Tumorigenesis and Neoplastic Progression

Antibody GD3G7 Selected against Embryonic Glycosaminoglycans Defines Chondroitin Sulfate-E Domains Highly Up-Regulated in Ovarian Cancer and Involved in Vascular Endothelial Growth Factor Binding

Gerdy B. ten Dam,* Els M.A. van de Westerloo,*
Anurag Purushothaman,[†] Radu V. Stan,[‡]
Johan Bulten,[§] Fred C.G.J. Sweep,[¶]
Leon F. Massuger,^{||} Kazuyuki Sugahara,[†]
and Toin H. van Kuppevelt*

From the Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, and the Departments of Pathology,[§] Chemical Endocrinology,[¶] and Obstetrics and Gynaecology,^{||} Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands; the Department of Biochemistry,[†] Kobe Pharmaceutical University, Kobe, Japan; and the Department of Pathology,[‡] Dartmouth Medical School, Lebanon, New Hampshire*

Chondroitin sulfate (CS) is abundantly present in the tumor stroma, and tumor-specific CS modifications might be potential targets to influence tumor development. We applied the phage display technology to select antibodies that identify these tumor-specific CS modifications. Antibody GD3G7 was selected against embryonic glycosaminoglycans, and it reacted strongly with CS-E (rich in GlcA-GalNAc4S6S units). In ovarian adenocarcinomas, strong expression of this CS-E epitope was found in the extracellular matrix, and occasionally on tumor cells. No expression was found in normal ovary and cystadenomas. Differential expression was found in ovarian carcinoma cell lines, which correlated with the gene expression of the GalNAc4S-6st enzyme, involved in biosynthesis of CS-E. Vascular endothelial growth factor (VEGF)-sensitive fenestrated (in normal tissues) and tumor blood vessels were both identified by antibody GD3G7, which might implicate a role for CS-E in VEGF biology. VEGF bound to CS-E and antibody GD3G7 could compete for binding of VEGF to CS-E. In conclusion, antibody GD3G7 identified rare CS-E-like structures that were strongly expressed in ovarian adenocarcinomas. This antibody might therefore be instrumental for identifying tumor-related CS

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Chondroitin sulfate (CS) and dermatan sulfate (DS) are sulfated linear polysaccharides, which are synthesized as side-chains of proteoglycans (PGs) and are found in the extracellular matrix (ECM) or bound to cell surfaces.¹ CS/DSPGs are involved in many biological and pathological processes. In cancer, alterations in the expression pattern and levels of the protein cores as well as of the carbohydrate moiety are observed. Expression of decorin, a small leucine-rich PG, is decreased in ovarian cancer,² whereas overexpression is observed in other types of cancer.^{3,4} Versican, a large ECM CSPG is overexpressed in the stroma of ovarian cancer⁵ and many other cancers. Correlation of versican expression and increased risk has been observed in, eg, prostate cancer but not in ovarian cancer.⁵ The CS amount in tumors is often increased. In gastric cancer, a 2.5-fold net increase was observed. Moreover, the composition or CS-sulfation pattern is changed in pancreatic and gastric carcinomas as altered levels of the nonsulfated and 6-O-sulfated disaccharide

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Current address for K.S.: Laboratory of Proteoglycan Signaling and Therapeutics, Faculty of Advanced Life Science, Graduate School of Life Science, Hokkaido University, Frontier Research Center for Post-Genomic Science and Technology, Kita-ku, Japan.

Address reprint requests to Gerdy B. ten Dam, Department of Biochemistry 280, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, PO. Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: g.tendam@ncmls.ru.nl.

units were observed.^{6,7} Moreover, it was demonstrated that chondroitinase-AC and -B (CS/DS-degrading enzymes) treatment of melanoma cells resulted in decreased proliferation and invasiveness of tumor cells suggesting a role for CS/DS in metastasis.⁸ Besides their role in pathological processes, CS/DSPGs participate in normal biological processes such as neuronal development, morphogenesis, growth factor binding, and signaling.^{9–12} Highly sulfated CS structures show specific binding interactions with heparin-binding growth factors such as hepatocyte growth factor, fibroblast growth factor-2 (FGF2), pleiotrophin, midkine, and heparin co-factor II.^{9,12} Moreover, they are involved in leukocyte trafficking by binding to P- and L-selectin.¹³ Despite the important roles of these highly sulfated CS structures in diverse biological processes, there is a limited number of reports that focus on CS/DSPGs that contain these disaccharide units.

The sugar backbone of CS consists of repetitive disaccharide units composed of D-glucuronic acid (GlcA) and N-acetyl galactosamine (GalNAc) residues, which are variably modified by O-sulfation (2-O, 4-O, and 6-O). DS is a stereo-isomeric variant of CS with varying amounts of GlcA epimerized into L-iduronic (IdoA) acid residues. The structural variability is generated by several sulfotransferases creating monosulfated, disulfated or, very rarely, trisulfated disaccharide units.^{1,14} The monosulfated A or C unit consists of GlcA-GalNAc4S or GlcA-GalNAc6S disaccharide units, respectively. DS is the isomeric variant of CS-A and contains IdoA-GalNAc4S (iA). The disulfated disaccharide units consist of GlcA2S-GalNAc6S (D unit), IdoA2S-GalNAc6S (iD unit), GlcA-GalNAc4S6S (E unit), or IdoA-GalNAc4S6S (iE or H unit).^{1,15} The monosulfated disaccharide units (A and C) are common, major components of CS chains, whereas the oversulfated disaccharides units like the iD/D and iE/E units are rather rare, although significant proportions of these units have been detected in mammalian tissues. To study CS alterations in ovarian cancer, we selected, using the phage display technology, antibody GD3G7 that strongly reacted with CS-E epitopes. These CS-E epitopes are expressed in specific vascular moieties in normal tissues and in ovarian carcinomas and are involved in vascular endothelial growth factor (VEGF) binding.

Materials and Methods

Materials

HS from bovine kidney and CS-C (contains considerable amounts of CS-A) from shark cartilage were from Sigma (St. Louis, MO). DS from porcine intestinal mucosa was from Celsus Laboratories Inc. (Cincinnati, OH). CS-A from sturgeon notochord, CS-D from shark cartilage, and CS-E from squid cartilage were from Seikagaku (Tokyo, Japan). Chondroitinase ABC (C-3667), AC (C-2780), and B (C-8058) were from Sigma-Aldrich (St. Louis, MO). The anti-VSV-tag mouse hybridoma cell line P5D4 was obtained from the American Type Culture Collection (Rock-

ville, MD; IgG). Anti-rat VEGF (AF564) was obtained from R&D Systems (Minneapolis, MN). The chicken anti-rat PV-1 (plasmalemmal vesicle protein-1, diluted 1:500) antibody was kindly provided by Dr. Radu V. Stan (Dartmouth Medical School, Lebanon, NH).

Animals, Human Tumor Tissues, and Cell Lines

Rats (Wistar, embryos E18 and males 8 weeks) and mouse embryos (E18) were obtained from the Central Animal Laboratory (Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands). Human ovarian tissues from normal ovary ($n = 1$), cystadenomas ($n = 2$), and cystadenocarcinomas [$n = 15$; subdivided into endometrioid ($n = 3$), clear cell ($n = 2$), serous ($n = 8$), and mucinous ($n = 2$)] were obtained from the archives of the Institute of Pathology of the Radboud University Nijmegen Medical Center. All samples were handled in a coded manner according to local ethical guidelines. All ovarian tissue sections were reviewed by an experienced gynecopathologist (J.B.). The human ovarian carcinoma cell lines SKOV-3 (clear cell adenocarcinoma), OVCAR-3 (poorly differentiated serous adenocarcinoma), and OVCAR-4 (ovarian adenocarcinoma) were generously provided by Dr. L.G. Poels (Radboud University Nijmegen Medical Center). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Paisley, UK).

Selection, Expression, and Purification of Anti-Glycosaminoglycan (GAG) Antibodies

GAGs from rat embryos (E18) and ovarian adenocarcinomas were isolated using standard procedures and analyzed by agarose gel electrophoresis.¹⁶ The human semisynthetic single-chain variable fragment (scFv) library no. 1¹⁷ was generously provided by Dr. G. Winter (Medical Research Council Molecular Biology, Cambridge, UK) and used to select single chain variable fragment (scFv) antibodies. The selection of phages displaying scFv antibodies and scFv antibody production and purification were performed as described previously.^{18–20}

Evaluation of Specificity by Enzyme-Linked Immunosorbent Assay (ELISA)

To study the specificity of antibody GD3G7, an indirect ELISA was performed using different GAGs (HS, CS-A, DS, CS-C, CS-D, and CS-E) as described previously.²¹ To determine further the specificity, a competition ELISA was performed. Fixed amounts of antibody GD3G7 were mixed with increasing amounts of CS-E or CS-A (0.5 to 50 $\mu\text{g/ml}$) and added to CS-E-coated ELISA plates. Bound antibody was detected as described previously. All assays were performed at least three times, and representative results are shown.

Evaluation of Specificity by Immunohistochemistry

Immunofluorescence analysis with antibody GD3G7 on cryosections of rat and human ovarian tissues was performed as described before.^{20,22} As a control, primary antibodies were omitted, or an irrelevant antibody was used. Double-labeling experiments on rat tissue sections were performed using antibody GD3G7 and chicken anti-PV-1 antibody. Ovarian carcinoma cells were identified using anti-keratin 7 antibody OV-TL 12/30²³ generously provided by Dr. L.G. Poels, and CS was identified using antibody CS-56 (Sigma). To evaluate the specificity of the antibody, tissue sections were pretreated with chondroitinase-ABC (digest CS/DS), chondroitinase-AC (digest CS), or chondroitinase-B (digest DS) (all obtained from Sigma) to remove all CS/DS according to standard procedures. As a control, tissue sections were incubated with reaction buffer without enzyme. After CS/DS removal, tissue sections were washed and processed for immunofluorescence analysis as described. Tissue sections were also incubated with mixtures of antibody and GAG (CS-A or CS-E; 1, 10, and 20 $\mu\text{g/ml}$) and processed for immunofluorescence analysis as described.

For immunohistochemical analysis human ovarian tumor cryosections were fixed with 4% paraformaldehyde, washed in Tris-based saline, and blocked in 2% bovine serum albumin in phosphate-buffered saline. Next, tissue sections were incubated with antibody, and bound antibodies were detected by anti-VSV antibodies. Rabbit anti-mouse antibodies were used to detect the anti-VSV antibodies. The bound antibodies were detected by anti-mouse antibodies followed by incubation with the peroxidase anti-peroxidase mouse complex (Dakopatts, Glostrup, Denmark), which was visualized using diaminobenzidine substrate (Dakopatts). Tissue sections were counterstained using hematoxylin solution according to Mayer (Sigma-Aldrich Chemie, Steinheim, Switzerland) and permanently mounted with entellan (Merck, Darmstadt, Germany).

GalNAc4S-6st Gene Expression Analysis

RNA was isolated from ovarian tumor cell lines using the RNA-bee (Tel-Test Inc., Friendswood, TX) method, and 2 μg of RNA was reverse-transcribed for 1 hour at 42°C using 200 U of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and 100 ng of random hexamer primers (Invitrogen). cDNA fragments were amplified by polymerase chain reaction (PCR) with the forward and reverse GalNAc4S6st (sense: 5'-CATCCCCAACAAATTCCTTCC-3'; anti-sense: 5'-GCGCAGTGAATAATCAAGCATGC-3') or the GAPDH primers (sense: 5'-GGTATCGTGGAAGGACTCAT-3' anti-sense: 5'-ACCACTGGTGCTCAGTGTA-3') (Bioscience BV, Maarsen, The Netherlands) using *Taq* DNA polymerase (Roche, Indianapolis, IN) according to the manufacturer's protocol. Samples were amplified using the following protocol: 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C for 35 cycles. The PCR product was analyzed by agarose gel electrophoresis.

Binding of VEGF to CS-E

To study the binding capacity of VEGF to GAGs, we performed an indirect ELISA. CS-A, CS-C, CS-E, and HS were immobilized in microtiter plates and incubated with increasing amounts of recombinant rat VEGF (1 to 10 $\mu\text{g/ml}$ ²⁴), and bound VEGF was detected using anti-VEGF antibodies (AF654; R&D Systems) and alkaline phosphatase-conjugated antibodies. Enzyme activity was detected as described. To determine whether antibody GD3G7 could compete with VEGF for binding to CS-E, a competition ELISA was performed. Therefore, increasing amounts of antibody (0 to 100 $\mu\text{g/ml}$) were incubated with a constant amount of VEGF (1 $\mu\text{g/ml}$), and bound VEGF was detected as described.

Results

Selection of Antibodies against Tumor-Specific GAGs

Antibodies were selected using the phage display technology directed against rat embryo (E18) GAGs as a source for carcinoembryonic antigens. These GAGs were mainly composed of CS and contained only small amounts of HS and DS (Figure 1A) as analyzed by agarose gel electrophoresis. GAGs from ovarian tumor tissues (Figure 1B) contained increased amounts of CS as compared with normal ovary, which contained mainly HS and DS. Antibody GD3G7 was selected and showed in initial immunofluorescence analysis positive reactivity with the tumor matrix of ovarian tumors and with cartilage present in the embryo (mouse E18, no reactivity was observed with other structures). DNA sequence analysis revealed that antibody GD3G7 belongs to the V_H3 family, has a DP 38 germline gene segment, and contains the heavy chain complementarity-determining region 3 (CDR3) amino acid sequence GRWTQMT.

Specificity of Antibody GD3G7

Ovarian carcinoma cryosections were treated with chondroitinase-ABC, -AC, and -B and were subsequently stained with antibody GD3G7 to determine the specificity of antibody GD3G7 (Figure 1C). Strong reactivity was still observed in the tissue sections treated with no lyase and with chondroitinase-B (digest DS), whereas the reactivity was completely abolished after treatment with chondroitinase-ABC (digest CS and DS) and chondroitinase-AC (digest CS) indicating that antibody GD3G7 reacted with a CS-like epitope. To differentiate between various CS preparations, an indirect ELISA approach was used to determine the specificity of antibody GD3G7 (Figure 1D and Table 1). Antibody GD3G7 reacted strongly with CS-E, which consists of 61.5% GalNAc4S6S disaccharide units,²⁵ and to a minor extent with CS-A. No reactivity was observed with DS, CS-C, CS-D, and HS. These results suggested that disulfated GalNAc (E units) residues were important for antibody recognition. Competition ELISA demonstrated that antibody GD3G7 reacted much

Figure 1. Evaluation of the specificity of antibody GD3G7. GAGs were isolated from rat embryo E18 (**A**) and ovarian tissue samples (**B**) and analyzed by agarose gel electrophoresis. Rat embryo GAGs, eluted with 0.5 mol/L and 1.0 mol/L NaCl, contained mainly CS. GAGs from normal ovary contained mainly HS and DS, and increased amounts of CS were observed in the ovarian carcinomas (p1, serous adenocarcinoma; p2, endometrioid adenocarcinoma; p3, serous adenocarcinoma). **C:** The specificity of antibody GD3G7, selected against rat embryo GAGs, was determined by lyase digestion of ovarian carcinoma tissue sections. Cryosections were incubated with buffer (without enzyme), chondroitinase-ABC (ABCcase), chondroitinase-AC (ACase), and chondroitinase-B (Base) and subsequently stained with antibody GD3G7. Reactivity of antibody GD3G7 was lost after chondroitinase-ABC and -AC digestion. **D:** Next, the reactivity of antibody GD3G7 with immobilized HS, CS-A, DS, CS-C, CS-D, and CS-E was analyzed by indirect ELISA. A strong reactivity was observed with CS-E. Bars represent mean $\pm$ SD ($n = 3$). Scale bar = 50 μ m.

stronger with CS-E compared with CS-A, and competition studies on ovarian carcinoma and rat kidney tissue sections revealed that only 1 μ g/ml CS-E was able to com-

Table 1. Immunoreactivity of Antibody GD3G7 with Various CS/DS Subtypes

CS/DS	Isolated from	Structure	Reactivity with GD3G7
CS-A	Sturgeon notochord	GlcA-GalNAc4S	+
CS-B/DS	Pig intestinal mucosa	IdoA-GalNAc4S	—
CS-C	Shark cartilage	GlcA-GalNAc6S	—
CS-D	Shark cartilage	GlcA2S-GalNAc6S	—
CS-E	Squid cartilage	GlcA-GalNAc4S6S (61.5%) ²⁵	+++
CS-H	Hagfish notochord	IdoA-GalNAc4S6S (54%) ²⁶	++ ²⁸
SS-DS	Shark skin	GlcA/IdoA-GalNAc4S6S (10%) ²⁷	++ ²⁸

Reactivity of antibody GD3G7 is indicated as +++, strong; ++, moderate; +, weak; and —, no reactivity.

pete for binding of antibody GD3G7 to the tissue sections, whereas up to 20 μ g/ml CS-A was unable to do the same (data not shown). Moreover, antibody GD3G7 also showed strong reactivity with CS-H^{1,26} and SS-DS (shark skin DS²⁷), of which both are rich in E and iE units (Table 1), indicating that GlcA-GalNAc4,6S as well as IdoA-GalNAc4,6S disaccharide units could be recognized.²⁸

Immunolocalization of the CS Structures Recognized by Antibody GD3G7 in Embryo and Normal Rat Tissues

Normal mouse embryo tissue sections (E18) were analyzed for reactivity with antibody GD3G7. Very intense staining of all cartilage structures was observed whereas no staining was observed in all other tissues (liver, lung, intestine, muscle, and so forth) except for minor staining observed in the developing glomeruli in the kidney and in the hair follicles in the skin (Figure 2 and Table 2). Normal rat tissue sections (ear, intestine, kidney, liver, pancreas, spleen, and testis) were analyzed for reactivity with antibody GD3G7. Like in the embryo, strong staining was observed in the cartilage (ear, data not shown). Furthermore, a very restricted staining pattern was observed, ie, a subset of small blood vessels and capillaries stained positive with antibody GD3G7 (Figure 3, left; and Table 2). Immunofluorescence analysis using anti-rat CD31 and GD3G7 antibodies confirmed that all blood vessels and capillaries identified by antibody GD3G7 were CD31-positive, but not all CD31-positive blood vessels were GD3G7-positive (data not shown). In villi of the intestine, the capillaries directly beneath the intestinal epithelium stained positive (Figure 3). In the kidney specific capillaries stained positive, whereas other capillaries, larger blood vessels, and the glomeruli were not reactive with antibody GD3G7. Capillaries in the islets of Langerhans of the pancreas stained very strong with antibody GD3G7, whereas weak staining of capillaries in the exocrine part of the pancreas was observed (Figure 3). The specific location of the capillaries identified by antibody

Figure 2. Immunolocalization of CS structures recognized by antibody GD3G7 in mouse embryo. Cryosections of mouse embryo E18 (cartilage, kidney, skin, and lung-diaphragm-liver) were stained with antibody GD3G7. Very strong staining was observed in all cartilage structures in the embryo, and weak staining was observed in the developing glomeruli in the kidney and in the hair follicles in the skin. No staining was observed in the lung, diaphragm (muscle), and liver. Scale bar = 50 μ m.

GD3G7 suggested that these were fenestrated capillaries. To confirm this observation, double-staining experiments were performed with an antibody against PV-1 (Figure 3, right). PV-1 (plasmalemmal vesicle 1) is a component of fenestral and stomatal diaphragm in fenestrated endothelia, which is not present in continuous endothelium of muscle and brain or in nondiaphragm fenestrated endothelium of the kidney glomeruli.^{29,30} Co-localization was observed in capillaries identified by antibody GD3G7 and anti-PV-1 antibodies in rat intestine, kidney, and pancreas. The anti-PV-1 antibody recog-

Figure 3. CS structures recognized by antibody GD3G7 co-localize with PV-1-positive capillaries in normal rat tissues. Cryosections of normal rat tissues (intestine, kidney, and pancreas) were double-stained with GD3G7 and anti-PV-1 (fenestrated capillaries) antibodies. Antibody GD3G7 recognized CS structures in capillaries that co-localize with PV-1-positive capillaries. Not all PV-1-positive blood vessels express the CS structures recognized by antibody GD3G7. Other structures such as BMs, stroma, and cells were not reactive with antibody GD3G7. Scale bar = 50 μ m.

Table 2. Immunoreactivity of Antibody GD3G7 in Normal and Embryonic Tissues

	Location	Staining intensity
Embryo E18 (mouse)	Cartilage	+++
	Developing glomeruli in the kidney	+
	Hair follicles	+
	Liver	—
	Intestine	—
	Lung	—
Adult tissues (rat)	Muscle	—
	Ear	+++
	Intestine (small)	+++
	Subepithelial capillaries in the villi	—
	Stroma villi	—
	Kidney	+++
	Peritubular capillaries	—
	Glomeruli	—
	Liver	—
	Pancreas	+++
Spleen	Endocrine capillaries	+
	Exocrine capillaries	—
	Testis	—
	Interstitial capillaries	+++
	Interstitial larger blood vessels	—

Tissue localization and staining intensity of antibody GD3G7 are indicated. +++, strong; ++, moderate; +, weak; and —, no staining.

nized peritubular capillaries as well as other blood vessels (eg, vasa recta) in the rat kidney (Figure 3, right).³¹ In kidney only a subset of PV-1-positive capillaries was identified by antibody GD3G7, whereas in intestine and pancreas almost all PV-1-positive capillaries were identified by antibody GD3G7. Note that no staining was observed in the rat spleen and liver. Moreover, no reactivity was observed with antibody GD3G7 in CS-rich structures like the interstitial stroma of the tissues.

Table 3. Immunoreactivity of Antibody GD3G7 in Ovarian Tumor Tissues

	Surface epithelium	BM of surface epithelium	Stroma
Normal ovary	—	—	—
Cystadenomas	—	—†	—
	Tumor cells	BM of tumor cells	Stroma
Adenocarcinomas			
Endometrioid	—*	+++	+++
Clear cell	—*	+++	+++
Serous	—	+++	+++
Mucinous	—	++	++

The immunoreactivity of antibody GD3G7 was analyzed in ovarian carcinoma tissues, the location and staining intensity are indicated. +++, strong; ++, moderate; +, weak; and —, no staining. BM, basement membrane.

*Sporadic positive staining of the surface of tumor cells is observed.

†Depending on the grade of epithelial alterations slight increase in staining near the basement membrane is observed.

Figure 4. Immunolocalization of CS structures recognized by antibody GD3G7 in ovarian tumors. Immunohistochemical analysis of ovarian cystadenoma and cystadenocarcinoma cryosections with antibody GD3G7. **A:** No staining was observed in the ovarian cystadenomas (**a** and **b**) except for some very minor staining of the stroma directly underlining the cyst epithelium (**a** and **b**, arrow). Very strong staining of the stroma and the BMs of tumor cells and blood vessels was observed in ovarian adenocarcinomas as demonstrated for endometrioid (**c**), serous (**d**), clear cell (**e**), and mucinous (**f**) adenocarcinomas. Tumor cells of ovarian endometrioid and clear cell adenocarcinoma stained positive with antibody GD3G7 as illustrated in **B**. Positive tumor cells of an endometrioid adenocarcinoma are indicated by an arrow. Scale bars: 100 μ m (**A**); 25 μ m (**B**).

Immunolocalization of CS Structures Recognized by Antibody GD3G7 in Human Ovarian Tumors

A panel of 18 ovarian tissue samples including one normal ovary, two ovarian cystadenomas, and 15 ovarian cystadenocarcinomas were analyzed for reactivity with antibody GD3G7 (Table 3 and Figure 4A). No staining was observed in the normal ovary and in the cystadenomas whereas strong staining was observed in all carcinomas analyzed. In normal ovary, no staining was observed in the stromal compartments (cortex and medulla), and, likewise, no staining was observed in the basement membrane (BM) of the mesothelial cells, the epithelial cell layer covering the ovaries. Both structures are rich in GAGs (not shown). However, very weak expression was observed in the stroma directly underlining epithelial cells of particular parts of the cyst-epithelium that showed alterations in cell morphology and cell number (although no malignant alterations were observed; Figure 4A, arrows in **a** and **b**). Very strong staining of the ECM was observed in endometrioid, serous, clear cell, and mucinous (Figure 4A, **c**–**f**) adenocarcinomas. The staining was present in the stromal compartments and associated with the BM of the tumor cells. Detailed analysis revealed that tumor cells showed occasionally weak ex-

pression of the CS structure recognized by antibody GD3G7 as observed in endometrioid and serous adenocarcinomas (Figure 4B, arrow; and Table 3). In lymph node metastasis of serous adenocarcinomas, strong staining of antibody GD3G7 was found in the tumor stroma and in stroma closely associated with the tumor mass, which might suggest that the epitope recognized by antibody GD3G7 is up-regulated in activated stroma (Figure 5). Normal (lymph node or surrounding) stroma not associated with the tumor mass was not stained with antibody GD3G7. All stroma (or ECM) components were rich in CS as demonstrated with antibody CS-56 (Figure 5).

Gene Expression of the GalNAc4S-6ST Enzyme in Ovarian Carcinoma Cell Lines Coincides with Antibody GD3G7 Expression

The GalNAc4S 6-O-sulfotransferase (GalNAc4S-6st) catalysis is one of the final steps in the CS pathway creating disulfated GalNAc residues, and these disaccharides were specifically recognized by antibody GD3G7. Therefore, the gene expression pattern of GalNAc4S-6st was analyzed in a panel of ovarian carcinoma cell lines and compared with the GD3G7 expression pattern. The ovar-

Figure 5. Immunolocalization of CS structures recognized by antibody GD3G7 in ovarian metastasis. Serial cryosections of two lymph node metastasis of serous ovarian adenocarcinomas (top and bottom) were stained with antibody GD3G7, antibody CS-56, which recognizes a common CS epitope (CS-A and CS-C), and antibody OV-TL12/30, which is specific for keratin-7 and detects ovarian tumor cells. Both anti-CS antibodies detect the tumor stroma (arrow). Moreover, antibody GD3G7 was reactive with activated stroma near the tumor mass (asterisk) and not with normal stroma away from the tumor mass (triangle), whereas antibody CS-56 was reactive with all types of stroma. Scale bar = 50 μ m.

ian carcinoma cell lines OVCAR-3, OVCAR-4, and SKOV-3 showed different gene expression levels of the GalNAc4S-6st enzyme with low levels of expression observed in the OVCAR-4 cells and the highest expression observed in the SKOV-3 cells (Figure 6B). Antibody GD3G7 expression showed similar profiles. Hardly any expression was found in the OVCAR-4 cell line, whereas the highest expression was found in the SKOV-3 cell line. The other cell line, OVCAR-3, stained weak to moderately with antibody GD3G7 (Figure 6A). SKOV-3 ovarian carcinoma cells showed a matrix-like staining pattern, whereas the other cell line, OVCAR-3, showed a cell membrane-staining pattern.

Blockage of VEGF Binding to CS-E by Antibody GD3G7

The observation that fenestrated capillaries and tumor blood vessels, both of which are VEGF-dependent and

leaky, were immunoreactive with antibody GD3G7 led us to investigate the binding-capacity of VEGF with CS-E. VEGF was able to bind to CS-E, in a similar manner as to HS, but no binding was observed with CS-A and CS-C (Figure 7A). Furthermore, antibody GD3G7 could compete with VEGF for binding to CS-E, whereas an irrelevant scFv antibody (MPB49) could not (Figure 7B).

Discussion

CS alterations in ovarian cancer were investigated using antibody GD3G7, which was selected using the antibody phage display technique. This antibody was selected against GAGs from rat embryos (E18) as a source for carcinoembryonic antigens. Antibody GD3G7 reacted very strongly with tumor-specific CS-E epitopes in ovarian adenocarcinomas. The expression in ovarian carcinomas was mainly located in the ECM, ie, the (interstitial)

Figure 6. CS-E expression, recognized by antibody GD3G7, coincidence with GalNAc4S-6st expression in ovarian carcinoma cell lines. **A:** Ovarian carcinoma cells, OVCAR-3, SKOV3, and OVCAR4, were immunostained with antibody GD3G7. Note the strong, matrix-like, staining of antibody GD3G7 in SKOV-3 cells, the weak cellular staining in OVCAR-3 cells, and the absence of staining in OVCAR-4 cells. **B:** Semiquantitative reverse transcription-polymerase chain reaction was performed with RNA isolated from the ovarian carcinoma cells using the GalNAc4S-6st and GAPDH primers. Weak gene expression is observed for the OVCAR-4 cells and strong gene expression was found in the SKOV-3 cells. Scale bar = 50 μ m.

Figure 7. Antibody GD3G7 inhibits VEGF binding to CS-E. **A:** Recombinant VEGF was able to bind to immobilized CS-E and HS and not to CS-A and CS-C, as analyzed by ELISA. **B:** Moreover, antibody GD3G7 was able to compete for binding of VEGF to CS-E in a dose-dependent manner, as analyzed by competition ELISA. The irrelevant antibody MPB49 was not able to do so.

stromal compartments, or associated with the BM of tumor cells and tumor blood vessels. Occasionally, expression of tumor cells was observed. No reactivity was found in normal ovary and also not in the (benign) cystadenomas analyzed. However, increased numbers of normal ovary and ovarian cystadenomas need to be analyzed to confirm this observation. Versican, a large CSPG, is strongly enriched in the malignant stroma of ovarian carcinomas and is associated with unfavorable prognosis but is not an indicator of patient survival.⁵ Decorin a small, leucine-rich PG is found in the stroma of ovarian carcinomas and not on ovarian cancer cells, and it was proposed that decorin might be a tumor suppressor gene that is inactivated during epithelial cell development.² Little is known about the alteration in the fine structure of CS, like the sulfation pattern, in ovarian cancer. In other types of cancer, structural alteration in HS (mostly) and CS have been described.^{6,7,32–34} In addition, anti-GAG antibodies were applied to demonstrate structural changes in the GAG profile between normal skin or nevi and metastatic melanomas.^{35–37} Recently, it was demonstrated that CS epitope levels, detected by antibody WF6, in serum of patients with ovarian cancer were raised.³⁸ The WF6 epitope has recently been determined as CS octasaccharides containing a D unit (P. Pothacharoen, K. Kalayanamitra, S.S. Deepa, T. Hardingham, P.

Kongtawelert, and K. Sugahara, manuscript in preparation), and this study illustrates the potential of new anti-CS antibodies such as antibody GD3G7 that detect CS epitopes raised in ovarian cancer.

Very restrictive staining patterns, unlike that found in the ovarian adenocarcinomas, of antibody GD3G7 were observed both in embryo and normal tissues. Remarkable was the change in expression from that observed in embryonic tissues to that in tissues from the adult. Staining of cartilage, very rich in CS, observed in the embryo remained present in the adult. No staining, however, of blood vessels was observed in the embryonic stage, whereas almost an exclusive expression in capillaries was found in the adult stage, suggesting that these CS-E-like structures are not expressed during the development of the vascular system (vasculogenesis) in the embryo but emerge in the adult vascular system (angiogenesis). Remarkably, not all blood vessels were reactive with antibody GD3G7. Only (a subset of) capillaries in intestine, kidney, and pancreas were strongly reactive. Not all organs were analyzed but the data strongly suggest that the capillaries detected in intestine, kidney, and pancreas were fenestrated capillaries. Fenestrated capillaries (with a diaphragm) are found in these tissues at similar locations.³⁹ Co-localization with PV-1 confirmed this observation. GD3G7-positive capillaries were indeed fenestrated capillaries. PV-1 is a component of fenestral and stomatal diaphragms in fenestrated endothelia and is considered as a marker for fenestrated capillaries.^{29,30} In the kidney, PV-1 is present in peritubular capillaries but also in continuous vessels with stomatal diaphragms such as the vasa recta.³¹ PV-1 is, however, not present in the endothelium of the glomeruli, like the CS structures recognized by antibody GD3G7. In the kidney antibody, GD3G7 identified a subset of these peritubular capillaries maybe suggesting that these capillaries are functionally different. Beside reactivity with these fenestrated capillaries, no reactivity was observed with other structures in the tissues analyzed. Normally, CS expression is found in stroma, as was shown for phage display anti-CS antibodies.³⁶

Fenestrated capillaries and tumor blood vessels both are leaky (fenestrated) and VEGF-dependent.^{40–42} Both types of blood vessels express the CS-E epitope recognized by antibody GD3G7, suggesting a role for CS-E in VEGF biology. We demonstrated that VEGF could bind to CS-E and HS but not to monosulfated CS such as CS-A and CS-C, suggesting that both the 4-O and 6-O sulfates of CS-E are essential for VEGF binding. Whether both modifications are essential for VEGF-mediated signaling, such as 6-O-sulfates present in the S-domains of HS,⁴³ remains to be elucidated. In addition, we demonstrated that antibody GD3G7 could compete with VEGF for binding to CS-E. Growth factors and chemokines such as FGF2, FGF10, and midkine were shown to bind to CS-E,⁹ and now we report that VEGF could also interact with CS-E. It is noteworthy that VEGF weakly binds to CS-H rich in iE units (H units).¹²

The role, however, of CS-E in VEGF biology is still speculative. Recently, it was demonstrated that GAG side chains of neuropilin-1, a co-receptor for VEGF that

enhances the angiogenic signals cooperatively with VEGFR2, are important for responsiveness to VEGF in endothelial cells and smooth muscle cells. The composition of the GAG chains can either be HS or CS, differing between cell types. In endothelial cells, neuropilin modified with HS enhanced VEGF-VEGFR2 signaling, whereas neuropilin expressed by smooth muscle cells modified with CS (50%) down-regulated VEGFR2 expression, suggesting that it acted as a decoy receptor rather than a co-receptor.⁴⁴ The CS chains of neuropilin expressed by endothelial cells and smooth muscle cells do not contain CS-E-like modifications although CS-E is the only CS capable of enhancing VEGF binding to neuropilin in endothelial cells in a similar manner as heparin.⁴⁴ In conclusion, we demonstrate that CS-E is expressed (in large amounts) by blood vessels that are strongly influenced by VEGF and that CS-E is able to sequester VEGF. It will be very interesting to investigate the role of CS-E in VEGF biology, and in that respect, the nature of the GAG chains of neuropilin expressed by the fenestrated capillaries or the tumor blood vessels. Moreover, overexpression of CS-E in ovarian carcinomas might have important implications for growth factor signaling (VEGF) in cancer, especially because CS-E, and not monosulfated CS, was able to compete for binding of VEGF to HS (data not shown). Increased sequestering and most likely increased signaling of growth factors (VEGF) in the tumor might increase the potential of tumor cells to grow and metastasize.

Antibody GD3G7 reacted strongly with CS-E, which consists of GlcA-GalNAc4S6S disaccharide units. Recently, it was demonstrated that antibody GD3G7 also reacted with IdoA-GalNAc4S6S disaccharide units (present in CS-H and shark skin DS) and with a decasaccharide containing a minimum of three consecutive E units,²⁸ suggesting that the GalNAc4S6S residue was most important for antibody recognition irrespective of the presence of a GlcA or IdoA residue. The precise structural composition of the CS epitope recognized by antibody GD3G7 remains to be determined. Immunoprecipitation of CS-oligosaccharides derived from, eg, ovarian cancer and sequence analysis of the precipitated oligosaccharides might reveal the precise nature of the epitope recognized by antibody GD3G7. These experimental procedures will be explored in the near future. Previously, our laboratory selected single chain antibodies against CS-C using the phage display technology. The selected antibodies reacted with CS structures present in CS-A, CS-C, and CS-E (IO3H9, IO3H12, and IO4C2), or more specifically with CS structures present in CS-E (IO3D9).³⁶ All antibodies displayed different (but abundant) staining patterns in normal rat tissues, unlike antibody GD3G7, and strong immunoreactivity was found in melanoma and psoriasis in skin. Monoclonal antibodies, generated according to the conventional method in the mouse, were directed against CS. Ito and colleagues⁴⁵ described the structural characterization of CS epitopes recognized by monoclonal antibodies 473HD, CS-56, and MO-225. Initially antibody 473HD was characterized as an antibody recognizing a CS/DS hybrid structure (referred as the DSD-1 epitope), antibody CS-56 recognized CS-A and

CS-C, and antibody MO-225 was described to react strongly with CS-D. Similar was that they all showed reactivity with CS epitopes containing the D unit (GlcA2S-GalNAc6S). Recently, antibody 2A12 was selected against DS from ascidian *Ascidian nigra* and was specific for iD unit (IdoA2S-GalNAc6S) enriched oligosaccharides, the main structural component of ascidian DS.⁴⁶ Detailed structural characterization of the epitopes recognized by these anti-CS antibodies is essential to determine the natural occurrence and function of these specific CS structures.

In conclusion, we have developed a single chain antibody that strongly reacts with CS-E-like structures and highly depends on the presence of GlcA/IdoA-GalNAc4S6S disaccharide units. The expression of this epitope, recognized by antibody GD3G7, is strongly up-regulated in ovarian carcinomas, and its expression is very restricted in normal tissues. Moreover, expression of this epitope is found in fenestrated and tumor blood vessels, both of which are VEGF-dependent. This raises the suggestion, which needs further exploration, that CS-E plays a role in VEGF biology.

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