Enhanced Tumorigenic Potential of Colorectal Cancer Cells by Extracellular Sulfatases

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Abstract

Heparan sulfate endosulfatase-1 and -2 (SULF1 and SULF2) are two important extracellular 6-O-endosulfatases that remove 6-O sulfate groups of N-glucosamine along heparan sulfate (HS) proteoglycan chains often found in the extracellular matrix. The HS sulfation pattern influences signaling events at the cell surface, which are critical for interactions with growth factors and their receptors. SULFs are overexpressed in several types of human tumors, but their role in cancer is still unclear because their molecular mechanism has not been fully explored and understood. To further investigate the functions of these sulfatases in tumorigenesis, stable overexpression models of these genes were generated in the colorectal cancer cells, Caco-2 and HCT-116. Importantly, mimicking overexpression of these sulfatases resulted in increased viability and proliferation, and augmented cell migration. These effects were reverted by shRNA-mediated knockdown of SULF1 or SULF2 and by the addition of unfractionated heparin. Detailed structural analysis of HS from cells overexpressing SULFs showed reduction in the trisulfated disaccharide UA(2S)-GlcNS(6S) and corresponding increase in UA(2S)-GlcNS disaccharide, as well as an unexpected rise in less common disaccharides containing GlcNAc(6S) residues. Moreover, cancer cells transfected with SULFs demonstrated increased Wnt signaling. In summary, SULF1 or SULF2 overexpression contributes to colorectal cancer cell proliferation, migration, and invasion.

Implications: This study reveals that sulfatases have oncogenic effects in colon cancer cells, suggesting an important role for these enzymes in cancer progression. Mol Cancer Res; 13(3); 510–23. © 2014 AACR.

Introduction

Heparan sulfate proteoglycans (HSPG) consist of a limited range of core proteins to which sugar chains of heparan sulfate (HS) are covalently attached (1, 2). The HS chains are linear polysaccharides consisting of repeating disaccharide units of glucuronic (GlcA) or iduronic acid (IdoA) linked to glucosamine (GlcN; ref. 3). HSPGs are present on the cell surface of most animal cells and are major components of the extracellular matrix (ECM; ref. 4). HSPGs are involved in cell signaling as coreceptors owing to their capability to bind diverse molecules, such as growth factors, cytokines, chemokines, morphogens, matrix ligands, and cell surface molecules (4), and these functionalities are strongly influenced by the HS sulfation pattern.

During biosynthesis, disaccharide units of HS chains are submitted to a complex set of polymer modifications involving deacetylation and N-sulfation of N-acetylglucosamine (GlcNAc), epimerization of D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), and O-sulfation (5). Through the action of Golgi-resident sulfotransferases, four different sulfation modifications (denoted S) are generated on HS chains: at the N-, 3-O, and 6-O positions of glucosamine and at the 2-O position of the iduronic acid residue (6). The result is complex sequence heterogeneity, which is not explained by a simple linear biosynthetic scheme. Once synthesized, additional processing may also occur at the membrane/ECM following action by another class of enzymes, the endosulfatases, sulfatase-1 (SULF1) and sulfatase-2 (SULF2).

Initially cloned in 2002 (7, 8), the SULFs are endoglucosaminine-6-sulfatases that hydrolyze 6-O-S groups mainly within the trisulfated disaccharide units (IdoA2S-GlcNS6S) along the S domains of heparin/heparan sulfate chains (9). The SULFs have been shown to modulate the interaction of a number of protein ligands with heparin or heparan sulfate (9, 10–12). Following the cloning of the human SULF cDNAs, recent studies employing quantitative PCR or gene microarray analyses have reported the overexpression of SULFs in a wide range of human tumors: hepatocellular carcinoma (13), pancreatic carcinoma (14), head and neck squamous cell carcinoma (15), gastric cancer (16), and lung adenocarcinoma (17) for SULF1, and hepatocellular carcinomas (13, 18), breast cancer (19), glioblastoma (20), and lung squamous cell carcinoma (17) for SULF2. However, the literature is ambiguous about the function of SULFs in cancer, and the enzymes are reported both as anti- and as protumorigenic. SULF1 has been associated with tumor suppressor effects in various models of cancer (21–23), whereas SULF2 dysregulation was in relation with protumorigenic effects (8, 24). Therefore, the precise role of SULFs in cancer is still not fully understood, suggesting the complexity of their action in the tumor microenvironment (24, 25).
Role of SULFs in Colorectal Cancer Cells

One important consequence of SULFs overexpression is higher activation of the Wnt canonical pathway (9). Studies have demonstrated that activation of SULFs leads to weakened association of Wnt ligands with HSPGs in ECM, which allows the ligands to activate signal transduction receptors Frizzleds, resulting in accumulation of β-catenin (8, 13, 18, 26, 27). Unphosphorylated β-catenin accumulates in the cytoplasm and is then translocated into the nucleus, where β-catenin–targeting genes are activated (28, 29). These genes include cell-cycle–regulating genes and those related to metastasis and cancer cells invasion (30, 31).

Interestingly, the deregulation of the Wnt/β-catenin signaling pathway has been considered to play an important role in colon carcinogenesis (32, 33).

Colorectal cancer is one of the leading causes of cancer-related human morbidity and mortality worldwide. Although surgery is the most effective treatment for advanced colon cancer, recurrence frequently occurs within a few years (34). Thus, it is relevant to better understand the molecular mechanisms involved in this disease.

This study aimed to analyze the consequences of the over-expression of HS 6-O-endosulfatasases SULF1 and SULF2 in colorectal cancer cell lines. To do so, Caco-2 and HCT-116 cell lines were transfected with either SULF1- or SULF2-expressing vector and submitted to proliferation, migration, and adhesion assays. Moreover, differences in HS structure and their correlation with changes in Wnt signaling pathway in these cells were also studied.

Materials and Methods

Cell culture

The colorectal adenocarcinoma cell line Caco-2 and the colorectal carcinoma cell line HCT-116 were purchased from the ATCC. HCT-116 cells were grown in RPMI (Life Technologies) and Caco-2 cells were grown in DMEM (Gibco; Life Technologies). Both mediums were supplemented with 10% (v/v) FBS and antibiotics. After 8 hours of incubation, the medium was replaced by the respective culture medium of each cell line. The gene silencing was observed 48 hours after transfection. Viability and cell migration assays were performed at different times to analyze the consequences of SULF1 and SULF2 silencing.

RNA extraction and real-time PCR

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen; Life Technologies) according to the manufacturer’s guidelines. The total RNA extracted was used as the template for the reverse transcriptase reaction. Aliquots of cDNA were amplified using the primers described in Supplementary Table S2. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix and Amplicon-GOLD polymerase (Applied Biosystems). Reactions were performed in triplicate on the ABI PRISM 7500 Real Time PCR System (Applied Biosystems). The relative expression levels of genes were calculated using the 2−ΔΔCT method. The levels of target gene expression in each sample were normalized to the average expression level of the endogenous control, GAPDH, and β-actin.

Protein preparation and Western blotting

Adhered cells were removed from the culture dishes with the aid of a cell scraper in cell lysis buffer (Cell Signaling Technology) containing proteinase inhibitor cocktail (Roche). The lysate proteins were separated according to molecular mass by SDS-PAGE using a 7.5% to 10% polyacrylamide gel (Merck) and transferred to polyvinylidene difluoride membranes (Merck). The membranes were quenched for 1 hour with 5% nonfat dry milk in TBST buffer and then incubated overnight at 4°C with primary antibodies: goat anti-SULF1 (Santa Cruz Biotechnology), rabbit anti-SULF2 (Santa Cruz Biotechnology), rat anti-Wnt3A (R&D Systems), mouse anti-β-catenin (R&D Systems), rabbit phospho-β-catenin (R&D Systems), mouse anti-β-actin (Santa Cruz Biotechnology), diluted in 1% BSA in TBST. Thereafter, the membranes were further incubated for 1 hour at room temperature with the appropriate secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology). Chemiluminescence signal detection was performed using the gel documentation system G:Box Chemi HR16 (Syngene). Densitometric analysis was performed using the Scion Imaging software (Scion Corporation), using β-actin as a control for each sample.

Immunocytochemistry

For immunocytochemical staining, cells were fixed with 2% parafomaldehyde for 30 minutes and blocked with 5% FBS. The cells were then incubated for 2 hours with the following primary antibodies: goat anti-SULF1 (Santa Cruz Biotechnology) and rabbit anti-SULF2 (Santa Cruz Biotechnology). After washing with PBS, the sections were incubated at room temperature for 1 hour with the appropriate secondary antibody: anti-rabbit IgG or anti-goat IgG, all produced in donkeys and conjugated to Alexa 594 or Alexa 488 (1:200 Invitrogen). Nuclei were stained using DAPI (0.5 μg/mL; Molecular Probes). The cells were mounted using FluoromountG (Electron Microscopy Sciences) and analyzed using a Zeiss LSM510 scanning confocal inverted
microscope. Colocalization images were generated using Zeiss LSM Image Browser.

Cell viability and proliferation assays
For 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays, cells were plated into 96-well plates at 3 \times 10^3 cells per well and incubated in 10% FBS for 24 to 72 hours. Cell viability was then assessed by the MTT-reducing capacity. After 2 hours at 37°C, the medium was carefully removed and formazan crystals produced were solubilized by addition of DMSO (MP Biomedicals). The absorbance was measured in the EXL800 ELISA plate reader and Universal MICROPLATE Reader (Bio-TEK Instruments, Inc.) at 540 nm. The bromodeoxyuridine (BrdUrd) incorporation assay was performed according to the manufacture’s instructions (Roche). Each experiment was performed in six replicates at least three times.

Wound-healing assay
A total of 2 \times 10^5 cells per well were plated in 24-well plates. After reaching confluence, a straight scratch simulating a wound was made with the aid of a 200 µL tip in the center of the plate. The closure of the wound was monitored using an inverted optical microscope images obtained by camera (Sony) attached to the microscope (Zeiss).

Cell migration assay
A total of 2 \times 10^4 cells were plated in RPMI medium in the top of transwell membrane chambers (Costar; Corning, 8-µm pore size). In the bottom chamber, medium supplemented with 10% FBS was added. Migration assays were carried out for 24 hours in the tissue culture incubator. The cells were fixed by replacing the culture medium in both bottom and top chambers with 4% formaldehyde dissolved in PBS. After fixation for 15 minutes at room temperature, cells were stained with 0.2% crystal violet for 10 minutes. After the chambers were washed five times by dipping in a large beaker of dH2O and cells in the upper chamber were removed with several Q-tips, remaining cells were solubilized in 10% acetic acid, and the absorbance was measured at 560 nm.

Cell adhesion assay
Petri dishes were precoated with ECM proteins: collagen I, laminin, or fibronectin (50 µg/mL). The plates remained in the incubator at 37°C under 5% CO_2 pressure for 2 hours. The nonspecific binding sites were with 1% BSA in PBS for 1 hour. As a control, plates not coated with matrix proteins, containing only 1% BSA, were used. Finally, cells were trypsinized and plated at a concentration of 2 \times 10^5 cells per mL medium. After incubation for 6 hours, nonadherent cells were aspirated, and those adherent were stained with DAPI, viewed, and photographed in a fluorescence microscope (Zeiss). Adherent cells were counted using the ImageJ software (http://rsb.info.nih.gov/ij/).

Colony formation on soft agar
Twenty-four-well plates were coated with 300 µL of 0.7% agarose and kept at 4°C for 30 minutes. Cells (6 \times 10^5) were resuspended in medium containing 0.35% agarose and plated on 24-well plates previously covered with agarose. The plate was kept at 37°C with 5% CO_2 for 1 hour, and then augmented with the respective culture medium of each cell. Formation of colonies was followed for 20 days. The colonies were counted and measured using an inverted optical microscope (Zeiss).

[^35S]-sulfate metabolic radiolabeling of glycosaminoglycans
Caco-2 and HCT-116 cells were incubated with [35S]Na_2SO_4 (100 µCi/mL) for 24 hours for glycosaminoglycans (GAG) labeling. The medium was removed, and the cells were washed with PBS. The cells were then detached with 0.025% EDTA, and the remaining ECM was extracted with 0.01% trypsin. The cells were lysed by treatment with 3.5 mol/L urea in PBS. Proteoglycans from the medium, cell extract, and matrix were precipitated with 3 volumes of ethanol, dried, and subjected to proteolysis with maxatase, a protease from Sporobolus, (4 mg/mL) in 0.05 mol/L Tris-HCl, pH 8.0, for 24 hours at 60°C. Labeled GAGs were dried and resuspended in 100 µL of water. The composition of GAGs in each compartment was analyzed by agarose gel electrophoresis.

GAGs analysis
Samples were applied to agarose gel slabs in 0.05 mol/L 1,3-diaminopropane acetate buffer (35, 36). Following electrophoresis, GAGs were precipitated with 0.2% cetyltrimethylammonium bromide. ^35S-radiolabeled GAGs were visualized after exposure to CycloneTM Storage Phosphor Screen (Packard Instrument Company Inc.), and images were acquired using a CycloneTM Storage Phosphor System (Packard Instrument Company Inc.). Chondroitin Sulfate (CS) and HS were identified after agarose gel electrophoresis, based on the difference in migration under this electrophoresis condition, as previously described (37). Quantitative analysis was performed by densitometry using the Opti-Quanti Software. For HS disaccharide analysis, each sample was digested using heparitinases I and II from Flavobacterium heparinum (Sigma-Aldrich), and analyzed on a Phenosphere 5 µm SAX 80 Å LC Column 150 × 4.6 mm. The column was previously calibrated with standard HS Δ-disaccharide produced by extensive heparin digestion with heparin lyases, followed by purification on gel permeation chromatography and strong anion exchange. Cells, ECM, and medium HS Δ-disaccharides were eluted with a linear gradient of NaCl (0–1 mol/L) over a 30-minute period at a flow rate of 1 mL per min. Individual fractions (0.5 mL) were collected and counted using a micro-β counter. HS disaccharides were generated for three independent experiments and the products of digestion were combined before analysis to allow detection. Hence, the results represent an overall trend, but cannot be further analyzed statistically.

Analysis of biotinylated FGF2 binding by flow cytometry
To analyze the binding of basic fibroblast growth factor FGF-2, we used the Fluorokine Biotinylated Human FGF basic (R&D Systems) according to manufacturer's specifications. Cells were detached from their substrate with 0.025% EDTA in PBS. Biotinylated FGF-2 were added to 25 µL of the cell suspension. For competition assay, 5 µg/mL of heparin or chondroitin-6-sulfate was added. The cells were incubated for 30 to 60 minutes at 8°C. A total of 10 µL of avidin–FITC were added and incubated for 30 minutes at 4°C in the dark. The cells were washed twice with RDF1 buffer to remove unreacted fluorescein–avidin. The cells were resuspended in RDF1 for analysis by flow cytometry.

Flow cytometry
For each sample, 10^5 cells were used. The cells were washed with PBS and fixed with 2% paraformaldehyde in PBS for 30 minutes. Staining was performed by incubating cells with primary...
antibody for 2 hours, followed by incubation with anti-IgG conjugated to Alexa 488 (1:300 dilution; Invitrogen) for 40 minutes. Data were collected using the FACSCalibur flow cytometer (Becton Dickinson).

Statistical analysis
Statistical significance was determined by the paired Student t test on Microsoft Excel. All experiments were performed in triplicate, and repeated three times, unless stated otherwise. P values < 0.05 were considered significant; error bars indicate SD.

Results
SULFs are upregulated in human colorectal cancer cell lines
SULF1 and SULF2 mRNA expressions were evaluated in non-transfected Caco-2 and HCT-116 colorectal cancer cell lines by real-time RT-PCR, compared with a normal colon cell line, FHCC. Both cancer cell lines expressed SULFs at high levels when compared with the normal cells (Fig. 1A). We then transfected SULF1 or SULF2 plasmid construct or control plasmid vector into Caco-2 and HCT-116 cells and isolated clones stably expressing SULF1 or SULF2. Quantitation of SULFS expression by real-time RT-PCR showed that SULF1 transfection increased by 200 times the relevant SULF1 mRNA in Caco-2 and 250 times in HCT-116 cells. The overexpression of one isoform of SULF did not affect the endogenous expression of the other in both cell lines (data not shown). The expression of SULF2 was increased by 150 times in Caco-2 and by 200 times in HCT-116 (Fig. 1B). The overexpression of SULFs was also confirmed by flow cytometry (Fig. 1C) and by Western immunoblotting, where we observed an augmentation of protein mostly, as expected, in the conditioned medium of both cells (Fig. 1D). It is noteworthy that despite the large increase in mRNA levels for both SULFs, the increase in protein levels was more modest (50%–100% increase). These data show that there was no gain in unknown signaling and highlight the importance of using SULF-positive cells.

Overexpression of SULFs promotes colorectal cancer cells proliferation and migration
After verifying the efficiency of transfection, the selected cells were tested for cell viability using the MTT method, and cell proliferation by BrdU incorporation. As shown in Fig. 2, there was an increase in cell viability in both cells transfected with SULF1 as well as in cells transfected with SULF2 at 24 and 48 hours (Fig. 2A). Moreover, it was possible to observe—for except for Caco-2 cells overexpressing SULF1—an increase in cell proliferation in both transfections performed at 24 hours (Fig. 2B). The effects of SULFs overexpression were more pronounced on the HCT-116 cell line, which is derived from a highly metastatic cancer, than in the Caco-2 cell line, which has low metastatic potential. It is important to mention that, although derived from a colon carcinoma, when cultured under specific conditions, Caco-2 cells become differentiated and polarized, resembling normal enterocytes from small intestine (38).

To determine whether forced expression of SULFs increases colorectal cancer cell migration, we measured cell migration using the scratch wound–healing assay in cell monolayers. HCT-116 cells with forced expression of SULFs presented increased migration in comparison with cells transfected with empty vector (Fig. 2C). However, scratch assay results were not significant for Caco-2 cells overexpressing SULF1, agreeing with the results of cell proliferation for the same condition.

Knockdown of SULFs decreases cell proliferation and migration
To confirm our previous results, we investigated the effects of downregulation of SULFs on cell growth and migration. Therefore, we knocked down SULF1 and SULF2 in Caco-2 and HCT-116 cells using shRNA targeting those mRNAs. SULF mRNA expression in the stable clones was quantified by real-time RT-PCR, and the efficacy of the knockdown was at least 80% (Supplementary Fig. S3). Decreased SULF expression inhibited cell viability on both colorectal cancer cell lines, measured by MTT (Fig. 3A). Knockdown of SULFs also decreased significantly the cell migration in Caco-2 and HCT-116 cells (Fig. 3B). Moreover, to confirm that the previous observation about cell proliferation and migration could be caused specifically by the S6 removed by SULFs, we incubated the cells with heparin and heparin that had been selectively 6-O–desulfated. The colorectal cancer cells were then submitted to viability and migration assays (Fig. 3C) to determine whether heparin concentration could be toxic to the cells. Interestingly, the addition of 6-O–desulfated heparin increased even more the cell viability of Caco-2 cells overexpressing SULF2, as well as of HCT-116 overexpressing both SULFs (Fig. 3C). Moreover, the addition of heparin to the medium of colorectal cancer cells overexpressing SULFs was sufficient to reverse the increased migratory behavior of both cells as in the case of the knockdown experiments. There was also an increase in migration of HCT-116 control cells, transfected with empty vector, only by the addition of 6-O–desulfated heparin (Fig. 3D), suggesting that 6-OH glucosamine residues could act as facilitators for cell migration or could indirectly interfere in cell signaling events that may control cell proliferation and migration (2).

SULFs increase cancer cells adhesion and invasiveness
We next investigated the effects of the overexpression of SULFs on the adhesion and invasiveness of colorectal cancer cells. For the adhesion assay, Caco-2- and HCT-116–transfected cells were plated in culture dishes coated with fibronectin, laminin, or collagen I and allowed to adhere for 6 hours. Nonadherent cells were removed by aspiration, and those that remained on the plate were stained with DAPI and counted. A significant increase on adhesion of both colorectal cancer cells transfected with SULF1 and SULF2 (Fig. 4A) was observed. Interestingly, this increased adhesion was more significant when cells overexpressing SULFs were plated on fibronectin, which is known to bind HS and participate in the adhesion of colorectal cancer cells (38).

Transmigration assays were performed with Caco-2 and HCT-116 cells transfected with SULF1 or SULF2. Cancer cells were seeded on transwell membranes and allowed to migrate for 24 hours. Migratory cells on the bottom chamber of the transwell were stained, solubilized, and the migration was measured by absorbance. Cells in which SULFs were overexpressed migrated more through the transwell membrane (Fig. 4B). Therefore, the results indicated that colorectal cancer cells overexpressing SULF1 or SULF2 are able to adhere more rapidly to proteins from the ECM, and that they are capable of migrating more, suggesting a possible increase of invasiveness.

Overexpression of SULFs results in larger colony formation by colorectal cancer cells
To further analyze the tumorigenic potential of SULFs in colorectal cancer cells, we performed the colony formation, anchorage-independent growth, assay in soft agar. Caco-2– and
HCT-116–transfected cells were embedded in 0.35% agar, and the number and size of the colonies were monitored by 20 days. We did not observe an increase in the number of colonies formed by cancer cells overexpressing SULF1 or SULF2 (Fig. 5). Yet, the colonies formed were at least twice the size of the ones formed by the nontransfected cells, demonstrating that the overexpression of SULFs increases the tumorigenic potential of colorectal cancer cells in vitro. Moreover, by immunofluorescence, we observed an intense staining for SULFs in the colonies formed by the cells, indicating a strong presence of the enzymes after the soft agar assay, which simulates the tumor formation in vitro.
SULFs alter HS substitution pattern

After the cellular assays, we analyzed the structure of HS in colorectal cancer cells with forced expression of SULFs. GAGs were radiolabeled with $[^{35}\text{S}]\text{Na}_2\text{SO}_4$ and analyzed by agarose gel electrophoresis in PDA buffer. For disaccharide analysis, HS produced by cancer cells was digested using a mixture of heparin lyases, and the products were analyzed by HPLC using a SAX column. First, we noticed a decrease of sulfated heparan in Caco-2 and HCT-116 cancer cells overexpressing SULF 1 or 2, in all the compartments analyzed, the medium, cell extract, and ECM (Fig. 6A). The disaccharide analysis of HS from cells overexpressing SULFs showed a trend toward reduction of the trisulfated disaccharide UA(2S)-GlcNS(6S) in Caco-2 as well as in HCT-116 cancer cells (Fig. 6B) and a corresponding increase.
in the disulfated disaccharide UA(2S)-GlcNS. Our results are in agreement with the data from the literature, which points to the trisulfated disaccharide as the main substrate for SULFs (10, 25). However, differences in other disaccharides composing HS from cancer cells overexpressing SULFs were also observed. Interestingly, in some cases, a substantial increase in the less common disaccharides containing GlcNAc(6S) was also observed (Fig. 6B). Such observation was more evident for Caco-2 overexpressing both SULF1 and -2, where 7 of 12 GlcNAc(6S)—UA-GlcNAc(6S) and UA2S-GlcNAc(6S)—containing disaccharides were increased. Furthermore, there was a preferred increase in Caco-2 overexpressing both SULF1 and -2 of UA-GlcNAc(6S), whereas UA2S-GlcNAc(6S) was preferably increased in HCT-116 overexpressing SULF1 exclusively.

Owing to the changes in HS substitution pattern, we analyzed the levels of mRNA of the HS biosynthetic enzymes: 2OST, 6OST1, and NDST 1–4 (Supplementary Fig. S4). Interestingly, there were changes in the expression of the enzymes analyzed, including a decrease of 6OST1 in HCT-116 cells. There was also a reduction in the expression of NDST2, which is the main isoform expressed in both cells. Caco-2 cells do not present any NDST3 and NDST4 expression, and HCT-116 cells do not present NDST4.

**Figure 3.**
Knockdown of SULFs decreased cancer cells viability and migration, and addition of heparin reverses the effects of SULFs overexpression. Cells were transfected with shRNA targeting SULF1 or SULF2 as described in Materials and Methods. SULFs mRNA levels were quantified by real-time PCR (not shown). MTT viability (A) and (B) wound-healing assays were performed as described previously. To study the effects of heparin and 6-O-desulfated heparin on viability and migration of Caco-2 and HCT-116 colorectal cancer cells overexpressing SULFs, 100 μg/mL of heparin were added to cell medium for 24 hours. The cells were then submitted to (C) MTT and (D) wound-healing assays. CTRL NEG, scramble shRNA sequence; SULF1: shRNA targeting SULF1, SULF2: shRNA targeting SULF2. VECTOR, cells transfected with empty vector; SULF1, cells transfected with SULF1-expressing plasmid; SULF2, cells transfected with SULF2-expressing plasmid.

\* P \leq 0.05 compared with empty vector.

Next, we tried to correlate the structure of HS with changes in signaling pathways. The binding of FGF-2 to colorectal cancer cells overexpressing SULFs was analyzed by flow cytometry. Moreover, we examined changes in Wnt3A canonical signaling pathway, which is known to be modified by SULF activity and is involved in colorectal cancer (22, 30). There was a decrease in the binding of FGF-2 to the transfected cells (Fig. 7A). To confirm the specificity of FGF-2 binding to HSPGs, cells were incubated with FGF-2 in the presence of heparin or chondroitin-6-sulfate. Heparin decreased FGF-2 binding to Caco-2 and HCT-116 cells, whereas chondroitin did not. Furthermore, we observed an increase in the Wnt signaling pathway, evinced by the accumulation of active unphosphorylated β-catenin (Fig. 7C) and also overexpression of glypicans...
Fig. 7B), which have been shown to be involved in Wnt signaling (39). By flow cytometry analyses, the proportion of cells presenting both Wnt3A and β-catenin (Fig. 7D) increased. Caco-2 cancer cells overexpressing SULF1 or SULF2 showed 16.6% and 19.3% of cells double stained for Wnt3A and β-catenin, respectively, in comparison with 9.5% in control cells transfected with empty vector. HCT-116 cells overexpressing SULF1 or SULF2 showed 29.7% and 31.2% of cells double stained for Wnt3A and β-catenin, respectively, compared with 18.4% in control cells transfected with empty vector.

Figure 4.
Forced expression of SULFs increased cell adhesion to different ECM proteins and cell migration. A, HCT-116 and Caco-2 cells stably transfected with SULF1 or SULF2 were seeded on coverslips precoated with collagen-I, laminin, or fibronectin (50 μg/mL). After 6 hours, nonadherent cells were aspirated and adherent cells were stained with DAPI and counted over fluorescent microscope using Image J software. B, for transwell migration assay, cells were seeded on the top chamber of transwell membranes (8 μm pore size). Migrating cells were fixed with 4% formaldehyde and stained with crystal violet. The graphics represent the relative migration. SULF1, cells transfected with SULF1-expressing plasmid; SULF2, cells transfected with SULF2-expressing plasmid; VECTOR, cells transfected with empty vector. *P < 0.05 compared with empty vector.
SULF2 in colorectal cancer cells. The major mechanisms underlying their regulation remain unclear. Cancers, their precise role in cancer pathogenesis and the molecules have demonstrated the involvement of SULFs in various types of cancers, their precise role in tumor development and the role of HSPGs on cell adhesion, which is dependent on the interaction of HS chains with ECM proteins such as fibronectin, has already been well established. Apparently the decrease of the sulfation status of HS facilitated the adhesion and migration of colorectal cancer cells. These effects were reverted through the knockdown of SULF1 or SULF2 using shRNAs; therefore, the effects on cell migration occurred by the increased activity of SULFs. Moreover, the overexpression of SULFs resulted in larger colonies formed by colorectal cancer cells on soft agar. This result demonstrates that the forced expression of SULFs increases the tumorigenic potential of Caco-2 and HCT-116 colorectal cancer cell lines.

As stated, although SULFs are described as being upregulated in several types of cancers, their precise role in tumor development remains unclear, and some studies have reported that overexpression of SULFs in cancer cell lines leads to decreased cell growth. It is important to note that the cell lines investigated in this regard were SULF negative. Perhaps, forced expression of large amounts of SULFs could compromise signaling pathways, which the cells rely upon. Therefore, we opted to overexpress the enzymes in Caco-2 and HCT-116 colorectal cancer cells, which already had a high expression of SULFs, compared with the normal colorectal cell line FHC.

Forced expression of SULF1 and SULF2 increased cell growth in most cases apart from SULF1 overexpression in Caco-2 cells which, indeed, in some cases may seem as a normal cell line. This result is consistent with previous data from the literature reporting the overexpression of SULF-enhanced cell growth in pancreatic cancer, gastric cancer, glioma cancer cells, and lung cancer in vitro and in vivo. Besides increasing cell proliferation, the forced expression of SULFs also resulted in much higher cell migration. Again, as for cell growth, SULF1 overexpression in Caco-2 cells did not result in robust migratory behavior, fact that reinforces its so-called “normal” status in some specific cases. One of the possible reasons that could explain such behavior is that the cancer cells overexpressing SULF1 or SULF2 showed increased adhesion to collagen I, laminin, and fibronectin.

The role of HSPGs on cell adhesion, which is dependent on the interaction of HS chains with ECM proteins such as fibronectin, has already been well established. Apparently the decrease of the sulfation status of HS facilitated the adhesion and migration of colorectal cancer cells. These effects were reverted through the knockdown of SULF1 or SULF2 using shRNAs; therefore, the effects on cell migration occurred by the increased activity of SULFs. Moreover, the overexpression of SULFs resulted in larger colonies formed by colorectal cancer cells on soft agar. This result demonstrates that the forced expression of SULFs increases the tumorigenic potential of Caco-2 and HCT-116 colorectal cancer cells in vitro.

SULFs liberate 6-O-S mainly from trisulfated disaccharide units (IdoA2S-GlcNS6S) within S domains of heparin/HS chains. Our HS disaccharide analysis from cells overexpressing SULFs also showed a trend toward reduction of the trisulfated disaccharide UA(2S)-GlcNS(6S) and a corresponding rise in the disulfated disaccharide UA(2S)-GlcNS in Caco-2 and HCT-116 cancer cells. An increase in less common disaccharides containing GlcNAc(6S) residues was also observed in some cases. Our results cannot rule out that such difference could be the result of enzyme substrate specificity. Selfouh and colleagues have demonstrated that SULF1 and SULF2 share the same desulfation process, whereas SULF1 showed a much stronger potency for in vitro desulfation of HS substrates. Moreover, data from the literature indicate that the substrate specificity of the SULFs remains unclear, because some methods are not sensitive enough to detect less abundant disaccharide units (26, 43). However, in vivo and in vitro data indicate that these enzymes essentially exert their activity on UA2S-GlcNS6S units, which...
Role of SULFs in Colorectal Cancer Cells

Figure 6.
The content and structural composition of HS labeled with [35S]Na2SO4. A, GAGs were purified from the cultured medium (MEDIUM), the cancer cells (CELL) extracted with EDTA, and the matrix (MATRIX) produced by cells. The content of GAGs of these compartments was analyzed through agarose gel electrophoresis in 1,3-diaminepropane acetate buffer (50 mmol/L, pH 9.0). GAGs were purified from the cultured medium (MEDIUM), the cancer cells (CELL) extracted with EDTA, and the matrix (MATRIX) produced by cells. The content of GAGs of these compartments was analyzed through agarose gel electrophoresis in 1,3-diaminepropane acetate buffer (50 mmol/L, pH 9.0). The gel was exposed to a screen and the bands identified using an image analysis system, Ciclone (Storage Phosphor System-Packard Instrument). Quantification was performed by densitometry with Opti-Quanti Software. B, each sample was digested using a mixture of heparin lyases and analyzed on a PhenoSphere 5 μm SAX 80 Å LC Column 150 × 4.6 mm. Hexa-disaccharide were eluted with a linear gradient of NaCl (0–1 mol/L) over a 30-minute period at a flow rate of 1 mL per minute. Individual fractions (0.5 mL) were collected and counted using a micro-β counter. The bars indicate the average of three independent experiments. The arrows show the trisulfated disaccharide UA(2S)-GlcNac(6S). SULF1, cells transfected with SULF1-expressing plasmid; VECTOR, cells transfected with empty vector. *, P < 0.05 compared with empty vector.

are found within HS S-domains, and to a much lesser extent on (UA-GlcNS6S) disulfated disaccharides. Therefore, our results are then most likely linked to specific cell response via regulation of HS biosynthetic machinery in response to increased SULF activity.

To further test this hypothesis, using real-time PCR, we observed a decrease in the mRNA expression of 6OST1 and in NDS2, which is the major expressed isoform in Caco-2 and HCT-116 cells. Recently, a scheme for the biosynthesis at the level of disaccharides has been proposed (44), in which two branches,
one comprising disaccharides containing N-acetylated glucosamine and another, containing N-sulfated glucosamine, are suggested. The branch point is the action of the NDST enzyme, and the subsequent epimerase activity is much more efficient in the conversion of GlcA-GlcNS than GlcA-GlcNAc (45), resulting in a branch containing the less common, IdoA/GlucA-C6-C6S-GlcNAc disaccharides. Again, the increased trend in GlcNAc(6S) disaccharides observed here could be a cellular response to increased SULF activity employing this alternative pathway, attempting to compensate for the decrease in 6-O-sulfated residues within the trisulfated disaccharide. However, the normal behavior—robust migration—could not be recovered, possibly because the NAc to NS modification strongly affects the overall geometry of the HS chain (46).

Lamanna and colleagues (26) have also demonstrated that loss of SULFs has dynamic effects on the expression of each of the three 6-O-sulfotransferase isoforms present in mammalian cells, HS6ST1, HS6ST2, and HS6ST3. Interestingly, both colorectal cancer cells do not express NDST4, which is believed to be a tumor suppressor candidate (27).

It is important to point out, however, that discrepancies were previously observed between in vitro and in vivo studies involving SULFs (26, 43). In vitro, the enzyme activity of the SULFs has been studied after near-exhaustive digestion of the polysaccharide. However, in tissues, the extent of HS 6-O-desulfation by the SULFs is likely to depend on critical environmental factors, such as the amount of enzymes expressed, their ability to diffuse throughout tissues, or the structure and sulfation pattern of HS present in these tissues (43). The physiologic activity of the SULFs may therefore lead to partially desulfated HS chains, which may differentially affect binding of some HS ligands but not others, depending on the extent of desulfation.

Figure 7. Signaling pathways modified due to the overexpressing of SULFs. A, FGF-2 binding was performed according to the manufacture’s instruction (R&D). Caco-2 and HCT-116 cells transfected or not with SULF1 or SULF2 were incubated with 10 nmol/L biotinylated FGF2 ligand with or without 5 μg/mL heparin or 5 μg/mL chondroitin-6-sulfate for 60 minutes at 4°C. After staining with streptavidin, cells were counted and analyzed by flow cytometry. B, glypicans expression was measured by real-time PCR using GAPDH as control. C, Wnt signaling pathway was verified by Western blot and quantified using β-actin as control. Inactive phospho-β-catenin and active β-catenin were stained with specific antibodies (R&D). Anti-β-catenin antibody recognizes only the unphosphorylated protein. D, Caco-2 and HCT-116 cells overexpressing SULFs were stained with Wnt-3A and β-catenin antibodies and analyzed by flow cytometry, as described in Materials and Methods. Representative photographs are shown, indicating the percentage of double-stained cells. SULF1, cells transfected with SULF1-expressing plasmid; SULF2, cells transfected with SULF2-expressing plasmid; VECTOR, cells transfected with empty vector. *P ≤ 0.05 compared with empty vector.
The differences in HS structure due to the overexpression of SULFs in colorectal cancer cells resulted in alterations of signaling pathways. We observed a decrease in the binding of FGF-2 to the cells with forced expression of SULFs and, more importantly, we observed an increase in Wnt3A signaling. The decreased FGF-2 binding does not match the increased tumorigenic potential presented by colorectal cancer cells overexpressing SULFs, which indicates that probably other signaling pathways, such as Wnt, overrule the decreased FGF binding effect. A similar effect was noticed by Nikolova and colleagues (47) by the knockdown of the HSPG syndecan-1 in breast cancer cells that led to decreased MAPK signaling, whereas cell adhesion and integrin-related signaling were increased.

Ai and colleagues (9) have proposed a model in which SULF promotes Wnt signaling. The model posits that the action of SULF is to weaken the association of Wnt ligands with HSPGs, allowing the ligands to activate signal transduction receptors (Frizzled’s). When Wnt proteins are present, they bind to the Frizzled receptor and to low-density lipoprotein-related receptors 5 and 6. These events lead to the dissociation of β-catenin from the APC/Axin/GSK3β destruction complex. Thus, unphosphorylated β-catenin accumulates in the cytoplasm and translocates into the nucleus, where β-catenin interacts with T-cell factor and lymphoid-enhancing factor to activate the transcription of Wnt/β-catenin–mediated target genes. The target genes include cell-cycle–regulating genes and genes related to metastasis and the invasion of cancer cells (33). The dysregulation of the Wnt/β-catenin signaling pathway has been considered to play an important role in colon carcinogenesis (12, 33).

Finally, and showing the importance of 6-O-sulfates for normal cell behavior, the addition of heparin to the medium of colorectal cancer cells overexpressing SULFs was sufficient to reverse the robust migratory effects caused by SULF overexpression. The abundance of exogenous substrate—heparin—in the medium available to the enzymes, possibly resulted in less endogenous HS 6-OS degradation, hence generating "normal" behavior, which was not the case when selectively 6-O-desulfated heparin was used. Such data highlight the pivotal role of 6-O-sulfate groups to these cellular characteristics. In addition, there was also an increase in migration of HCT-116 control cells, transfected with empty vector, only by the addition of 6-O-desulfated heparin. Furthermore, the fact that treatment of HCT-116 control cells with 6-O-desulfated heparin resulted in migratory behavior gain, which was not observed for Caco-2 cells, suggests that 6-OH glucosamine residues may interfere in cell signaling events that are dependent on this specific sulfate leading, indirectly, to increased cell migration/proliferation (1–6). Different studies had already demonstrated that changes in HS sulfation pattern, caused by SULFs, have dynamic effects on cell signaling response to growth factors (8, 26). Together, these results establish the importance of the HS sulfation pattern in cancer cells.

Therefore, the presented data indicate a possible protumorigenic role of SULFs in colorectal cancer. However, due to the limitations of in vitro experiments using cell lines, further studies, including in vivo assays, are necessary to better understand the complex function of SULFs in cancer. Different studies had already described the increase of SULF1 expression in human colon mucinous adenocarcinoma (n = 13) versus normal tissue (n = 5; refs. 8, 48), human colon adenocarcinoma (n = 41) versus normal tissue (n = 5; refs. 8, 48), human colon adenocarcinoma (n = 50) versus normal tissue (n = 28; refs. 8, 49), and human cecum adenocarcinoma (n = 17) versus normal tissue (n = 5; refs. 8, 48). Thus, our in vitro study indicates a possible mechanism in which SULFs are contributing to colorectal cancer progression, providing new molecular insights into the role of SULFs and heparan sulfate in colon cancer pathogenesis. Moreover, the potential for therapeutic interventions with heparinoids provides an interesting perspective for patients. Further in vivo and ex vivo studies are being undertaken by us to investigate SULFs relevance in colorectal cancer pathogenesis.

It is noteworthy that an inhibitor of SULFs has already been identified, PI-88 (8). This agent consists of a mixture of chemically sulfated yeast oligosaccharides with a molecular weight range of 1,400 to 3,100 Da and has been shown to inhibit the enzymatic activity of both SULFs against 4-MUS, heparin, and HSPGs. This compound has been tested in clinical trials for advanced melanoma, liver cancer, lung cancer, and prostate cancer. Yet, these studies have demonstrated recurring problems of immune-mediated thrombocytopenia in a significant number of patients associated with the use of PI-88 (50). Once PI-88 presents a wide range of different biological activities, it becomes difficult to interpret either beneficial or adverse effects of the drug and, perhaps, heparin derivatives that have been proved to be safe and that lack significant residual side effects, such as thrombocytopenia and bleeding could be tested as specific inhibitors of SULFs, thereby providing more information about their role in cancer as well as providing a more promising treatment of cancer where SULFs overexpression plays an important role for disease progression.

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

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