Heparan Sulfate Synthesized by Mouse Embryonic Stem Cells Deficient in NDST1 and NDST2 Is 6-O-Sulfated but Contains No N-Sulfate Groups

Received for publication, August 5, 2004, and in revised form, August 19, 2004
Published, JBC Papers in Press, August 19, 2004
DOI 10.1074/jbc.C400373200

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Heparan sulfate structure differs significantly between various cell types and during different developmental stages. The diversity is created during biosynthesis by sulfotransferases, which add sulfate groups to the growing chain, and a C5-epimerase, which converts selected glucuronic acid residues to iduronic acid. All these modifications are believed to depend on initial glucosamine N-sulfation carried out by the enzyme glucosaminyl N-deacetylase/N-sulfotransferase (NDST). Here we report that heparan sulfate synthesized by mouse embryonic stem cells deficient in NDST1 and NDST2 completely lacks N-sulfation but still contains 6-O-sulfate groups, demonstrating that 6-O-sulfation can occur without prior N-sulfation. Reverse transcriptase-PCR analysis indicates that all three identified 6-O-sulfotransferases are expressed by the cells, 6-O-sulfotransferase-1 being the dominating form. The 6-O-sulfated polysaccharide lacking N-sulfate groups also contains N-unsubstituted glucosamine units, raising questions about how these units are generated.

Some biological functions of heparan sulfate (HS), such as anticoagulant activity through interactions with antithrombin and regulation of cell proliferation through binding of FGF-2, are strictly dependent on special arrangements of HS modifications (1). On the other hand, most tissues are formed in apparently normal fashion in mice with dramatically altered HS structure such as in mice lacking 2-O-sulfotransferase, C5-epimerase, or N-deacetylase/N-sulfotransferase 1 (NDST1; refs. 2–4). However, when the HS-polymerase EX11 is knocked out, apparently leading to a complete lack of HS, the embryos die at gastrulation (5). Early embryonic lethality is also seen in embryos devoid of both NDST1 and NDST2 (6).

In HS biosynthesis, NDST activity appears to be a key regulator, since all types of modifications, 2-O-, 6-O-, and 3-O-sulfation and C5 epimerization, are found in N-sulfated regions (7). However, very little is known about the actual organization of the HS biosynthesis machinery, and an increasing amount of evidence suggests that NDST activity is influenced by what has been thought to be subsequent modifications (4, 8). In addition, the HS polymerases are apparently influenced by N-sulfation (9). While the presence of N-unsubstituted glucosamine residues in native HS has been documented (10, 11), the mechanism responsible for their generation is not known. It has been speculated that they may arise from incomplete action of NDSTs (12). In this paper we have studied HS biosynthesis in mouse embryonic stem (ES) cells deficient in NDST1 and NDST2. These cells synthesize 6-O-sulfated HS, devoid of N-sulfate groups, containing some N-unsubstituted glucosamine residues. We conclude that 6-O-sulfation as well as the generation of N-unsubstituted glucosamine residues may occur independently of N-sulfation.

EXPERIMENTAL PROCEDURES

Establishment of ES Cell Lines—Murine blastocysts of different genotypes (NDST1−/−/NDST2−/−, A1, A3, B5, NDST1−/−/NDST2−/− control, C57, 129) were isolated and plated on primary mouse embryonic fibroblast in embryonic stem cell medium consisting of Dulbecco’s modified Eagle’s medium with glutamax-1, sodium pyruvate, 4500 mg/liter glucose, and pyridoxine (Dulbecco’s modified Eagle’s medium, Invitrogen) supplemented with 1× non-essential amino acids (Invitrogen), 20% fetal calf serum (Invitrogen), 10−4 M β-mercaptoethanol (Sigma), and 1000 units/ml leukemia inhibitory factor (LIF) (mouse recombinant, Chemicon). After 5–6 days in culture the inner cell mass was isolated and dispersed after exposure to 1× trypsin/EDTA in phosphate-buffered saline (Invitrogen). The dispersed cells were then plated in a fresh well coated with mouse embryonic fibroblast. ES cell-like colonies appeared after 2–8 days of culture and were isolated and dispersed as described above until a stable ES cell line was established. The cells were genotyped by PCR using the following primers: forward NDST-1, 5′-GTCGTCGTCGTCACCTTCATCCTCTTC-3′; reverse NDST-1/NDST-2 knockout primer (located in the NDST-2 intron), 5′-TTCCTC-3′. RT-PCR Analysis—Total RNA was isolated from ES cells with the RNeasy midi kit (Qiagen) and converted to cDNA using the GeneAmp RNA PCR kit (PerkinElmer Life Sciences). For PCR analysis of NDST expression, the AmpliTaq Gold PCR kit (Applied Biosystems) was used. PCR reactions were performed by denaturing the DNA at 95 °C for 8 min before amplification for 35 cycles, each cycle consisting of 95 °C, 30 s; 60 °C, 30 s; 72 °C, 40 s, with a final elongation step at 72 °C for 10 min.

The cDNA was amplified using the following primers: NDST-1 (1007 bp), forward 5′-GGCTACAAACAGGAAATGAGGAAAGTGAAG-3′ and reverse 5′-GGCGTTCCAGGCATCCAGGTT-3′; NDST-2, forward 5′-CTCTGCTTGTGTTGTCTGTG-3′ and reverse wild type 5′-CGTTTCAGCCATACAGGAGGAG-3′; reverse NDST-1NDST-2 knockout primer (located in the neomycin cassette), 5′-CTGCTCTTATCTGAAGGCCCTC-3′.

RT-PCR Analysis—Total RNA was isolated from ES cells with the RNeasy midi kit (Qiagen) and converted to cDNA using the GeneAmp RNA PCR kit (PerkinElmer Life Sciences). For PCR analysis of NDST expression, the AmpliTaq Gold PCR kit (Applied Biosystems) was used. PCR reactions were performed by denaturing the DNA at 95 °C for 8 min before amplification for 35 cycles, each cycle consisting of 95 °C, 30 s; 60 °C, 30 s; 72 °C, 40 s, with a final elongation step at 72 °C for 10 min.

1 The abbreviations used are: HS, heparan sulfate; NDST, N-deacetylase/N-sulfotransferase; ES, embryonic stem; RT, reverse transcriptase; RIP, reverse phase ion pair; HPLC, high performance liquid chromatography; CS, chondroitin sulfate.

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This paper is available on line at http://www.jbc.org
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**Heparan Sulfate Biosynthesis**

**Metabolic Labeling of Glycosaminoglycans**

Glycosaminoglycans were isolated from the solubilized cell lysate on a 0.3-ml column of DEAE-Sepharose (Amersham Biosciences). One-quarter of the sample was saved for analysis. The remaining part was acetylated. After addition of methanol to a final concentration of 10%, repeated additions of acetic acid anhydride were made during 1 h. The pH was kept at 5.6 for 15 min at 4°C, the supernatant was deacetylated by hydrazinolysis for 5 h, followed by combined deamination at pH 1.5 and 3.9 (18) and reduction with NaBH₄. The resulting ³H-labeled HS chains were desalted using YM3 filters (Amicon Bioseparations). One-quarter of the sample was saved for nitrous acid treatment, while the remaining part was acetylated. After addition of methanol to a final concentration of 10%, repeated additions of acetic acid anhydride were made during 1 h. The pH was kept at 7–7.5 by addition of saturated Na₂CO₃ in 10% methanol. The resulting ³H-labeled glycosaminoglycans were recovered by ethanol precipitation and centrifugation. The pellet was dissolved in water and subjected to nitrous acid treatment at pH 1.5 and 3.9 (17). The resulting ³H-labeled oligosaccharides were separated by gel chromatography on a Superose 6 10/30 column (Amersham Biosciences), eluted in 0.5 mM NH₄HCO₃.

**Total Disaccharide Composition**

Radiolabeled glycosaminoglycans were desalted using YM3 filters and subjected to chondroitinase ABC and alkali treatment as described above. Isolated ³H-labeled HS was chemically N-deacylated by hydrazinolysis for 5 h, followed by combined deamination at pH 1.5 and 3.9 (18) and reduction with NaBH₄. The resultant disaccharides, containing terminal idurandinitrile (αMan₃) residues, were recovered by gel filtration on a column (1×180 cm) of Sephadex G-15 (Amersham Biosciences) in 0.2 mM NH₄HCO₃. The disaccharides were characterized by anion-exchange chromatography on a Partisil-10 SAX column (Whatman Inc.) eluted with a stepwise gradient of KH₂PO₄ (19).

**RESULTS**

**NDST-1 and NDST-2 Are the Main NDST Isoforms in Embryonic Stem Cells**

In ES cells derived from C57 and 129 ES cells were metabolically labeled for 16 h with 50 μCi of [³H]glucosamine in 6 ml of embryonic stem cell medium. After 16 h of incubation, the medium was recovered, and the cells were washed with cold phosphate-buffered saline followed by incubation for 30 min in 2 ml of solubilization buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.10 M NaCl), containing the protease inhibitors EDTA (2 mM), N-ethylmaleimide (2 mM), phenylmethylsulfonyl fluoride (1 mM), and pepstatin A (10 μg/ml). After centrifugation at 8000 × g for 15 min at 4°C, the supernatant (solubilized cell lysate) containing radiolabeled macromolecules was recovered.

**Glycosaminoglycan Isolation**

Glycosaminoglycans were isolated from the solubilized cell lysate on a 0.3-ml column of DEAE-Sepharose (Amersham Biosciences), equilibrated in 50 mM Tris-HCl pH 7.4, 0.1% Triton X-100, 0.10 M NaCl, and 0.10 of the concentration of the protease inhibitors added to the solubilization buffer (see above). The column was first washed with equilibration buffer and subsequently with 50 mM acetate buffer, pH 4.0, containing 0.1% Triton X-100 and 0.10 M NaCl. The glycosaminoglycans were eluted with 50 mM acetate buffer, pH 4.0, containing 0.1% Triton X-100 and 2 M NaCl.

**HS Structural Analysis**

Radiolabeled glycosaminoglycans were alkali-treated as described previously (15). After desalting in water on PD10 columns (Amersham Biosciences) followed by lyophilization, the ³H-labeled glycosaminoglycans were subjected to digestion with 0.1 unit of chondroitinase ABC (Seikagaku) as described previously (16). The resulting ³H-labeled HS chains were desalted using YM3 filters (Amicon Bioseparations). One-quarter of the sample was saved for nitrous acid treatment, while the remaining part was acetylated. After addition of methanol to a final concentration of 10%, repeated additions of acetic acid anhydride were made during 1 h. The pH was kept at 7–7.5 by addition of saturated Na₂CO₃ in 10% methanol. The resulting ³H-labeled glycosaminoglycans were recovered by ethanol precipitation and centrifugation. The pellet was dissolved in water and subjected to nitrous acid treatment at pH 1.5 and 3.9 (17). The resulting ³H-labeled oligosaccharides were separated by gel chromatography on a Superose 6 10/30 column (Amersham Biosciences), eluted in 0.5 mM NH₄HCO₃.

**FIG. 1. Analysis of NDST and 6-O-sulfotransferase expression in control, NDST−/−/NDST−/−, and NDST−/−/NDST−/− mouse ES cells. mRNA was isolated and subjected to RT-PCR using gene-specific primers for NDST1–NDST4, the housekeeping gene hprt, and 6-O-sulfotransferases 1–3 as described under "Experimental Procedures." Three ES cell clones devoid of NDST1 and NDST2 obtained from three different blasticysts (A1, A3, and B5) were analyzed along with NDST−/−/NDST−/− ES cells and two control cell lines (C57, 129). CDNA from mouse E11.5 was used as a positive control. PCR amplification was carried out for 35 cycles.**

**FIG. 2. Reverse phase ion pair chromatography of HS disaccharides from ES cells. HS was isolated and degraded to disaccharides by cleavage with heparitinases I, II, and III. The disaccharides obtained were separated by reverse phase ion pair chromatography, followed by labeling with 2-cyanoacetamide (see "Experimental Procedures"). All chromatograms have been normalized with respect to the height of the non-sulfated disaccharide peak eluting after 4 min (data not shown). The elution positions of known disaccharide standards are indicated by arrows.**
NDST1 and NDST2 are the only NDST isoforms expressed in significant amounts, as judged by RT-PCR (Fig. 1). Low levels of a PCR product corresponding to NDST3 were obtained after 35 cycles of amplification using mRNA from 129 control ES cells as template (Fig. 1). Extending the number of cycles to 40, small amounts of NDST3 transcript were also detected in the other ES cell lines (data not shown). The identity of the PCR product as NDST3 was confirmed by sequencing (data not shown). No NDST4 was detected in any of the ES cells even after 40 cycles of PCR amplification.

6-O-Sulfation without N-Sulfation—To characterize the HS produced by ES cells devoid of NDST1 and NDST2, glycosaminoglycans were isolated from these cells and from control cells. After chondroitinase ABC digestion, an aliquot was saved for analysis of CS structure with RPIP-HPLC. HS was isolated from the digest on a DEAE spin column and subjected to analysis of CS structure with RPIP-HPLC. HS was isolated from control C57 and 129 control blastocysts, containing both NDST1 and NDST2, synthesize a polysaccharide resembling "an average" low sulfated mouse tissue HS (Fig. 2, Table I), indicating that HS biosynthesis in ES cells shares general properties of HS biosynthesis with more differentiated cells. The amount of HS synthesized in the different cultures was roughly the same for cells of all genotypes. Also the HS/CS ratios were similar in the different cultures with the control with effects resembling those of NDST1 deficiency in liver,\(^2\) with 2-O-sulfation more severely affected than 6-O-sulfation. The lowered HS sulfation in these cells compared with that in C57 and 129 ES cells could reflect a dose dependence for NDST1 transcript. Alternatively, NDST2 has a role in HS biosynthesis in ES cells. In ES cells deficient in NDST1 and NDST2, no disaccharides containing N-sulfate groups were present (Fig. 2, Table I). Interestingly, about half of the 6-O-sulfate groups remaining were compared with HS from control ES cells. In the RPIP-HPLC protocol, the structure of disaccharides obtained after enzymatic cleavage is based on retention time compared with known standards (14). To exclude the possibility that an unrelated structure could be eluted as the same position as standard 6'-sulfated disaccharides, another method for structural analysis was performed. \(^3\)H-Labeled HS from control C57 and NDST1’/’/NDST2’/’ (B5) cells were subjected to extensive deamidative cleavage (17) followed by reduction with NaBH\(_4\). The obtained reduced products were then separated on an anion-exchange SAX column as described (19). The \(^3\)H-labeled HS disaccharides obtained from NDST1’/’/NDST2’/’ (B5) cells were of two kinds, non-sulfated or GlcA/GlcNAc6S, confirming the results obtained with RPIP-HPLC method. The two methods generated quantitatively similar results, and also

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It is known that excess amounts of low pH nitrous acid reagent will efficiently cleave HS also at positions of free amino groups (20), and the \(^3\)H-labeled HS was therefore chemically acetylated before treatment with nitrous acid at pH 1.5. The acetylation did not result in any change in elution position on Superose 6 (data not shown). While acetylated \(^3\)H-labeled HS from control C57 cells was sensitive to treatment with nitrous acid at pH 1.5 (Fig. 3A), no degradation of acetylated \(^3\)H-labeled HS from B5 NDST1\(^{-/-}\)/NDST2\(^{-/-}\) cells could be detected (Fig 3B), confirming the lack of N-sulfate groups in HS from these cells. Acetylated \(^3\)H-labeled HS from both cell types were resistant to treatment with nitrous acid at pH 3.9 (Fig. 3, C and D), as could be expected. In contrast, the unacetylated \(^3\)H-labeled HS preparations were both sensitive to this treatment (Fig. 3, E and F), demonstrating the presence of N-unsubstituted glucosamine residues in control as well as in NDST1\(^{-/-}\)/NDST2\(^{-/-}\) cells. It may also be noted in Fig. 3 that the \(^3\)H-labeled HS polysaccharide chains from NDST1\(^{-/-}\)/NDST2\(^{-/-}\) cells are longer (\(K_{AV} 0.58\)) than the control polysaccharides (\(K_{AV} 0.65\)).

**DISCUSSION**

We have shown that ES cells deficient in NDST1 and NDST2 synthesize 6-O-sulfated HS lacking N-sulfate groups. This was an unexpected finding, since 6-O-sulfate groups have always been found in close vicinity to N-sulfate groups (21), and, defects in *Drosophila sulfateless*, a gene encoding an NDST homolog, result in the biosynthesis of a completely non-sulfated HS polysaccharide (22). In addition, in *vitro* experiments with recombinant 6-O-sulfotransferases have shown that N-sulfated disaccharides are preferred substrates for these enzymes (23, 24). Our results may suggest a more independent role for 6-O-sulfotransferases in HS biosynthesis. It is also noteworthy that almost nothing is known about the substrate specificity of the NDST enzymes and what factors that may influence the length of the N-sulfated regions. If some 6-O-sulfation is initiated before or at the same time as N-sulfation, it is possible that the positions of the 6-O-sulfate groups may influence the N-sulfation pattern. Further studies with recombinant NDST enzymes are required to investigate if and how O-sulfation regulates NDST substrate recognition.

In addition, we show that the generation of free amino groups appears to be independent of NDST1 and NDST2, as similar amounts of N-unsubstituted glucosamine residues are found in HS from both control and NDST1\(^{-/-}\)/NDST2\(^{-/-}\) cells (Fig. 3). With sensitive PCR conditions, we could detect small amounts of NDST3 mRNA. It has been demonstrated previously that NDST3 has a high N-deacetylating capacity, while the N-sulfotransferase activity is the lowest among the four NDSTs (25). NDST3 is therefore a tentative generator of free amino groups. However, we know from previous studies of NDST1 mutants overexpressed in 293 cells that free amino groups generated by an NDST1 mutant lacking N-sulfotransferase activity are efficiently sulfated by N-sulfotransferase-expressing NDSTs in the same cell (15). Therefore, since both control cells and NDST1\(^{-/-}\)/NDST2\(^{-/-}\) cells contain N-unsubstituted glucosamine residues, NDST3 is likely to perform its action in a Golgi compartment trans of the compartment where NDST1 and/or NDST2 reside. The minimal amounts of NDST3 transcript detected in the cells may also suggest that a so far unidentified N-deacetylase could be responsible for deacetylation of N-acetylg glucosamine residues.

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