Defective Heparan Sulfate Biosynthesis and Neonatal Lethality in Mice Lacking N-Deacetylase/N-Sulfotransferase-1*

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Heparan sulfate is a sulfated polysaccharide present on most cell surfaces and in the extracellular matrix. In vivo functions of heparan sulfate can be studied in mouse strains lacking enzymes involved in the biosynthesis of heparan sulfate. Glucosaminyl N-deacetylase/N-sulfotransferase (NDST) catalyzes the first modifying step in the biosynthesis of the polysaccharide. This bifunctional enzyme occurs in several isoforms. We here report that targeted gene disruption of NDST-1 in the mouse results in a structural alteration of heparan sulfate. Biochemical analysis of heparan sulfate purified from fibroblast cultures, lung, and liver of NDST-1-deficient embryos demonstrated a dramatic reduction in N-sulfate content. Most NDST-1-deficient embryos survive until birth; however, they turn out to be cyanotic and die neonatally in a condition resembling respiratory distress syndrome. In addition, a minor proportion of NDST-1-deficient embryos die during the embryonic period. The cause of the embryonic lethality is still obscure, but incompletely penetrant defects of the skull and the eyes have been observed.

Heparan sulfate (HS)† is a sulfated polysaccharide that is present covalently attached to different core proteins in HS proteoglycans. HS proteoglycans are found in the extracellular matrix, mainly in basement membranes (BMs) (e.g. perlecan, collagen XVIII, and agrin), whereas syndecans and glypicans are HS proteoglycans present on the surface of most cells (1–4). The HS chains interact with a large number of different molecules primarily in the extracellular space (e.g. growth factors, extracellular matrix proteins, and enzymes). Some of these molecules recognize a specific pattern of sulfate groups, which are added to the HS chain during biosynthesis (5). A spatially and temporally regulated biosynthesis will therefore indirectly determine the localization of other molecules, such as growth factors/cytokines, enzymes, enzyme inhibitors, and extracellular matrix components (6, 7).

The biosynthesis of HS is a complex process, involving polymerization and concomitant modification, including sulfation, of the chain (5–7). The product of the polymerization reactions is a polysaccharide consisting of repeating D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) residues. The first modification step is N-deacetylation and N-sulfation of selected GlcNAc units, carried out by the bifunctional enzyme N-deacetylase/N-sulfotransferase (NDST). Because all further modifications occur in the vicinity of N-sulfated GlcN units (5), NDST has a key role in determining the final structure of the polysaccharide chain. Four related NDSTs, NDST-1–NDST-4, have been identified (8–12). Both NDST-1 and NDST-2 transcripts are widespread in different organs and cells (13, 14), whereas the distribution of NDST-3 and NDST-4 appears to be more restricted (11, 12).

Studies on mutations in Drosophila genes affecting HS biosynthesis have demonstrated that HS proteoglycans play a critical role in developmental processes (15). Identification of Drosophila strains carrying mutant alleles for sugarless (16–18), sulfateless (19), or tout-velu (20) (the Drosophila homologues of mammalian UDP glucose dehydrogenase (necessary for UDP-GlcUA production), NDST, and EXT-1 (HS polymerase), respectively) have revealed that HS is crucial for embryonic development. Furthermore, it has been demonstrated in these mutants that HS interacts with the different signaling pathways Wingless (Wnts in vertebrates), Decapentaplegic (transforming growth factor β family), Hedgehog, and Branchless (fibroblast growth factor) (16–21). Some of these interactions have been ascribed to the Drosophila homologue of glypicans (21, 22). When HS recently was analyzed in sulfateless, sugarless, and tout-velu mutants, a marked alteration in the biosynthesis of the polysaccharide was evident (23, 24).

The disruption of the NDST-2 gene in mouse was recently reported by our group (25) and by Humphries et al. (26). The lack of NDST-2 does not affect normal life span and fertility, but NDST-2-deficient mice display a severe mast cell defect, caused by a complete lack of sulfated heparin in the secretory granules. However, analysis of HS from liver (25) and kidney2 caused by a complete lack of sulfated heparin in the secretory granules. However, analysis of HS from liver (25) and kidney2 of NDST-2-deficient mice did not reveal any structural alterations in the polysaccharide. The lack of HS defects in NDST-2-deficient mice indicates that other NDSTs are more important in HS biosynthesis or that these isoforms are able to...
compensate for the lack of NDST-2. In the present paper, we describe the generation of NDST-1-deficient mice. It is demonstrated that NDST-1, in contrast, plays a critical role in HS biosynthesis in most parts of the body. Furthermore, it is shown that a lack of normally sulfated HS leads to a fully penetrant abnormality of the lungs. The lung phenotype of NDST-1-deficient mice was recently described by Fan et al. (27). In addition, we have observed incomplete penetrance of defects of the skull and the eyes and that a minor proportion of the NDST-1-deficient embryos die during the embryonic period.

**EXPERIMENTAL PROCEDURES**

**Construction of the Targeting Vector**—A genomic clone containing the 5′ end of the NDST-1 gene was isolated from a bacteriophage mouse (strain 129SvJ) genomic library (Stratagene). A 7-kb SacI/KpnI fragment located 3′ of exon 1 was subcloned into a bluestrip plasmid (BSIKS+, Stratagene) to serve as a long arm of homology to the NDST-1 locus. Subsequently, a PGK-neo cassette was inserted into a SalI/XhoI site 5′ of the SacI/KpnI fragment in the opposite direction relative to the transcription of the NDST-1 gene. Finally, a 3.1-kb BamHI fragment located 5′ of the coding region in exon 1 was ligated upstream of the PGK-neo cassette to create a second arm of homology to the endogenous gene. Following homologous recombination, a deletion of the first 513 nucleotides of the coding region of the NDST-1 gene is created.

**Homologous Recombination in ES Cells and Generation of NDST-1-deficient Mice**—The targeting vector was linearized at a NotI site and electroporated into R1 embryonic stem (ES) cells (28). G418 (350 μg/ml; Life Technologies, Inc.) was used to select for cells that had taken up the targeting vector. Clones that had undergone homologous recombination (3 of 360 clones) were identified by Southern blot analysis using an external 0.4-kb SacI/XbaI fragment as probe (Fig. 1A). These were injected into C57BL/6 blastocysts, and chimeric male founder mice from two of the clones were shown to transmit the mutation to the offspring when crossed with C57BL/6 females. Tail biopsies were genotyped as described (29), and heterozygous mice were intercrossed to produce NDST-1-deficient mice. Phenotypic studies were performed on mice with mixed genetic background (129/SvJ/Sv and C57BL/6).

**Northern Blot Analysis**—Total RNA from lung and liver was isolated using the RNeasy kit (Qiagen). Northern blot analysis was carried out as described previously (9) using a cDNA probe spanning nucleotides 1–435 of the mouse NDST-1 coding region.

**Immunofluorescence**—Embryos were embedded in Tissue-Tek (Miles Inc.) and snap-frozen in liquid nitrogen. Six-μm sections were cut using a cryostat. Tissue sections were stained with the antibodies HepSs-1 (Seikagaku) and RB4CB9 (30), which recognize epitopes in HS, and antibodies against surfactant proteins (SPs) A and B. For RB4CB9, stainings were performed as described previously for other HS-recognizing phage display antibodies (31). For HepSs-1, tissue sections were rehydrated for 5 min in 0.05% (v/v) Tween-20 in phosphate-buffered saline (Tweens phosphate-buffered saline), blocked with 0.1% (v/v) bovine serum albumin in Tween/phosphate-buffered saline for 30 min and incubated with HepSs-1 primary antibody diluted 1:200 in blocking buffer for 90 min at room temperature. The HepSs-1 primary antibody was detected by incubation with fluorescein isothiocyanate-conjugated goat-anti-mouse IgM (Jackson ImmunoResearch) diluted 1:100 in blocking buffer. After each incubation, sections were washed three times for 10 min each in Tween/phosphate-buffered saline. For SP-A and SP-B, stainings were performed as described previously (32).

**Isolation of [3H]Glucosamine-labeled HS**—Fibroblasts were prepared from minced and trypsin treated E14.5 embryos, essentially as described previously (33), and stored at −70 °C. After thawing, the cells were cultured in Dulbecco's modified Eagle's medium/Nutrient Mix (Life Technologies, Inc.) in the presence of 10% fetal calf serum and passaged once before use. Subconfluent fibroblast cultures were preincubated 1–2 days in low glucose medium (Dulbecco's modified Eagle's medium with the addition of 5% fetal calf serum) and then metabolically labeled for 25 h with 250 μCi of [3H]glucosamine in 5 ml of the same medium. The medium fractions were applied to DEAE-Sephadex columns (Amersham Pharmacia Biotech) equilibrated in 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1% Triton X-100. [3H]Glycosaminoglycans were recovered and digested with papain as described previously (34). After desalting in water on PD10 columns (Amersham Pharmacia Biotech) followed by lyophilization, the [3H]glycosaminoglycans were subjected to digestion with 0.1 units of chondroitinase ABC (Seikagaku) as described previously (34). The resultant [3H]-labeled HS chains were recovered in the void volume after gel filtration on a column (100 × 0.5 cm) of Sephadex G-50 (superfine grade, Amersham Pharmacia Biotech) in 0.2 M NH4HCO3.

**Analytical Ion Exchange Chromatography**—Isolated [3H]HS (8000 cpm) was applied to a MonoQ ion exchange column (Amersham Pharmacia Biotech) eluted in 50 mM Tris-HCl buffer, pH 8.0, with a NaCl gradient ranging from 0.3 to 1.5 M. Fractions of 0.5 ml were collected and assayed for [3H] radioactivity. [3H]-Labeled capsular polysaccharide from E. coli K5 (a gift from U. Lindahl, Uppsala University), with the same (GluNAc1–6GlcNAc1–4) structure as the initial polymerization product in HS biosynthesis, was used as a standard.

**HS Structural Analysis**—Isolated [3H]HS preparations from fibroblast cultures were cleaved at N-sulfated GlcN residues by desamination at pH 1.5 (35). The resultant [3H]-labeled oligosaccharides were separated by gel chromatography on a column (1 × 140 cm) of Bio-Gel P-10 (fine grade, Bio-Rad).

**Histological Analysis**—Embryos were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Hematoxylin and eosin stainings followed standard protocols.

**RESULTS**

NDST-1-deficient mice were generated by gene targeting in ES cells. A targeting vector designed to create a functional mutation was constructed (Fig. 1A). Following homologous recombination, 2 kb of the gene, including the first 171 amino acids comprising both the cytoplasmic and trans-Golgi membrane domains, are deleted. The vector was electroporated into R1 ES cells (28), and clones that had undergone homologous recombination were identified by Southern blot analysis (Fig. 1B) using the external probe indicated in Fig. 1A. Three of 360 analyzed clones contained the targeted allele and were micro-injected into C57BL/6 blastocysts, giving rise to highly chimeric animals. Germ line transmission of the mutation was achieved from two of the clones. No apparent defects were observed in heterozygous animals. Genotype analysis of embryos from heterozygous crossings at E18.5 showed a ratio of 33% wild type, 50% heterozygous, and 17% homozygous embryos (n = 305, p = 0.05), indicating that a minor proportion of the NDST-1-deficient embryos die during the prenatal period, whereas most NDST-1-deficient individuals survive the embryonic period. To confirm a correct targeting event of the wild type allele, Northern blot analysis of E18.5 embryos was performed (Fig. 1C). The expected 8-kb transcript was present in wild type animals, but it was absent in homozygous individuals.

**Structural Alteration of HS**—In order to analyze the role of NDST-1 in HS biosynthesis, sections of embryos (E18.5) were
stained with two different antibodies, HepSS-1 and RB4CB9, that recognize HS. Both antibodies specifically bind to HS; the RB4CB9 antibody was recently characterized and is dependent on N-sulfated GlcN-residues for epitope recognition (30), as is the HepSS-1 antibody. Both RB4CB9 and HepSS-1 antibodies systemically stained most BMs in wild type animals. For example, HS recognized by the RB4CB9 antibody was present in BMs of lung, liver, and kidney from wild type animals (Fig. 2, A, E, I, and J). With the exception of some blood vessels, this antibody showed no positive staining in mutant lung and liver (Fig. 2, B and F). In wild type kidney, the antibody strongly recognized HS in Bowman’s capsule BM and HS in the BMs of tubuli or collecting ducts, whereas the staining of the glomerular BM was more diffuse. In mutant kidney, the staining was lost in the BMs of Bowman’s capsule and glomerulus, but a weak staining that recognized HS in Bowman’s capsule BM and HS in the BMs of tubuli or collecting ducts was still present in mutant lung and liver (Fig. 2, A, E, I, and J). With the exception of some blood vessels, this antibody showed no positive staining in mutant lung and liver (Fig. 2, B and F).

The antibodies used were RB4CB9 (A, B, E, F, I, and J) and HepSS-1 (C, D, G, H, K, and L). For example, HS recognized by the RB4CB9 antibody was present in BMs of lung, liver, and kidney from wild type animals (Fig. 2, A, E, I, and J). With the exception of some blood vessels, this antibody showed no positive staining in mutant lung and liver (Fig. 2, B and F). In wild type kidney, the antibody strongly recognized HS in Bowman’s capsule BM and HS in the BMs of tubuli or collecting ducts, whereas the staining of the glomerular BM was more diffuse. In mutant kidney, the staining was lost in the BMs of Bowman’s capsule and glomerulus, but a weak staining that was present in wild type was still present in BMs of tubuli or collecting ducts (Fig. 2). HepSS-1 showed the same staining pattern as RB4CB9 in the wild type tissues (Fig. 2, C, G, and K) and left no or only very weak traces of staining in a few BMs (Fig. 2, D, H, and L). Structural analysis of [3H]glucosamine-labeled HS from cultured embryonic fibroblasts (E14.5) further supported these observations. A clearly reduced charge density of HS in cultured fibroblasts from NDST-1–/– embryos compared with wild type fibroblasts was evident, indicating a lowered sulfation of HS from mutant mice (Fig. 3A). Similar results were obtained with [3H]glucosamine-labeled HS isolated from embryonic liver and lung (E18.5; data not shown). To study the degree of N-sulfation, the [3H]-labeled HS preparations from fibroblasts were treated with nitrous acid at pH 1.5, followed by gel chromatography on Bio-Gel P-10 (Fig. 3B). During nitrous acid treatment, the polysaccharide chain is cleaved at N-sulfated glucosamine units; the extent of depolymerization thus correlates with the degree of N-sulfation. Calculations based on peak areas indicated that the degree of N-sulfation was lowered from >40% in HS from NDST-1+/– fibroblasts to <15% in NDST-1–/– HS (Fig. 3B). HS from NDST-1–/– fibroblasts was indistinguishable from wild type HS (data not shown).

**DISCUSSION**

We have by homologous recombination in ES cells created mice deficient in NDST-1. Structural analysis of HS from cultured fibroblasts from NDST-1–/– and NDST-1–/– embryos demonstrated that the N-sulfation of HS in these cells was greatly reduced (Fig. 3). Similar results were obtained when metabolically [3H]glucosamine-labeled HS from embryonic (E18.5) liver and lung was studied (data not shown). Immunohistochemical staining of E18.5 lung sections for SP-A and SP-B suggests an altered homeostasis of both surfactant proteins in NDST-1–/– embryos (Fig. 4, C and D; shown only for SP-A); although less secreted surfactant appeared to be present in lungs of NDST-1–/– embryos, an increased number of the cells producing these proteins could be noted. Because we had indications that a minor proportion of NDST-1-deficient embryos die during the embryonic period, pups were genotyped at E9.5–E14.5. At these stages, the expected Mendelian ratio was found: 20% wild type, 55% heterozygous, 25% mutant embryos (Fig. 4A). With the exception of some blood vessels, this antibody showed no positive staining in mutant lung and liver (Fig. 2, B and F). In wild type kidney, the antibody strongly recognized HS in Bowman’s capsule BM and HS in the BMs of tubuli or collecting ducts, whereas the staining of the glomerular BM was more diffuse. In mutant kidney, the staining was lost in the BMs of Bowman’s capsule and glomerulus, but a weak staining that was present in wild type was still present in BMs of tubuli or collecting ducts (Fig. 2J). HepSS-1 showed the same staining pattern as RB4CB9 in the wild type tissues (Fig. 2, C, G, and K) and left no or only very weak traces of staining in a few BMs (Fig. 2, D, H, and L). Structural analysis of [3H]glucosamine-labeled HS from cultured embryonic fibroblasts (E14.5) further supported these observations. A clearly reduced charge density of HS in cultured fibroblasts from NDST-1–/– embryos compared with wild type fibroblasts was evident, indicating a lowered sulfation of HS from mutant mice (Fig. 3A). Similar results were obtained with [3H]glucosamine-labeled HS isolated from embryonic liver and lung (E18.5; data not shown). To study the degree of N-sulfation, the [3H]-labeled HS preparations from fibroblasts were treated with nitrous acid at pH 1.5, followed by gel chromatography on Bio-Gel P-10 (Fig. 3B). During nitrous acid treatment, the polysaccharide chain is cleaved at N-sulfated glucosamine units; the extent of depolymerization thus correlates with the degree of N-sulfation. Calculations based on peak areas indicated that the degree of N-sulfation was lowered from >40% in HS from NDST-1+/– fibroblasts to <15% in NDST-1–/– HS (Fig. 3B). HS from NDST-1–/– fibroblasts was indistinguishable from wild type HS (data not shown).

**Neonatal and Embryonic Lethality of NDST-1-deficient Mice**—A major proportion of the HS-deficient embryos completed, as mentioned, development until E18.5. When litters from heterozygous crossings were genotyped at P21, however, no homozygous mice could be identified, indicating perinatal (or early postnatal) lethality. When observed at birth, pups that later were shown to be NDST-1-deficient attempted to breathe but remained cyanotic and died neonatally. Lungs from these newborn NDST-1–/– animals were severely atelectatic (Fig. 4B), whereas lungs from surviving pups, which always were wild type (Fig. 4A) or heterozygous (not shown) for the mutation, were inflated. Immunohistochemical staining of E18.5 lung sections for SP-A and SP-B suggests an altered homeostasis of both surfactant proteins in NDST-1–/– embryos (Fig. 4, C and D; shown only for SP-A); although less secreted surfactant appeared to be present in lungs of NDST-1–/– embryos, an increased number of the cells producing these proteins could be noted. Because we had indications that a minor proportion of NDST-1-deficient embryos die during the embryonic period, pups were genotyped at E9.5–E14.5. At these stages, the expected Mendelian ratio was found: 20% wild type, 55% heterozygous, 25% mutant embryos (n = 148, p = 0.05) indicating lethality between E14.5 and E18.5.
stainings using monoclonal antibodies against HS illustrated that a structural alteration of HS occurs in most BMs of the embryo (Fig. 2). In contrast, indistinguishable immunostaining patterns in wild type and mutant embryos were obtained for the BM-components nidogen-1, laminin γ1-chain, and perlecan (not shown), indicating no severe structural alteration in BMs. It is interesting to note that whereas the HepSS-1 staining is uniformly absent or very weak in BMs of mutants in lung, liver and kidney, the RB4CB9 staining is still left in some blood vessels and in some kidney structures. These results demonstrate that the two antibodies recognize different HS epitopes, even if N-sulfation is required for both. More importantly, the different stainings indicate a selective alteration of HS structure in the mutant animals. In addition to HepSS-1 and RB4CB9, a panel of HS recognizing phage display antibodies3 were also tested (not shown). The staining patterns observed with these antibodies confirmed that some structural epitopes had been lost in the mutant, whereas some still remained, indicating that different isoforms of NDST might be expressed in a fine-tuned manner in different cell populations.

The rapid death after birth of the NDST-1+/− pups and the observation that newborn homozygous pups gasp for air and turn cyanotic indicate lung failure as the primary cause of the neonatal lethality. Examination of histological sections from both neonates and E18.5 embryos further supported this idea. Atelectasia seen in lungs from NDST-1+/− pups indicated that their lungs had never been properly inflated (Fig. 4B). Even though breathing reflexes are present, the possibility cannot be fully excluded that the respiratory failure may be due to disturbances in the brain stem region. The compact structure of the lungs from NDST-1-deficient embryos indicates that they contain an elevated number of cells. Immunostainings using antibodies against SP-A (Fig. 4, C and D) and SP-B (data not shown) indeed support this notion. Interestingly, overproliferation of type II epithelial cells has been observed in vitro as a consequence of undersulfated HS (36). Furthermore, differentiation of these cells seems to be delayed because extracellular deposits of surfactant are less frequently observed in NDST-1-deficient embryos compared with their wild type littersmates. The condition of the NDST-1-deficient newborns therefore resembles the respiratory distress syndrome seen in premature infants (37). This observation is in accordance with a recent report describing NDST-1 mutant mice (27). However, we found, in addition, that a minor proportion of our HS-defective mice died during the embryonic period. Discrepancies might be due to differences in the genetic background of the embryos. Alternatively, the sulfation degree of HS, not investigated by Fan et al. (27), might differ in the two strains.

The phenotype of NDST-1-deficient mice differs from that of mice lacking the HS 2-O-sulfotransferase gene (38), although a not fully penetrant eye defect may be similar between the strains. The 2-O-sulfotransferase-deficient mice are reported to die at a later time after birth than the NDST-1-deficient mice, due to bilateral renal agenesis and they also show skeletal abnormalities (38). Mice lacking the core protein of the HS bearing proteoglycan perlecan display stress-sensitive BMs and develop exencephali and chondrodysplasia (39, 40). Mice devoid of the cell surface HS proteoglycan glypic-an-3 show various phenotypes related to overgrowth (41), which in some aspects resemble the human overgrowth condition Simpson-Behmel-Golabi syndrome, in which glypic-an-3 mutations have been found (42). Further demonstrations of the function of HS come from analysis of the autosomal dominant disorder hereditary multiple exostoses, caused by mutations in HS-polymersases (43). The condition of hereditary multiple exostoses is the most common cause of benign skeletal tumors in humans. In light of the many suggested functions ascribed to HS, it is surprising that NDST-1-deficient embryos carrying structurally altered HS in most parts of the body are able to complete the embryonic period with only minor histological defects. Genotype analysis of embryos at stage E18.5 showed, as mentioned, that a minor population of the NDST-1-deficient embryos die at an earlier stage during development. The cause of the embryonic lethality is still unclear, but we have observed not fully penetrant phenotypes of the skull and the eyes. As the number of individuals displaying morphological skull defects found among NDST-1-deficient embryos at each developmental stage is low, 4 of 21 at E14.5 and 7 of 52 at E18.5, we cannot, at this point, judge whether this is a reason for the lethality between these two stages.

Although NDST-1 obviously is important for HS biosynthesis, NDST-2 appears to be involved primarily in heparin production (25, 26). However, lack of both NDST-1 and NDST-2 in the mouse causes earlier developmental lethality,4 indicating that NDST-2 might also be involved in HS biosynthesis and that NDST-2 may to a certain extent compensate for the loss of NDST-1 in NDST-1−/− mice. Further analysis of mice deficient in NDST-1 or NDST-2 and analysis of mice lacking both NDST-1 and NDST-2 will give more insights into the role of these two enzymes in HS biosynthesis.

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