Immuno-Electron Microscopical Demonstration of Lysosomes in Human Blood Platelets and Megakaryocytes Using Anti-Cathepsin D

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Immunocytochemistry with affinity-purified anti-human cathepsin D was applied to ultrathin frozen sections of human bone marrow megakaryocytes and of blood platelets from peripheral blood. The fixative used was paraformaldehyde (concentration gradient 2→8%). Protein A/colloidal gold (5 and 8) particles were used as second label. Cathepsin D was localized in primary and secondary lysosomes in blood platelets and in primary and secondary lysosomes in megakaryocytes. Primary lysosomes in mega-

HUMAN BLOOD PLATELETS contain several types of granules: α-granules, containing various proteins; dense granules, containing serotonin, adenosine triphosphate (ATP), adenosine diphosphate (ADP) calcium, and pyrophosphates; peroxisomes, and lysosomes. Lysosomes were initially thought to be similar to α-granules, but the secretion pattern for lysosomal enzymes is different; patients lacking α-granules have normal contents of lysosomal enzymes, and cell fractions containing α-granules, contain almost no lysosomal enzymes. Recently, electronmicroscopic histocytochemistry demonstrated the presence of acid phosphatase and aryl sulphatase in vesicles, which were smaller than α-granules. The precise morphology of the vesicles could not be described, because they were covered with reaction product. We confirm and extend these data with immunocytochemistry on cryosections using anti-cathepsin D. We have found that megakaryocytes contain primary as well as secondary lysosomes. The primary lysosomes are smaller than the α-granules and of equal density or somewhat lighter with often a characteristic submembrane halo. The secondary lysosomes are larger and show inclusions. Blood platelets contain primary lysosomes similar to those in megakaryocytes.

MATERIALS AND METHODS

Tissue Fixation: Blood Platelets

Fifteen milliliters of blood was collected from each of three normal individuals into 35 mL of 2% paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.4 at ambient temperature (20 °C) and centrifuged (ten minutes, 150 g, 20 °C) to obtain a platelet suspension. The platelets were pelleted (15 minutes, 1,500 g, 20 °C), and the paraformaldehyde concentration was gradually raised to 8% in one hour. After another hour of fixation, the platelet pellet was resuspended in 10% gelatin on 0.1 mol/L phosphate buffer, pH 7.4, equilibrated for five minutes, and pelleted again (five minutes, 10,000 g, 20 °C). Blocks of gelatin with tissue were stored in 8% paraformaldehyde for up to two weeks. In order to obtain thrombin-treated platelets, blood platelets were first washed by gelfiltration in calcium-free Tyrode. The platelet suspension was warmed for ten minutes at 37 °C. Human thrombin (Sigma, St Louis) was added to a final concentration of 1.0 U/mL. The suspension was incubated for three minutes at 37 °C and subsequently fixed as described above.

Megakaryocytes

Fragments from diagnostic sternum punctures in three patients were collected into 4 mL of 2% paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.4 at room temperature. The small tissue fragments were collected into a pellet by pipetting off the excess fixative. The further procedure was as described above for blood platelets and according to Geuze and Slot. Fixation in paraformaldehyde alone was selected, because the usual fixation mixture of 2% paraformaldehyde with 0.1% glutaraldehyde did not preserve the antigenicity of cathepsin D.

Immunocytochemistry

Electron microscopic immunocytochemistry was performed as described previously. Ultrathin cryosections were indirectly labeled with 5-nm or 8-nm colloidal gold particles conjugated to proteins. We used affinity purified rabbit anti-human placental cathepsin D. The antibody recognizes the precursor and native forms of the enzyme and has been extensively characterized. Following immunolabeling, the sections were stained with uranyl acetate and embedded in methylcellulose according to Tokuyasu. Affinity-purified rabbit anti-rat pancreas amylase was used as control.

RESULTS

Bone Marrow

A positive reaction for cathepsin D was found in megakaryocytes, macrophages, monocytes, granulocytes, endothelial cells, and fat cells. The strongest reaction was found in macrophages where cathepsin D was localized predominantly in primary and secondary lysosomes. This will be published elsewhere.

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Fig 1. Overview of Golgi region of a megakaryocyte. Primary lysosomes are indicated by single arrows. A secondary lysosome is indicated by a double arrow. α, Typical α-granules; N, nucleus. Areas (A) and (B) are shown at higher magnification in the insets. The bars in the overview and inset (A) correspond to 0.50 μm; in inset (B) to 0.25 μm.
In megakaryocytes, label was found in primary lysosomes, which were characterized by a smaller diameter than \( \alpha \)-granules with often a submembranous translucent halo and by their frequent association with the trans-side of the Golgi complex (Fig 1), where primary lysosomes develop.

Secondary lysosomes, characterized by their larger size and the heterogeneity of the contents, were also labeled (Fig 1, inset A). No label was observed in the Golgi complex or in the rough endoplasmic reticulum. Control sections incubated with anti-rat pancreas amylase showed no reaction at all.

**Blood Platelets**

A positive reaction was observed on granules of a homogenous density, which were somewhat lighter and in general smaller than \( \alpha \)-granules (Figs 2, 3). These granules were scarce. In general, label was found only on one single granule in every three platelet profiles. Label was also found in occasional dilated surface-connected tubules, presumably as a result of secretion. This was confirmed in experiments in which cathepsin D was localized in surface-connected tubules after release has been induced with thrombin (Fig 4). Control sections incubated with anti-rat pancreas amylase were completely negative.

**DISCUSSION**

The presence of lysosomes as secretory granules in human blood platelets is well documented. Secretion of acid hydrolases requires a stronger stimulus as well as constant occupancy of membrane receptor for thrombin in comparison to the secretion of the contents of \( \alpha \)-granules and dense bodies. Patients with the gray platelet syndrome lack \( \alpha \)-granules and have a normal lysosomal content. Likewise, in the combined \( \alpha \)-\( \delta \) storage disease, both the contents of the dense granules and \( \alpha \)-granules are diminished but the lysosomal enzymes remain unchanged.

Recent histochemical studies of acid phosphatase and aryl sulphatase localization in normal platelets and megakaryocytes and the platelets in the gray platelet syndrome demonstrated the presence of reaction product in vesicles in blood platelets and megakaryocytes and also of acid phosphatase in some cisternae of the Golgi complex. Our studies extend these observations.
We found cathepsin D to be localized in granules that were smaller than α-granules and had the same or slightly lower density. In the vicinity of the Golgi complex, still-smaller granules were found, some of which were associated with the trans-side of the Golgi complex. This localization is in agreement with our present-day concept of lysosome formation. No label was seen over the Golgi complex in contrast to the findings of Bentfeld-Barker and Bainton, who demonstrated acid phosphatase but not aryl sulphatase in the Golgi complex. These data are also in contrast to the findings in macrophages where some label was found over the Golgi stack. It may well be that the amount of cathepsin D in the Golgi cisternae is below the detection level for immunoelectronmicroscopy (H.J. Geuze, personal communication). This absence of label may be due to the low local concentration or increased sensitivity to fixation of cathepsin D in the Golgi complex.

Our immunocytochemistry studies with anti-cathepsin D also visualized granules in megakaryocytes, which were evidently secondary lysosomes. As no evidence of active phagocytosis has been found in blood platelets, the presence of secondary lysosomes was somewhat surprising. The most likely explanation is that secondary lysosomes are caused by autophagy. This may indicate the presence of a rapid breakdown of cellular material in megakaryocytes.

The presence of cathepsin D in surface-connected tubules is in accordance with the idea that secretion of lysosomes occurs via the surface-connected tubules, as has previously been reported for proteins stored in α-granules such as fibrinogen and platelet factor 4. Prolonged retention of acid hydrolases in the surface-connected tubules may be responsible for the relatively slow and partial release observed for acid hydrolases. A similar mechanism has been suggested for platelet factor 4.

The granules labeled with anti-cathepsin D are not necessarily representative for all lysosomal granules. Two types of acid hydrolase-containing granules have been recognized with the digitonin lysis technique and in patients with storage pool deficiency. This lysosomal heterogeneity may interfere with the identification of lysosomes. Cathepsin D may be present in one type of lysosomes and not in another. The appearance of the granules that are labeled with anti-cathepsin D in platelets is not sufficiently different from that of α-granules to exclude the possibility that cathepsin D is present in a subclass of α-granules. Theoretically, double-label experiments should offer direct evidence. We have previously used two different gold labels to demonstrate the contribution of albumin and β-thromboglobulin in α-granules. We have also used double-immunofluorescent labeling to demonstrate the codistribution of fibrinogen, β-thromboglobulin, and platelet factor 4. Experiments in which we tried double labeling at the electron microscopical level with two gold labels failed for unexplained reasons. The immunofluorescent studies had insufficient resolution in order to ascertain that the occasional granules stained with anti-cathepsin D were different from the many α-granules stained with anti-fibrinogen. The presence of cathepsin D in a subclass of α-granules or conversely the presence of α-granule proteins in lysosomes seems less likely, however, because of the different secretion patterns for α-granule proteins and lysosomal enzymes, and because of the normal presence of lysosomal enzymes in patients with the gray platelet syndrome. Moreover,
in megakaryocytes the granules stained with anti-cathepsin D differ morphologically from α-granules.

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REFERENCES

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