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Short communication

Analysis of the Potent Platelet Glycoprotein IIb-IIIa Antagonist from Natural Sources

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Adhesive interaction of the platelet glycoprotien IIb-IIIa (GP IIb-IIIa) with a plasma protein, such as fibrinogen, plays an important role in thrombosis and hemostasis. The specific sequence Arg-Gly-Asp (RGD) is critical for the binding of fibrinogen to platelet. To examine and characterize the GP IIb-IIIa antagonist from natural sources, we have developed a simple enzyme-linked immunosorbant assay (ELISA) system. The GP IIb-IIIa complex was purified to homogeneity from platelet lysates by the combination of two affinity chromatographic methods using the synthetic RGD peptide (GRGDSPK)-immobilized Sepharose and wheat germ lectin-Sepharose. The synthetic peptide GRGDSP inhibits GP IIb-IIIa binding to immobilized fibrinogen with an IC_{50} of 1.5 μM. Venoms of three different snake species and a Korean scolopendra extract have strong antagonistic activities for the binding of human fibrinogen to the platelet GP IIb-IIIa complex. The IC_{50} values of the snake venoms and scolopendra were in the range of 5.5 μg to 60 μg. These results provide meaningful information for developing antiplatelet agents.

Keywords: GP IIb-IIIa antagionist, Platelet.

Introduction

Platelets play a central role in hemostasis. They are activated by various physiological agonists via different pathways and undergo a series of morphological and functional changes (Hawjger *et al.*, 1994). Platelet activation results in aggregation by the interaction of fibrinogen with the platelet membrane glycoprotein IIb-IIIa (GP IIb-IIIa) (Phillips *et al.*, 1988; Plow *et al.*, 1989). The platelet surface receptor, the GP IIb-IIIa complex, is a major plasma membrane protein complex involved in the attachment, spreading, and aggregation of platelets. The GP IIb-IIIa complex is a member of the integrin family (Hynes, 1987; Ginsburg *et al.*, 1988), several of which bind multiple ligands containing the Arg-Gly-Asp (RGD) sequence (Pierschbacher and Ruoslahti, 1984a; 1984b). The RGD sequence is a key structural element in the recognition and binding of GP IIb-IIIa to its *in vivo* ligands fibrinogen, fibronectin, vitronectin, and von Willebrand factor (Ginsburg *et al.*, 1983; Gardner and Hynes, 1985; Pytela *et al.*, 1986). The C-terminal non-RGD region of the fibrinogen γ-chain is also important in mediating platelet aggregation (Farrel *et al.*, 1992; Hettasch *et al.*, 1992; Hoekstra and Beavers, 1995). Thus, the binding of fibrinogen to GP IIb-IIIa is a final common event of all activators leading to aggregation and is an excellent target for therapeutic intervention in thrombotic diseases (Stein *et al.*, 1989). Most of the potent inhibitors of platelet aggregation acting via antagonism of the GP IIb-IIIa receptor have been isolated from natural sources. Snake venom is composed of complex protein species including various hydrolytic enzymes (Chung *et al.*, 1992; 1993a; 1993b; Yum *et al.*, 1993a; 1993b). The GP IIb-IIIa antagonists, named disintegrin, that are chiefly identified as small proteins of 5 to 9 kDa, and cysteine-rich polypeptides which essentially contain the RGD or Lys-Gly-Asp (KGD) sequence, are mainly derived from a wide spread of snake venoms (Huang *et al.*, 1987; Gan *et al.*, 1988; Chao *et al.*, 1989; Huang *et al.*, 1989; Dennis *et al.*, 1990; Seymour *et al.*, 1990; Williams *et al.*, 1990; Mazur *et al.*, 1991; Scarborough *et al.*, 1991; Maruyama *et al.*, 1997). In this communication, we report on the purification of the GP IIb-IIIa receptor from platelet membrane, development of a solid phase fibrinogen/GP IIb-IIIa enzyme-linked immunosorbant assay (ELISA) system, and screening of the material that has the GP IIb-IIIa antagonistic activity from natural sources.

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Materials and Methods

Materials Fresh venom of *Agkistrodon halys brevicaudus* was obtained directly from the local snake farm in Korea. Snake venoms of *Agkistrodon halys blomhoffi* and *Agkistrodon rhodostoma* were purchased from Sigma. Korean scolopendra and lumbricus were from a Korean local market. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and molecular weight markers were obtained from NOVEX (San Diego, USA). Capcell pak C18 reverse phase HPLC column was from Shiseido Research Center (Tokyo, Japan); TSK G-2000 SWXL gel filtration column was from Tosoh Co. (Tokyo, Japan); CNBr-activated Sepharose was from Pharmacia-LKB Biotechnology Inc. (Upsala, Sweden). The peptides (GRGDSP or GRGDSPK) were synthesized using f-Moc chemistry (Fields *et al.*, 1992) and purified on a reverse phase C18 column using 0.1% trifluoroacetic acid/acetonitrile gradient

Preparation of platelet lysates Human platelet concentrates (5 Unit) were pooled into 250-ml centrifuge bottles and centrifuged at 300 × *g* for 5 min to remove red blood cells. This procedure was repeated if red cells were still evident. The platelet supernatant was removed and centrifuged at 1800 × *g* for 15 min to precipitate the platelets. This pellet was subsequently washed three times at 4°C in phosphate-buffered saline (PBS). The platelets in the final pellet were lysed by resuspending in PBS containing 50 mM octylthioglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 mM phenylmethanesulphonyl fluoride (PMSF) at 4°C for 15 min. The lysate was centrifuged at 30,000 × *g* for 20 min at 4°C and the supernatant was frozen at −20°C until processed further.

Preparation of GRGDSPK-affinity resin CNBr-activated Sepharose resin (10 ml) was washed with 300 ml of ice-cold 50 mM sodium acetate, pH 4.5, in a coarse sintered glass funnel. After removing excess buffer, the washed resin was transferred to a 15-ml conical tube containing 100 mg of the synthetic RGD peptide GRGDSPK. The peptide-conjugated resin was transferred to a sintered glass funnel and washed with 0.1 M $NaHCO_3$, pH 8.3, followed by 0.1 M sodium acetate, pH 4.0, each containing 0.5 M NaCl. Finally, the coupled GRGDSPK-resin was washed with 100 ml of PBS containing 25 mM octylthioglucoside, 1 mM PMSF, and 2 mM $CaCl_2$ (column buffer). This preparation was stored at 4°C prior to use.

Purification of GP IIb-IIIa receptor The platelet lysate was mixed with GRGDSPK-Sepharose resin (bed volume, 10 ml) and then incubated overnight at 4°C. The mixture was packed into a column and washed with PBS containing 25 mM octylthioglucoside and 1 mM PMSF. Elution of the bound components was accomplished by washing the column with 10 ml of the column buffer containing GRGDSPK-peptide (1.0 mg/ml) and then with 10 ml of the column buffer. Active fractions eluted from the column were pooled and further purified by wheat germ lectin-Sepharose chromatography preequilibrated with 50 mM Tris-Cl, pH 7.4. Purified GP IIb-IIIa receptor was stored frozen in TACTS (20 mM Tris-Cl, pH 7.5/ 0.02% NaN_3/ 2 mM $CaCl_2$/0.05% Tween 20/150 mM NaCl).

Fibrinogen/GP IIb-IIIa ELISA The fibrinogen/GP IIb-IIIa ELISA was performed by a modified method of Nachman and Leung (1982). Microtiter plates were coated with purified human fibrinogen (10 μg/ml). After blocking with 1% bovine serum albumin in TACTS for 1 h, the plate was washed, and samples to be evaluated were added, followed immediately by addition of purified GP IIb-IIIa (40 μg/ml) in TACTS containing 0.5% bovine serum albumin. After 2 h incubation, the plate was washed and mouse anti-human GP IIIa antibody was added. Following an additional 1 h incubation and washing, goat anti-mouse IgG conjugated to horseradish peroxidase was added. A final wash was performed and developing substrate solution was added. Then, the plate was incubated for about 10 min until color developed. The reaction was stopped with 3 M HCl followed by absorbance measurement at 492 nm.

Results

Purification of the GP IIb-IIIa receptor from platelets The GP IIb-IIIa receptor was purified by affinity chromatography prepared with the Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) coupled to Sepharose. This peptide corresponds in sequence to the cell attachment site of fibronectin. Detergent extract of washed human platelets was applied to this affinity resin, and elution of the GP IIb-IIIa complex was carried out with the GRGDSP peptide solution (1.0 mg/ml). Analysis of the purified GP IIb-IIIa receptor by SDS-PAGE in the presence of 2-mercaptoethanol revealed two protein bands located at molecular weights of 124 kDa and 108 kDa corresponding to the heterodimeric structure of the receptor (Fig. 1).

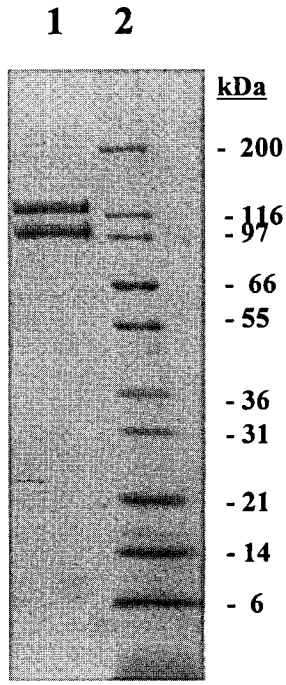

Fig. 1. SDS-PAGE analysis of purified GP IIb-IIIa receptor from platelet. The purified GP IIb-IIIa receptor was analyzed by SDS-PAGE using 4–20% polyacrylamide gel. Lane 1, purified GP IIb-IIIa; Lane 2, molecular mass markers.

Screening of GP IIb-IIIa antagonists from natural sources The interaction of GP IIb-IIIa with fibrinogen, as measured by the solid-phase fibrinogen/GP IIb-IIIa ELISA, was inhibited by synthetic peptide GRGDSP. The synthetic peptide was able to inhibit the binding of fibrinogen to the integrin GP IIb-IIIa in a concentration-dependent manner with an IC_{50} of 1.5 μM (Fig. 2). To examine the platelet GP IIb-IIIa antagonist activity from natural sources, including snake venoms, scolopendra, holotrichia, and lumbricus, it was investigated whether natural sources affect the interaction of purified GP IIb-IIIa receptor with immobilized fibrinogen via solid phase fibrinogen/GP IIb-IIIa ELISA. The binding of purified GP IIb-IIIa with immobilized fibrinogen was inhibited by three different snake venom species and by the extract of Korean scolopendra. As shown in Fig. 3, the IC_{50} values determined with crude snake venoms for the inhibition of the GP IIb-IIIa/fibrinogen binding were in the range of 5.5–24 μg/ml while the IC_{50} for the extract of Korean scolopendra was 60 μg/ml. On the other hand, no inhibition of the GP IIb-IIIa/fibrinogen interaction was observed in the extract of holotrichia and lumbricus (Fig. 3). These results strongly suggest that snake venoms from these three different species and the scolopendra extract have potent activities which inhibit platelet aggregation mediated by the integrin GP IIb-IIIa.

Discussion

Disintegrins which bind to the GP IIb-IIIa integrin receptor have been mainly isolated from a wide variety of snake venoms thus far. In this work, we have analyzed the potent disintegrins, using solid phase fibrinogen/GP IIb-IIIa ELISA, from natural sources including snake venoms and a scolopendra extract, which may have antagonistic

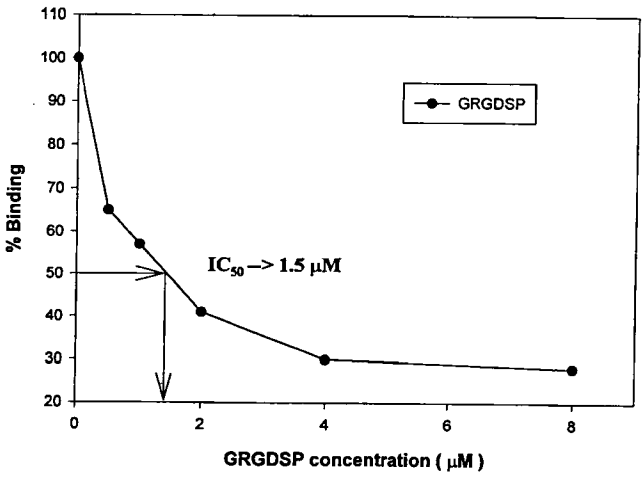

Fig. 2. Inhibition of fibrinogen binding to GP IIb-IIIa receptor by synthetic RGD peptide GRGDSP. Binding assay was performed using solid phase fibrinogen/GP IIb-IIIa ELISA as described in Materials and Methods.

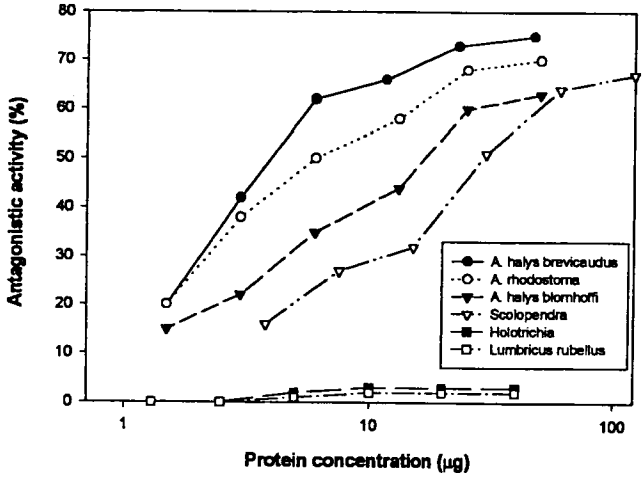

Fig. 3. Screening of GP IIb-IIIa antagonist from natural sources. Platelet GP IIb-IIIa antagonistic activities of natural sources were determined by solid-phase integrin binding assay as described in Materials and Methods. Each point represents the mean value of triplicates.

activities for GP IIb-IIIa receptor. The tested snake venoms and scolopendra extract showed inhibitory action on platelet aggregation by blocking the GP IIb-IIIa integrin. On the other hand, the extracts of holotrichia and lumbricus were not able to affect the interaction of fibrinogen to the GP IIb-IIIa receptor. In our recent work, we have isolated and characterized a novel GP IIb-IIIa antagonist, salmosin, that strongly inhibits platelet aggregation (Kang *et al.*, 1998). The protein contains the RGD sequence that was identified as the common binding site to the integrin receptor. It is interesting to note that Korean scolopendra has been used for the treatment of thrombolytic diseases as a traditional medicine. However, no information about scolopendra-derived disintegrin related to the platelet aggregation is available. Therefore, further investigation to identify and characterize the scolopendra-derived novel disintegrin would be valuable in developing anti-thrombotic agents.

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