Chemically Oversulfated Glycosaminoglycans Are Potent Modulators of Contact System Activation and Different Cell Signaling Pathways *§

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Contaminated heparin was associated with adverse reactions by activating the contact system. Chemically oversulfated/modified glycosaminoglycans (GAGs) consisting of heparan sulfate, dermatan sulfate, and chondroitin sulfate have been identified as heparin contaminants. Current studies demonstrated that each component of oversulfated GAGs was comparable with oversulfated chondroitin sulfate in activating the contact system. By testing a series of unrelated negatively charged compounds, we found that the contact system recognized negative charges rather than specific chemical structures. We further tested how oversulfated GAGs and contaminated heparins affect different cell signaling pathways. Our data showed that chemically oversulfated GAGs and contaminated heparin had higher activity than the parent compounds and authentic heparin, indicative of sulfation-dominant and GAG sequence-dependent activities in BaF cell-based models of fibroblast growth factor/fibroblast growth factor receptor, glial cell line-derived neurotrophic factor/c-Ret, and hepatocyte growth factor/c-Met signaling. In summary, these data indicate that contaminated heparins intended for blood anticoagulation not only activated the contact system but also modified different GAG-dependent cell signaling pathways.

Heparin is prepared from crude heparin that consists of ~50% heparin and ~50% less sulfated GAGs, including heparan sulfate, dermatan sulfate, and chondroitin sulfate (5). The less sulfated GAGs are removed from crude heparin into two heparin by-products named GAG waste and tank bottom (6). We have analyzed the same contaminated heparin preparations and concluded that heparin is contaminated either with chemically oversulfated/desulfated GAG waste or chemically oversulfated/desulfated GAG waste plus oversulfated tank bottom (7, 8).

OSCS has been suggested to be the cause of adverse reactions by inducing anaphylatoxins, bradykinin, C3a, and C5a, through contact system activation (9). The established contact system in human plasma consists of two serine protease prozymes as follows: prekallikrein and factor XII; a cofactor, high molecular weight kininogen; and a regulator, C1 inhibitor. Activated kallikrein produces bradykinin from high molecular weight kininogen (10), whereas thrombin produces C3a and C5a (11, 12). These established molecular mechanisms suggest that heparin contaminants might induce thrombin generation, which produced the observed C3a and C5a through contact system activation. It was recently reported that dextran sulfate, another artificially sulfated polysaccharide, greatly enhances protein aggregate-dependent contact system activation and also facilitates protein aggregate-independent factor XI activation (13). Activated factor XI leads to thrombin propagation (14).

In our current studies, we found that oversulfated heparan sulfate (OSHS) and oversulfated dermatan sulfate (OSDS) were as potent as OSCS in activating the contact system. We further compared the ability of heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, several contaminated heparins, various oversulfated GAGs, and a variety of negatively charged molecules, such as bacteria lipopolysaccharides (LPS), yeast zymosan, phospholipids, DNA, and sulfatide, to induce kallikrein- and thrombin-like activities in pooled normal human plasmas. We found that oversulfated GAGs were the most potent kallikrein- and thrombin-like activity inducers.

Heparin regulates multiple growth factor signaling pathways (15), including but not limited to fibroblast growth factor (FGF)/FGFR (16), hepatocyte growth factor (HGF)/c-Met (17–20), glial cell line-derived neurotrophic factor (GDNF)/c-Ret/GFRα1 (21, 22), vascular endothelial growth factor/vascular endothelial growth factor receptor (23, 24), platelet-derived growth factor/platelet-derived growth factor receptor (25),
Contaminated Heparin, Contact System, and Cell Signaling

BAFF/TACI (26), Indian hedgehog (Ihh), Wnt, and bone morphogenetic protein signaling pathways where genetic studies revealed the absolute requirement for GAGs (27, 28). Because of the amplification power of cell signaling pathways, we presumed that contaminated heparin might also affect certain cell signaling pathways. We made stable BaF cell lines that expressed c-Met or c-Ret/GFRα1. We used the BaF cell lines expressing FGFR1c, S252WFGFR2b (16), c-Met, and c-Ret/GFRα1 to test how chemically oversulfated GAGs and contaminated heparins affect the BaF cell proliferation. We found that contaminated heparins were more potent than authentic heparin in the BaF cell-based models of FGFR/FGFR2, GDNF/c-Ret, and HGF/c-Met signaling.

EXPERIMENTAL PROCEDURES

Materials—Heparin (H4784), LPS, sulfatide, herring DNA, lipid A, zymosan, egg lecithin, kallikrein substrate, N-benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride, and thrombin substrate, N-methylglycine-Pro-Arg-p-nitroanilide dihydrochloride, were obtained from Sigma. Heparan sulfate and dermatan sulfate from porcine intestinal mucosa were purchased from Celsus Laboratories. OSHS, OSDS, and oversulfated heparin by-products (OSHB) were synthesized in our laboratory. The contaminated heparin, cartilage OSCS, and heparin by-product used in this study were supplied by the United States Food and Drug Administration.

Each GAG (20 mg) was weighed and dissolved in 5 ml of H2O to make 4 mg/ml stock solution. Aliquotted solutions (0.5 ml) for each GAG were stored at −20 °C before use.

Pooled normal human plasma from 30+ normal donors was purchased from George King Biomedical, Inc. The plasma was aliquoted and stored at −70 °C before use.

Chemical Synthesis of OSHS, OSDS, and OSHB—Heparan sulfate, dermatan sulfate, and heparin by-products were converted to tetrabutylammonium salt by a previously reported procedure (29). The resulting salt (100 mg) was dissolved in 2 ml of N,N-dimethylformamide to which 2.3 g of N,N-dimethylformamide-sulfur trioxide complex dissolved in 16 ml of N,N-dimethylformamide was added. After 1 h at 70 °C, the reaction was stopped by adding 5 ml of cold water. The pH was adjusted with 10 N NaOH to pH 9. The oversulfated material was precipitated with 3 volumes of ethanol saturated with sodium acetate and then collected by centrifugation. The resulting pellet was washed with 30 ml of 75% ethanol three times using ultrasound to break up the pellet followed by centrifugation. The oversulfated material was dissolved in 10 ml of water, dialyzed to remove salt, and lyophilized.

Capillary Electrophoresis (CE)—CE analysis was conducted on a Hewlett-Packard three-dimensional capillary electrophoresis instrument equipped with a diode array detector set at a wavelength of 200 nm (bandwidth 10 nm). Separations were performed in a bare fused silica capillary, internal diameter 50 mm, 64.5 cm total length, 56 cm effective length with a column temperature of 25 °C. The polarity was negative with a voltage of 30 kV. Samples were dissolved in Milli-Q water at a concentration of ~10 mg/ml and filtered through 0.2-μm cellulose acetate membrane filters (Micro-Spin filter tubes, Alltech Associates, Deerfield, IL). The sample solutions were injected using hydrodynamic pressure at 50 mbars for 10 s. The electrolyte solution was 36 mM phosphate buffer (pH 3.5) filtered with a 0.2-μm cellulose acetate syringe filter (Grace, Deerfield, IL). The capillary column was preconditioned at the beginning of each day by flushing with 1 M NaOH, 0.1 M NaOH, and water, each for 2 min, and prior to running each sample by flushing with water for 2 min and electrolyte solution for 2 min.

Kallikrein- and Thrombin-like Activity Assays—Plasmas were activated in vitro by adding 20 μl of samples to be tested into 30 μl of plasma at 37 °C for 5 min with a final concentration of 0, 2, 20, and 200 μg/ml, respectively, in a 96-well plate (catalog no. TP9296, MIDSCI, Inc., St. Louis, MO). TBS buffer (50 mM Tris, pH 7.6, 50 mM NaCl) containing 2 mM Ca2+ was then added to each well to a total volume of 150 μl. Absorbance at 405 nm was recorded as blank by Spectramax M2 plate reader (Molecular Devices). Absorbance at 405 nm was recorded every 90 s for 40 min after adding substrate (50 μl in TBS) to the activated plasmas in each well. For kallikrein and thrombin assays, 0.5 mM N-benzoyl-Pro-Phe-Arg-p-nitroanilide dihydrochloride and 1 mM N-methylglycine-Pro-Arg-p-nitroanilide dihydrochloride substrates were used as substrates, respectively. Both substrates were dissolved in TBS buffer.

GAG-dependent Cell Proliferation Assay—96-Well tissue culture plates (catalog no. TP9296, MIDSCI, Inc., St. Louis, MO) were dispersed with 100 μl of BaF cell culture media (30) containing different kinds of GAGs at 0, 0.064, 0.32, 1.6, 8, 0, and 200 μg/ml. BaF3 cells (30,000 live cells/well in 50 μl of BaF cell culture media) expressing FGF1c, S252WFGFR2b, Met, and c-Ret/GFRα1 were then added. FGFs, HGF, or GDNF (50 μl in BaF cell culture media) were then added to a final concentration of 8 nM. Cells were cultured for 40 h, followed by adding 20 μl of resazurin (2 mg/ml dissolved in water, catalog no. R7017, Sigma) to the media for 16 h. The fluorescent signal was monitored using 544 nm excitation wavelength and 595 nm emission wavelength by Spectramax M2 plate reader (Molecular Devices). The relative fluorescence unit (RFU) generated from the assay was proportional to the number of living cells in each well.

RESULTS

Chemically Oversulfated GAGs Were More Sulfated than Heparin—To compare the biological activities of chemically sulfated GAGs with heparin, we first chemically sulfated the heparin by-product, heparan sulfate, and dermatan sulfate purified from porcine mucosa crude heparin. We compared the degree of sulfation of these chemically sulfated compounds with heparin and purified heparin contaminant by capillary electrophoresis analysis. Theoretically, heparin should always migrate faster than any other naturally occurring GAGs, whereas only chemically oversulfated GAGs can migrate faster than heparin by capillary electrophoresis analysis. As shown in Fig. 1A, heparin eluted at 5.6 min, heparin by-product at 6.5 min (Fig. 1C), porcine mucosa dermatan sulfate at 6.5 min (Fig. 1E), and porcine mucosa heparan sulfate at 7.6 min with a broad elution peak (Fig. 1G), which indicates that it had a broader sulfation distribution than that of porcine mucosa dermatan sulfate. All chemically oversulfated GAGs migrated faster than heparin by using the sulfation conditions described under
**Contaminated Heparin, Contact System, and Cell Signaling**

Heparan sulfate but not chondroitin sulfate nor dermatan sulfate contains N-sulfated residues. It was reported that de-N-sulfation occurs during the chemical modification process of heparin/heparan sulfate (31–33), which generates free amine residues in OSHS. We did not perform re-N-sulfation reactions for OSHS and OSHB to be consistent with the same sulfation condition for other oversulfated GAGs. The presence of free amine groups in oversulfated heparan sulfate might explain the broader OSHB (Fig. 1D) and OSHS (Fig. 1H) elution peaks observed.

By analyzing 34 contaminated heparin samples in our published studies (see Ref. 7, supplemental Table S1), we found that OSCS is not a major galactosamine containing heparin contaminants in any of the 34 samples when taking into account that the UV_{200 nm} absorbance response for OSCS is three times greater than that of heparin based on both CE- and galactosamine-based heparin contaminant quantifications (see Ref. 7, supplemental Table S1). These data suggest that less sulfated heparin contaminants might co-migrate with heparin, which makes such contaminants undetectable by CE-based and anion exchange HPLC-based heparin quality control assays. To test the hypothesis, we used three different chemical sulfation conditions to make highly (H) sulfated, intermediately (M) sulfated, and less (L) sulfated DS and HS (OSDS-H, OSDS-M, OSDS-L and OSHS-H, OSHS-M, OSHS-L), respectively. The protocol is detailed in the legend of Fig. S2.

We then performed NMR (supplemental Fig. S2A), anion exchange HPLC (supplemental Fig. S2B), and CE (data not shown) analysis on these chemically sulfated GAGs. We discovered that highly sulfated DS and HS (OSDS-H, OSHS-H) as evidenced by simpler NMR proton profiles shown in supplemental Fig. S2A) co-migrated with heparin, whereas less sulfated HS and DS (OSDS-M, OSDS-L, OSHS-M, and OSHS-L) migrate like OSCS when analyzed by both CE (data not shown) and anion exchange HPLC analysis (supplemental Fig. S2B). Consistent with our published observations that the heparins are not contaminated with OSCS alone (7, 8), the published CE data (6, 34) also showed that an OSHS co-migrated with heparin (see Ref. 6.

**FIGURE 1. CE profiles of normal and chemically sulfated GAGs.** The CE analysis was performed as described under “Experimental Procedures.” A, standard heparin; B, oversulfated chondroitin sulfate; C, heparin by-products; D, oversulfated heparin by-products; E, heparan sulfate; F, oversulfated heparan sulfate; G, dermatan sulfate; and H, oversulfated dermatan sulfate. NMR profiles of these GAGs can be found in supplemental Fig. S1.

“Experimental Procedures,” where OSCS (Fig. 1B), OSHB (Fig. 1D), OSDS (Fig. 1F), OSHS (Fig. 1H), and purified heparin contaminants all eluted at 5.2 min, the typical elution time observed in contaminated heparins.

OSCS had the sharpest elution peak by capillary electrophoresis analysis and simplest NMR proton profile (supplemental Fig. S1B), indicating it was fully sulfated. Oversulfated forms of heparin by-product, heparan sulfate, and dermatan sulfate had a broader elution peak at 5.2 min compared with OSCS and more complicated NMR proton profiles (supplemental Fig. S1, D, F, and H), indicating they were not as fully sulfated as OSCS.

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supplemental Fig. S9, last panel), and there are two heparin contaminant peaks that migrated before heparin in contaminated heparin by employing a high resolution CE method (see Ref. 34, Fig. 2). One peak was named fully sulfated CS and the other was named partially sulfated OSCS in the published report (34).

In addition, we found that less sulfated DS and HS (OSDS-M, OSDS-L and OSHS-M, OSHS-L) had several proton signal peaks such as 2.04, 2.07, 2.10, and 2.12 ppm for OSHS-M and OSHS-L and/or OSDS-M and OSDS-L, which made the 2.07 ± 0.02 and 2.12 ± 0.02 ppm proton chemical shifts not specific for DS and OSDS, respectively, in contrast to the published reports (6, 34). Because the heparin contaminant that migrated before heparin, based on CE analysis, was defined as OSCS or heparin contaminant during the past 2 years, we decided to choose all the OSGAGs that migrated like OSCS (Fig. 1) to compare their biological activities.

Charge-dependent Induction of Kallikrein- and Thrombin-like Activities—We used a variety of negatively charged molecules to induce kallikrein- and thrombin-like activities through contact system activation in pooled normal human plasmas. It was reported that negatively charged molecules can exert both activating and inhibitory effects on kallikrein-like activities in a time-dependent manner (35), and we measured the kinetics instead of a single time point used in previous studies (9). To this end, the human plasma was incubated with a series of negatively charged molecules at 37 °C for 5 min. The compounds tested include different bacterial LPS, lipid A, yeast zymosan, DNA, phospholipids, sulfatide, chondroitin sulfate-A, chondroitin sulfate-E, porcine mucosa dermatan sulfate, heparan sulfate, and heparin by-products, over-sulfated forms of heparin by-products, heparan sulfate, chondroitin sulfate, dermatan sulfate, and five different types of contaminated heparin described previously (8), including heparin containing oversulfated contaminant (OSCH4), heparin containing both low sulfated and oversulfated contaminants (OSLSCH5), heparin containing OSHS (lot S1), heparin containing chemically sulfated/desulfated GAGs (G1), heparin containing low sulfated heparin contaminant (LSCH6), and authentic heparin control purchased from Sigma. Kinetics of kallikrein- and thrombin-like activity induction was measured colorimetrically by kallikrein or thrombin substrate cleavage, respectively.

We found that each negatively charged molecule had distinct characteristics in terms of the lag time, rate, and pattern of induced kallikrein- and thrombin-like activities compared with the control (supplemental Figs. S3 and S4). Because the plasma control exhibited a 7-min delay in kallikrein activation induced by the plate surface in the presence of 2 mM CaCl₂ in the TBS buffer, we chose optical density readings at 3 min after adding protease substrate to the activated plasmas as a measure of the rate of kallikrein- and thrombin-like activity generation by tested compounds. We chose OD readings at 40 min as a measure of overall kallikrein- and thrombin-like activity generation by both the tested compound and plate surface (Fig. 2).

FIGURE 2. Negatively charged molecule-induced kallikrein- and thrombin-like activities. Normal pooled human plasma (30 µl) was incubated with 20 µl of negatively charged molecules at a final concentration of 0, 2, 20, and 200 µg/ml at 37 °C for 5 min. Kallikrein-like activities were assessed by addition of 150 µl of the kallikrein chromogenic substrate, 0.5 mM N-benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride dissolved in TBS. OD readings were taken every 90 s for 40 min (supplemental Fig. S3). Thrombin-like activity was assessed by the addition of the thrombin chromogenic substrate, 1 mM N-methylglycine-Pro-Arg-p-nitroanilide dihydrochloride dissolved in TBS. OD readings were taken every 90 s for 40 min (supplemental Fig. S4). A, OD readings of kallikrein-like activities at 3 min from two independent experiments. B, OD readings of thrombin-like activities at 3 min from two independent experiments. C, OD readings of kallikrein-like activities at 40 min from two independent experiments. D, OD readings of thrombin-like activities at 40 min from two independent experiments. (One set of kinetics data is shown in the supplemental Figs. S3 and S4). Because the plasma control exhibited a 7-min delay in kallikrein activation induced by the plate surface in the presence of 2 mM CaCl₂ in the TBS buffer, we chose optical density readings at 3 min after adding protease substrate to the activated plasmas as a measure of the rate of kallikrein- and thrombin-like activity generation by tested compounds. We chose OD readings at 40 min as a measure of overall kallikrein- and thrombin-like activity generation by both the tested compound and plate surface (Fig. 2).
Contaminated Heparin, Contact System, and Cell Signaling

All forms of oversulfated GAGs induced substantial kallikrein- and thrombin-like activities (supplemental Figs. S3 and S4), which are reflected in the 3-min OD readings shown in Fig. 2A (lanes 11–15) and Fig. 2B (lanes 11–15) after the protease substrate was added to the plasmas that have been activated by different compounds for 5 min. No kallikrein- and thrombin-like activities were induced by LPS, lipid A, or zymosan (Fig. 2, A and B, lanes 1–4 and 7) at 3 min, but a low degree of kallikrein-like activity was induced by herring DNA (lane 6) at 3 min. Interestingly, these compounds enhanced overall kallikrein-like activities by 10–30% compared with the blank control (0 μg/ml) at 40 min (Fig. 2C, lanes 1–4, 6, and 7 compared with lane 22). Sulfatide (Fig. 2, lane 5) induced both kallikrein- and thrombin-like activities (Fig. 2, A–D). Interestingly, low concentration of sulfatide was preferred for inducing kallikrein-like activities (Fig. 2, A and C, lane 5), whereas high concentration of sulfatide was preferred for inducing thrombin-like activities.

Natural GAGs, such as dermatan sulfate and heparin, induced kallikrein-like activities but induced no or low thrombin-like activities at 3 min (Fig. 2, A–D, lanes 12–15). The contaminated heparins had decreased levels of oversulfated GAG contamination in the order of OSCH4, OSLSCH5, S1, LSCH6, and G1 (8), which correlated with their ability in inducing kallikrein- and thrombin-like activities at the 200 μg/ml level (Fig. 2, A–D, lanes 16–20).

LPS, lipid A, and zymosan were kallikrein-like activity enhancers once the contact system was activated (Fig. 2C, lanes 1–4 and 7). Herring DNA was a prekallikrein activator and a kallikrein-like activity enhancer (Fig. 2, A and C, lane 6 compared with the control lane 22). E. coli LPS and lipid A did not induce thrombin- and kallikrein-like activities at 3 min (Fig. 2B, lanes 2 and 4); however, they enhanced thrombin-like activities at high concentration (200 μg/ml) (Fig. 2D, lanes 2 and 4 compared with the control lane 22).

We conclude that the kallikrein-like activities were induced by a variety of structure-unrelated compounds in pooled normal human plasmas. Chemically oversulfated GAGs were the most potent kallikrein- and thrombin-like activity inducers. Natural GAGs served as kallikrein- and thrombin-like activity inducers and inhibitors at different times of contact system activation in a structure- and concentration-dependent manner (Fig. 2, A–D, lanes 8–11 and 21). Chondroitin sulfate-E (Fig. 2, A–D, lane 11) was the most potent kallikrein- and thrombin-like activity inducer among all natural occurring GAGs tested.

Oversulfated GAGs Enhanced FGF/FGFR Signaling—We previously reported that the single amino acid mutation S252W in fibroblast growth factor receptor 2 (FGFR2) of Apert syndrome patients leads to abnormal GAG/FGF/FGFR2 signaling (16). The Apert syndrome is one of the most severe types of human craniosynostosis syndromes. We discovered that different GAGs can either activate or inhibit specific FGF/ S252WFGR2 signaling in a BaF cell-based model of Apert syndrome. BaF3 cells are dependent on cytokine interleukin-3 for normal growth. When stably transfected to express FGFs, BaF3 cells die or do not grow well if only FGF or GAG is added to the interleukin-3 free cell culture media. BaF3 cell growth can only be achieved in the presence of both GAGs and proper FGFs (16).

To test how natural GAGs, including heparan sulfate, chondroitin sulfate, and dermatan sulfate, and chemically oversulfated forms of heparan sulfate, chondroitin sulfate, and dermatan sulfate affect FGFs/FGFR1c and FGFs/S252WFGR2b signaling in BaF cells, we incubated FGFR1c- and S252WFGR2b-expressing BaF cells with FGF-1, -2, -7, -9, -10, and -18 in the presence of 0–200 μg/ml of tested GAGs and oversulfated GAGs. FGFR1c signaling is expected to be activated by FGF-1, -2, -9, and -18, and S252WFGR2b signal is expected to be activated by all six FGFs in the presence of GAGs based on established FGF/FGF receptor affinity and BaF cell test results (16, 30). We used resazurin to replace previously used [3H]thymidine to measure FGF- and GAG-dependent BaF cell proliferation (16). Resazurin is a metabolic indicator dye. In its normal oxidized state, it shows very little fluorescence. Once the dye penetrates the cell, it becomes reduced to the highly fluorescent resorufin. The rate of resazurin reduction directly correlates with cell numbers and thus reflects overall cell growth induced by GAGs and FGFs.

Fig. 3 shows that high concentrations of heparan sulfate, chondroitin sulfate, and dermatan sulfate induced FGFR1c-expressing BaF cell proliferation in the presence of FGF-1 and -2 as evidenced by the increased relative fluorescence units. In contrast, oversulfated forms of heparan sulfate, chondroitin sulfate, and dermatan sulfate induced FGFR1c-expressing BaF cell proliferation in all six FGFs tested. It was unexpected that these oversulfated GAGs activated FGF-7/FGFR1c and FGF-10/FGFR1c signaling because FGF-7 and -10 are not natural FGFR1c ligands (30).

Fig. 4 shows that heparan sulfate, chondroitin sulfate, and dermatan sulfate enhanced S252WFGR2b-expressing BaF cell proliferation in the presence of FGF-1, -7, -9, and -10 better than the FGFR1c-expressing BaF cells, which is consistent with the report published previously (16). Oversulfated forms of heparan sulfate, chondroitin sulfate, and dermatan sulfate greatly enhanced the BaF cell proliferation in all six FGFs tested compared with the natural heparan sulfate, chondroitin sulfate, and dermatan sulfate. These results indicate that oversulfated GAGs promote FGF/FGFR signaling in the BaF cell proliferation system in a sulfation-dominant manner. These results predict that contaminated heparin would enhance FGF/FGFR signaling in the BaF cell systems.

Contaminated Heparins Were More Potent than Heparin in Enhancing FGF/FGFR, HGF/Met, and GDNF/c-Ret Signaling in BaF Cells—To compare how contaminated heparin affects FGF/FGFR, HGF/Met, and GDNF/c-Ret signaling, we first made stable BaF cell lines that expressed Met or c-Ret/FGFR1 by transfecting the corresponding cDNAs into wild-type BaF
cells, and we then performed the BaF cell proliferation assay with FGF1, HGF, and GDNF in the presence of different GAGs, including heparin and contaminated heparins.

HGF/Met signaling is regulated by GAGs (17). It was reported that thrombin activates the zymogen of the HGF activator (36). The HGF activator is a serine protease and

FIGURE 3. Chemically oversulfated GAGs (OSHs, OSDS, and OSCS) were more potent than natural GAGs (heparan sulfate, dermatan sulfate, and chondroitin sulfate) in stimulating cell proliferation in BaF cell-based FGF/FGFR1c signaling systems. 96-Well plates were dispersed with GAGs at 0, 0.064, 0.32, 1.6, 8, 40, and 200 μg/ml. FGFR1c-expressing BaF cells (30,000 live cells/well) were added to the GAGs. Six different FGFs were then added at a final concentration of 8 nM. Cells were cultured for 40 h, followed by adding resazurin to the media for 16 h. The fluorescent signal is monitored using 544 nm excitation wavelength and 595 nm emission wavelength by Spectramax M2 plate reader (Molecular Devices). The relative fluorescence unit (RFU) generated from the assay is proportional to the number of living cells in each well. Data are mean ± range of duplicates. The results are representative of at least two independent experiments.

FIGURE 4. Chemically oversulfated GAGs (OSHs, OSDS, and OSCS) were more potent than natural GAGs (heparan sulfate, dermatan sulfate, and chondroitin sulfate) in stimulating cell proliferation in BaF cell-based FGF/S252WFGFR2b signaling systems. 96-Well plates were dispersed with GAGs at 0, 0.064, 0.32, 1.6, 8, 40, and 200 μg/ml. S252WFGFR2b-expressing BaF cells (30,000 live cells/well) were added to the GAGs. Six different FGFs were then added at a final concentration of 8 nM. Cells were cultured for 40 h, followed by adding resazurin to the media for 16 h. The fluorescent signal is monitored using 544 nm excitation wavelength and 595 nm emission wavelength by Spectramax M2 plate reader (Molecular Devices). The relative fluorescence unit (RFU) generated from the assay is proportional to the number of living cells in each well. Data are mean ± range of duplicates. The results are representative of at least two independent experiments.
Contaminated Heparin, Contact System, and Cell Signaling

FIGURE 5. Contaminated heparins were more potent than heparin in enhancing FGF/FGFR, HGF/Met, and GDNF/c-Ret signaling in BaF cells. 96-Well plates were dispersed with GAGs at 0, 0.064, 0.32, 1.6, 8, 40, and 200 μg/ml. BaF cells expressing FGFR1c, S252WFGR2b, c-Met, and c-Ret/GFRα1 (30,000 live cells/well) were added to the GAGs. FGF-1, HGF, and GDNF were then added to each plate at a final concentration of 8 nM. Cells were cultured for 40 h, followed by adding resazurin to the media for 16 h. The fluorescent signal is monitored using 544 nm excitation wavelength and 595 nm emission wavelength by Spectramax M2 plate reader (Molecular Devices). The relative fluorescence unit (RFU) generated from the assay is proportional to the number of living cells in each well. Data are mean ± range of duplicates. The results are representative of at least two independent experiments.

Potently activates pro-HGF to HGF. Kallikrein also activates pro-HGF to HGF in an in vitro assay (37). HGF is a major signaling molecule in the liver where all contact, complement, and coagulation proteins are produced. HGF is critically involved in tissue morphogenesis, regeneration, and tumor progression through Met receptor signaling (38). We suspected that the contact system activation might be also accompanied by HGF induction through either thrombin or kallikrein. Therefore, contaminated heparin might affect HGF/Met signaling.

GDNF/c-Ret signaling is also a GAG-regulated signaling system (39). GDNF/c-Ret signaling is associated with chronic kidney disease (40). The adverse events of contaminated heparin are mostly associated with hemodialysis patients with kidney failure. GDNF binds to its co-receptor GDNF family receptor α1 (GFRα1) and signals through the receptor tyrosine kinase c-Ret.

Fig. 5 shows the cell proliferation results. In the absence or at low concentration of GAGs, all growth factors were weak at inducing BaF cell proliferation (Fig. 5, A–D). OSHB was much more potent than heparin, contaminated heparins, and other GAGs in stimulating cell proliferation in FGFR1c- and S252WFGR2b-expressing BaF cells. Two contaminated heparins (lot S1, which had passed both capillary electrophoresis and NMR-based heparin quality controls, and lot OSCH4, which was associated with anaphylactic reactions) (2) stimulated overall cell growth better than heparin in all four BaF cell systems tested. Heparin stimulated cell proliferation better than heparin by-products in FGFR1c-, S252WFGR2b- and c-Ret/GFRα1-expressing BaF cells, whereas the heparin by-product worked better than heparin and OSHB in stimulating cell proliferation in c-Met-expressing BaF cells. Heparin pentasaccharides stimulated cell proliferations in FGFR1c- and FGFR2b-expressing BaF cells at high concentrations. In contrast, the contaminated heparins (S1 and OSCH4) stimulated cell proliferation better than heparin in all four GAG- and growth factor-dependent BaF cell systems. The most preferred GAGs for HGF/c-Met and GDNF/c-Ret signaling were not the same for FGF1/FGFR1c and FGF1/S252WFGR2b signaling, suggesting that both the degree of sulfation and GAG sequence might be involved in HGF/c-Met and GDNF/c-Ret signaling regulation.

DISCUSSION

Contact System Is a Pattern Recognition System—It has been reported that in vitro contact system activation occurs on physiologically relevant surfaces such as articular cartilage, skin, sodium urate crystals, and calcium pyrophosphate (41). In vivo contact system activation occurs on developing thrombus, RNA and DNA from degrading cells, enriched polysomes from platelet membranes, β-amyloid, sulfatides, fatty acids, cholesterol sulfate, GAGs, activated platelet surface during platelet transfusion (42), ionic contrast media (43), and also under conditions of sepsis, where bacteria lipopolysaccharides provide a negatively charged surface (44–47). It was recently reported that protein aggregates activate FXII and prekallikrein. However, FXII activation is greatly enhanced in the presence of dextran sulfate, suggesting that negatively charged protein aggregates are better contact system-activating surfaces (13). Based upon these established facts (41) and our data (Fig. 1), we propose that the contact system is a broad pattern recognition system, recognizing negative charges rather than specific chemical structures. Different surfaces have different capacities in inducing kallikrein- and thrombin-like activities. Negatively charged molecules, especially chemically oversulfated GAGs,
Contaminated Heparin, Contact System, and Cell Signaling

were potent contact system activators. They also induced thrombin-like activities. We assume that oversulfated GAGs may resemble dextran sulfate in that they induce thrombin generation through FXI activation (13).

OSCS has been suggested to be responsible for the adverse events by producing anaphylatoxins, complement C3a, C5a, and bradykinin, through contact system activation (9). Our data showed that all oversulfated forms of GAGs activated the contact system (Fig. 2, lanes 13–15), which confirmed that the contact system is a pattern-dependent but not a structure-dependent signaling system (Fig. 2). Therefore, the evidence that OSCS induces contact system activation was insufficient to prove that it is the sole harmful contaminant in heparin.

The naturally occurring heparin activated the contact system initially but then rapidly inhibited both kallikrein- and thrombin-like activities (Fig. 2 A–D, lanes 21). It has been well established that the specific 3-O-sulfate-containing sequence in heparin is required for antithrombin activation and thrombin inhibition. We suspected that heparin might inhibit kallikrein activities through activating other serine protease inhibitors through specific heparin sequences as well. Indeed, we found that the contact system protein, C1 inhibitor, like antithrombin, bound only to Chinese hamster ovary cell surfaces expressing 3-O-sulfated heparan sulfate structures.4

All the oversulfated GAGs induced less kallikrein activities in pooled normal human plasma at high concentrations (200 µg/ml) than at low concentrations (20 µg/ml) (Fig. 2A, lanes 12–15). These phenomena were also observed in the report published previously (9). Because high concentrations of contaminated heparin did not induce kallikrein activities in normal human plasma, it was presumed that the contact system was not activated by high concentrations of contaminated heparin due to the known bell-shaped concentration dependence of GAG activities (9).

Because activation of the contact system is accompanied by proteolytic cleavage of prekallikrein, factor XII, high molecular weight kininogen, and C1 inhibitor as well as deactivation of kallikrein and FXII via covalent attachment to the C1 inhibitor or other serine protease inhibitors, we employed Western analysis to systematically monitor the contact system proteins in human plasmas with or without oversulfated heparin by-product induction. We discovered that oversulfated heparin by-products at 200 µg/ml consumed all the C1 inhibitor and high molecular weight kininogen in both normal and hemodialysis patient plasmas (48). These observations indicate that oversulfated GAGs activated the contact system. However, the kallikrein activities might be inhibited by the oversulfated GAGs through activating plasma serine protease inhibitor(s) or through a yet unknown molecular mechanism.

Degree of Chemical Sulfation May Affect the Potency of OSGAGs in Inducing Kallikrein- and Thrombin-like Activities in Human Plasma—We discovered that highly sulfated DS and HS co-migrated with heparin, and less sulfated HS and DS migrated like OSCS when analyzed by both CE (data not shown) and anion exchange HPLC analysis (supplemental Fig. S2B). Because heparin contaminant migrated before heparin by CE analysis was defined as OSCS during the past 2 years, we decided to show that all the OSGAGs with proper degree of sulfation could migrate like OSCS based on CE analysis (Fig. 1). We used the same OSGAG CE migration rate as a sample selection criterion in our current studies. The drawback of our experimental design is that OSDS and OSHS used in our studies were not as fully sulfated as the cartilage OSCS based on both CE and NMR data (Fig. 1 and supplemental Figs. S1 and S2), so the most sulfated OSCS was most potent in activating the contact system in our current studies (Fig. 2 and supplemental Figs. S3 and S4).

**Thrombin-like Activity Generation Through the Contact System Activation**—Thrombin plays multiple roles not only in hemostasis and thrombosis but also in tissue repair, development, and inflammation (49). Unlike the constitutive loss of clotting, platelet function, immune B-cells or T-cells, the loss of prothrombin leads to rapid death of adult mice (50), suggesting that thrombin is a key survival factor and is constantly generated in blood circulation. Our data showed that endogenous molecules such as chondroitin sulfate-E (Fig. 2, B and D, lane 11), induced thrombin-like activities through the contact system activation in human plasma (Fig. 2), suggesting such GAGs might be involved in in vivo thrombin generation through contact system activation.

Our data in Fig. 2 indicate that other factors may amplify or inhibit thrombin-like activities during the contact system activation. We observed that *Escherichia coli* LPS, sulfatide, and lipid A amplified thrombin-like activities (Fig. 2D, lanes 2, 4, and 5). In contrast, herring DNA, zymosan, heparan sulfate, dermatan sulfate, and heparin-induced kallikrein- but not thrombin-like activities (Fig. 2, A–D, lanes 6–9 and 21). Thrombin propagation in vivo that leads to blood coagulation requires Ca²⁺ and phospholipids. Therefore, it is predictable that thrombin-like activity generated through the contact system activation in the absence of Ca²⁺ and phospholipid or in the presence of high concentrations of heparin or other thrombin inhibitors will not result in blood coagulation (13).

**Contaminated Heparin-induced Cell Signaling Events**—Our data in Figs. 3–5 indicate contaminated heparins (S1 and OSCH4) and oversulfated GAGs, including OSHS, OSCS, OSDS, and OSHB, were more potent than heparin in up-regulating FGF/FGFR, HGF/Met, or GDNF/c-Ret signaling in BaF cell-based signaling system. Based on established knowledge, contaminated heparin could impact cell signaling either in contact system activation-dependent or -independent fashion.

Heparin administration is associated with FGF release in the blood circulation (51), which suggests that heparin administration might regulate FGF/FGFR signaling directly. In contrast, contact system activation might up-regulate HGF/Met signaling through either thrombin-facilitated (36) or kallikrein-facilitated (37) HGF generation. Because HGF is a major signaling molecule in the liver where all contact, complement, and coagulation proteins are produced, how heparin and contaminated heparin affect liver function through HGF/Met signaling might be an interesting research subject.

It has been established that artificially sulfated polysaccharides, such as dextran sulfate, and naturally occurring sulfated polysaccharides, such as fucoidan, work better than heparin in...
Initiating specific FGF signaling (52), suggesting that sulfation dominates the FGF signaling. We have replicated the FGF signaling experiment in FGFR1c-expressing BaF by using FGF1 and heparin, dextran, fucoidan, and two dextran sulfates with different molecular weights (data not shown). We found that native dextran with no sulfates had no mitogenic activities. Fucoidan and dextran sulfates stimulated higher mitogenic activities than heparin at low concentrations; however, the activities started to drop at higher concentrations (data not shown). Only heparin had a constant mitogenic activity from low to high concentrations in the BaF cell systems, which is consistent with our previously published results (16). We found that OSHS, OSCS, and OSDS were similar to the artificially sulfated polysaccharides but were different from heparin in that they did not stimulate FGF signaling in most of FGF/FGFR pairs tested at high concentrations (Fig. 3).

Our observation is also consistent with the observation made by Rapraeger and co-workers (53) that heparin at high concentrations disrupts FGF/FGFR complex formation but still stimulates FGF signaling. These data suggest that heparin-dependent FGF signaling is beyond the simplified FGF-1/FGFR-heparin tertiary signaling complex model supported by co-crystal structures (54, 55), which makes it difficult to predict if heparin contaminants in different contaminated heparin lots stimulate or inhibit endogenous cell signaling. However, it is probable that contaminated heparin would have a different impact on endogenous cell signaling compared with heparin based on our data (Figs. 3–5).

Complexity of Heparin Biology—Heparin is one of the most used drugs in modern medicine and remains a time-honored drug for the treatment and prevention of thromboembolic disorders (1), the leading cause of human death. Heparin is different from traditional medicines. It affects human physiology in a complicated way through interactions with 22% plasma proteins (15, 56) in contrast to a single target for most clinically used drugs. Our current studies showed that the degree of sulfation in GAGs dominated contact system activation (Fig. 2). In contrast, both the degree of sulfation and the specific GAG sequences contributed to GAG-dependent growth factor signaling (Fig. 5) and to heparin-catalyzed thrombin inhibition (15). Therefore, appreciating the complex role of sulfation and sequence in endogenous GAGs in the regulation of human development, health, and diseases and understanding the multiple roles heparin and chemically oversulfated GAGs play in human physiology may open up new therapeutic avenues.

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