In vivo, platelets associate with neutrophils at sites of hemorrhage or inflammation. In vitro, stimulated platelets bind to neutrophils in a Ca\(^{2+}\)-dependent manner. GMP-140, an integral membrane glycoprotein found in secretory granules of platelets and endothelium, is rapidly translocated to the cell surface after cellular activation. It shares sequence similarity with two leukocyte adhesion molecules, ELAM-1 and a lymphocyte homing receptor. We have recently shown that neutrophils bind to purified GMP-140 in a Ca\(^{2+}\)-dependent fashion, and that GMP-140 participates in adhesion of neutrophils to activated endothelium. In this study we demonstrate that GMP-140 also mediates adhesion of neutrophils to stimulated platelets. Fixed thrombin-activated human platelets, but not unstimulated platelets, formed rosettes around neutrophils in the presence of Ca\(^{2+}\). The binding of platelets to neutrophils was inhibited by a monoclonal antibody to GMP-140 and by purified GMP-140. By promoting close cell–cell contact, GMP-140 may recruit both platelets and neutrophils to sites of tissue injury as well as modulate the function of each cell type by the other.

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### MATERIALS AND METHODS

**Materials.** Hanks balanced salt solution (HBSS) without added divalent cations and HBSS containing 1.26 mmol/L Ca\(^{2+}\) and 0.81 mmol/L Mg\(^{2+}\) (HBSS/Ca) were obtained from Flow Laboratories (Bio-rad Laboratories, Richmond, CA). The Ca\(^{2+}\) concentration of HBSS was further depleted by passing buffer through a 2.5 × 30 cm column (Pharmacia LKB Biotechnology Inc, Piscataway, NJ) containing Chelex-100 resin (Bio-rad Laboratories, Richmond, CA). The Ca\(^{2+}\) concentration of Chelex-treated HBSS (HBSS/CLX) was 15.5 μmol/L as determined by atomic absorption spectrophotometry (Varian Techtron, Model 1200, Sugarland, TX).

**Antibodies.** All antibodies were used as purified immunoglobulin G (IgG) dissolved in phosphate buffered saline (PBS), pH 7.4. The monoclonal antibodies (MoAbs) S12, G1, and T10 are all IgG, antibodies.

**Proteins.** GMP-140, purified from human platelet lysates by immunoaffinity chromatography on S12-Sepharose\(^{18}\) and ion-exchange chromatography on a Mono-Q column (FPLC, Pharmacia LKB Biotechnology, Piscataway, NJ), was dissolved in 20 mmol/L Tris-HCl, pH 7.5 (TBS), containing 0.01% Lubrol PX at a concent...
Platelet isolation. Platelet-rich plasma was isolated from normal human blood at 300 x g for 15 minutes and the platelet pellet was resuspended in 5 mmol/L PIPES, 145 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl2, 0.5 mmol/L Na2HPO4, 5.5 mmol/L glucose, 0.1% human serum albumin (Miles Inc, Elkhart, IN), 10 mmol/L PGE2, pH 6.8. After centrifugation at 500 g for 15 minutes, the platelets were resuspended in 20 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, 0.05 mmol/L Na2HPO4, 1 mmol/L MgCl2, 0.1 mmol/L glucose, pH 7.5. The cell count was measured with a model ZBI counter from Coulter Electronics Inc, Hialeah, FL. Seven milliliters of the platelet suspension, adjusted to a final concentration of 1 x 108 cells/mL, was exposed to bovine thrombin (0.1 U/mL final concentration) or diluent for 10 minutes at 37°C. The platelets were then fixed with 1 mL of 8% paraformaldehyde for 60 minutes at room temperature. Fixation was stopped by the addition of 1 mL of a solution containing 250 mmol/L Tris and 500 mmol/L glycine. After 15 minutes at room temperature, the platelets were washed three times with HBSS/CLX.

Neutrophil isolation. Blood from the platelet donor was also collected into bovine lung heparin (Upjohn Co., Kalamazoo, MI) at a final concentration of 10 U/mL. Neutrophils were isolated using Mozo-poly Resolving Media (Flow Laboratories, Maclean, VA), which is composed of Ficoll-400 and Hypaque 85 in a ratio that yields a density of 1.114. This method rapidly resolves neutrophils from other blood components without the need for hypotonic lysis of red blood cells. Cells were washed and resuspended to a concentration of 0.9 to 1.4 x 106 cells/mL in either HBSS/CLX or HBSS/Ca. Neutrophil suspensions were greater than 98% pure and greater than 95% viable as assessed by trypan blue exclusion.

Rosette assay. The binding of platelets to neutrophils was examined using minor modifications of the method of Jungi et al.34 Thirty-microliter aliquots of fixed platelets (1.5 to 2.0 x 108 cells/mL in HBSS/CLX) were placed in sterile microtiter wells. Then 10 µL of either HBSS/CLX, HBSS/Ca, or MoAb was added and incubated for 15 minutes at room temperature. Then 30 µL of neutrophils, yielding a final concentration of 4.5 to 6.0 x 106 platelets/mL and 2.6 to 4.2 x 105 neutrophils/mL (platelet/neutrophil ratio of 18 to 27:1) was added to the platelet suspension. The final concentration of each MoAb was 10 µg/mL. In some experiments EDTA or EGTA (final concentration of 1 mmol/L) was added. In other experiments, buffers containing 1 mg/mL of bovine serum albumin were used. To determine whether GMP-140 could inhibit binding of platelets to neutrophils, 10 µL of either purified GMP-140, the control membrane proteins glycoprotein IIb-IIIa or thrombomodulin, or buffer containing an equivalent amount of nonionic detergent were preincubated with 30 µL of neutrophils for 30 minutes at room temperature before addition of 30 µL of platelets. The final concentration of purified protein in each case was 35 µg/mL and the final concentration of Ca2+ was 1.6 mmol/L. After 30 minutes, 17 µL of the contents of each platelet-neutrophil suspension were placed on a microscope slide over which a glass coverslip was positioned. The cell suspension was examined under water immersion with a Zeiss Photomicroscope 2 (Thornwood, NJ). One hundred neutrophils were scored for the presence (two or more platelets per neutrophil) or absence (zero or one platelet per neutrophil) of platelets. Thus, neutrophils bearing two or more platelets were defined as rosettes.

RESULTS

As previously noted,14 relatively few unstimulated platelets bound to neutrophils. Only 15% of neutrophils rosetted two or more unstimulated fixed platelets (Fig 1). In contrast, over 60% of neutrophils bound two or more platelets stimulated with thrombin. Furthermore, more stimulated platelets bound to each neutrophil than did unstimulated platelets. The adherence of stimulated platelets to neutrophils was dependent on added Ca2+, confirming previous results.14 In the absence of Ca2+, the number of neutrophils bearing stimulated platelets was reduced to the levels seen for unstimulated platelets. Addition of 1 mmol/L EGTA or EDTA did not further reduce the number of platelet-neutrophil rosettes, although the average number of platelets on each neutrophil was diminished (not shown).

To test the involvement of GMP-140 in platelet-neutrophil interactions, we added neutrophils in Ca2+-containing buffers to stimulated platelets in the presence of MoAbs to GMP-140 or control MoAbs. Two different MoAbs to GMP-140 were used: G1, which blocks adhesion of neutrophils to purified GMP-14030 or to activated endothelium,31 and S12, which does not inhibit adhesion of neutrophils in either of these systems. As shown in Fig 1, G1, but not S12, inhibited the interaction of neutrophils with stimulated platelets. T10,33,34 an MoAb to the platelet membrane glycoprotein IIb-IIIa complex that blocks platelet aggregation, did not prevent the association of stimulated platelets with neutrophils. Similar results were seen when higher concentrations of antibodies were used and when human serum albumin (1 mg/mL) was added to all buffers (not shown).

To further test the role of GMP-140 in mediating interaction of stimulated platelets with neutrophils, we preincubated neutrophils with purified fluid-phase GMP-140 or with

![Fig 1. Binding of platelets to neutrophils requires platelet stimulation and Ca2++](image)
the mean age of neutrophils binding to endothelium. In this study we demonstrate that it also
defined lymphocyte homing is homologous to a
catalytic lectin family of Ca2+-dependent lectins." While formal proof is not
responsible for at least part of the Ca2+-dependent interaction of
products may inhibit certain neutrophil functions such as
superoxide production. Transcellular metabolism of
intermediates from one cell type by the other has also
be known whether direct contact between platelets and neutrophils facilitates the modulating
dominant role of these soluble products. The ability to block binding of
activated platelets to neutrophils with antibodies to GMP-140 may allow this issue to be addressed.
Monocytes also bind to both platelets and endothelium, suggesting that GMP-140 could promote monocyte adhesion
to these cells. In preliminary studies, we found that human
monocytoid U937 cells bind to stimulated platelets in a Ca2+-dependent manner and that this adhesive interaction is
inhibited by MoAbs to GMP-140. While this paper was
under review, Larsen et al reported that GMP-140 mediates binding of stimulated platelets to both neutrophils and monocytes. The relative roles of GMP-140 and other adhesive proteins such as thrombospondin in platelet-leukocyte interactions remain to be determined. By promoting
rapid binding of leukocytes to both activated platelets and endothelium, GMP-140 may facilitate communication between the hemostatic and inflammatory responses to tissue injury.

ACKNOWLEDGMENT

We are grateful to Drs Kevin Moore, Greg Bliss, and Jian-Guo Geng for helpful discussions and review of the manuscript. We thank Drs Paul W. Kincaid for assistance in the use of his phase contrast microscope, Leslie Parise for purified platelet glycoprotein IIb-IIIa, and Naomi Esmon for purified thrombomodulin.

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