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Inhibition of human platelet aggregation by eosinophils

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ABSTRACT

Aims: The relationship between the activity of eosinophils and platelets has been observed in recent decades by many scientists. These observations include increased numbers of eosinophils associated with platelet disorders, including changes in the coagulation cascade and platelet aggregation. Based on these observations, the interaction between eosinophils and platelets in platelet aggregation was analyze.
Main methods: Human platelets were incubated with eosinophil cytosolic fraction, promyelocytic human HL-60 clone 15 cell lineage, and eosinophil cationic protein (ECP). Platelet rich plasma (PRP) aggregation was induced by adenosine diphosphate, platelet activating factor, arachidonic acid, and collagen, and washed platelets (WP) were activated by thrombin.
Key findings: Aggregation induced by all agonists was dose dependently inhibited by eosinophil cytosolic fraction. This inhibition was only partially reversed by previous incubation of the eosinophils with L-Nitro-Arginine-Methyl-Ester (L-NAME). Previous incubation with indomethacin did not prevent the cytosolic fraction induced inhibition. The separation of eosinophil cytosolic fraction by gel filtration on Sephadex G-75 showed that the inhibitory activity was concentrated in the lower molecular weight fraction. HL-60 clone 15 cells differentiated into eosinophils for 5 and 7 day were able to inhibit platelet aggregation. The ECP protein inhibited the platelet aggregation on PRP and WP. This inhibition was more evident in WP, and the citotoxicity MTT assay proved the viability of tested platelets, showing that the observed inhibition by the ECP protein does not occur simply by cell death.
Significance: Our results indicate that eosinophils play a fundamental role in platelet aggregation inhibition.

Introduction

Acquired platelet dysfunction with eosinophilia or nonthrombocytopenic purpura with eosinophilia is an acquired bleeding disorder of unknown etiology associated with platelet dysfunction and eosinophilia (Mitrakul, 1975; Suvatte et al., 1979).

Many authors have reported associations between the increased numbers of eosinophils with platelet dysfunctions, such as increased bleeding time, reduction in platelet aggregation induced by various agonists, among others disorders (Chin and Koong, 1990; Hathirat et al., 1993; Laosombat et al., 2001; Lim et al., 1989; Lucas and Seneviratne, 1996; Muthiah et al., 1984; Poon et al., 1995; Ramanathan and Duraisamy, 1987; Suvatte et al., 1979).

In most cases the bleeding symptoms are mild and transient with spontaneous recovery, and eosinophilia remains only a few weeks after the onset (Lee, 2012; Lim et al., 1989; Poon et al., 1995; Ramanathan and Duraisamy, 1987). The eosinophil count can vary from 3 to 6% of total white blood cells (Suvatte et al., 1979). Bleeding time is prolonged in about 60% of patients (Suvatte et al., 1979; Hathirat et al. 1982), but the platelet count is normal. Platelet adhesiveness is abnormally low in 60% of patients. Aggregation in response to stimulation by ADP, thrombin, and collagen is decreased, but the response to ristocetin is normal (Laosombat et al., 2001; Lim et al., 1989; Suvatte et al., 1979).

A distinctive group of proteins comprise the eosinophil granule (Gleich and adolphson, 1993; Peters et al., 1986). Among these, the eosinophil major basic protein (MBP) on a molar basis is the most abundant eosinophil granule protein. The surrounding granule matrix contains other proteins, including eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) (Abu-Ghazaleh et al., 1992; Gleich et al., 1993).

The eosinophils are transported in the bloodstream in a resting state and can be activated at sites of inflammation or when an activated endothelium is found. Only a small amount of activated eosinophils seems to be found in the bloodstream. However, in some circumstances, such as in idiopathic eosinophilic syndrome (HES), where there are an increase of eosinophils, and consequently a higher level of activated cells in the bloodstream (Egesten et al., 2001).

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The protein synthesis in eosinophils may occur in a constitutive manner, where new proteins are constantly secreted or regulated (degranulation) in response to specific stimuli.

In recent years, non-classical forms of eosinophils degranulation have been suggested. These include piecemeal degranulation, which is based on morphological changes of granules or cytolytic degranulation (e.g., necrosis). This phenomenon is present in atopic dermatitis and allergic inflammation of the upper airways (Egesten et al., 2001).

Based on evidence about the involvement between eosinophils and platelet disorders, this work describes the effect of eosinophil cytosolic fraction, HL-60 clone 15 cells lineage differentiated into eosinophil, and the ECP protein on the in vitro platelet aggregation induced by different agonists.

Materials and methods

Materials

Adenosine diphosphate (ADP), platelet-activating factor (PAF), collagen, arachidonic acid, thrombin, Nω-nitro-L-arginine methyl ester (L-NAME), [3-(4,5-dimethythiazol-2-yl)-2,5 diphenyl tetrazolium bromide] (MTT), minimum essential medium (MEM), butyric acid and sephadex G-75 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The promyelocytic human HL-60 clone 15 cell lineage was obtained from the American Type Culture Collection (Rockville, MD). Eosinophil cationic protein (ECP) was purchased from Lee biosolutions laboratory (St. Louis, MO, USA). The other materials and chemicals were obtained from commercial sources.

Platelet rich plasma (PRP) preparation

The platelet rich plasma was prepared as previously described with minor modifications (Donato et al., 1996).

Blood from healthy donors, who had not taken any medication for at least ten days, was anticoagulated with 3.8% sodium citrate (1:9 v/v). PRP was obtained by centrifuging whole blood at 200 g × 15 min at room temperature. The supernatant (PRP) was collected and left at room temperature until the aggregation assay was performed. An aliquot (1 ml) of PRP was centrifuged at 2000 ×g for 15 min at room temperature to obtain the platelet poor plasma (PPP) used to calibrate the aggregometer for the maximum aggregation.

Washed platelet (WP) preparation

Blood was collected in plastic tubes containing the ACD-C anticoagulant solution (citric acid/citrate/dextrose) at the proportion of 1:9 (v/v). The mixture was centrifuged at 200 ×g for 15 min at room temperature, and the supernatant (PRP) was collected in a clean tube. Iloprost (0.8 μM) was added to the PRP/ACD-C, which was then centrifuged at 800 ×g for 12 min. The supernatant was discarded and the platelet pellet was carefully resuspended in calcium-free Krebs–Ringer solution (composition in mM: NaCl, 118; Mg $SO_4.7H_2O$, 1.7; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 5.6). Iloprost (0.8 μM) was added again and the final cell suspension was centrifuged at 800 ×g for 10 min. The precipitated platelets were finally resuspended in calcium-free Krebs–Ringer solution to a final volume sufficient for a platelet suspension containing 2×10^8 platelets/ml. The platelet suspension was kept at room temperature, and $CaCl_2$ (1.0 mM) was added to the platelet suspension immediately before the aggregation assay.

Platelet aggregation

Platelet samples (0.4 ml) were incubated at 37 °C for 1 min and constantly stirred at 900 rpm in a double-channel Lumi-Aggregometer (Chrono-log 560 CA; Chronolog Corp., Havertown, PA, USA), before the addition of 10 μl of different agonists. Platelet aggregation using either PRP or WP was monitored for at least 5 min. The results were expressed as the percentage of maximum light transmission obtained when the aggregometer was calibrated for 100% transmission with PPP (PRP platelet aggregation assays) or Krebs solution (WP aggregation assays).

Preparation of the eosinophil cytosolic fraction

Eosinophils were obtained from the peritoneal cavity of 20–30 male Wistar rats (200–230 g) and purified on a discontinuous metrizamide gradient (Vadas et al., 1979). Briefly, the animals were killed with an overdose of halothane anesthesia, and the peritoneal cavities were washed with 20 ml of Phosphate-buffered saline (PBS; pH 7.2) containing heparin (20 units/ml). The peritoneal washings obtained from the animals were collected in a clean plastic tube immersed in an ice bath and centrifuged at 1000 ×g for 10 min at 20 °C. The metrizamide discontinuous gradient was prepared by carefully layering 2.5 ml of decreasing concentrations of metrizamide dissolved in minimum essential media (MEM; pH 7.2) containing 0.1% gelatin (23.5, 20, and 18%, wt/vol) into a conical propylene tube. The resulting leukocyte-rich pellet was gently resuspended in 2.5 ml of 18% metrizamide and layered over the top of the metrizamide gradient. The gradient tube was first centrifuged at 90 ×g (11 min at 4 °C) and then at 1000 ×g (14 min at 4 °C). The gradient zone containing the eosinophils (between the 23.5% and 20% gradients) was removed and washed twice in MEM containing 1 mg/ml of hydrolyzed ovalbumin. The May–Grünwald dye exclusion test show that the final cell suspension contained 80–90% eosinophils and a cell viability above 90%. Before testing, the eosinophil suspension was diluted in Hanks balanced salt solution (pH 7.2) to give a final concentration of 5×10^7 cells/ml.

To avoid the interference of eosinophil nitric oxide (NO) release or PG_1 synthesis on platelet aggregation, eosinophils were incubated with L-NAME (10 μM) and indomethacin (10 μM) for 60 and 20 min at 37 °C, respectively. Cells were prior disrupted by sonication (Sonic Dismembrator, Model 100; Fischer Scientific) in an ice bath using 3 cycles of 10 s at full intensity and immediately stored at −80 °C. After complete freezing, cell homogenate was removed from the freezer and kept at room temperature until the samples were thawed. After this freezing and thaw process, samples were centrifuged at 12,000 ×g for 5 min at 4 °C and the supernatant was collected and used to assay the inhibitory effect over platelet aggregation. The two inhibitors were used separately or in association before the eosinophil lysis.

To check the temperature stability of the active compound, the eosinophil cytosolic fraction was incubated at 100 °C for 20 min and subsequently tested for activity over the platelet aggregation process.

Gel filtration

The active fraction was isolated using gel filtration chromatography on Sephadex G-75. Lyophilized rat eosinophil cytosolic fraction was dissolved in Tris–HCl (0.05 M), pH 7.2 and centrifuged at 10,000 ×g for 5 min. The clear supernatant was applied to a Sephadex G-75 column (1.5 × 24 cm) and eluted with the same buffer at a flow rate of 10 ml/h. The eluted material was collected at 0.5 ml/fraction and assayed for the inhibitory activity on platelet aggregation. The active fractions were pooled and stored at −20 °C for further analysis.

Liquid chromatography analysis (RP-HPLC)

Prior to injecting the eosinophil sample into the analytical column, a desalting step (3 min at 30 μl/min with Water and 0.1% Trifluoroacetic acid; TFA) was performed using a pre-column cartridge packed with C-18 pepmap resin in line with an analytical column. Desalted sample was loaded on a 75 μm C-18 pepmap column operating at 200 nl/min. The buffers used for separation was Water and Acetonitrile, both with