Mice Deficient in Heparan Sulfate 6-O-Sulfotransferase-1 Exhibit Defective Heparan Sulfate Biosynthesis, Abnormal Placentation, and Late Embryonic Lethality*

Received for publication, August 4, 2006, and in revised form, March 27, 2007 Published, JBC Papers in Press, April 3, 2007, DOI 10.1074/jbc.M607434200

Hiroko Habuchi †, Naoko Nagai ‡, Noriko Sugaya ‡, Fukiko Atsumi ‡, Richard L. Stevens §, and Koji Kimata ¶

From the † Institute for Molecular Science of Medicine and ‡ Laboratory Animal Research Center, Aichi Medical University, Nagakute, Aichi 480-1195, Japan and the § Department of Medicine, Harvard Medical School and Brigham and Women’s Hospital, Boston, Massachusetts 02115

Heparan sulfate (HS) plays critical roles in a variety of developmental, physiological, and pathogenic processes due to its ability to interact in a structure-dependent manner with numerous growth factors that participate in cellular signaling. The divergent structures of HS glycosaminoglycans are the result of the coordinate actions of several N- and O-sulfotransferases, C5-epimerase, and 6-O-endosulfatases. We have shown that 6-O-sulfation of the glucosamine residues in HS are catalyzed by the sulfotransferases HS6ST-1, -2, and -3. To determine the biological and physiological importance of HS6ST-1, we now describe the creation of transgenic mice that lack this sulfotransferase. Most of our HS6ST-1-null mice died between embryonic day 15.5 and the perinatal stage, and those mice that survived were considerably smaller than their wild-type littermates. Some of these HS6ST-1-null mice exhibited development abnormalities, and biochemical and molecular analyses of these mice revealed an ~50% reduction in the number of fetal microvessels in the labyrinthine zone of the placenta relative to that in the wild-type mice. Because we observed a modest reduction in VEGF-A mRNA and protein in the tissues of HS6ST-1-null mice, an HS-dependent defect in cytokine signaling probably contributes to increased embryonic lethality and decreased growth. Biochemical studies of the HS chains isolated from various organs of our HS6ST-1-null mice revealed a marked reduction of GlcNAc(6SO₄) and HexA-GlcNSO₃(6SO₄) levels and a reduced ability to bind Wnt2. Thus, despite the presence of three closely related 6-O-sulfotransferase genes in the mouse genome, HS6ST-1 is the primary one used in HS biosynthesis in most tissues.

† This work was supported by a Japan Society for the Promotion of Science grant-in-aid for the promotion of science, by Grants-in-aid for Scientific Research on Priority Areas 14082206 and 17570099 from the Ministry of Education, Culture, Sport, Science, and Technology of Japan, by National Institutes of Health Grant HL036110 (to R. L. S.), and by a special research fund from Seikagaku Corp. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan. Tel.: 81-52-264-4811; Fax: 81-561-63-3532; E-mail: kimata@aichi-med-u.ac.jp.

§ The abbreviations used are: HS, heparan sulfate; NDST, N-deacetylase/N-sulfotransferase; GlcNSO₃, N-sulfoglucosamine; FGF, fetal growth factor; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; ES, embryonic stem; MLI, mean linear intercept; HPLC, high pressure liquid chromatography; En, embryonic day n.

‡‡ The abbreviations used are: HS, heparan sulfate; NDST, N-deacetylase/N-sulfotransferase; GlcNSO₃, N-sulfoglucosamine; FGF, fetal growth factor; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; ES, embryonic stem; MLI, mean linear intercept; HPLC, high pressure liquid chromatography; En, embryonic day n.
can complexes in their secretory granules (19–23). Thus, NDST-2-null mice have defects in some mast cell-dependent reactions. HS2ST-deficient mice exhibit bilateral renal agenesis (24), and homozygous knock-out of Hspsi leads to lethality, also associated with renal agenesis (25). HSST-1 knock-out mice are seemingly healthy (26). Thus, the inactivation of the genes involved in HS modification reactions leads to distinct phenotypes, suggesting that the specific structure of HS regulates the activity of growth factors and morphogens distinctly in vivo.

Only one Hs6st (HS 6-O-sulfotranserase) gene has been identified in Drosophila and Caenorhabditis elegans, whereas at least two Hs6st genes are present in zebrafish. It has been concluded that Hs6st participates in tracheal development in Drosophila by regulating fibroblast growth factor (FGF)-dependent signaling pathways (27). A Hs6st in zebrafish also appears to control Wnt-dependent signaling pathways (28). In C. elegans, mutation of its Hs6st gene leads to defects in axonal and cellular guidance of the worm’s neurons (29). Thus, the defects of Hs6st appear to vary in a species-specific manner.

We demonstrated previously that in mammals, heparan sulfate 6-O-sulfotransferases exist in three isoforms (Hs6st-1, -2, and -3) and one alternatively spliced form (Hs6st-2S) (30, 31). Although the substrate specificities of these isoforms largely overlap, the individual isoforms exhibit a characteristic preference for the uronic acid residue neighboring the O-sulfate content. We also show that Hs6st-null mice exhibit increased neonatal lethality and decreased growth.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparitinase I (Flavobacterium heparinum; EC 4.2.2.8), heparitinase II (F. heparinum; no number assigned), heparinase (F. heparinum; EC 4.2.2.7), and the unsaturated disaccharides kit for HS were purchased from Seikagaku Corp. Anti-CD31 (PECAM-1) monoclonal antibody was from BD Biosciences, anti-VEGF antibody was from Upstate and Calbiochem, and/or PCR. Genomic DNA from the ES cell clones or mouse tails was isolated using a DNA purification kit (Dneasy tissue kit; Qiagen), digested with SpHl, and hybridized to 5′ external probes (shown in Fig. 1A). The PCR amplification was performed by using the following three pairs of primers: forward primer p1 (5′-GCTCCTCCCAAACCCAGGACAAATG-3′) and reverse primer p2 (5′-GCCAGGGGTGTTGGGGCTTGG-3′) for the analysis of the ES cell clones; forward primer (p1, as mentioned above) and reverse primer p3 (5′-TGCACGAGGTGTTGGGGCTTGG-3′) for the analysis of the wild-type offspring; and forward primer p4 (5′-ATGAACTGCAGGACATTCG-3′) and reverse primer p5 5′-CCACCGCTATGTCCTGATTGGC-3′ for the analysis of the mutant mice.

**Histological Analysis, Immunohistochemistry, and Staining of Various Tissues**—Tissues except for lung were fixed with 10% formaldehyde and embedded in paraffin according to standard procedures.
Roles of HS 6-O-Sulfation in Microvessel Formation

histological procedures. Six-micrometer sections were stained with hematoxylin/eosin. For the immunohistochemical staining, the tissues were either fixed with 4% paraformaldehyde at 4 °C for 4 h and then embedded in an OCT compound or fixed with IHC zinc fixative (BD Biosciences Pharmingen), according to the manufacturer’s recommendation and then embedded in paraffin. Placental tissues were stained with anti-CD31 (PECAM-1) monoclonal antibody, anti-VEGF antibody, anti-HIF-1α monoclonal antibody, and anti-actin antibody and processed as usual for immunohistochemistry. Semiquantitative analysis by immunohistochemistry was performed by counting numbers of stained microvessels or cells in a certain area as indicated in the figure legends. The mean values ± S.D. were obtained from the three independent experiments using different sections that were performed in triplicate. Lungs were also perfused with 4% paraformaldehyde via trachea under a constant pressure of 20 cm H2O, and then tracheas were ligated, perfused with 4% paraformaldehyde overnight. Lungs were embedded in paraffin. Morphometric analysis of lungs was done as follows. The intra-alveolar distance was measured as the mean linear intercept (MLI), which was determined by dividing the total length of 36 lines drawn across the lung sections by the number of intercepts encountered as determined by the investigator (34, 35). Skeletons of newborn mice were prepared and stained as described by Peters (36). Images were captured using an Olympus BX50 microscope. Confocal images were captured using a Zeiss LSM5 Pascal confocal microscope.

Expression levels of Wnt2, VEGF-A, HS6STs, and HS2ST mRNA—The placentas were dissected in cold phosphate-buffered saline from the wild-type, heterozygous, and homozygous embryos of the intercross between heterozygous mice. The maternal deciduas were removed as much as possible in order to exclude the maternal contributions under a stereoscopic microscope. Therefore, the placental samples used for analyses should be almost free from maternally derived tissues. The animals’ livers also were dissected in cold phosphate-buffered saline. Total RNA from the placentas of E15.5 embryos and the livers of 10-week-old mice were isolated using an RNeasy kit (Qiagen). A reverse transcription reaction was performed using a high capacity cDNA archive kit (ABI) and 1.0 μg of total RNA as template. PCR was performed using primers and commercial probes specific for Wnt2 (Mm00470018), VEGF-A (Mm00437304), HS6ST-1 (Mm01229698), HS6ST-2 (Mm00479296), HS6ST-3 (Mm00479297), and HS2ST (Mm00478684) from TaqMan gene expression assays (Applied Biosystems, Foster, CA). Normalization of expressions was performed using a primer pair specific for rodent glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems, Foster, CA). The PCR products were analyzed in real time using the ABI Prism 7700 system. Three independent experiments were performed in triplicate to obtain the values.

Expression Levels of VEGF-A Protein and HIF-1α in Placenta—Placental tissues from E15.5 embryos were also isolated by removing most of the maternal decidua and washed twice with cold phosphate-buffered saline and then homogenized with a Polytron homogenizer in extract buffer containing 10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 1.5 mM EDTA, 1 mM Na2PO4, 23 mM NaF, 1 mM Na3VO4, and one tablet/10 ml of protease inhibitor mixture tablets (Complete Mini; Roche Applied Science). The homogenates were stirred gently at 4 °C for 1 h and clarified by centrifugation at 10,000 × g for 30 min. Thirty micrograms of protein was subjected to 10% SDS-PAGE and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). Western blots were probed with the antibodies against VEGF-A, HIF-1α, and actin, and the bands were detected using a horseradish peroxidase-conjugated secondary antibody and Western Blotting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) according to the manufacturer’s instructions. The band density of VEGF-A and HIF-1α were measured using Image Gauge software and normalized against the actin bands. Three independent experiments were performed in triplicate to obtain the values.

Preparation and Structural Analysis of HS—Various tissues from newborn mice and E15.5 placentas were defatted with acetone and dried. The maternal deciduas were removed from the placentas as much as possible. Each tissue was suspended in 10 volumes of 0.2 M NaOH per wet tissue weight, incubated for 16 h at room temperature, neutralized with 4 M acetic acid, and then incubated with DNase I, RNase A, and 10 mM MgCl2 at 37 °C for 2 h. Proteinase (½ 500 of tissue wet weight, actinase E; Kaken Seiyaku) was added, and incubation was continued at 37 °C for 16 h. The reaction was stopped by heating at 100 °C for 5 min, and samples were centrifuged at 13,000 rpm for 10 min to remove insoluble material. The supernatants were diluted with an equal volume of 20 mM Tris-HCl buffer (pH 7.2) and loaded onto a DEAE-Sephalac column equilibrated with 20 mM Tris-HCl buffer. The columns were washed with 10 column volumes of 20 mM Tris-HCl buffer containing 0.2 M NaCl and then eluted with 4 column volumes of 2 M NaCl in Tris-HCl buffer. The eluates were precipitated with 2.5 volumes of cold 95% ethanol containing 1.3% potassium acetate, and the glycosaminoglycans were recovered by centrifugation. Using an aliquot of the glycosaminoglycans, the disaccharide compositions of HS were analyzed as described previously (32), according to the method of Toyoda et al. (37), with a slight modification of the elution conditions. The HSs from spleen were subjected to nitrous acid degradation at pH 1.5. The products degraded with nitrous acid were first labeled with [3H]sodium borohydride and then subjected to gel filtration, and the labeled O-sulfated disaccharide fractions were then subjected to HPLC on a column of Partisil-10 SAX. The nitrous acid degradation and the gel filtration and subsequent HPLC were performed as described previously (32).

Surface Plasmon Resonance Analysis—The interaction of growth factors with HS was achieved using a BIACore 2000 SPR biosensor. A streptavidin-conjugated sensor chip SA was used to immobilize HS prepared from wild-type and HS6ST-1-deficient embryonic placentas. Ten micrograms of HS was incubated with 6.7 μg of NHS-LS-Biotin (Pierce) in 120 μl of 50 mM sodium bicarbonate buffer (pH 8.5) for 30 min at room temperature. An excess of NHS-LS-Biotin was reacted with 0.1 mM ethanolamine for 30 min at room temperature. The biotinylated
Roles of HS 6-O-Sulfation in Microvessel Formation

TABLE 1
Lethality in the HS6ST-1+/− mice from E15.5 to the perinatal stage

| Age     | Number of mice | HS6ST-1 genotype |
|---------|----------------|------------------|
| P1      | 133            | +/+              |
| E17.5–E18.5 | 27          | +/−              |
| E15.5   | 23             | −/−              |
| E14.5   | 18             | −/−              |
| E12.5   | 23             | −/−              |

* Resorbed embryo was homozygous. Genotypes of the offspring of the intercrosses between C57BL/6 and C3H/He strain were determined.

RESULTS

Targeted Disruption of the HS6ST-1 Gene—The construction of the targeting vector and the strategy for screening mice for homologous recombination are shown in Fig. 1A. In the recombinant allele, two 3’-phosphoadenosine 5’-phosphosulfate binding sites were deleted. We generated chimeric mice from an ES clone that had undergone homologous recombination at the HS6ST-1 locus and that were confirmed to contain no additional sites of vector integration. The chimeric mice were subsequently crossed with C57BL/6 mice to facilitate the germ line transmission. We confirmed the complete destruction of the HS6ST-1 gene by Southern and Northern blot analyses. We first analyzed an SphI digest of the genomic DNA from wild-type mice and mutant mice using a 5’-external probe. Digests from wild-type mice contained a single fragment of 3.8 kb that hybridized to the probe, whereas digests from the homozygous mutant mice contained a hybridizing fragment of 2.7 kb, and digests from the heterozygous mutant mice contained two fragments corresponding in size to the fragments detected individually in the digests from the wild-type and homozygous mutant mice (Fig. 1B). The HS6ST-1 transcript was not detected in the homozygous mutant mice (Fig. 1C). In contrast, levels of the HS6ST-2 and HS2ST transcripts in the homozygous mice were similar to those observed in wild-type mice. These results indicate that the expression of the HS6ST-1 gene was abolished in the homozygous mutant mice.

Lethality of HS6ST-1 Deficient Mice at a Later Embryonic Stage—The genotype analysis of the progeny derived from intercrosses of heterozygous mice (C57BL/6 strain, 3F–4F) is shown in Table 1; this indicates that most of the HS6ST-1 null mice died between E15.5 and the perinatal stage. The genotypes of E12.5 and E14.5 agreed well with the Mendelian rule, indicating that HS6ST-1 null mice at the early embryonic stage appeared to be fully viable. By E15.5, however, one of the five homozygotes had died. From E17.5 to E18.5, the number of homozygous embryos was ~30% of that expected from the Mendelian rule. The accumulated data suggest that HS6ST1 is more important in the late stages of embryonic development in C57BL/6 mice than the early stages. On the 21st postnatal day, less than 4% of the offspring from the heterozygotes were homozygous mutants; these attained adulthood, albeit at a rather small size, and were fertile. In the C3H/He strain, ~7% of the offspring of the heterozygote (4F) intercrosses were homozygotes at the same stage. However, in the BALB/c strain, no HS6ST-1-deficient mice were found at birth in the offspring of the heterozygotes (3F–4F) intercrosses. The survival rate appeared to depend on the genetic backgrounds, although the homozygous mice have not yet had a pure genetic background. One of factors affecting the survival rate might be the number of littermates; this is based on the observation that the number of littermates usually decreases in the order of BALB/c > C57BL/6 > C3H/He strain. In the case of HS3ST-1 mice, the

glycosaminoglycans were precipitated once with 3 volumes of cold 95% (v/v) ethanol containing 1.3% potassium acetate and 1 mM EDTA, and then the precipitates were washed twice with 3 volumes of cold 95% (v/v) ethanol containing 1.3% potassium acetate and 1 mM EDTA. Glycosaminoglycans were immobilized on the sensor tips to produce ~500 resonance units. All measurements were carried out at room temperature using various concentrations of growth factors. Sensograms were evaluated using BIACore evaluation software (version 2) according to the manufacturer’s instructions.

RESULTS

Targeted Disruption of the HS6ST-1 Gene—The construction of the targeting vector and the strategy for screening mice for homologous recombination are shown in Fig. 1A. In the recombinant allele, two 3’-phosphoadenosine 5’-phosphosulfate binding sites were deleted. We generated chimeric mice from an ES clone that had undergone homologous recombination at the HS6ST-1 locus and that were confirmed to contain no additional sites of vector integration. The chimeric mice were subsequently crossed with C57BL/6 mice to facilitate the germ line transmission. We confirmed the complete destruction of the HS6ST-1 gene by Southern and Northern blot analyses. We first analyzed an SphI digest of the genomic DNA from wild-type mice and mutant mice using a 5’-external probe. Digests from wild-type mice contained a single fragment of 3.8 kb that hybridized to the probe, whereas digests from the homozygous mutant mice contained a hybridizing fragment of 2.7 kb, and digests from the heterozygous mutant mice contained two fragments corresponding in size to the fragments detected individually in the digests from the wild-type and homozygous mutant mice (Fig. 1B). The HS6ST-1 transcript was not detected in the homozygous mutant mice (Fig. 1C). In contrast, levels of the HS6ST-2 and HS2ST transcripts in the homozygous mice were similar to those observed in wild-type mice. These results indicate that the expression of the HS6ST-1 gene was abolished in the homozygous mutant mice.

Lethality of HS6ST-1 Deficient Mice at a Later Embryonic Stage—The genotype analysis of the progeny derived from intercrosses of heterozygous mice (C57BL/6 strain, 3F–4F) is shown in Table 1; this indicates that most of the HS6ST-1 null mice died between E15.5 and the perinatal stage. The genotypes of E12.5 and E14.5 agreed well with the Mendelian rule, indicating that HS6ST-1 null mice at the early embryonic stage appeared to be fully viable. By E15.5, however, one of the five homozygotes had died. From E17.5 to E18.5, the number of homozygous embryos was ~30% of that expected from the Mendelian rule. The accumulated data suggest that HS6ST1 is more important in the late stages of embryonic development in C57BL/6 mice than the early stages. On the 21st postnatal day, less than 4% of the offspring from the heterozygotes were homozygous mutants; these attained adulthood, albeit at a rather small size, and were fertile. In the C3H/He strain, ~7% of the offspring of the heterozygote (4F) intercrosses were homozygotes at the same stage. However, in the BALB/c strain, no HS6ST-1-deficient mice were found at birth in the offspring of the heterozygote (4F) intercrosses. Thus, the survival rate appears to depend on the genetic backgrounds, although the homozygous mice have not yet had a pure genetic background. One of factors affecting the survival rate might be the number of littermates; this is based on the observation that the number of littermates usually decreases in the order of BALB/c > C57BL/6 > C3H/He strain. In the case of HS3ST-1 mice, the
Roles of HS 6-O-Sulfation in Microvessel Formation

FIGURE 2. Reduction of 6-O-sulfate in the HS of various tissues taken from mutant mice. HS was isolated from various tissues of neonatal mice from the ears of 8-week-old mice as described under "Experimental Procedures." HS was digested with a heparitinase-I, heparitinase-II, and heparinase mixture; these digests were subjected to reverse-phase ion pair chromatography with postcolumn fluorescence labeling as described under "Experimental Procedures." The histograms show the percentage compositions of unsaturated disaccharide in the HS isolated from the organs of wild-type, heterozygous, and homozygous mutant mice. The values were obtained from three independent experiments, and each bar represents the mean ± S.D. from three independent experiments. *, p < 0.01; **, p < 0.05; ***, p < 0.001. ΔDi-2-acetamide-2-deoxy-4-O-(4-deoxy-a-l-threo-hex-4-enepyranosyluronic acid)-D-glucose; ΔDi-6S, ΔDi-NS, ΔDi-(N,6)diS, ΔDi-(N,2)diS, and ΔDi-(N,6,2)triS indicate the position and number of sulfate residues in the ΔDi unsaturated disaccharide.

Reduction of 6-O-Sulfate Content in the HS Isolated from HS6ST-1−/− Mice—We next examined the structure of HS in the HS6ST-1-deficient mice to determine whether the neonatal death of these mice was correlated with structural changes in their HS. We prepared HS from various organs isolated from newborn wild-type, heterozygous, and homozygous mice. The isolated HS was then subjected to disaccharide composition analysis (Fig. 2). The following changes in the structures of HS were observed in some of the homozygote tissues. There was a marked decrease in the relative contents of GlcNAc(6SO4) and HexA-GlcNSO3(6SO4) residues in the HS isolated from the kidneys and lungs; these tissues normally express relatively high levels of HS6ST-1 but rather low levels of HS6ST-2 (30). In contrast, the decrease in 6-O-sulfation was more modest in the HS isolated from the spleens and brains, where HS6ST-2 is expressed at high levels. The structural alterations of heparan sulfate from homozygous tissues were considerably consistent with the expected ones from the expression levels of HS6ST-1 in wild-type tissues by Northern blot analysis (30). Interestingly, the decrease in the trisulfated disaccharide unit, IdoA(2SO4)-GlcNSO3(6SO4) was not marked when compared with the decreases in GlcNAc(6SO4) and HexA-GlcNSO3(6SO4) units, even in the liver, kidneys, and lungs. In the spleen, the trisulfated disaccharide unit rather increased. These results appear to be consistent with the previously observed substrate specificities of the HS6ST isoforms. The apparent slight decrease in the trisulfated disaccharide unit in HS6ST-1-deficient mice may be due to the action of HS6ST-2; this is because HS6ST-2 exhibited a higher activity for the 6-O-sulfation of the IdoA(2SO4)-GlcNSO3 residue than HS6ST-1 and HS6ST-3 (30). To determine the isomers of the hexuronate residues (GlcA or IdoA) in the HS isolated from the homozygous mice, the HS derived from the spleen of these mice was cleaved with nitrous acid at pH 1.5, and the resulting disaccharide derivatives were analyzed by HPLC. The ratio of IdoA-GlcNSO3(6SO4) to GlcA-GlcNSO3(6SO4) in the HS from homozygotes was found to be lower than that observed in the HS from their wild-type littermates (data not shown). These results are also consistent with the properties of HS6ST-1 in vitro, where it has been shown to preferentially catalyze the 6-O-sulfation of IdoA-GlcNSO3 residues over GlcA-GlcNSO3 residues (30). In addition, to examine whether HS6ST-1 is involved in the 6-O-sulfation of heparin, we prepared heparin-enriched glycosaminoglycans from the ears of 8-week-old mice, in which mast cells are abundant, as observed by the staining with Alcian blue and safranine O (data not shown), and analyzed the disaccharide composition. The content of the trisulfated disaccharide unit was more than 45% in the ears of both wild-type and HS6ST-1-deficient mice, suggesting that the major portion of the trisulfated disaccharide unit was derived from heparin (Fig. 2). The nearly identical content of trisulfated disaccharide unit in the wild-type and HS6ST-1-deficient mice suggests that HS6ST-1 does not contribute to the 6-O-sulfation of heparin, although the possibility that isoforms other than HS6ST-1 may compensate for the deficiency of HS6ST-1 cannot be ruled out.

We also examined whether there was any up-regulation of HS6ST-2, HS6ST-3, and HS2ST transcripts in the homozygous mice as a compensation for the loss of HS6ST-1 mRNA. Analysis by real time reverse transcription-PCR of these mRNAs in liver from 8-week-old mice revealed that the expression levels of HS6ST-2, HS6ST-3, and HS2ST in homozygote were 0.9-, 1.1-, and 1.1-fold, respectively, of those in the wild-type. The finding that the 2-O-sulfation of HS prepared from the homozygous tissues was almost the same as that of wild-type HS was consistent with the above data (Fig. 2).

Tissue Abnormality and Growth Retardation of HS6ST-1-deficient Mice—The HS6ST-1-deficient mice tended to be smaller than the wild-type mice at both the embryonic and
neonatal stages, prompting us to compare the skeletons of both mice at birth. We prepared the skeletons as described by Peters (36) and the skeletal preparations of the wild-type and the homozygous mice were doubly stained with Alcian blue for cartilages and alizarin red for bones (Fig. 3). Systemic growth retardation was observed in the HS6ST-1-deficient mice. The hind limb phalange was missing in the HS6ST-1-deficient mice, and the hind limb tarsus was either smaller than that in the wild-type mice or completely lacking a tarsus. D–F, higher magnification of the respective hind limbs shown in A–C. Abnormal eye morphology was observed in approximately one-half of the HS6ST-1-deficient mice. The arrows indicate the tarsus (t), phalanges (p), and eyes (e).

Aberrant Angiogenesis in the Placenta—Impaired placental function appeared to be implicated in homozygous mouse growth retardation and/or perinatal lethality, which we observed in the HS6ST-1-deficient mice. Thus, we first analyzed the HS structure of placenta (Fig. 4). The placenta were isolated from 15.5-day embryos derived from intercrosses between heterozygous mice, and the maternal deciduas were removed as much as possible. Compared with other tissues, the HS from the placentas of wild-type animals exhibited the higher content of Di-OS and the lower contents of Di-(N,2)diS, Di-(N,6)diS, and Di-(N,6,2)triS (compare Fig. 2 with Fig. 4). In the placentas from the homozygous embryos, with the exception of the trisulfated disaccharide unit, 6-O-sulfated disaccharide units almost disappeared. Thus, HS6ST-1 plays a particularly important role in HS biosynthesis in the placenta.

We next compared the placental development of the homozygous and wild-type embryos by staining with hematoxylin/eosin (Fig. 5). Embryo-derived nucleated red blood cells were abundant in the placentas of wild-type embryos (Fig. 5, A and C) but were scarce in the placentas of the HS6ST-1-deficient embryos (Fig. 5, B and D), suggesting that angiogenesis was impaired in the placentas of the HS6ST-1-deficient embryos. Therefore, we stained the endothelial cells of the placental blood vessels with an anti-CD31 antibody, an antibody specific for vascular endothelial cells, to examine the degree of angiogenesis (Fig. 6A). In the wild-type placentas, intense staining of numerous microvessels was observed in the labyrinthine zone, where the exchange of the nutrients and gases occurs. In contrast, a weak staining of microvessels was observed in the labyrinthine zone of the HS6ST-1-deficient placentas, indicating the presence of fewer microvessels in the homozygous mice.
Roles of HS 6-O-Sulfation in Microvessel Formation

FIGURE 5. Comparison of placenta morphology in HS6ST-1/+ (A and C) and HS6ST-1−/− (B and D) mice. The E15.5 placentas were stained with hematoxylin/eosin. The lower panels show a higher magnification of the rectangle in the upper panels. The orange arrowheads indicate nucleated fetal red blood cells, and the green arrows indicate maternal red blood cells. In the labyrinthine zone of HS6ST-1−/− embryo placentas, there were a reduced number of fetal red blood cells. The yellow arrow indicates the labyrinthine zone. De, maternal decidua; Ch, chorionic plate; La, labyrinthine zone; Sp, spongiosarcomatous layer.

Consistent with this result, the analysis of these tissues by confocal microscopy revealed that the number of microvessels in the homozygous placenta was reduced to ~60% of the level observed in the wild-type placentas (Fig. 6, B and C). Despite the apparent reduction of angiogenesis in the placenta of homozygous mice, vascularization in the embryo body itself, including in the yolk sac, appeared unaffected. These results indicate that the nutritional supply and gas exchange in the labyrinthine zone may be jeopardized in the HS6ST-1-deficient placentas due to reduced angiogenesis. The reduction in the number of microvessels in the placentas of HS6ST-1-deficient embryos suggests the presence of hypoxia. HIF-1α is a marker for hypoxia. Thus, to test this supposition, we used an anti-HIF-1α antibody, a marker molecule for hypoxia, to identify (Fig. 7, A and B) and quantitate (Fig. 7C) the level of this protein in the placentas of the homozygous and wild-type embryos. As predicted, HIF-1α-positive cells were considerably more abundant in the labyrinthine zone of the placentas of the HS6ST-1-deficient mice than in those of wild-type mice. These observations provide further evidence for the reduced microvessel formation in the homozygous placentas.

Reduction of VEGF-A mRNA and Its Protein in the Placenta—We next examined how this abnormal angiogenesis occurred in the homozygous embryos. Wnt-2 knock-out mice (38) have been reported to exhibit abnormal placentation, growth retardation, and perinatal lethality, grossly similar to the phenotype that we have observed in HS6ST-1-deficient mice. Wnt2 is known to induce the expression of VEGF-A, a strong inducer of angiogenesis (39). Members of the Wnt family and some spliced forms of VEGF-A have an affinity for heparin, and evidence is accumulating that HS regulates signaling through these factors. Therefore, we next examined the expression of VEGF-A mRNA and Wnt2 mRNA by real-time reverse transcription-PCR. The level of VEGF-A mRNA in HS6ST-1-deficient placentas was ~60% of that in the wild-type placentas, whereas there was no significant change in the expression of Wnt2 mRNA in the deficient placentas (Fig. 8A). We also examined whether the expression of the VEGF-A protein in the placenta was reduced by immunohistochemical staining with an anti-VEGF-A antibody. The labyrinthine zones of both the wild-type and HS6ST-1-deficient placentas were obviously stained by this antibody, although the intensity of the staining in the HS6ST-1-deficient placentas was reduced compared with that in the wild-type placentas (Fig. 8B). Furthermore, we have confirmed the lower expression of VEGF-A protein in homozygous placentas by Western blot analysis (Fig. 8, C and D). The amount of VEGF-A protein in HS6ST-1-deficient placentas was ~73 ± 6% of that in the wild-type placentas and was significantly decreased compared with the heterozygous placentas. These observations suggest that the decreased VEGF-A expression in the HS6ST-1-deficient placentas results in impaired vascular development and thereby leads to growth retardation and lethality. It is known that HIF1-α induces VEGF-A expression (40). However, at present, it is difficult to provide a rational explanation for the
Thus, we analyzed the intra-alveolar distance by measuring the number of 1α expressing cells relative to the wild-type (WT) placenta (\( p < 0.001 \)).

**Interaction of Wnt2 with Mutant HS**—As described above, VEGF-A expression was decreased in the homozygous placentas. The expression of VEGF-A is known to be induced by Wnt (39), which is bound to heparin and regulated by heparin and HS. We thus analyzed the interaction of Wnt2 with wild-type HS and the homozygous HS using a BIAcore system. The sensorgrams and their dissociation constants (K_D) are shown in Fig. 9, A and B. The 330 nM K_D value for Wnt2/wild-type placenta-HS was approximately half the 430 nM K_D value for Wnt2/homozygous placenta-HS. Although these interactions were weak, the differences were significant. Thus, these studies suggest that the 6-O-sulfate residues in HS participate in its recognition of Wnt2.

**Aberrant Lung Morphology**—The formation of lung alveoli is known to be regulated by Wnt5a, FGF10, and BMP4 (41, 42), which bind to heparin/HS. Furthermore, HS6ST-1 is expressed strongly at the tips of branching tubules in the developing lung (43), and FGF10 is preferentially bound to 6-O-sulfate residues rather than 2-O-sulfate residue in HS (7). Thus, a deficiency in HS6ST-1 is potentially relevant to impaired lung development. To determine if lung development is affected by the loss of HS6ST-1, we examined the morphology of 7-day-old mice (Fig. 10, A and B) and adult mouse lung (10 months old) (Figs. 10, C and D) by staining with hematoxylin/eosin. Alveolar enlargement was seen in the homozygous mice lung (Fig. 10, B and D). Thus, we analyzed the intra-alveolar distance by measuring the MLI (Fig. 10, E and F). In 7-day-old mice, MLI of the homozygous mice was significantly increased compared with the heterozygous mice (40 ± 5 vs. 30 ± 4 μm, n = 3). In adult mice, the difference was more marked between the homozygous mice (42 ± 6 μm) and the wild-type mice (29 ± 4 μm). Since it has been reported that an increase in MLI is inversely proportional to the internal lung surface area (35), these results suggest that HS6ST1-deficient mice have impaired alveolarization. These observations raise the possibility that decreased 6-O-sulfation adversely affects the signaling of undefined growth factors and possibly extracellular matrix proteins in the lung, which adversely impact alveolarization. Although we cannot rule out the possibility that the aberrant lung morphology is caused by growth retardation, it is unlikely, because 10-month-old mice also revealed abnormal lungs. Nevertheless, our data revealed that HS6ST-1 is considerably less important than NDST-1 in controlling the development and function of the lung.

**DISCUSSION**

We previously noted that diminished levels of HSST in *Drosophila* lead to abnormal tracheal branching (27). This is thought to be a model for mammalian vasculogenesis, because
Roles of HS 6-O-Sulfation in Microvessel Formation

Figure 9. Lower affinity of Wnt2 for the homozygous placental HS. Various concentrations of Wnt2 were injected onto the surface of the wild-type placenta-HS (A) or homozygous placenta-HS (B) immobilized sensor chips. 

Figure 10. Aberrant morphology of lungs in the HS6ST-1-deficient mice. Lungs were fixed via trachea with 4% paraformaldehyde under a constant pressure of 20 cm H2O as described under “Experimental Procedures.” Paraffin sections from 7-day-old mice (C and D) were stained with hematoxylin/eosin. A, heterozygous mice; C, wild-type mice; B and D, homozygous mice. Shown are the results at ×100 magnification. The lungs of the HS6ST-1-deficient mice had apparently enlarged alveoli. E and F, the intra-alveolar distance of the lungs from 7-day-old (E) and adult mice (F) was measured as the MLI, as described under “Experimental Procedures.” MLI was increased in the homozygous mice compared with the wild-type (WT) mice (*, p < 0.001; **, p < 0.001).

Both Drosophila tracheogenesis and vertebrate vasculogenesis share many common molecules, such as FGFs, VEGF, cadherin, integrins, and HIF-1-like factors (5). Using an antisense approach to reduce the levels of HS6ST in zebrafish, we also showed that this sulfotransferase is required for optimal Wnt signaling (28). More recently, these morphants have been reported to exhibit defective branching morphogenesis of the caudal vein angiogenesis (45). C. elegans lacking HS6ST exhibit axonal and cellular guidance defects in specific neuron classes. In this case, the 6-O-sulfate residues in the HS chains appear to be involved in axon branching via signaling mediated by Kal-1/Anosmin-1 (29). Thus, 6-O-sulfation appears to regulate the signaling of many growth factors in lower organisms. Nevertheless, no definitive evidence for the functional roles of a HS6ST or 6-O-sulfated HS in mammalian development has been reported. Our study is therefore the first to demonstrate the physiological importance of the 6-O-sulfation of HS in mouse development.

Disruption of the Hs6st-1 gene in our mice resulted in growth retardation and developmental abnormalities, with most embryos dying between the E15.5 and perinatal stages. These phenotypes apparently were partly due to abnormal placentaion caused by a reduction in the microvessels in the labyrinthine zone. The structure of the HS glycosaminoglycans isolated from various organs of the HS6ST-1-null mice differed markedly in their pattern of 6-O-sulfation from those isolated from their wild-type littermates.

Placental development requires the organized interaction of vascular growth factors (VEGF-A and PlGF) and their receptors (VEGFR-1 and -2) (46). We discovered that in the placentas of our HS6ST-1-null mice, the expressions of VEGF-A mRNA and protein were reduced to ~60 and 75%, respectively, of their normal levels (Fig. 8, A and D). HS stimulates VEGF signaling (47, 48, 49), and mouse embryos genetically engineered to express an isoform of VEGF that is unable to recognize heparin and HS have a noticeable defect in the vascular branch formation (50, 51). We recently showed that chlorate-treated endothelial cells are less responsive to VEGF_165. This in vitro finding was associated with changes in the structure of HS, resulting in a decrease in O-sulfation but not in N-sulfation (49). VEGF-A in the placentas of our HS6ST-1-null mice might be reduced not only in the level of expression but also in its activity. This is due to the alteration of HS structures, resulting in aberrant placenta angiogenesis.

VEGF-A is a downstream target of Wnt signaling (39), and the activities of Wnts are regulated by HS (52, 53). At the E14.5 stage of embryonic development, Wnt2-null mice have a phenotype similar to that of our HS6ST-1-null mice, namely diminished growth, increased perinatal lethality, and numerous placental defects, including the presence of decreased numbers of fetal capillaries (38). Frizzled-5 is one of the receptors for the Wnt family. Mice lacking this receptor also exhibit embryonic lethality and defects in placental vasculogenesis (54). We observed fewer microvessels in the labyrinthine zone of the placentas in our HS6ST-1-null mouse embryos. The accumulated data suggest that 6-O-sulfated HS has critical functions during the late stages of placental development due to its ability to optimally interact with heparin/HS-binding growth factors,
such as Wnt2. Since the expression of Wnt2 at the mRNA level was similar in HS6ST-1-null mice and their +/+ littermates (Fig. 8A), we examined the affinity of Wnt2 binding to placental HS. Those results revealed diminished binding of Wnt2 to HS isolated from our HS6ST-1-null mice (Fig. 9). Such a difference in $K_D$ values might be functionally significant. This is because the activity of VEGF-A was stimulated by heparin, although the $K_D$ value for VEGF165/heparin was 165 nM, a value similar to the $K_D$ for Wnt2/placenta-HS, (7, 49). Further, Wnt signaling mediated by Frizzled receptors is defective in organisms lacking HS (52). On the other hand, desulfation of HS by HS 6-O-endosulfatase (a sulfatase capable of releasing sulfate from the trisulfated IdoA(2SO$_4$)-GlcNSO$_3$(6SO$_4$) disaccharide in HS/heparin (11–13)) appears to stimulate Wnt signaling (55–57). Taken together, these observations suggest that Wnt activity might be regulated either negatively or positively by different HS structures. Further studies are necessary to clarify whether the different affinities of Wnt2 for the HS chains isolated from HS6ST-1-null mice and their wild-type littermates are biologically significant in vivo. However, placental vasculogenesis also might be regulated by an unknown signaling pathway. In this regard, it is well known that HS also interacts with matrix proteins. For example, mice lacking the laminin α5 chain that binds to HS exhibit dysmorphogenesis of the placental labyrinth (58). We therefore cannot rule out the possibility that the abnormal placentation might be caused by the disregulated interaction of HS with extracellular matrix proteins.

Analysis of the HS structures from various organs of our HS6ST-1-null mice revealed a marked reduction in the levels of HexA-GlcNSO$_3$(6SO$_4$)$_3$. In contrast, the decrease in the content of the trisulfated disaccharide IdoA(2SO$_4$)$_2$-GlcNSO$_3$(6SO$_4$)$_3$ was less pronounced even in the HS chains isolated from liver, kidney, and lungs. These results are consistent with the reported in vitro substrate specificity of HS6ST-1 (30, 31), which prefers to sulfate the HexA-GlcNSO$_3$ disaccharide over the IdoA(2SO$_4$)$_2$-GlcNSO$_3$(6SO$_4$)$_3$ disaccharide. Furthermore, the content of GlcNAc-6SO$_4$ residues in the HS isolated from our HS6ST-1-null mice was markedly reduced. We previously noted that HS6ST-1 exhibits less activity toward GlcNAc residues but that cells induced to overexpress HS6ST-1 generate HS chains that contain considerably more GlcA-GlcNAc(6SO$_4$) than the HS in the nontransfected cells (31). Holmborn and co-workers (59) also reported that ES cells lacking NDST-1 and NDST-2 generate HS with 6-O-sulfated GlcNAc residues. Taken together, these observations suggest that HS6ST-1 can efficiently transfer sulfate to position 6 of GlcNAc residues and that in the process of HS modification, some GlcNAc residues are likely to be 6-O-sulfated before they are N-sulfated.

Four NDSTs have been identified in mice and humans, and N-sulfation of heparin in mast cells is catalyzed primarily by NDST-2 (20). Unlike the NDSTs, it is not known which HS6ST is preferentially involved in the 6-O-sulfation of heparin. Our preliminary data suggest that the fine structure of the heparin glycosaminoglycans in the skin mast cells of our HS6ST-1/−/− mice is not altered. These data suggest that skin mast cells preferentially rely on HS6ST-2 and/or HS6ST-3 for 6-O-sulfation of their heparin. However, the possibility of redundancy between the HS6ST isoforms should not be excluded. Furthermore, mast cells are heterogeneous cells regulated by numerous factors in their tissue microenvironments. It is therefore possible that HS6ST-1 plays a more significant role in the sulfation of heparin in the mast cells that reside at sites other than the skin.

To date, it has been reported that mice deficient in many of the enzymes involved in HS biosynthesis have abnormal phenotypes. The defects observed in each mutant often are characteristic of the missing or mutated enzyme. Nevertheless, significant overlapping phenotypes have been observed between some HS mutants. For example, HS2ST−/− and Hsepi−/− mice lack kidneys (24, 25), whereas NDST-1−/− and HS6ST-1−/− mice have normal kidney development. N-Sulfation in the HS from NDST-1−/− mice was reduced only to one-half of their +/+ littermates. Epimerization and 2-O- and 6-O-sulfation also occurred partially in the NDST-1−/− mice (60, 61). Morphogenesis of the kidney is dependent on heparin-binding growth factors, such as FGFs, Wnt4, Wnt11, and bone morphogenetic protein 7 (24). Thus, these factors possibly require the IdoA(2SO$_4$)$_2$-GlcNSO$_3$ or IdoA(2SO$_4$)$_2$-GlcNSO$_3$(6SO$_4$)$_3$ disaccharide units in HS. An abnormal lung phenotype also was observed in NDST-1−/− (19, 20), Hsepi−/−, and HS6ST-1−/− mice but not in HS2ST−/− or NDST-2−/− mice. The content of the IdoA-GlcNSO$_3$(6SO$_4$)$_3$ unit in the HS was apparently decreased in NDST-1−/−, Hsepi−/−, and HS6ST-1−/− mice but not HS2ST−/− mice. Therefore, IdoA-GlcNSO$_3$(6SO$_4$)$_3$ might be the more important structural unit for normal organogenesis of the lung, which is known to involve FGF10 and Wnt5a signalings (40, 41). Furthermore, the following facts appear to support the notion that HS6ST-1 in the developing lung is expressed strongly at the tips of branching tubules (41) and that FGF10 preferentially binds to 6-O-sulfate residues over 2-O-sulfate residues in HS (7). However, to definitively clarify the function of 6-O-sulfate in HS/heparin, it probably will be necessary to phenotype mice lacking HS6ST-2, HS6ST-3, and varied combinations of the three HS6STs. Moreover, an investigation into how the cells obtained from these sulfotransferase-null mice respond to various heparin-binding proteins would provide valuable information that would lead to a better understanding at the molecular level of how HS controls organogenesis.

While we were preparing this manuscript, Pratt et al. (44) reported the generation of HS6ST-1−/− mice using a gene trap approach. They demonstrated that the homozygous mice exhibited navigation errors of retinal ganglion cell axons from each eye and suggested that HS6ST-1 at least in part modulates the response of the navigating growth cone to Slit protein.

Acknowledgments—We thank Drs. Takashi Kobayashi, Keisuke Kamimura, and Satoko Ashikari-Hada for fruitful discussion; Yukako Nishimura and Hiromi Fuwa for skillful technical assistance; and Drs. Alan C. Rapraeger, Jeffrey D. Esko, and Osami Habuchi for stimulating discussion and critical reading of the manuscript.

REFERENCES

1. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincum,J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
