Brief Definitive Report

Characterization of GMP-140 (P-selectin) as a Circulating Plasma Protein

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Summary

GMP-140 is a 140-kD granule membrane protein, found in the α granules of platelets and the Weibel-Palade bodies of endothelial cells, that is surface expressed on cell activation and mediates neutrophil attachment. Cloning data for GMP-140 from an endothelial library predict a soluble form of the protein, the transcription message for which is also found in platelets. In this study, we report the detection by enzyme-linked immunosorbent assay of soluble GMP-140 in plasma centrifuged for 3 h at 100,000 *g* (to remove platelet microparticles) and confirm its identity by purification from plasma. Plasma concentrations were found to be 0.251 ± 0.043 μg/ml ($\bar{x}$ ± SD, n = 10) in normal male controls and 0.175 ± 0.063 μg/ml ($\bar{x}$ ± SD, n = 10) in normal female controls. The purified protein had an identical molecular mass (nonreduced) to platelet membrane GMP-140 (~3 kD lower, reduced) and was immunoblotted by polyclonal anti-GMP-140, and the anti-GMP-140 monoclonal antibodies AK4 and AK6. Analytical gel filtration studies indicated that the plasma GMP-140 eluted as a monomer whereas detergent-free, platelet membrane GMP-140 eluted as a tetramer consistent with plasma GMP-140 lacking a transmembrane domain. Purified plasma GMP-140 bound to the same neutrophil receptor as the membrane-bound form, and when immobilized on plastic, bound neutrophils equivalently to immobilized platelet membrane GMP-140. Since it has been shown that fluid-phase GMP-140 is antiinflammatory and downregulates CD18-dependent neutrophil adhesion and respiratory burst, its presence in plasma may be of major importance in preventing the inadvertent activation of neutrophils in the circulation.

GMP-140 (P-selectin) is a member of a family of cell adhesion receptors termed selectins, which mediate one or more aspects of leukocyte adhesion (1, 2). Selectins share a common structural theme: an NH_2-terminal calcium-dependent lectin domain, an epidermal growth factor motif, a variable number of repeats of a sequence found in complement regulatory proteins, a transmembrane domain, and a short cytoplasmic tail (3). For GMP-140, each of these domains is encoded by separate exons (4). Cloning data from an endothelial cell library predicts the existence of three separate forms of GMP-140, two of which differ in the number of complement regulatory protein repeats, while a third form lacks a transmembrane domain, and hence predicts a soluble form of GMP-140 (3). In support of this, human platelets have been found to contain approximately equal amounts of mRNA encoding for GMP-140 with and without the transmembrane domain (4).

Surface-expressed membrane GMP-140 mediates the binding of activated platelets to myeloid cells and the transient adhesion of neutrophils to thrombin-stimulated endothelial cells (2, 5). In contrast, we have found that GMP-140 can also serve an antiinflammatory function in that exposure of TNF-α-activated neutrophils to fluid-phase platelet membrane GMP-140 inhibits their CD18-dependent adhesion to resting endothelium (6) and superoxide production (7). In the present study, we demonstrate that soluble GMP-140 circulates in normal plasma in a soluble and potentially functional form and is secreted from activated platelets.

Materials and Methods

Monoclonal and Polyclonal Anti-GMP-140 Antibodies. The murine mAbs AK4 and AK6, affinity-purified rabbit polyclonal anti-GMP-140 antibody, and preparation of nonimmune rabbit IgG have been previously described (8, 9). LeoA1 is a murine mAb directed against the 65-kD PTA1 antigen (10).

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Secretion of GMP-140 from Thrombin-stimulated Platelets. Washed platelets were isolated from anticoagulated venous blood either in the presence or absence of PGE_1 (3 μM, final concentration). Platelets (10^9/ml) isolated in the absence of PGE_1 were subsequently activated by the addition of α thrombin (1 U/ml, final concentration). Resting (PGE_1) and activated (α thrombin) platelets were pelleted by centrifugation at 1,000 *g*. The platelet supernatants were recentrifuged at 1,500 *g* to remove residual platelets, and divided in two, with one sample being further centrifuged at 100,000 *g* for 3 h. Samples were analyzed under nonreducing conditions on a 5–20% gradient SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted for GMP-140 using affinity-purified rabbit anti–GMP-140 antibody (1 μg/ml) or rabbit nonimmune IgG (1 μg/ml) as control.

Detection and Quantitation of Plasma GMP-140 by ELISA. Venous blood was collected from normal laboratory volunteers who had been medication free for 10 d before study. Blood (nine parts) was collected using a 19-gauge Terumo-winged infusion set directly into 3.2% trisodium citrate (one part) as anticoagulant and processed immediately. Alternatively, in some experiments in order to mimic normal hospital collection procedures, venous blood collected with a 21-gauge needle was added to a 5-ml buffered sodium citrate vacutainer (Becton Dickinson & Co., Mountain View, CA) and allowed to sit 2 h before processing. Platelet-poor plasma was prepared by centrifugation at 1,500 *g* for 20 min, then microfuged at 10,000 *g* for 5 min, and finally ultracentrifuged at 100,000 *g* for 3 h. GMP-140-depleted plasma was prepared by batch absorption overnight of ultracentrifuged plasma with AK4-Affigel-10 followed by centrifugation at 1,500 *g* for 20 min. Surface-expressed GMP-140 on platelets was quantitated as previously described (8).

Plasma GMP-140 levels were quantitated using a sandwich ELISA that employed immobilized affinity-purified rabbit anti-GMP-140 antibody and detection with the murine anti-GMP-140 mAb AK6. Briefly, 200 μl of affinity-purified rabbit anti-GMP-140 antibody (0.4 μg/ml) in 0.1 M sodium bicarbonate buffer, pH 8.3, was added to each well of an EIA microtiter plate (Linbro/Titertek, McLean, VA) and incubated overnight at 4°C. After three washes with ELISA buffer (0.12 M NaCl, 0.02 M imidazole, 0.005 M citric acid, 0.1% [wt/vol] BSA, pH 7.3), each well was blocked with 5% (wt/vol) BSA in ELISA buffer for 1 h at room temperature. After washing three times, 20 μl of each plasma was added to each of three wells containing 180 μl of ELISA buffer. For each experiment, a standard curve was generated using purified platelet membrane GMP-140 (0–1.6 μg/ml, final concentrations). For the standard curve, 20 μl of serially diluted platelet membrane GMP-140 was added in triplicate to 160 μl of ELISA buffer and 20 μl of GMP-140-depleted plasma. After 1 h, the plates were washed four times, and 200 μl of AK6 IgG in ELISA buffer (2.5 μg/ml, final) was then added. After 1 h, the plate was rewashed five times and 200 μl of a 1 in 1,000 dilution of horseradish peroxidase–conjugated rabbit anti- mouse IgG was added for 1 h. The plate was washed five times and color developed using 200 μl/well of 0.42 M 3,3',5,5'-tetramethylbenzidine dihydrochloride in 0.1 M acetate buffer, pH 6, containing 1.3 mM hydrogen peroxide. The reaction was stopped after 10 min by the addition of 50 μl of 2 M sulphuric acid. Absorbance was then read on an automated reader (EL312; Biokinetics, Winooski, VT) at 450 vs. 490 nm.

Purification of Plasma GMP-140. Fresh platelet-poor plasma obtained on the day of collection from the Red Cross Blood Bank (Parramatta, Sydney) was centrifuged at 10,000 *g* for 1.5 h in order to remove residual platelets. Buffers used for the purification of plasma GMP-140 included 0.1% (wt/vol) Triton X-100, due to the marked propensity of plasma GMP-140 to irreversibly absorb to surfaces during its isolation. The plasma was loaded onto an AK6-Affigel-10 column that had been equilibrated with 0.01 M Tris, 0.15 M NaCl, 0.1% (wt/vol) Triton X-100, and 0.02% (wt/vol) sodium azide, pH 7.4 (buffer A). After exhaustive washing with buffer A and buffer A made 1 M in NaCl and 1 mM in EDTA, the column was eluted with 0.1 M glycine, 0.1% (wt/vol) Triton X-100, pH 2.4, and immediately neutralized by the addition of one-fifth volume of 1 M Tris, pH 8.0. GMP-140-rich fractions as evaluated by SDS-PAGE were pooled and dialyzed overnight against buffer A. BSA (10 mg/ml) was then added to help prevent nonspecific absorption of contaminating proteins in the next immunoaffinity step. The crude GMP-140 was then loaded onto sequential LeoA1-Affigel-10 and AK4-Affigel-10 columns. After thorough washing of the AK4 column, bound GMP-140 was eluted, neutralized, and then dialyzed against buffer A as described above. GMP-140-rich fractions were then loaded onto a column of heparin-Sepharose CL-6B. Purified GMP-140 was eluted using a 50-ml linear 0.15–1.0 M NaCl gradient in buffer A. Platelet membrane GMP-140 was purified from human platelets as previously described in detail (8). Triton-X-100 was removed from purified platelet membrane and plasma GMP-140 using Extractigel-D resin as previously described (9). Platelet membrane and plasma GMP-140 were ^{125}I-labeled as described (9).

Interaction of Plasma GMP-140 with Neutrophils. Neutrophil isolation, binding of ^{125}I-labeled GMP-140 to neutrophils, and adhesion of neutrophils to GMP-140-coated plastic were all performed as previously described in detail (6–8).

Analytical Methods. NH_2-terminal peptide sequence analysis, analytical gel filtration, SDS-PAGE, gel staining, and Western blot analysis were performed as previously described in detail (9, 11, 12).

Results

Secretion of GMP-140 from Platelets. Since cDNA and platelet mRNA analysis both predict a soluble form of GMP-140 with a deleted transmembrane domain (3, 4), we initially examined whether GMP-140 was secreted from platelets on activation. GMP-140 could be detected by Western blot analysis in the supernatant of thrombin-stimulated platelets but not control platelets (Fig. 1 *B*, lane *2* vs. Fig. 1 *A*, lane *2*), even after the platelet supernatant had been centrifuged at 100,000 *g* for 3 h (Fig. 1 *B*, lane *3*). The majority of the platelet GMP-140, however, remained platelet-associated after thrombin stimulation (Fig. 1 *B*, lane *1* vs. Fig. 1 *A*, lane *1*). Since platelet microparticles that contain GMP-140 would sediment at 100,000 *g* (13), this experiment suggests that GMP-140 can exist in soluble form.

Detection of GMP-140 in Plasma. A sensitive ELISA for the measurement of GMP-140 levels in ultracentrifuged plasma was developed. Addition of known amounts of purified platelet membrane GMP-140 to either GMP-140-depleted plasma or normal plasma indicated that the determined level of GMP-140 in plasma was strictly additive at 0–1.0 μg/ml. Plasma from 10 normal male and 10 normal female donors (age range, 20–40 yr) gave GMP-140 levels of 0.251 ± 0.043 and 0.175 ± 0.063 μg/ml ($\bar{x}$ ± SD), respectively ($p < 0.01$, unpaired student's *t* test). Western blot analysis of serially diluted plasma and known amounts of platelet membrane GMP-140 were also consistent with a plasma level of GMP-140 of ~0.2 μg/ml (data not shown). Under our standard conditions for venous

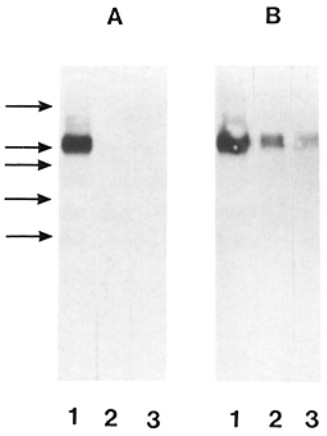

Figure 1. Secretion of GMP-140 from platelets. Control (*A*) and thrombin-activated platelets (*B*) were separated on a 5–20% exponential SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with affinity-purified rabbit anti-GMP-140 antibody. Lane *1*, platelet pellet; lane *2*, platelet supernatant; lane *3*, platelet supernatant centrifuged at 100,000 *g* for 3 h. Molecular weight markers in decreasing order are myosin (200,000), β-galactosidase (130,000), phosphorylase B (94,000), BSA (68,000), and OVA (43,000).

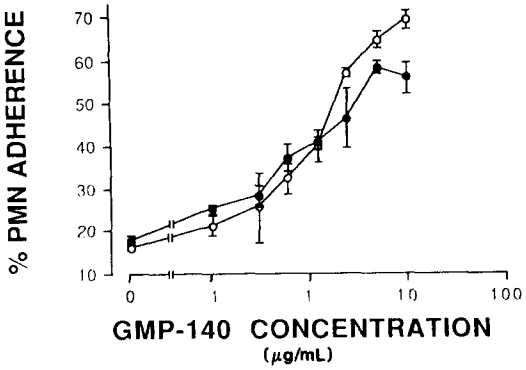

Figure 3. Neutrophil adherence to GMP-140-coated plastic. Neutrophil adherence to increasing concentrations of immobilized platelet membrane GMP-140 (○) and plasma GMP-140 (●).

blood sampling, the platelets in platelet-rich plasma had <100 copies of surface-expressed GMP-140 compared with ~10,000 copies on the surface of fully activated platelets (1), indicating that the plasma GMP-140 did not arise due to platelet activation during sample collection. In addition, the plasma GMP-140 level was insensitive to different collection procedures (Materials and Methods), indicating that the assay could be applied to routinely collected, hospital-citrated plasma samples. Finally, the plasma GMP-140 level in plasma centrifuged at 10,000 *g* for 5 min vs. plasma centrifuged at 100,000 *g* for 3 h was not significantly different, indicating that plasma GMP-140 could not be accounted for as microparticle contamination. In this regard, it can be calculated from the data of George et al. (13) that microparticle-associated GMP-140 in normal plasma would account for <0.05 ng/ml of plasma GMP-140.

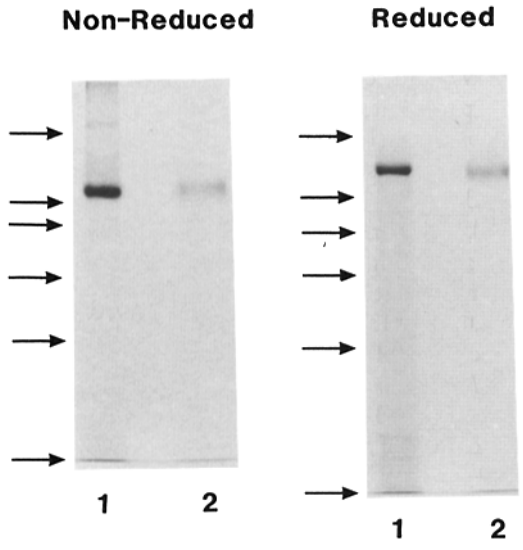

Figure 2. Analysis of purified GMP-140 by SDS-PAGE. Purified platelet membrane GMP-140 (10 μg, lane *1*) and plasma GMP-140 (5 μg, lane *2*) were analyzed on a 5–15% exponential SDS-polyacrylamide gel under nonreducing and reducing conditions and stained for protein with coomassie brilliant blue. Molecular weight markers in decreasing order are myosin (200,000), β-galactosidase (130,000), phosphorylase B (94,000), BSA (68,000) and OVA (43,000). Bottom arrow indicates the position of the dye front.

Purification of Plasma GMP-140. GMP-140 was purified to homogeneity from pooled normal human plasma (see Materials and Methods) with an overall yield of ~100 μg from 6 liters of starting plasma. Relative to platelet membrane GMP-140, the purified protein had similar binding characteristics to heparin, a similar molecular mass on SDS-PAGE under nonreducing and reducing conditions (Fig. 2), cross-reacted with monoclonal and polyclonal anti-GMP-140 antibodies (data not shown), and had an identical NH_2-terminal peptide sequence, WTYHYSTKAYSWXFSR (3). Plasma GMP-140, however, differed from platelet membrane GMP-140 in that its reduced molecular mass on SDS-polyacrylamide gels was ~3 kD lower (Fig. 2), and in that on analytical gel filtration under detergent-free conditions, it did not elute as tetramers (9) but remained monomeric. Both these results are consistent with the plasma form of GMP-140 lacking a transmembrane domain. Given that platelets contain mRNA encoding soluble GMP-140 (4) and that soluble GMP-140 is secreted from activated platelets (this study), it is probable that plasma GMP-140 represents the alternatively spliced, soluble form of GMP-140 lacking the transmembrane sequence encoded by exon 14 (4). It remains possible, however, that plasma GMP-140 is proteolytically derived from the membrane-bound form of GMP-140.

Plasma GMP-140 Is Functional. Since we have previously established that fluid-phase GMP-140 regulates various aspects of neutrophil function, it was important to establish whether plasma GMP-140 retained the capacity to interact with neutrophils. Plasma GMP-140 bound specifically to neutrophils. As for binding of platelet membrane GMP-140 to neutrophils (9, 14), this binding was divalent-cation dependent, as it was inhibited by excess EDTA. In addition, binding was completely inhibited by affinity-purified rabbit anti-GMP-140 Fab fragments, inhibited by ~50% by the anti-GMP-140 mAb AK4, but not by AK6 (9). Excess plasma GMP-140 completely inhibited the binding of radioiodinated platelet membrane GMP-140 to neutrophils (and vice versa) suggesting, as expected, that both the platelet membrane and plasma forms

of GMP-140 bind to the same receptor (data not shown). Finally, neutrophils bound equally well to platelet membrane and plasma GMP-140 immobilized on plastic (Fig. 3).

Discussion

Current evidence suggests that GMP-140 plays a fundamental role in both limiting and mediating the inflammatory responses of neutrophils (2, 6, 7). In the present study, we demonstrate that GMP-140 circulates in a soluble and potentially functional form in plasma and is secreted in soluble form from activated platelets. In normals, the plasma concentration of GMP-140 was found to be ~0.15–0.30 μg/ml. Based on the measured binding characteristics of fluid-phase platelet membrane GMP-140 to neutrophils (9), this would correspond to 20–40% saturation of neutrophil GMP-140 receptors, potentially allowing both an antiinflammatory action (6, 7) as well as the focal adhesion of neutrophils to GMP-140 expressed on activated endothelium (2).

GMP-140 has only been localized to the α granules of platelets (and megakaryocytes) and the Weibel-Palade bodies of endothelium (1), and is surface expressed on both cell types on cellular activation. Since mRNA encoding the soluble form exists in both endothelium (3) and platelets (4), plasma GMP-140 must derive from either one or both of these sources. One would therefore predict that the plasma level of GMP-140 could provide a useful marker of either platelet activation and/or vascular endothelial perturbation in thrombotic and inflammatory diseases. This topic is under current investigation.

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