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Anti-tumor activities of chondroitinase AC and chondroitinase B: inhibition of angiogenesis, proliferation and invasion

Elizabeth M. Denholm*, Yong-Qing Lin, Paul J. Silver

Department of Cellular Biology, IBEX Pharmaceuticals, Inc., 5485 Pare, Montreal, Quebec, Canada H4P 1P7

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Abstract

In the current study, two specific glycosaminoglycan lyases, chondroitinase AC and chondroitinase B, were utilized to examine the roles of chondroitin sulfates and dermatan sulfate in tumor metastasis and angiogenesis. Melanoma cells (SK-MEL) or endothelial cells were treated with either medium or chondroitinase enzyme. Chondroitinase AC inhibited melanoma invasion and proliferation as well as endothelial proliferation and angiogenesis. Apoptosis of melanoma and endothelial cells, as measured by the activity of caspase-3, was also increased by chondroitinase AC, but not by chondroitinase B. Chondroitinase B inhibited endothelial and melanoma proliferation and invasion, but to a lesser extent than chondroitinase AC. Neither chondroitinase had a detectable effect on gelatinase secretion by melanoma cells. These results indicate that both chondroitin and dermatan sulfates regulate many cellular activities related to metastasis.

Keywords: Chondroitin sulfate; Dermatan; Chondroitinase; Metastasis; Proliferation; Apoptosis

1. Introduction

The growth of tumors requires tumor cell proliferation as well as the formation of new blood vessels. Metastasis of the primary tumor and growth of secondary tumors involve a complex sequence of events, which has been referred to as the "metastatic cascade" (Sneath and Mangham, 1998). While many growth factors, enzymes and extracellular matrix components are involved in these events, works by other investigators have suggested that chondroitin sulfate proteoglycans may play a key role in tumor growth and metastasis.

In examining the functions of chondroitin sulfates in metastasis, two different approaches have been used: inhibiting the formation of chondroitin sulfate proteoglycans with β-D-xyloside (ρ-nitrophenyl-β-D-xylopyranoside), and enzymatically removing chondroitin sulfates from cells with chondroitinase ABC. Both methods have similar results and effectively decrease tumor cell invasion and endothelial migration and adhesion (Henke et al., 1996; Faassen et al., 1992, 1993).

Taken together, studies utilizing inhibitors or enzymes, which degrade chondroitin sulfates, indicate that chondroitin sulfates play important roles in the processes of tumor growth and metastasis (Iida et al., 1996; Sneath and Mangham, 1998). However, it is not clear from these prior studies which chondroitin sulfate proteoglycans were involved. Chondroitin sulfate proteoglycans exist in three major forms: chondroitin sulfate A, chondroitin sulfate B (dermatan sulfate) and chondroitin sulfate C. Recent work has demonstrated that the different forms of chondroitin sulfates have different functions. Dermatan sulfate is important in controlling fibroblast proliferation, and chondroitin sulfates A and C regulate integrin-mediated cell adhesion (Denholm et al., 2000; Moyano et al., 1999).

The purpose of the present study was to determine the relative importance of the different forms of chondroitin sulfate in cellular activities related to metastasis. To accomplish this, two specific glycosaminoglycan lyases were utilized: chondroitinase AC (substrates are chondroitin sulfates A and C) and chondroitinase B (substrate is chondroitin sulfate B or dermatan sulfate). The effects of these two glycosaminoglycan lyases on several tumor and endothelial cell functions were compared, and the mechanisms underlying the inhibition of these activities were examined.

* Corresponding author. Tel.: +1-514-344-4004 ext. 122; fax: +1-514-344-8827.

E-mail address: edenholm@ibexpharma.com (E.M. Denholm).

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2. Materials and methods

2.1. Materials

Human melanoma (SK-MEL-2) and calf pulmonary artery endothelial cells were from ATCC, Manassas, VA. Dulbecco's minimal essential medium, Fischer's medium, phosphate buffered saline (PBS) and fetal bovine serum were from Gibco, Grand Island, NY. Dermatan sulfate, chondroitin sulfates A and C, and heparan sulfate were purchased form Celsus Laboratories, Cincinnati, OH. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were purchased from Peprotech, Rocky Hill, NJ. Calcein acetoxymethyl (AM) ester, CyQuant proliferation kits and Gelatinase/collagenase kits were from Molecular Probes, Eugene, OR.

2.2. Enzymes

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

2.3. Angiogenesis

Calf pulmonary artery endothelial cells were grown in minimal essential medium (MEM) with 20% fetal bovine serum. Cells were treated for 1 h at 37°C with 0.5% serum-MEM alone, or with the addition of the indicated concentration of chondroitinase AC or B. Endothelial cells were then mixed with growth factor-reduced Matrigel (15 mg/ml, Collaborative Biochemicals, Cambridge, MA) at a concentration of 3×10^5 cells/ml. Ten microliters of this cell suspension were added to the center of each well of a 48-well culture dish, and incubated for 30 min at 37°C. Following gel formation, MEM, containing 0.5% serum and 20 ng/ml of VEGF, was added either without addition or with the addition of the indicated concentration of chondroitinase AC or B. Controls consisted of cells treated with medium alone and 1 mM β-xyloside.

Angiogenesis was assessed as the formation of capillary-like structures after incubation for 6 days. For optimal visualization of capillary-like structures, endothelial cells were labeled with 1 μM calcein AM (Molecular Probes, Portland, OR). Capillary-like structures were quantified by counting the number of capillary-like structures in 100× fields in the center of the well.

2.4. Proliferation assays

Calf pulmonary artery endothelial cells were cultured at 2500 cells/well in 96-well plates, with MEM containing 10% serum. After 24 h, the medium was replaced with MEM containing 1% serum, 20 ng/ml VEGF and the indicated concentration of chondroitinase AC or B. The number of cells per well was quantified 3 days later using the CyQuant assay method from Molecular Probes, Eugene, OR. The fluorescence of each well was determined and the cell numbers were calculated from a standard curve prepared with a known number of cells.

Human melanoma cells (SK-MEL-2) were suspended in Matrigel (15 mg/ml) to a concentration of 3×10^5 cells/ml and plated in 48-well culture dishes at 3000 cells/well, with MEM containing 1% serum, and the indicated concentration of chondroitinase AC or B. On day 8, the cells were labeled with 1 μM calcein AM and the fluorescence of each well was determined and cell numbers were calculated from a standard curve prepared with a known number of cells.

2.5. Tumor cell invasion

Human melanoma cells (SK-MEL-2) were grown in MEM with 10% serum. Cells were resuspended at a concentration of 1×10^5 cells/ml in 0.5% BSA-MEM. Cells were pretreated in either medium (controls) or medium containing the indicated concentration of chondroitinase AC or B, for 1 h at 37°C, before loading into cell culture inserts.

The invasion assay was performed in 8-μm pore polycarbonate filter cell culture inserts (Falcon, Franklin Lakes, NJ), as described previously (Durko et al., 1997), with modifications. Matrigel was diluted to 0.2 mg/ml in cold serum-free MEM and 60 μl was added to each filter. The coated filters were dried overnight and equilibrated with serum-free medium and placed in a 24-well plate. Fifty thousand tumor cells in 500 μl of medium alone, or a medium containing the indicated concentration of chondroitinase AC or B were placed on top of the filters, and fibroblast-conditioned medium (Inokuchi et al., 1990) was placed below the filter. The plates were incubated for 24 h at 37°C. The cells on top of the filters were then removed with a cotton swab and the filters were stained using the Diff-Quik staining set (Baxter, Miami, FL). Invasion was assessed as the number of cells that migrated through Matrigel to the underside of the filters. For each filter, a total of 10 random 400× fields was counted. Controls consisted of cells treated with the medium alone, 1 mM β-xyloside, or 5 μg/ml rat anti-human CD44 immuno-

globulin (IgG_{2b}), from Calbiochem–Novabiochem, La Jolla, CA.

2.6. Gelatinase assays

Gelatinase activity was determined using an assay utilizing fluorescein-conjugated gelatin. Melanoma (SK-MEL-2) cells were plated at a density of 1×10^6 cells/well in six-well plates, with MEM with 10% serum. After an overnight incubation, cells were washed with serum-free MEM without phenol red (medium), then given fresh medium containing no additions, or a medium containing 10 ng/ml of human tumor necrosis factor α (TNF-α) with 0, 1 or 10 IU/ml of chondroitinase AC. Following a 24-h incubation at 37°C, medium was removed for assay.

Gelatinase and collagenase activity was quantitated using the Gelatinase/Collagenase assay kit from Molecular Probes, Eugene, OR. Medium samples were collected and the microgram of cell protein per well was determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Medium samples were incubated with 100 μg/ml gelatin fluorescein conjugate (DQ™ gelatin) substrate for 24 h at room temperature in 96-well plates. Gelatinase activity was measured as the increase in fluorescent units (excitation, 485 nm and emission, 530 nm). Enzyme activity was quantitated based on a standard curve prepared with 0.5–50 ng of bacterial collagenase. The metalloproteinase inhibitor, 1,10-phenanthroline (10 μM) was used as control. Enzyme activity was expressed as nanogram collagenase per 25 μg cell protein.

2.7. Apoptosis assays

Human melanoma cells (SK-MEL-2) or calf pulmonary artery cells were plated in six-well dishes at a density of 1×10^6 cells/ml/well, with MEM containing 10% serum. Cells were allowed to adhere for 2 h at 37°C, then medium was changed to MEM plus 10% serum alone, or with the addition of 0.1–10 IU/ml of chondroitinase AC or B, or 40 μg/ml of glycodeoxycholic acid (Genistein, Calbiochem–Novabiochem). Cells were incubated for 48 h, then trypsinized and counted on a hemacytometer. Cells were then pelleted and resuspended in lysis buffer to a concentration of 2×10^7 cells/ml. Cells lysis and quantitation of caspase-3 activity were performed according to manufacturer's directions, with the Caspase-3 Fluorometric kit from Biosource International, Camarillo, CA. Caspase activity was measured in fluorescent units (excitation, 360 nm and emission, 460 nm), and expressed as the percentage of untreated control cells.

2.8. Agarose gel electrophoresis of glycosaminoglycans

Glycosaminoglycans were labeled by incubation of melanoma cells with [^{35}S]sodium sulfate for 48 h at 37°C, as previously described (Denholm et al., 2000). Briefly, glycosaminoglycans were labeled by incubating melanoma cells 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 AC. 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 sulfates A and C, and heparan sulfate at a concentration of 125 μg in 5 μl of sample buffer, and disaccharide fragments of chondroitin sulfate A, chondroitin sulfate C, dermatan sulfate and heparan sulfate. The disaccharide fragments were prepared by pre-incubating 10 mg/ml of the indicated glycosaminoglycan and 10 IU/ml of the corresponding glycosaminoglycan lyase for 1 h at 37°C. Enzyme protein was removed from the dissaccharide preparations by filtration through Centricon (Amicon, Beverly, MA) concentrators with size exclusion filters of 10 kDa. Following electrophoresis and drying with a gel-airdrying system (BioRad), agarose gels were stained with 0.2% toluidine blue to visualize standards. Film was then applied to gels for detection of [^{35}S]-labeled glycosaminoglycans.

2.9. Cytotoxicity assays

Melanoma and endothelial cells were plated in 96-well plates at a density of 2.5×10^4 cells/well in medium with 10% serum, and incubated overnight at 37°C. Medium was changed to phenol red-free medium with 1% serum, with or without the addition of 1.0 or 10 IU/ml of chondroitinase AC or B. Incubation was continued for 18 h, after which, medium was collected, centrifuged and assayed for lactate dehydrogenase (LDH). LDH in the medium was determined using the CytoTox 96 LDH assay kit from Promega, Madison, WI.

2.10. Statistics

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. Angiogenesis and endothelial cell proliferation

Capillary endothelial cells were treated with 0.1–10 IU/ml of chondroitinase AC or chondroitinase B, and angiogenesis, as measured by the formation of capillary-like structures, was examined. Endothelial cells treated with chondroitinase AC contained fewer capillary-like

structures than untreated controls did (Figs. 1 and 2). Angiogenesis was inhibited by 46% and 72%, following treatment with 1.0 and 10 IU/ml of chondroitinase AC, respectively. In comparison, β-D-xyloside, which blocks the formation of chondroitin sulfate proteoglycans, inhibited angiogenesis by $39 \pm 3\%$ ($n = 3$; data not shown). There was no significant inhibition of angiogenesis in parallel endothelial cultures treated with 0.1–10 IU/ml of chondroitinase B.

Since angiogenesis involves both the migration and proliferation of endothelial cells, the effects of the two chondroitinases on proliferation were also examined. The concentrations of chondroitinase AC necessary to cause significant inhibition of proliferation were the same as those that inhibited capillary-like structure formation. Endothelial proliferation was inhibited by 16–49% following treatment with chondroitinase AC at concentrations ranging from 1.0 to 10 IU/ml (Fig. 3).

Chondroitinase B was less effective in inhibiting endothelial proliferation when compared to the same concentrations of chondroitinase AC. The maximal inhibition with chondroitinase B was $20 \pm 2\%$, at a concentration of 10 IU/ml.

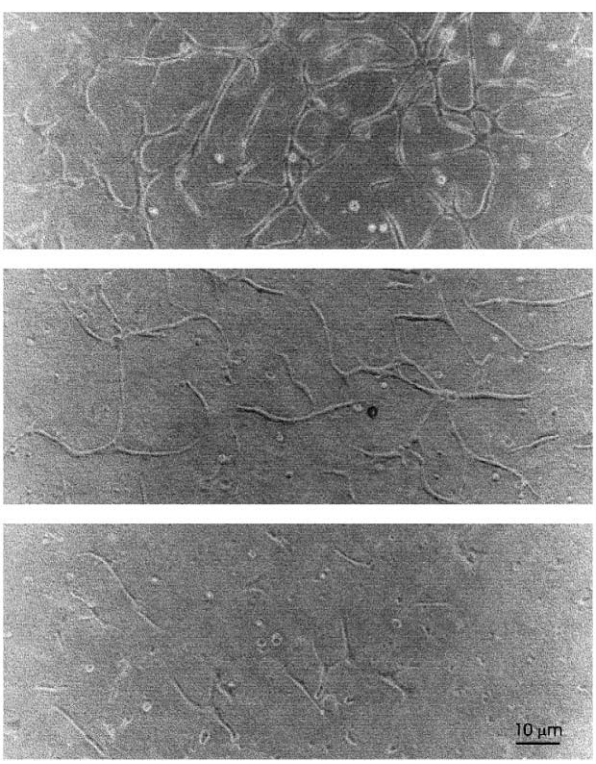

Fig. 1. Dose-dependent effects of chondroitinase AC on endothelial cell capillary formation. Cells were treated for 1 h at 37°C with 0.5% serum-MEM alone, or with the addition of the indicated concentration of chondroitinase AC. Endothelial cells were then mixed with growth factor-reduced Matrigel and plated in MEM containing 0.5% serum and 20 ng/ml of VEGF either without addition or with the addition of the indicated concentration of chondroitinase AC. Angiogenesis was assessed as the formation of capillary-like structures after incubation at 37°C for 6 days. Capillary-like structure formation in: top panel. Untreated control cultures; middle panel, cultures treated with 1.0 IU/ml of chondroitinase AC; bottom panel, cultures treated with 10.0 IU/ml chondroitinase AC.

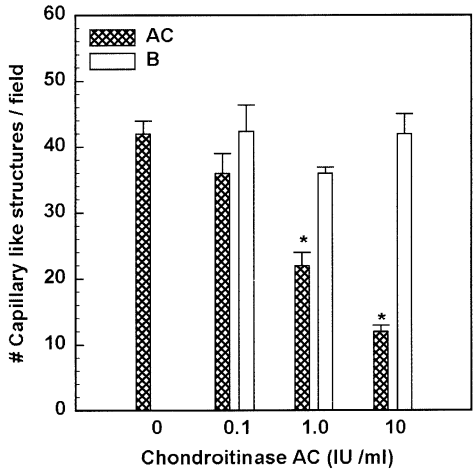

Fig. 2. Dose-dependent inhibition of endothelial cell capillary-like structure formation by chondroitinase AC and chondroitinase B. Cells were treated as described in Fig. 1 and Section 2, with the indicated concentration of either chondroitinase AC or B. After 6 days in culture, cells were labeled with calcein and capillary-like structures were quantitated by counting 100× fields. Data are the means ± SEM of five such assays performed in duplicate. The (*) indicates cell numbers significantly less than that in untreated controls, $P < 0.05$.

3.2. Melanoma cell proliferation and invasion

Chondroitinase AC and chondroitinase B also inhibited the proliferation of SK-MEL human melanoma cells (Fig. 4). The relative effectiveness of the two enzymes was

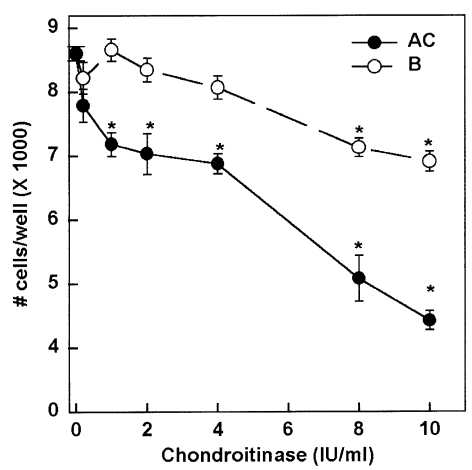

Fig. 3. Dose-dependent effect of chondroitinase AC and chondroitinase B on endothelial cell proliferation. Endothelial cells were pretreated for 1 h at 37°C with the indicated concentration (dose range of 0.01–10.0 IU/ml) of either chondroitinase AC or B in serum-free medium. The number of cells per well was determined after 72 h in the presence of medium containing 20 ng/ml VEGF and 1% serum, with or without the addition of the indicated concentration of either chondroitinase AC or B. Data are the means ± SEM of five such experiments performed in quadruplicate. The (*) indicates cell numbers significantly less than that in untreated controls, $P < 0.05$.

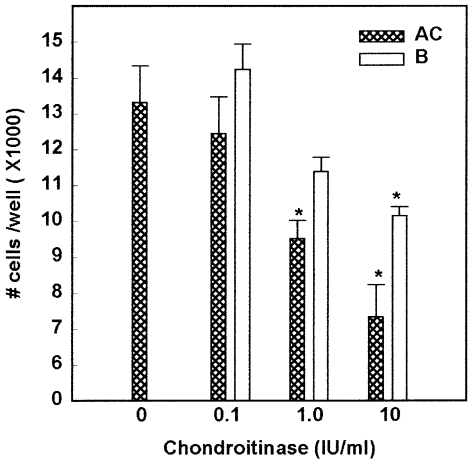

Fig. 4. Dose-dependent inhibition of melanoma cell proliferation by chondroitinase AC or B. Melanoma cells were suspended in Matrigel and plated in culture dishes containing medium with 1% serum and the indicated concentration of either chondroitinase AC or B (dose range of 0.1–10.0 IU/ml). The number of cells per well was determined after 8 days incubation at 37°C. Data are the means ± SEM of four such experiments performed in duplicate. The (*) indicates cell numbers significantly less than that in untreated controls, $P < 0.05$.

similar to that observed with endothelial proliferation. In these experiments, maximal inhibition of 45% was attained by treatment with 10 IU/ml of chondroitinase AC, while the same dose of chondroitinase B inhibited melanoma proliferation by only 22%.

Since the ability of tumor cells to invade tissues is essential to the metastatic process, the effects of chondroitinase AC and chondroitinase B on invasion were also examined. Similar to the results in melanoma proliferation assays, chondroitinase AC inhibited invasion to a greater extent than did chondroitinase B (Fig. 5, left panel). For both melanoma proliferation and invasion, chondroitinase AC was significantly more effective than chondroitinase B at a concentration of 10 IU/ml.

Chondroitin and possibly, dermatan sulfates, are linked to the CD44 membrane protein, which plays a role in both cell invasion and adhesion (Faassen et al., 1992; Henke et al., 1996). Further experiments found that removing cell surface chondroitin or dermatan sulfates with chondroitinase AC or B (respectively), and inhibiting CD44 function with antibodies, had nearly the same effects on melanoma cell invasion. Anti-CD44 inhibited invasion by 34 ± 1%, while 1.0 IU/ml of chondroitinase AC or B inhibited invasion by 28 ± 2% or 21 ± 3%, respectively (Fig. 5, right panel). Treating cells with the combination of antibodies to CD44 and either chondroitinase AC or B, had nearly the same effect as that of antibodies or chondroitinase alone.

3.3. Tumor cell secretion of gelatinase

To determine if the chondroitinase enzymes might inhibit invasion by decreasing tumor cell secretion of gelatinase, melanoma cells were treated with chondroitinase AC or B. Chondroitinase AC, at concentrations of 0.10 to 10 IU/ml, had no effect on the amount of gelatinase secreted by melanoma cells (Table 1). Chondroitinase B, at a

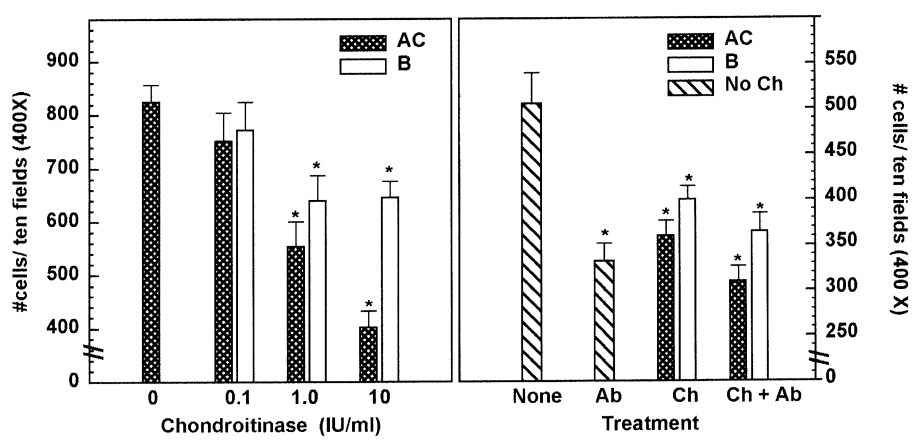

Fig. 5. Inhibition of melanoma cell invasion into Matrigel. Left panel: dose-dependent effect of chondroitinase AC and chondroitinase B. Melanoma cells were pretreated for 1 h at 37°C in serum-free medium with or without the indicated concentration of either chondroitinase AC or B (dose range of 0.1–10.0 IU/ml). Cells were then placed into Matrigel-coated 8-μm pore nitrocellulose filter inserts and incubated for 24 h. The number of cells that migrated to the underside of the Matrigel-coated filter inserts in response to fibroblast-conditioned medium was quantitated as the number of cells per 10 fields at 400×. Data are the means ± SEM of four such experiments performed in duplicate. The (*) indicates cell numbers significantly less than that in untreated controls, $P < 0.05$. Right panel: comparative effects of antibody to CD44 (Ab), chondroitinase (Ch) AC or chondroitinase B, and chondroitinase AC or B plus antibody to CD44 (Ch + Ab). Melanoma cells were pretreated with medium alone (No Ch), 1.0 IU/ml of the indicated chondroitinase, 5 μg/ml of antibody, or both, at the indicated concentrations, for 1 h at 37°C, prior to being placed into invasion chambers. Invasion assay and quantitation were as described above and in Section 2. Data are the means ± SEM of four such experiments performed in duplicate. The (*) indicates cell numbers significantly less than that in untreated controls, $P < 0.05$.

Table 1
Effects of chondroitinase enzymes on gelatinase secretion

Treatment		Gelatinase activity
Control		13 ± 1
Chondroitinase AC	1.0	15 ± 1
	10	15 ± 1
Chondroitinase B	10	14 ± 2
Control + phenanthroline		1 ± 2

Melanoma cells were incubated in medium containing 1.0 or 10.0 IU/ml of either chondroitinase AC or B for 24 h. Medium was removed from cells and incubated with 100 μg/ml gelatin fluorescein conjugate (DQ™ gelatin) substrate for 24 h at room temperature. Gelatinase activity was measured as the increase in fluorescent units (excitation, 485 nm and emission, 530 nm). Controls were cells treated with medium alone, control medium was assayed with or without the addition of the metalloproteinase inhibitor 1,10 phenanthroline (10 μM). Enzyme activity was expressed as nanogram per milliliter of gelatinase, calculated from a standard curve prepared with 5.0–50.0 ng/ml of bacterial collagenase.

concentration of 10 IU/ml, also failed to enhance or inhibit gelatinase secretion by these cells.

3.4. Tumor and endothelial cell apoptosis

Other experiments were done in order to determine if the chondroitinases might inhibit cellular activities via the induction of apoptosis. Capillary endothelial and melanoma cells were incubated with 0.10–10 IU/ml of chondroitinase AC or B. Apoptosis, as measured by the increase in caspase-3 activity, was increased in both endothelial and melanoma cells by chondroitinase AC (Fig. 6). Caspase-3 activity in endothelial cells (Fig. 6, left panel) was increased by up to 171% compared to untreated controls. Melanoma cells treated with chondroitinase AC had greater increases in caspase activity than did endothelial cells (Fig. 6, right panel). A dose of 10.0 IU/ml of chondroitinase AC increased caspase activity of melanoma cells by 256% over that of controls. In comparison, the apoptosis inducer Genistein (Kwo et al., 1995) increased caspase activity by 189% in endothelial cells and by 269% in melanoma cells. Caspase-3 activity in either endothelial or melanoma cells was not significantly increased by chondroitinase B at any of the concentrations tested.

The release of the cytoplasmic enzyme, lactate dehydrogenase, was also measured to determine if the chondroitinases had cytotoxic effects. Melanoma and endothelial cells were incubated with medium alone, or with 10 IU/ml of chondroitinase AC or B for 24 h. There were no significant differences in the amount of lactate dehydrogenase released by cells treated with either chondroitinase, compared to cells treated with medium alone (data not shown). These results were in agreement with previous studies (Denholm et al., 2000), which had shown that chondroitinase AC and chondroitinase B were not cytotoxic for fibroblasts.

In all the cellular activities assayed, the effects of heat-inactivated chondroitinase AC and chondroitinase B were also tested. When compared to controls treated with medium alone, there were no significant increases or decreases in any of the activities assayed using the heat-inactivated enzymes (data not shown).

3.5. Analysis of glycosaminoglycans removed from cells by chondroitinase AC

To ensure that the effects observed on cellular activities were due to the removal only of chondroitin sulfates,

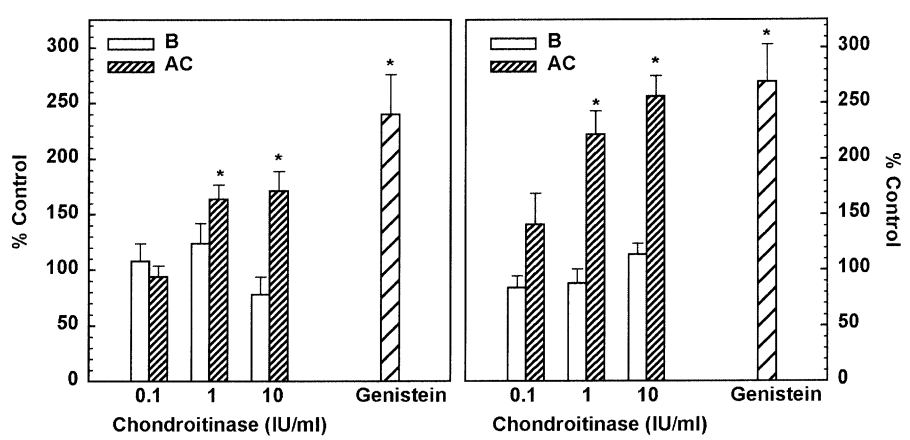

Fig. 6. Induction of apoptosis in endothelial and melanoma cells by chondroitinase AC and chondroitinase B. Left panel: dose-dependent effects of chondroitinases on capillary endothelial cells. Cells were incubated for 48 h at 37°C with the indicated concentration of chondroitinase AC or B (dose range of 0.01–10.0 IU/ml), or Genistein (40 μg/ml) in medium containing 10% serum. Control cells were incubated in serum containing medium alone. Apoptosis was assessed as the caspase-3 activity in 1×10^6 cells. Data shown are the percentages of control [fluorescence of enzyme or Genistein-treated cells)/fluorescence of medium-treated cells) × 100]. Each bar represents the mean ± SEM of five such experiments performed in triplicate. The (*) indicates activity significantly greater than that of corresponding controls, $P < 0.05$. Right panel: dose-dependent effects of chondroitinases on melanoma cells. Melanoma cells were treated with the indicated concentration of chondroitinase AC or B. Treatments and assays for apoptosis were performed, as described, for endothelial cells. Each bar represents the mean ± SEM of four such experiments performed in triplicate. The (*) indicates activity significantly greater than that of corresponding controls, $P < 0.05$.

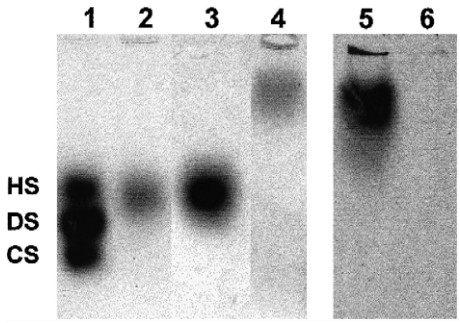

Fig. 7. Agarose gel analysis of glycosaminoglycans removed from cells by chondroitinase AC. Glycosaminoglycans were labeled by incubation of melanoma cells with [^{35}S]$NaSO_4$ for 48 h at 37°C. Cells were treated with chondroitinase AC (1.0 IU/ml) or medium for 1 h. Glycosaminoglycans released into the medium were precipitated with Cetavalon, resuspended in sample buffer and applied to agarose gels along with unlabeled standards. Lanes 1–4: migration of unlabeled glycosaminoglycan standards on toluidine blue stained gels; (1) heparan sulfate (HS), dermatan sulfate (DS) and chondroitin sulfate (CS); (2–4) disaccharide fragments of: (2) DS, (3) HS, and (4) CS. Lanes 6 and 7: autoradiograph of [^{35}S]glycosaminoglycans isolated from medium of 4×10^6 cells treated with (6) 1.0 IU/ml chondroitinase AC or (7) medium alone.

glycosaminoglycans released into the medium following enzyme treatment were examined. Melanoma cell glycosaminoglycans were labeled with ^{35}S, and cells were treated with medium or chondroitinase AC. Autoradiography of glycosaminoglycans released into the medium demonstrated that chondroitinase AC specifically removed chondroitin sulfates from cells (Fig. 7, right panel). No glycosaminoglycans were detectable in medium from control cells. Chondroitin sulfates released into the medium were degraded to disaccharides, so that migration on gels was comparable to that of the disaccharides rather than to intact chondroitin sulfate (Fig. 7, left panel). Previous work had demonstrated that chondroitinase B is also specific, and removes only dermatan sulfate from cells (Denholm et al., 2000).

4. Discussion

Comparison of the relative effects of chondroitinase AC with those of chondroitinase B, indicated that both chondroitin and dermatan sulfates are involved in regulating a number of cellular activities linked to metastasis. At the start of these experiments, it had been thought that the use of the two specific chondroitinase enzymes would reveal a clearly predominant role for either chondroitin or dermatan sulfates in each of these various activities. It would appear that chondroitin sulfates A and C have more prominent roles in regulating these activities, since removal of these chondroitin sulfates was consistently more effective in inhibiting metastasis-related activities than the removal of dermatan sulfate. In addition, for all the cellular activities examined, treating endothelial or melanoma cells with chondroitinase AC alone produced, essentially, the same effect as that of treating cells with equal concentrations of both chondroitinase AC and chondroitinase B (data not shown).

The inhibition of angiogenesis and invasion, shown here, was similar to the effects obtained in previous studies, which demonstrated that chondroitinase ABC inhibited endothelial cell migration and tumor cell motility (Faassen et al., 1992; Henke et al., 1996). All suggest that dermatan and chondroitin sulfates regulate these cellular activities and may be important in the metastatic process. Additional evidence for the involvement of these glycosaminoglycans in metastasis are contained in studies utilizing β-D-xyloside, which has very similar effects as those of the chondroitinases on cell adhesion, invasion and angiogenesis (Faassen et al., 1993; Henke et al., 1996).

A significant difference in the roles of chondroitin and dermatan sulfates was apparent in experiments examining endothelial and melanoma cell proliferation. Removal of chondroitin sulfates A and C with chondroitinase AC, inhibited proliferation of both cell types by nearly twice as much as the removal of dermatan sulfate with chondroitinase B. These relative effects of the two chondroitinase enzymes on proliferation were the opposite of those which had been observed in a previous study of fibroblast proliferation (Denholm et al., 2000).

In the present work, the comparative effects of the two chondroitinases on proliferation of endothelial and melanoma cells were reflected in their ability to induce apoptosis. For both cell types, chondroitinase AC significantly increased apoptosis and decreased proliferation. While chondroitinase B also decreased proliferation, this enzyme did not enhance apoptosis. However, it seems unlikely that chondroitinase AC inhibited cell proliferation solely via the activation of caspase-3. The observed increases in caspase-3 activity were significant, but did not correlate well with the corresponding decreases in proliferation at the same concentrations of enzyme. This suggests that the induction of apoptosis may only be one of several mechanisms by which chondroitinases inhibit cell proliferation and activation.

It seems likely that the degree of influence that chondroitin and dermatan sulfates have on proliferation and other cellular activities is determined by the relative abundance of these glycosaminoglycans in different cell types and by their linkage to various receptors. In this regard, chondroitin and/or dermatan sulfate proteoglycans regulate the binding of platelet factor 4, basic fibroblast growth factor, hepatocyte growth factor, pleiotrophin and interferon-γ to their respective receptors in a variety of cell types (Denholm et al., 2000; Hurt-Camejo et al., 1999; Lyon et al., 1998; Maeda et al., 1996; Petersen et al., 1998). Other studies have shown that chondroitinase treatment also decreases cell activation mediated by these receptors. Chondroitinase B decreases basic fibroblast growth factor-induced fibroblast proliferation (Denholm et

al., 2000), and chondroitinase ABC decreases platelet factor 4-stimulated degranulation in neutrophils (Petersen et al., 1998).

Glycosaminoglycans may regulate cell activation through effects on different signal transduction pathways. Dermatan, heparan and chondroitin sulfates can, with varying degrees, inhibit cyclic adenosine monophosphate-dependent protein kinase (Dittmann et al., 1999). Conversely, enzymatic removal of heparan sulfates from smooth muscle cells inhibits phosphorylation of receptors and substrates in smooth muscle cells and inhibits proliferation (Nguyen et al., 1998; Silver et al., 1998). It is not clear at this time, whether removal of chondroitin and dermatan sulfates with chondroitinases might also limit cell activation by inhibiting protein phosphorylation.

Removing cellular and extracellular chondroitin sulfates can also reduce cell activation, which occurs during adhesion. Two cell surface antigens, involved in tumor growth and invasion, have associated chondroitin sulfates. Both of these proteins, CD44 and melanoma chondroitin sulfate proteoglycan (MCSP), can stimulate $\alpha 4\beta 1$ integrin function and subsequent cell activation (Eisenmann et al., 1999; Moyano et al., 1999). CD44 stimulation of integrin function has shown to be mediated by chondroitin sulfate linked to the CD44 protein (Moyano et al., 1999).

It seems likely that chondroitinase (AC and B) inhibition of many of the cellular activities described here can be ascribed to the effects of removing chondroitin and/or dermatan sulfates from CD44. The CD44 protein is important in cell adhesion, migration and invasiveness (Iida et al., 1996; Sneath and Mangham, 1998). The linkage of chondroitin and/or dermatan sulfates to CD44 has been demonstrated through the use of chondroitinase ABC (Faassen et al., 1992; Henke et al., 1996; Hurt-Camejo et al., 1999). Many of the activities affected by chondroitinases are likewise affected by disrupting CD44 function with either antibodies or soluble CD44. In vitro, anti-CD44 inhibits endothelial cell migration, proliferation and angiogenesis (Faassen et al., 1993; Henke et al., 1996; Trochon et al., 1996). Similarly, blocking CD44 function with soluble CD44 has been shown to inhibit tumor growth and invasion, and to induce apoptosis of mammary carcinoma cells in vivo (Yu et al., 1997). In addition, in the present study, combining chondroitinase treatment with anti-CD44 did not have an additive inhibitory effect on invasion, suggesting that the enzymes and anti-CD44 are acting in a similar fashion.

In summary, both chondroitin and dermatan sulfates were important in controlling many of the cellular activities related to metastasis. However, chondroitin sulfates A and C had greater impacts on all of these activities. It is possible that chondroitinase AC alone or in combination with chondroitinase B, may also be able to inhibit these same activities in vivo. Thus, the chondroitinases could prove useful as therapeutic agents to prevent or inhibit tumor growth, neovascularization and metastasis.

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