

Short communication

**SULFOCHITOSAN INHIBITS P-SELECTIN-MEDIATED HL-60
LEUKOCYTE ADHESION UNDER FLOW CONDITIONS**JINFENG HUANG, RUIFEI WANG, XIMING LIU, XIANLU ZENG*
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Abstract: Excessive trafficking of leukocytes can lead to serious tissue injury. Here, four regioselectively sulfated chitosans were assessed as inhibitors of HL-60 leukocyte binding to P-selectin, by investigating their effect on leukocyte adhesion to CHO cells expressing human P-selectin under static and flow conditions. The results show that the sulfochitosans exhibit inhibitory activity in this general order: heparin > N-sulfated/6-O-sulfated chitosan \geq 3-O,6-O-sulfated chitosan > 6-O-sulfated chitosan \gg N-sulfated chitosan. This suggests that the sulfation of the double site in chitosan is essential for efficient inhibition of P-selectin-mediated HL-60 leukocyte adhesion and that such sulfochitosans may have potential as therapeutic agents against inflammatory disease.

Key words: Chitosan, Sulfated chitosan, P-selectin, Inhibition, Cell adhesion, Flow condition, Heparin

INTRODUCTION

Leukocyte migration is a characteristic feature of the host defense against pathogens. However, excessive leukocytes in tissues can cause a significant amount of tissue damage, especially in some disease states, such as ischemia-reperfusion injury, rheumatoid arthritis and inflammatory bowel disease, and after allogeneic organ transplantation [1-3]. Data obtained in clinical trials have

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Abbreviations used: AC1.2 – a non-blocking mAb against P-selectin; DMF – dimethylformamide; FBS – fetal bovine serum; HPLC – high performance liquid chromatography; N-S-C – N-sulfated chitosan; N,6-S-C – N-sulfated/6-O-sulfated chitosan; P-Fc – recombinant human P-selectin/Fc chimera protei; 3,6-S-C – 3-O,6-O-sulfated chitosan; 6-S-C – 6-O-sulfated chitosan; 9E1 – a leukocyte adhesion blocking IgG mAb against P-selectin

convincingly shown that inhibiting leukocyte migration into the target organs is an effective therapeutic approach [4].

Leukocyte migration is a complicated interaction process between the leukocytes and endothelium. It involves multiple steps, including the rolling phase, the firm adhesion phase and the final phase of transendothelial migration [5]. Several families of cell adhesion molecules, such as selectins, selectin ligands, integrins, and the IgG superfamily of cell adhesion molecules, participate in different phases independently or collaboratively [6]. It has been largely accepted that the interaction of P-selectin with its natural ligand, P-selectin glycoprotein ligand-1 (PSGL-1) [7, 8], mediates the earliest rolling phase of leukocyte migration [6, 9]. Therefore, blocking P-selectin with antagonists may effectively reduce undesired leukocyte trafficking, and thus prevent vascular and tissue injury.

Previous studies have demonstrated that heparin can function as an excellent ligand for P-selectin and effectively block P-selectin from binding to its native ligand [10, 11]. Moreover, the sulfate groups on the glucosamine unit of heparin have been shown to be critical for P-selectin binding [12, 13]. We prepared four regioselectively sulfated chitosans – N-sulfated chitosan (N-S-C), 6-O-sulfated chitosan (6-S-C), N-sulfated/6-O-sulfated chitosan (N,6-S-C) and 3-O,6-O-sulfated chitosan (3,6-S-C) – to mimic the glucosamine unit of heparin and to allow us to evaluate their potential to inhibit P-selectin-mediated HL-60 leukocyte adhesion. In our previous study, these compounds were tested for their inhibition of the binding of P-Fc (recombinant human P-selectin/Fc chimera protein) to human melanoma A375 cells [14]. However, malignant cells and inflammatory cells have different P-selectin ligands, which prompted us to explore the inhibitory activity of these compounds against leukocyte cell adhesion and evaluate their anti-inflammatory potential. It was also crucial to assess their inhibition of cell-cell interaction under physiological conditions.

MATERIALS AND METHODS

Materials

Chitosan and sulfation reagents were purchased from Fluka Chemical Co. Other reagents and chemicals were from local commercial sources. N,N-dimethylformamide (DMF) was purified using a 4 Å molecular sieve before use.

Proteins and antibodies

Recombinant human P-selectin/Fc chimera protein (P-Fc) and the blocking mAb 9E1 against P-selectin were purchased from R&D Systems, Inc. A non-blocking mAb AC1.2 against P-selectin was obtained from BD PharMingen, Inc. Human IgG isotype control and FITC-conjugated goat anti-human IgG were purchased from Jackson Immuno Research Laboratories, Inc.

Cells

Human myelocytic leukemia (HL-60) cells were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Science (China). They

were nourished in RPMI 1640 (Gibco) with 10% heat-inactivated fetal bovine serum (FBS) at 37°C under a 5% CO₂ atmosphere. CHO cells were cultured in IMDM (Invitrogen) supplemented with 10% heat-inactivated FBS at 37°C in the presence of 5% CO₂. Human P-selectin-expressing CHO cells (CHO-P) were provided by Dr. Fei Rui. They were cultured and harvested in the same way as the CHO cells.

Modification of chitosan

The regioselective sulfation of chitosan was carried out as follows. Water-insoluble chitosan was further hydrolyzed and deacetylated with diluted chlorhydric acid to give a water-soluble, low-molecular-weight precursor chitosan. N-sulfation of chitosan was achieved with a sulfation reagent of SO₃·NMe₃ at approximately pH 9.0 [15]. The Cu²⁺ protection technique was employed for the synthesis of 6-S-C as described before [16]. N,6-S-C was obtained using the N-sulfation method on 6-S-C. The synthesis of 3,6-S-C was a three-step synthetic approach [17]. First, the phthalimido group was introduced as a selective protection group for -NH₂. Then, sulfation was carried out with a SO₃-pyridine complex. Finally, the reagent hydrazine hydrate was used to remove the phthalimido group. All of the derivatives were purified using a dialysis tube with a pore size of 15-20 Å against deionized water.

Structure determination

The molecular weights of the derivatives were evaluated using high performance liquid chromatography (HPLC; Shimadzu, Japan). Runs were performed on a TSK-GEL G3000 PWXL column and an RID-10A Refractive Index Detector with 0.7% Na₂SO₄ as the mobile phase at 0.5 ml/min. The column was calibrated with standard T-series Dextran with different molecular weights (T-200, T-80, T-40, T-20 and T-10). FT-IR spectra were recorded on a Nicolet Nexus 470 IR spectrometer with KBr pellets. ¹³C NMR spectra were recorded on a Bruker AV-600 spectrometer in D₂O. The sulfate content of modified chitosan was determined by ion chromatography (Dionex, California, USA).

Flow cytometric assay

Flow cytometric analysis was performed as described previously, to check the inhibitory effect of sulfochitosans on the adhesion of P-Fc to HL-60 cells [18]. HL-60 cells were collected by centrifugation (1000 × g) for 5 min, washed twice, counted and resuspended in culture medium to a final concentration of 5×10⁶ cells/ml. For the P-selectin binding assay, cells (100 μl) were incubated with 3 μg/ml of P-Fc or human IgG for 30 min at 4°C, washed once, and resuspended in 100 μl of RPMI 1640 medium containing FITC-labeled goat anti-human IgG (2 μg/ml). After incubation for another 30 min at 4°C, cells were washed twice, and 10,000 cells were collected for flow cytometric analysis with a FACScan (Beckman-Counter, USA). For the inhibition experiments, P-Fc was preincubated with 10 μg/ml of mAbs 9E1 or AC1.2, or different concentrations of heparin or sulfochitosans for 30 min at room temperature.

Static adhesion assay

The adhesion of HL-60 cells to CHO-P cells was assayed as described previously [19]. HL-60 cells were labeled with 2 μM calcein AM, at 37°C for 30 min. The labeled cell suspension (6×10^5 cells/ml, 200 μl) in RPMI 1640/1% BSA was loaded on a monolayer of CHO-P cells in a 48-well culture plate. After incubation at 4°C for 30 min, non-adherent cells were removed. After the well was gently washed three times with PBS/1% BSA, the fluorescence intensity was measured with a Gemini EM fluorescence spectrophotometer (ex 485 nm, em 515 nm). For the inhibition assay, 9E1, AC1.2 or different concentrations of heparin or sulfochitosans were incubated with CHO-P cells for 30 min before the addition of labeled HL-60 cells.

Flow adhesion assay

The adhesion of HL-60 cells to CHO-P cells under flow conditions was assessed using a parallel-plate flow chamber (GlycoTech, USA) at a shear stress of 1 dyn/cm^2 . For visualization, the chamber was immobilized on an Olympus Optical inverted microscope equipped with a Panasonic camera connected to a computer monitor. The HL-60 cells were washed twice, resuspended in RPMI 1640 medium (1×10^6 cells/ml) and maintained in ice water during the experiment. For a single run, HL-60 cells were injected through the CHO or CHO-P cell monolayer with a syringe pump and the interaction events were recorded. The total number of interacting cells (tethering or rolling on CHO-P cells) in a single $\times 10$ field of view (0.127 mm^2) over a 3-min perfusion period was quantified by digital image processing. For the inhibition experiments, CHO-P cell monolayers were pretreated with mAbs (20 $\mu\text{g}/\text{ml}$), heparin or sulfochitosans at different concentrations for 20 min at 37°C.

RESULTS

Sulfochitosans inhibit P-selectin binding to HL-60 cells

Using several well established methods, we prepared a series of chitosan sulfated derivatives, including N-S-C, 6-S-C, N,6-S-C and 3,6-S-C. Representative saccharide units of sulfochitosans and heparin are given in Fig. 1.

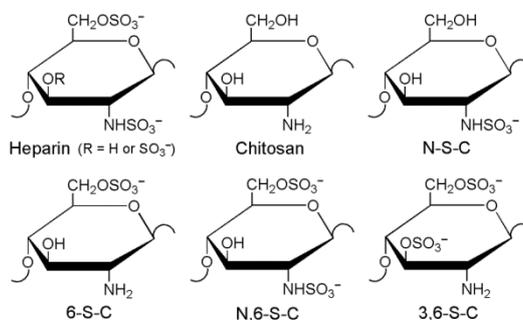


Fig. 1. Representative saccharide units of heparin, chitosan and sulfochitosans.

HPLC analysis indicated that their molecular weights were about 40-56 kD. FT-IR and ^{13}C -NMR analysis proved the reliability of structures of the modified chitosans [14]. The sulfate contents of N-S-C, 6-S-C, N,6-S-C and 3,6-S-C, determined by ion chromatography, were respectively 29.41, 35.12, 39.21 and 40.01% (w/w).

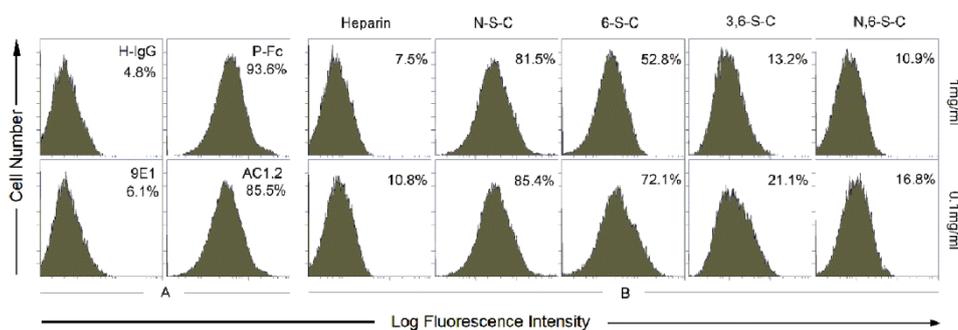


Fig. 2. Effects of sulfochitosans on the inhibition of P-selectin binding to HL-60 cells. A – P-selectin binding to HL-60 cells. The assay was performed in the presence of blocking mAb (9E1) or non-blocking mAb (AC1.2) and analyzed by flow cytometry. An isotype-matched human IgG was used as the negative control. B – Inhibition of P-selectin binding to HL-60 cells by heparin and sulfochitosans. P-selectin recombinant protein was preincubated with heparin or sulfochitosans at concentrations of 1 mg/ml or 0.1 mg/ml for 30 min at room temperature and then incubated with HL-60 cells for another 30 min. The results are representative of three independent experiments.

We first confirmed the binding specificity of P-selectin to HL-60 cells. As expected, P-Fc bound to HL-60 cells effectively. Preincubation of P-Fc with 9E1 inhibited this binding, but preincubation with AC1.2 did not (Fig. 2A). To examine the inhibitory effect of sulfated chitosans, sulfated chitosans at final concentrations of 1 mg/ml or 0.1 mg/ml were preincubated with P-Fc. Flow cytometry analysis showed that N-S-C sulfochitosans exhibited poor inhibitory effects and 6-S-C exhibited moderate inhibitory effects at both concentrations, whereas N,6-S-C and 3,6-S-C had significant inhibitory effects at both concentrations. Furthermore, the inhibitory activity of 6-S-C, N,6-S-C and 3,6-S-C was concentration dependent. The results implied that sulfochitosans may act as P-selectin antagonists (Fig. 2B).

Sulfochitosans inhibit HL-60 cells adhering to CHO-P cells under static and flow conditions

The inhibitory effects of the sulfochitosans on CHO cells expressing transfected human P-selectin cDNA (CHO-P cells) were examined under static conditions using a 48-well culture plate. The adhesion of fluorescently labeled HL-60 cells to the monolayer of CHO-P cells was inhibited by 9E1, AC1.2, heparin and chitosan derivatives. As shown in Fig. 3, HL-60 cells bound to CHO-P cells, but not to CHO cells. Preincubation of CHO-P cells with 9E1, but not AC1.2,

inhibited the adhesion, indicating the binding specificity of HL-60 cells to CHO-P cells. N-S-C had no inhibitory effect at all and 6-S-C had a slight effect, whereas N,6-S-C and 3,6-S-C exhibited an impressive inhibitory behaviour.

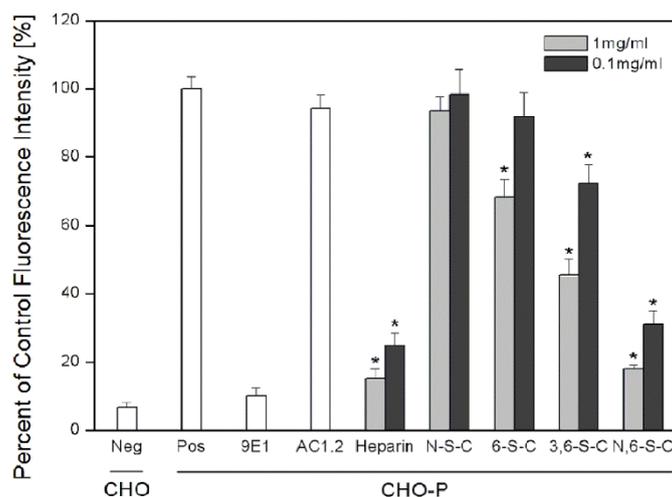


Fig. 3. Effects of sulfochitosans on the adhesion of HL60 cells to CHO-P cells under static conditions. Calcein-labeled HL-60 cells were loaded on CHO (negative) or CHO-P cells (positive) pretreated with or without inhibitors in a 48-well culture plate and incubated at 4°C for 30 min. The quantity of adherent cells was determined based on fluorescence intensity with a fluorescence spectrophotometer (ex = 485 nm, em = 515 nm) after the removal of non-adherent cells by washing with PBS. *P < 0.01 compared with non-blocking mAb (AC1.2) to P-selectin.

To corroborate the above findings, we used the flow adhesion system, mimicking the fluid mechanical environment of the microcirculation and post-capillary venules. HL-60 cells were perfused through a parallel plate flow chamber with its lower plate coated with a layer of CHO-P cells at the appropriate flow rate to obtain wall shear stresses of 1.0 dyn/cm². The interaction events of HL-60 cells and CHO-P cells can be observed when the HL-60 cells run through the surface of CHO-P cells. As shown in Fig. 4, the adhesion events happened between HL-60 cells and CHO-P cells, but not between HL-60 cells and CHO cells. Preincubation of CHO-P cells with 9E1, but not AC1.2, inhibited the occurrence of adhesion events, indicating the binding specificity of HL-60 cells to CHO-P cells under flow conditions. We then preincubated the sulfochitosans with CHO-P cells. The statistics for the cell adhesion events showed that incubation of CHO-P cells with N,6-S-C and 3,6-S-C significantly decreased the interacting HL-60 cell numbers under flow conditions. By contrast, the adhesion events were not significantly shuttled down after incubation of CHO-P cells with N-S-C or 6-S-C, except in the case of 6-S-C at a concentration of 1 mg/ml (Fig. 4).

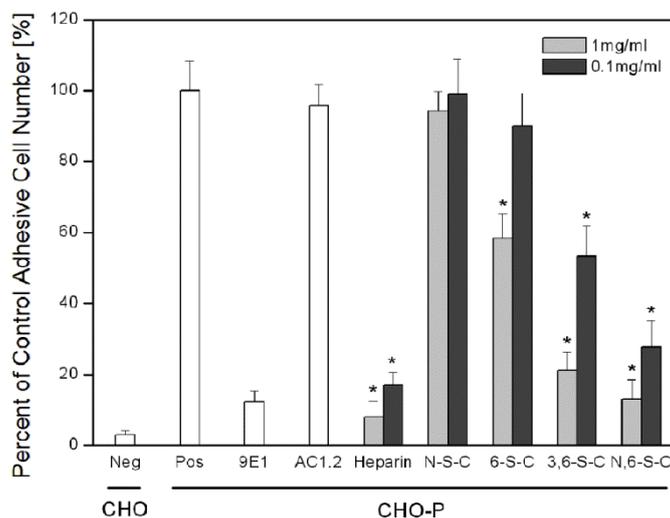


Fig. 4. Effects of sulfochitosans on the adhesion of HL-60 cells to CHO-P cells under dynamic flow conditions. Adhesion of HL-60 cells to immobilized CHO cells (negative) or immobilized CHO-P cells (positive) at 1.0 dyn/cm² was measured by video microscopy. For inhibition experiments, CHO-P cells were preincubated with 9E1, AC1.2, heparin or modified chitosans. Values are calculated as percentages of the positive control with no inhibitors. *P < 0.01 compared with non-blocking mAb (AC1.2) to P-selectin.

DISCUSSION

Blocking leukocyte adhesion to the endothelium has been shown to be an effective way to moderate vascular and tissue injury in a wide variety of animal models of inflammatory and immune disease [20]. Various P-selectin antagonists have been discovered, such as mAbs, sLe^x, sLe^a, recombinant PSGL-1, and heparin [21]. However, all these inhibitors have various drawbacks, including narrow cross-reactivity, weak affinity, short circulating half-life, great expense, potential antigenicity, and highly variable complex structure. Here, we selected chitosan, a linear homopolysaccharide of β -(1-4)-linked D-glucosamine, as a precursor of the P-selectin antagonist because of its biocompatibility, biodegradability and non-toxicity [22]. Most importantly, after the replacement of the sulfate group at the positions of C2-NH₂, C3-OH or C6-OH, it can take on a more similar structure to that of the glucosamine unit of heparin, which has been demonstrated to be critical for P-selectin binding.

In our previous study, we investigated the inhibitory effects of the sulfochitosans on the binding of P-selectin to melanoma cells. In this study, the consistent inhibitory potency of these compounds was also found in the case of P-selectin-mediated inflammatory cell adhesion. The sulfochitosans show inhibitory activity in this general order: heparin > N-sulfated/6-O-sulfated chitosan \geq 3-O,6-O-sulfated chitosan > 6-O-sulfated chitosan \gg N-sulfated chitosan. These results indicate that the action mechanism of these derivatives relies on the

blocking of the P-selectin functional domain, which is a prerequisite for the binding of both melanoma cells and HL-60 cells. Furthermore, the previous study only evaluated the inhibition of the binding of P-selectin/Fc chimera protein with the melanoma cell in a static state. The inhibitory effects of the synthetic derivatives are also evaluated at a cell-to-cell level in a dynamic state in this study. In the anti-P-selectin-mediated HL-60 leukocyte adhesion assay, N,6-S-C and 3,6-S-C, but not N-S-C and 6-S-C, exhibited strong inhibitory activity under static and flow conditions, indicating that the sulfation of double sites is more efficient than the sulfation of a single site for improving the inhibition of P-selectin adhesion by chitosan. It may also be inferred that the binding of P-selectin requires plentiful negative charge groups, such as sulfate groups, or that the spatial synergy of the N- or 3-O-sulfate group with the 6-O-sulfate group in glucosamine units is probably critical to meet the demand of recognition by P-selectin.

In summary, our studies demonstrated that N,6-S-C and 3,6-S-C could significantly inhibit P-selectin-mediated inflammatory cell adhesion and such analogs may have value as therapeutic inhibitors of inflammatory disease, especially when P-selectin is targeted. The structural pattern of double sulfation may also adapt to other sulfated glycan ligands of P-selectin.

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