Ultrastructural Demonstration of Tubular Inclusions Coinciding With von Willebrand Factor in Pig Megakaryocytes

By Elisabeth M. Cramer, Janine Breton-Gorius, Julian E. Beesley, and John F. Martin

The appearance of von Willebrand factor (vWF) in bone marrow megakaryocytes was studied by standard electron microscopy (EM) and immuno-EM using an original purification technique. Eighty percent pure megakaryocytes were isolated from porcine rib bone marrow using Percoll gradients followed by counterflow centrifugation. Gold particles were located eccentrically at one pole of the α-granule, labeling only its periphery or outlining one side of an elongated granule. Tubule profiles could be seen underlying the immunolabeling and were usually located at one side of the granule. In conclusion, this study demonstrates the presence of tubular structures in megakaryocyte α-granules, their association with vWF, and the appearance of both in the Golgi-associated vesicles.

© 1988 by Grune & Stratton, Inc.

The large glycoprotein von Willebrand factor (vWF) in porcine platelets was shown to be the source of plasma and subendothelial vWF, and in megakaryocytes before circulating in platelets. In endothelial cells, vWF is synthesized as a large precursor of mol wt 0.26 x 10^6 Kd. After forming dimers, it is then transferred to the Golgi apparatus where interdimer disulfide bond formation begins. It is stored in endothelial cell-specific organelles called the Weibel-Palade bodies. The process of biosynthesis of vWF in megakaryocytes has been shown recently to be very similar to that in endothelial cells. In addition, we demonstrated that in human and porcine platelet α-granules the immunolocalization of vWF coincides with tubular structures similar to those seen in the Weibel-Palade bodies. These structures are absent in severe von Willebrand disease (vWD) when vWF is also absent. Little is known of the localization of vWF in megakaryocytes during maturation. We used electron microscopy (EM) to study the appearance of the immunoreactivity of vWF and the production of vWF-associated tubules in maturing pig megakaryocytes. The porcine model was chosen as a consequence of our recent findings of prominent immunolabeling for vWF and numerous intragranular tubules in porcine platelets. This study was facilitated by use of a new technique for isolation from the bone marrow of a representative megakaryocyte population of high purity and yield.

MATERIALS AND METHODS

Separation of megakaryocytes. Ribs from a normal pig were obtained immediately after the animal was killed. The ribs were broken with bone forceps, and the marrow was flushed out using a solution of 3 mmol/L EDTA, 7.5 mmol/L glucose, 5 mmol/L potassium chloride, 1 mmol/L sodium sulfate in phosphate-buffered saline (PBS) containing prostacyclin (PGI2) (Wellcome Research Laboratories, Beckenham, Kent, England) 1 mg L^-1, pH 7.0, 290 mOsmol/kg H2O. The resulting marrow suspension was dispersed by passing it once through a 21-gauge needle using a polypropylene syringe. The marrow suspension was prefixed in the above buffer containing 0.01% glutaraldehyde and then layered onto 30% Percoll (vol/vol with 70% of the above buffered solution). This was then centrifuged at 190 g at 2°C for 20 minutes.

Megakaryocytes and WBCs and their precursors remained at the top of the gradient, whereas the RBC lines sedimented at the bottom of the gradient. The megakaryocyte-containing fraction was decanted and suspended in the initial buffer containing 0.1% glutaraldehyde. The suspension was then pumped through a Beckman J6B counter current flow centrifugal elutriator fitted with a Sander-son chamber (Beckman, High Wycombe, England). The flow rate was 15 mL/min with a rotor speed of 2,400 rpm. The elutriator was allowed to run until a pellet of cells was visualized in the stroboscopically illuminated chamber, ~5 minutes. The chamber was then removed, and its contents were washed out using the same buffer solution. The procedure time was ~40 minutes. The final recovery of cells, assessed with a Neubauer chamber, was 86% as compared with the whole marrow. Megakaryocyte purity was 80%. Megakaryocyte nuclear DNA content distribution was measured on a representative aliquot of cells before and after elutriation by fluorescent-activated cell sorter (FACS-420; Becton-Dickinson, Oxford, England) following staining with thymamycin and ethidium bromide.

Processing for EM. Cells were fixed for 1 hour in 3% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, washed three times in the phosphate buffer, postfix in 1% osmium tetroxide, stained en bloc with 2% aqueous uranyl acetate, and embedded in Epon. For immunocytochemistry, cells were prepared as previously described, and sections were incubated for 2 hours in the rabbit antiporcine vWF serum at a dilution of 1:20. To demonstrate the specificity of vWF labelling, control sections were incubated with preimmunized rabbit antiserum. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

From Service d'Hématologie et d'immunologie biologiques, CHU Bichat, Paris; Hôpital Henri Mondor, Creteil, France; and Wellcome Research Laboratories, Beckenham, Kent, England.


Supported in part by the Wellcome Trust.


The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1988 by Grune & Stratton, Inc.

0006-4971/88/7106-0010$3.00/0

Blood, Vol 71, No 6 (June), 1988: pp 1533-1538

© 1988 by Grune & Stratton, Inc.
the staining, control sections were incubated with nonimmune rabbit serum, followed by the antoglobulin coupled to colloidal gold.

RESULTS

There was no significant difference in megakaryocyte nuclear DNA distribution before and after the elutriation step.

Light microscopy of megakaryocyte populations. Smears of whole bone marrow before enrichment indicated the presence of megakaryocytes in all maturation stages. These constituted <0.5% of the total cell population. After the purification step, the cell sample contained an average of 80% megakaryocytes in different maturation stages (Fig 1). Most cells had well-preserved morphology.

Standard EM of megakaryocytes. The purified megakaryocytes were ultrastructurally well preserved, and the plasma membrane of most of the cells was intact. The most fragile cells appeared to be the large mature megakaryocytes, which were sometimes fractured, with leakage of their contents.

The early megakaryoblasts were identified, according to the criteria of Levine, by their small size relative to mature megakaryocytes, their low nuclear segmentation, high nucleocytoplasmic ratio, few demarcation membranes, numerous ribosomes and rough endoplasmic reticulum profiles, and scarcity or absence of mature α-granules. The Golgi zone of these cells displayed some spherical or elongated tubule profiles in the small immature granules and budding off the Golgi saccules (Fig 2).

EM examination of the megakaryocytes allowed good visualization of the tubular structures that had been recognized initially in the normal platelet α-granules. In early megakaryoblasts, in the cytoplasm of which no α-granules could be identified, small vesicles generally located in the vicinity of the Golgi complex could be observed (Fig 2). Within these vesicles, transverse sections of tubules were occasionally detected. In more mature megakaryocytes (Fig 3), each of the numerous large α-granules contained many tubules arranged in parallel and grouped eccentrically. In transverse section, the tubules had a constant diameter of 20 nm and were regularly arranged in parallel to one another. They were observed either transversely or longitudinally sectioned, disposed at one pole of the α-granule, lining one of its sides, or coiled along its periphery. Sometimes they filled the entire matrix. Groups of tubules with different orientations occasionally could be observed within one single granule, and groups of up to 30 tubules could be identified.

Immunogold localization of vWF in megakaryocytes. In early megakaryoblasts (Fig 4A) immunolabeling for vWF was observed in small vesicles and granules arising from the Golgi complex. In some of the immature granules, underlying the immunolabeling, small round, clear profiles, corresponding to the transverse section of tubular structures, could be seen even in this early stage of granule maturation. In more mature megakaryocytes (Fig 4B) vWF immunolabeling was prominent and restricted to the α-granules, which were labeled in the pattern previously described in porcine platelets. Immunolabeling was extensive, generally

Fig 1. Semithin section of the isolated population of megakaryocytes showing the large proportion of megakaryocytes (arrows), the variety of developing stages and the good morphological preservation of the cells. (H&E.)
being asymmetrically located at one pole of the granule or outlining one of its sides parallel to its long axis. Again, underlying the immunolabeling, some tubular structures could be identified within the granules (Fig 4C). No labeling was observed in any other organelle, specifically the demarcation membrane system, the mitochondria, and the plasma membrane. The control sections were consistently negative.

**DISCUSSION**

This study describes a new method for isolating 80% pure fixed megakaryocytes that would have a general application to immunocytochemical studies of other platelet proteins and to the development of α-granules. Indeed, the ultrastructural study of megakaryocytes of various stages of maturity requires preparation of adequate numbers of well-preserved cells. Because megakaryocytes are the largest cells in the bone marrow, they may be separated with relative ease from other cell lines by countercurrent flow centrifugation. This method separates cells primarily according to size, with cellular density playing a very small part (as defined by the Stokes flow equation). We have modified the technique described originally by Worthington and Nakell so that high yield and purity of megakaryocytes are combined with little damage to the cells. Their activation was prevented by use of prostacyclin in the buffer used to wash the marrow out of the rib and the introduction of a low concentration of aldehyde fixative in the Percoll gradient used for initial preparation of the bone marrow cells.

Combining these purification techniques with immuno-EM, we demonstrated the presence of vWF within the megakaryocytes α-granules with a specific eccentric distribution pattern. This completes several immunocytochemical studies and two previous ultrastructural reports that showed the association of vWF with megakaryocyte α-granules but were not able to demonstrate its specific distribution. This is the first study to use the high-resolution immunogold technique for localization of a megakaryocyte intracellular protein.

Another original observation in the present study is the association in megakaryocytes between vWF antigenic activity and some tubular structures similar to those seen in Weibel-Palade bodies, the vWF storage compartment specific to endothelial cells. Although endothelial cells store vWF, they are also able to secrete it, and this secretion is associated with the disappearance of Weibel-Palade bodies. Megakaryocytes have not been considered secretory cells; thus, the tubules they contain may be functionally related to storage of vWF. However, the nature of these tubules has not yet been elucidated.

The detection of vWF within the Golgi-associated vesicles of immature precursors is in accord with previous biochemical evidence that vWF is synthesized by megakaryocytes and then processed through the Golgi complex. Some immunofluorescent and immunohistochemical studies performed on cultured and fresh bone marrow megakaryocytes also showed that vWF has a granular distribution in the mature cells, whereas megakaryoblasts show a diffuse pat-
Fig 3. (a) Standard EM appearance of maturing megakaryocytes. Part of a partially mature megakaryocyte whose granules are still of intermediary size: numerous tubular structures occur within the granules (g), sometimes occupying the entire matrix. Tubules are also found within vesicles (v) scattered within the cytoplasm. (b) Part of a mature megakaryocyte: numerous, large α-granules each contain many tubules, arranged in parallel and grouped eccentrically. They are either transversely (arrowheads) or longitudinally (arrows) sectioned. (c) Groups of tubules with different orientations are occasionally found within a single granule (arrows). (d) Tubules can be coiled along the periphery of the granule (arrow), but more often are gathered at one pole of the α-granule (arrowhead). (e) The high number of tubules within α-granules are readily apparent in transverse section (here there are at least 20 within one granule). (f) Tubules generally occupy the lucent part of the matrix, gathered at one side of the granule (arrowhead) or orientated parallel to its long axis (arrow).
Fig 4. Immunogold localization of vWF in maturing megakaryocytes. (a) Golgi region of a megakaryoblast that already displays the tubular structures. The gold particles are associated with the tubules, which are eccentrically positioned within the new formed granule (arrow). Nearby vesicles (v) arising from the Golgi complex (G) are immunolabeled; m, mitochondria. (b) In the mature megakaryocyte, immunolabeling for vWF is prominent and located eccentrically either along the long axis of the α-granule (arrows) or at one pole (arrowhead). (c) Mature α-granule displays tubules at different orientations decorated with gold probes (arrows).

tern of labeling, most prominent in the region of the Golgi apparatus.

In this study, we showed that the first appearance of tubules associated with vWF occurs within the vesicles of the Golgi complex in immature megakaryocytes. This is consistent with previous ultrastructural autoradiographic studies indicating participation of the Golgi apparatus in the formation of α-granules. Similarly, in endothelial cells, the tubular structures of Weibel-Palade bodies have been shown by EM to arise from the Golgi complex. If we assume that vWF is the constitutive molecule of the tubules and know that dimerization of the molecule occurs in the pre-Golgi compartment of the cell, determination of which of the vWF protomeric or multimeric forms gives rise to these structures will be of interest. This could be ascertained by the study of platelets from patients with various subtypes of vWD in which differing multimeric forms of the vWF molecule are reduced or absent. Examination of the effect of monensin and ammonium chloride on the ultrastructure of cultured megakaryocytes would also be of interest, since these substances not only impair the intracellular polymerization of dimers but also the formation of the Weibel-Palade bodies in the endothelial cells. Finally, it appears from this study that the pig is an ideal model for the study of vWF in megakaryocytes.

ACKNOWLEDGMENT

We gratefully acknowledge Annie Higgs for careful review of the manuscript, Drs Ludovic Drouet and Dominique Meyer for providing the porcine anti-vWF serum, Dr G. F. Savidge for discussion of the manuscript, and Mark P. Betts for expert technical assistance.

REFERENCES

3. Wagner DD, Marder VJ: Biosynthesis of von Willebrand...
15. Stokes GG: On the effect of the internal friction of fluids on pendulums. Trans Camb Phil Soc 98, 1830
Ultrastructural demonstration of tubular inclusions coinciding with von Willebrand factor in pig megakaryocytes

EM Cramer, J Breton-Gorius, JE Beesley and JF Martin