Gray Platelet Syndrome: Immunoelectron Microscopic Localization of Fibrinogen and von Willebrand Factor in Platelets and Megakaryocytes

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An immunogold method was used for investigating the subcellular localization of von Willebrand factor (vWF) and fibrinogen (Fg) in platelets and cultured megakaryocytes from normal subjects and from three patients with the gray platelet syndrome (GPS), a rare congenital disorder characterized by the absence of α-granules.

In normal platelets at rest, vWF was detected exclusively in α-granules, with a characteristic distribution: gold particles were localized at one pole of each labeled granule, outlining the inner face of its membrane. vWF was distributed similarly in the α-granules of megakaryocytes at day 12 of culture, where it was also found in small vesicles near the Golgi complex. In contrast, Fg was observed in the whole matrix of all platelet α-granules but not in the nucleoids.

In platelets from three patients with GPS, vWF and Fg were distributed homogeneously in the rare normal α-granules, which could be recognized by their size, and also in small granules identified as abnormal α-granules, which were similar in size to the small, possibly immature granules present in normal megakaryocytes. In addition, in some unstimulated platelets, Fg labeling was associated with dense material in the lumen of the surface-connected canalicular system (SCCS). At day 12 of culture, megakaryocytes from the patients with GPS contained some small α-granules labeled for Fg and vWF identical to those found in mature platelets. The majority of α-granules of normal size appeared partially or completely empty. Thus, we conclude that vWF is distributed differently from Fg in normal α-granules, and that unstimulated platelets from patients with GPS contain Fg and vWF in a population of small granules identifiable as abnormal α-granules only by immunoelectron microscopy. In addition, the presence of Fg in the SCCS of gray platelets suggests a spontaneous release of the α-granule content.

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Fig 1. (A) Control platelet incubated with normal rabbit serum and antirabbit immunoglobulin coupled to colloidal gold: no labeling is observed on the α-granules (A). Original magnification ×57,000. (B) Normal platelet treated with an anti-vWF antibody, followed by immunogold: the gold particles label part of the periphery of the α-granules (A), forming a crescent shape along the inner face of the granule membrane. Dense bodies (db), small granules (g), mitochondria (m), SCCS, and plasma membrane (pm) are not stained. Original magnification ×58,500. (C) Normal platelet treated with an anti-Fg antibody and immunogold: gold particles are localized on the electrlucent matrix of all the α-granules (A). The dense nucleoids (n) are devoid of labeling, as are the SCCS, plasma membrane (pm), mitochondria (m), and small granules (g). Note the absence of background staining. Original magnification ×56,550.
in 0.1 mol/L phosphate buffer, pH 7.2, for two hours at 22 °C, washed three times in the same buffer, and embedded in glycol methacrylate (GMA) by the method of Leduc and Bernhard.23

Immunologic staining. The immunocytochemical reactions were performed on thin sections by the method of De Mey.24 Antihuman vWF, a polyclonal rabbit antibody (IgG fraction), was purchased from Dakopatts (Copenhagen) and used at 1:10 dilution. Antihuman Fg, a rabbit polyclonal antibody (IgG fraction), was purchased from Cappel (Cochranville, Pa) and used at 1:100 dilution. Goat antirabbit immunoglobulins fractions coupled to 5-nm colloidal gold particles (GARGS) were purchased from Janssen Pharmaceutica (Beerse, Belgium) and used at 1:10 dilution.

Controls. To demonstrate the specificity of the staining, we performed the following control studies: (1) Sections were incubated with nonimmune normal rabbit serum, followed by GARGS; (2) before they were applied to the sections, antisera were adsorbed with an excess of the corresponding antigens prepared as previously described25 for one hour at 37 °C and for 17 hours at 4 °C.

RESULTS

The fine structure of platelets fixed in glutaraldehyde and embedded in GMA was well preserved (Fig 1). Plasma and SCCS membranes, mitochondria, dense bodies, and α-granules (between 0.2 and 0.4 μm in diameter, with dense nucleoids) could easily be identified.

When nonimmune rabbit serum, followed by immunogold, was applied to platelet sections, α-granules showed no specific labeling (Fig 1A).

vWF in normal platelets. When platelet sections were processed with an anti-vWF antibody, followed by immunogold, vWF was detected in the α-granules in a specific distribution. Gold particles outlined the inner face of the α-granule membrane in an asymmetrical way (Fig 1B). Most of the labeled granules showed a crescent-shaped pattern of labeling, sparing the nucleoid. A few granules were uniformly labeled, but they may have been tangentially sectioned. Some α-granules were not labeled, but this finding can be explained by the focal distribution of vWF observed in most of the labeled granules. Plasma and SCCS membranes were not stained either in washed, anticoagulated platelets or in platelets from fixed whole blood. Similarly, mitochondria, small granules, and granules identified as dense bodies did not display any consistent labeling. A few gold particles were present outside the α-granules. Granules in other blood cells (neutrophils, monocytes, lymphocytes) were consistently negative.

Fg in normal platelets. Fg was detected in all α-granules of every platelet; numerous gold particles were observed over the entire matrix of the granules, except for the nucleoids, which were never labeled (Fig 1C). The labeled granules were heterogeneous in shape. Some were markedly elongated, spanning the whole width of the cell (Fig 2A); some were rod-shaped (Fig 2B); and others possessed a long tail (Fig 2C). Neither platelets from fixed whole blood nor washed platelets from anticoagulated blood displayed any...
Fig 3. Localization of vWF and Fg in platelets from patients with GPS. (A) Gray platelet showing vWF in some phantom α-granules of normal size (A₁) and in a population of small granules (A₂), which are especially numerous in this platelet. Note that gold particles are randomly distributed on the matrix of those abnormal small granules that do not display the focal localization of vWF detected in normal α-granules. The sccs, the plasma membrane (pm), and some small granules (g) of other types are not labeled. Original magnification ×47,400. (B) Part of a large highly vacuolated gray platelet processed for Fg localization; gold label is present inside α-granules that are of normal size but partially empty and lacking nucleoids (A₁) and in some very small, electron-dense α-granules (A₂) that can only be recognized as such by the presence of Fg. The SCCS, mitochondria (m), and some small granules (g) are not labeled. Original magnification ×47,400.
specific labeling of their plasma or SCCS membranes. Platelets in control experiments showed no labeling.

**GPS platelets.** The ultrastructure of the platelets from two of the patients with GPS has been previously reported. Platelets from the third patient presented the same morphological abnormalities, ie, they were often large and vacuolated and virtually lacked normal α-granules. The immunologic findings concerning vWF and Fg were identical in the three patients.

Immunologic localization of vWF in the platelets (Fig 3A) revealed the presence of two types of abnormal α-granules. The first type were normal in size but lacked nucleoids and were partially empty; the scant material remaining in their matrices was labeled for vWF. Surprisingly, the second type were very small granules (less than 0.1 μm in diameter), which were frequently elongated and were intensely labeled for vWF. Their size, much smaller than normal α-granules, made them resemble other platelet granules, lysosomes or microperoxisomes. These granules could be identified as abnormal α-granules only by immunoelectron microscopy. In contrast to the α-granules of normal platelets, which showed asymmetrical labeling that spared the nucleoid (Fig 1B), the abnormal granules of platelets from patients with GPS showed randomly scattered labeling throughout the matrix. We used platelets from fixed whole blood for the immunologic localization of Fg and thus avoided stimulating them in vitro. Labeling for Fg was identical to that for vWF (Fig 3B), localized in two types of abnormal α-granules, among which the small granules were the more numerous. There were zero to 12 granules, with a mean of 1.0 (CO), 1.5 (HB), and 3.0 (MS) per platelet section. Occasionally, the lumen of SCCS contained dense material that stained strongly for Fg (Fig 4).

**Normal cultured megakaryocytes.** In megakaryocytes at day 12 of culture, vWF was detected either in small vesicles near the Golgi complex (Fig 5A) or in mature α-granules (Fig 5B), with the same peripheral and asymmetric distribution in both sites. The vesicles may therefore correspond to immature α-granules. These findings support the idea that vWF is synthesized in megakaryocytes. Perinuclear cisternae, endoplasmic reticulum, and Golgi cisternae were not labeled. Surprisingly, under our technical conditions, we never detected Fg in cultured megakaryocytes, although we could find it in the α-granules of normal bone marrow megakaryocytes (not shown).

**Cultured megakaryocytes from GPS.** Megakaryocytes from two patients with GPS (HB and MS) looked identical in the 12th day of culture (Fig 5C and D). Their general morphological appearance has been previously reported, the most striking features being poor granulation and numerous vacuoles. Immunolabeling for vWF stained small granules (<0.1 μm in diameter) identical to those found in mature platelets; gold particles were distributed randomly in their matrix, in contrast to the focal distribution in immature granules from normal megakaryocytes. Some vacuoles the size of normal α-granules contained scanty material that was labeled for vWF; they were, therefore, identified as abnormal phantom α-granules. Normal α-granules like those in control megakaryocytes were not observed. In addition, numerous small vesicles in the vicinity of the Golgi complex were labeled for vWF (Fig 5C).

**DISCUSSION**

We have used an immunoelectron microscopic technique to compare the subcellular distribution of Fg and vWF in human platelets. Recent ultrastructural studies using immunoperoxidase, or using immunogold on frozen thin sections or preembedded material, have shown that Fg and vWF are present in platelet α-granules. Our technique...
Fig 5. Megakaryocytes on the 12th day of culture, processed for vWF localization as in Fig 1b. (A) Part of a megakaryocyte cultured from a normal subject. Near the Golgi complex (G) some dense vesicles (v) and small granules (g) are asymmetrically labeled for vWF. Original magnification ×53,700. (B) The same cell also displays mature α-granules showing the characteristic distribution of vWF (A). Original magnification ×56,100. (C) Cultured megakaryocyte from a patient with GPS. Numerous vesicles (arrowheads) near the Golgi complex (G) are labeled for vWF. A few gold particles are also present on the large, partially empty α-granules of normal size (A). Original magnification ×53,700. (D) Cultured megakaryocyte from a patient with GPS. vWF is detected in some small α-granules and in the scant material remaining in the phantom α-granules of normal size (A). Original magnification ×56,100.
provides better preservation of protein antigenicity and ultrastructural morphology because the use of hydrosoluble GMA as an embedding medium avoids the need for organic solvents and allows polymerization at 4 °C.31,32 This technique permits the identification of dense bodies and small, unreactive granules corresponding to lysosomal granules11 and microperoxisomes.19 This technique also permits combined ultrastructural cytochemistry and immunologic detection of proteins, as shown for neutrophils.22 Thus, we have been able to demonstrate that all the α-granules of every platelet contain Fg, which is distributed throughout the electrondense matrix of the granule but is absent from the nucleoid. Typical α-granules were identified by their size, their nucleoids, and the presence of Fg. The visualization of Fg has revealed the heterogeneous shape of this population of granules in unstimulated platelets. We were able to identify some structures as atypical α-granules, whereas by standard electron microscopy they might have been confused with dense bodies.23

Using the same technique, we have demonstrated that platelet α-granules contain vWF in a specific, nonrandom distribution. This pattern cannot be ascribed to redistribution of the antigenic sites during sectioning because we used fixed and plastic-embedded tissue. Also, because the technique we used stains only the antigenic sites at the surface of the section,24 there could have been no penetration problem. Moreover, we observed a random, rather than an asymmetric, distribution of vWF in the normal α-granules of platelets from patients with GPS (Fig 3A). Previous investigators did not observe the specific asymmetric localization of vWF in normal platelet α-granules.27,30; this discrepancy can be explained by the higher background staining of their immunocytochemical reaction, which hinders precise protein localization. In order to avoid background staining, we used the lowest concentration of antibodies still allowing good sensitivity, and we extensively washed the sections, especially between incubations with the primary and secondary antibodies. The distribution of vWF is thus clearly distinct from that of Fg. This finding may support the observation that Fg and vWF are not released identically after thrombin stimulation.15

Our observation of vWF in cultured megakaryocytes is in accordance with a previous ultrastructural demonstration of this protein in megakaryocytic cells.35 However, two original findings arise from our study. First, the presence of vWF in small vesicles arising from the Golgi complex strongly suggests that it is synthesized by the megakaryocyte. This observation is in accordance with a previous biochemical study36 showing that cultured megakaryocytes from guinea pig synthesize vWF. In addition, another study of the maturation of human megakaryocytes in vitro19 revealed a sequential pattern of immunofluorescent labeling for vWF: diffuse at five to seven days of culture, predominant in the nuclear concavity at six to eight days, and microgranular at eight to 12 days. These findings suggest that vWF is synthesized by the megakaryocyte in the endoplasmic reticulum and Golgi complex, and then packaged in α-granules. However, we were unable to detect vWF in the endoplasmic reticulum and Golgi cisternae by immunoelectron microscopy. An identical observation had been made using the same technique for the study of lactoferrin and myeloperoxidase in granulocytes,22 and we believe that we are dealing with the threshold of sensitivity of the technique. Our study also showed that vWF first appears in megakaryocyte granules in a peripheral and focal distribution, which remains similar during the maturation of the α-granules.

Because platelets from patients with GPS lack α-granules,14-18,38 they have been used as a model to assess the role of these organelles in platelet function.16-18,29 However, biochemical analyses have shown a severe decrease in, but not a complete lack of, α-granule proteins in gray platelets.17,18 The localization of the residual proteins inside small abnormal granules is consistent with this finding and rules out the possibility that the proteins detected biochemically represent an extragranular pool. Indeed, we found that the platelets of all three patients with GPS contained a population of small granules, less than 0.1 μm in diameter, which, by standard electron microscopy, resembled lysosomes and microperoxisomes but which could be identified as abnormal α-granules by their Fg and vWF content. These granules may correspond to the small granules arising from the Golgi complex observed in megakaryocytes and may be precursors of α-granules. We detected a dense material labeled for Fg within the dilated channels of the SCCS of unstimulated gray platelets; it looked very much like the dense material found inside the dilated demarcation membranes of bone marrow megakaryocytes from the same patients.19 We therefore conclude that it represents a release of α-granule content, occurring in the early maturation of α-granules. The presence of vWF inside small vesicles near the Golgi complex of GPS megakaryocytes, as in normal cells, supports the concept that α-granule content is normally synthesized by megakaryocytes GPS.

Finally, the finding of small, possibly immature α-granules in gray platelets suggests that the primary deficiency in GPS is not a general defect of synthesis of α-granule content; instead, the normally synthesized content seems to be lost during megakaryocyte maturation by a mechanism that remains to be elucidated.

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NOTE ADDED IN PROOF

Subsequent to the submission of this work, we have made progress in the determination of the localization of vWF within normal platelets. This has been the subject of a concise report published in the September 1985 issue of Blood (Cramer EM, Meyer D, Le Menn R, Breton-Gorius J: Eccentric localization of von Willebrand factor in tubular structures of normal platelet α-granules resembling those of Weibel-Palade bodies. Blood 66:710, 1985).
REFERENCES


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