Distinct Effects of N-Acetylgalactosamine-4-sulfatase and Galactose-6-sulfatase Expression on Chondroitin Sulfates*

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The sulfatase enzymes, N-acetylgalactosamine-4-sulfatase (aryl sulfatase B (ASB)) and galactose-6-sulfatase (GALNS) hydrolyze sulfate groups of CS. Deficiencies of ASB and GALNS are associated with the mucopolysaccharidoses. To determine if expression of ASB and GALNS impacts on glycosaminoglycans (GAGs) and proteoglycans beyond their association with the mucopolysaccharidoses, we modified the expression of ASB and GALNS by overexpression and by silencing with small interference RNA in MCF-7 cells. Content of total sulfated GAG (sGAG), chondroitin 4-sulfate (C4S), and total chondroitin sulfates (CSs) was measured following immunoprecipitation with C4S antibodies and treatment with chondroitinase ABC. Following silencing of ASB or GALNS, total sGAG, C4S, and CS declined significantly. Following overexpression of ASB or GALNS, total sGAG, C4S, and CS increased significantly. Following silencing of ASB or GALNS, total sGAG, C4S, and CS declined significantly. Measurements following chondroitinase ABC treatment of the cell lysates demonstrated no change in the content of the other sGAG, including heparin, heparan sulfate, dermatan sulfate, and keratan sulfate. Following overexpression of ASB and immunoprecipitation with C4S antibody, virtually no sGAG was detectable. Total sGAG content increased to 23.39 (±1.06) μg/mg of protein from baseline of 12.47 (±0.68) μg/mg of protein following ASB silencing. mRNA expression of core proteins of the CS-containing proteoglycans, syndecan-1 and decorin, was significantly up-regulated following overexpression of ASB and GALNS. Soluble syndecan-1 protein increased following increases in ASB and GALNS and reduced following silencing, inversely to changes in CS. These findings demonstrate that modification of expression of the lysosomal sulfatases ASB and GALNS regulates the content of CSs.

N-Acetylgalactosamine-4-sulfatase (aryl sulfatase B, ASB)2 and galactose-6-sulfatase (GALNS) are two important lysosomal enzymes that hydrolyze sulfate groups from the glycosaminoglycans (GAGs) chondroitin sulfate (CS) and dermatan sulfate. Deficiencies of ASB and GALNS activity are associated with the congenital mucopolysaccharidoses (MPS) Maroteaux-Lamy syndrome (MPS VI) and Morquio syndrome (MPS IVA), respectively. In these lysosomal storage diseases, there is accumulation of highly sulfated, unmetabolizable glycosaminoglycans and proteoglycans. The impact of sulfatase activity on metabolism of GAGs and the proteoglycans (PGs) with which they are associated has not been widely explored, beyond the congenital storage diseases.

Recently, we reported that ASB activity is reduced in uncorrected cystic fibrosis cells, consistent with the accumulation of highly sulfated GAG in pulmonary secretions in cystic fibrosis patients. Following correction of cystic fibrosis transmembrane conductance regulator in pulmonary epithelial cell lines, ASB activity increased significantly (1). Also, we found marked reductions in arylsulfatases A and B, galactose-6-sulfatase, and steroid sulfatase in malignant mammary cell lines (MCF-7, T47D, and HCC 1937), compared with normal primary mammary epithelial and myoepithelial cells in tissue culture (2). Decline in sulfatase activity in malignant mammary cells suggests a possible role for diminished sulfatases in the evolution of mammary malignancy, because an increase in the highly negatively charged extracellular sulfates might affect cell-cell interactions and signaling processes.

Specified sulfations of GAG side chains may produce unique binding motifs that selectively recognize and mediate interactions between growth factors and cellular receptors. The interaction of heparin sulfate (HS), fibroblast growth factor (FGF), and the FGF receptor are required for FGF signaling, and deficiency of 2-O-sulfotransferase, which sulfates iduronic acid in HS, results in marked abnormalities of FGF signaling (3). Increase in 2-O-sulfotransferase leads to enhanced FGF responsiveness (4).

Alterations in specific sulfations by modifications of sulfotransferase expression and activity are also known to modulate syndecan function (5, 6). Syndecan has vital roles in cell-cell interactions and development, influencing the adhesion capacity of neoplastic cells with the stroma and, thereby, the growth and invasion of neoplastic cells (7). Inhibition of syndecan-1 expression in epithelial cells results in loss of cell polarity, thereby affecting epithelial-mesenchymal transition during development (8). Recent studies demonstrated that the physiological functions of syndecan-1 not only depend on its temporal and spatial expression pattern, but also on the configuration of its HS and CS side chains, which cooperate with HS chains in binding to the heparin-binding growth factors (9).
ASB and GALNS Modify Chondroitin Sulfates

Although sulfotransferases have been regarded as the predominant intracellular mechanism for the modification of the extent of sulfation of the GAG, the effects of extracellular sulfatase enzymes have also been recognized. The mammalian sulfatases (Sulf1 and Sulf2) are secreted and act as specific heparin 6-O-sulfatases (10, 11). They can mediate heparin-binding growth factor signaling by modification of GAG chains extracellularly (12). Hsulf1 has been reported to modulate hepatocyte growth factor-mediated tumor cell invasion (13). Qsulf1, the avian homolog of Sulf, has been shown to remodel HS on the cell surface and to promote Wnt-1 signaling (14).

In this report we present distinct effects of the activity of the lysosomal sulfatases ASB and GALNS on modification of CSs in MCF-7 cells, hypothesizing a role for these enzymes in cellular metabolism, beyond that associated with the mucopolysaccharidoses. To clarify if modification of lysosomal sulfatase expression can affect chondroitin sulfations in cells unaffected by genetic deficiency of sulfatase activity, we have considered the effects of silencing and overexpression of the lysosomal enzymes ASB and GALNS on total sulfated GAG (sGAG), total CS (C4S, C6S, CS-D, and CS-E), and chondroitin 4-sulfate (C4S) content, in MCF-7 cells in tissue culture. We have also determined the effects of modification of sulfatase enzymes on syndecan-1 and decorin mRNA expression and soluble syndecan-1 protein, because ASB and GALNS can hydrolyze the sulfate groups of the chondroitin component of the PG formed by these core proteins.

EXPERIMENTAL PROCEDURES

Cell Culture of MCF-7 Cells—MCF-7 cells from American Type Culture Collection (ATCC) were thawed, cultured as recommended, and sub-cultured in phenol red-free RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum (Invitrogen). Cells were maintained at 37 °C in humidified, 5% CO2 environment. After 24 h, medium was exchanged with fresh growth medium, and cells were maintained in culture for an additional 24 h. Intracellular localization of the transfection complexes was ascertained by observing fluorescence of the cells that had been transfected with rhodamine-tagged control siRNA by fluorescence microscopy. Fluorescence was detected in over 95% of the cells.

ASB and GALNS Overexpression—ASB (NCBI NM_000046) and GALNS (NCBI NM_000512) plasmids in pCMV6-XL4 vector were obtained (Origene) and overexpressed in MCF-7 cells by transient transfection using 2 μg of the plasmid and Lipofectamine™ 2000 (Invitrogen). Combined overexpression of ASB and GALNS was also performed. Controls included untransfected cells, cells transfected with vector only, and cells transfected with ASB or GALNS and vector control. Media were changed after 6 h, and cells were incubated for 48 h in humidified, 37 °C, 5% CO2 environment, and then harvested.

ASB and GALNS Activity Assays—Control, transfected, or siRNA-treated cells were harvested, and cell homogenates were prepared for measurement of ASB activity. ASB activity in the samples was determined by 4-methylumbelliferyl sulfate as substrate in 0.05M acetate buffer, pH 5.6, as previously described (1, 2). GALNS assay was also performed as previously reported (1, 2). Activity is expressed as nanograms/mg of cellular protein per hour. Protein was measured by BCA™ protein assay kit, using bovine serum albumin as standard.

Measurement of Sulfated GAGs—Total sulfated glycan content, including chondroitin 4-sulfate, chondroitin 6-sulfate, keratan sulfate, dermatan sulfate, HS, and heparan, were measured in cell lysates by sulfated GAG assay (Blyscan™, Biocolor Ltd., Newtownabbey, Northern Ireland), using labeled 1,9-dimethylmethyleny blue. The sulfated polysaccharide component of PG and protein-free sulfated GAG chains are measured, whereas degraded disaccharide fragments or hyaluronan are not. The reaction is performed in the presence of excess unbound dye. The cationic dye and GAG at acid pH produce an insoluble dye-GAG complex, and the GAG content is determined by the amount of dye that is recovered from the test sample following exposure to Blyscan dissociation reagent. Absorbance maximum of 1,9-dimethylmethyleny blue is 656 nm. Concentration is expressed as micrograms/mg of protein of cell lysate.

Chondroitinase ABC Digestion of MCF-7 Cell Lysates—Cell lysates were prepared from the harvested MCF-7 cells following overexpression and silencing by siRNA of ASB and GALNS. Chondroitinase ABC, 0.1 unit (Sigma-Aldrich) was added to each tube, and lysate was digested for 1 h at 37 °C. After digestion, the mixture was dialyzed overnight in a Dispo-Biodialyzer.
(Sigma) with 1-kDa molecular mass limit, to separate undigested GAGs from the products of chondroitinase digestion. The undigested molecules were recovered from the tube and subjected to sulfated GAG assay.

Keratanase Digestion of MCF-7 Cell Lysates—Content of keratan sulfate (KS) was calculated following digestion of MCF-7 cell lysates by keratanase (0.1 unit for 1 h at 37 °C; Sigma). Following digestion, the mixture was dialyzed overnight at 4 °C in a dispo-biodialyzer (Sigma) with 1-kDa molecular mass limit, to separate undigested GAGs from the products of keratanase digestion. The undigested molecules were recovered from the tube and sGAG content determined, as previously described. Similar analysis was performed in cell lysates prepared following overexpression of ASB and of GALNS for 48 h.

Immunoprecipitation of MCF-7 Cell Lysates by C4S and CS Antibodies—Cell lysates were prepared from treated and control cells. C4S (4D1) and CS (5C6) antibodies were procured (Santa Cruz Biotechnology, Santa Cruz, CA). These antibodies are not specific for the CS stubs, but instead detect native C4S or total CS. There is no prior enzymatic digestion of the cell samples by chondroitinase ABC with these antibodies.

To exclude cross-reactivity of the anti-C4S with CS-E and to determine the yield from immunoprecipitation, we tested the immunoprecipitation of C4S (10 μg, Biocolor Ltd.) and of CS-E (10 μg, Cape Cod Associates, East Falmouth, MA) with the C4S antibody (1 μg). Minimal reactivity with CS-E (recovery of 0.71 ± 0.03 μg increasing to 0.84 ± 0.04 μg with 2 μg of antibody) was demonstrated. Recovery of C4S was 93.3 ± 2.7%. The CS-56 antibody is a mouse monoclonal antibody, raised against ventral membranes of chicken gizzard fibroblasts, that does not detect dermatan sulfate (15).

The antibodies were added to the cell lysates (C4S antibody at a concentration of 1 μg/mg of protein; CS to a final dilution of 1:50), and tubes were rotated overnight in a shaker at 4 °C. Next, 100 μl of pre-washed Protein L-agarose (Santa Cruz Biotechnology) was added to each tube, and the tubes were incubated overnight at 4 °C. The Protein L-agarose-treated beads were then washed three times with phosphate-buffered saline containing Protease Inhibitor Mixture. The precipitate was eluted with dye-free elution buffer and subjected to sulfated GAG assay as described above.

Soluble Syndecan-1 Enzyme-linked Immunosorbent Assay— Syndecan-1 in the MCF-7 cell lysate and spent medium was measured by commercially available enzyme-linked immunosorbent assay Kit for human soluble syndecan-1 (hCD138, Cell Sciences, MA). At the termination of overexpression or RNA silencing procedures (48 h of incubation), MCF-7 cells were harvested, and spent medium was collected and stored at −80 °C. Cell lysates were prepared in radioimmune precipitation assay buffer (50 mm Tris-HCl containing 0.15 m NaCl, 1% Nonidet P40, 0.5% deoxycholic acid, and 0.1% SDS, pH 7.4), microcentrifuged at 14,000 rpm for 10 min at 4 °C, and clear supernatant used as cell extract containing solubilized syndecan-1.

Syndecan-1 in the MCF-7 cell extracts and spent medium was determined by solid-phase sandwich enzyme-linked immunosorbent assay, following the manufacturer’s instructions. Syndecan-1 molecules in the samples were captured in the wells of a microtiter plate that was pre-coated with specific monoclonal antibody to syndecan-1. Immobilized syndecan-1 molecules were detected by a second monoclonal antibody to syndecan-1 that was conjugated with biotin and streptavidin-horseradish peroxidase complex. The enzyme activity bound to the syndecan-1 molecules was determined by chromogenic reaction using hydrogen peroxide/tetramethylbenzidine. The color developed due to enzymatic activity was stopped by 1.8 N sulfuric acid, and intensity of color was measured at 450 nm in an enzyme-linked immunosorbent assay Plate reader (SLT, Spectra) with a reference filter of 620 nm. Syndecan-1 concentrations in the samples were extrapolated from a standard curve derived using known syndecan-1 quantities. Sample values were normalized with total cell protein concentrations determined using BCA Protein assay kit.

Quantitative RT-PCR—Total RNA was prepared from control, vector control, and plasmid transfected MCF-7 cells using an RNaseasy Mini Kit (Qiagen). Equal amounts of RNA from all samples were reverse transcribed and amplified in a one-step reaction using Brilliant SYBR Green QRT-PCR Master Mix kit (Stratagene). QRT-PCR was performed using a Mx3000P system (Stratagene). Syndecan-1 and decorin were amplified with specific primers that were designed using NCBI data base and Primer 3 software. hβ-Actin was used as an internal control. Primers were as follows. Syndecan-1 (NM_001202): Forward, 5′-CTCTGTGCTCTTGCTTTC3′, Reverse, 5′-CCACCTTCCTTGCGCATTT3′; Decorin (NM_001202): Forward, 5′-GCCGTTCGGTGTGTGGC3′, Reverse, 5′-TGGC-TAAGTTGGGATTGTGAGTTT3′; Syndecan-1 and decorin were amplified with specific primers that were designed using NCBI data base and Primer 3 software. hβ-Actin was used as an internal control. Primers were as follows. Syndecan-1 (NM_001202): Forward, 5′-CTCTGTGCTCTTGCTTTC3′, Reverse, 5′-CCACCTTCCTTGCGCATTT3′; Decorin (NM_001202): Forward, 5′-GCCGTTCGGTGTGTGGC3′, Reverse, 5′-TGGC-TAAGTTGGGATTGTGAGTTT3′; hβ-actin (NM_001101): Forward, 5′-GCCCTTCAGGAAAGCAGATCA3′, Reverse, 5′-CCAGGAAGGACGCTGAA3′.

Optimal Tm of the primers was calculated from Primer 3 software. The -fold changes of specific mRNAs following transfection were determined from the difference between the cycle thresholds of amplification (Ct) by using Equations 1–3.

\[
\text{Fold changes} = 2^{\Delta \Delta C_t} \quad (\text{Eq. 1})
\]

\[
\Delta C_t = C_{t \text{control target gene}} - C_{t \text{control actin}} \quad (\text{Eq. 2})
\]

\[
\Delta C_{t} = C_{t \text{treated target gene}} - C_{t \text{treated actin}} \quad (\text{Eq. 3})
\]

Immunoprecipitation with Syndecan-1 and Decorin Antibodies—To determine the fraction of cellular sGAG associated with syndecan-1 and with decorin, MCF-7 cell lysates were prepared and immunoprecipitated using antibodies to syndecan-1 (SC-5632, Santa Cruz Biotechnology) and to decorin (SC-22753, Santa Cruz Biotechnology) at a concentration of 2 μg/mg of protein. The antibodies were added to the cell lysates, and tubes were rotated overnight in a shaker at 4 °C. Next, 40 μl of pre-washed Protein A/G PLUS-agarose (Santa Cruz Biotechnology) was added to each tube, and the tubes were incubated overnight at 4 °C. The treated beads were washed three times with phosphate-buffered saline containing Protease Inhibitor Mixture. The precipitate was eluted with dye-free elution buffer and sulfated GAG measured as described above.
RESULTS

ASB and GALNS Activity in Mammary Cells and following Overexpression in MCF-7 Cells—Determinations of ASB and GALNS activity were performed in normal mammary epithelial cells grown in tissue culture, malignant mammary tissue from surgical specimens, and MCF-7 cells. In normal mammary epithelial cells in tissue culture, ASB activity was 233.9 (±10.1) nmol/mg of protein/h and GALNS activity was 8.44 (±0.26) nmol/mg of protein/h. In malignant mammary tissue, ASB activity was 20.87 (±8.08) nmol/mg of protein/h and GALNS activity was 2.06 (±0.78) nmol/mg of protein/h. At baseline, MCF-7 cells had ASB activity of 54.07 (±4.44) and GALNS of 3.85 (±0.14) nmol/mg of protein/h. Transfection of MCF-7 cells with 2 µg of ASB-pCMV6-XL-4 vector increased the ASB activity in the cells 110% to 113.82 (±7.68) nmol/mg of protein/h, compared with control (p < 0.001, one-way analysis of variance with Tukey-Kramer post-test) (Table 1). Transfection of MCF-7 cells with 2 µg of GALNS increased the GALNS activity in the cells to 8.15 (±0.20), a 117% increase, and statistically significant compared with controls (p < 0.001, one-way analysis of variance and Tukey-Kramer post-test). Combined silencing of ASB and GALNS and combined overexpression of ASB and GALNS did not further change the decline or increase in enzymatic activity achieved by modification of ASB or GALNS alone.

Reduced Sulfatase Activity following Silencing by siRNA in MCF-7 Cells—Silencing of ASB and GALNS activity by siRNA reduced the associated enzyme activity significantly (p < 0.001, one-way analysis of variance followed by Tukey-Kramer post-test) (Table 1). ASB activity declined to 14.03 (±1.05) nmol/mg of protein/h, a decline of 74% following silencing by siRNA. GALNS activity declined to 0.97 ± 0.04 nmol/mg of protein/h, a decline of 75% when MCF-7 cells were transfected with GALNS siRNA. Combined silencing of both ASB and GALNS did not lead to further reduction in either the ASB or GALNS activity. When ASB was silenced, GALNS activity remained at control level, and reciprocally, reflecting high specificity of the silencing. Vector control for siRNA did not change the ASB or GALNS activity in MCF-7 cells, compared with the normal basal activity.

Total Sulfated GAG Content following Immunoprecipitation with C4S and CS Antibodies—To distinguish the specific GAG affected by modification of sulfatase, immunoprecipitations with C4S antibody and CS antibody were performed, prior to sGAG assay (Table 2 and Fig. 2, A–C). Increases and declines are statistically significant (p < 0.001).

Following silencing of ASB and immunoprecipitation with C4S antibody (Fig. 2A), sGAG measured 14.53 (±0.89) µg/mg of protein, an increase of 11.01 µg/mg of protein over the baseline value. Similarly, when immunoprecipitated with CS antibody, sGAG increased to 17.55 (±1.58) µg/mg of protein, an increase of 11.05 over the control value. Total sGAG increased by 11.11 µg/mg of protein following ASB silencing, consistent with the other values.

Following silencing of GALNS and immunoprecipitation with CS antibody (Fig. 2A), sGAG increased to 13.27 (±0.86) µg/mg of protein, an increase of 6.77 µg/mg of protein. When immunoprecipitated with C4S, no increase was detected. Total sGAG increased by 6.26 µg/mg of protein, indicating that the increase in sGAG following silencing of GALNS was less than that observed following silencing of ASB.

Following overexpression of ASB and immunoprecipitation with C4S antibody (Fig. 2B), sGAGs were virtually absent (0.12 ± 0.01 µg/mg of protein), indicative of the impact of ASB on C4S content. Following immunoprecipitation with CS antibody, sGAG measured 3.00 (±0.30) µg/mg of protein, consistent with the content of C6S and dermatan sulfate derived from the difference in sGAG following immunoprecipitation with CS antibody (6.50 ± 0.30 µg/mg of protein) or C4S antibody (3.52 ± 0.28 µg/mg of protein) or C4S antibody in the controls.

| TABLE 1 | ASB and GALNS activity following silencing and overexpression in MCF-7 cells |
|----------|-------------------------------------------------|
| ASB (nmol/mg protein/h) | GALNS (S.D.) |
| Control | 54.07 (0.44) | 8.15 (0.20) |
| Control siRNA | 52.12 (0.27) | 7.68 (0.50) |
| ASB siRNA | 14.03 (1.05)* | 2.06 (0.30) |
| GALNS siRNA | 55.03 (3.48) | 0.95 (0.04)* |
| Vector control OE | 56.31 (5.22) | 3.71 (0.12) |
| ASB OE | 108.00 (7.53)* | 3.53 (0.13) |
| GALNS OE | 54.04 (5.12) | 8.15 (0.20)* |
| Combined siRNA | 13.83 (0.86)* | 0.90 (0.02)* |
| Combined OE | 119.67 (11.04)* | 8.24 (0.45)* |

* Differences in ASB and GALNS activity in the MCF-7 cells following overexpression and silencing are statistically significant compared to control (p < 0.001, one-way analysis of variance, Tukey-Kramer post-test).
Following overexpression of GALNS and immunoprecipitation with CS antibody (Fig. 2C), sGAG measured 3.40 (±0.29) μg/mg of protein, consistent with the baseline CS content of 3.52 (±0.28) μg/mg of protein and the fact that the CS content should not be modified by the overexpression of GALNS. Following immunoprecipitation with CS and overexpression of GALNS, sGAG measured 3.32 (±0.21) μg/mg of protein, consistent with the other measurements of CS.

Sulfated GAG Content following Treatment with Chondroitinase ABC—To determine if modification of expression of ASB or GALNS had any effect on the non-chondroitin sGAG, including KS, heparin, or HS, cell lysates were treated with chondroitinase ABC, and low molecular mass (=1 kDa) degradation products were dialyzed. sGAG assay was performed of the GAG not digested by chondroitinase ABC. Results (Table 2) demonstrate that silencing of ASB or GALNS or overexpression of ASB or GALNS does not significantly affect the quantity of the non-chondroitin sGAG. Control value is 5.40 (±0.21) μg/mg of protein, compared with 6.14 (±0.54) μg/mg of protein following ASB silencing. Similarly, silencing of GALNS or overexpression of ASB or GALNS does not affect the non-chondroitin sGAG content.

**Variation in Syndecan-1 following Changes in Expression of ASB and GALNS—**Overexpression of ASB in MCF-7 cells increased soluble syndecan-1 protein in the cell extract 65% over vector control (Fig. 3A). Similarly, overexpression of GALNS increased the syndecan-1 in the cell extract 68% over the vector control (Fig. 3B). Increases were statistically significant (p < 0.001). No significant differences in syndecan-1 were detected in the spent media following ASB or GALNS transfection.

Reduction in ASB and GALNS activity in the MCF-7 cells following silencing by siRNA was associated with significant declines in soluble syndecan-1 in the cell extract (Fig. 3C) and the spent media (Fig. 3D). When ASB was silenced, syndecan-1 in the cell extract declined from 215.0 (±3.9) ng/mg of protein to 173.0 (±3.0) ng/mg of protein. Similarly, GALNS silencing decreased the syndecan-1 by 28.4% in the cell extract. Combined silencing of ASB and GALNS reduced syndecan-1 by 30.0% in the cell extract. Similar results were found for syndecan-1 in the spent media (Fig. 3D). The observed differences are statistically significant (p < 0.001) and indicate that silencing of ASB and GALNS produces increases in CSs that are associated with decline in soluble syndecan-1 in the MCF-7 cells.

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**Table 2**

| Experiment | Total sGAG | CS IP | CS4 IP | Other sGAG |
|------------|------------|-------|--------|------------|
| Control    | 12.47 ± 0.68 | 6.50 ± 0.30 | 3.52 ± 0.28 | 5.48 ± 0.21 |
| Cn siRNA   | 12.28 ± 0.96 | 6.50 ± 0.59 | 3.52 ± 0.27 | 5.42 ± 0.35 |
| ASB siRNA  | 23.39 ± 1.06 | 17.55 ± 1.58 | 14.53 ± 0.89 | 6.14 ± 0.54 |
| GALNS siRNA| 18.73 ± 1.53 | 13.29 ± 0.86 | 3.52 ± 0.11 | 5.87 ± 0.50 |
| ASB vector | 12.15 ± 0.91 | 6.50 ± 0.48 | 3.51 ± 0.14 | 5.37 ± 0.34 |
| control OE | ASB OE      | 8.39 ± 0.15 | 3.00 ± 0.30 | 0.12 ± 0.01 | 5.46 ± 0.37 |
| GALNS vector control OE | 12.67 ± 0.97 | 5.50 ± 0.49 | 3.53 ± 0.20 | 5.38 ± 0.38 |
| GALNS OE   | 8.59 ± 0.59 | 3.40 ± 0.29 | 3.32 ± 0.21 | 5.36 ± 0.40 |

* ± S.D. Units are micrograms/mg of cellular protein. All results are mean of three experiments with technical duplicates of each measurement. Measured in cell lysate.

* Measured following immunoprecipitation with CS antibody (CS-56).

* Measured following immunoprecipitation with CS4 antibody (4D1).

* Measured following treatment of cell lysate with chondroitinase ABC.

* p < 0.001, one-way analysis of variance with Tukey-Kramer post-test.
Increased mRNA Expression of Syndecan-1 and Decorin following Overexpression of ASB and GALNS—Quantitative RT-PCR demonstrated 1.24-fold increase (2.24 ± 0.25 times baseline) in syndecan-1 expression and 4.23-fold increase (5.23 ± 1.00 times baseline) in decorin expression 24 h after transfection of ASB into the MCF-7 cells (Fig. 4A). Similarly, GALNS transfection increased the expression of syndecan-1 to 3.00 (±0.33) times the baseline and decorin to 2.24 (±0.20) times baseline (Fig. 4B), compared with cycle thresholds of vec-
ASB and GALNS Modify Chondroitin Sulfates

The intracellular lysosomal enzymes ASB and GALNS have been associated with the occurrence of the mucopolysaccharidosis or lysosomal storage diseases. We have recently reported increased ASB activity in association with correction of the CFTR defect in cystic fibrosis cells and reduced activity of ASB, GALNS, steroid sulfatase, and arylsulfatase A in malignant mammary cell lines (1, 2). However, the impact of ASB or GALNS activity on other cellular processes has not been reported previously. In this report, we have examined the effects of silencing and overexpression of ASB and GALNS in MCF-7 cells on sulfated GAG content and on expression of syndecan-1 and decorin and found significant changes induced by modification of sulfatase activity.

The study findings provide evidence for a significant role of ASB and GALNS in the determination of CS content and in the core protein expression of two proteoglycans with CS GAG attachments. These effects may be related to direct effects of these enzymes on the lysosomal catabolism of C4S, C6S, and dermatan sulfate. ASB and GALNS are lysosomal enzymes that undergo post-translational modification for functional activation. When synthesized, they are targeted by mannose 6-phosphate and incorporated into transport vesicles that separate from the Golgi and proceed to fuse with the endosomes/lysosomes. The observation that syndecan-1 accumulates in lysosomes in mammary carcinoma cells is consistent with reduced metabolism by the lysosomal sulfatases ASB and GALNS in malignant cells (16). Co-localization experiments have demonstrated the presence of intact endocytosed syndecan-1 in lysosomes, and inactivation of lysosomes in MCF-7 and T47D mammary epithelial cells was associated with the accumulation of intact syndecan-1 in the lysosomes (16). Degradation of the small leucine-rich proteoglycan decorin has also been localized to a lysosomal pathway in explant cultures of tendon (17), ligament (18), and other cells (19–21). Although the studies that we report demonstrate a decline in syndecan-1 protein in association with reduction in sulfatase activity and accumulation of sGAG, the intracellular localization of syndecan was not determined. Endosomal/lysosomal accumulation might arise in association with reduced degradation of the sGAG, even though syndecan-1 protein is reduced. In association with overexpression of ASB and GALNS, syndecan-1 protein and mRNA increased, coincident with decline in sGAG. Imaging studies of syndecan localization in this circumstance might demonstrate mobilization and accumulation of syndecan in extralysosomal sites, as well as in the lysosomal sites previously observed.

The immunoprecipitation studies using C4S and CS antibodies have permitted selective detection of C4S, C6S, and dermatan sulfate, and KS, did not vary significantly following modification of expression of ASB or GALNS, in contrast to the

tor controls. Differences are statistically significant (p < 0.001, two-tail t test between control-transfected and sulfatase-transfected groups).

**TABLE 4**
sGAG content following immunoprecipitation with decorin or syndecan-1 antibody and following treatment of syndecan immunoprecipitate with chondroitinase ABC

| sGAG       | Percentage of total sGAG |
|------------|--------------------------|
| Total      | 12.18 ± 0.92*; 12.41 ± 0.25a   |
| Decorin IP | 1.10 ± 0.09               |
| Syndecan-1 IP | 0.62 ± 0.04   |
| Syndecan-1 IP + chondroitinase ABC | 0.37 ± 0.02 |

*Total sGAG determination following immunoprecipitation (IP) with decorin antibody.

aTotal sGAG determination following immunoprecipitation with syndecan-1 antibody.

FIGURE 4. Expression of syndecan-1 and decorin in MCF-7 cells following overexpression of ASB and GALNS. A, syndecan-1 and decorin transcripts were increased by overexpression of ASB (2.24 ± 0.22 and 5.22 ± 0.52 times baseline) at 48 h. B, similarly, GALNS overexpression produced increases of syndecan-1 (3.04 ± 0.25 times baseline) and decorin (2.32 ± 0.21 transcripts at 48 h. Differences are statistically significant (p < 0.001, two-tail t test between control-transfected and sulfatase-transfected groups).

**DISCUSSION**

The intracellular lysosomal enzymes ASB and GALNS have been associated with the occurrence of the mucopolysaccharidosis or lysosomal storage diseases. We have recently reported increased ASB activity in association with correction of the CFTR defect in cystic fibrosis cells and reduced activity of ASB, GALNS, steroid sulfatase, and arylsulfatase A in malignant mammary cell lines (1, 2). However, the impact of ASB or GALNS activity on other cellular processes has not been reported previously. In this report, we have examined the effects of silencing and overexpression of ASB and GALNS in MCF-7 cells on sulfated GAG content and on expression of syndecan-1 and decorin and found significant changes induced by modification of sulfatase activity.

The study findings provide evidence for a significant role of ASB and GALNS in the determination of CS content and in the core protein expression of two proteoglycans with CS GAG attachments. These effects may be related to direct effects of these enzymes on the lysosomal catabolism of C4S, C6S, and dermatan sulfate. ASB and GALNS are lysosomal enzymes that undergo post-translational modification for functional activation. When synthesized, they are targeted by mannose 6-phosphate and incorporated into transport vesicles that separate from the Golgi and proceed to fuse with the endosomes/lysosomes. The observation that syndecan-1 accumulates in lysosomes in mammary carcinoma cells is consistent with reduced metabolism by the lysosomal sulfatases ASB and GALNS in malignant cells (16). Co-localization experiments have demonstrated the presence of intact endocytosed syndecan-1 in lysosomes, and inactivation of lysosomes in MCF-7 and T47D mammary epithelial cells was associated with the accumulation of intact syndecan-1 in the lysosomes (16). Degradation of the small leucine-rich proteoglycan decorin has also been localized to a lysosomal pathway in explant cultures of tendon (17), ligament (18), and other cells (19–21). Although the studies that we report demonstrate a decline in syndecan-1 protein in association with reduction in sulfatase activity and accumulation of sGAG, the intracellular localization of syndecan was not determined. Endosomal/lysosomal accumulation might arise in association with reduced degradation of the sGAG, even though syndecan-1 protein is reduced. In association with overexpression of ASB and GALNS, syndecan-1 protein and mRNA increased, coincident with decline in sGAG. Imaging studies of syndecan localization in this circumstance might demonstrate mobilization and accumulation of syndecan in extralysosomal sites, as well as in the lysosomal sites previously observed.

The immunoprecipitation studies using C4S and CS antibodies have permitted selective detection of C4S and total CS, and treatment with chondroitinase ABC has enabled isolation of the non-chondroitin sGAG content in the cell lysates. The non-chondroitin sGAG content, including heparin, HS, dermatan sulfate, and KS, did not vary significantly following modification of expression of ASB or GALNS, in contrast to the
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marked changes observed in the C4S and CS contents. Because ASB and GALNS can modify the sulfation of N-acetylgalactosamine residues of CSs, changes in expression of ASB or GALNS would not be expected to affect the sulfations of idurionate or of N-acetylglucosamine present in HS, heparin, or KS. The studies have demonstrated no change from baseline in KS following the overexpression of ASB or GALNS, indicating that the galactose residues in the KS disaccharide structural unit were not modified by the overexpression of GALNS. Modifications of the sulfation of CS-D or CS-E by GALNS or ASB have not been addressed by the experiments that we report. The findings with regard to detection of changes in C4S sulfation by modification of ASB expression and immunoprecipitation with the C4S (4D1) antibody appear to be highly specific.

The changes identified in syndecan-1 expression following modification of sulfatase activity resemble those observed following alteration of heparanase in human myeloma and breast cancer cell lines (22, 23). Hence, the study findings suggest that both modifications of CS and HS can affect the production of syndecan-1.

Silencing the activity of ASB or of GALNS in the mammary MCF-7 cells significantly increased content of CSs and inhibited syndecan-1 protein expression. In contrast, overexpression of ASB and GALNS reduced CS content and produced increases in syndecan-1 and decorin expression. These reciprocal changes in core protein expression and CS content suggest that the extent of chondroitin sulfation may affect the regulation of core protein expression, by as yet unexplained mechanisms. The changes found in the chondroitins following overexpression or knockdown of the ASB and GALNS may not be limited to changes in the chondroitins associated with syndecan or decorin, but may include changes in chondroitin associated with other PGs, such as biglycan, versican, aggrecan, or decorin, but may include changes in chondroitin associated with syndecan-1.

Increases in syndecan-1 and decorin expression following overexpression of ASB and GALNS, in association with declines in CSs and no change in non-chondroitin sGAG, suggest that the increased catabolism of CS and the concomitant liberation of sulfate, may, by an as yet unexplained sulfate signaling mechanism, stimulate core protein biosynthesis. The decorin-associated C4S comprises a substantial fraction (~17%) of the total CS, indicating the likely importance of this PG in cell-matrix and cell-cell interactions.

Previously, we have found marked decline in sulfatase enzymes in malignant mammary cell lines and malignant mammary tissue (13). Current findings, that sulfatase activity modifies expression of syndecan-1 and decorin, suggest that activity of ASB and GALNS may be of critical importance in defining cell surface recognition markers. This ability may be diminished in malignant cells, if sulfatase activity is diminished. The extent of chondroitin sulfation may affect the interactions of the PGs with growth factors, cations, and membrane receptors. Modification of chondroitin sulfations by ASB and GALNS may have effects on cell-cell signaling and cell-matrix interactions that are mediated by the PGs. Specific mechanisms of intracellular sulfate/sulfatase signaling, such as those leading to increases in syndecan and decorin expression, require further investigation. Recognition of the high specificity of ASB and GALNS may facilitate clarification of these vital signaling mechanisms.

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