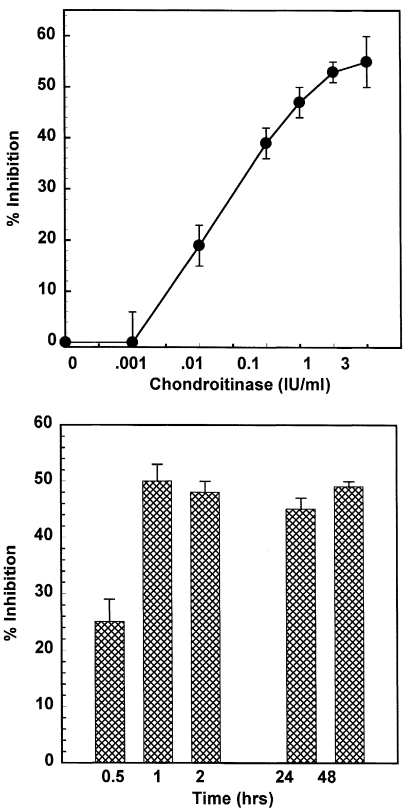

Fig. 1. Top panel. Dose-dependent effect of chondroitinase B (ChB) on fibroblast proliferation to 10% fetal bovine serum. Fibroblasts were pretreated for 1 h at 37°C with the indicated concentration (dose range of 0.001–3.0 IU/ml) of ChB in serum-free medium. The # cells/well was determined after 48 h in the presence of medium with 10% serum. Data were expressed as % inhibition, calculated by comparison of cell numbers in ChB-treated wells compared to medium-treated controls. Each point is the mean ± S.E.M. of four experiments performed in triplicate. Bottom panel. Time-dependent effects of ChB (dose of 1.0 IU/ml) on fibroblast proliferation to 10% serum. Cells were treated for the indicated time (range of 0.5–48 h) with either medium (medium + 10% serum) or medium containing ChB; following enzyme treatment, cells were given fresh medium without ChB and incubation continued for a total of 48 h. Data were expressed as % inhibition calculated by comparison of cell number in control and ChB-treated wells at each time point. Each bar represents the mean ± S.E.M. of three experiments.

Fibroblasts were subsequently treated with 1.0 IU/ml of chondroitinase B for varying amounts of time. Maximal inhibition of proliferation was achieved following a 1 h exposure to the enzyme (Fig. 1, bottom panel). Similar inhibition was observed after incubation with chondroitinase B for up to 48 h.

In further experiments, heat-inactivated enzyme was compared with active enzyme. There was no effect on proliferation when fibroblasts were treated for 48 h with 1.0 or 10 IU/ml of heat-inactivated chondroitinase B. Inhibition of proliferation with 1.0 and 10 IU/ml of heat-inactivated enzyme was $0 \pm 1\%$ and $0 \pm 3\%$, respectively (mean ± S.E.M., $n = 4$, data not shown).

3.2. Analysis of the resynthesis of dermatan sulfate in chondroitinase B-treated fibroblasts

The proliferation assay determined growth 48 h after removal of dermatan sulfate by chondroitinase B. Since dermatan sulfate would likely be replenished over this time period, we examined the rate of replacement of dermatan sulfate on cells following chondroitinase B treatment. Fibroblast glycosaminoglycans were labeled with ^{35}S, and cells were treated with either medium or chondroitinase B. Immediately following enzyme treatment and for intervals up to 72 h afterwards, cell-associated glycosaminoglycans were analyzed to determine the relative amount of dermatan sulfate present. In untreated controls, the percentage of dermatan sulfate ranged from 28% to 39% over the 72 h incubation (Fig. 2). In chondroitinase B-treated cells, dermatan sulfate was nearly undetectable immediately following enzyme treatment, and returned slowly to the cell surface. After 48 h, the percentage of dermatan sulfate in chondroitinase B-treated cells was half of that observed in control cells (30%). Dermatan sulfate on enzyme treated cells was still slightly lower 72 h after treatment with chondroitinase B.

In additional studies, exogenous dermatan sulfate (concentration range of 1 pg/ml–50 μg/ml) was added back to chondroitinase B-treated fibroblasts to determine if proliferative capacity could be restored. There was no stimulatory effect of exogenous dermatan sulfate at any of these concentrations, either in response to serum or to bFGF (data not shown).

3.3. Analysis of glycosaminoglycans removed from cells by chondroitinase B

To ensure that the effects observed on proliferation were due to the removal of only dermatan sulfate, glycosaminoglycans released in to the medium following enzyme treatment were examined. Fibroblast glycosaminoglycans were labeled with ^{35}S, and cells were treated with medium or chondroitinase B. Autoradiography of glycosaminoglycans released into the medium demonstrated

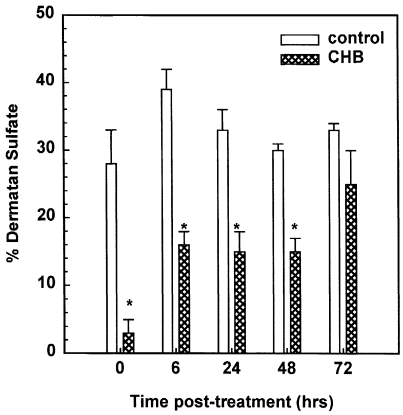

Fig. 2. Re-expression of dermatan sulfate (DS) on fibroblasts following treatment with ChB. Glycosaminoglycans were labeled by incubating fibroblasts with $[^{35}S]NaSO_4$ for 72 h in Fischer's medium with 10% serum; cells were then treated for 1 h with either medium alone (control), or medium containing 1.0 IU/ml of ChB. After treatment and washing to remove enzyme, medium was replaced with fresh Fischer's medium containing 10% serum, and incubation continued for 6 to 72 h. Cells were washed, then lysed with 10 mM Tris–HCl pH 8.0, 8 M urea, 0.1% Triton X-100. Lysate equivalent to 10^4 cells was added to each slot of the dot blot. Blots were cut into segments and treated with either phosphate buffered saline with 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$ (PBS), or PBS containing 1.0 IU/ml of ChB for 1 h at 37°C, then washed and counted. The relative amount of DS was calculated as:

$$\%\,\mathrm{DS} = \frac{(\mathrm{cpm\,PBS\,treated\,lysate}) - (\mathrm{cpm\,enzyme\,treated\,lysate})}{(\mathrm{cpm\,PBS\,treated\,lysate})}.$$

Each bar represents the mean ± S.E.M. of four experiments performed in duplicate. The (*) is the amount of DS on ChB-treated cells was significantly less than that of corresponding controls; $P < 0.05$.

that chondroitinase B specifically removed dermatan sulfate from cells (Fig. 3, top panel). No glycosaminoglycans were detectable in medium from control cells. Dermatan sulfate released into the medium was degraded, so that migration on gels was comparable to degraded rather than intact dermatan sulfate.

Since there was some overlap in the migration of degraded dermatan sulfate and that of heparan sulfate, the specificity of chondroitinase B was further examined by Western blot analysis. An antibody, which recognizes the protein core protein of heparan sulfate proteoglycans only after heparan sulfate removal, was used. Lysates from cells treated with medium, chondroitinase B or heparinase III were compared. Heparan sulfate removal was demonstrated in cells treated with heparinase III (Fig. 3, bottom panel). In contrast, heparan sulfate was not removed from cells treated with medium alone or with 1 or 10 IU/ml of chondroitinase B.

3.4. Effects of chondroitinase B treatment of fibroblast response to bFGF

Additional experiments were done to examine the mechanism by which the chondroitinases affect fibroblast proliferation. To determine if chondroitinase treatment might affect the binding of growth factors to their receptors, we repeated the fibroblast proliferation experiments using bFGF in place of fetal bovine serum. Removal of dermatan sulfate with chondroitinase B inhibited the proliferative response of fibroblasts to bFGF (Fig. 4, left panel). However, higher concentrations of enzyme were required to inhibit fibroblast proliferation to bFGF, and the amount of inhibition was less than observed with cells grown in serum. No significant inhibition of proliferation was observed at chondroitinase B concentrations below 1.0 IU/ml; maximal inhibition was 26 ± 4% at a concentration of 10 IU/ml.

The effects of chondroitinase B on fibroblast proliferation to bFGF suggested that removal of dermatan sulfate from the cell surface might interfere with the binding of

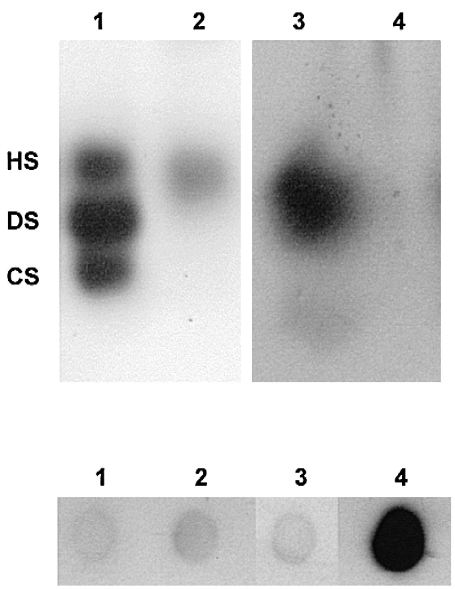

Fig. 3. Top panel. Agarose gel analysis of glycosaminoglycans removed from cells by ChB. Glycosaminoglycans were labeled by incubation of fibroblasts with $[^{35}S]NaSO_4$ for 48 h at 37°C. Cells were treated with ChB (1.0 IU/ml) or medium for 1 h. Glycosaminoglycans released into the medium were precipitated with Cetavalon, resuspended in sample buffer and applied to agarose gels along with unlabeled standards. Lanes 1 and 2: migration of unlabeled on glycosaminoglycan standards on toluidine blue stained gels (1) heparan sulfate (HS), dermatan sulfate (DS) and chondroitin sulfate (CS); and (2) disaccharide fragments of DS. Lane 3 and 4: autoradiograph of $[^{35}S]$glycosaminoglycans isolated from medium of 4×10^6 cells treated with 1.0 IU/ml ChB (3) or medium alone (4). Bottom panel Western blot demonstrating that ChB does not remove HS from fibroblasts. Fibroblasts were treated with medium alone (control) or medium containing ChB (1.0 or 10 IU/ml) or heparinase III (1.0 IU/ml) for 1 h at 37°C. After enzyme treatment, cells were washed and lysed with extraction buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM Na orthovanadate, 1% Triton X-100, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaFl, 10 μg/ml leupeptin). Ten micrograms of cell protein for each dot was applied to Immobilon filters using a dot blot apparatus. Primary antibody was 3G10, which recognizes the core protein of HS proteoglycans after the removal of HS. Lanes: (1) control, (2) 1.0 IU/ml ChB, (3) 10 IU/ml ChB, and (4) 1.0 IU/ml heparinase III.

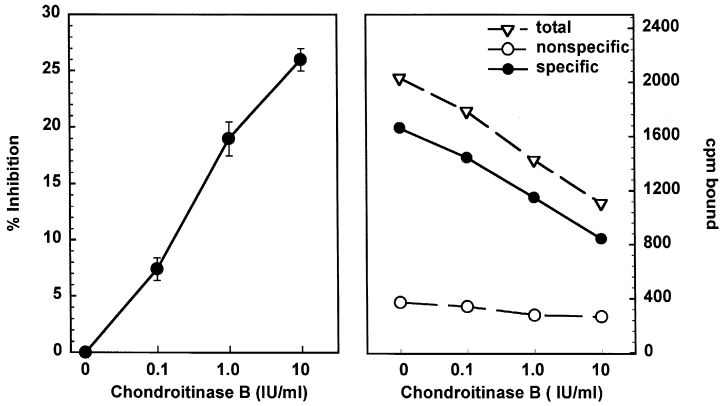

Fig. 4. Left panel. Dose-dependent effect of ChB on fibroblast proliferation to 1.0 ng/ml bFGF. Fibroblasts were pretreated for 1 h at 37°C with the indicated concentration (dose range of 0.01 to 10 IU/ml) of ChB in serum-free medium. The # cells/well was determined after 48 h in the presence of medium with bFGF, and % inhibition calculated by comparison of cell number in control and ChB-treated wells. Each point is the mean ± S.E.M. of four experiments performed in triplicate. Right panel. Dose-dependent effect of ChB on binding of [^{125}I]bFGF to fibroblasts. Fibroblasts were pretreated for 1 h with the indicated concentration of ChB (0.10 to 10 IU/ml). Following enzyme treatment, cells were chilled, then incubated with 25 ng/ml [^{125}I]bFGF alone or with the addition of 25 μg/ml of unlabeled bFGF for 1 h at 4°C. Cells were washed and the amount of [^{125}I]bFGF bound to receptors determined as described in Materials and methods. Data are from a representative of five such experiments performed in triplicate.

this growth factor to its receptor. The binding of iodinated bFGF to chondroitinase B-treated fibroblasts was therefore examined. On chondroitinase B-treated cells, the amount of bFGF bound to both cell surface glycosaminoglycans and receptors decreased as the concentration of enzyme increased (Fig. 4, right panel). Specific binding to receptors was significantly decreased at 1.0 and 10 IU/ml. Receptor binding was decreased by 32 ± 9% and 31 ± 8% ($n = 3$) at 1.0 and 10 IU/ml, respectively; these were the same concentrations found to effectively inhibit proliferation with bFGF.

Binding of bFGF to both untreated and chondroitinase B-treated fibroblasts was saturable (Fig. 5, left). Scatchard plot analysis of bFGF binding data (Fig. 5, right) found a decrease in the number of receptors on chondroitinase B-treated cells when compared to controls, with no change in binding affinity. Chondroitinase B-treated fibroblasts had $1.8(\pm 0.6) \times 10^5$ receptors while untreated fibroblasts had $3.0(\pm 0.8) \times 10^5$ receptors. The binding affinity in chondroitinase-treated fibroblasts was 15.3 ± 3.6 nM, compared to 16.7 ± 2.9 nM in controls, (mean ± S.E.M., $n = 5$).

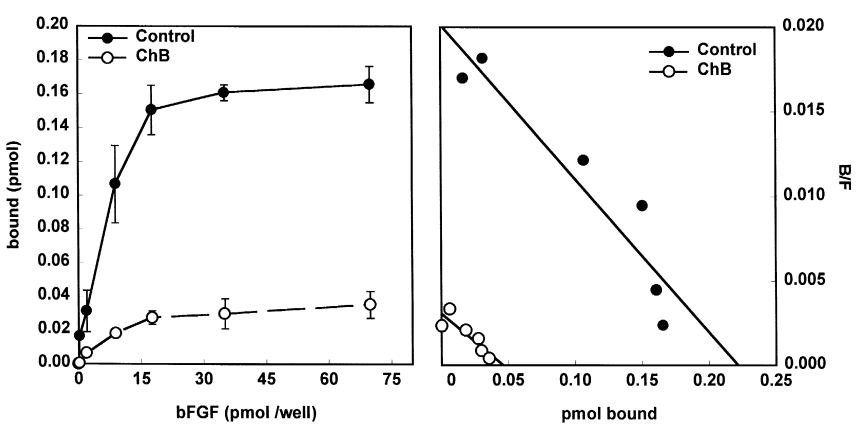

Fig. 5. Left panel. Concentration-dependent binding of [^{125}I] bFGF to fibroblasts. Fibroblasts were pretreated for 1 h with medium alone (control) or medium containing 1.0 IU/ml of ChB. Following enzyme treatment, cells were chilled, then incubated with 25 ng/ml [^{125}I]bFGF alone or with the addition of 0.025 to 25 μg/ml of unlabelled bFGF for 1 h at 4°C. Data are from a representative of five such experiments performed in triplicate. Right panel. Scatchard analysis of bFGF bound to receptors of fibroblasts treated with medium or ChB. All procedures were as described in the left panel. Data are from a representative of five such experiments performed in triplicate. Scatchard analysis of data indicated that binding of bFGF to control ($r^2 = 0.90$) and ChB ($r^2 = 0.97$) treated cells fit a single site model.

3.5. Comparison of the effects of other glycosaminoglycan lyases

The relative effects of two other glycosaminoglycan lyases, heparinase III and chondroitinase AC, on fibroblast proliferation and binding were also investigated.

Removal of heparan sulfate from fibroblasts by heparinase III reduced bFGF binding by 73 ± 2% (Table 1). In contrast to this marked effect on bFGF binding, heparinase III treatment had a relatively small effect on proliferation. Fibroblast proliferation in response to bFGF was inhibited by 21 ± 1%, in comparison to untreated cells. Although fibroblast proliferation to serum was also reduced (27 ± 2%) by heparinase III, inhibition was much less than that which had been observed when cells were treated with an equal concentration of chondroitinase B.

The effects of chondroitinase AC, which cleaves chondroitin sulfate A and chondroitin sulfate C, were also examined. Fibroblast proliferation to fetal bovine serum was inhibited by 25 ± 8% following treatment with chondroitinase AC (Table 1). In contrast, neither fibroblast proliferation to bFGF, nor binding of bFGF to its receptor were affected by this concentration of chondroitinase AC.

3.6. Effects on adherence and viability

The effects of chondroitinase B treatment on cell adherence and viability were also examined. Enzyme treatment had no effect on cell attachment. There was no difference in the number of cells adherent to wells following treatment with medium alone or with 1 and 10 IU/ml of chondroitinase B (Table 2). There was also no decrease in cell viability as assessed by measuring the release of LDH into the medium, at zero or 18 h following enzyme treatment. The lack of effect of the chondroitinases on adherence and viability indicated that the observed inhibitory effects of this enzyme on proliferation and binding were not due to cell detachment or death.

Table 1
Comparison of the effects of chondroitinase B, chondroitinase AC and heparinase III on proliferation and bFGF receptor binding

Enzyme	% Inhibition		
	Proliferation		Binding
	serum	bFGF	bFGF
Chondroitinase B	53 ± 2	20 ± 2	25 ± 2
Chondroitinase AC	25 ± 8	0 ± 5	1 ± 1
Heparinase III	27 ± 2	21 ± 1	73 ± 3

Cells were treated with 1.0 IU/ml of the indicated enzyme for 1 h. Proliferation medium was either medium w/10% serum or 1.0 ng/ml bFGF; cell numbers were measured 48 h after treatment. In binding studies, cells were chilled to 4°C and specific binding of [^{125}I]bFGF to receptors determined as described in Materials and methods. % Inhibition was calculated as described in Materials and methods, based on the # cells, or cpm in untreated cells. The means ± S.E.M. of four proliferations and four binding experiments performed in triplicate are shown.

Table 2
Effects of chondroitinases on adherence and viability

Chondroitinase B (IU/ml)	Adherence (% control)	Viability (units)	
	0 h	0 h	18 h
None	100	16 ± 11	24 ± 6
1	105 ± 8	20 ± 5	17 ± 11
10	103 ± 4	18 ± 2	22 ± 1

Effects of enzyme treatment on adherence and viability. Fibroblasts were labeled with 1 μM calcein for 20 min at 37°C, then treated for 1 h with either serum-free medium (none) or medium containing 1.0 IU/ml of chondroitinase B. After enzyme treatment cells were given fresh medium w/10% serum. Adherence and viability were assessed immediately (0 h) and 18 h after treatment. Fluorescence was determined at an excitation of 485 nm and emission of 530 nm, and # cells/well was calculated from a standard curve. Data shown for adherence are the % control (# cells in enzyme treated wells/medium treated wells). Viability was assessed by the amount of lactate dehydrogenase (LDH) released into the medium; data are in units of LDH activity from a spectrophotometric assay. Data for both adherence and viability are the mean ± S.D. of two experiments, performed in sextuplets.

4. Discussion

The results of the present study suggest that dermatan sulfate proteoglycans on fibroblasts are important in controlling fibroblast response to multiple growth factors. Removal of dermatan sulfate from cells with chondroitinase B significantly decreased proliferation to serum. This inhibitory effect could not be reversed by adding dermatan sulfate back to the medium. This finding implies that the activities of dermatan sulfate in controlling cellular proliferation require functional dermatan sulfate-containing proteoglycans, and that the structural incorporation of dermatan sulfate into the cell membrane is an important component of this regulatory process. These results are in contrast to those reported in studies of heparan sulfate proteoglycans. Both bFGF binding and proliferative activity can be restored by the addition of exogenous heparan sulfate or heparin to cells deficient in this glycosaminoglycan (Rapraeger et al., 1991; Yayon et al., 1991).

It has been well documented that heparin and heparan sulfate proteoglycans bind a large number of growth factors, and through this greatly influence the subsequent biological activities of such factors. Recent studies of growth factors and proteoglycans in nervous tissue indicate that cellular activation and responses to growth factors may be determined by the interactions with proteoglycans made up of more than one type of glycosaminoglycan. These studies have demonstrated that chondroitinase ABC treatment decreased binding of pleiotrophin (Maeda et al., 1996) and bFGF to the proteoglycan phosphocan (Milev et al., 1998), and have suggested that both the chondroitin

sulfate chains and the core protein to which they are attached, may control growth factor binding. Another growth factor, hepatocyte growth factor (HGF/SF), has also been shown to bind to both dermatan and heparan sulfate (Lyon et al., 1998).

In the current study, the comparative studies with heparinase III shown an interesting relationship between the specific glycosaminoglycans, heparan and dermatan sulfate, growth factor binding, and the proliferative response, within human dermal fibroblasts. Whereas heparinase III treatment greatly reduced basic bFGF binding (> 70%), the proliferative response to either serum or bFGF was reduced by only 21–27%. The same concentration of chondroitinase B reduced binding and proliferation to bFGF by roughly the same percentage (20–25%). This suggests that there is a different relationship between these glycosaminoglycans and growth factor receptors in affecting signal transduction. Furthermore, the relative ineffectiveness of chondroitinase AC suggests that neither chondroitin sulfate A or C plays a predominant role in modulating bFGF receptor activity in human dermal fibroblasts.

Mechanistically chondroitinase B inhibited bFGF-mediated fibroblast proliferation by reducing the number of functional receptors without altering the affinity of bFGF. It is possible that chondroitinase B treatment had a pronounced inhibitory effect on proliferation to fetal bovine serum by simultaneously decreasing the binding of a number of growth factors to their respective receptors. Although this hypothesis has yet to be tested, preliminary observations indicate that chondroitinase B treatment also inhibits fibroblasts proliferative responses to heparin-binding epidermal growth factor (HB-EGF), interleukin-8 and fibronectin (data not shown).

Aside from the effect that removing dermatan sulfate might have on the binding of various growth factors, other mechanisms should also be considered.

Chondroitinase B treatment of fibroblasts may inhibit proliferation by regulating the interaction of cells with the extracellular matrix as well as regulating matrix secretion. In support of this hypothesis, chondroitin sulfate proteoglycans have been found to both enhance (Moyano et al., 1999) and inhibit (Yamagata et al., 1989) cell adhesion to fibronectin and other non-proteoglycan molecules. Reduction of cell-associated chondroitin sulfate proteoglycans has been linked to a reduction in fibronectin and laminin in the extracellular matrix, and to decreased actin filament formation and growth of smooth muscle cells (Hamati et al., 1989). Changes in extracellular matrix composition not only affect the cell cytoskeleton, but also influence secretion of growth factors (Blomme et al., 1998). The presence of serum and cytokines can in turn regulate fibroblast expression and secretion of growth regulating proteoglycans such as decorin (Brown et al., 1999). Likewise, extracellular matrix composition can indirectly upregulate secretion of bFGF and vascular endothelial growth factor (VEGF) via cytokines in an autocrine fashion (Samaniego et al., 1998). It is possible that altering the composition of cellular proteoglycans may influence cellular cytokine production and thus regulate secretion of growth factors and proliferation.

In summary, the results of this study show that selective removal of dermatan sulfate form human dermal fibroblasts by a selective glycosaminoglycan lyase, chondroitinase B, can significantly reduce growth factor binding and growth factor-mediated proliferation. This effect likely entails a different relationship between dermatan sulfate and growth factor receptors, than that which has been observed with heparan sulfate and growth factor receptors (Jackson et al., 1991; Kjellen and Lindahl, 1991; Vlodavsky et al., 1995; Yayon et al., 1991).

While the use of a specific glycosaminoglycan lyase has made it possible to examine the activities of dermatan sulfate proteoglycans, it remains to be determined which if any of these proposed mechanisms explain how chondroitinase B treatment inhibits fibroblast proliferation. Further studies of the interactions of different types of sulfated glycosaminoglycans with each other and with various receptors should help define how these individual components contribute to and synchronize cellular responses to growth factors and other stimuli.

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