Eccentric Localization of von Willebrand Factor in an Internal Structure of Platelet α-Granule Resembling That of Weibel-Palade Bodies

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Immunogold staining was used to study the ultrastructural distribution of von Willebrand factor (vWF) in unstimulated platelets. vWF was detected in the α-granules with a specific eccentric distribution pattern opposite the nucleoids. Similar findings were obtained with a polyclonal antibody or a pool of monoclonal antibodies to human vWF. This labeling coincided with the presence of tubular structures located at the periphery of the α-granules. These structures were better visualized on platelets treated for standard electron microscopy: they formed a group of one to four tubules ranging from 200 Å to 250 Å in diameter. They closely resembled the internal tubular structures found in Weibel-Palade bodies, which are the storage organelles of vWF in endothelial cells.

**PLATELET ADHERENCE** to the damaged vessel wall is one of the earliest events in hemostasis and thrombosis. von Willebrand factor (vWF) is a large glycoprotein of complex multimeric structure with an estimated range of molecular weight (mol wt) from 1 to 20 million\(^1\) that mediates attachment to the subendothelium. vWF is present in platelets and in endothelial cells, where it is stored in Weibel-Palade bodies; these are membrane-bound elongated organelles that contain regularly spaced tubular structures aligned parallel to their longitudinal axis.\(^4\) In unstimulated platelets, vWF has been demonstrated by subcellular fractionation,\(^3\) immunofluorescence,\(^6\) or immunoelectron microscopy\(^13\) to be stored in the α-granules with other proteins of hemostasis, such as fibrinogen or thrombospondin, and released during platelet aggregation at different rates.\(^15\) In the present study, an immunoelectron microscopic technique for the demonstration of antigenic sites on thin sections of fixed and embedded tissue\(^16\) has allowed us to study the distribution of vWF in unstimulated platelets. The high sensitivity and resolution of our technique led to the recognition of a specific eccentric localization of vWF in the α-granules not previously reported.\(^13\) This localization coincides with the presence of an internal tubular structure identifiable by standard electron microscopy and resembling that of Weibel-Palade bodies.

**MATERIALS AND METHODS**

**Cells.** Blood samples were taken from healthy volunteers by venipuncture on sodium EDTA. Platelet-rich plasma was centrifuged and platelets were fixed and processed for standard electron microscopy as previously described,\(^17\) stained en bloc in uranyl acetate, and embedded in Epon. Alternatively, they were fixed in 1% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2, for one hour at 22 °C, washed three times in the same buffer, and embedded in glycol methacrylate (GMA) according to Leduc and Bernhard.\(^18\) Thin sections were collected on nickel grids coated with formvar.

**Antibodies.** Two different sources of antibodies to vWF were used. A rabbit polyclonal antiserum (IgG fraction) to human vWF was purchased from Dakopatts (Copenhagen) and used at a 1:10 dilution. A pool of five mouse monoclonal antibodies to human vWF (B301, B302, 7, 9, 10) was also used, at a 1:2 dilution. The specificity of these antibodies was assessed as previously described.\(^13\) The marker for electron microscopy was either goat anti-rabbit IgG (GARG5) and goat anti-mouse IgG (GAMG5) or protein A (protAG5) coupled to 5 nm colloidal gold (Janssen Pharmaceutica, Beerse, Belgium).

Immunocytochemical labeling was performed according to De Mey\(^19\) as described previously.\(^14\) The controls were the following: (1) replacement of the primary antisera by normal rabbit or mouse serum and (2) absorption of the polyclonal anti-vWF antiserum with a fivefold excess of vWF prepared as previously described\(^18\) before application to the sections.

**RESULTS**

When a polyclonal antiserum to vWF followed by an antigloubin coupled to gold was applied to the platelet sections, vWF was detected exclusively in the α-granules opposite the nucleoids, according to a focal and asymmetric pattern of distribution (Figs 1 and 2). The nucleoids were always free of labeling. A similar distribution of vWF was observed when the polyclonal antisem was replaced by a pool of five monoclonal antibodies to human vWF (Fig 3), or when the antigloubin coupled to gold was replaced by protein A–gold complex (Fig 4); in both cases, the same asymmetric and peripheral localization of vWF was observed. However, when the pool of monoclonal antibodies was used, the labeling was weaker and less consistent; a few particles per α-granule were present in a low proportion of granules.

The most original finding of this study is that labeling for vWF was sometimes observed on regularly spaced small tubular structures, often grouped by three or four and located opposite the nucleoids (Figs 4 and 5). These structures were better visualized on platelets processed for standard electron microscopy and stained en bloc by uranyl acetate (Figs 6 and 7); they were located at the periphery of the α-granules in an electron-lucent zone and away from the nucleoids. They were found either isolated or grouped by two, three, or four. Their diameters ranged from 200 to 250 Å. Longitudinal sections of these tubules were eventually observed (Fig 6), and this corresponded to a special elongated pattern of labeling for vWF found in some α-granules.
Fig 1. Section of a platelet stained with a polyclonal anti-vWF antibody followed by GARG5. The gold particles are eccentrically located on the α-granules (A) and label part of their periphery. Dense bodies (db), small granules (g), mitochondria (m), surface connected canalicular system (SCCS), and plasma membranes (pm) are not stained (original magnification ×19,500; current magnification ×18,525).

Fig 2. Higher magnification of the same preparation as in Fig 1: the α-granules (A) display the characteristic focal distribution of gold particles. The nucleoid (n) are always free of labeling (original magnification ×82,500; current magnification ×82,500).

Fig 3. Part of a platelet treated as in Fig 1 except that a pool of five different monoclonal antibodies was used as a primary antibody. Gold particles are located at one side of the α-granule (A) away from the nucleoids (n); the labeling is consistently weaker than with a polyclonal antibody (original magnification ×82,500; current magnification ×83,325).

Fig 4. Part of a platelet treated as in Fig 1 except that protein A gold was used as the labeling agent. Gold particles are eccentrically located over the α-granules, and it is interesting to note that in one of them, this localization coincides with the presence of three light tubular structures (arrows) (original magnification ×82,500; current magnification ×78,787.5).

DISCUSSION

This study clearly indicates that vWF displays a specific non-random distribution pattern inside platelet α-granules. The presence of vWF inside α-granules had previously been shown by several techniques including immunoelectron microscopy. The different distribution of vWF inside platelet α-granules as compared to other platelet-specific

(Fig 5). However, since these tubules could only be seen when transversally sectioned, they were rarely identifiable (one group of tubules was seen in approximately ten platelet sections). But when observed, these structures coincided with the immunolocalization of vWF.

Several types of control experiments were performed as described above, and in all cases, α-granules did not display any staining. Neutrophil and monocyte granules were never stained.
proteins was first suspected by Wencel-Drake et al\(^2\); these authors noted that the fluorescence staining pattern for vWF was finer than for the other \(\alpha\)-granule proteins, although co-localized with them. However, this was not confirmed by their immunoelectron microscopy study performed on frozen thin sections\(^4\); this discrepancy may be due to redistribution of the antigenic sites during sectioning. Another immunoelectron microscopic study of vWF\(^5\) failed to show consistent labeling of platelet \(\alpha\)-granules; the authors show the presence of vWF inside megakaryocyte \(\alpha\)-granules, but the precise definition of the labeling pattern is hindered by a high background staining. In the present study, two types of primary antibodies (polyclonal and monoclonal) and two different labeling agents (protein A and goat anti-rabbit IgG coupled to gold) led to similar results (except that labeling was weaker with the monoclonals, although a pool of five monoclonal antibodies was used to increase the number of epitopes susceptible to be recognized). Redistribution of the antigenic sites during the sectioning cannot be implicated because this study was performed on fixed and plastic embedded tissue. Moreover, the immunolocalization of fibrinogen in normal platelets performed by the same technique has allowed us to show that fibrinogen was randomly distributed in the matrix of the \(\alpha\)-granules.

The specific distribution of vWF inside \(\alpha\)-granules may support the observation that fibrinogen and vWF are not released identically following thrombin stimulation\(^6\); this may influence the way vWF and fibrinogen react with their respective receptors glycoprotein Ib\(^23\) and glycoprotein IIb–IIIa\(^24\) or compete with their common receptor glycoprotein IIb–IIIa at the platelet surface.

The localization of vWF in the \(\alpha\)-granules coincides with a tubular structure described by White at the periphery of some platelet \(\alpha\)-granules\(^26\) and found by Behnke in megakaryocyte \(\alpha\)-granules.\(^27\) It is also known that in endothelial cells, vWF is located in the Weibel-Palade bodies,\(^3\) which have a specific recognizable internal structure made of an assembly of fine elongated tubules about 200 \(\text{Å}\) in diameter.\(^4\) These tubular elements closely resemble, by their size and their pattern of association, the tubules found in the platelet \(\alpha\)-granules. These tubular structures are rarely observed in the \(\alpha\)-granules but this might be explained first by the fact that only transversal sections can be accurately identified and then by their special sensitivity to fixation; indeed, they are poorly preserved using only glutaraldehyde fixation (Figs 4 and 5) and better visualized when osmium postfixation is used. A good counterstaining including en bloc uranyl acetate is also needed to allow their visualization in the dense matrix of the \(\alpha\)-granules. Their appearance is thus related to technical conditions, since White\(^6\) could observe them in most platelet profiles and found that their incidence rises after digitonin treatment.

Thus, we have shown that in the platelet \(\alpha\)-granules vWF is co-localized with internal tubular structures similar to those found in the Weibel-Palade bodies of endothelial cells; knowing the large size of vWF, these structures may represent the vWF molecule itself.
REFERENCES

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