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• BASIC RESEARCH •

Effects of dermatan sulfate derivatives on platelet surface P-selectin expression and protein C activity in blood of inflammatory bowel disease patients

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Abstract

AIM: To investigate the effect of dermatan sulfate (DS) derivatives on platelet surface P-selectin expression and blood activated protein C (APC) activity in patients with inflammatory bowel disease (IBD), and to clarity the anti-inflammatory mechanism of DS derivatives.

METHODS: Dermatan sulfate (DS) was sulfated with chlorosulfonic acid to prepare polysulfated dermatan sulfate (PSDS). The major disaccharides of DS and PSDS were determined by ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) and ^{13}C-NMR. Both DS and PSDS were depolymerized with hydrogen peroxide. The fragments were separated by gel filtration chromatography. The effects of DS derivatives on P-selectin expression were assayed by ELISA method, and blood APC activity was assayed by the synthetic chromogenic substrate method.

RESULTS: The major disaccharides of DS and PSDS were IdoA-1→3-GalNAc-4-SO_3 and IdoA-2SO_3-1→3-GalNAc4, 6-diSO_3, respectively. Compared with the adenosine diphosphate stimulated group and IBD control group, DS and its derivatives all had significant inhibitory effects on P-selectin expression (P<0.01), but there was no difference between DS-derived oligosaccharides (DSOSs) and PSDS-derived oligosaccharides (PSDSOSs). The experiments on APC activity showed that DS and its derivatives all enhanced APC activity. The most active DSOS was the one with a relative molecular weight (M_r) of 4 825, which enhanced the APC activity from 106.5±11.5% to 181.8±22.3% (P<0.01). With the decrease of M_r, the activity of DSOSs decreased gradually. The effect of PSDS on APC activity enhancement was more significant than that of DS, and the APC activity was raised to 205.2±22.1% (P<0.01). All the PSDSOSs were more active than DSOSs on the basis of comparable M_r. With the decrease of M_r, the activity of PSDSOSs increased gradually, and the most active PSDSOS was PSDSOS$_3$ with M_r of 2 749, which enhanced the APC activity to 331.2±27.8% (P<0.01), then the activity of PSDSOSs decreased gradually.

CONCLUSION: DS and its derivatives can significantly inhibit P-selectin expression on platelet surface, but the effect has no correlation with DS molecular mass and sulfation. The effect of DS or its derivatives on APC activity at molecular level involves complex mechanisms that depend on the molecular mass, the degree of sulfation, and the heterogeneous composition of DS. On the same molecular size, the higher the degree of DS sulfation, the more significant the effect on enhancing APC activity.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic non-specific intestinal inflammation. Recent researches have revealed a number of links between inflammation and coagulation, patients with IBD may be at increased risk of having venous thromboembolism[1-9]. Abnormal platelet activity has been reported in patients with ulcerative colitis (UC) and Crohn's disease (CD), and plays an important role in inflammation aggravation[10-12]. P-selectin, a membrane glycoprotein expressed on activated platelets and endothelial cells, plays a crucial role in thrombosis and inflammatory response[13]. P-selectin antagonism could decrease thrombosis and inflammation in animal models of venous thrombosis prophylaxis[14]. The protein C anticoagulant pathway appeares to be the major pathway involved in the cross link between inflammation and coagulation. Activated protein C (APC) contributes to systemic anticoagulant and anti-inflammatory activities[15], and may reduce organ damage by inhibiting thrombin generation and leukocyte activation[16]. To inhibit the release of P-selectin and enhance APC activities are helpful for the treatment of IBD and related thrombosis[17].

Dermatan sulfate (DS) is a member of the family of structurally complex, sulfated and linear polysaccharides called glycosaminoglycans (GAGs). It is mainly composed of [4-α-L-IdoA-1→3-β-D-GalNAc-1]$_n$ and [4-α-L-GlcA-1→3-β-D-GalNAc-1]$_n$ and their derivatives with sulfate groups at C-2 of L-IdoA, C-4 or/and C-6 of D-GalNAc (IdoA, iduronic acid, GalNAc, *N*-acetylgalactosamine, GlcA, glucuronic acid). The relative molecular weight (M_r) of natural DS is within 15 ku to 45 ku[18]. Polysulfated DS is the sulfated product of DS reacting with chlorosulfonic acids. DS-derived oligosaccharides (DS-Oligs) are the oligosaccharides from depolymerizing DS and PSDS. DS is an important glycosaminoglycan found in a wide variety of tissues

in animals. Recently, growing evidence has suggested that DS, like the better studied heparin and heparan sulfate, is an important cofactor in a variety of cell behaviors[18]. Many of the biological activities of DS proteoglycans are associated with their glycosaminoglycan structure[19]. In addition to its anticoagulant and antithrombotic activities[20,21], DS has also been reported possessing anti-inflammatory activities[22-24]. The mechanisms of anti-inflammation of DS are unknown. To the author's knowledge, the relationships between the molecular structure of DS-Oligs and their effects on P-selectin and APC activity have not been studied. The present study was designed to observe the effects of DS-Oligs with different M_r and structure on platelet surface P-selectin expression and blood APC activities in patients with IBD, and to clarify the anti-inflammatory mechanisms of DS in the treatment of IBD.

MATERIALS AND METHODS

Materials

Dermatan sulfate was donated by Dongying Tiandong Biochemical Industrial Co. Ltd and 300 mL/L hydrogen peroxide (H_2O_2) was purchased from Laiyang Fine Chemical Reagent Co. Ltd. Adenosine diphosphate (ADP) was purchased from Sigma Co., USA. Superdex-30 was purchased from Amersham Biosciences. Human P-selectin ELISA kit (96 tests) and protein C CSA were purchased from Shanghai Sun Diagnostics, China.

Preparation of DS-Oligs

PSDS was prepared by the reaction of DS with chlorosulfonic acids. DS and PSDS were degraded by oxidative depolymerization with hydrogen peroxide[25] respectively. All DS-Oligs were obtained from the products separated by gel filtration chromatography with Superdex 30 column (26 mm×1 200 mm), and the DS-Oligs were eluted with 0.25 mol/L ammonium bicarbonate and dried with lyophilization.

Determination of the relative molecular weight of DS-Oligs

The relative molecular weight (M_r) of the Oligs was determined by high performance liquid chromatography (HPLC)[26].

^{1}H-NMR and ^{13}C-NMR analysis of DS and PSDS

^{1}H-NMR and ^{13}C-NMR spectra of DS and PSDS were assayed using a 600 MHz instrument as previously described[27].

Patients and controls

Ten healthy blood-donors served as a control group and blood samples from 10 IBD patients, including five patients with UC and five patients with CD were used for experiments.

Collection of blood samples

Blood used to prepare platelets was collected in silicone-coated tubes containing 32 g/L sodium citrate as anticoagulant just before preparation and analysis.

Preparation of isolated platelets (IP)

Nine milliliter of blood with sodium citrate as anticoagulant blood was centrifuged at 500 r/min for 20 min at room temperature to obtain platelet rich plasma (PRP). The upper PRP was taken out, and the remaining plasma was centrifuged at 3 000 r/min for 15 min again and the platelet poor plasma (PPP) was obtained. The PRP was diluted with PPP to the platelet density of 3.0×10^8 cells/mL for P-selectin test.

Preparation of P-selectin standard calibration curve

Standard *P*-selectin was diluted to 80, 40, 20, 10, 5 and 2.5 ng/mL with assay buffer, and then added to the standard wells. The standard curve was tested according to the test kit manufacturer's instructions.

Measurement of P-selectin in IP by ELISA

A total of 225 μL of afore mentioned PRP was added to tubes, 30 μL of saline was added to the control group and adenosine diphosphate (ADP) control tubes, and then 30 μL of 2.50 mg/mL DS, PSDS and DS-Oligs saline solution were added to the sample tubes. All the tubes were incubated for 10 min at 37 ℃. Then 25 μL of 4.68 μmol/L ADP solution was added to the ADP and sample tubes and an equal volume of saline was added to the blank control tubes. Then all the tubes were incubated for 5 min at 37 ℃, followed by centrifugation for 15 min at 3 000 r/min, and finally the P-selectin in the upper solution was tested by ELISA according to the test kit manufacturer's instructions.

Specimen collection and preparation for APC test

Five milliliter of venous blood, containing 32 g/L sodium citrate as an anticoagulant, was collected from ten patients with IBD and the sample was centrifuged immediately. The plasma was collected and stored at 2-8 ℃ until use.

Preparation of protein C activity standard calibration curve

Normal plasma was dissolved in 200 μL of buffer and the APC activity was defined as 200%. The solution was diluted three times to make the activities 100%, 50% and 25%, respectively. The activity of buffer was 0% criterion. Twenty five microliters of the four standards was added to the standard wells of the test plate, then 50 μL of activator at the concentration of 0.1 mg/mL was added to activate protein C. These points were determined according to the manufacturer's instructions at 405 nm. The absorbance (A_{405nm}) was plotted versus the known percentage of standard APC activity to give a standard curve.

Detection of APC activity

APC activity in the plasma pre-incubated with DS and its derivatives was determined according to test kit manufacturer's instructions with minor modifications. Fifty microliters of DS and its derivatives solution at the concentration of 0.20 g/L was used to produce 50 μL of activator. The APC enhancement by 50 μL of DS at the concentration of 0.20 g/L was defined as 100%. Fifty microliters of buffer served as a blank control without any activator. The APC activities of the DS-Oligs were calculated according to the calibration curve.

Statistical analysis

Data were expressed as mean±SD. Student's *t* test and one-way analysis of variance were used for statistical analysis according to the data obtained. *P* values less than 0.05 were considered statistically significant.

RESULTS

Analysis of ^{1}H-NMR and ^{13}C-NMR spectra of DS and PSDS

The ^{1}H-NMR and ^{13}C-NMR spectra of DS and PSDS and the main disaccharides in their structure are shown in Figure 1. The major disaccharide sequence in DS was IdoA-1→3-GalNAc-4-SO_3. In the ^{1}H-NMR spectra of PSDS (Figures 1B, D), the chemical shift of all the protons was shifted downfield in PSDS with respect to the DS. Similar effects could also be seen in the ^{13}C-NMR spectra (Figures 1 A, C). The chemical shift of C-6 in GalNAc was shifted downfield (from 63.89 ppm to 68.60 ppm), while that of C-5 in GalNAc was shifted upfield (from 74.63 ppm to 69.98 ppm). The same phenomenon occurred in IdoA. The spectra showed that the hydroxyl group of C-6 in GalNAc and C-2 in IdoA of PSDS was sulfated, and the major disaccharide sequence was IdoA-2SO_3-1→3-GalNAc4, 6-diSO_3.

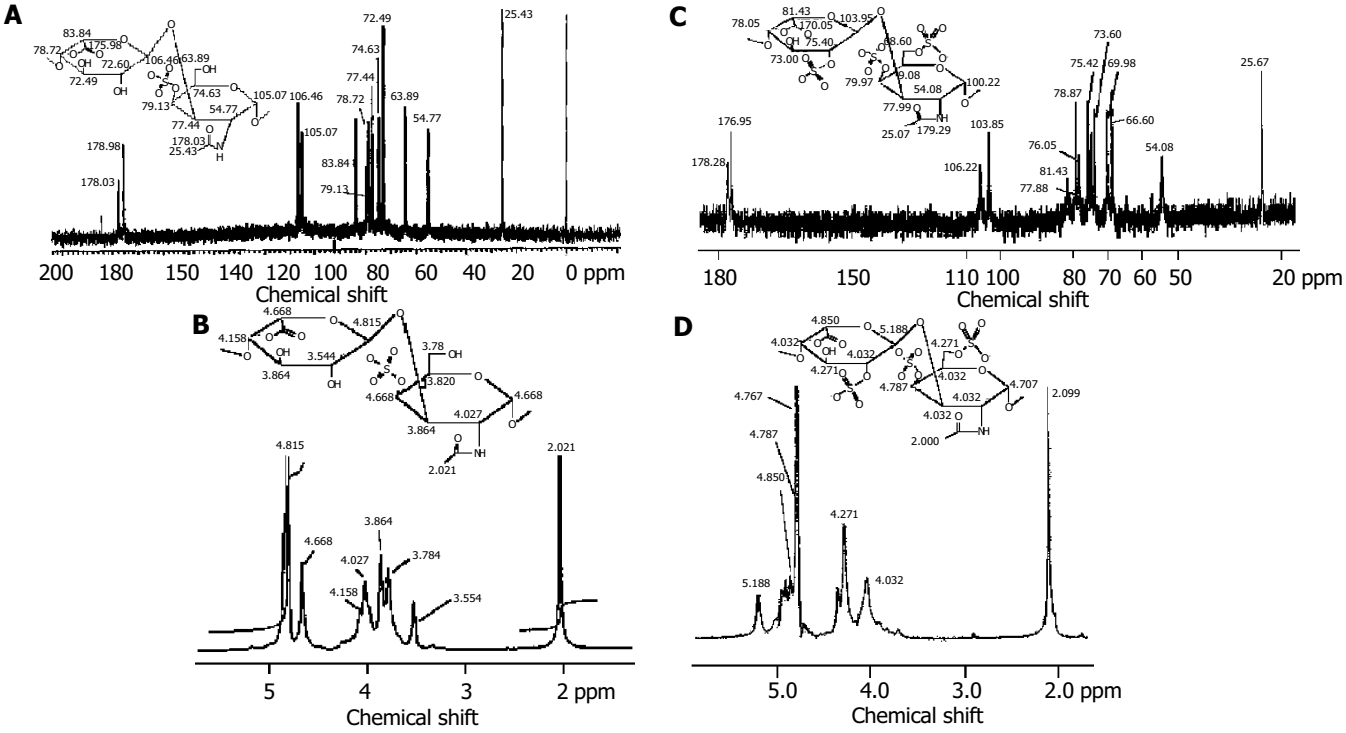

Figure 1 ^{1}H-NMR and ^{13}C-MNR spectra of DS and PSDS. A and B: ^{13}C-MNR and ^{1}H-NMR spectra of DS and the chemical shift of major disaccharides in DS; C and D: ^{13}C-MNR and ^{1}H-NMR spectra of PSDS and the chemical shift of the major disaccharides in PSDS.

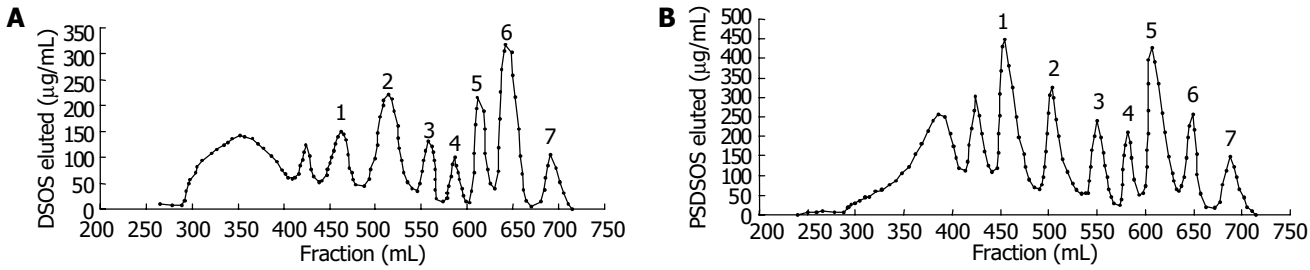

Figure 2 Fragment size separation of DS-Oligs. The products were separated on a Superdex 30 size exclusion column (26 mm×1 200 mm) at a flow rate of 0.5 mL / min in 0.25 mol / L ammonium bicarbonate. Elution profiles were monitored by carbazole assay. A and B: depolymerized products of DS and PSDS.

Fractionation of DS-Oligs

The DS-Oligs prepared from DS and PSDS degradation and separated by gel filtration chromatography are shown in Figure 2. The peaks numbered were DS-Oligs collected for the experiments on P-selectin and APC, and their M_r are listed in Table 1.

Table 1 M_r of DS-Oligs

DS oligosaccharides	M_r	PSDS oligosaccharides	M_r
$DSOS_1$	4 825	$PSDSOS_1$	4 959
$DSOS_2$	3 926	$PSDSOS_2$	4 280
$DSOS_3$	2 254	$PSDSOS_3$	2 749
$DSOS_4$	1 696	$PSDSOS_4$	2 107
$DSOS_5$	1 309	$PSDSOS_5$	1 594
$DSOS_6$	1 038	$PSDSOS_6$	1 021
$DSOS_7$	709	$PSDSOS_7$	785

Effects of DS derivatives on the expression of platelet surface P-selectin

Expression of platelet surface P-selectin on IP of IBD control group was significantly increased from 13.92±1.69 ng/mL (control group) to 23.95±2.51 ng/mL, and the ADP group showed a significant increase in P-selectin expression after stimulation with ADP (P<0.01). All DS and its derivative groups showed a significant decrease in P-selectin expression compared with the ADP-stimulated group and IBD control group (P<0.01), but no significant differences could be seen as compared with the control group, and between DSOS and PSDSOS groups (Figure 3).

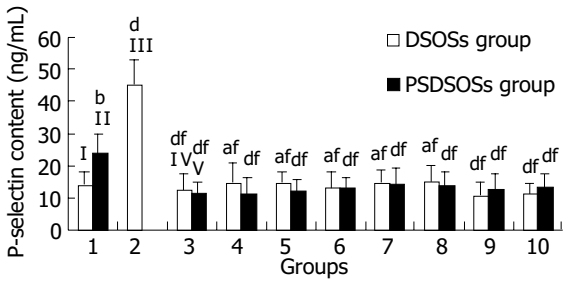

Figure 3 Effects of DS derivatives on expression of P-selectin on IP surface. Bars I, II, III, IV and V represent control group, IBD control group, ADP group, DS group and PSDS group, respectively. Groups 4 to 10 represent DS-Oligs from 1 to 7 listed in Table 1. ^{b}P<0.01 *vs* control group; ^{a}P<0.05, ^{d}P<0.01 *vs* IBD control group; ^{f}P<0.01 *vs* ADP group (n = 10 for each group).

Effects of DS derivatives on APC activity

The experimental results showed that all DS and its derivatives enhanced APC activity. The most active DSOS was $DSOS_1$ with M_r of 4 825, which enhanced the APC activity from 106.5±11.5% (DS group) to 181.8±22.3% (P<0.01). With the decrease of M_r, the activity of DSOSs decreased gradually. PSDS was more active than DS, which enhanced the APC activity from 106.5±11.5% (DS group) to 205.2±22.1% (P<0.01). On the same molecular size basis, PSDSOSs were more active than DSOSs. With the decrease of M_r, the activity of PSDSOSs increased, and the most active one was $PSDSOS_3$ with M_r of 2 749, which enhanced the APC activity to 331.2±27.8%, then decreased gradually (Figure 4).

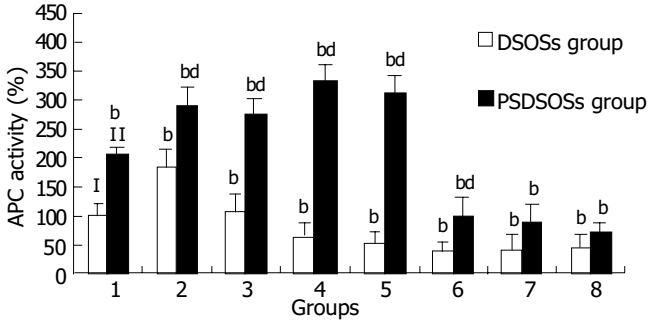

Figure 4 Effects of DS and its derivatives on APC activity. Bars I and II represent DS group and PSDS group, respectively. Groups 2 to 8 represent DS-Oligs from 1 to 7 listed in Table 1. ^bP<0.01 *vs* DS group, ^dP<0.01 *vs* PSDS group (n = 10 for each group).

DISCUSSION

IBD is a gastrointestinal disorder of unknown aetiology. Current research has paid attention to platelet dysfunction as a possible contributor to the disease, and the prevalence of IBD seems to be lower among patients with coagulation disorders[28]. Platelets play a crucial role in hemostasis and inflammatory response. Normally, platelets circulate in a quiescent state, but after stimulation with agonists, such as thrombin, ADP and epinephrine, platelets begin to express P-selectin and release inflammatory mediators and factors, thereby promoting hemostasis[29]. P-selectin acts as a cell-adhesion molecule in activated platelets and endothelial cells, and its main actions are to adhere to neutrophils and monocytes[30] and to facilitate diapedesis. It has been reported that DS acts as a ligand to bind to P-selectin, and the sulfation plays an important role in the interactions between L- or P-selectin and DS[31,32]. Our results showed that DS, PSDS and their derivatives significantly inhibited the P-selectin expression on IP surface, but there was no difference between DS and PSDS, and the effect seemed to be independent of molecular weight.

The increased risk of thrombosis in IBD patients has been attributed to a hypercoagulable state[33-37]. Investigations of the hypercoagulation state and clinical risk factors in patients with IBD are of interest because thromboembolism is a significant and serious complication in IBD patients, with a high morbidity and mortality. The incidence of arterial and venous thromboembolic disease in patients with UC and CD is between 1% and 8%, and was 39% in some postmortem studies[38]. Deficiencies and functional abnormalities of protein C, antithrombin III and protein S are the well-recognized causes of thrombotic disease[8,39]. Blood coagulation is carefully controlled *in vivo* by several anticoagulant systems. Protein C pathway is one of these natural anticoagulant systems. It could regulate the coagulation process through proteolytic cleavage and inactivation of the two cofactors in the coagulation cascade, the activated forms of factor V and VIII[40-42]. The response of UC to heparin therapy[43] and the low risk of IBD in patients with hemophilia and von Willebrand's disease[29] also suggested that thrombosis and vascular occlusion might be important in the pathogenesis of IBD.

DS has been widely used as an antithrombotic drug. Its anticoagulant activity may involve multiple mechanisms. One of the mechanisms is that DS could promote the inhibition of thrombin activity by heparin cofactor II[44]. The present experiments were designed to investigate the relationships between the APC activity enhancement and structures of DS or its derivatives *in vitro*. The results showed that DS and its derivatives all enhanced APC activity. Furthermore, a variety of experiments based on enhancement of APC activity compared the activity of various DS derivatives and defined the influences of DS molecular size and degree of sulfation on this activity. Two important variables of DS derivatives are the relative molecular weight and the degree of sulfation of the molecules. To assess the influence of molecular size, we studied different DS and PSDS fragments with M_r ranging from 0.7 ku to 5.0 ku. A clear correlation between the molecular size of DS and PSDS fragments and the degree of APC activity enhancement was observed by the synthetic chromogenic substrate method. With the decrease of molecular weight, the activity of DS-Oligs increased, and then decreased gradually. DSOS with M_r of 4 825, containing 16 to 24 monosaccharide residues, had more significant activity than undegraded DS. The same tendency was observed on PSDSOSs. But the most active PSDSOS was $PSDSOS_3$ with M_r of 2 749 containing about 8 to 10 monosaccharide residues.

To study the influence of the sulfate groups in DS molecules on APC enhancement, a chemically modified DS with a high degree of sulfation was prepared and compared with its parent DS. PSDSOSs were also compared with DSOSs with comparable M_r. The APC activity enhancement of PSDSOSs was much higher than that of DSOSs.

In conclution interactions between APC and DS-Oligs at the molecular level involve complex mechanisms that depend on the M_r, the degree of sulfation, and the heterogeneous composition of DS, and are of great importance in the development of new anti-thrombotic DS-derived drugs.

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