Dermatan Sulfate Is Involved in the Tumorigenic Properties of Esophageal Squamous Cell Carcinoma

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Abstract

Extracellular matrix, either produced by cancer cells or by cancer-associated fibroblasts, influences angiogenesis, invasion, and metastasis. Chondroitin/dermatan sulfate (CS/DS) proteoglycans, which occur both in the matrix and at the cell surface, play important roles in these processes. The unique feature that distinguishes DS from CS is the presence of iduronic acid (IdoA) in DS. Here, we report that CS/DS is increased five-fold in human biopsies of esophageal squamous cell carcinoma (ESCC), an aggressive tumor with poor prognosis, as compared with normal tissue. The main IdoA-producing enzyme, DS epimerase 1 (DS-epi1), together with the 6-O- and 4-O-sulfotransferases, were highly upregulated in ESCC biopsies. Importantly, CS/DS structure in patient tumors was significantly altered compared with normal tissue, as determined by sensitive mass spectrometry. To further understand the roles of IdoA in tumor development, DS-epi1 expression, and consequently IdoA content, was downregulated in ESCC cells. IdoA-deficient cells exhibited decreased migration and invasion capabilities in vitro, which was associated with reduced cellular binding of hepatocyte growth factor, inhibition of pERK-1/2 signaling, and deregulated actin cytoskeleton dynamics and focal adhesion formation. Our findings show that IdoA in DS influences tumorigenesis by affecting cancer cell behavior. Therefore, downregulation of IdoA by DS-epi1 inhibitors may represent a new anticancer therapy.

Introduction

Malignant tumors of the esophagus, comprising the 2 dominating forms, esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC), are the seventh leading cancer-related cause of death worldwide (1). The poor prognosis of ESCC and other types of squamous cell carcinoma (SCC) has motivated research on new treatment strategies. SCC antigen recognized by cytotoxic T lymphocytes 2 (SART2) was cloned as a gene with unknown functions highly expressed in SCC of different origins (2). Subsequently, a phase I clinical trial was conducted with prostate cancer patients by immunization with SART2 peptides (3). Our group found that SART2 is identical to dermatan sulfate epimerase 1 (DS-epi1), an enzyme involved in the biosynthesis of the complex polysaccharide DS (4).

CS/DS polysaccharides are unbranched polymers consisting of repeated alternating hexuronic acid [either glucuronic acid (GlcA) or iduronic acid (IdoA)] and N-acetyl-galactosamime (GalNAc). DS-epi1 and DS-epi2 convert GlcA to its epimer, IdoA, to a variable degree. The presence in a chain of even a single IdoA dictates the name of DS, which is invariably composed of a mixture of the 2 epimers. GalNAc residues in CS/DS are almost quantitatively sulfated at the hydroxyl groups either in the C4 or C6 position (4-OS and 6-OS), or occasionally in both positions. Similarly, GlcA/IdoA can be sulfated to a certain extent at the C2 position. Four 4-O-sulfotransferases, three 6-O-sulfotransferases, and a single 2-O-sulfotransferase catalyze the reactions. As a result of these modifications, CS/DS contains structural microdomains endowed with biological information. For instance, less predominant structures containing GlcA/IdoA(2-OS)-GalNAc(6-OS) and IdoA(2-OS)-GalNAc(4-OS) are important for neurite outgrowth and coagulation, respectively (5, 6).

CS/DS proteoglycans (PGs) consist of CS/DS chains attached to different core proteins. They play a recognized role in the extracellular matrix of host organ stroma, in tumor extracellular matrix, and on the cell surface of cancer cells (7). Functions of CS/DS-PGs in tumor progression can be attributed not only to the core proteins but also to the CS/DS chains. Removal of the CS/DS chains has been shown to inhibit angiogenesis, proliferation, and invasion of melanoma cells.
GlcA-GalNAc(4-O- and 6-O-disulfated)-containing CS structures on the cell surface of lung carcinoma and osteosarcoma cells are important in the metastatic process (9, 10). In addition, IdoA-containing domains bind the fibroblast growth factors (FGF), FGF2 and FGF7, and hepatocyte growth factor (HGF; refs. 11–13). HGF, together with its receptor c-MET, is a major player in cancer migration and invasion (14). Fibroblast-secreted HGF was shown to promote ESCC invasion through the c-MET receptor in an in vivo-like organotypic 3-dimensional cell culture (15). It is known that HGFs bind DS in vitro (12), but the functions of HGF binding to DS and its possible role in cancer are still unknown.

The high expression of DS-epi1 in human SCC prompted us to analyze the amount, structure, and localization of DS in ESCCs. Furthermore, using in vitro models of ESCC, we aimed to elucidate whether IdoA in DS could have a functional role in critical aspects of tumor development.

**Materials and Methods**

Materials, and descriptions of primary antibodies and chemicals, immunohistochemical DS-epi1 staining, characterization of metabolically labeled CS/DS chains from the cell layer and cell culture medium, immunoblot analysis and phosphokinase array, quantitative real-time PCR, digital holographic imaging, O-sulfotransferase assay, micro array RNA analysis, and characterization of the position of the incorporated sulfates are listed in Supplementary I Materials and Methods.

**Patient material**

Thirty-two male and 9 female patients, aged 39 to 83 years (mean 65 years) diagnosed with ESCC (n = 14), esophageal adenocarcinoma (n = 19), or gastric adenocarcinoma (n = 8) were randomly selected among the patients which underwent primary surgery at Lund University Hospital, Sweden, during 2001 to 2005 (Supplementary Table S1). None of the patients had received neoadjuvant radiotherapy or chemotherapy. Biopsies were taken from the normal and carcinoma-affected part of the removed tissues and directly snap frozen. Written informed consent was provided by all patients, and the study was approved by the Lund University Ethics Committee. Results presented in Figs. 1, 5, and 6 were obtained from biopsies randomly selected among the ESCC patients.

**Cell culture**

TE cell lines were a gift from Dr. Dinjens, Rotterdam, The Netherlands (17). The TE-1 cell line was authenticated by short tandem repeat profiling by Boonstra and colleagues (17). TE-1 cells were routinely cultured in RPMI 1640 supplemented with 10% FBS.

**DS-epi1 assay**

Lysates were prepared from 50 to 200 mg of biopsy tissues for epimerase and O-sulfotransferase assays (16). Protein concentration was estimated by the Bradford assay (Bio-Rad). The first step of the epimerization reaction is the abstraction of the C5-hydrogen from the chondroitin substrate and the subsequent formation of water in the reaction buffer. The epimerase assay measures the presence of $^3$H$_2$O derived from in vivo labeled $^3$H-C5 labeled substrate, and was carried out as described previously (4).
Lentivirus-mediated gene silencing

TE-1 cells were infected according to the manufacturer’s instructions, with MISSION Lentiviral Transduction Particles (SIGMA code: SHVRS) containing 2 short hairpin RNA (shRNA) sequences specific for DS-epi1 (TRCN0000121967 (shRNA-a) and TRCN0000122101 (shRNA-b)) or nontarget control shRNA (SHC002V). Following selection for puromycin resistance, different isolated clones were tested for epimerase activity.

Immunocytochemistry

DS-epi1 staining was carried out with immunopurified anti–DS-epi1 antibody. Briefly, TE-1 cells were grown in chamber slides, fixed with methanol for 10 minutes, permeabilized for 10 minutes in 0.2% Triton X-100, and blocked for 1 hour with 5% bovine serum albumin (BSA) in TBS. Immunopurified anti–DS-epi1 (2 μg/mL) and anti-GM130 (1:100) antibodies were incubated overnight at 4°C and visualized with goat anti-rabbit IgG AF488 (Invitrogen A11008) and goat anti-mouse IgG AF546 (Invitrogen A11003), respectively, at 1:200 dilutions. The presence of IdoA on the cell surface was visualized with a single-chain phage display antibody (GD3A12) that specifically recognizes DS (18). Cells were fixed with methanol for 2 minutes and stained as previously described by Li and colleagues (9). Immunostaining of pFAK and F-actin was done after a wound scratch assay in chamber slides. Cells were fixed in 4% PFA, permeabilized in Triton 0.1%, blocked in 1% BSA for 30 minutes and stained with anti-pY397FAK (1:1,000) overnight at 4°C in 1% BSA. pFAK was visualized with goat-anti mouse-AF488 at 1:500 dilution (Invitrogen) and subsequently counterstained with phalloidin-TRITC (P-1951; Sigma). HGF surface staining was carried out according to the manufacturer’s instructions followed by IdoA staining (see above). Briefly, cells were fixed in methanol, and recombinant, biotinylated HGF (1:10) was added followed by visualization with avidin-FITC (1:10). Controls without biotinylated HGF or primary anti-IdoA antibody were included in all experiments. Cells were analyzed by a Zeiss LSM 710 confocal scanning microscope equipped with a ×20 and ×63 objectives.

Figure 2. Downregulation of DS-epi1 in ESCC TE-1 cells results in decreased IdoA. TE-1 cells were infected with lentiviral particles containing DS-epi1 shRNA-a, shRNA-b, or nontarget control sequences. A, DS-epi1 protein in control, DS-epi1 shRNA-a, and shRNA-b cell lysates. Immunopurified anti–DS-epi1 antibody was used at 1 μg/mL. B, confocal microscopy immunofluorescence analysis of DS-epi1 (green) and the cis Golgi marker GM130 (red) shows reduced DS-epi1 levels in shRNA-a cells as compared with control. Scale bar, 20 μm. C, size distribution on Superdex Peptide column of metabolically labeled CS/DS chains from TE-1 cells transduced with shRNA control (black line) or DS-epi1 shRNA-a (grey, broken line) cleaved at IdoA residues by chondroitinase B before column separation. CS/DS derived from the large PG versican released into the medium is shown. Two shRNA-a clones were isolated and gave similar results (one is shown for brevity). Similar patterns were obtained from the CS/DS chains of the small PGs decorin and biglycan and from PGs present in the cell layer (see Table 1). D, surface staining of nonpermeabilized TE-1 cells with the anti-DS antibody GD3A12 that specifically recognizes IdoA residues. Bars, mean ± SD of triplicate stainings from flow cytometry analyses. E, confocal microscopy immunofluorescence analysis of IdoA from a similar experiment as in D. F and G, qRT-PCR of DS-epi1 (F) and DS-epi2 (G) on DS-epi1 shRNA-a and control shRNA cells.
Flow cytometry
Quantification of cell surface IdoA was done after detachment of the cells with PBS supplemented with 0.5 mmol/L EDTA. Sequential application of antibodies to DS (GD3A12; 1:80), rabbit anti-tag VSV-G (1:400), and donkey anti-rabbit IgG (1:200; Jackson) was carried out for 30 minutes at 4°C. Binding experiments of human biotinylated HGF were done after 24 h cell starvation in 0.1% serum followed by cell detachment in 2 mmol/L EDTA/PBS and analyzed according to the protocol provided by the manufacturer. A FACS-Calibur instrument integrated with CellQuest software (BD Biosciences), and Flowjo were used for analysis.

In vitro wound scratch assay
TE-1 cells were grown to confluency in 6-well culture plates and starved in medium supplemented with 0.1% serum for 48 hours. Confluent cell monolayers were scratched and cells were washed twice followed by the addition of fresh medium supplemented with 0.1% FBS in the absence or presence of 50 ng/mL of HGF or, alternatively, 1 or 10 ng/mL of FGF2 (HGF and FGF2; Sigma H1404 and F0291, respectively). Closure of the scratch was monitored with a ×4 objective at 0, 24, 48, and 72 hours. Digital pictures were analyzed by AutoCad software, and scratched cell-free areas were calculated.

Migration and invasion assays
Cells were starved in 0.1% serum for 24 hours, detached by 2 mmol/L EDTA/PBS, and 80,000 cells were seeded in serum-free medium on 24-Transwell membranes (8 μm pores). For invasion assay, the upper side of the membrane was coated with 70 μL of 1 mg/mL Matrigel. The lower chamber was filled with medium, supplement with 0.1% FBS with or without 50 ng/mL HGF. After 48 hours, membranes were fixed in 1% glutaraldehyde and stained with 0.5% crystal violet. Membranes were punched out with a 6 mm diameter dermal biopsy punch, and crystal violet was solubilized in 10% acetic acid and measured. Percentage of migrated cells was calculated as: absorbance 595 nm from migrated cells/absorbance 595 nm from migrated + nonmigrated cells.

Mass spectrometric analysis
Approximately 30 mg (wet weight) biopsies of normal tissue and carcinoma-affected tissue that also contained surrounding stroma were freeze dried and digested for 48 hours at 55°C in 50 mmol/L Tris/HCl, pH 8, 1 mmol/L CaCl2, 1% Triton X-100, containing 0.5 mg DTT. GAGs (Glycosaminoglycans (GAG)) were recovered after incubation of the GAG sample with 0.5 mol/L NaOH at 4°C for 24 hours. The alkaline solution was neutralized to pH 6 by acetic acid and centrifuged at 11,000 × g for 10 minutes. The supernatants containing GAGs were recovered by a weak anion exchange workup, with 1.5 mL DEAE-Sepharose columns. Samples were loaded and washed with 25 mL of 0.1 mol/L NaCl, 20 mmol/L NaAc pH 6.0 buffer. GAGs were eluted with 2.5 mL of 1 mol/L NaCl, 20 mmol/L NaAc pH 6.0. These fractions were desalted with PD-10 columns and vacuum dried. The exhaustive depolymerization was accomplished by dissolving one-sixth of each sample in 10 μL of water, followed by adding 8 μL of 100 mmol/L Tris/HCl pH 7.45, 2 μL of 1 mol/L ammonium acetate, 20 μl of chondroitinase ABC, 10 μu of chondroitinase AC-L, and 1.25 μu of chondroitinase B. In a distinct incubation, 2 μl of chondroitinase B only was added to the GAG samples, in the presence of 0.1% BSA and 1 μmol/L CaCl2 to specifically cleave at IdoA residues of DS. In both cases, the digest solutions were incubated at 37°C, and after 2 hours, another aliquot of lyases was added.

The disaccharide and oligosaccharide analysis with SEC liquid chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry was conducted as reported previously (19, 20). Briefly, the separation was accomplished by a Superdex Peptide (GE Healthcare) column (3.2 mm × 300 mm), with isocratic solvent (0.016 mL/min, 12.5 mmol/L formic acid, pH titrated to 4.4 by ammonia, in 10% acetonitrile) delivered by a Waters Acquity UPLC. The effluent was coupled with an Applied Biosystem Sciex QSTAR mass spectrometer by TurbolonSpray interface.

Statistical analysis
The Student t test and Wilcoxon signed-rank test were conducted using the GraphPad Prism 5.0c software.
Results

**DS-epi1 is expressed in an active form in ESCC biopsies**

CS has been shown to be increased in several cancer types (21), although little is known about DS. It is produced by DS-epi1 and DS-epi2, of which the former is predominant in vivo (16). DS-epi1 is highly expressed in SCC tumors, but in which cells is currently unknown. Immunohistochemical staining showed that DS-epi1 was expressed in normal esophageal epithelium and connective tissue as well as in cancer cells and tumor stroma (Fig. 1A and B). Specificity of the anti–DS-epi1 antibody was ascertained on DS-epi1−/− mouse esophagus (Fig. 1C and D; ref. 16). Epimerase activity was subsequently measured in ESCC biopsies and compared with normal esophageal tissue derived from the same patient. We found that DS epimerase activity was 4-fold upregulated in cancer tissue as compared with normal tissue (Fig. 1E). Increased DS epimerase activity was also found in biopsies from esophageal adenocarcinoma and gastric adenocarcinoma patients (Supplementary Fig. S1). Consistently, DS-epi1 protein expression was highly upregulated (Fig. 1F). At least 3 isoforms of DS-epi1 were present in tumor samples, possibly corresponding to differences in N-glycosylated isoforms (22), while in normal tissues DS-epi1 was below detection level. We thus concluded that functional DS-epi1 is elevated in human ESCC.

**DS-epi1 downregulation decreases IdoA in CS/DS in the ESCC cell line TE-1**

Elevated DS-epi1 levels in human ESCC prompted studies on the functional effects of DS-epi1 in vitro. To evaluate the role of IdoA in tumorigenesis, we used TE-1 cells, a well-established cellular model of ESCC (15, 23, 24). TE-1 cells displayed the highest DS epimerase activity among the TE-1, TE-2, TE-4, TE-5, TE-8 cell lines tested. (Supplementary Table S2). TE-1 cells were infected with lentiviruses containing 2 DS-epi1 shRNA sequences (shRNA-a and shRNA-b) and 1 control shRNA sequence. Two clones containing shRNA-a or shRNA-b were studied. Epimerase activity was downregulated by approximately 90% in shRNA-a–transduced cells and approximately 82% in shRNA-b–transduced cells, as compared with control nontarget shRNA-transduced clones (Supplementary Table S2). Accordingly, DS-epi1 protein was substantially reduced in shRNA-a and shRNA-b cells (Fig. 2A). In addition, confocal microscopy analysis showed that DS-epi1 colocalized with the cis Golgi marker GM130 in control cells, while it was virtually below detection level in shRNA-a cells (Fig. 2B). To study the effect of DS-epi1 downregulation on DS structure, cells were labeled with [35S]-sulfate and PGs derived either from the cell layer or released into the medium were fractionated by size exclusion chromatography. Isolated [35S]-sulfate–labeled CS/DS chains were specifically degraded at IdoA residues with chondroitinase B, and size fractionated (Fig. 2C). Calculation based on the degradation pattern showed that the IdoA content was 7% to 15% in the control CS/DS-PGs, and was approximately 80% reduced upon DS-epi1 silencing (Table 1). In accordance with these results, IdoA-containing epitopes at the cell surface, measured by an anti-DS antibody, were reduced by 64% and 47% upon DS-epi1 downregulation by shRNA-a and shRNA-b, respectively (Fig. 2D). These data were corroborated by visualization of cell surface IdoA with an anti-DS antibody (Fig. 2E). The remaining IdoA upon DS-epi1 downregulation could be formed by the action of DS-epi2, the mRNA expression of which increased 5-fold in DS-epi1–downregulated cells (Fig 2G), while, as expected, DS-epi1 mRNA was decreased by approximately 90% (Fig. 2F).

**IdoA is involved in HGF-mediated ERK signaling**

HGF has previously been shown to bind DS with higher affinity than CS (13). Control shRNA TE-1 cells were stained with recombinant, biotinylated HGF, and anti-IdoA antibody and visualized with confocal microscopy. In support of a direct interaction of HGF with IdoA of DS, partial colocalization on the cell surface was observed (Fig. 3A), and HGF binding was significantly reduced in shRNA-a and shRNA-b cells, as compared with control shRNA cells (Fig 3B). Notably, these cells expressed equal amounts of the MET receptor, which supports a direct role of IdoA in HGF binding (Fig. 3C).

To further address the signaling pathways activated by HGF in TE-1 cells, we initially carried out a phosphokinase antibody array on shRNA control cells with or without HGF stimulation (Fig. 3D). HGF appeared to specifically induce phosphorylation of ERK-1/2 and its downstream target RSK-1/2/3 under the conditions used. HGF-mediated induction of pERK-1/2 was confirmed by Western blotting and, more importantly, the induction was substantially reduced in shRNA-a and shRNA-b cells as compared with control shRNA cells (Fig. 3E). These data indicate that unperturbed IdoA formation is required for efficient HGF-mediated signaling through ERK-1/2 in TE-1 cells.

**Cancer cell migration and invasion depend on IdoA**

To investigate possible functional roles of IdoA, we next conducted migration and invasion assays with shRNA control, shRNA-a and shRNA-b TE-1 cells. In a wound scratch assay, the capacity of control cells to migrate was greatly enhanced by the addition of HGF (Fig. 4A and B). On the contrary, FGF2 added at 1 or 10 ng/mL did not stimulate migration of TE-1 cells (data not shown). Interestingly, shRNA-a and shRNA-b cells were found to migrate significantly less than shRNA control cells, and this difference was more pronounced in the context of HGF stimulation (Fig. 4A and B). Migration and invasion were next studied in Transwell assays and, again, HGF increased TE-1 cell migration (Fig. 4C), although to a lesser extent than in wound scratch experiments. shRNA-a and shRNA-b cells presented significant reduction in migration as compared with control cells, both in the absence and in the presence of HGF. Control cell invasion was enhanced approximately 2-fold by the addition of HGF, and shRNA-a cells had significantly reduced invasive capacity in the context of HGF stimulation (P < 0.01), whereas with border-line significance (P = 0.051) in nonstimulated cells (Fig. 4D). shRNA-b, however, had no effect on the invasion of nonstimulated cells, whereas there was a strong trend (P = 0.054) toward inhibition of HGF-driven invasion of TE-1 cells (Fig. 4D). Cell migration requires a continuous cycle of protrusion, attachment, and traction at...
Experimental studies, including the data presented above, clearly indicate that the biological functions of CS/DS chains depend on their fine structure. However, clinical data are still lacking to validate these in vitro experiments. For the first time, we have investigated the structure of CS/DS chains derived from small amounts of pathologic samples with a sensitive mass spectrometric approach. To this end, CS/DS was purified and extensively degraded into disaccharides by a mixture of chondroitinases ABC, AC-1, and B enzymes. Interestingly, sulfated disaccharides were increased 5-fold in ESCC patient tumors compared with their normal counterparts (Fig. 5A). We further analyzed the type of sulfation on the disaccharides. Normal tissues contained approximately 82% monosulfated, 4-O-sulfated disaccharides and 18% monosulfated, 6-O-sulfated disaccharides, whereas cancer tissues contained approximately 65% monosulfated, 4-O-sulfated disaccharides and 35% monosulfated, 6-O-sulfated disaccharides (Fig. 5B). Disulfated disaccharides within CS/DS chains are less abundant components that have been associated with biological functions. We present data showing that these disaccharides represented approximately 7% of the total sulfated disaccharides in normal esophageal tissues. Their relative content in all cancer biopsies decreased approximately 4-fold compared with normal tissues (Fig. 5C).

Next, CS/DS was digested by chondroitinase B alone, resulting in disaccharides from DS regions containing at least 2 adjacent IdoA residues. The absolute content of IdoA disaccharides was unaffected in cancer biopsies compared with normal tissues (amount of IdoA/weight of tissue; Fig 5D). However, there was a 5-fold increase of CS/DS content in tumor tissues (Fig 5A). Collectively, these data show that the relative amount of IdoA/chain [% of IdoA/(IdoA + GlcA)] decreased in tumor compared with control tissues. In summary, total CS/DS levels were increased in ESCC samples. The average structure of the chains derived from tumor tissues was altered: 6-O-sulfated disaccharides were increased, and 4-O-monosulfated disaccharides as well as disulfated disaccharides and IdoA-containing structures were decreased.

ESCC tumors express an altered CS/DS structure as compared with normal tissue

The leading edge that depends on the coordinated dynamics of the actin cytoskeleton and focal adhesions (25). Indeed, IdoA was found to have a role in cell attachment and spreading, as well as in cytoskeleton dynamics; shRNA-a cells exhibited an approximately 40% greater cell area compared with control shRNA cells (Supplementary Fig. S2B), which was associated with an approximately 2-fold induction of p-FAK and total FAK as compared with control cells (Fig. 4E). Moreover, p-FAK seemed to be increased and more homogeneously distributed at the cell membrane as compared with control cells in shRNA-a and shRNA-b cells after 48 hours of HGF stimulation in a wound scratch assay (Fig. 4F). Furthermore, DS-epi1–downregulated shRNA-a and shRNA-b cells displayed an altered morphology with less prominent plasma membrane protrusions (Fig. 4F, top), and with relatively few cytoplasmic stress fibers compared with control cells (Supplementary Fig. S2C). In conclusion, these data show that cell surface located IdoA is involved in the migratory and invasive behavior of ESCC cells, especially in the context of HGF signaling, and suggest a novel role of DS-epi1 in the regulation of cell motility and cytoskeleton modulation.
Increased O-sulfotransferase activities in ESCC biopsies

There are 3 major 4-O- and two 6-O-sulfotransferases that add a sulfate group at the C4 and C6 positions of the GalNAc residue, respectively (26, 27). The activities of these sulfotransferases were measured by incubating tissue extracts with the substrates chondroitin [(GlcA-GalNAc)n] or dermatan [(IdoA-GalNAc)n] together with the labeled sulfate donor [35S]-PAPS. The incubations with chondroitin (Fig. 6A) showed a 13-fold upregulation of 4-O-activity in tumor tissue as compared with normal tissue (P = 0.043), while the 6-O-activity was increased 4-fold (P = 0.08). These assays measure 4- and 6-O-sulfotransferases activities but not DS 4-O-sulfotransferase 1 (D4ST-1) activity, which requires dermatan as substrate. D4ST-1 activity was upregulated in 3 out of 4 patients (Fig. 6B). Moreover, 6-O-sulfation, carried out by C6ST1 (27), was also detected on dermatan and increased in 3 patients out of 4. Next, CS/DS biosynthetic enzymes, beyond the epimerases and O-sulfotransferases, were analyzed by cDNA microarray (Supplementary Fig. S3; the data obtained have been deposited in the Gene Expression Omnibus database, accession number GSE27040). The mRNA levels in ESCC biopsies were compared with a reference pool of 10 human cancer cell lines to give a broad representation of transcripts. Therefore, the values do not represent a comparison between cancer and normal tissues. The data showed that tumors from 37 patients with esophageal and gastric carcinomas had similar mRNA expression patterns. Altogether, the activity results showed that the main O-sulfotransferase activities are upregulated in human ESCC, which is in line with the increased production of CS/DS observed in tumors.

Discussion

The main finding of this report is that tumor-promoting functions can be attributed to IdoA in DS chains. DS-epi1 and DS-epi2 are the enzymes responsible for conversion of GlcA to IdoA, and ultimately for the formation of DS. DS-epi1 is the main enzyme in vivo (16) and, because of its overexpression in most cancers, it has been suggested to be a cancer-associated antigen (2). In support of this idea, we found that DS-epi1 is...
highly expressed in ESCC cancer cells together with increased DS-epimerase activity in cancer tissue. Interestingly, we provide evidence that DS-associated IdoA seems to have a functional role in cancer cell behavior. Previous data suggest a synergistic effect of exogenously added DS and HGF in the stimulation of muscle cell proliferation and migration (28). The profound action of HGF on invasion has been shown in a variety of cancer cells, including ESCC (15). DS and HGF could potentially form a receptor binding signaling complex in analogy with FGF2 and heparan sulfate (29, 30). Previous reports with different cell lines have shown that migration and invasion are enhanced in the presence of HGF, through its downstream target pERK-1/2 (14). Here, we show partial cell surface colocalization between HGF and IdoA, significantly reduced binding of HGF in DS-epi1–downregulated, IdoA-deficient cells, and a strong dependence on unper-turbed IdoA formation for efficient HGF-mediated signaling through ERK-1/2. These effects were associated with less migration and invasion of DS-epi1–downregulated cells compared with control cells, especially in the context of HGF stimulation. It is noteworthy that a single IdoA seems to be sufficient for HGF binding to DS chains (12). Our findings that IdoA-deficient cells displayed only limited reduction of HGF binding (Fig. 3B), and more substantial reduction of HGF signaling (Fig. 3E) as well as inhibition of functional effects (Fig. 4), underscore the biological relevance of the DS-bound HGF subfraction. It may be speculated that although HGF binding remains relatively intact upon IdoA deficiency, HGF is presented in an altered microenvironment and conformation that does not allow efficient downstream signaling activation.

Migration and invasion are intriguing processes with constant formation and disassembly of adhesion. Adhesion occurs at protrusions while disassembly of the focal adhesion complex takes place at the cell rear and at the base of protrusions. Focal adhesion kinase (FAK) is an important regulator of cytoskeleton dynamics involved in adhesion and migration (31). Our results suggest that DS-epi1–downregulated cells have a malfunctioning disassembly of adhesion complexes and abnormal actin cytoskeleton architecture. The induction of FAK and pFAK could be due to increased spreading of IdoA-deficient cells (32).

Figure 5. Mass spectrometry analysis of CS/DS from human ESCC biopsies and normal tissue. CS/DS was purified from ESCC biopsies and adjacent normal tissue and extensively degraded by a mixture of chondroitinases ABC, AC-I, and B (A–C), or specifically degraded at IdoA residues by chondroitinase B alone (D), as described in Materials and Methods. The degradation products were separated by size permeation and on line injected into the mass spectrometer. Monosulfated (m/z = 458) and disulfated (m/z = 538) disaccharides were quantified. Nonsulfated disaccharides (m/z = 378) were not considered due to contamination from hyaluronic acid–derived disaccharides after chondroitinase ABC digestion, and as they constituted a minor component (mean 1.8%) of the predominant monosulfated disaccharides after chondroitinase B alone digestion. Measurements were carried out in triplicate. P values were obtained by Wilcoxon signed-rank test (A–D; P < 0.05 cancer vs. normal). Results from analyses of normal tissue were set to 1 in A and D.
Altered signals could originate from a modified ECM, deposited by shRNA-a and shRNA-b cells over the time of cultivation. The actual presence of IdoA in CS/DS or CS has been overlooked in many cases, especially when considering cell surface bound PGs. An exception is the part time PG CD44 that has been shown to contain IdoA under certain circumstances (33). CD44 is localized in the focal adhesions of invadopodia (34) and has a functional role in the anchoring of cytoskeleton elements to cell membrane in connection with ECM molecules and in concentrating metalloproteases. In conclusion, the altered distribution of actin cytoskeleton and focal adhesions showed in DS-epi1-downregulated cells is consistent with decreased directionality in cellular movements. Future studies clearly have to elucidate which type of PG/PGs are specifically involved in these functions.

The changed composition and structures of CS/DS polysaccharide chains in malignant tumors could play distinct roles in tumor development (7, 35). We found that the expression patterns of CS/DS biosynthetic enzymes and the structure of the CS/DS chains are consistent among the patients examined. In ESCC biopsies, the activities of epimerases, 4-O-, and 6-O-sulfotransferases were increased. It is plausible that these activities are needed to stand the 5-fold increase in CS/DS production. Previous studies have shown that CS/DS increases in most of the cancers examined (21, 36). We extend previous data and show that the average composition of CS/DS chains produced by tumor biopsies is altered in many different aspects as compared with normal tissues. IdoA residues can be found in CS/DS chains in 3 major patterns: isolated, where IdoA is surrounded by GlcA; in alternating IdoA-GlcA structures; or in long blocks of adjacent IdoA residues. Using mass spectrometry analysis of purified CS/DS, we reveal that the relative content of IdoA is decreased in blocks and in alternating structures. Intriguingly, there is thus an apparent discrepancy between higher epimerase activity in tumor tissue and lower relative content of alternating and blocks of IdoA structures. This discrepancy could simply be explained by the presence of isolated IdoA structures that were not included in the mass spectrometry analyses. Alternatively, increased turnover of IdoA-containing DS oligosaccharides in tumors may cause the release of sequestered HGF and additional growth factors from CS/DS PGs in the stroma resulting in increased accessibility to and stimulation of tumor cells.

In summary, we show that critical aspects of cancer cell function are dependent on the presence of IdoA in DS, and specific features of the CS/DS structure are altered in ESCC patient tumors. This work shows the potential to pharmacologically block DS-epi1 and expectably down-regulate HGF signaling, which may provide new avenues for cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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