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RO-Heparin Inhibits β_2-Integrin(Mac-1)-mediated Neutrophils Adhesion*

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Abstract The adhesion of leukocytes to vascular endothelium is crucial for the generation of inflammatory responses. The selectins and β_2-integrin (Mac-1) play a major role in the process. Recently, it was reported that RO-heparin can inhibit selectin-mediated leukocyte adhesion. The effect of RO-heparin on the Mac-1-mediated neutrophils adhesion were further tested. The results showed that RO-heparin could effectively inhibit neutrophils binding to ICAM-1, adhering to COS-7 cells expressing human ICAM-1, and adhering to human umbilical vein endothelial cells (HUVECs) under static and flow conditions. The findings suggest that the effect of RO-heparin on leukocyte adhesion is mainly due to its inhibition on the interaction between selectins or Mac-1 and their ligands and that RO-heparin might be useful in preventing inflammation diseases.

Key words RO-heparin, β_2-integrin (Mac-1), human umbilical endothelial cells (HUVECs), inflammation

Recruitment of neutrophils into areas of inflammation is a key event during inflammatory responses [1,2]. Extravasation of neutrophils from the blood stream proceeds through three coordinated steps: rolling and tethering, firm adhesion, and transmigration. The first step depends on the selectin molecules expressed on both neutrophils and endothelial cells. The second step is mediated through the interactions of β_2-integrin (Mac-1) on neutrophils and their counter receptors on endothelial cells[3].

Mac-1, a heterodimeric receptor primarily expressed on neutrophils and monocytes/macrophages, is comprised of a specific α chain (CD11b) and a β_2 chain (CD18) which is common to the other members of the β_2-integrin family [4]. As is the case for other integrins, Mac-1 (also known as CD11b/CD18, $\alpha_M\beta_2$, Mo-1 or CR3) activation is required for efficient binding to its ligands [5]. Mac-1 is one of the most versatile adhesion molecules with ligands of very different biological functions. ICAM-1, a transmembrane adhesion molecule which is constitutively expressed by endothelial cells and upregulated rapidly during inflammation, is a member of the immunoglobulin superfamily and can interact with Mac-1 [6]. In addition to ICAM-1, Mac-1 also binds to a number of other ligands, including ICAM-2, iC3b, factor X, fibrinogen, HSPGs and many denatured proteins[7]. Furthermore, it was reported that heparin is also a ligand for Mac-1 and can inhibit the ligands of Mac-1 binding to leukocytes. The findings suggest that heparin may influence leukocyte recruitment in acute and chronic inflammatory response by blocking the interaction of Mac-1 with its ligands[8].

The strong anticoagulant properties and the potential of bleeding contraindicated its use in anti-inflammatory disease, though the heparin has anti-inflammation activity. Many investigators have therefore been trying to find heparin derivatives with low anticoagulant activity but preserving anti-inflammatory property. It has been reported that several heparin derivatives (N-desulfated heparin, N-acetyl heparin, O-desulfated heparin or carboxyl-reduced heparin) with effects on

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inflammatory reactions have been prepared by chemical modification [9]. Recently, we reported that periodate-oxidized, borohydride-reduced heparin (RO-heparin) can inhibit L-selectin and P-selectin-mediated acute inflammation[10, 11]. In the present study, we further characterized the effect of RO-heparin on Mac-1-mediated neutrophil binding to ICAM-1 under static conditions, or to COS-7 cells expressing ICAM-1 and HUVECs under flow conditions. We found the capacity of RO-heparin in blocking Mac-1-mediated adhesive events is similar to that of heparin. These results suggest that RO-heparin can function in inflammation via interference with Mac-1-dependent leukocyte adhesion and may be useful as an inhibitor of inflammatory disease.

1 Materials and methods

1.1 Reagents

Recombinant human ICAM-1/Fc chimera protein and a blocking mAb to ICAM-1, BBIG-I1, were obtained from R&D Systems (Minneapolis, MN, USA). Blocking mAb to CD11b (CBRM1/5) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human IgG isotype control, FITC-labeled goat anti-human IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. Dextran T-500 used for neutrophil isolation was purchased from Sigma (Saint Louis, MO, USA). Porcine intestinal heparin (M_r=180 008～20 000) was purchased from Sigma-Aldrich Inc. Periodate-oxidized, borohydride-reduced heparin (RO-heparin) was prepared according to the method of Casu *et al* [12]. The anticoagulant activity of heparin or RO-heparin was analyzed by aPTT assay. Clotting times were determined by using an ACL200 Automated Coagulation Laboratory (Japan) and Lyophilized silica aPTT kit. The main structure integrality is measured by means of Sepharose CL-6B Gel chromatograph. ^{13}C NMR experiments were performed on a Bruker AV-400 spectrometer (100 MHz).

1.2 Cell culture

The COS-7 cells were obtained from the Cell Bank of Type Culture Collection of The Chinese Academy of Science (Shanghai, China). They were cultured in IMDM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 mg/L penicillin and 100 mg/L streptomycin (Life Technologies) in a humiditied atmosphere containing 5% CO_2 at 37℃. Cells were passaged by mild trypsinization (0.25% trypsin) and harvested with 2 mmol/L EDTA. Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase (Sigma) treatment of human umbilical vein as described previously. COS-7 cells expressing human ICAM-1 was obtained by transient transfection of COS-7 cells with PolyFect transfection reagent (Qiagen) according to instructions from the manufacturer. HUVECs were passaged by mild trypsinization (0.25% trypsin/0.02 EDTA) and cultured to confluence in 1% gelatin-coated 35 mm tissue culture dishes.

1.3 Isolation of neutrophils

Neutrophils were isolated by standard procedures after sedimentation of erythrocytes by dextran T-500 and centrifugation of leukocytes over Ficoll-hypaque gradients as described in our previous work. After lysis of the contaminating erythrocytes with hypotonic saline (0.17 mol/L Tris, 0.16 mol/L NH_4Cl, pH 7.2), neutrophils were washed and resuspended in cold PBS containing 0.1% bovine serum albumin (BSA). The isolated neutrophils were adjusted at concentration of 0.5×10^6 cells/ml and stored at 4℃ until use. More than 95% of the isolated cells were polymorphonuclear leukocytes, and viability was determined to be > 98% by trypan blue exclusion.

1.4 Flow cytometry

For flow cytometric analysis, 5×10^5 cells were washed twice with PBS/FBS (PBS supplemented with 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 1% heat-inactivated FBS), and pellets were resuspended in 100 μl of RPMI 1640 medium. For the cell surface ICAM-1 binding assay, neutrophils were stimulated with PMA (0.1 mg/L) for 15 min. The cells were incubated with 0.3 μg of ICAM-1-Fc or human IgG for 30 min at 4℃, and washed once, then resuspended in 100 μl of RPMI 1640 medium containing FITC-labeled goat anti-human IgG (2 mg/L). After incubation for another 30 min at 4℃, cells were washed twice and 10 000 cells were collected for flow cytometric analysis (FACScan, Beckman-Counter, USA). For inhibition experiments, the neutrophils were preincubated with 1 μg of CD11b blocking mAb CBRM1/5, or RO-heparins for 30 min at room temperature.

1.5 Adhesion assay under flow conditions

Neutrophils adhesion to HUVECs was performed under flow conditions by using a parallel-plate flow chamber (GlycoTech, Rockville, MD). Neutrophils (1×10^6 cells/ml) were washed twice and resuspended in

RPMI 1640 medium. At a shear stress of 0.5×10^{-5} N/cm^2, cells were perfused over the activated HUVECs monolayer via a syringe pump. Interactions of neutrophils with endothelial cells were visualized and recorded by using an inverted microscope (Olympus Optical, Tokyo, Japan) equipped with a camera (Panasonic, Yokohama, Japan) connected to a VCR and a TV monitor. The number of bound cells was quantified from videotape recordings of 10～20 fields of view obtained at the end of 3 min perfusion. For inhibition assays, neutrophils were pretreated with mAbs (20 mg/L), heparin or RO-heparin with various concentrations at RT for 30 min. The given data represent the average number of arrested cell in the fields of view.

1.6 Adhesion assay under static conditions

Adhesion of neutrophils to fibrinogen was determined in 96 well tissue culture plates (Costar Mesa, CA) coated with fibrinogen. Prior to the addition of neutrophils, the plates were incubated with 100 μl/well of human fibrinogen (50 mg/L in PBS) at 37℃ for 2 h. The wells were washed once with HBSS, blocked with 1% BSA (Sigma, USA) in PBS at 37℃ for 1 h, and washed twice with PBS. Neutrophils (5×10^5 cells/ml in PBS) at a concentration of 100 μl/well was then added to individual wells. After stimulation with PMA (0.1 mg/L) at 37 ℃ for 15 min, non-adherent cells were removed by aspiration and the wells were gently washed twice with warm PBS containing 1 mmol/L Ca^{2+}. The adherent cells could then be counted by using an inverted microscope (Olympus Optical, Tokyo, Japan). The numbers of adherent neutrophils were quantified from 10 ～20 fields of view under a ×10 objective. For some inhibition experiments, PMA-stimulated neutrophils were preincubated with mAbs (20 mg/L), heparin or RO-heparin with various concentrations at 22 ℃ for 30 min. Adhesion of neutrophils to COS-7-ICAM-1 cells was determined in 96 well tissue culture plates (Costar Mesa, CA). COS-7-ICAM-1 cells (5×10^5 cells/ml), 36 h after transfection, were seeded into 96 well tissue culture plates and after 5 h nonspecific binding sites were blocked with 0.1% heat-treated BSA solution at 37℃ for 1 h. The neutrophils binding assay was quantitated as described above.

1.7 Statistics

Data are expressed as $\bar{x} \pm s$. Statistical significance of differences between means was determined by one-way ANOVA. If the means were shown significantly different, multiple comparisons by pairs were performed by the test. Probability values of $P < 0.01$ were considered statistically significant.

2 Results

2.1 Preparation of RO-heparin

In this study, we prepared RO-heparin by using the wel1-established chemical modification methods [12]. Representative saccharide units of RO-heparin and heparin are given in Figure 1. The Sepharose CL-6B gel analysis indicated that the integrity and the molecular mass of the main structure of the RO-heparin remained, and the NMR analysis indicated that heparin has undergone the expected oxidation-reduction reaction (data have been shown previously). To determine the *in vitro* anticoagulant activity of RO-heparin, we measured its aPTT value. As shown in Table 1, the aPTT value of RO-heparin has been reduced 50 folds. When the content is increased to 4 mg/L, the aPTT value of heparin can not be measured anymore，while that of RO-heparin can still be measured. Based on the data above, we chose RO-heparin as a heparin derivative with low anticoagulant activity and applied it to the following experiments.

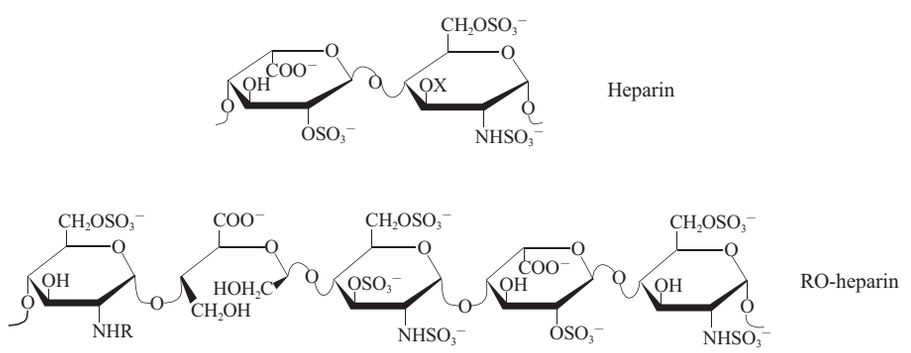

Fig. 1 Representative saccharide units of heparin and RO-heparin

Each saccharide illustrates a characteristic unit in the indicated preparation and does not represent the overall structure of the chains. X = H or SO_3^-, R = SO_3^- or $COCH_3$(Ac).

Table 1 Anticoagulant activity[1] of RO-heparin

Concentration	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	6 mg/L	8 mg/L	100 mg/L	1 000 mg/L
Control	48.4	48.4	48.4	48.4	48.4	48.4	48.4	48.4
Heparin	63.6	96.1	> 120[2]	–	–	–	–	–
RO-heparin	49.9	54.7	64.4	70.8	71.1	80.1	– > 120[2]	–

[1] aPTT (activated partial thromboplastin time) of human plasma containing heparin or RO-heparin at various concentrations. [2] Values that are more than 120 s cannot be determined.

2.2 RO-heparin inhibits the ligands of Mac-1 binding to neutrophils

The Mac-1 is important for firm adhesion of neutrophils. Previously published work has shown that heparin can function as a ligand for Mac-1 and block Mac-1 binding to its nature ligands[6, 8]. ICAM-1 is one of the ligands of Mac-1. In this work, therefore, we examined whether RO-heparins could inhibit ICAM-1-Fc binding to Mac-1 under static conditions. Firstly, ICAM-1 binding to PMA-stimulated neutrophils was confirmed by flow cytometry and this binding could be blocked by the Mac-1 blocking mAb (Figure 2a). Next, we conducted the inhibition experiment of RO-heparin. As shown in Figure 2b and 2c, ICAM-1 binding to PMA-stimulated neutrophils was blocked by heparin or RO-heparin, and both compounds exhibited a strong inhibitory effect on ICAM-1 binding (Table 2). Furthermore, fibrinogen also is one of the ligands of Mac-1[13]. In the following experiments, we tested the effects of RO-heparin on fibrinogen binding to neutrophisl. Similar to the above results, RO-heparin also strongly inhibited the interactions between neutrophils expressing Mac-1 and fibrinogen (Figure 3). These results indicate that RO-heparin can inhibit Mac-1 binding to its ligands.

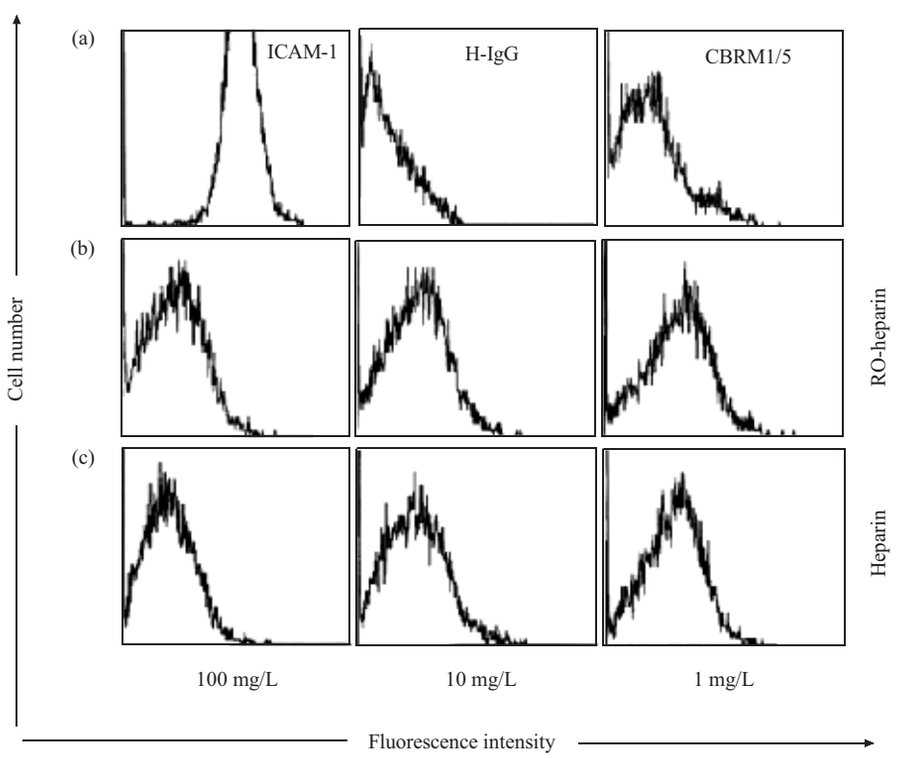

Fig. 2 RO-heparin inhibits ICAM-1 binding to neutrophils

(a) PMA-stimulated neutrophils were incubated with ICAM-1-Fc, human IgG or Mac-1 blocking mAb (CBRM/15) followed by a FITC-conjugated Ab against human IgG. (b) PMA-stimulated neutrophils was preincubated with RO-heparin. (c) PMA-stimulated neutrophils was preincubated with heparin. For inhibition experiments, PMA-stimulated neutrophils was preincubated with mAb, heparin or RO-heparin for 30 min at 4℃ before ICAM-1-Fc was added. The binding events were analyzed by flow cytometry. Results are presented as histograms of the log fluorescence intensities from 10^4 cells.

Table 2 Inhibition of ICAM-1 binding to neutrophils by heparin and RO-heparin

ρ/(mg•L^{-1})	Heparin(%)	RO-heparin(%)
100	70.1	66.7
10	67.2	62.5
1	51.3	48.6

Values for inhibition of ICAM-1 binding to neutrophils were obtained from Figure 2 and expressed as percentages of the positive control.

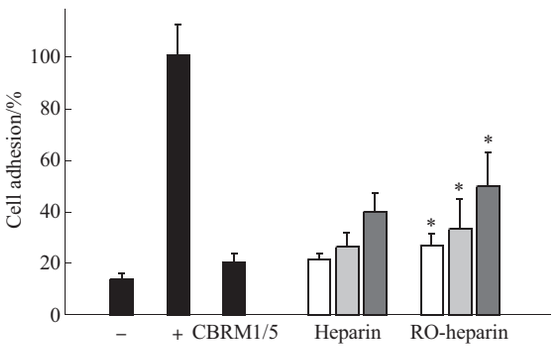

Fig. 3 RO-heparin inhibits fibrinogen binding to neutrophils

The adhesion experiments were performed as described in **Materials and methods**, without fibrinogen (designated as –) or with fibrinogen (designated as +). For inhibition assay, PMA-stimulated neutrophils were pretreated with mAb or indicated concentrations of heparin or RO-heparin. The values are calculated as percentage of positive control. The total number of the interacting cells in 10～15 fields of view (0.127 mm^2) under a ×10 objective was counted by digital image processing. All values were expressed as the $\bar{x} \pm s$. All results are representative of 3～6 separate experiments. Statistical significance of differences between means was determined by one-way ANOVA. *P < 0.01 with respect to positive control. ■: Control; □: 100 mg/L; ▨: 10 mg/L; ▩: 1 mg/L.

2.3 RO-heparin inhibits neutrophil adhesion to COS-7-ICAM-1 cells

The previous experiment proved that RO-heparin can inhibit ICAM-1 binding to PMA-stimulated neutrophils. However, in the experiment above, recombinant human ICAM-1/Fc chimera protein (ICAM-1-Fc) was used instead of the cells expressing ICAM-1. Therefore, these experiments can not completely reflect the binding of neutrophils to ICAM-1 under physiological conditions. To corroborate the findings above, COS-7 cells expressing human ICAM-1 (COS-7-ICAM-1) were used as the carrier of ICAM-1. Surface expression of ICAM-1 on the transfected COS-7 cells averaged 50％～60% while vector-transfected COS-7 cells showed no expression (data not shown). In following adhesion experiments, we found that PMA-stimulated neutrophils can adhere to COS-7 cells expressing ICAM-1, but can not adhere to the cells transfected with vector alone. The binding is specific as it is blocked completely with mAb to Mac-1. To investigate whether RO-heparin can inhibit Mac-1-dependent adhesion of neutrophils to COS-7-ICAM-1 cells, we investigated the effect of heparin or RO-heparin on the interactions between neutrophils and COS-7-ICAM-1 cells. The results showed that heparin or RO-heparin could significantly inhibit the adhesion of Mac-1-mediated neutrophils to COS-7-ICAM-1 cells (Figure 4).

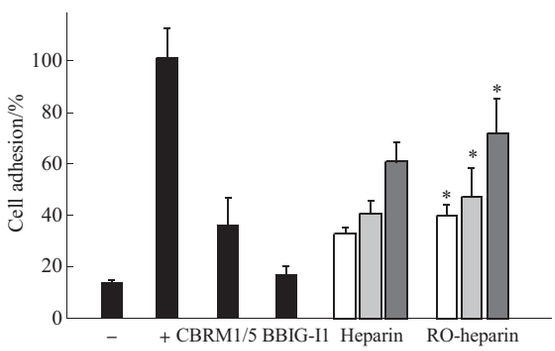

Fig. 4 RO-heparin inhibits neutrophil adhesion to COS-7-ICAM-1 cells

The adhesion experiments were performed as described in **Materials and methods**. Empty vector transfected cells were designated as – and human ICAM-1 cDNA transfected cells were designated as +. For inhibition assay, neutrophils were incubated with mAb or indicated concentrations of heparin or RO-heparin. The values are calculated as percentage of positive control (transfected human ICAM-1 cDNA). The total number of the interacting cells in 10～15 fields of view (0.127 mm^2) under a ×10 objective was counted by digital image processing. All values were expressed as the $\bar{x} \pm s$. All results are representative of 3～6 separate experiments. Statistical significance of differences between means was determined by one-way ANOVA. *P < 0.01 with respect to positive control. ■: Control; □: 100 mg/L; ▨: 10 mg/L; ▩: 1 mg/L.

2.4 RO-heparin inhibits the interactions between neutrophils and HUVECs

The interaction between neutrophils and endothelial cells is crucial for inflammatory process[1]. To investigate whether heparin and RO-heparin can inhibit the Mac-1-mediated adhesion of neutrophils to the vascular endothelial cells under flow condition, neutrophils were perfused through a parallel plate flow chamber whose lower plate was coated with a layer of HUVECs. It has been reported that neutrophil attachment to HUVEC was mediated by Mac-1 at shear stresses of 0.5×10^{-5} N/cm^2[14]. So we conducted the inhibition experiment at 0.5×10^{-5} N/cm^2 under flow

conditions. As illustrated in Figure 5, both BBIGI-1 (Mac-1 blocking antibody) and CBRM1/5 (ICAM-1 blocking antibody) obviously decreased the number of adherent neutrophils. The results showed that the firm adhesion of neutrophils to HUVECs principally depend on the interactions between Mac-1 and its ligand. Next, we examined the effect of heparin and RO-heparin on inhibiting the adhesion of neutrophils to HUVECs monolayers. In the experiments, the concentration of the heparin or RO-heparin was 100 mg/L. The results showed that heparin and RO-heparin could significantly inhibit the adhesion of neutrophils to HUVECs under flow condition. Compared with the positive control, the preincubation of heparin or RO-heparin reduced the percentage of neutrophil adhesion to HUVECs by 74% and 67.2%, respectively.

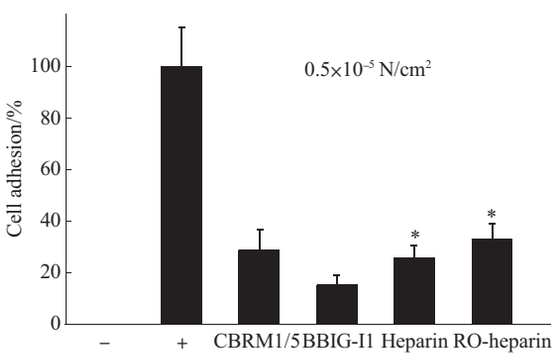

Fig. 5 RO-heparin inhibits the interactions of neutrophils and HUVECs

The adhesion experiments were performed as described in **Materials and methods**. Without HUVECs (designated as –) or with HUVECs (designated as +). For inhibition assay, PMA-stimulated neutrophils were pretreated with mAb or indicated concentrations of heparin or RO-heparin. The values are calculated as percentage of positive control (with HUVECs). The number of bound cells was quantified from the videotape recordings of 10 ～20 fields of view under a ×10 objective obtained at the end of 3 min perfusion. All values were expressed as the $\bar{x} \pm s$. All results are representative of 3 ～6 separate experiments. Statistical significance of differences between means was determined by one-way ANOVA. $^{*}P < 0.01$ with respect to positive control.

3 Discussion

Selectin and integrin play a major role in the inflammatory process. Therefore, blocking the interaction of selectin and integrin with their ligands can affect leukocyte recruitment in acute and chronic inflammatory responses[2, 6, 15]. For this aim much work has been done to find inhibitors to interrupt abnormal leukocyte emigration into tissues during pathological situations. Among the various inhibitors, heparin has been shown to have strong inhibitory effects in experimental inflammation models. However, its potent anticoagulant activity limits its clinical use. To solve this problem, the chemical or enzymatic modification to heparin to reduce its anticoagulant activity has been developed[8, 9, 16, 17]. In the present study, we prepared a heparin derivative, RO-heparin, by chemical modification according to the well-established method[12]. The anticoagulant activity of RO-heparin have been dramatically reduced (Table 1).

RO-heparin contains GlcA-Aman (3,6-$(SO_4)_2$) sequences that lie outside of the antithrombin-binding pentasaccharide. It has been reported that RO-heparin can inhibit the adhesion of P-selectin-mediated tumor cells[18]. Recently, we reported that RO-heparin can inhibit L-selectin and P-selectin-mediated leukocyte adhesion[10, 11]. However, whether the RO-heparin also can inhibit Mac-1-mediated leukocyte adhesion to vascular endothelium remains unclear. To answer the question, in our experiments, on the basis of the static cell surface binding of neutrophils with ICAM-1 and the cell adhesion of neutrophils on COS-7-ICMA-1 cells, we made a further observation of the inhibitory effect of heparin or RO-heparin on the adhesion of neutrophils to HUVECs under flow conditions. We found that RO-heparin exhibit excellent ability in blocking Mac-1-mediated neutrophil adhesion. Furthermore, we demonstrated that both heparin and RO-heparin can also inhibit the adhesion of PMA-stimulated neutrophils to fibrinogen. The findings suggested that in addition to L-selectin and P-selectin, the inhibitory role of RO-heparin in inflammation may also partially due to the interference of Mac-1-mediated leukocyte adhesion.

Previous studies have shown that the individual subunits of Mac-1 are able to mediate specific and distinct functions of the heterodimeric integrin. The α_M subunit appears to impart the firm adhesion mediated by the receptor, and the β_2 subunit is critical for cell migration[19]. The I-domain on the α chain of Mac-1 is an important recognition site for several ligands such as ICAM-1, fibrinogen, and iC3b[20]. The role of the I-domains in the ligand binding has been well established. Diamond, *et al*, found that the binding of C3bi and ICAM-1 to Mac-1 can be blocked by mAbs to the α I-domain, suggesting a spatial proximity between these ligand-binding sites[21]. Based on the

ability of a whole set of monoclonal antibodies to inhibit Mac-1-mediated neutrophil adhesion on immobilized heparin, Diamond also suggested the I-domain as the binding site for heparin[22]. Therefore, heparin can block the binding of fibrinogen, ICAM-1, and iC3b to Mac-1, and this is consistent with the mapping of the ligand binding sites to the I-domain of Mac-1 [7]. It has been reported that either N- or O-sulfation is sufficient for heparin to bind efficiently to Mac-1[22]. RO-heparin under conditions of cleaveage of all the C(2)-C(3) bonds of non-sulfated uronic acid residues preserves almost the original molecular mass of heparin and the original content of the sulfate residues [12]. Therefore, we believe that RO-heparin which had greatly reduced anticoagulant activity still preserve the efficient binding ability to Mac-1. The inhibition of Mac-1-mediated cell adhesion on ICAM-1 by RO-heparin is expected to impair Mac-1-dependent leukocyte adhesion on endothelial cells.

In conclusion, our studies showed that RO-heparin has a potent activity in anti-leukocyte adhesion while has lost or partly lost its anticoagulant activity. The anti-leukocyte adhesion activity of RO-heparin in part depends on its blockade of the interaction between Mac-1 and its ligands. All the combined properties appear to suggest that RO-heparin may act as a potential safer drug for the treatment of inflammatory disease.

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高碘酸氧化肝素抑制 β_2-整合素(Mac-1)介导的嗜中性粒细胞黏附 *

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摘要　已有的研究结果表明，肝素可以作为 β_2- 整合素(Mac-1)的配体抑制炎症过程中 Mac-1 介导的嗜中性粒细胞与血管内皮细胞的黏附．通过选择性化学修饰方法制备了具有低抗凝血活性的高碘酸氧化 - 硼氢化钠还原肝素(RO-肝素)，系统地研究了它对 Mac-1 介导的嗜中性粒细胞黏附的抑制作用．结果表明，显著失去抗凝血活性的 RO- 肝素仍能有效地抑制 Mac-1 介导的嗜中性粒细胞与 ICAM-1 重组蛋白、转染 ICAM-1 cDNA 的 COS-7 细胞和人脐静脉内皮细胞黏附．为深入阐明拮抗 Mac-1 介导的白细胞黏附的分子机制和筛选抗炎症药物提供了有价值的实验证据．

关键词　高碘酸氧化肝素，β_2- 整合素(Mac-1)，嗜中性粒细胞，人脐静脉内皮细胞
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