Guinea pig neutrophils adhered to adherence-inhibiting factor (AIF)-coated plastic; the adherence was completely inhibited by the addition of AIF, but partly inhibited by type IV collagen. Binding of 125I-labeled AIF to neutrophils was inhibited by unlabeled AIF, but partly inhibited by type IV collagen. Scatchard analysis showed that neutrophils have two classes of binding sites for AIF, high-affinity binding sites (kd = 860 pmol/L) numbering 6,400 per cell. Type IV collagen increased the kd of low-affinity binding sites for AIF, but did not alter the kd of high-affinity binding sites. We have isolated and characterized the AIF-binding proteins. Using AIF affinity chromatography, the radioactive fraction containing six proteins of molecular mass 45, 63, 87, 90 to 105, 145, and 195 Kd was isolated from 125I surface-labeled neutrophil extracts. This radioac-
tive fraction was further separated into two fractions using type IV collagen affinity chromatography, ie, one fraction was adsorbed on the type IV collagen column and contained the 45-, 63-, and 87-Kd proteins, whereas another fraction was not adsorbed on the column and contained the 45-, 63-, 90- to 105-, 145-, and 195-Kd proteins. To isolate the type IV collagen-binding proteins, 125I surface-labeled neutrophil extracts were applied to a type IV colla-
gen-Sepharose column; the isolated radioactive fraction contained the 45-, 63-, and 87 Kd proteins and bound to an AIF-Sepharose column. Taken together, these results suggest that the AIF-binding proteins, which bind to type IV collagen, are the type IV collagen-binding proteins of neutrophils.

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**MATERIALS AND METHODS**

**Materials.** Glycogen, Trizma base (Tris), phenylmethylsulfonyl fluoride (PMSF), FMLP, bovine serum albumin (BSA; fraction V), and type IV collagen (from human placenta) were obtained from Sigma Chemical, St Louis, MO. Lactoperoxidase and glucose oxidase were from Boehringer Mannheim, Mannheim, Germany. Fibronectin active fragment Arg-Gly-Asp-Ser (RGDS-peptide) was from the Peptide Institute, Osaka, Japan. Other reagents were of analytical reagent grade. Fibronectin was purified from guinea pig platelet-free plasma by gelatin-Sepharose affinity chromatography as described previously.11

**Isolation of cells.** Hartley male guinea pigs weighing approximately 350 g were used as the source of platelets and neutrophils. Platelets were isolated from citrate-anticoagulated blood by differential centrifugation as described previously.10 Neutrophils were isolated from the peritoneal cavity 13 to 15 hours after intraperitoneal injection of 0.17% glycogen in saline as described previously.10 The purities of isolated cells were more than 96% for neutrophils and almost 100% for platelets. Neutrophils were suspended in buffer A (8.1 mmol/L Na2HPO4, 1.5 mmol/L KH2PO4, 2.7 mmol/L KCl, 1.0 mmol/L MgCl2, 1.0 mmol/L CaCl2, and 137 mmol/L NaCl, pH 7.4).

**Purification and 125I-labeling of AIF.** AIF-I was purified from guinea pig platelet granules as described previously.11 The purified AIF-I in 50 mmol/L sodium phosphate buffer (pH 7.5) and 0.2 mol/L NaCl was stored at -80°C until use. For 125I-labeling, AIF-I (10 μg/500 μL) was incubated with 1 Iodo-Bead (Pierce Chemical, Rockford, IL) and 500 μCi Na2125I (317 mCi/mL; Nordion International, Kanata, Ontario, Canada) at room temperature for 30 minutes. The 125I-labeled AIF was separated from free Na2125I by gel filtration through a NAP-10 column (Pharmacia, Uppsala, Sweden) equilibrated with buffer A containing 10 mg/mL BSA. The specific activity of the labeled AIF was 700 to 810 cpm/fmol AIF. The neutrophil adherence-inhibiting activity of AIF was not affected by the labeling. On polycrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-PAGE (SDS-PAGE), the mobility of labeled AIF was the same as that of unlabeled AIF. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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NEUTROPHIL-BINDING PROTEINS FOR AIF

Fig 1. Effects of the addition of AIF, type IV collagen, and fibronectin on neutrophil adherence to AIF-coated plastic. Polystyrene plastic tubes were coated with 2 μg AIF or 10 mg heat-inactivated BSA per tube. Neutrophils were incubated in AIF-coated plastic tubes with various concentrations of AIF (●), type IV collagen (■), and fibronectin (△) or were incubated in BSA-coated tubes with AIF (◇) at 37°C for 30 minutes. Each bar represents the mean ± SE of six experiments.

beled AIF described in the previous report, indicating that AIF was not degraded by the labeling procedure.

Fig 2. Effects of the addition of AIF, type IV collagen, fibronectin, and RGDS-peptide on 125I-AIF binding to neutrophils. Neutrophils (5 × 10^6 cells) were incubated with 125I-AIF (8,000 cpm/tube) in the absence or presence of various concentrations of unlabeled AIF (●), type IV collagen (■), fibronectin (△), and RGDS-peptide (△) for 30 minutes at 37°C. Specific binding was defined as the difference between the total binding and nonspecific binding that was obtained in the presence of 1 μmol/L (2.8 μg/mL) unlabeled AIF. Each bar represents the mean ± SE of five experiments.

Fig 3. Scatchard plot analysis of AIF binding to neutrophils. Binding experiments were performed in the absence (A) or presence (B) of 10 μg/mL type IV collagen. Specific binding of 125I-AIF to neutrophils was determined as described in the Materials and Methods. Data were analyzed in terms of a two-site model using a modified LIGAND program. Predicted by linear regression of the data and used for the calculation of Kd values and the numbers of binding sites; the curvilinear best fits for a two-site model. Each point is the average of two determinations in a single experiment; two additional experiments gave similar results.

for 2 hours at 37°C. After washing with buffer A, tubes were treated with 10 mg/mL heat-denatured BSA in buffer A without divalent cations at 37°C for 30 minutes to block nonspecific adsorption sites. After three washings with buffer A, coated tubes were used immediately for the cell adherence assay. Under our experimental conditions, AIF was found to bind to uncoated plastic tubes by more than 95%, but to BSA-coated tubes by less than 0.1% using 125I-AIF.

Binding assay. Under the standard condition, the binding reaction was performed by incubating 5 × 10^6 neutrophils and various concentrations of 125I-AIF in the presence or absence of unlabeled AIF in 100 μL buffer A containing 10 mg/mL BSA in a 1.5-mL polypropylene tube. After incubation for 30 minutes at 37°C, the mixture was layered over 750 μL 75% fetal calf serum in a 1.5-mL polypropylene tube and centrifuged at 6,000g for 2 minutes at 4°C. After aspirating the supernatant, the tube was cut 2 to 3 mm above the cell pellet, and cell-associated 125I-AIF was counted using a γ-counter (Pharmacia, model 1270 Rack Gamma II). Nonspecific binding was determined in the presence of more than 200-fold excess of unlabeled proteins. Control experiments without cells demonstrated that less than 0.2% of labeled AIF was detected in the layer corresponding to the cell pellet after centrifugation. In some

binding assays were performed by estimating the number of cells adhered to the substrata in terms of the cell protein concentration as described previously. In some experiments, neutrophil adherence assays were performed in the presence of 10^-4 mol/L FMLP. Polystyrene plastic tubes (Falcon 20 17; Becton Dickinson, Oxnard, CA) were coated with 2 pg/mL AIF or 4 pg/mL collagen for 2 hours at 37°C. After washing with buffer A, tubes were treated with 10 mg/mL heat-denatured BSA in buffer A without divalent cations at 37°C for 30 minutes to block nonspecific adsorption sites. After three washings with buffer A, coated tubes were used immediately for the cell adherence assay. Under our experimental conditions, AIF was found to bind to uncoated plastic tubes by more than 95%, but to BSA-coated tubes by less than 0.1% using 125I-AIF.

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experiments, the binding reaction was performed in the presence of 10\(^{-8}\) mol/L FMLP, type IV collagen, fibronectin, and RGDS-peptide. Binding of \(^{125}\)I-AIF to the cells reached equilibrium by 30 minutes, and the addition of unlabeled AIF reversibly dissociated bound \(^{125}\)I-AIF from cells (data not shown). Binding of labeled AIF at 4°C was essentially the same as that at 37°C (data not shown). The data of saturation binding experiments were analyzed using a modified LIGAND program.\(^{14,15}\)

Cell surface labeling and preparation of cell extract. Neutrophils were labeled with \(^{125}\)I by the lactoperoxidase/glucose oxidase method\(^{16}\) with a slight modification. Briefly, neutrophils were suspended in buffer A containing 20 mmol/L glucose and 0.1 mmol/L PMSF at 5 \(\times\) 10\(^5\) cells/mL and then iodinated by incubation with 1 mCi/mL Na\(^{125}\)I, 20 pg/mL lactoperoxidase, and 100 mU/mL glucose oxidase for 30 minutes. Cells were washed three times with buffer A containing 0.1 mmol/L PMSF, 5 mmol/L glucose, and 0.1 mmol/L NaI. Labeled cells were lysed with 3 mL lysis buffer (50 mmol/L sodium phosphate buffer, pH 7.5, containing 0.5% Nonidet P-40 (NP-40), 1 mmol/L Mg\(_2\)+, 0.07 mmol/L Ca\(_2\)+, 2 mmol/L PMSF, and 20 \(\mu\)g/mL chymostatin) for 30 minutes at 4°C because the major chymotrypsin-like protease activity reported to be present in whole-neutrophil lysates can be substantially inhibited by 1 mmol/L PMSF together with 20 \(\mu\)g/mL chymostatin.\(^{16}\) Insoluble materials were removed by centrifugation at 1,500 for 15 minutes at 4°C, and resultant supernatants were further centrifuged at 15,000 for 30 minutes to obtain detergent-solubilized materials. In some experiments, neutrophils were treated with 1 mmol/L diisopropylfluorophosphate (DFP) for 15 minutes on ice before the labeling procedure, based on the observation that treatment of neutrophils with DFP before solubilization markedly inhibits proteolysis.\(^{17}\) Independent of the DFP treatment, the same results were obtained on SDS-PAGE (data not shown).

Isolations of AIF- or type IV collagen-binding proteins. The AIF- or type IV collagen-Sepharose column was prepared by the coupling of AIF (10 \(\mu\)g/mL) or type IV collagen (2 mg/mL) to 1 mL CNBr-activated Sepharose 4B (Pharmacia) as described by the manufacturer, followed by treatment with 10 mg/mL BSA to decrease nonspecific binding.

AIF- or type IV collagen-binding proteins were isolated from neutrophils using the affinity chromatography procedure\(^{18,19}\) with modification. All purification procedures were performed at 4°C. To remove nonspecific binding proteins, detergent-solubilized materials were first applied to a Sepharose 4B column (1.5 \(\times\) 5.7 cm, 10 mL) equilibrated with buffer B (50 mmol/L sodium phosphate buffer, pH 7.5, containing 0.25% NP-40, 1 mmol/L Mg\(_2\)+, 0.07 mmol/L Ca\(_2\)+, 2 mmol/L PMSF, and 20 \(\mu\)g/mL chymostatin). The flow-through fraction of the Sepharose 4B column was pumped onto the AIF-Sepharose column (0.5 \(\times\) 1.3 cm, 1 mL) or the type IV collagen-Sepharose column (0.5 \(\times\) 6.4 cm, 5 mL) equilibrated with buffer B and then cycled over the column for 15 hours. Then the column was sequentially washed with approximately 10 column vol buffer B, the 2 mL of 10 mg/mL BSA in buffer B, and buffer B. Binding proteins were eluted from the affinity column with a linear 0 to 2 mol/L NaCl gradient in buffer B. In some experiments, binding proteins were eluted from the AIF- or type IV collagen-Sepharose column with 20 \(\mu\)g/mL AIF or 2 mg/mL type IV collagen, respectively.

To obtain the binding proteins that recognize both AIF and type IV collagen, binding proteins obtained from the AIF-Sepharose column were desalted using NAP-10 columns equilibrated with buffer B containing 10 mg/mL BSA, and then applied to the type IV colla-

### Table 1. Effects of Divalent Cations and FMLP Stimulation on \(^{125}\)I-AIF Binding to Neutrophils

<table>
<thead>
<tr>
<th>Condition (mmol/L)</th>
<th>High-Affinity Binding Sites</th>
<th>Low-Affinity Binding Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kd Value (pmol/L)</td>
<td>No. (sites/cell)</td>
</tr>
<tr>
<td>Ca(_2)+ Mg(_2)+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 1</td>
<td>5.0 ± 0.4</td>
<td>500 ± 46.0</td>
</tr>
<tr>
<td>0.001 1</td>
<td>33.7 ± 2.1</td>
<td>580 ± 31.0</td>
</tr>
<tr>
<td>0 1</td>
<td>51.7 ± 4.8</td>
<td>490 ± 15.8</td>
</tr>
<tr>
<td>1 0.001</td>
<td>21.1 ± 1.2</td>
<td>453 ± 26.0</td>
</tr>
<tr>
<td>1 0</td>
<td>35.0 ± 2.4</td>
<td>427 ± 33.3</td>
</tr>
<tr>
<td>0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 1</td>
<td>7.2 ± 3.5</td>
<td>727 ± 24.8</td>
</tr>
</tbody>
</table>

### Fig 4. Effects of divalent cation concentrations on neutrophil adherence to AIF-coated plastic. Neutrophils were incubated in AIF-coated tubes in the presence of various concentrations of calcium and magnesium at 37°C for 30 minutes. Each bar represents the mean ± SE of six experiments.
Fig 5. Isolation of AIF-binding proteins using AIF-Sepharose chromatography. (A) Elution profile of AIF-Sepharose chromatography. Surface-labeled neutrophils were extracted and subjected to an AIF-Sepharose column, and then bound proteins were eluted with a linear 0- to 2-mol/L NaCl gradient. Fractions (each 1.0 mL) were collected and monitored for radiologic activity. The arrow indicates the position of the washing with 10 mg/mL BSA. (B) SDS-PAGE analysis of proteins obtained from AIF-Sepharose chromatography. The eluted fraction at about 0.5 mol/L NaCl was treated with (R) or without (NR) 10 mmol/L dithiothreitol (DTT) at 100°C for 5 minutes and electrophoresed on 5% to 15% linear gradient SDS-PAGE followed by autoradiography. Positions of molecular mass standards are shown at the side of each panel.

RESULTS

Binding interactions of neutrophils to AIF. To clarify whether neutrophils have binding sites for AIF, we first examined neutrophil adherence to AIF-coated plastic. Neutrophils adhered to AIF-coated plastic by 46.7 ± 3.2% (mean ± SE of six experiments), but hardly adhered to BSA-coated plastic (3.2 ± 0.6%). As shown in Fig 1, AIF inhibited neutrophil adherence to AIF-coated plastic in a concentration-dependent fashion, with complete inhibition at 1 pg/mL. Next, we examined the binding of 125I-AIF to neutrophils. As shown in Fig 2, unlabeled AIF inhibited the binding of 125I-AIF to neutrophils. It has been demonstrated that AIF affects neutrophil adherence to type IV collagen-coated plastic, but not to fibronectin-coated plastic. Type IV collagen inhibited AIF-binding to neutrophils by 45.1 ± 2.8% (Fig 1) and 125I-AIF binding to neutrophils by 87.1 ± 2.3% (Fig 2).
Effects of divalent cations on interactions of AIF to neutrophils. To determine whether the binding of AIF to neutrophils is divalent cation–dependent, we studied the effect of various concentrations of calcium and magnesium on neutrophil adherence to AIF-coated plastic and on 125I-AIF binding. As shown in Fig 4, neutrophil adherence was diminished with decreasing calcium or magnesium concentrations. In the absence of divalent cations, neutrophil adherence to AIF-coated plastic was decreased to the same level as that of BSA-coated plastic. Scatchard analysis indicated that FMLP increased the numbers of high- and low-affinity binding sites without affecting the $K_a$ of these binding sites (Table 1). In fact, FMLP stimulation increased neutrophil adherence to AIF-coated plastic by 116.5% ± 3.2%. Therefore, AIF-binding sites of neutrophils are likely upregulated by FMLP stimulation.

Affinity chromatography. Next, we attempted to isolate AIF-binding proteins from 125I surface-labeled neutrophil extracts using affinity chromatography. A single radioactive peak was eluted with a linear 0 to 2 mol/L NaCl gradient (Fig 5A) or 10 µg/mL AIF (data not shown) from the AIF-Sepharose column. SDS-PAGE analysis of the eluate under the reducing condition showed the four major bands of molecular mass, 45, 63, 87, and 90 to 105 kDa, and the two minor bands of molecular mass, 145 and 195 kDa (Fig 5B). In an attempt to determine whether the affinity chromatography columns used here bind proteins nonspecifically, a BSA-Sepharose column was run under the same conditions. SDS-PAGE of the eluate with either 10 mg/mL BSA or a linear 0–2 mol/L NaCl gradient showed only minor smearing with autoradiography (data not shown), suggesting that the radioactive fraction isolated by the AIF-Sepharose column contained the specifically bound proteins.

Because Scatchard plot analysis showed that neutrophils had the AIF-binding sites that bound to both AIF and type IV collagen, we determined whether FMLP changes the affinity and numbers of AIF-binding sites on neutrophils. Scatchard analysis indicated that FMLP increased the numbers of high- and low-affinity binding sites without affecting the $K_a$ of these binding sites (Table 1). In fact, FMLP stimulation increased neutrophil adherence to AIF-coated plastic by 116.5% ± 3.2%. Therefore, AIF-binding sites of neutrophils are likely upregulated by FMLP stimulation.

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Because Scatchard plot analysis showed that neutrophils had the AIF-binding sites that bound to both AIF and type IV collagen (Fig 3), the eluate of the AIF-Sepharose column with the NaCl gradient was chromatographed on a type IV collagen–Sepharose column. The radioactivities were recovered in both the nonadsorbed and adsorbed fractions (Fig 6A), and each fraction showed smearing with different migration on PAGE (Fig 6B). On SDS-PAGE under the reducing condition, the adsorbed fraction was found to contain the 45-, 63-, and 87-Kd proteins and the nonadsorbed fraction the 45-, 63-, 90- to 105-, 145-, and 195-Kd proteins (Fig
NEUTROPHIL-BINDING PROTEINS FOR AIF

The 45- and 63-Kd proteins were common in both the nonadsorbed and adsorbed fractions.

Because neutrophils have type IV collagen receptors, we attempted to isolate the type IV collagen–binding proteins from neutrophils. When \(^{125}\)I surface-labeled neutrophil extracts were applied to a type IV collagen–Sepharose column, a single radioactive peak was eluted with a linear 0 to 2 mol/L NaCl gradient or 2 mg/mL type IV collagen (data not shown). The eluate was found to contain three protein bands of molecular mass 45, 63, and 87 Kd under the reducing condition on SDS-PAGE (Fig 7, lane 3). When the eluate from the type IV collagen–Sepharose column was chromatographed on an AIF-Sepharose column after desalting, the radioactivity was recovered, with a linear 0 to 2 mol/L NaCl gradient, as a single peak containing the 45-, 63-, and 87-Kd proteins on SDS-PAGE (Fig 7, lane 4). These results indicate that the fraction isolated using type IV collagen affinity chromatography binds to not only type IV collagen but also to AIF, suggesting a similarity between the type IV collagen–binding protein and the AIF-binding protein adsorbed on the type IV collagen–Sepharose column.

**DISCUSSION**

When a blood vessel wall is injured at inflammatory sites, neutrophils adhere to an extracellular matrix such as type IV collagen through the specific receptors. Type IV collagen is the predominant component of basement membranes and serves as a scaffold for the adherence and migration of leukocytes. Thus type IV collagen receptors are thought to play an important role in extravasation of neutrophils; however, little is known about the type IV collagen receptors of neutrophils. Recently, the 67-Kd type IV collagen–binding protein has been purified from human neutrophils using a type IV collagen–Sepharose chromatography procedure. We have already demonstrated that AIF inhibits neutrophil adherence to type IV collagen, suggesting that AIF directly and/or indirectly affects the interaction between neutrophil type IV collagen receptors and type IV collagen.

The present study demonstrated that neutrophils have two classes of AIF-binding sites on Scatchard analysis, as follows: high-affinity AIF-binding sites, which recognize only AIF, and low-affinity AIF-binding sites, which recognize both AIF and type IV collagen (Fig 3). The binding of AIF to high- and low-affinity binding sites is dependent on calcium and magnesium concentrations (Table 1). Using AIF affinity chromatography, a single radioactive peak was isolated that comprised six proteins of molecular mass 45, 63, 87, 90 to 105, 145, and 195 Kd on SDS-PAGE (Fig 7). The radioactive fraction eluted from the AIF-Sepharose column was further separated into two fractions, adsorbed and nonadsorbed, by type IV collagen affinity chromatography. The adsorbed fraction contained the 45-, 63-, and 87-Kd proteins on SDS-PAGE. Because the adsorbed fraction bound to both the AIF- and type IV collagen–Sepharose columns, this fraction seems to contain low-affinity AIF-binding molecules.

Using type IV collagen affinity chromatography, type IV collagen–binding proteins were isolated from guinea pig neutrophils. These binding proteins also bound to the AIF-Sepharose column and contained the 45-, 63-, and 87-Kd proteins, as observed in the AIF-binding proteins adsorbed on the type IV collagen–Sepharose resin (Fig 7). These findings suggest that type IV collagen–binding proteins are identical to low-affinity AIF-binding molecules.

The AIF-binding fraction, which was adsorbed on the AIF-Sepharose column but not on the type IV collagen–Sepharose column, was composed of the five proteins of molecular mass 45, 63, 90 to 105, 145, and 190 Kd. Based on its binding only to AIF, this fraction seems to contain high-affinity AIF-binding molecules. Among the five proteins, the 90- to 105-, 145-, and 190-Kd proteins were found only in this fraction, suggesting that these three proteins may be important for the specific binding of neutrophils to AIF.

Besides type IV collagen–binding proteins, many other molecules have been identified as adhesion receptors of neutrophils; most of these adhesion molecules are also expressed on monocytes and macrophages. Previously, we demonstrated that AIF did not affect human and guinea pig monocyte adherence. In preliminary experiments, AIF-binding proteins were not obtained from guinea pig peritoneal macrophages using the AIF-Sepharose affinity chromatography procedures described in this study. Therefore, the AIF-binding proteins may be expressed on neutrophils but not on monocytes and macrophages, and neutrophil extravasation may be regulated by AIF released from platelets through AIF-binding proteins. Further studies are required to elucidate the detailed functions of the AIF-binding proteins.

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Isolation and characterization of the neutrophil-binding proteins for platelet-derived adherence-inhibiting factor

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