Chemically modified heparins inhibit fibrinogen-bridged indirect adhesion between tumor cells and platelets

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Abstract. The interaction between platelets and tumor cells is critical for the hematogenous metastasis of tumor cells. We recently reported that fibrinogen was capable of bridging and enhancing the interaction of platelets and tumor cells under conditions of physical shear force. In the present study, we aimed to detect the effects of 8 chemically modified heparins on the binding of fibrinogen to platelets or tumor cells using flow cytometry assays, as well as the fibrinogen-bridged adhesion of platelets and tumor cells using flow chamber assays. The results showed that fibrinogen binds to platelets and tumor cells in a $\beta3$ integrin-dependent manner and bridges the adhesion between platelets and tumor cells. Heparin and certain chemically modified heparins, including borohydride-reduced (RO)-, carboxyl-reduced (CR)- and 2-O, 3-O-desulfated (2/3ODS)-heparins, inhibited the $\beta3$ integrin-dependent adhesion of fibrinogen to platelets or tumor cells, and consequently blocked the fibrinogen-bridged indirect adhesion of platelets to tumor cells. These data indicate that chemically modified heparins should be potential inhibitors for the fibrinogen-bridged indirect adhesion of platelets and tumor cells, which provides a novel explanation of the anti-adhesion property of heparin and proposes a new anti-metastatic target for cancer treatment.

Introduction

Platelets are known to enhance the hematogenous metastasis of tumor cells through various mechanisms. Platelets are capable of forming a protective cloak by adhering to tumor cells to protect them from natural killer (NK) cytotoxicity (1,2), enhancing tumor angiogenesis by forming growth factors 3-5) and promoting the adhesion of tumor cells on the endothelium and the invasion of tumor cells into the surrounding tissues (6-8). The common basis for these carcinogenic effects is the interaction of platelets and tumor cells. P-selectin and $\alpha IIb\beta3$ integrin, expressed in platelets, and their heparan sulfate-like proteoglycan (HSPG) ligands, expressed in tumor cells, have been reported to be crucial for this interaction (9-12). A number of studies have focused on inhibitors of adhesive molecules or their ligands, including blocking antibodies, oligosugars, polysugars and peptites (13,14). Among these inhibitors, a traditional clinical reagent, heparin, may be one of the most valuable candidates in blocking the adhesion of platelets and tumor cells (15,16). However, the hemorrhagic dangers caused by the marked anticoagulant effect of heparin has limited its application as an anti-metastatic drug. Previously, we demonstrated that certain chemically modified heparins with low anticoagulant activity were capable of blocking the P-selectin and $\alpha IIb\beta3$ integrin-mediated adhesion of platelets and tumor cells (17-19).

Since the adhesion of platelets and tumor cells occurs in the blood, the effect of plasma proteins cannot be ignored. In a recent study, we found that fibrinogen strongly bridged and enhanced the adhesion of platelets and tumor cells under various shear forces (20). Fibrinogen is a major component of the plasma; thus, the identification of blockers to the adhesion of platelets and tumor cells bridged by fibrinogen is necessary, as identifying such blockers may have greater significance for clinical tumor treatment. In the present study, we aimed to detect the effects of 8 chemically modified heparins on the binding of fibrinogen to platelets or tumor cells using flow cytometry assays, as well as the fibrinogen-bridged adhesion of platelets and tumor cells using flow chamber assays. The results revealed that borohydride-reduced (RO)-, carboxyl-reduced (CR)- and 2-O, 3-O-desulfated (2/3ODS)-heparins inhibited the binding of fibrinogen to platelets or tumor cells, and that fibrinogen bridged indirect adhesion between platelets and tumor cells.

Materials and methods

Cells. B16F10, a murine melanoma cell line, A375, a human melanoma cell line, and CHO cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The cells were grown in Iscove's Modified Dulbecco's Media (IMDM; Gibco; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum in the presence of

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100 U/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were passaged by mild trypsinization (0.25% trypsin) and harvested with 2 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Tumor cells were washed twice with PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) (pH 7.4), resuspended in serum-free culture medium containing 0.1% bovine serum albumin (BSA), and stored at 4°C for no longer than 5 h prior to use.

Antibodies and reagents. The monoclonal antibodies (mAbs) LM609 recognizing human β3 integrins (blocking mAb) and 2C9.G2 recognizing murine β3 integrins (blocking mAb) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HMb1-1 recognizing murine β1 integrins (blocking mAb) was purchased from BioLegend, Inc. (San Diego, CA, USA). Armenian hamster IgG isotype control and fluorescein isothiocyanate (FITC)-conjugated antibody against Armenian hamster IgG were purchased from Santa Cruz Biotechnology, Inc. Human IgG and mouse IgG isotype control, FITC-labeled goat anti-human IgG and anti-mouse IgG were purchased from Jackson Immune Research Laboratories, Inc. (West Grove, PA, USA). Human fibrinogen, 3-aminopropyltriethoxylsilane (APES) and porcine intestinal heparin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Periodate-oxidized, RO-, CR-, 2/3ODS-, N-desulfated, 2/3ODS- (N/2/3DS-), carboxyl-reduced sulfated- (CRS-), N-desulfated/N-acetylated- (NDS-), 6-O-desulfated- (6ODS-) and N-desulfated 6-O-desulfated-heparin (N/6DS-heparin) were prepared in our laboratory (18).

Preparation of platelets. Specific pathogen-free C57BL/6 J mice (male, 6-8 weeks old) were obtained from the animal center of Jilin University (Changchun, China). Blood of healthy mice injected with prostaglandin E1 (PGE-1) was collected retro-orbitally, and then immediately mixed with sodium citrate anticoagulant buffer (0.38% w/v). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 300 x g for 10 min, and platelet-poor plasma (PPP) and platelets were separated by centrifugation of PRP at 1,300 x g for 10 min. The density of platelets was adjusted to $2x10^8$/ml by the HEPES Tyrode buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM NaH_2PO_4, 5 mM HEPES, 5 mM glucose, 1% BSA, pH 7.4) for further use. To avoid any unwanted activation of platelets, 2 μM PGE-1 was added to the buffers, and PGE-1 was washed off by HEPES Tyrode buffer prior to the use of platelets (21). The study protocols were approved by the Animal Care and Use Committee of Northeast Normal University, (Changchun, China).

Flow cytometry assay. To assess the adhesion of fibrinogen to tumor cells, flow cytometry was performed using a flow cytometer (Coulter Epics XL; Beckman-Coulter, FL, USA). In brief, tumor cells were harvested and washed as previously described, counted and resuspended in culture medium at a final concentration of $5x10^6$ cells/ml. For the fibrinogen binding assay, $5x10^5$ cells were incubated in 100 μl of culture medium containing 0.02 mg of fibrinogen conjugated with Alexa Fluor488 green (no. F13191; Molecular Probes, Invitrogen, OR,

USA) at room temperature for 30 min. Subsequently, cells were washed twice, and 10,000 cells were collected for flow cytometry. For the adhesion inhibition experiments, the cells were preincubated with 1 μg/ml of 2C9.G2, HMB1-1 or LM609, or with 1 mg/ml of chemically modified heparins for 30 min.

Static adhesion assay. To assess the adhesion of fibrinogen to platelets, human and murine platelets were stained with calcein acetoxymethyl ester (AM) (no. 32805; Molecular Probes, Invitrogen, OR, USA), and the stained platelets were added into the wells, which were pre-coated with fibrinogen following washing twice with HEPES Tyrode buffer. Adhesions were performed for 30 min, non-specific adhered platelets were removed by mild rinsing, and the fluorescence density of each well was measured using a microplate reader (MD VersaMax, Hamilton-Molecular Devices driver; Hamilton Company, Höchst, Germany), which reflected the number of stably adhered platelets. For the inhibition assay, platelets were preincubated with various antibodies or chemically modified heparins for 30 min prior to addition into the wells.

Flow chamber adhesion assay. Platelet monolayers were prepared as previously described (18). Non-specific binding was blocked with 0.1% BSA at 37°C for 10 min. Cell adhesion to platelets under flow conditions was measured as previously reported (21). In brief, cells were washed, resuspended and adjusted to $1x10^6$ cells/ml in serum-free medium containing 0.1% BSA. Surface-adhered platelets were incubated with 100 μl of thrombin (1 U/ml in PBS/0.1% BSA) at 37°C for 1 min. Circular glass slides coated with platelets were assembled in a flow chamber and mounted on the stage of an inverted microscope (Olympus Optical, Tokyo, Japan) equipped with a camera (Panasonic; Yokohama, Japan) connected to a personal computer by a TV monitor card. After washing the platelet layer with PBS/0.1% BSA for approximately 2 min, tumor cells were perfused through the chamber for 3 min at appropriate flow rates to obtain wall shear stresses of 0.3 to 1.2 dyn/cm^2 at 22°C using a syringe pump (Cole-Parmer Instrument Co.; Montreal, Canada), thereby mimicking the flow mechanical environment of microcirculation in postcapillary venules. Interactions between tumor cells and surface-adherent platelets (or fibrinogen) were visualized in real-time by phase-contrast video microscopy. The number of bound cells was quantified from the digital recordings of 10 random fields of the views obtained.

Statistics. Data are shown as the means ± standard deviation (SD). Statistical significance of differences between the means was determined by one-way ANOVA. If the means were revealed to be significantly different, multiple comparisons using pairs were performed using the Tukey test. Probability values of $P<0.01$ were considered to indicate statistically significant differences.

Results

Fibrinogen binds to tumor cells and platelets in a β3 integrin-dependent manner. Although a number of studies (6,9) have demonstrated that platelets directly adhere to tumor cells, the effects of plasma protein cannot be ignored as the adhesion that occurs in blood and fibrinogen is one of the major components
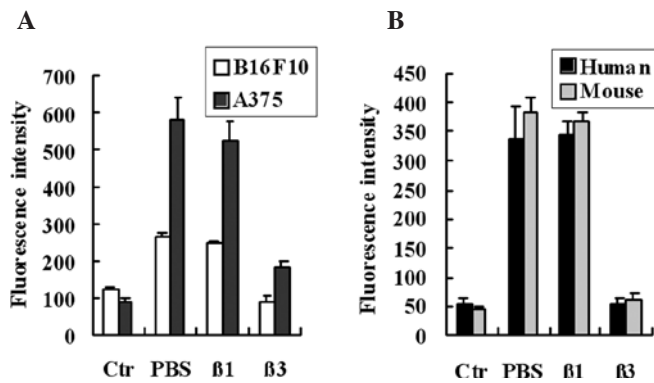
Figure 1. Fibrinogen binds to tumor cells and platelets in a β3 integrin-dependent manner. (A) A total of 100 μl of 2 mg/ml fibrinogen (Alexa Fluor488 conjugated) were incubated with 100 μl of 2x10^6/ml A375 or B16F10 cells in phosphate-buffered saline (PBS) containing Ca^{2+} (PBS samples) or EDTA (Ctr samples) for 30 min. Tumor cells adhered with fibrinogen were assessed by flow cytometry after being washed three times with PBS. Preincubation of melanoma cells with integrin antibodies was performed prior to the adhesion of fibrinogen to tumor cells (β1 or β3 samples). (B) Adhesion of murine or human platelets to fibrinogen layers were analyzed by static adhesion assays. A total of 100 μl of 2x10^9/ml platelets (Calcein AM dyed) in PBS containing Ca^{2+} (PBS samples) or EDTA (Ctr samples) were added to the wells that were precoated with 100 μl of 2 mg/ml fibrinogen, and adhesion was carried out for 30 min. Platelets adhered to fibrinogen were assessed using a plate reader after washing three times with PBS. Preincubation of platelets with integrin antibodies was performed prior to the adhesion of platelets to fibrinogen (β1 or β3 samples). Ctr, control; EDTA, ethylenediaminetetraacetic acid.
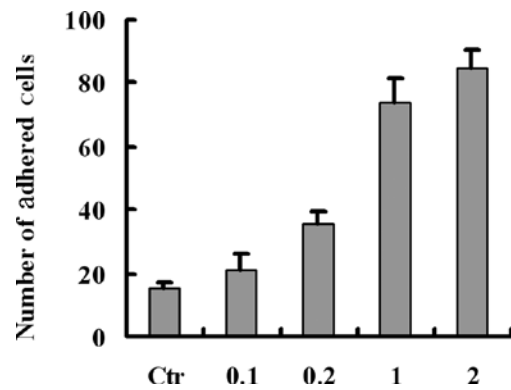
Figure 2. Fibrinogen enhances the adhesion of tumor cells and platelets in a concentration-dependent manner. Platelet monolayers on glass slides were washed with PBS/0.1% bovine serum albumin for 2 min. A375 cells were preincubated with or without (Ctr) various concentrations of fibrinogen for 30 min, the treated cells were then perfused over the layers under 1.2 dyn/cm^2. The adhered tumor cells on platelet monolayers were counted in 10 randomly selected fields after 3 min. PBS, phosphate-buffered saline; Ctr, control.

results showed that the adhesion of fibrinogen to human and murine platelets were blocked by the blocking antibody against the β3 integrin (Fig. 1B). These results indicate that fibrinogen binds to tumor cells and platelets, and that β3 integrins, expressed in tumor cells and platelets, are the major receptors of fibrinogen.

of the plasma. In a recent study, we found that fibrinogen bridged and enhanced the adhesion of platelets and tumor cells under physiological conditions (20). In the present study, flow cytometry and static adhesion assays were performed to assess the adhesive ability of fibrinogen to tumor cells and platelets. As shown in Fig. 1, fibrinogen bound to tumor cells (A) and platelets (B). As previously reported the receptors of fibrinogen on melanoma cells are the αvβ3 and α5β1 integrins, we assessed these integrins to determine whether they play crucial roles in the binding process by using blocking antibodies. The results showed that the binding of fibrinogen to B16F10 and A375 cells was blocked by the antibody against the β3 integrin, but not the β1 integrin (Fig. 1A). We also assessed the adhesion of fibrinogen to platelets using a static adhesion assay. The

Fibrinogen bridges the indirect adhesion between tumor cells and platelets in a β3 integrin-dependent manner. To confirm the role of fibrinogen in the adhesion of tumor cells and platelets, melanoma cells were preincubated with various concentrations of fibrinogen for 30 min prior to perfusion over the platelet monolayers. Results revealed that fibrinogen mediated and enhanced the adhesion between tumor cells and platelets in a concentration-dependent manner (Fig. 2). Previously, we demonstrated that the preincubation of tumor cells with a β3 integrin-blocking antibody inhibited the indirect adhesion between tumor cells and platelets (19). In the present study, we investigated the contribution of β3 integrins, expressed in platelets, as well as β3 and β1 integrins, expressed in tumor cells. Results from the flow chamber assay revealed
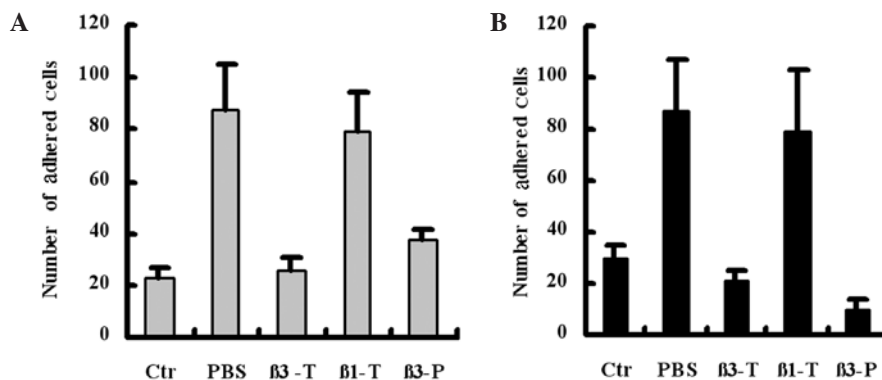

Figure 3. β3 integrins expressed in tumor cells and platelets are involved in indirect adhesion. The effects of β3 integrin-blocking antibodies on the indirect adhesions were assessed using flow chamber assays. (A) B16F10 and (B) A375, tumor cells (β3-T or β1-T) or platelets (β3-P) were incubated with mAbs or PBS (PBS). Ctr were the control samples, which were the direct adhesions of platelets and tumor cells, performed without fibrinogen. mAbs, monoclonal antibodies; PBS, phosphate-buffered saline; Ctr, control.
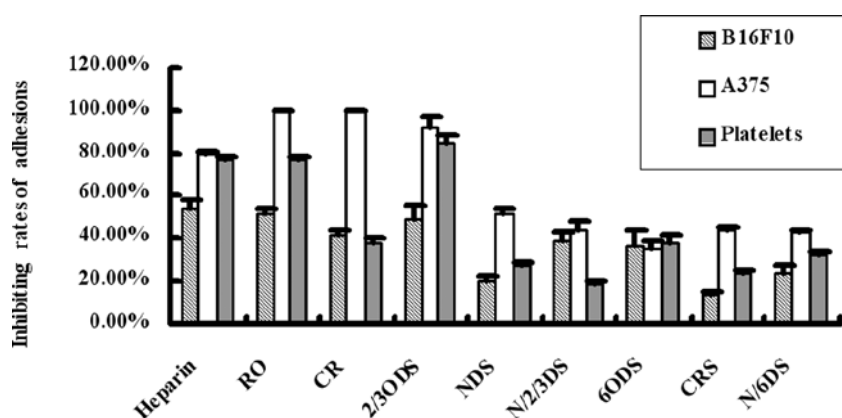
Figure 4. Chemically modified heparins inhibit the binding of fibrinogen to tumor cells or platelets. The binding of fibrinogen (2 mg/ml) to melanoma cells (A375 and B16F10) or platelets was assessed by flow cytometry or static adhesion assays (platelets). Prior to the assays being performed, cells were incubated with 1 mg/ml of 8 chemically modified heparins or PBS for 30 min, and the inhibitory rates of adhesions were calculated using the results from these assays. PBS, phosphate-buffered saline; NDS, N-desulfated/N-acetylated; CRS, carboxyl-reduced sulfated; CR, carboxyl-reduced; RO, borohydride-reduced.
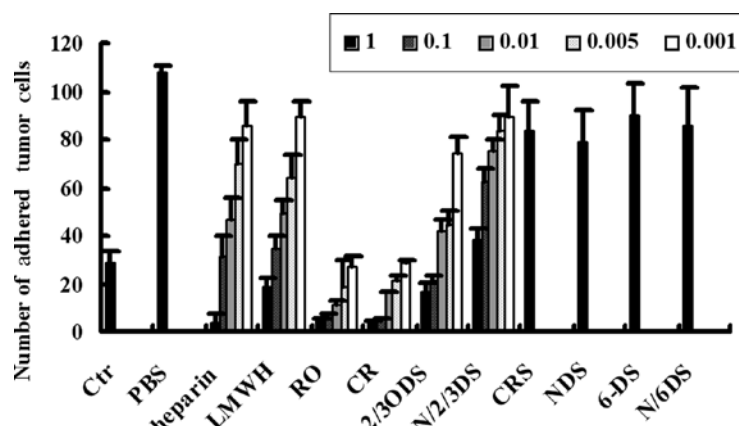

Figure 5. Chemically modified heparins inhibit the fibrinogen-mediated indirect adhesion of tumor cells and platelets. The indirect adhesion of A375 cells to platelet monolayers mediated by fibrinogen were assessed using flow chamber assays. Prior to incubation with fibrinogen, tumor cells were preincubated with PBS or varying concentrations of heparin, LMWH or chemically modified heparins. The adhesions of tumor cells to platelet monolayers were carried out following incubations, and the number of adhered tumor cells was quantified. Ctr were the control samples of direct adhesion of tumor cells and platelets, without the addition of fibrinogen or heparins. LMWH, low molecular weight heparin; Ctr, control; PBS, phosphate-buffered saline; NDS, N-desulfated/N-acetylated; CRS, carboxyl-reduced sulfated; CR, carboxyl-reduced; RO, borohydride-reduced.

that the preincubation of platelets (β3-P) or tumor cells (β3-T) with β3 integrin-blocking antibodies blocked the indirect adhesion of B16F10 (Fig. 3A) or A375 (Fig. 3B) to the platelet monolayer under flow conditions. The results confirm the bridging role of fibrinogen in mediating the adhesion of tumor cells and platelets, and indicate that β3 integrins, expressed in tumor cells or platelets, are involved in indirect adhesion.

Chemically modified heparins inhibit the direct binding of fibrinogen to tumor cells and platelets. Heparin has been reported to be a powerful inhibitor of the adhesions between cells and cells, or between cells and fibrinogen, and the adhesive molecules involved in the adhesion include selectins and integrins. Fibrinogen is capable of binding to tumor cells and platelets through β3 integrin and bridging the adhesion of tumor cells and platelets. In the present study, we examined the blocking effects of 8 chemically modified heparins (1 mg/ml) on the binding of fibrinogen to tumor cells and platelets. As shown in Fig. 4, heparin, RO-, CR- or 2,3ODS-heparins

strongly blocked the binding of fibrinogen to A375 cells (inhibition rate, >75%). RO- and 2/3ODS-heparin blocked the binding of fibrinogen to platelets to the levels of heparin (inhibition rate, >75%). In addition, RO- and 2/3ODS-heparin blocked the binding of fibrinogen to B16F10 cells (inhibition rate, >50%). These data suggest that certain types of chemically modified heparins, including RO-, CR- or 2,3ODS-heparins, act as effective blockers for direct adhesions.

Chemically modified heparins inhibit the fibrinogen-bridged indirect adhesion of tumor cells and platelets. As demonstrated previously, fibrinogen adhered to tumor cells and platelets and bridged their adhesion, and certain chemically modified heparins blocked the direct binding of fibrinogen to tumor cells or platelets. The aim of the present study was to detect whether the chemically modified heparins were capable of blocking the fibrinogen-mediated indirect adhesion of tumor cells and platelets using flow chamber assays. As shown in Fig. 5, compared to the direct adhesion (Ctr), pre-incubation of tumor cells with

fibrinogen notably enhanced the adhesion of tumor cells and platelets (PBS), and this indirect adhesion was inhibited by certain types of chemically modified heparins. RO-, CR- and 2/3ODS-heparins have been found to be more effective than other types of heparins in that their inhibitory abilities were similar to, or even greater than, heparin and low molecular weight heparin (LMWH), the well-known clinical reagents. Moreover, the inhibitory effects of RO-, CR- and 2/3ODS-heparins were concentration-dependent. These data suggest that RO-, CR- and 2/3ODS-heparins have a greater potential for cancer treatments due to their limited anticoagulant activity.

Discussion

The adhesion between platelets and tumor cells is known to enhance tumor hematogenous metastasis. Studies have focused on identifying the adhesive mechanism and searching for inhibitory drugs. P-selectin and αIIbβ3 integrin, expressed in platelets, and HSPGs, expressed in tumor cells, are the major receptors for the direct adhesion between tumor cells and platelets, and various inhibitors targeting these adhesive molecules have been developed (13-17). Among the inhibitors, heparins and their derivatives have been reported to be the most powerful candidates for mediating direct adhesion, in that they markedly inhibit the direct adhesion mediated by P-selectin and αIIbβ3 integrin (18,19). In the present study, we demonstrated that fibrinogen adhered to tumor cells and platelets in a β3 integrin-dependent manner (Fig. 1), and that the adhesion of melanoma cells to the platelet monolayer was increased by fibrinogen in a concentration-dependent manner (Fig. 2). β3 integrin, expressed in tumor cells and platelets, is essential for this indirect adhesion (Fig. 3), and fibrinogen mediated the indirect adhesion between melanoma cells and platelets as a bridge (20).

Since fibrinogen is rich in blood, developing inhibitors to the indirect adhesion between tumor cells and platelets is of significance. In this study, we detected the effects of heparin and its derivatives on indirect adhesion. We first examined the effects of heparin derivatives on direct adhesions between melanoma cells and fibrinogen or platelets and fibrinogen, and found that RO-, CR- and 2/3ODS-heparins blocked the direct adhesion of fibrinogen to melanoma cells or platelets (Fig. 4). The blocking effects to the indirect adhesion were then detected by flow chamber assay, and we found that RO-, CR- and 2/3ODS-heparins blocked the indirect adhesion, and that this blocking effect was almost identical to that of heparin, and even higher than LMWH (Fig. 5). The blocking effects of heparin derivatives to the indirect adhesion were multiple, although their inhibitory rates to the fibrinogen-B16F10 were no more than 60%, and to the fibrinogen-platelets were no more than 80%. RO-, CR- and 2/3ODS-heparins inhibited the indirect adhesion up to 95% (Fig. 5). These results confirmed the bridging role of fibrinogen in the indirect adhesion of tumor cells and platelets, and the stronger inhibitory activity of these heparin derivatives. In this study, a structure comparison demonstrated that the coexistence of N-sulfate and 6-sulfate was crucial for the blocking activity of chemically modified heparins, and that desulfation of 2-/3-/O-sulfate and the reduction of carboxyl has a slight effect, while the sulfation of reduced carboxyl (-CH2OH) should be avoided (17).

In conclusion, along with their function in inhibiting the direct adhesion of tumor cells and platelets mediated by selectins, integrins and HSPGs, these data suggest that RO-, CR- and 2/3ODS-heparins should be considered as valuable candidates for blocking direct and indirect adhesions of tumor cells and platelets, and that the low anticoagulant character of the chemically modified heparins is likely to benefit their clinical application in anti-tumor metastasis (18).

Acknowledgements

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