Identification of Primary Lysosomes in Human Megakaryocytes and Platelets

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The presence of lysosomal enzymes in human platelets is well documented; the identity of the "lysosome," however, has been the subject of some disagreement. In order to determine the time of appearance and subcellular localization of two lysosomal enzymes in megakaryocytes (MK) and platelets, we examined normal human bone marrow and blood by electron microscopy and cytochemistry. Acid phosphatase (AcPase) was present in the Golgi region in the youngest recognizable MK, as well as in those with a considerable degree of cytoplasmic maturation. Heavy reaction product was usually confined to one or two Golgi-associated cisternae and coated vesicles; other Golgi cisternae were sometimes lightly reactive. In mature MK, reaction product was limited to vesicles of variable size, but smaller than α-granules. Another lysosomal enzyme, arylsulfatase (AS), was localized in similar small vesicles in MK of all stages; it could not be demonstrated in the Golgi complex. Vesicles containing AS were also found in about 25% of platelet profiles, whereas vesicles containing AcPase were found in only about 15% of platelet profiles. The α-granules of all MK and platelets examined were negative for both enzymes. We conclude that the enzyme-containing vesicles in these cells constitute the lysosomes and that they are distinct from other platelet organelles. Since there was no evidence that they had participated in any digestive event, we believe that they are primary lysosomes, whose contents are secreted during platelet aggregation and the release reaction.

BLOOD PLATELETS of man and other mammals contain a variety of storage organelles. Dense bodies and α-granules are morphologically distinct, and many of their components have been identified. Although α-granules are often considered to be lysosomes, we concluded from a previous cytochemical study that rat megakaryocytes (MK) and platelets store lysosomal enzymes in vesicles distinct from both dense bodies and α-granules. Recent cell fractionation studies of human platelets also indicate that lysosomes and α-granules are separable. Furthermore, analyses of platelets from patients with storage pool disease and the "gray platelet syndrome" support this conclusion since these platelets have lysosomal enzymes but reduced or absent α-granule markers. In the present article, we present cytochemical evidence that human MK and platelets contain two lysosomal enzymes, acid phosphatase (AcPase) (with B-glycerophosphate and trimetaphosphate as substrates) and arylsulfatase (AS), in small vesicles similar to those we reported in the rat, and that these vesicles are distinct from α-granules from the time of their formation.

MATERIALS AND METHODS

Human bone marrow was collected from rib fragments of patients undergoing chest surgery. Cells were expressed from the marrow cavity directly into fixative by pressure on the bones. Blood was drawn from the antecubital vein of normal volunteers into either acid-citrate-dextrose (ACD) or a heparinized (50–100 U heparin/10 ml blood) syringe, or without the use of anticoagulants directly into fixative. When heparin or ACD was used, the blood was centrifuged for 10 min at 250 g on a table-top clinical centrifuge; platelet-rich plasma was then pipetted into 5–10 vol of fixative. The fixative was either 1.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4, with 1% sucrose, or a modification of Karnovsky's fixative containing 1.4% glutaraldehyde and 1.9% paraformaldehyde in 0.2 M Na-cacodylate buffer with 0.025% CaCl₂. When anticoagulants were omitted, blood was dripped into fixative; cells were fixed for 30 min at 37°C, allowed to cool at 22°C, and then fixed for 3 hr more at that room temperature. Prior to cytochemical incubations, fixation was carried out at 4°C for 30 min. After fixation, the cells were thoroughly washed (1 hr to overnight) in 0.1 M Na-cacodylate buffer, pH 7.4, containing 7% sucrose.

The incubation procedures for AcPase and AS are described in detail elsewhere. Cells were incubated for trimetaphosphatase (TMPase) according to the method of Oliver. Cells in control preparations were either incubated without substrate or the cells (while suspended in the postfixation wash buffer) were heated in a water bath at 90°C for 10 min before incubation. Cells were postfixed in OsO₄; stained with uranyl acetate in veronal acetate buffer, pH 6.0; dehydrated in ethanol; and embedded in Epon. Sections were cut with a diamond knife, stained briefly with lead citrate, and examined with a Siemens 101 electron microscope.

RESULTS

Megakaryocytes constitute 0.1%–0.5% of the nucleated cells of the bone marrow. The general features of MK maturation in human bone marrow resemble those described for other species. Maturation in this cell line involves increasing nuclear polyplody and the development of a unique set of cytoplasmic organelles; electron microscopy has shown that cytoplasmic maturation begins during the ploidyization phase, but continues after nuclear development is complete. Since the two processes are neither synchronous nor sequential, classification of the maturational stages of MK is difficult. The nomenclature used in the

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Fig. 1. Golgi region of an immature human MK tested for AcPase. Several stacks of Golgi cisternae (Gc) are evident. In most stacks, heavy reaction product is confined to one cisterna (arrows) and nearby coated vesicles (cv), while the remaining cisternae show little or no reaction. No typical α-granules can be identified in this field; many of the mitochondria (m) have been disrupted by cold fixation or the acid incubation medium. Tissue fixed in 1.5% glutaraldehyde for 30 min at 4°C and incubated in the Barks-Anderson medium for 3 hr at 30°C (n, nucleus; nu, nucleolus; ×22,000). Lower Inset: Higher magnification of part of same cell. Reactive and unreactive coated vesicles can be identified (×36,000). Upper Inset: Light micrograph of 1 μm Epon section of an immature MK similar to the cell shown in the electron micrograph. It has scant cytoplasm and a bilobed nucleus with several large nucleoli (×2000).
present study is based on the system proposed by Paulus and used in our previous paper on rat MK. Briefly: (1) "immature MK" are small (~15–18 µm in diameter) cells that have a nucleus:cytoplasm ratio greater than 1 and few cytoplasmic organelles other than large numbers of polyribosomes; (2) "maturing MK" are heterogeneous in size, have a lobulated nucleus, a well developed Golgi complex, abundant rough endoplasmic reticulum (RER), and variable numbers of α-granules and demarcation membranes; (3) "mature MK" are also heterogeneous in size, the nuclear lobes are closely apposed, and the cytoplasm is fully granulated and divided into platelet fields.

Immature cells constitute a minority of the total MK population, and are very difficult to identify by morphological criteria, especially in thin sections. The youngest cell we identified by electron microscopy (not shown) had a bilobed nucleus similar to that of the cell shown in the upper inset of Fig. 1. It measured 18 µm in diameter and showed AcPase reaction in the Golgi region. A slightly larger cell, containing more nuclear lobes, but also showing AcPase activity limited to the Golgi region, is depicted in Fig. 1. Forming lysosomes can be identified as small AcPase-positive vesicles (~80–120 nm) that are usually coated (Fig. 1, lower inset). Reaction product is also evident in each Golgi stack, chiefly in one or two cisternae, probably equivalent to GERL (Golgi, endoplasmic reticulum, lysosome), although a small amount may be present in some of the other cisternae. The RER, including the perinuclear cisterna, is unreactive. Maturing MK (Figs. 2 and 3) have a large well developed Golgi region with accumulating α-granules, vesicles, demarcation membranes, and well developed RER. As can be seen in Fig. 3A and B, they showed a similar distribution of AcPase. In favorable sections, reaction product was found in vesicles and a few Golgi cisternae, but the RER and α-granules were never reactive. In more mature cells, the AcPase-positive vesicles were more variable in size (175–250 nm), suggesting that smaller vesicles may fuse to form larger vesicles. All appeared homogeneous showing no cellular debris within their boundaries. As we had found with rat MK, AcPase was difficult to demonstrate in mature cells by the lead-salt methods used for electron microscopy. Nevertheless, a few small reactive vesicles could usually be seen in thin sections of such cells. At this stage of maturation, Golgi complexes have generally decreased considerably in size and are difficult to sample. Control preparations incubated without substrate or heated showed no reaction.

In MK of all maturational stages, from the youngest recognizable cells to the most mature, AS was always present, confined to small vesicles. Although reactive vesicles were often present in the Golgi region, we never saw reaction product in any component of the secretory apparatus. We presume that this may reflect limited sampling, since we previously observed AS in the Golgi region of MK from thrombocytopenic rats, which have a higher proportion of young MK. Part of the highly demarcated cytoplasm of a typical mature MK is shown in Fig. 4A. Figure 4B illustrates a cell at the same stage reacted for AS. Reaction product is localized in vesicles of variable size throughout the cytoplasm. At higher magnification (inset, Fig. 4B), it can be seen that the reactive structures are distinct from the α-granules, which are unreactive and usually larger. Dense bodies (illustrated in Fig. 5, inset) are normally not seen in routine electron microscopy of MK; therefore, the densities seen in MK after incubation for AS (Fig. 4B) are very probably reaction product for lysosomal enzyme. The reaction product could not be completely abolished in controls incubated without substrate, suggesting the presence of some unidentified endogeneous substrate. However, cells heated in a water bath for 10 min at 90°C prior to incubation in complete medium were totally devoid of reaction product, indicating enzyme inactivation; morphological preservation under these conditions was excellent.

Vesicles reactive for both enzymes were also demonstrable in circulating platelets. Figure 5 shows a platelet from blood dripped directly into fixative without anticoagulant. The disc shape of the platelet is well preserved, as are numerous α-granules and one dense body.* Figures 6, 7, and 8 show platelets incubated for AS, AcPase, and TMPase, respectively. All three enzymes were present in vesicles somewhat smaller than the α-granules. We did not test MK for TMPase. Cytochemical identification of lysosomes in platelets poses problems because the density of reaction products for lysosomal enzymes and the platelet dense bodies are similar (see Fig. 5). When present, the characteristic light halo is helpful in identifying the dense body (Fig. 5, inset). In preparations incubated for AS, we noted densities that we consider due to reaction product in about 25% of platelet profiles. In preparations processed with our standard fixation and embedding techniques, but not incubated for enzymatic activity, we saw the densities that we assume represent serotonin-containing dense bodies in only about 7% of platelet profiles. Thus, in preparations incubated for AS, even if all the dense bodies were

*In our earlier paper we identified these organelles as serotonin granules; although this amine may contribute to their electron density, other components appear to form a major portion of their contents, and therefore, we have dropped this designation.
Fig. 2. Golgi region of a maturing MK. Surrounding a centriole (ce) in the perinuclear area are numerous stacks of Golgi cisternae (Gc) and abundant vesicles similar to, but more extensive than those seen early in maturation. The cytoplasm is replete with α-granules (α), demarcation membranes (dms) and rough endoplasmic reticulum (rer) (×16,000). Inset (×38,000): Higher magnification of a few Golgi cisternae with numerous adjacent vesicles (ve), some of which are coated (cv). Occasionally, a Golgi cisterna is swollen and contains flocculent material (arrow). Fixed in glutaraldehyde at 4°C, postfixed in OsO₄, and stained en bloc with uranyl acetate (n, nucleus).
ical tests to define GERL in MK more precisely, we here refer to the reactive sites as Golgi-associated cisternae.

Fig. 3. (A) Golgi complex of a maturing MK. AcPase reaction product is confined to two cisternae (arrows) and coated vesicles (cv), which appear to bud from a reactive cisterna. Alpha granules (α) are unreactive. Tissue prepared as in Fig. 1 (×35,000). Inset: Light micrograph of a cell at a similar maturation stage. Cell size has increased, but the nucleus:cytoplasm ratio has decreased as compared with the immature cell in Fig. 1 (×1500). (B) Golgi complex of another maturing MK, but at a more advanced stage of maturation than Fig. 3A. Distribution of reaction product (arrows) is similar to that shown in Fig. 3A. Note negative α-granules (α). Tissue prepared as in Fig. 1 (×33,000). Inset: Light micrograph of a similar cell, which exhibits a lobulated nucleus and a greatly decreased nucleus:cytoplasm ratio (×1500).

misclassified as lysosomes (which is unlikely), they would account for less than 30% of the total lysosomes. When platelets were incubated for AcPase, dense deposits similar to those seen after incubation for AS were found in only about 15% of platelet profiles. Since more limited experiments were performed with TMPase, we have not attempted to quantitate our results. With our methods, it is impossible to determine whether the sites of these different enzyme activities are the same. The smaller percentage of AcPase-reactive platelets may reflect a narrower distribution of this enzyme as compared with AS or it may be due to technical problems, such as partial cytochemical latency.

DISCUSSION

These studies provide further evidence that human platelets and MK contain a distinct class of organelles that can be designated primary lysosomes. AcPase was shown to be present in the secretory apparatus of immature and maturing MK, as well as in vesicles of mature MK and some platelets. As we had found in rat MK, heaviest reaction product was confined to one or two cisternae and coated vesicles in the Golgi complex.† Although AS was never found in any Golgi cisterna, it was always present in vesicles of variable size in both MK and platelets. Unfortunately, we could not determine whether the same organelles are reactive for both enzymes or whether there are two populations of reactive organelles. Nevertheless, it is clear that α-granules were not reactive for either enzyme. Previous cytochemical studies23–26 have reported AcPase to be present in what appeared to be platelet α-granules; however, this demonstration usually required platelet activation or injury that may have altered enzyme distribution. Furthermore, some published micrographs of platelets25 clearly show a distribution of the enzyme outside the α-granules as well, and none of these investigations was extended to MK, where identification of reaction product is less equivocal than in platelets (see Fig. 4).

Recent biochemical studies of fractionated platelets also indicate that platelet lysosomes are distinct from platelet α-granules. Although some of the early cell fractionation studies of platelets27,28 demonstrated the presence of lysosomal enzymes in the “granule fraction,” new separation procedures25 have made it possible to obtain α-granule fractions of much greater purity. Such fractions show little lysosomal activity, but rather are rich in β-thromboglobulin, platelet factor 4, platelet growth factor, and fibrinogen.10,29 The platelet lysosomal enzymes that have been studied

†In our previous paper13 we designated the reactive cisternae as GERL. However, since other Golgi cisternae of MK in both species also appeared reactive in some cases, a clear-cut distinction was not always possible. Inasmuch as similar uncertainties have now been reported in other cell types,22 and we have done no further cytochemical tests to define GERL in MK more precisely, we here refer to the reactive sites as Golgi-associated cisternae.
Fig. 4. (A) Mature human MK. Note the numerous α-granules (α) and demarcation system (dms) (x 17,000). Inset: Light micrograph of a mature MK showing morphology typical of a thrombogenic or mature MK. The nuclear lobes are close together at one pole of the cell, and the voluminous cytoplasm appears to be fragmenting (x 1000). (B) Mature human MK processed for AS. The cytoplasm is replete with channels of the demarcation system (dms). Numerous α-granules (α) are also present. AS is found in vesicles of variable size (ly), but usually somewhat smaller than the α-granules, which are unreactive. Tissue fixed in paraformaldehyde-glutaraldehyde for 30 min at 4°C and incubated for 2 hr at 250°C in Goldfischer’s medium for AS. Inset: Higher magnification of small portion of another MK showing numerous unreactive α-granules and two lysosomal vesicles (ly) containing AS reaction product (x 17,000).
sediment in fractions of lower density, which have been shown by electron microscopy to be composed mostly of mitochondria and membrane vesicles.\textsuperscript{11} We believe that these vesicles probably contain the lysosomal enzymes found in that fraction. However, cytochemistry has not yet been performed on the various fractions.

In clinical investigations of patients with storage pool deficiencies, at least one patient has been described whose platelets lack both $\alpha$-granules and dense bodies, but contain normal amounts of lysosomal enzymes.\textsuperscript{14} The "gray platelet syndrome," first described in 1971\textsuperscript{30} and since then identified in several other patients,\textsuperscript{15,31} is another disorder in which the platelets contain either very few $\alpha$-granules or none at all, while the platelet lysosomal enzymes are normal by both biochemical\textsuperscript{12} and cytochemical criteria.\textsuperscript{15} These "experiments of nature" also point to distinct lysosomal organelles in platelets.

Studies of secretion kinetics indicate that thrombin, but not ADP, causes platelets to secrete a certain amount of each lysosomal enzyme, generally about
Although acid β-glycerophosphatase appears to be distributed in the same subcellular fractions as the other enzymes tested, only about 10% of it is secreted. Accordingly, Fukami and Salganicoff have postulated two classes of platelet lysosomes: one containing β-glycerophosphatase, and the other the remaining lysosomal enzymes. This is certainly possible, but the secretion kinetics could also reflect differential binding of β-glycerophosphatase to the lysosome membrane.

Unlike the primary lysosomes of leukocytes, which form a population of morphologically identifiable granules, MK and platelet lysosomes appear to be similar to those of most other cell types. They are vesicles of variable size, which apparently are pinched off from certain Golgi cisternae, perhaps GERL membranes. Compared with α-granules, they constitute a minor proportion of the MK or platelet organelles, and they probably are not recognizable without specific cytochemical stains. Their contents (without the cytochemical stains) may have an electron density similar to that of α-granule contents; however, especially as seen in MK, they are in no way identical to α-granules.

Little is known about the formation of α-granules in MK. On the basis of morphological observations in osmium-fixed tissue, Jones proposed that they arise in the Golgi complex. However, since no cytochemical marker for them has yet been found, the results of subsequent studies have been equivocal. We hope that immunocytochemical procedures now being developed in our laboratory (Halverson et al.) will soon make it possible to follow the formation of α-granules and thus further clarify their relationship to lysosomes. It is clear that lysosomes are present very early in MK maturation. A few α-granules may also be seen in immature MK, but they become more prominent later in maturation.

The lysosomes of both rat and human MK and platelets show none of the debris or other evidence of digestive activity that is normally seen in secondary lysosomes. We therefore believe that they are primary lysosomes in both cells. Although several investigators have attempted to demonstrate phagocytosis in platelets, most have concluded that substances that appear to be taken up are actually retained in the canalicular system. Digestion of exogenous material by platelets has never been demonstrated, and the function of platelet lysosomal enzymes (which appears to be extracellular) is still unknown.

Our current concept of the organelles of human platelets, as revealed by ultrastructural cytochemistry, is summarized in the diagram in Fig. 9. We have diagrammed and labeled lysosomes as distinct entities. Cytochemically, they are small acid-hydrolase-con-
taining vesicles that appear similar to primary lysosomes of most other cell types. On the basis of the several lines of evidence discussed above, we believe that they should be recognized as separate organelles and not be considered a subpopulation of α-granules.

REFERENCES


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