Detection of 2-O-Sulfated Iduronate and N-Acetylglucosamine Units in Heparan Sulfate by an Antibody Selected against Acharan Sulfate (IdoA2S-GlcNAc)ₙ*

Received for publication, April 14, 2004, and in revised form, June 29, 2004 Published, JBC Papers in Press, July 7, 2004, DOI 10.1074/jbc.M404166200

Gerdy B. ten Dam‡, Els M. A. van de Westerlo§, Toon F. C. M. Smetsers‡, Marieke Willems‡, Goos N. P. van Muijen§, Catherine L. R. Merry¶, John T. Gallagher¶, Yeong S. Kim¶, and Toin H. van Kuppevelt**

From the Departments of ‡Biochemistry and §Pathology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, Nijmegen 6500 HB, The Netherlands, ¶Cancer Research UK, Department of Medical Oncology, University of Manchester, Manchester, M20 4BX, United Kingdom, and ¶Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea

The snail glycosaminoglycan acharan sulfate (AS) is structurally related to heparan sulfates (HS) and has a repeating disaccharide structure of α-N-acetylgluco- cosaminyl-2-O-sulfo-α-L-iduronic acid (GlcNAc-IdoA2S) residues. Using the phage display technology, a unique antibody (MW3G3) was selected against AS with a V₃,4, DP 47, and a CDR3 amino acid sequence of KKKRPFR. Antibody MW3G3 did not react with desulfated, N-deacetylated or N-sulfated AS, indicating that reactivity depends on N-acetyl and 2-O-sulfate groups. Antibody MW3G3 also had a high preference for (modified) heparin oligosaccharides containing N-acetylated glucosamine and 2-O-sulfated iduronic acid residues. In tissues, antibody MW3G3 identified a HS oligosaccharide epitope containing N-acetylated glucosamine and 2-O-sulfated iduronic acid residues as enzymatic N-deacetylation of HS in situ prevented staining, and 2-O-sulfotransferase-deficient Chinese hamster ovary cells were not reactive. An immunohistochemical survey using various rat organs revealed a distinct distribution of the MW3G3 epitope, which was primarily present in the basal laminae of most (but not all) blood vessels and of some epithelia, including human skin. No staining was observed in the glycosaminoglycan-rich tumor matrix of metastatic melanoma. In conclusion, we have selected an antibody that identifies HS oligosaccharides containing N-acetylated glucosamine and 2-O-sulfated iduronic acid residues. This antibody may be instrumental in identifying structural alterations in HS in health and disease.

Acharan sulfate (AS) is a glycosaminoglycan (GAG) abundantly present in the giant African snail *Achatina fulica* (1). It largely consists of the repeating disaccharide structure of α1-4)α-N-acetamido-2-deoxyxyloglycopyranosyl(1→4)-α-L-idopyrano-syluronic acid-2-sulfate(1→ and is closely related to heparan sulfates. AS is located in large granules present in the outer surface of the snail and is secreted onto the surface as mucus. This mucus consists of 26% proteins, and the GAG moiety is entirely formed by AS. Whether AS is present as a proteoglycan is unclear (2). The pattern of adjacent N-acetyl-glucosamine and 2-sulfodiuronic acid residues is unusual and suggests interesting biological activities. Among the proposed biological functions of AS in snails are binding, uptake, and transport of divergent cations and functioning as an antidesicant (1). AS inhibits the mitogenic activity induced by basic fibroblast growth factor (3), angiogenesis (4), and tumor growth (5).

To study the cell biological importance of specific HS epitopes and their location in tissue, single chain antibodies have been generated as described by our group (6–9). These antibodies were selected against HS from various sources and stained differentially in tissues sections from various organs. Defined HS oligosaccharides were used to reveal details of the epitopes recognized by the antibodies (6). Although some chemical groups in HS essential for antibody reactivity could be determined, the oligosaccharide sequence recognized by these antibodies remains largely unknown. The homogenous structure of AS and its resemblance to HS, together with its biological activities, especially its anti-tumor potential, made us decide to select single chain variable fragment (scFv) antibodies directed against AS using the phage display technology.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—The human synthetic scFv library no. 1 (10) was generously provided by Dr. G. Winter (Cambridge University, Cambridge, UK).

Rats (Wistar, male, 8 weeks) were obtained from the Central Animal Laboratory (University Medical Center Nijmegen). The anti-V5V tag mouse hybridoma cell line 5D14 was obtained from the American Type Culture Collection (IgG, Manassas, VA). Wild-type CHO K1 and mutant CHO pgsF17 cell lines were kindly provided by Prof. Dr. J. Esko (Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA). N-deacetylase/N-sulfotransferase (NDST) 1 (clone 11) and 2 (clone 55) stably transfected human kidney 293 cell lines were kindly provided by Prof. Dr. L. Kjellen (Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden).

N-deacetylase/N-sulfotransferase; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; K5, K5 polysaccharide (GlcA-GlcNAc)ₙ.
Chemically Modified AS, Heparin, and Heparin Oligosaccharides—Large Scale Preparation of Antibodies—Antibody against Acharan Sulfate (IdoA2S-GlcNAc)n

All chemicals used were purchased from Merck unless stated otherwise. Bacterial medium (2 × TY) was from Invitrogen. Cell culture media were from Invitrogen. Isopropyl-β-D-thiogalactopyranoside and bovine serum albumin (fraction V) were from Sigma. Protease inhibitor mixture was from Roche Applied Science. Mowiol (4-88) was obtained from Calbiochem. The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit was from PerkinElmer Life Sciences. Microlon 96-well microplates from Greiner (Frickenhausen, Germany). Polystyrene maxisorp immunotubes were from Nunc (Roskilde, Denmark). Multipoint slides were from Nutacon (Leimuiden, The Netherlands). Protein A-agarose beads were from Kem-Tec (Copenhagen, Denmark).

Heparin and HS from porcine intestinal mucosa, HS from bovine kidney, chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, dermatan sulfate from porcine intestinal mucosa, and HS from human umbilical cord, and DNA from Staphylococcus were from Sigma. (GlcA-GlcNAc)n, (K5) and K5-O-S were a kind gift from the Department of Nephrology, University Medical Center Nijmegen (11). Recombinant heparinase III (from Flavobacterium heparinum) was a kind gift from IBEX Technologies (Montreal, Canada).

Commercially Modified AS, Heparin, and Heparin Oligosaccharides—Isolation of Specificity—Evaluation of Specificity by Immunohistochemistry—Tissue sections were snap frozen in liquid nitrogen and stored at −80 °C. Tissue sections (5 μm) were incubated with periplasmic fractions of anti-AS antibodies in 1% (w/v) bovine serum albumin in PBS with 0.05% (v/v) Tween 20. Bound antibodies were detected using anti-tag antibodies (anti-VSV, PDS4, and IgG1) followed by Alexa-labeled (488) anti-mouse antibodies.

Evaluation of Specificity by Immunohistochemistry—Tissue sections were preincubated for 2 min in PBS before adding to the tissue sections. Tissue sections were probed with antibodies specific for AS and stained with an irrelevant antibody. After washing with PBS, tissue sections were incubated with a mixture of anti-AS antibodies and AS or AS oligosaccharides (14-mers) and evaluated for inhibition of antibody staining. Mixtures of anti-AS antibodies and oligosaccharides (10 and 50 μg/ml) were preincubated for 2 min before adding to the tissue sections. Tissue sections were processed for immunofluorescence analysis as described above.

Staining patterns were analyzed by fluorescence microscopy.

**RESULTS**

**Acharan Sulfate—**AS was analyzed by agarose gel electrophoresis followed by silver staining of the gel (Fig. 1). AS migrates somewhat faster than HS from bovine kidney but slower than dermatan sulfate.

**Selection of Antibodies against Acharan Sulfate—**Four rounds of panning were performed against AS using the semisynthetic scFv library 1, which resulted in a total increase of phage titer from 6 × 10⁶ colony-forming units in the first round to 1 × 10⁸ colony-forming units in the fourth round. Supernatants containing antibodies of the third and fourth selection rounds were tested for reactivity with AS by ELISA. Of the 188 clones tested for reactivity with AS by ELISA. Of the 188 clones selected for further analysis, this antibody belongs to the VH3 family, has a DP 47 germline gene segment, and contains the

**RESULTS**

**Acharan Sulfate—**AS was analyzed by agarose gel electrophoresis followed by silver staining of the gel (Fig. 1). AS migrates somewhat faster than HS from bovine kidney but slower than dermatan sulfate.

**Selection of Antibodies against Acharan Sulfate—**Four rounds of panning were performed against AS using the semisynthetic scFv library 1, which resulted in a total increase of phage titer from 6 × 10⁶ colony-forming units in the first round to 1 × 10⁸ colony-forming units in the fourth round. Supernatants containing antibodies of the third and fourth selection rounds were tested for reactivity with AS by ELISA. Of the 188 clones screened, four reacted with AS, which were all from the third selection round. DNA sequence analysis revealed that all clones expressed identical antibodies. Clone MW3G3 was selected for further analysis. This antibody belongs to the VH3 family, has a DP 47 germline gene segment, and contains the

**RESULTS**

**Acharan Sulfate—**AS was analyzed by agarose gel electrophoresis followed by silver staining of the gel (Fig. 1). AS migrates somewhat faster than HS from bovine kidney but slower than dermatan sulfate.

**Selection of Antibodies against Acharan Sulfate—**Four rounds of panning were performed against AS using the semisynthetic scFv library 1, which resulted in a total increase of phage titer from 6 × 10⁶ colony-forming units in the first round to 1 × 10⁸ colony-forming units in the fourth round. Supernatants containing antibodies of the third and fourth selection rounds were tested for reactivity with AS by ELISA. Of the 188 clones screened, four reacted with AS, which were all from the third selection round. DNA sequence analysis revealed that all clones expressed identical antibodies. Clone MW3G3 was selected for further analysis. This antibody belongs to the VH3 family, has a DP 47 germline gene segment, and contains the

**RESULTS**

**Acharan Sulfate—**AS was analyzed by agarose gel electrophoresis followed by silver staining of the gel (Fig. 1). AS migrates somewhat faster than HS from bovine kidney but slower than dermatan sulfate.

**Selection of Antibodies against Acharan Sulfate—**Four rounds of panning were performed against AS using the semisynthetic scFv library 1, which resulted in a total increase of phage titer from 6 × 10⁶ colony-forming units in the first round to 1 × 10⁸ colony-forming units in the fourth round. Supernatants containing antibodies of the third and fourth selection rounds were tested for reactivity with AS by ELISA. Of the 188 clones screened, four reacted with AS, which were all from the third selection round. DNA sequence analysis revealed that all clones expressed identical antibodies. Clone MW3G3 was selected for further analysis. This antibody belongs to the VH3 family, has a DP 47 germline gene segment, and contains the
An Antibody against Acharan Sulfate (IdoA2S-GlcNAc)_n

Evaluation of the Specificity of Anti-AS Antibody MW3G3—

Antibody MW3G3 was analyzed for reactivity with various GAG preparations (ELISA). Bound antibodies were visualized by anti-tag antibodies followed by alkaline phosphatase-conjugated anti-mouse IgG antibodies. Enzymatic activity was measured using p-nitrophenyl phosphate as a substrate. Bars, mean ± S.D. (n = 3). Hep, heparin from porcine intestinal mucosa; HS, heparan sulfate from bovine kidney; HS\textsubscript{im}, heparan sulfate from porcine intestinal mucosa; CSA, chondroitin sulfate C; CSA, chondroitin sulfate A; DS, dermatan sulfate; AS, acharan sulfate; Hyal, hyaluronate; DNA, deoxyribonucleic acid.

**Table I. Reactivity of antibody MW3G3 with various GAG preparations and tissues**

| Name                      | MW3G3 reactivity |
|---------------------------|------------------|
| AS                        | ++               |
| NS AS                     | −                |
| N-de-Ac AS                | −                |
| de-O-S AS                 | −                |
| Heparin (porcine intestinal mucosa) | + +          |
| Heparin (bovine lung)     | +                |
| N-de-S, N-Ac heparin (bovine lung) | + + +         |
| 2-O-de-S heparin (bovine lung) | −          |
| N-de-S, N-Ac partially 2-O-de-S heparin (bovine lung) | −  |
| N-de-S, N-Ac heparin (porcine intestine) | + + +       |
| De-S, N-S heparin (porcine intestine) | −        |
| De-S, N-Ac Heparin (porcine intestine) | −        |
| K5                        | −                |
| K5 O-S (1.2 O-sulfates/disaccharide) | + + +       |
| HS (bovine kidney)        | +/−              |
| HS (kidney section)       | +                |
| HS (kidney section) after NDST treatment | − |
| HS (CHO cells)            | +                |
| HS (CHO pgsf17, 2-O-sulfotransferase-deficient) | −  |

* Heparin form porcine intestinal mucosa and bovine lung contain 3.0% and 0.8% IdaA2S-GlcNAc(6S) units, respectively (33).

* α* 80% of the disaccharides consist of IdaA2S-GlcNAc(6S) units.

* α* 80% of the disaccharides consist of IdaA,N,6-O-disulfo-α-D-glucosamine (GlcNS6S) units.

* α* 80% of the disaccharides consist of IdaA-GlcNAc(6S) units; 15% of the disaccharides are 2-O-sulfated (IdoA2S).

* α* Staining is heparinase III-sensitive.

* α* <1% 2-O-sulfates (22, 26).

**N-Acetylation Is Essential for Binding of Antibody MW3G3 in Situ as Determined by HS Deacetylation of Rat Kidney Tissue Sections**—The necessity of N-acetyl groups for antibody recognition was further analyzed by enzymatic removal of N-acetyl groups using cell lysates of human kidney 293 cells stably transfected with NDST 1 and NDST 2 cDNA (23, 24), followed by anti-HS antibody staining of the tissue section (25) (Fig. 4, Table I). After incubation, hardly any staining with

**FIG. 1. Agarose gel electrophoresis of acharan sulfate.** AS was analyzed by barium acetate-agarose gel electrophoresis followed by silver staining of the gel. Lane 1, GAG markers: HS (40 ng), dermatan sulfate (DS) (20 ng), and chondroitin sulfate (CS; 20 ng). Lane 2, AS (50 ng).

**FIG. 2. Evaluation of the specificity of anti-AS antibody MW3G3.** Antibody MW3G3 was analyzed for reactivity with various GAG preparations (ELISA). Bound antibodies were visualized by anti-tag antibodies followed by alkaline phosphatase-conjugated anti-mouse IgG antibodies. Enzymatic activity was measured using p-nitrophenyl phosphate as a substrate. Bars, mean ± S.D. (n = 3). Hep, heparin from porcine intestinal mucosa; HS, heparan sulfate from bovine kidney; HS\textsubscript{im}, heparan sulfate from porcine intestinal mucosa; CSA, chondroitin sulfate C; CSA, chondroitin sulfate A; DS, dermatan sulfate; AS, acharan sulfate; Hyal, hyaluronate; DNA, deoxyribonucleic acid.

**TABLE I. Reactivity of antibody MW3G3 with various GAG preparations and tissues**

| Name                      | MW3G3 reactivity |
|---------------------------|------------------|
| AS                        | ++               |
| NS AS                     | −                |
| N-de-Ac AS                | −                |
| de-O-S AS                 | −                |
| Heparin (porcine intestinal mucosa) | + +          |
| Heparin (bovine lung)     | +                |
| N-de-S, N-Ac heparin (bovine lung) | + + +         |
| 2-O-de-S heparin (bovine lung) | −          |
| N-de-S, N-Ac partially 2-O-de-S heparin (bovine lung) | −  |
| N-de-S, N-Ac heparin (porcine intestine) | + + +       |
| De-S, N-S heparin (porcine intestine) | −        |
| De-S, N-Ac Heparin (porcine intestine) | −        |
| K5                        | −                |
| K5 O-S (1.2 O-sulfates/disaccharide) | + + +       |
| HS (bovine kidney)        | +/−              |
| HS (kidney section)       | +                |
| HS (kidney section) after NDST treatment | − |
| HS (CHO cells)            | +                |
| HS (CHO pgsf17, 2-O-sulfotransferase-deficient) | −  |

* Heparin form porcine intestinal mucosa and bovine lung contain 3.0% and 0.8% IdaA2S-GlcNAc(6S) units, respectively (33).

* α* 80% of the disaccharides consist of IdaA2S-GlcNAc(6S) units.

* α* 80% of the disaccharides consist of IdaA,N,6-O-disulfo-α-D-glucosamine (GlcNS6S) units.

* α* 80% of the disaccharides consist of IdaA-GlcNAc(6S) units; 15% of the disaccharides are 2-O-sulfated (IdoA2S).

* α* Staining is heparinase III-sensitive.

* α* <1% 2-O-sulfates (22, 26).
An Antibody against Acharan Sulfate (IdoA2S-GlcNAc)α

MW3G3

1+2 +
1 2 3+4 3 4
1) Hep dp6
2) Hep dp6, N-acetylated
3) Hep dp8
4) Hep dp8, N-acetylated

Fig. 3. Immunoprecipitation analysis of (modified) heparin oligosaccharides using antibody MW3G3. Heparin dp6 (hep dp6; 1) and dp8 (hep dp8; 2) and N-desulfated reacetylated heparin dp6 (hep dp6, N-acetylated; 2) and dp8 (hep dp8, N-acetylated; 4) oligosaccharides were combined and incubated with antibody MW3G3, followed by incubation with protein A-agarose beads loaded with anti-tag antibody. Immunoprecipitated fragments were analyzed by 33% polyacrylamide gel electrophoresis followed by alcian blue fixation and silver staining. Individual oligosaccharides (indicated by 1, 2, 3, and 4) and the immunoprecipitated fragments (indicated by 1 + 2 + 3 + 4) were loaded on the gel. Note that there is a high preference for N-acetylated heparin oligosaccharides of 8 saccharides in length.

Fig. 4. Immunolocalization of the MW3G3 epitope in rat kidney section after treatment with N-deacetylae/N-sulfotransferase enzymes. Cryosections of rat kidney were incubated without (A and B) and with (C and D) cell lysates of 293 cells stably transfected with NSDT enzymes to remove N-acetyl groups from GlcNAc residues (note that no sulfotransferase activity is present due to a lack of sulfate donor). After incubation for 16 h at 37 °C with NDST 1 cell lysate (500 μg of protein/ml), cryosections were washed and stained using antibody MW3G3 (A and C) and HS4C3 (B and D; control). Staining with antibody MW3G3, but not HS4C3, is almost completely abolished after NDST incubation. No differences were observed between cell lysates of NDST 1- or 2-transfected cells. Bar, 25 μm.

antibody MW3G3 was observed anymore (Fig. 4, A and C). Staining with anti-HS antibody HS4C3, which recognizes highly sulfated HS structures (9), was not affected (Fig. 4, B and D). No differences were observed between NDST 1 or NDST 2 cell lysates. These data indicated that the presence of N-acetyl groups is essential for staining of antibody MW3G3.

2-O-Sulfation Is Essential for Binding of Antibody MW3G3 in Situ as Determined by 2-O-Sulfotransferase Mutant CHO Cells—The necessity of 2-O-sulfates for antibody recognition was determined by the analysis of immunoreactivity of antibody MW3G3 for CHO cells defective in HS 2-O-sulfation (26). An average of 3.1% of ΔHexA(2S)-GlcNAc disaccharide units were found in wild type CHO cells (CHO1) as determined by lyase digestion and disaccharide analysis, whereas no ΔHexA(2S)-GlcNAc disaccharide units were found in mutant CHO cells (pfsF17). Disaccharide compositional analysis of HS in wild type and mutant CHO cells is given in Table II. Wild type CHO cells showed high expression of MW3G3 epitopes (Fig. 5A), whereas mutant CHO cells were negative (Fig. 5B). We used antibody AO4B08 as a control antibody for the presence of 2-O-sulfation (6) (Fig. 5, C and D).

Immunofluorescence Detection of the MW3G3 Epitope in the Giant Snail A. fulica and Rat Tissues—To ascertain that antibody MW3G3 recognizes HS, tissue sections of rat kidney and intestine and of human skin, atypical nevi, and metastatic melanoma (see below) were pretreated with heparinase III. A total loss of staining was observed, indicating that indeed HS was recognized by antibody MW3G3 (data not shown). Antibody staining was completely inhibited by incubation of the antibody with AS and AS oligosaccharides (14-mers) at a concentration of 50 μg/ml (data not shown). At a concentration of 10 μg/ml, staining was greatly reduced. In the snail A. fulica, large granules present in the outer surface of the snail, which contain AS, stained very intensely (Fig. 6A), whereas the control showed no staining at all (Fig. 6B). Rat tissue sections (intestine, liver, pancreas, kidney, testis, and tongue) were analyzed for reactivity with antibody MW3G3 (Fig. 7, Table III). In general, blood vessels were stained, whereas the con-...
metastatic melanoma (Fig. 8). In normal skin and atypical nevi, the basal lamina of the epidermis strongly stained; however, no staining of blood vessels in the dermis was observed (Fig. 8, Skin and AN, left). An anti-chondroitin sulfate scFv antibody IO3H10 (27) (Fig. 8, right) showed strong staining associated with the basal lamina of the epidermis and of the blood vessels. No staining was observed in metastatic melanoma with antibody MW3G3, whereas the anti-chondroitin sulfate antibody intensely stained the extracellular matrix surrounding nests of tumor cells and the blood vessels (Fig. 8, MM).

DISCUSSION

The disaccharide structure of AS is quite homogenous and largely consists of repeating GlcNAc-IdoA2S disaccharides (1). This uniform GAG structure seemed ideal to select antibodies against, since this might predict the epitope structure recognized by the antibody. Therefore, the antibody phage display technology was applied to obtain antibodies against AS. Antibody MW3G3 (DP47, V_{H}3, complementarity-determining region 3 QKKPRPF) was selected and reacted strongly with AS. Both the N-acetyl and 2-O-sulfate groups were found essential for antibody recognition. In general, anti-GAG single chain antibodies react well with oligosaccharides consisting of five or more monosaccharides (6), and therefore the most compatible epitope in AS may be (IdoA2S-GlcNAc)$_n$.

The antibody also reacts with HS and heparin, and, as in AS, the essential groups are N-acetylated glucosamine and 2-O-sulfated uronic acid residues, as was demonstrated by ELISA, immunoprecipitation, NDST enzyme treatment on tissue sections, and mutant cell lines (2-O-sulfotransferase-deficient cells). HS as well as heparin contain IdoA2S-GlcNAc disaccha-
rides, albeit in low amounts (22, 28–33). Since heparin octasaccharides bind much better to the antibody than hexasaccharides, it is unlikely that one single IdoA2S-GlcNAc disaccharide forms the epitope. However, a repeating disaccharide structure as found in AS has not been demonstrated in HS/heparin and appears biosynthetically impossible, since N- and 2-O-sulfation reactions are interdependent such that N-sulfation on the non-reducing side is a prerequisite for 2-O-sulfation on the reducing side (34).

To create IdoA2S-GlcNAc units, epimerization has to occur. The epimerase acts on GlcA only when it is located at the reducing side of a GlcNS residue, and the enzyme does not react with GlcA that is O-sulfated or that is adjacent to an O-sulfated glucosamine (35–37). Therefore, epimerization starts after N-deacetylation and N-sulfation but before 6-O- and 3-O-sulfation (38). The iduronic acid residue may then be 2-O-sulfated by the 2-O-sulfotransferase, which acts on both IdoA and GlcA units but prefers the former (39, 40). The percentage of 2-O-sulfation of the GlcA units is very low (32), and GlcA2S-GlcNAc disaccharide units have not been reported in HS/heparin. Taken together, biosynthetic constraints make it unlikely that in HS/heparin the epitope of antibody MW3G3 is (IdoA2S-GlcNAc)n (n > 1), although it cannot be excluded that such an epitope, be it very rare, occurs. The epitope may therefore be an oligosaccharide with a core of IdoA2S-GlcNAc, flanked by other disaccharides, or an oligosaccharide in which IdoA2S and GlcNAc residues are scattered.

Given the notion that at least one IdoA2S and one GlcNAc residue are present in the epitope, one can speculate which sites in HS/heparin chains are most reactive with the antibody. An HS/heparin chain is made up of N-acetylated (NA), N-sulfated (NS), and N-acetylated/N-sulfated (NA/NS) or mixed domains. The total number of IdoA is almost evenly distributed over NS and mixed domains. However, almost all IdoA are 2-O-sulfated in the NS domains, whereas almost none are in the NA/NS domains. Since 2-O-sulfated IdoA are largely restricted to NS domains, it is predicted that the location of the MW3G3 epitope is at the interface of NS domains and the adjacent NA/NS regions (41) in sequences of the type GlcNS-IdoA2S-GlcNAc-GlcA-.

Immunohistochemical staining using antibody MW3G3 revealed a very specific expression pattern in rat tissues. The MG3G3 epitope is almost exclusively found in basal lamina of most, but not all, blood vessels and of some epithelia, indicating expression of IdoA2S- and GlcNAc-containing glycosaminoglycans in these structures. Remarkable is the absence of the epitope in extracellular matrix structures present in melanoma metastases, which are very rich in GAGs (42). The absence of IdoA2S/GlcNAc residues in melanoma metastasis is in line with the

| Tissue sections of snail and rat were analyzed by immunofluorescence for reactivity with antibody MW3G3. Reactivity is indicated as follows: +++, strong; +, moderate; +/-, weak; −, negative. |
| Tissue | MW3G3 reactivity |
|-------|------------------|
| A. fulica | + |
| AS-containing vesicles | + |
| Rat | + |
| Intestine | ++ |
| Cryptic epithelium | — |
| Villus epithelium | ++ |
| Blood vessel network in villi | — |
| Paneth cells (granules) | ++ |
| Liver | — |
| Interlobular vein | — |
| Interlobular artery | — |
| Interlobular bile ductule | — |
| Sinusoids | — |
| Central vein | ++ |
| Pancreas | — |
| Acini | — |
| Intercalating duct | + |
| Capillaries (in between acini) | ++ |
| Large blood vessels | — |
| Islets of Langerhans | — |
| Capillaries | ++ |
| Cells at the periphery (most likely α-cells) | ++ |
| Kidney | — |
| Glomerulus | — |
| Bowman’s capsule | — |
| Peritubular capillaries | — |
| Tubuli | — |
| Transitional epithelium (calyx) | + |
| Capillaries (associated with transitional epithelium) | + |
| Testis | + |
| Seminiferous tubuli | — |
| Interstitial tissue | — |
| Tongue | — |
| Epithelium | + |
| Blood vessels in lamina propria | — |
| Secretory ducts | — |
| Blood vessels (perimysium) | — |
| Capillaries (endothelium) | — |
| Myelinated nerve | — |
| Capillaries (in nerve) | — |

**Fig. 8. Immunolocalization of the HS epitope recognized by antibody MW3G3 in human skin, atypical nevi and melanoma metastasis.** Cryosections of human skin, atypical nevi, and melanoma metastasis were stained with antibody MW3G3. The origin of the sections is indicated in the lower right corner (Skin, normal skin; AN, atypical nevi; MM, metastatic melanoma). MW3G3 staining patterns are shown on the left, and IO3H10 (anti-chondroitin sulfate) staining patterns are shown on the right. Note that antibody MW3G3 stained the basal lamina of the epithelium in normal skin and atypical nevi (arrows). No staining was observed in the extracellular matrix of metastatic melanoma, which is rich in glycosaminoglycans (as demonstrated by antibody IO3H10). In addition, antibody MW3G3 does not stain with blood vessels (arrowheads), whereas antibody IO3H10 does (arrowheads). Bar, 25 μm.
observation that AS can act as an anti-tumor agent (5), suggesting that AS-like residues are inhibitory for tumor growth.

In conclusion, we have developed an antibody that strongly reacts with AS and a rare oligosaccharide structure present in HS/heparin. Reduced levels of AS-like residues are inhibitory for tumor growth. AS-like residues are essential for antibody recognition.

**REFERENCES**

1. Kim, Y. S., Jo, Y. Y., Chang, I. M., Toida, T., Park, Y., and Linhardt, R. J. (1996) *J. Biol. Chem.* 271, 11750–11755
2. Jeong, J., Toida, T., Muneta, Y., Kusuhori, I., Imanari, T., Linhardt, R. J., Choi, H. S., Wu, S. J., and Kim, Y. S. (2001) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 130, 513–519
3. Wang, H., Toida, T., Kim, Y. S., Shin, K. H., Ryu, N., and Ohuchi, K. (2002) *Br. J. Pharmacol.* 137, 441–448
4. Lee, Y. S., Yang, H. O., Shin, K. H., Choi, H. S., Jung, S. H., Kim, Y. M., Oh, D. K., Linhardt, R. J., and Kim, Y. S. (2003) *Eur. J. Pharmacol.* 465, 191–198
5. Denissen, M. A., Jenniskens, G. J., Pfeffers, M., Versteeg, E. M., Petou, M., Veerkamp, J. H., and van Kuppevelt, T. H. (2002) *J. Biol. Chem.* 277, 10982–10986
6. Jenniskens, G. J., Oosterhof, A., Brandwijk, R., Veerkamp, J. H., and van Kuppevelt, T. H. (2000) *J. Neurosci.* 20, 4099–4111
7. van de Westerlo, E. M., Smetsers, T. F., Dennissen, M. A., Linhardt, R. J., Veerkamp, J. H., van Muijen, G. N., and van Kuppevelt, T. H. (2002) *Blood* 99, 2437–2443
8. van Kuppevelt, T. H., Dennissen, M. A., van Venrooij, W. J., Hoet, R. M., and Veerkamp, J. H. (1998) *J. Biol. Chem.* 273, 12680–12686
9. Nussinov, A., Hogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., and Winter, G. (1994) *EMBO J.* 13, 692–698
10. van den Born, J., Jann, K., Assmann, K. J., Linhardt, R., and Berden, J. H. (1996) *J. Biol. Chem.* 271, 22802–22809
11. David, G., Bai, X. M., Van der, S. B., Cassiman, J. J., and Van den Bergh, H. (1992) *J. Cell Biol.* 119, 961–975
12. van de Lest, O. C., Versteeg, E. M., Veerkamp, J. H., and van Kuppevelt, T. H. (1994) *Anal. Biochem.* 221, 356–361
13. Wu, S. J., Chun, M. W., Shin, K. H., Toida, T., Park, Y., Linhardt, R. J., and Kim, Y. S. (1998) *Thromb. Res.* 92, 273–281
14. Ishihara, M., Kariya, Y., Kikuchi, H., Minamisawa, T., and Yoshida, K. (1997) *J. Biol.Chem.* (Tokyo) 121, 343–349
15. Feyzi, E., Tryhula, E., Bergstrom, T., Lindahl, U., and Spillmann, D. (1997) *J. Biol. Chem.* 272, 24850–24857
16. Spillmann, D., Witt, D., and Lindahl, U. (1996) *J. Biol. Chem.* 271, 15457–15465
17. Goger, B., Halden, Y., Rek, A., Moi, R., Pye, D., Gallagher, J., and Kungl, A. J. (2002) *Biochemistry* 41, 1640–1646
18. Ostrovsky, O., Berman, B., Gallagher, J., Mulloy, B., Fernig, D. G., Delehedde, M., and Ron, D. (2002) *J. Biol. Chem.* 277, 2444–2453
19. ten Dam, G. B., Hafmans, T., Veerkamp, J. H., and van Kuppevelt, T. H. (2003) *J. Histochem. Cytochem.* 51, 727–739
20. ten Dam, G. B., Hafmans, T., Veerkamp, J. H., and van Kuppevelt, T. H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 2698–2703
21. Merry, G. L., Bullock, S. L., Swan, D. C., Backen, A. C., Lyon, M., Beldingdon, R. S., Wilson, Y. A., and Gallagher, J. T. (2001) *J. Biol. Chem.* 276, 35429–35434
22. Cheung, W. F., Eriksson, I., Kusche-Gullberg, M., Lindahl, U., and Kjellen, L. (1996) *Biochemistry* 35, 5250–5256
23. Pikas, D. S., Eriksson, I., and Kjellen, L. (2000) *Biochemistry* 39, 4552–4558
24. Pikas, D. S., Eriksson, I., and Kjellen, L. (2000) *Biochemistry* 39, 4552–4558
25. van den Born, J., Pikas, D. S., Pisa, B. J., Eriksson, I., Kjellen, L., and Berden, J. H. (2003) *Glycobiology* 13, 1–10
26. Bai, X. and Esko, J. D. (1996) *J. Biol. Chem.* 271, 17111–17117
27. Smetsers, T. F., van de Westerlo, E. M., ten Dam, G. B., Overes, I. M., Schalkwijk, J., van Muijen, G. N., and van Kuppevelt, T. H. (2004) *Invest. Dermatol.* 122, 707–716
28. Esko, J. D., and Selleck, S. B. (2002) *Annu. Rev. Biochem.* 71, 435–471
29. Jayson, G. C., Lyon, M., Parasekova, C., Turnbull, J. E., Deakin, J. A., and Gallagher, J. T. (1998) *J. Biol. Chem.* 273, 51–57
30. Kato, M., Wang, H., Bernfield, M., Gallagher, J. T., and Turnbull, J. E. (1994) *J. Biol. Chem.* 269, 18881–18890
31. Lyon, M., Deakin, J. A., and Gallagher, J. T. (1994) *J. Biol. Chem.* 269, 11209–11215
32. Maccarana, M., Sakuya, T., Tawada, A., Yoshida, K., and Lindahl, U. (1996) *J. Biol. Chem.* 271, 17804–17810
33. Biancati, P., Liverani, I., Masellini, G., and Parma, B. (1997) *Semin. Thromb. Hemost.* 23, 3–10
34. Lindahl, U., Kusche, G. M., and Kjellen, L. (1998) *J. Biol. Chem.* 273, 24979–24982
35. Backstrom, G., Hook, M., Lindahl, U., Feingold, D. S., Malmstrom, A., Roden, L., and Jacobsen, I. (1979) *J. Biol. Chem.* 254, 2975–2982
36. Hagner, M. A., Hannesson, H. H., Campbell, P., Westley, J., Roden, L., Lindahl, U., and Li, J. P. (2000) *Glycobiology* 10, 159–171
37. Jacobsen, I., Lindahl, U., Jensen, J. W., Roden, L., Prihar, H., and Feingold, D. S. (1984) *J. Biol. Chem.* 259, 1056–1063
38. Lindahl, U., Jacobsson, I., Hook, M., Backstrom, G., and Feingold, D. S. (1976) *Biochem. Biophys. Res. Commun.* 70, 492–499
39. Rong, J., Habuchi, H., Kimata, K., Lindahl, U., and Kusche, G. M. (2000) *Biochem.* 346, 463–468
40. Rong, J., Habuchi, H., Kimata, K., Lindahl, U., and Kusche-Gullberg, M. (2001) *Biochemistry* 40, 5548–5555
41. Murphy, K. J., Merry, C. L., Lyon, M., Thompson, J. E., Roberts, I. S., and Gallagher, J. T. (2004) *J. Biol. Chem.* 279, 27239–27245
42. Smetsers, T. F., van de Westerlo, E. M., ten Dam, G. B., Claris, R., Versteeg, E. M., van Gelof, W. L., Veerkamp, J. H., van Muijen, G. N., and van Kuppevelt, T. H. (2003) *Cancer Res.* 63, 2965–2970
Detection of 2-O-Sulfated Iduronate and N-Acetylglucosamine Units in Heparan Sulfate by an Antibody Selected against Acharan Sulfate (IdoA2S-GlcNAc)₄

Gerdy B. ten Dam, Els M. A. van de Westerlo, Toon F. C. M. Smetsers, Marieke Willemse, Goos N. P. van Muijen, Catherine L. R. Merry, John T. Gallagher, Yeong S. Kim and Toin H. van Kuppevelt

J. Biol. Chem. 2004, 279:38346-38352.
doi: 10.1074/jbc.M404166200 originally published online July 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404166200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 23 of which can be accessed free at http://www.jbc.org/content/279/37/38346.full.html#ref-list-1