Platelet-Derived Neutrophil Adherence-Inhibiting Factor in Humans

By Kazuhisa Iwabuchi and Tatsuhisa Yamashita

The effect of constituents of human platelets on leukocyte adherence was examined. Adherence-inhibiting factors (AIFs), which strongly inhibited neutrophil adherence to glass, were present in both cytosol and granule fractions of human platelets. On the Superose 6 gel chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden), the granular AIF was eluted as a single active peak (2,600 Kd), whereas cytosolic AIFs were eluted at two different positions (2,600 and 480 Kd). When platelets were stimulated by thrombin, granular AIF was released extracellularly without releasing a cytosolic marker. Using DE32 anion exchange chromatography and Superose 6 gel filtration, granular AIF was completely purified. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis suggests that granular AIF consists of two subunits with molecular masses of approximately 340 and 190 Kd. Purified granular AIF inhibited human neutrophil adherence to glass, plastic, and type IV collagen-coated plastic, whereas it did not affect monocyte adherence. These results suggest that granular AIF inhibits neutrophil adherence not only via nonspecific adsorption sites, but also via type IV collagen receptors.

The adherence of neutrophils to endothelium is an early step of the inflammatory response, which precedes their emigration into the extravascular compartment. Recent reports documented that platelets and/or their products modulated neutrophil adherence. We have already described that guinea-pig platelets contain adherence-inhibiting factors (AIFs), which strongly inhibit guinea-pig neutrophil adherence to glass, in both the cytosol and granule fractions. Granular AIF is easily released extracellularly by thrombin stimulation, and inhibits guinea-pig neutrophil adherence to type IV collagen-coated plastic surfaces, but not to fibronectin- or plasma-coated surfaces. These observations led us to the possible conclusion that granular AIF regulates neutrophil adherence via type IV collagen receptors in vivo.

In the present study, we examined whether AIF is present in human platelets as well as in guinea-pig platelets, and found that human platelets also contained AIFs in the cytosol and granule fractions. Therefore, we tried to purify and characterize human AIF.

MATERIALS AND METHODS

Materials. Phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA) (Fraction V), type IV collagen (type VI, from human placenta), and dithiothreitol (DTT) were obtained from Sigma Chemical Co, St Louis, MO. Bovine thrombin was obtained from Boehringer Mannheim, Mannheim, FRG. Peptatin, leupeptin, and fibronectin active fragment Arg-Gly-Asp-Ser (RGDS) were obtained from Peptide Institutes Inc, Osaka, Japan. Other reagents were of analytical reagent grade.

Isolation of cells. Peripheral blood obtained from normal volunteers by venipuncture was anticoagulated with 0.1 vol of 4% (wt/vol) citrate. Platelets were isolated by differential centrifugation as described previously. Such preparations contained almost 100% platelets.

Neutrophils were isolated from citrate anticoagulated blood of healthy donors by dextran sedimentation and Ficoll-Conray centrifugation as described previously, and suspended at $10^7$ cells/mL in buffer A (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na$_2$HPO$_4$, 1.5 mmol/L KH$_2$PO$_4$, 2.0 mmol/L MgCl$_2$, and 1.0 mmol/L CaCl$_2$, pH 7.4). Differential cell counts with Wright-Giemsa stain showed that more than 95% of the cells were neutrophils.

Monocytes were isolated from the same blood serving for neutrophil preparation. After Ficoll-Conray centrifugation, the interfaces were washed three times with buffer B (buffer A containing 1 mmol/L EDTA instead of divalent cations), then layered on top of self-generated gradient of Percoll in buffer B with density of 1.070. After centrifugation (500g for 15 minutes at 25°C), the monocyte fraction (the upper cell band) was harvested, washed with buffer B, and suspended at $10^7$ cells/mL in buffer A. The monocyte preparations were greater than 90% pure using Wright-Giemsa stain.

Guinea-pig neutrophils were isolated from the peritoneal cavity 13 to 15 hours after the intraperitoneal injection of sterilized 0.12% glycogen in 0.9% saline as described previously, and suspended at $10^7$ cells/mL in buffer A. The purity of these preparations were greater than 95% using Wright-Giemsa stain.

Guinea-pig monocytes were isolated from citrate anticoagulated blood by the same method as described above for human monocytes. The monocyte preparations were greater than 92% pure using Wright-Giemsa stain.

Preparation of coated tubes. Human platelet-free plasma was obtained from citrate-anticoagulated human blood as described previously, and 100-fold diluted with buffer A. Fibronectin was purified from guinea-pig platelet-free plasma by gelatin-Sepharose affinity chromatography as described previously and dissolved in buffer A at a final concentration of 30 μg/mL. Type IV collagen was dissolved in 0.5 mol/L acetic acid and diluted to a concentration of 20 μg/mL with 0.1 mol/L acetic acid. Polystyrene plastic tubes (Falcon 2017, Becton Dickinson Co, Oxnard, CA) were coated for 2 hours at 37°C with collagen, fibronectin solutions, or 1% plasma. Nonspecific adsorption sites were blocked by treatment of coated tubes with 10 mg/mL heat-denatured BSA in buffer A without divalent cations at 37°C for 30 minutes.

Adherence assay. Leukocyte adherence was assayed by estimating cells adhering to substrata in terms of cell protein concentration as described previously. Fifty microliters of platelet constituents and 325 μL of buffer A were placed in the assay tubes, and 125 μL of leukocyte suspension (1 × $10^7$ cells/mL) was added. The cells became attached to tubes by incubating for 30 minutes at 37°C, establishing monolayers. The tubes were then washed three times with 1 mL buffer A. The quantity of adherent cells remaining on
each tube was measured on a 0.5-mL sample by the method of Lowry et al., after the addition of 2 mL buffer A followed by sonication in ice for 1 minute, and expressed as the percent protein content of the total. The adherence assay without cells indicated that the proteins were not detected after three washings under our conditions. One unit of the activity of AIF was defined as the amount which in 1 mL caused 50% of the maximal adherence-inhibiting activity.

**Release of granular AIF.** The extracellular release of AIF from 1 U/mL thrombin-stimulated platelets was assayed as described previously. After incubation with thrombin at 37°C for 10 minutes, platelets were spun down and washed three times with ice-cold buffer, then centrifuged at 1,500g for 10 minutes. The resultant supernatant was ultracentrifuged at 200,000g for 1 hour, and assayed for adherence-inhibiting activity and for its molecular mass using a Superose 6 column (HR 10/30, Pharmacia LKB Biotechnology, Uppsala, Sweden).

**Subcellular fractionation of platelets.** Human platelets were suspended in sucrose buffer (0.25 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L PMSF, 10 μg/mL leupeptin, and 10 μg/mL pepstatin) at a concentration of 1 x 10^9 cells/mL and homogenized three times using the nitrogen decompression technique (Parr cell disruption bomb 4639, Parr Instrument Co, Moline, IL) with 1,250 psi at 0°C. The cell homogenate was centrifuged at 1,000g for 15 minutes and the resultant supernatant was centrifuged at 12,000g for 22 minutes. The resulting pellet was termed the granule fraction. The supernatant was further ultracentrifuged at 200,000g for 1 hour and the resulting supernatant and pellet were termed cytosol and membrane fractions, respectively. Activities of lactate dehydrogenase (a cytosol marker), N-acetyl-β-glucosaminidase (a lysosome marker), platelet factor 4 (an α-granule marker), and serotonin (a dense granule marker) of each fraction were assayed as described previously.

**Purification of granular AIF.** Platelet granule fraction in the sucrose buffer was sonicated on ice with intermittent pulses at 168 W for 150 seconds and then ultracentrifuged at 200,000g for 1 hour at 4°C. The supernatant was applied to a Superose 6 (HR 10/30) column equilibrated with 50 mmol/L potassium phosphate buffer (pH 7.5) containing 0.2 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L PMSF at a flow rate of 0.5 mL/min. Fraction (each 0.5 mL) was monitored for neutrophil adherence-inhibiting activity and protein (A 280 nm). The fractions containing adherence-inhibiting activity (AIF fraction) were collected and concentrated using an ultra filter unit USY-20 with a molecular mass cutoff of 200 Kd (Toyo Roshi Co, Tokyo, Japan). The concentrated AIF fraction was mixed with DTT at a final concentration of 0.5 mmol/L, and immediately applied to a Superose 6 (HR 10/30) column equilibrated with the same phosphate buffer at a flow rate of 0.5 mL/min. The inhibitory activity of AIF fraction from second Superose 6 chromatography was maintained at -80°C for at least 2 weeks. If granular AIF was prepared without DTT-treatment, the inhibitory activity of AIF fraction from second Superose 6 chromatography was lost within a few days. The pooled AIF fractions from second Superose 6 chromatography were dialyzed against 100 mmol/L Tris-HCl buffer (pH 7.5) containing 1 mmol/L EDTA and 1 mmol/L PMSF, and applied to a DE32 anion exchange column (7 x 0.6 cm, 2 mL; Whatman Biochemicals Ltd, Springfield Mill, England) previously equilibrated with the same Tris buffer. The column was washed with several column volumes of the equilibration buffer, followed by a linear gradient elution from 0 to 0.5 mol/L NaCl in 40 mL of the equilibration buffer at a flow rate of 7.2 mL/h at 4°C. Fractions (1.5 mL) were collected. The AIF fraction from DE32 chromatography was concentrated using USY-20, mixed with DTT at a final concentration of 50 μmol/L, and then immediately applied to a Superose 6 (HR 10/30) column equilibrated with 50 mmol/L potassium phosphate buffer (pH 7.5) containing 0.2 mol/L NaCl and 1 mmol/L EDTA at a flow rate of 0.5 mL/min. Finally, collected AIF fraction was rechromatographed on a Superose 6 (HR 10/30) column equilibrated with 50 mmol/L potassium phosphate buffer (pH 7.5) containing 0.2 mol/L NaCl at a flow rate of 0.5 mL/min. Purified granular AIF was stored at -80°C until used.

**Trypsin treatment and heat stability of granular AIF.** Purified granular AIF (1.5 μg/mL) was incubated with trypsin at a final concentration of 250 μg/mL at 37°C for 30 minutes in a total volume of 0.5 mL buffer A (pH 8.6). The reaction was stopped by addition of PMSF at a final concentration of 1 mmol/L and the resultant reaction mixture was assayed for adherence-inhibiting activity.

To assay the stability of the granular AIF activity, purified granular AIF (1.5 μg/mL) was incubated at 56°C for 30 minutes in buffer A. The reaction mixture was ultracentrifuged at 200,000g for 1 hour at 4°C to remove denatured materials, and assayed for adherence-inhibiting activity.

**Polyacrylamide gel electrophoresis.** Agarose-acrylamide composite slab gel (1.6% polyacrylamide, 0.5% agarose) electrophoresis and sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) with 2.5% to 10% linear gradient gel in 0.1% SDS were performed as described previously. Gels were silver-stained using a commercially available reagent kit (Daiichi Pure Chemicals Co, Tokyo, Japan).

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**Fig 1.** Superose 6 gel chromatography of subcellular fractions of human platelets. Cytosol or granule fractions of human platelets were obtained as described in Materials and Methods. These fractions were sonicated and ultracentrifuged, followed by Superose 6 gel chromatography. The adherence-inhibiting activity was measured by the inhibition of guinea-pig neutrophil adherence to glass at 25°C for 30 minutes. (A) Cytosol fraction; (B) granule fraction. AIF fractions are indicated by bars. Arrows indicate eluting positions of: 1. blue dextran (2,000 Kd); 2. thyroglobulin (669 Kd); 3. ferritin (440 Kd); 4. γ-globulin (160 Kd); 5. albumin (67 Kd); 6. cytochrome c (12.5 Kd).
Fig 2. Purification of granular AIF. The adherence-inhibiting activity was measured by the inhibition of guinea-pig neutrophil adherence to glass at 25°C for 30 minutes. (A) Second Superose 6 gel chromatography. After the first Superose 6 gel chromatography, AIF fraction indicated by a bar (Fig 1B) was concentrated and treated with 0.5 mmol/L DTT, followed by second Superose 6 gel chromatography. (B) DE32 cellulose column chromatography. Pooled AIF fractions of second Superose 6 chromatography were applied to the column and eluted with a gradient (0 to 0.5 mol/L) of NaCl. (C) Superose 6 gel rechromatography. AIF fraction indicated by a bar in (B) was concentrated and treated with 50 μmol/L DTT and immediately applied to a Superose 6 column (data not shown). Then, AIF fraction of third Superose 6 gel chromatography was subjected to rechromatography on a Superose 6 column. Arrows indicate eluting positions of: 1, blue dextran; 2, thyroglobulin; 3, ferritin; 4, γ-globulin; 5, albumin; 6, cytochrome c; 7, DTT (0.15 Kd).

RESULTS

Subcellular localization of AIF. In preliminary experiments, we observed that the soluble fraction of human platelets strongly inhibited the neutrophil adherence to glass. Thus, we examined subcellular localization of adherence-inhibiting activity in human platelets. In the cytosol fraction, the recoveries of lactate dehydrogenase, N-acetyl-β-glucosaminidase, platelet factor 4, and adherence-inhibiting activity were 89.6% ± 1.2%, 7.2% ± 0.4%, 3.6% ± 1.2%, and 52.2% ± 4.5% (means ± SE of three experiments), respectively. In the granule fraction, the recoveries of lactate dehydrogenase, N-acetyl-β-glucosaminidase, platelet factor 4, and adherence-inhibiting activities were 1.4% ± 0.2%, 73.2% ± 2.4%, 82.0% ± 4.3%, and 32.8% ± 3.9%, respectively. Lactate dehydrogenase, N-acetyl-β-glucosaminidase, platelet factor 4, and adherence-inhibiting activity in the membrane fraction were hardly detected, and the activities of these markers and AIF in the debris fraction were less than 10% of the total activity. These results indicate that AIFs are localized in the granule and cytosol fractions.

To determine whether the adherence-inhibiting activity of cytosol and granule fractions is the same or not, these fractions were analyzed by Superose 6 column chromatography (Fig 1). As for cytosol fraction, the adherence-inhibiting activities were eluted at two different positions with the

<table>
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<tr>
<th>Table 1. Summary of Granular AIF Purification</th>
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<tr>
<td>Protein (mg)</td>
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<td>---------------</td>
</tr>
<tr>
<td>1. Granule fraction</td>
</tr>
<tr>
<td>2. 1st Superose 6</td>
</tr>
<tr>
<td>3. 2nd Superose 6</td>
</tr>
<tr>
<td>4. DE32</td>
</tr>
<tr>
<td>5. 3rd Superose 6</td>
</tr>
<tr>
<td>6. Superose 6 rechromatography</td>
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One unit of the granular AIF activity is defined as the amount which in 1 mL causes 50% of the maximal adherence-inhibiting activity.
PLATELET-DERIVED ADHERENCE-INHIBITING FACTOR

Fig 4. Effect of granular AIF on neutrophil adherence to glass. Human (○) or guinea-pig (□) neutrophils (2.5 × 10⁶ cells/mL) were adhered to glass in the presence of different concentrations of purified granular AIF at 37°C for 30 minutes. The inhibition of the leukocyte adherence was expressed as percentage of the decreased adherent cells in the presence of granular AIF relative to total cells adhered in its absence. Each point shows the mean ± SE of five experiments.

molecular masses of about 2,600 and 480 Kd, respectively. In the case of the granule fraction, the adherence-inhibiting activity appeared as a single active peak with about 2,600 Kd.

Next, the release of AIF from stimulated human platelets was examined. Resting unstimulated platelets did not release N-acetyl-β-glucosaminidase, platelet factor 4, serotonin, AIF, and lactate dehydrogenase. In contrast, thrombin-stimulated platelets released extracellularly 46.9% ± 0.4% (mean ± SE of 6 experiments) of N-acetyl-β-glucosaminidase, 69.6% ± 2.1% of platelet factor 4, 74.0% ± 1.0% of serotonin, and 23.0% ± 1.2% of AIF, but lactate dehydrogenase was not detected, suggesting that the released adherence-inhibiting activity is originated from the granule fraction. When the supernatant from stimulated-platelet suspension was applied to a Superose 6 column, the adherence-inhibiting activity was eluted as a single peak with the molecular mass of about 2,600 Kd. These results support the granular origin of the released AIF.

Purification of granular AIF. Thrombin-stimulated platelets released granular AIF without releasing cytosolic components, indicating that granular AIF may be involved in the regulation of neutrophil adherence in vivo. Therefore, we attempted to purify granular AIF. In the first place, AIF fraction from first Superose 6 chromatography was treated with DTT, and immediately applied to a Superose 6 column (Fig 2A). Next, the AIF fraction from the second Superose 6 column was applied to a DE32 anion exchange chromatography column. The AIF activity was observed as a single active peak eluting with 0.1 mol/L NaCl (Fig 2B). The AIF fraction from DE32 chromatography was applied to a Superose 6 column. Finally, granular AIF was purified as a single peak with a molecular mass of about 2,600 Kd on Superose 6 rechromatography (Fig 2C).

A summary of the purification procedure from 8.2 mg of platelet granules is shown in Table 1. The overall increase in activity was 120-fold with a recovery of 28.5%.

Characterization of granular AIF. When purified granular AIF was subjected to agarose-acrylamide composite gel electrophoresis, a single protein band was observed (Fig 3A). On SDS-PAGE, 340-Kd protein band and the other protein bands which migrated slower than the 340-Kd band were observed (Fig 3B, NR). The molecular masses of large protein bands could not be determined because they migrated much slower than molecular mass standards. Under reducing condition, two protein bands of 340 and 190 Kd were observed. These results suggest that granular AIF is a complex of 190- and 340-Kd subunits, and 190-Kd subunits are joined by disulfide bonds.

Purified granular AIF inhibited human neutrophil adherence to glass in a concentration-dependent fashion (Fig 4). Half-maximal inhibition was observed with about 110 ng/mL granular AIF, and complete inhibition was observed with granular AIF in excess of 300 ng/mL. Granular AIF also inhibited the adherence of guinea-pig neutrophils. On the other hand, 200 ng/mL granular AIF affected neither human nor guinea-pig monocyte adherence to glass and plastic (Table 2).

The activity of purified granular AIF on neutrophil adherence to glass was completely lost by the heat treatment, the trypsin treatment, and the storage at −80°C for 1 month. Table 3 shows the effect of 200 ng/mL granular AIF on neutrophil adherence to several substrata. Granular AIF inhibited 69.4% of human neutrophil adherence to plastic, and 81.1% of the adherence to glass (Table 3). Granular AIF also inhibited 69.3% of human neutrophil adherence to type IV collagen-coated tubes. However, granular AIF hardly affected human neutrophil adherence to fibronectin- or plasma-coated (used as a source of vitronectin and thombo-

Table 2. Effect of Granular AIF on Monocyte Adherence

<table>
<thead>
<tr>
<th>Adherence (%)</th>
<th>Control</th>
<th>Granular AIF (200 ng/mL)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Human monocyte</td>
<td></td>
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<tr>
<td>Glass</td>
<td>51.5 ± 6.2</td>
<td>49.1 ± 5.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Plastic</td>
<td>31.2 ± 3.8</td>
<td>28.8 ± 4.2</td>
<td>7.7</td>
</tr>
<tr>
<td>Guinea-pig monocyte</td>
<td>50.8 ± 5.9</td>
<td>54.2 ± 3.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Glass</td>
<td>41.3 ± 3.3</td>
<td>38.3 ± 2.3</td>
<td>7.3</td>
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Data show the means ± differences of two independent experiments.
that granular AIF (2,600 Kd) is composed of 340- and fragment RGDS (0.5 mg/mL) inhibited 42.9% of human neutrophil adherence to fibronectin-coated tubes, but did not respondin as well as fibronectin. Tubes. Fibronectin active disulfide bonds. These data indicate that human granular subunits, and 190- and 165-Kd subunits are joined by AIF-I (2,800 Kd) is composed of 340-, 190-, and 165-Kd bonds. We have already reported that guinea-pig granular AIF lacks the 165-Kd subunit.

AIF is similar to guinea-pig AIF-I except that human expressed on both neutrophils and monocytes. The unstimulated adherence of CD11/CD18-deficient neutrophils to plastic, but did not affect the adherence to type IV collagen.

**DISCUSSION**

Human platelets have at least two kinds of AIF with molecular masses of 480 Kd and 2,600 Kd that are localized in the cytosol and granule fractions. We have shown here that granular AIF (2,600 Kd) is composed of 340- and 190-Kd subunits, and 190-Kd subunits are joined by disulfide bonds. We have already reported that guinea-pig granular AIF-I (2,800 Kd) is composed of 340-, 190-, and 165-Kd subunits, and 190- and 165-Kd subunits are joined by disulfide bonds. These data indicate that human granular AIF is similar to guinea-pig AIF-I except that human granular AIF lacks the 165-Kd subunit.

Recently, a family of three leukocyte adhesion molecules named LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18) was identified using monoclonal antibodies. These CD11/CD18 complexes are expressed on both neutrophils and monocytes. The unstimulated adherence of CD11/CD18-deficient neutrophils to plastic is the same as that of normal neutrophils, suggesting that CD11/CD18 complexes may not be involved in unstimulated neutrophil adherence to plastic. On the other hand, the unstimulated adherence of monocytes to plastic is reported to be primarily dependent on CD11/CD18 complexes. AIF completely inhibited unstimulated neutrophil adherence to plastic, but did not affect unstimulated monocyte adherence. These results suggest that AIF inhibits neutrophil adherence via the CD11/CD18-independent adherence mechanisms. It has been shown that neutrophils adhere to extracellular matrices such as fibronectin, collagen, and laminin via their receptors.

The physiologic role of AIF in vivo remains speculative. When a blood vessel wall is injured at an inflammatory site, platelets easily adhere to the basement membrane, which is mainly composed of collagen, and then are activated, followed by the extracellular release of granular components. Therefore, it is possible that released AIF may inhibit neutrophil adherence to exposed collagen at the injured vessel wall, resulting in interference with the migration of neutrophils across the matrix to the extravascular tissues, which would help to suppress the excessive inflammatory response caused by migrated neutrophils. However, the regulation of neutrophil adherence by platelets might be more complicated, because activated platelets themselves adhere to neutrophils via GMP-140, which is exposed to platelet-plasma membrane during degranulation. Further studies are likely to test these hypotheses and to identify the AIF receptors of neutrophils.

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K Iwabuchi and T Yamashita