Localization and Characterization of Melanoma-associated Glycosaminoglycans: Differential Expression of Chondroitin and Heparan Sulfate Epitopes in Melanoma


ABSTRACT

Glycosaminoglycans (GAGs) are anionic polysaccharides present on cells and in the extracellular matrix (ECM). They likely play a role in tumor formation because of their capacity to bind and modulate a variety of proteins including growth factors, cytokines, and proteases. Using a panel of (human) phage display-derived anti-GAG antibodies, the location and expression of GAG epitopes in human cutaneous melanocytic lesions was studied. Antibodies EW4E1 and EW4G2 identified a melanoma-associated chondroitin sulfate/heparan sulfate epitope, whereas antibody EW4B7 recognized a melanoma-associated heparan sulfate epitope. These antibodies showed a high reactivity with blood vessels and ECM in cutaneous melanoma tumors, whereas their reactivity with nevi was very low. Using a set of defined oligosaccharides it was established that sulfation groups are of main importance in the binding to the antibodies and that glycomimetics can mimic natural oligosaccharides. In xenografts of melanoma cell line MeL57, a strong association of GAG epitopes with an injected fluorescent fluid flow tracer was observed. In uveal melanoma antibody EW4E1 proved to be a sensitive probe for the detection of the geometry of ECM structures, known to have prognostic value. Taken together, data indicate that in melanoma a defined set and location of GAG epitopes are present with possible functional significance.

INTRODUCTION

GAGs are anionic polysaccharides that are located in the ECM and on the cell surface. They form the saccharide component of proteoglycan molecules (1). Especially HS and CS, two classes of GAGs, have been implicated in tumor formation, including melanoma, because of their capacity to bind and modulate a large number of biomolecules important for tumor development (2). These include basic fibroblast growth factor, vascular endothelial growth factor, and melanoma growth-stimulating factor. Many angiogenic factors belong to the class of heparin-binding growth factors, heparin being the glycomimetics can mimic natural oligosaccharides. In xenografts of melanoma cell line MeL57, a strong association of GAG epitopes with an injected fluorescent fluid flow tracer was observed. In uveal melanoma antibody EW4E1 proved to be a sensitive probe for the detection of the geometry of ECM structures, known to have prognostic value. Taken together, data indicate that in melanoma a defined set and location of GAG epitopes are present with possible functional significance.

MATERIALS AND METHODS

scFv Antibodies against GAGs

scFv antibodies with reactivity to heparin/HS were selected by phage display in a previous study (8). These antibodies originate from the semisynthetic antibody library #1, a generous gift from Dr. Greg Winter (Cambridge University, Cambridge, United Kingdom). The antibody genes were subcloned into vector pUC119 containing an 8× histidine stretch, and a VSV-G tag. Periplasmic fractions of bacteria, containing the antibodies, were prepared and used for immunohistochemistry as described (8). Six antibodies (Table 1) were selected based on their reactivity with various GAGs (8). All six of the antibodies recognize a different GAG epitope. An irrelevant scFv antibody (TSC01) without reactivity towards GAGs was used as a negative control (8).

Immunohistochemistry

Localization of GAG Epitopes. Human cutaneous melanocytic lesions, 10 MMs, 1 PM, 6 ANs, 3 NN, and 10 human uveal melanomas with differences in ECM constitution were used. Wistar rat kidney was also used. Tissue was snap-frozen in liquid nitrogen and stored at −80°C until use. Cryosections (5 μm) were prepared and incubated with various concentrations of anti-GAG antibodies. Bound antibodies were visualized by subsequent incubations with mouse anti-VSV antibody P5D4, a biotinylated horse antimouse antibody
Table 1 Characteristics of anti-GAG antibodies and their reactivity with human melanoma and nevi

<table>
<thead>
<tr>
<th>Antibody code</th>
<th>CDR 3</th>
<th>VH</th>
<th>DP</th>
<th>GAG class reactivity (ELISA)</th>
<th>MM/PM</th>
<th>AN</th>
<th>NN</th>
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<tr>
<td>EW4G1</td>
<td>GARLKR</td>
<td>3</td>
<td>42</td>
<td>Hep,* HS</td>
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<td>++</td>
<td>++</td>
</tr>
<tr>
<td>EW4G2</td>
<td>GKVLPN</td>
<td>3</td>
<td>38</td>
<td>Hep, HS, C 6-S, C 4-S</td>
<td>+/+</td>
<td>+/−</td>
<td>−/−</td>
</tr>
<tr>
<td>EW4B5</td>
<td>GRLHPK</td>
<td>3</td>
<td>45</td>
<td>Hep, HS</td>
<td>++</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>EW4C10</td>
<td>ARMTGIVR</td>
<td>3</td>
<td>45</td>
<td>Hep</td>
<td>Dots</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>EW4E1</td>
<td>GIRKLR</td>
<td>3</td>
<td>38</td>
<td>Hep, HS, C 6-S</td>
<td>+/+</td>
<td>+/−</td>
<td>−/−</td>
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<td>EW4B7</td>
<td>SSSRHRLHR</td>
<td>1</td>
<td>8</td>
<td>Hep, HS</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TSCO1</td>
<td>LGFHS</td>
<td>3</td>
<td>40</td>
<td></td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
</tbody>
</table>

* Hep, heparin. +/+ +, very strong staining; ++, strong staining; +, moderate staining; +/−, weak staining; −, no staining; dots, strong dot-like staining. C 4-S chondroitin 4-sulfate; C 6-S, chondroitin 6-sulfate. 4: no anti-GAG reactivity.

Anti-HMW-MAA

Reactivity of Anti-GAG Antibodies with Synthetic Oligosaccharides. Reactivity of the antibodies EW4E1 and EW4B7 with various synthetic oligosaccharides (a kind gift from Dr. Maurice Petitou, Sanofi-Synthélabo Research, Toulouse, France) was evaluated in a competition ELISA. Polystyrene 96-well plates were coated with heparin (8). Various amounts of oligosaccharides were added to the wells followed by anti-GAG antibodies. After incubation for 90 min, bound antibodies were detected by mouse anti-VSV monoclonal antibody P5D4, followed by alkaline phosphatase-conjugated rabbit antirat IgG. Enzyme activity was detected by p-nitrophenyl phosphate as a substrate. Absorbance was read at 405 nm. The amount of oligosaccharide resulting in 50% inhibition of the antibody binding to the heparin-coated wells was defined as the IC50.

Association of EW4E1 Epitope with Fluid Flow in Human Melanoma Xenografts

The human melanoma cell line Mel57 was cultured as described (9). For tumor growth, 2.5 × 10⁶ cells were injected s.c. into BALB/c nu/nu mice (9). When the tumors reached sizes between 100 and 700 mm³, mice were injected i.v. with 100 μl of a 3% (w/v) solution of FITC-BSA (12 mol FITC/mol BSA; Sigma, Brunschwig, Amsterdam, the Netherlands). Tumors were excised 60 min after the injection of tracer and snap-frozen in liquid nitrogen. Cryosections (4 μm) were incubated with antibody EW4E1 and rat antismouse CD31 (Mec7.46; Hy因ult Biotechnology, Uden, the Netherlands), or rabbit antilaminin (Sigma). Antibody binding was visualized using Cy3-conjugated mouse anti-VSV-tag P5D4 (Sigma), Alexa 594-conjugated goat antirat immunoglobulin (Molecular Probes), or Alexa 594-conjugated goat antirabbit immunoglobulin (Molecular Probes), respectively.

RESULTS

Anti-GAG Antibodies React Strongly with Melanoma. The location and expression of six different GAG epitopes, as defined by phage display-derived anti-GAG antibodies (8; Table 1) was investigated in 10 MMMs, 1 PM, 6 AN, and 3 NN. In melanomas the anti-GAG antibodies showed a strong reactivity with the ECM of blood vessels and with the ECM located around nests of tumor cells, and sometimes in between tumor cells (Fig. 1, A and C; Table 1). Anti-GAG stainings in melanomas corresponded with structures positive in AZAN staining, additionally indicating their location in the ECM (Fig. 1). Three antibodies (EW4E1, EW4B7, and EW4G2) showed a strong reactivity with melanomas, but poor or lack of staining with normal nevi and AN, with the occasional exception of the epidermal basement membrane (Fig. 1). This effect was most prominent for antibody EW4E1 (Fig. 1, A and B). Double staining with anti-CD31 confirmed the reactivity of these antibodies with blood vessels in melanoma (Fig. 2, A and B), and the absence of staining with blood vessels in AN (Fig. 2, C and D), in contrast to, e.g., antibody EW4G1 (Fig. 2, E and F). The anti-HMW-MAA antibody, reactive with the core protein of melanoma-associated CS proteoglycan, also strongly stained the ECM (Fig. 1E), but, in contrast with the anti-GAG antibodies, reacted with nevus cells (Fig. 1F).

Antibodies EW4E1 and EW4G2 Recognize Both HS and CS in Melanoma. To additionally characterize the epitopes defined by the antibodies EW4E1, EW4G2, and EW4B7, we performed enzyme digestions on cryosections. EW4B7 staining was completely lost after heparinase III treatment, whereas EW4E1 and EW4G2 stainings were only partially lost, indicating that these antibodies do not exclusively react with HS. Treatment with chondroitinase ABC as well as chondroitinase C alone did not completely abolish staining. A combined treatment with chondroitinase C (or chondroitinase ABC) and heparinase III did (data not shown). This indicates that, in contrast with EW4B7, antibodies EW4E1 and EW4G2 detect an epitope present in C 4-S, as well as in HS. The antibodies did not stain with AN, where

(Vector, Burlingame, CA) and avidin-biotin-peroxidase complex (Vectastain Elite kit; Vector) using aminoethyl carbazol as a substrate. Sections were counterstained with Meyer’s hematoxylin (Fluka, Buchs, Switzerland). To visualize blood vessels, anti-CD31 antibody clone JC70A (Dako Diagnostics, Glostrup, Denmark) was used. To detect HMW-MAA, hybridoma supernatant of antibody 2A7, kindly provided by Dr. Marcel Verbeek, University Medical Centre Nijmegen, Nijmegen, The Netherlands, was used. Colocalization studies were done using immunofluorescence (see below).

In Situ Characterization of GAG Epitopes. To establish the specificity of the antibodies for GAG epitopes, enzyme digestions were performed before immunohistochemistry. All of the enzyme digestions were performed in 25 mM Tris HCl (pH 8.0) for 16 h at 37°C. Digestion with heparinase III (digests HS; from Flavobacterium heparinum, a kind gift from IBEX Technologies Inc., Montreal, Quebec, Canada) was performed by incubating sections with 0.02 IU/ml of heparinase III. Treatment with chondroitinase ABC method (digests chondroitin sulfates and dermatan sulfates) and chondroitinase C (digests chondroitin 6-sulfate; Sigma, St. Louis, MO) were performed at 1 and 10 units/ml, respectively. Unit definitions of chondroitinase are according to the manufacturer. As a control, tissue sections were incubated in buffer without enzyme. After enzyme treatment, sections were incubated with anti-GAG antibodies together with antibody 3G10 (Seikagaku; Kogyo Co., Tokyo, Japan) to detect anti-GAG antibodies. Bound antibodies were visualized using goat antimouse IgG Alexa 594-conjugated goat antirat immunoglobulin (ICN Pharmaceuticals, Aurora, OH). CS was detected by antibody CS-56 (Sigma), which was visualized by goat antimouse IgM FITC (Sigma). For combined analysis of two different GAG epitopes, both c-myc and VSV-tagged antibodies were used, and detection was performed using P5D4 and rabbit anti-c-myc A-14 antibody (Santa Cruz Biotechnology). Bound antibodies were visualized using goat antirabbit IgG Alexa 488 (Molecular Probes) and goat antirabbit Ig Alexa 594 (Molecular Probes), respectively.

For colocalization studies, the anti-c-CD31 and anti-GAG antibodies were applied as described using a polyclonal anti-VSV-G tag antibody (MBL Biosciences, Nagano, Japan) to detect anti-GAG antibodies.

When the tumors reached sizes between 100 and 700 mm³, mice were injected i.v. with 100 μl of a 3% (w/v) solution of FITC-BSA (12 mol FITC/mol BSA; Sigma, Brunschwig, Amsterdam, the Netherlands). Tumors were excised 60 min after the injection of tracer and snap-frozen in liquid nitrogen. Cryosections (4 μm) were incubated with antibody EW4E1 and rat antismouse CD31 (Mec7.46; Hy因ult Biotechnology, Uden, the Netherlands), or rabbit antilaminin (Sigma). Antibody binding was visualized using Cy3-conjugated mouse anti-VSV-tag P5D4 (Sigma), Alexa 594-conjugated goat antirat immunoglobulin (Molecular Probes), or Alexa 594-conjugated goat antirabbit immunoglobulin (Molecular Probes), respectively.

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CS is abundantly present as indicated by the strong reactivity with a commercial anti-CS antibody (CS-56; data not shown). These data indicate that antibodies EW4E1 and EW4G2 define tumor-associated CS epitopes.

Antibodies Display Different Oligosaccharide-binding Profiles. To elucidate structural requirements in the epitopes necessary for antibody binding we investigated the reactivity of antibody EW4E1 and EW4B7 with a set of defined synthetic oligosaccharides. This set was based on the antithrombin-binding pentasaccharide in heparin/HS. Antibodies EW4E1 (reactive with HS/C 6-S) reacted strongly with a number of oligosaccharides, whereas EW4B7 (reactive with HS) did not, although its reactivity with HS was strong (Table 2). Data indicate that sulfate groups are essential for binding and that the minimal length for (strong) binding is a pentamer (Table 2). HS mimetics, in which N-sulfates were replaced by O-sulfates, and/or hydroxylic groups by methyl groups, were also capable to bind (compare, e.g., compounds 7 and 8, and 7 and 10). Sulfation as such was not enough for binding (see compound 14).

Fig. 1. Expression of GAG epitopes in MMs and AN. A. and B. anti-GAG antibody EW4E1; C and D. anti-GAG antibody EW4B7; E and F. anti-HMW-MAA; G and H. anti-CD31; I and J. Azan staining. Note the staining of antibodies EW4E1 and EW4B7 with MM. Also note the absence of reactivity of these antibodies with blood vessels in AN (see also Fig. 2). CD31 staining marks blood vessels in both melanomas and nevi. Bar, 100 μm.
GAG Epitope EW4E1 Is Associated with Fluid Flow in Xenografts. Since the ECM of melanoma may be involved with fluid flow, we investigated the location of the EW4E1 epitope as well as FITC-BSA as a marker for fluid flow in Mel57 xenografts in nude mice. There was a strong colocalization of EW4E1 staining with the fluid flow tracer (data not shown). This was not observed for laminin, a general ECM (basement membrane) component, which was present throughout the tumor (data not shown). Tracer was associated with some, but not all of the CD31-positive blood vessels.

Expression of EW4E1 Epitope in ECM of Uveal Melanomas. The geometry of ECM structures like arcs and loops has prognostic value in uveal melanoma (10). To evaluate whether antibody EW4E1

Table 2 Reactivity of anti-GAG antibodies with synthetic HS oligosaccharides and analogues

<table>
<thead>
<tr>
<th>Component name</th>
<th>Chemical structure</th>
<th>Reactivity (IC50) (μg/ml)</th>
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<td></td>
<td></td>
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<tr>
<td>Natural oligosaccharides</td>
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<tr>
<td>SR122579</td>
<td>IdoUA  GlcNS GlcUA GlcNS IdoUA2SaMe</td>
<td>35  40</td>
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<tr>
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<td>35  40</td>
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</tr>
<tr>
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<td>SR80535</td>
<td>Glc236triMe6S Glc236triMe6S Glc236triMe6S</td>
<td>35  40</td>
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</table>

a – no binding; GlcNS, N-sulfated glucosamine; Glc, glucose; GlcNac, N-acetylated glucosamine; GlcUA, glucuronic acid; IdoUA, iduronic acid; 2S, 2-O-sulfated; 3S, 3-O-sulfated; 6S, 6-O sulfated; Me, methyl.
is a sensitive marker for these structures, we studied 10 human uveal melanomas and compared antibody reactivity with AZAN staining (data not shown). Four specimens contained arcs and loops, 3 contained intermediate structures, and 3 had no detectable arcs and loops. Antibody EW4E1 staining correlated perfectly with the presence of arcs and loops as detected by AZAN staining but displayed a much brighter staining, resulting in easy recognition of the ECM geometry.

**DISCUSSION**

In this study we analyzed the expression of a number of GAG epitopes in different stages of melanocytic tumor development using phage display-derived anti-GAG antibodies. Differential expression in melanoma compared with nevi was observed for the HS epitope detected by antibody EW4B7, and the HS/CS epitopes detected by antibodies EW4G2 and EW4E1. Other epitopes were not differentially expressed. These data indicate that certain GAG epitopes are highly upregulated in melanoma. HS as well as CS have been implicated in tumorigenesis, including melanoma. For instance, in melanoma different HS species may differentially affect the interaction of growth factors with their receptors (11). Inhibition of melanoma lung colonization is dependent on the nature of modification of heparin (12). Heparinase III-digested HS (highly sulfated) inhibits tumor formation, whereas heparinase I-digested HS (moderately sulfated) promotes it (13). Next to HS, CS has been implicated in tumor formation. CS is involved in adhesive properties of melanoma cells (14), and enzymes degrading CS can inhibit metastasis formation in a mouse model (15). The melanoma-associated CS-proteoglycan HMW-MAA has been subject of many studies and has been used as basis for therapy in xenograft models (16). Also, CS-targeting, cationic liposomes have been successfully used to direct drug-containing liposomes to metastases (17). These studies indicate that CS and HS are important in tumorigenesis.

The antibodies presented here define specific modifications on the HS/CS molecules, which are expressed at a high level in melanoma. Preliminary data indicate that this also holds for other tumors like ovarian carcinoma. However, it should be noted that the reactivity of the anti-GAG antibodies is not limited to tumors but that these antibodies also react, be it at a lower level, with GAGs present in other tissues (8). The pattern of sulfation in HS/CS is likely to be crucial in binding to the antibodies. This pattern determines to a large extent the specificity and reactivity of GAGs with effector molecules like growth factors, cytokines, and proteases. The antibodies EW4G2 and EW4E1 recognize HS, as well as CS epitopes. The HS and CS epitopes likely share a similar configuration of sulfate groups. Next to overexpression of these epitopes in melanoma, a structural modification of HS or CS may be envisioned resulting in the formation of these epitopes. The availability of the antibodies now opens the way to isolate and sequence the HS/CS oligosaccharides involved, e.g., by affinity chromatography. Strategies to determine the GAG monosaccharide sequence have emerged recently (6). Knowing their structure, the specific GAG epitopes or suitable glycomimetics could be chemically synthesized and used for therapeutic purposes. This strategy has been successfully applied in the synthesis of a glycomimetic (Arixtra) resembling the specific heparin/HS pentasaccharide responsible for the activation of antithrombin III and resulting in the well-known anticoagulant clotting effect of heparin (18). This synthetic oligosaccharide replaces heparin as an anticoagulant with considerable less side effects (18). Interestingly, heparin by itself shows antitumor effects, indicating the possible use of GAG mimetics for treatment of tumors (4). Therapeutic effective GAGs or GAG analogues are thought to act by competing with GAG epitopes, important for tumor growth. The epitopes defined by the antibodies can be mimicked by artificial oligosaccharides (see Table 2), which are easier to synthesize than natural occurring GAG oligosaccharides.

Next to staining of ECM surrounding melanoma-associated blood vessels, the antibodies also stained matrix septa surrounding nests of melanoma cells. The ECM of blood vessels and septa are continuous with each other forming one large melanoma-associated network. These septa-like ECM structures have drawn much attention, because some consider them as vascular channels formed by melanoma cells and containing blood cells (vascular mimicry; Ref. 10). Although this is still a matter of debate, it seems that the ECM structures facilitate fluid flow thereby providing an alternative way of nourishment of tumors (9). The geometry of the septa does have prognostic significance (10). Some GAG epitopes were strongly associated with fluid flow pattern in xenografts of melanoma, in contrast with laminin, a general marker for ECM basement membranes. This indicates that the ECM septa may have a specialized molecular composition, perhaps analogous to the ECM of newly formed blood vessels. GAGs are known to be involved in trafficking of proteins. For instance they form a selective barrier for charged proteins in the glomerulus of the kidney (19). GAGs are also known to form depots and gradients of effector molecules like growth factors and cytokines. These characteristics may be used by the tumor to survive, grow, and metastasize.

The anti-GAG antibodies used here are human single-chain antibodies, which may have therapeutic and diagnostic capacity as described for other single-chain antibodies. Inhibition of tumor growth by an antilaminin single-chain antibody has been demonstrated, indicating that ECM-reactive antibodies are potential candidates for therapy (20). In conclusion, using (human) phage display-derived anti-GAG antibodies, HS/CS epitopes have been identified that are strongly associated with blood vessels and ECM of melanoma. The antibodies and/or their GAG epitopes may have potential in diagnosis and therapy of melanoma.

**ACKNOWLEDGMENTS**

We thank Ine Cornelissen and Han Zendman for technical assistance, Dr. Jos M. H. Raats (Department of Biochemistry, Faculty of Science, Nijmegen, The Netherlands) for providing the pUC 119 HIS VSV vector and Dr. Marcel Verbeek for providing the anti-HMW-MAA antibody. We thank IBEX Technologies Inc., (Montreal, Quebec, Canada) for providing the recombinant heparinase III, and Dr. Maurice Pettitou from Sanofi-Synthelabo Research, Toulouse, France, for providing the defined oligosaccharides.

**REFERENCES**


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