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Structural Characterization of Platelets and Platelet Microvesicles

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Abstract—Platelets are blood cells without nuclei, which, in conjunction with fibrin, cause bleeding to stop (hemostasis). Cellular microvesicles are microscopic particles released into extracellular space under activation and/or apoptosis of cells of different types. Platelet microvesicles form the main population of blood circulating through microvesicles and play an important role in the reactions of hemostasis, thrombosis, and many other (patho)physiological processes. Despite the large number of studies that have been devoted to the function of platelet microvesicles, the mechanisms of their formation and structural details remain poorly understood. The ultrastructure of the initial platelets and microvesicles formed in vitro from resting cells and platelets activated by arachidonic acid, ADP, thrombin, and calcium ionophore A23 187 is investigated in this study. The intracellular origin, stages of formation, structural diversity, and size of microvesicles were analyzed according to the results of transmission electron microscopy of human platelets and isolated microvesicles. It was shown that thrombin, unlike other activators, not only stimulates microvesiculation of the plasma membrane, but also causes decomposition of cells with the formation of subcellular particles that have sizes comparable with the size of the microvesicles from the outer membrane of the cells. Some of these microparticles are cellular organelles surrounded by a thin membrane. The size of isolated microvesicles ranges from 30 to 500 nm, but their size distribution depends on the nature of the activating stimulus. The obtained results contain new data on the formation of platelet microvesicles and their structural diversity, which are important for understanding of their multiple functions in health and disease.

Keywords: platelets, microvesicles, platelet activation, cell ultrastructure, electron microscopy

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INTRODUCTION

The activation or apoptosis of cells of different types, including platelets, is accompanied by the formation of microscopic extracellular structures called "microvesicles" (MVs) or "microparticles" regardless of the (patho) physiological stimulus that caused them (Burnouf et al., 2014). The most obvious feature of platelet MVs is their procoagulant effect, i.e., the ability to accelerate blood coagulation due to assembly of tenase and prothrombinase complexes on their surfaces (Zwicker, 2008). The ability of MVs to accelerate the generation of thrombin explains their important role in pathological conditions, which are accompanied with hemostatic and thrombotic disorders (Nomura and Shimizu, 2015). Furthermore, platelet MVs are involved in the immune response, inflammation, angiogenesis, regeneration, metastasis, and other reactions (Thushara et al., 2015; Varon and Shai, 2015). Due to their pathogenetic significance, platelet MVs are attracting the attention of physicians as biological markers (Owens and Mackman, 2011; Ayers et al., 2014). Despite the growing number of studies of MVs, including platelet MVs, data on the mechanisms of their formation, molecular composition, structure, and biological properties are not complete.

The morphological basis for the formation of platelet MVs and their structural characteristics are investigated in the present study.

MATERIALS AND METHODS

Platelets were isolated from blood samples of healthy donors who did not take medication for 2 weeks prior to blood sampling (according to resolution of the ethical committee of Kazan State Medical Academy no. 2/2012). The blood plasma rich in platelets was obtained by centrifugation of citrated whole blood at 200g for 10 min at room temperature. The platelets were isolated from plasma by gel filtration on Sepharose 2B (GE Healthcare, Sweden) equilibrated with Tyrode's buffer (4 mM HEPES, 135 mM NaCl, 2.7 mM KCl, 2.4 mM $MgCl_2$, 5.6 mM D-glucose, 3.3 mM NaH_2PO_4, 0.35 mg/mL bovine serum albumin, pH 7.4). The platelets were counted in Goryaev's chamber under a Primo Star microscope (Zeiss, Ger-

[1] *Abbreviations*: MVs—microvesicles, OCS—open canalicular system, PCD—programmed cell death.

many) at a magnification of 40x. The platelets were used within 3 h after blood sampling. Cell viability, determined by changes in mitochondrial potential using flow cytometry and MitoTracker DeepRed FM fluorescent dye (Invitrogen, United States), was about 97%.

The control platelets (45 million cells in 560 μL of Tyrode's buffer) were incubated at 37°C for 60 min (resting platelets). To activate the platelets 50 μM of arachidonic acid, 5 μM of ADP or 1 U/mL of thrombin were added, followed by incubation at 37°C for 60 min. The final volume of each sample was 560 μL with a cell concentration of about 80 million/mL.

Transmission electron microscopy of platelet preparations. Immediately after incubation, glutaraldehyde was added to the platelet suspension to a final concentration of 2%, with the resulting suspension being incubated for 30 min at room temperature and centrifuged at 1500 g for 5 min. The pellet was washed with Tyrode's buffer (pH 7.4). The samples were postfixed with 1% osmium tetroxide in the same buffer, containing sucrose (25 mg/mL) for 2 h. The samples were dehydrated in ethanol ascending with increasing concentration (30, 40, 50, 60, 70, and 96 vol %), and then in acetone and propylene oxide. Epon 812 was used as the embedding medium. Samples were polymerized for 3 days under increasing temperature from 37 to 60°C. The sections were obtained on an LKB-III ultramicrotome (Sweden). The sections were contrasted with saturated aqueous uranyl acetate for 10 min at 60°C and, then, with aqueous solution of lead citrate for 10 min. The preparations were examined using a Jem-1200EX electron microscope (Jeol, Japan).

Obtaining and electron microscopy of isolated platelet MVs. To stimulate the formation of MVs, 15 μM of calcium ionophore A23187 or 1 U/mL of thrombin was added to the platelets freshly isolated by gel filtration in Tyrode's buffer at room temperature, followed by incubation at room temperature for 15 min. The final volume of each sample was 300 μL and contained 1.5 million platelets. Immediately after the incubation, the cells were separated by centrifugation at 2000 g for 10 min. To remove cell debris, the supernatant was further centrifuged for 10 min at 7000 g. Twenty microliters of the supernatant fraction containing suspension of MVs was applied to a grid for electron microscopy with 2% Collodion (EMS, United States) as a support and dried in air. The preparation was contrasted with saturated aqueous solution of uranyl acetate for 15–20 s, followed by drying. The preparations were investigated using a Jem-1200 EX electron microscope (Jeol, Japan).

RESULTS

Ultrastructural characteristics of resting platelets. Resting (inactivated) platelets, depending on the projection, had a discoid or rounded shape. The average linear dimensions of rounded cells were 2–3 μm, while elongated discoid cells were 3 μm by the long axis and 0.8–1.2 μm by the short axis (Figs. 1a, 1b).

The central part of resting platelets contains several types of granules: α-granules, dense bodies (δ-granules), lysosomes (λ-granules), and microperoxisomes. Electron-dense α-granules of rounded shape were the most numerous. Their diameter ranged within 300–500 nm. The dense granules had a smaller diameter—200–300 nm. A darker central region surrounded by a light halo was typical for them. A small number of lysosomes in the form of bubbles with a diameter of 175–200 nm and microperoxisomes in the form of granules with a diameter of about 90 nm were found in resting platelets. Glycogen granules were randomly located in the cytoplasm or formed clusters in the form of fine-grained regions of the platelet body. An open canalicular system (OCS) was represented by numerous small vacuoles and tortuous canaliculi. The canaliculi formed a branching system that surrounded the platelet body and could be opened directly on the surface of the cytoplasmic membrane (Fig. 2c). The platelets had small rounded mitochondria with an electron-dense matrix and single cristae (Fig. 1b).

Ultrastructural characteristics of activated platelets. Platelet activation is accompanied by significant morphological changes, the nature of which depends on the nature of the stimulus. Under treatment with arachidonic acid (Fig. 1c), the platelet surface became tortuous due to invaginations of the plasma membrane and the formation of thin outgrowths—pseudopodia. The size of the platelet body did not change. Cell vacuolization by increasing the canal lumen of OCS with the formation of vacuoles of various sizes was observed. Most of the vacuoles contained various inclusions: α-granules, δ-granules, membrane components, and crumbly fine-grained inclusions. The formation of vacuoles of tortuous shape probably occurred as a result of enlargement and fusion of the canals.

Similar morphological features of platelet activation were observed under treatment with ADP (Fig. 1d). The platelets formed pseudopodia of different length; the number of vacuoles, which could also contain various inclusions, were increased. The zone of glycogen granule accumulation was clearly defined in the cells, and this zone could occupy up to half of the cell area.

The most significant changes in the ultrastructure of the platelets occurred during incubation with thrombin. The plasma membrane of the platelets formed deep invaginations and folds, which led to the rise of "amoeba-like" platelets and further breakdown of the platelet body into fragments (Fig. 1e). The cytoplasm of the cells became more electron-dense compared with the control inactivated cells, and hardly contain secretory granules. The whole platelet body was permeated with tortuous and narrow canaliculi of the open system with the access to the cell surface.

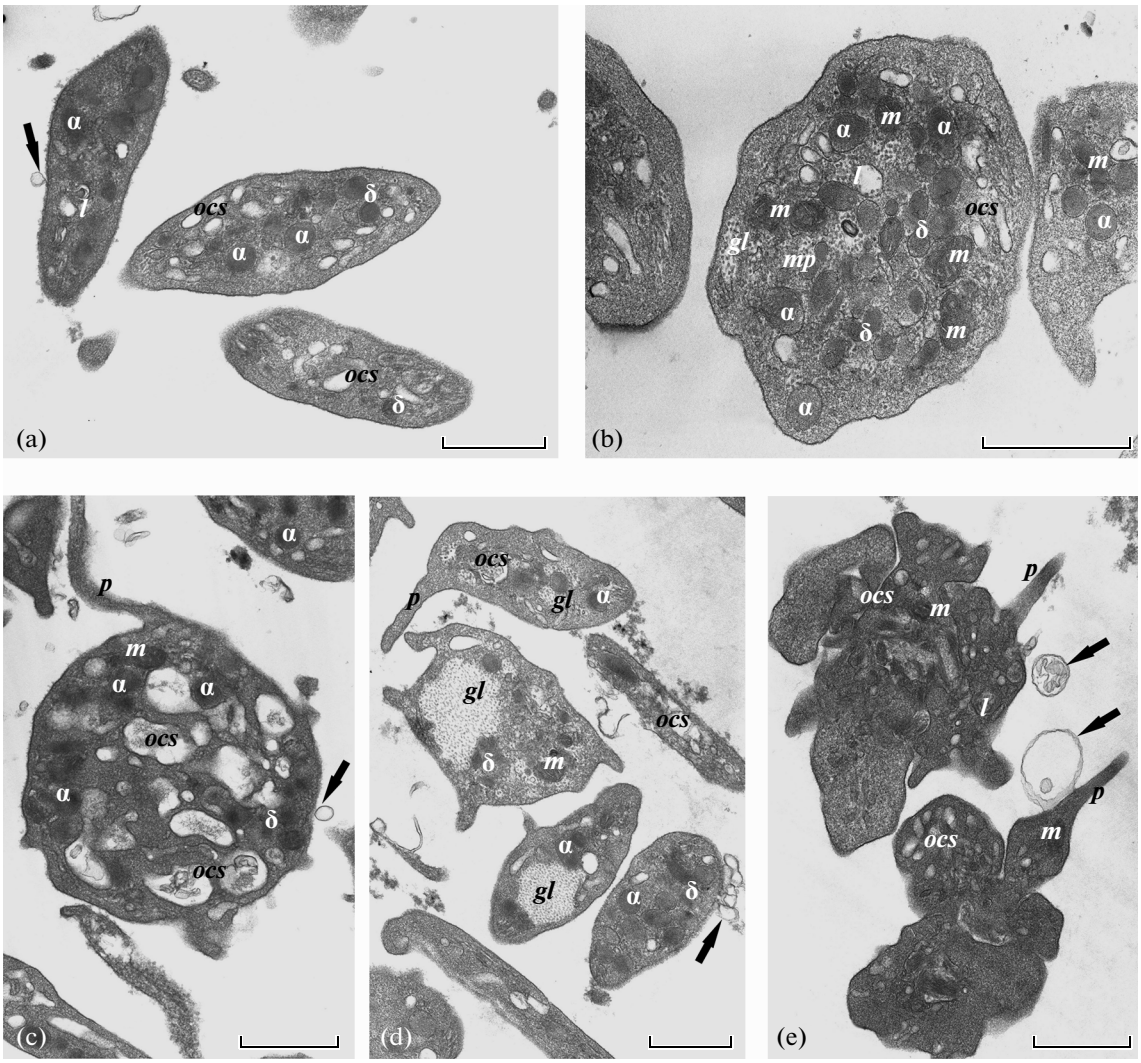

Fig. 1. (a, b) Ultrastructure of resting platelets and platelets activated by (c) arachidonic acid, (d) ADP, or (e) thrombin. Designations: α, α-granules; δ, δ-granules, *gl*, glycogen granules; *l*, lysosome; *m*, mitochondria; *mp*, microperoxisome; *OCS*, open canalicular system; *p*, pseudopodium. Microvesicles are indicated by arrows. The scale bar is 1 μm.

Fragments of the decomposed platelet were very different in size and shape and contained intracellular components, including organelles (Fig. 2d).

Formation of platelet MVs. When platelets were examined under a transmission electron microscope, we found a large number of MVs formed by the cells both at rest and during activation. In most cases, MVs were formed by invagination of the plasma membrane, followed by budding and release into the environment. MVs were budded from the surface of the platelet body, from pseudopodia and were formed with a participation of open canalicular system (Figs. 2a–2c).

The stages of formation and budding of MVs from the platelet body are shown in Figs. 2a-1–2a-4. It is clearly seen that, first, the invagination of the plasma membrane occurs, and then the contacting zone with the platelet body decreases, the edges of the bulging membrane are closed, and constriction and a spherical bubble develop. As a result, the newly formed MVs are budded from the platelet body. The formation of MVs may occur at the end of pseudopodia and also due to bulging of the plasma membrane. The structure of the newly formed MVs can be simple, i.e., take the form of a bubble restricted by a single membrane, as well as more complex, when smaller vesicles are arranged within a large vesicle (Figs. 2b and 3d).

Other mechanisms of MV formation are possible with the involvement of the platelet open canalicular system. Figures 2c and 2d show the release of intracellular granules into the environment and formation of MVs from OCS canaliculi. The canaliculus lumen opens directly on the cell surface, which enables outward release of the cytoplasm contents (Fig. 2c, increased fragment). In the second case, when acti-

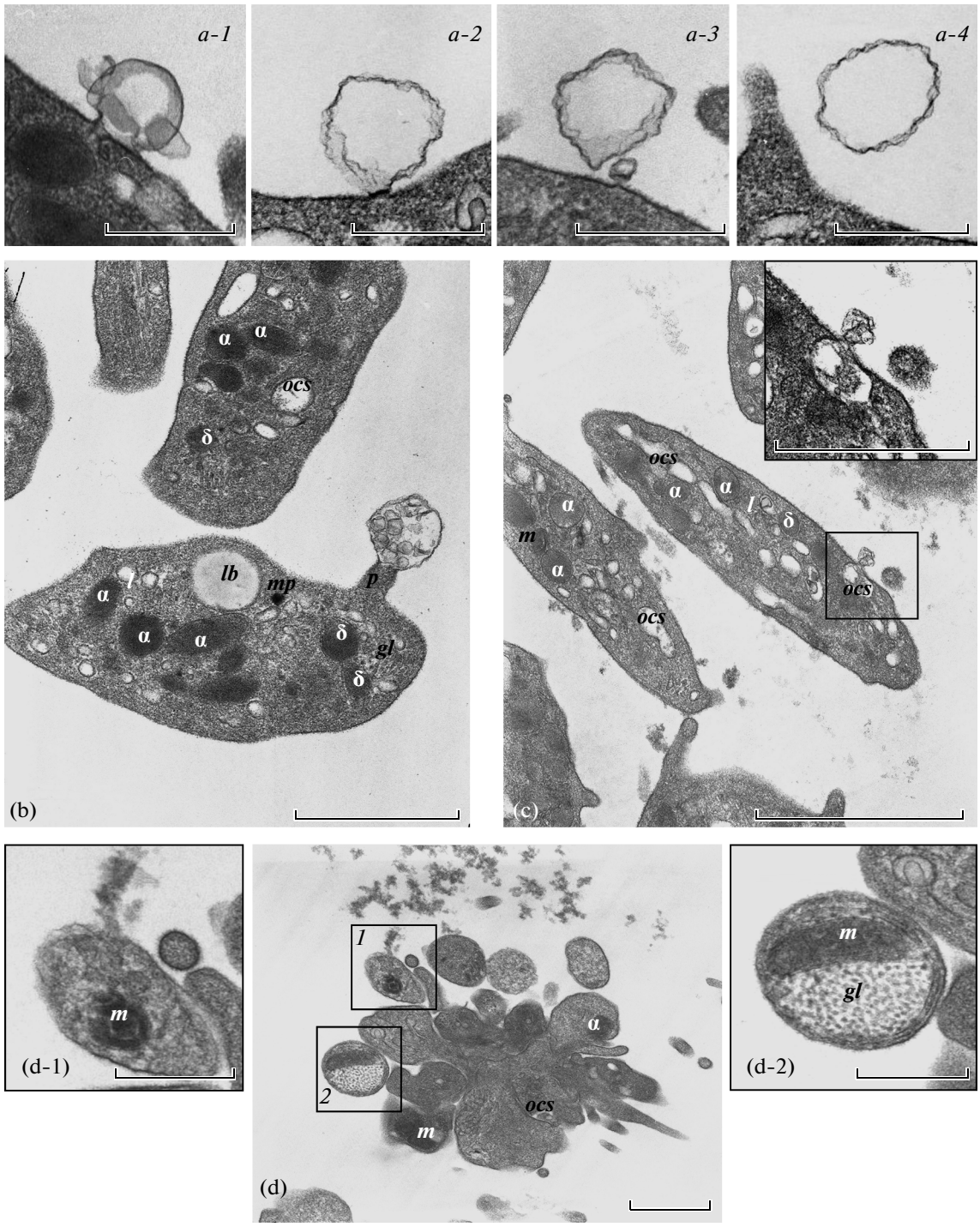

Fig. 2. Origin of platelet microvesicles (MVs). (a-1–a-4) Successive stages of MV formation by budding from the plasma membrane of the platelet body, (b) formation of MVs at the end of pseudopodia, (c) release of MVs from an open canalicular system; and (d) breakdown of the platelet after activation by thrombin into vesicles containing mitochondria (d-1) or glycogen granules and mitochondria (d-2); *lb*—lipid bubble; other designations are the same as in Fig. 1. The scale bar is 500 nm (for *a*, all inserts) and 1000 nm (for b–d).

vated by thrombin, interaction of OCS canaliculi with the plasmalemma leads to profound invaginations and compartmentalization of a part of the cytoplasm into individual fragments, which leads to platelet body fragmentation (Figs. 2d-1, 2d-2).

Structural heterogeneity of platelet MVs. MVs of platelet origin were found to be very heterogeneous in structure. According to structure, they can be divided into the following groups: (1) MVs restricted by a single membrane, (2) MVs consisting of several mem-

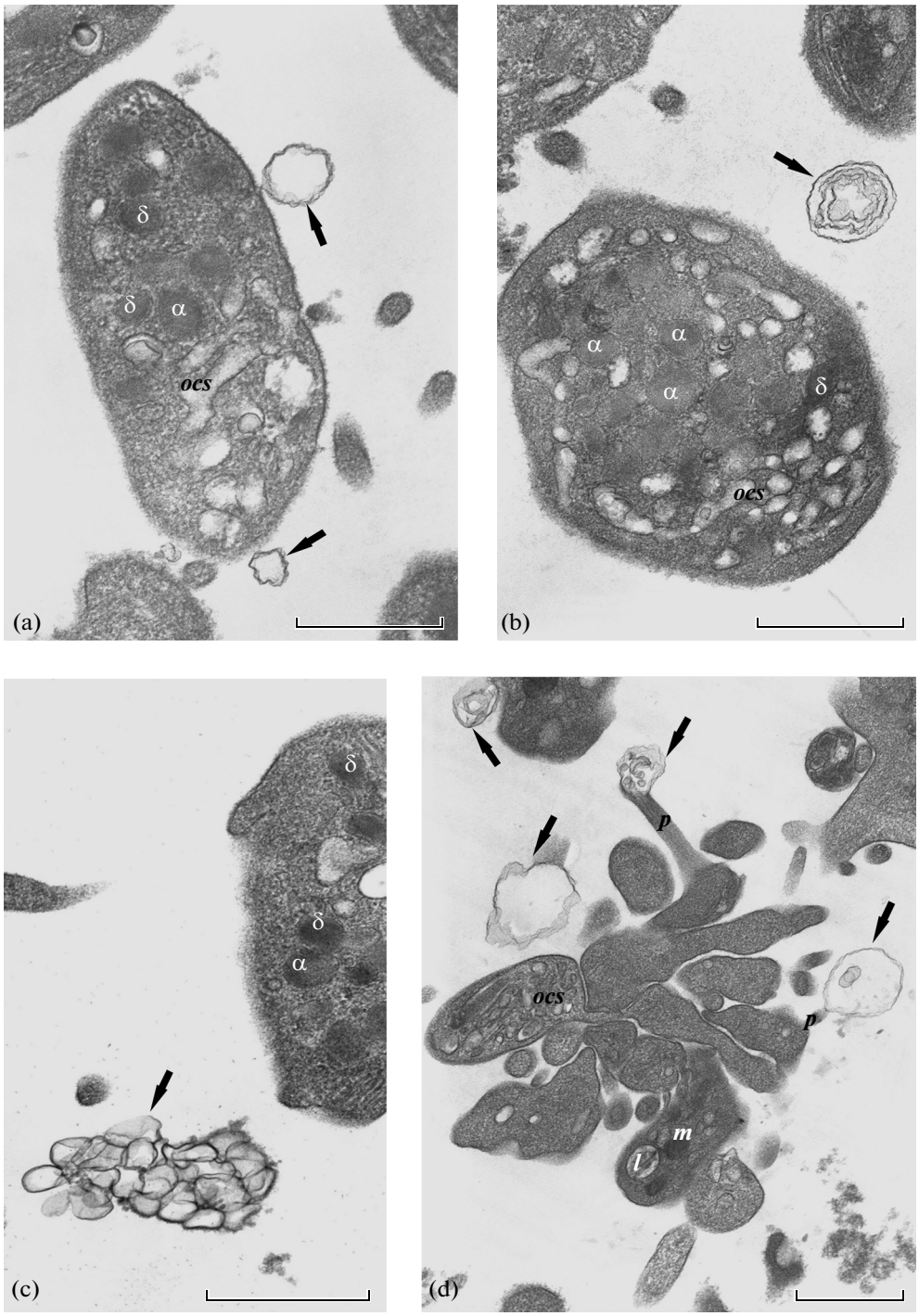

Fig. 3. Structural diversity of platelet MVs. (a) MVs with single membrane, (b) MVs with multilayer membrane, (c) multivesicular particle, and (d) MV formation of different structure after platelet fragmentation caused by incubation with thrombin. The designations of the structures are the same as in Fig. 1. Microvesicles are indicated by arrows. The scale bar is 1000 nm.

brane layers (multilayer MVs), (3) multivesicular particles, and (4) microparticles formed due to fragmentation of a platelet (Fig. 3). We observed the formation of all of the MV types from activated platelets, and of only the first three types from resting cells. MVs restricted by a single membrane with an electron-transparent content were the most frequent. These MVs were formed from the plasma membrane and had the greatest scatter of sizes (Figs. 2a, 3a). Figure 2 a-3 show two MVs: a large one with a size of 400 nm and a small one with a size of 80 nm. Multilayer MVs were formed from several closed membranes, while MVs of

different diameters were, roughly, put into one another and could have three to five membrane rings (Fig. 3b). Multivesicular particles consisted of several small vesicles that could be restricted by a common membrane or not have it. Small vesicles were arranged loosely and individually, if they were within one large MV (Fig. 2b and 3d), or, conversely, adhered to each other, forming a close contact with each other, if they had no common restricting membrane (Figs. 1d and 3c). Such aggregates could combine 10–15 individual MVs.

When platelets were activated by thrombin, the formation of all of the above MV types, as well as microparticles with electron-dense contents, was observed (Fig. 3d). Fragments of the decomposed platelet were restricted by the plasma membrane and contained the cytoplasm, various cellular components and organelles. Figure 2 shows fragments containing isolated mitochondria (d-1) or granules of glycogen and mitochondria (d-2).

The dimensions of platelet MVs detected in platelet preparations varied in a wide range of from 50 to 2000 nm with a predominance of particles of the size in the range of 50–130 nm (Fig. 3d). The dimensions of MVs did not depend on their structure and origin (the outer membrane or intracellular structures). MVs restricted by a single membrane could reach a value of 1500–2000 nm, but also could be much smaller (70–100 nm). Small vesicular particles (50–70 nm), which either were enclosed within the common vesicle with the size of 600–1000 nm (Fig. 3d) or did not have a restricting membrane, but were tightly grouped and formed aggregates of 1000–1500 nm (Fig. 3c), were observed. During the breakdown of a platelet into fragments, particles with the size of up to 1000 nm were formed; we considered them as one of the variants of microvesicular structures of platelet origin.

We used the method of negative contrast of isolated microparticles of platelet origin for additional evaluation of the dimensions of a large number of MVs (Fig. 4). MVs isolated from resting platelets had a rounded shape with sharp straight edges. MVs isolated from platelets activated by thrombin or calcium ionophore were heterogeneous. Two major populations in them can be distinguished visually: 1) relatively large MVs (greater than 200 nm) of regular spherical shape and 2) smaller vesicles (50–100 nm), which often have a rough surface and a thin outgrowths (Fig. 4). The distribution of platelet MVs in size is shown in Fig. 5. The size of MVs ranged from 30 to 500 nm with a distinct peak at 50–100 nm in the preparation of resting platelets (Fig. 5). The asymmetry of the peak with the shift to higher value indicates the presence of an MV fraction of 150–200 nm or more. After the activation of platelets by calcium ionophore, MVs become very different in size and additional peaks at 200–250 and 350–400 nm arise, reflecting an increase in the proportion of relatively large particles (Fig. 5), which were absent in resting cells. The activation of platelets by thrombin, in contrast, was accompanied by a significant increase in the fraction of small particles with a size less than 75 nm, presumably having an intracellular origin (Fig. 5).

It should be noted that MVs larger than 500 nm are absent in the histograms (Fig. 5), although, according to our data, platelet MVs may reach a size of 1000–1500 nm (Fig. 3). This is most likely related to a methodological feature of MV isolation, namely, larger MVs could sediment at the second stage of centrifugation at 7000 g, which is required to remove cell debris.

DISCUSSION

The ability of cells to form and release membrane microparticles is an important mechanism for regulation of physiological reactions, a method of intercellular communication, and a pathogenetic factor in many diseases (Kelton, 2005; Nomura et al., 2008; Varon and Shai, 2015). Platelets perform multiple functions, including a crucial involvement in the reactions of hemostasis and thrombosis, and so MVs of platelet origin also have diverse and important biological properties (Zubairov and Zubairova 2009; Aatonen et al., 2012). Platelet MVs constitute a large amount of the cell microparticles circulating in the blood of healthy people; under different types of pathology, their concentration in the blood increases in many times, which is of great clinical significance (Thushara et al., 2015). The present study is intended to partially fill the gap in our knowledge of platelet MV structure, which lags behind numerous studies on their functions in normal and pathological conditions.

The structure of daughter MVs is inseparable from the structure of the cells from which they originate, and so we initiated a description of the ultrastructure of resting and activated platelets, taking into account that they both can form MVs (Burnouf et al., 2014). In complete agreement with the literature (James and White, 1972; Zucker et al., 1974; Neumüller et al., 2013), we observed subcellular organization of normal resting platelets and typical morphological features of their activation, which included a change in cell shape due to the formation of pseudopodia, overgrowth of OCS canaliculi, and the release of secretory granules into the environment (Fig. 1). It is not clear to what extent "resting" platelets are intact, since the cells are inevitably exposed to various influences that can activate them during isolation from the blood. This nonspecific activation likely explains the observed formation of MVs from platelets even without the addition of specific promoters, although, on the other hand, microvesiculation can be a normal function of every cell in a state of physiological rest (Thushara et al., 2014).

The ultrastructural changes of activated platelets depended on the nature of the stimulus. Thus, the effects of arachidonic acid and ADP, physiological platelet activators, were relatively mild and similar to each other (Figs. 1c, 1d), while thrombin caused a

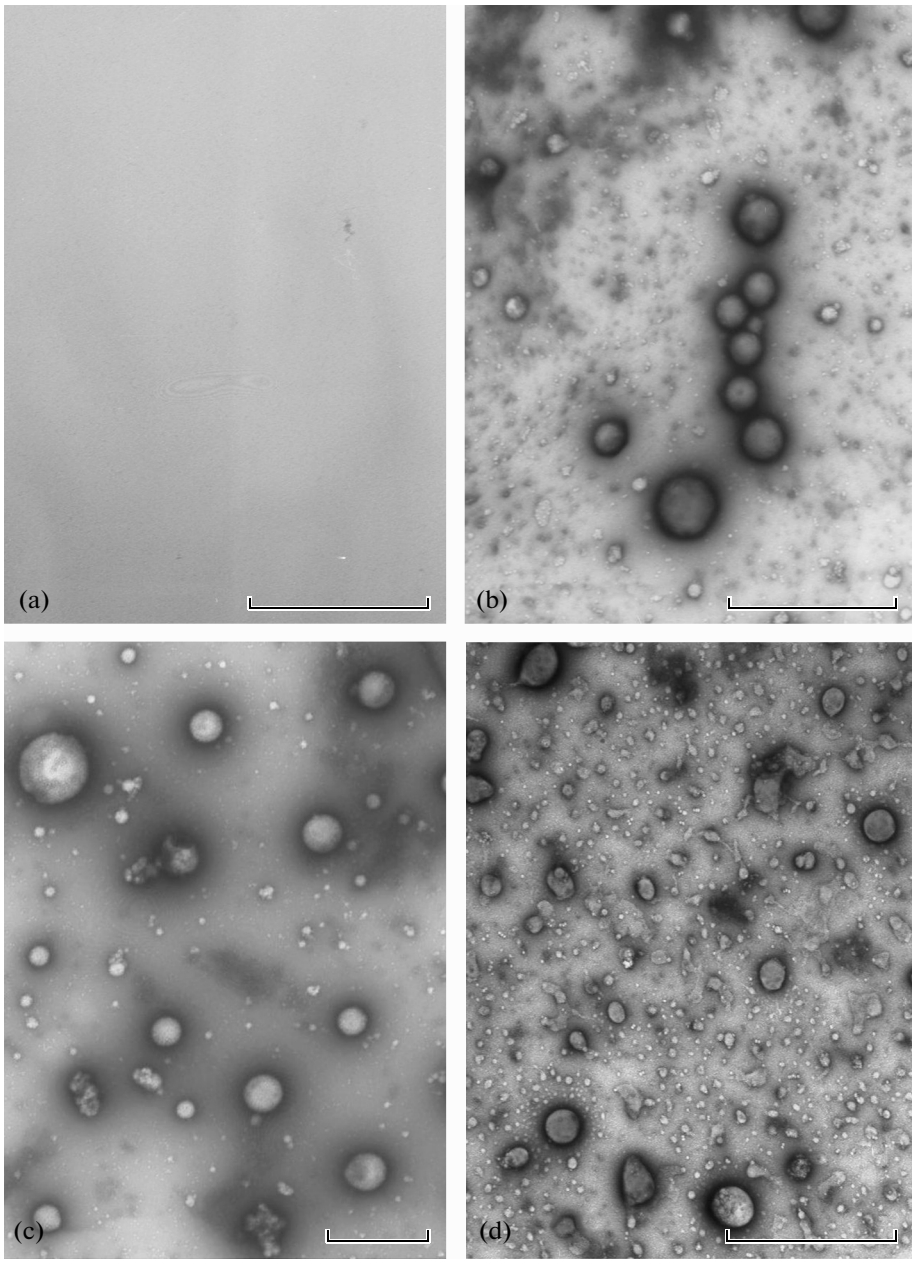

Fig. 4. Negative contrast of isolated platelet MVs. (a) Support material (negative control); (b) MVs isolated from the preparation of resting platelets; and (c, d) MVs isolated from preparations of platelets activated by calcium ionophore A23187 and thrombin, respectively. The scale interval is 1000 nm.

profound reorganization of the cell structure until breakdown (Figs. 1e, 2d, 3d). Fragmentation of platelets under activation by thrombin may reflect the later stages of apoptosis (Gyulkhandanyan et al., 2012; Akers et al., 2013). It has been shown that thrombin, in addition to the release of secretory granules and lysosomal enzymes, induces apoptosis, that can be determined by the expression of proapoptotic proteins Bax and Bak, the activation of caspase-3, the reduction of mitochondrial transmembrane potential, and the presence of phosphatidylserine on the plasma membrane (Leytin et al., 2006). In our experiments, thrombin induced induration and compression of platelet cytoplasm, disappearance of secretory vesicles in hyaloplasm, deep invaginations of the plasma membrane, and breakdown of the cell into heterogeneous small fragments (apoptotic bodies), which corresponds to morphological features of late apoptosis.

Cell MVs conditionally may be divided into two groups according to origin: ectosomes formed from

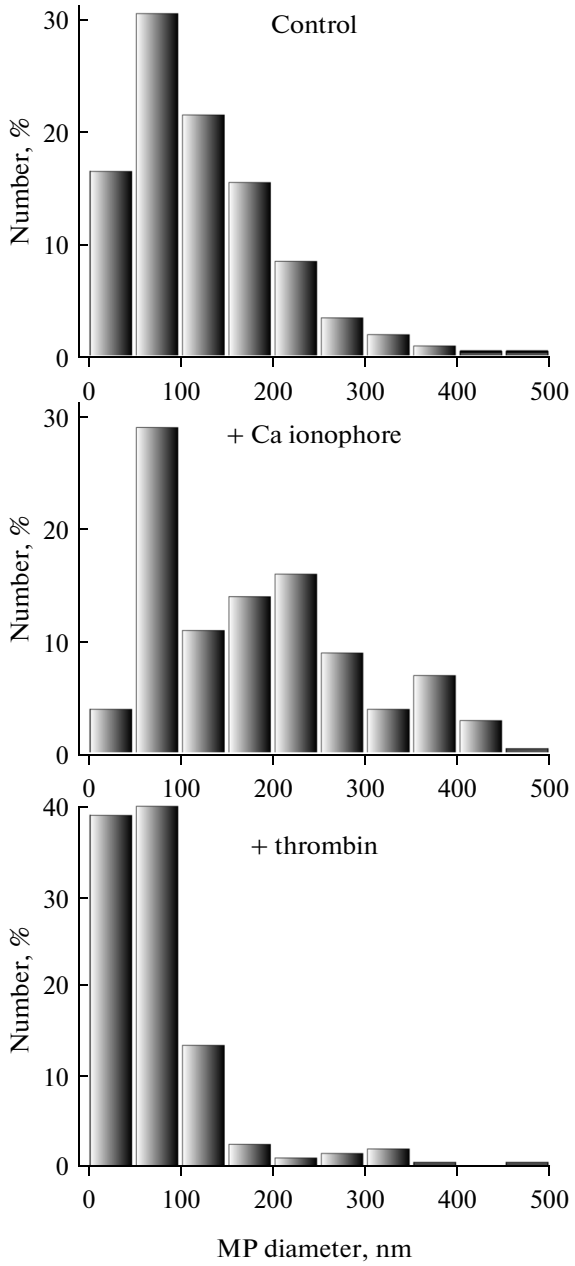

Fig. 5. Distribution of MVs isolated from the preparation of resting platelets on size in control (n = 200) and after activation by calcium ionophore (CI, n = 100) or thrombin (n = 200). Dimensions of MVs are determined using ImageJ software by electron microscopy with negative contrast. The total number of MVs in each group is taken as 100%.

the outer cell membrane (Stein and Luzio, 1991) and exosomes formed from inner membrane structures (Johnstone et al., 1987; Akers et al., 2013). Our research confirms the validity of this division with respect to platelet MVs. We revealed the successive stages of MV formation on the platelet body and showed that MV membrane is derived from plasmalemma (Fig. 2a). As an alternative, we observed the formation of MVs due to intracellular structures (Fig. 2c). Initially, exosomes of platelet origin were found in vitro, and recently they have been demonstrated in vivo (Nieuwland et al., 2013). To distinguish ectosomes and exosomes by other methods, except electron microscopy, is rather difficult, although exosomes are smaller (40–100 nm versus 100–1000 nm). Unfortunately, flow cytometry, which is widely used to determine MVs in biological fluids, generally does not detect particles smaller than 300–400 nm (Nieuwland et al., 2013; Ayers et al., 2014). In addition, flow cytometry does not provide information on the morphological diversity of cell microparticles (Heijnen et al., 1999; Leytin et al., 2006; Lacroix et al., 2010), which suggests the importance of ultrastructural studies on cell MVs.

We identified the following morphological variants of platelet MVs: single monolayer MVs, single multilayer MVs, multivesicular MVs (in the form of aggregates of several vesicles) and membrane fragments of a cell with organelles within. These fragments may include mitochondria, cytoskeleton elements, glycogen granules, small vacuoles and OCS components in addition to the cytoplasm. It should be noted that there are a few studies in which multilamellar structures (i.e., multilayer MVs) (George et al., 1982), multivesicular formations (Polasek, 1982), and platelet MVs containing mitochondria (Boudreau et al., 2014) have been described in addition to the classic single-walled "bubbles." Thus, our results are in agreement with data on the heterogeneity of platelet MVs from the literature. However, in contrast to previous papers, we defined the conditions of simultaneous formation of different types of MVs, traced the structural mechanism of their formation, and extended and characterized in detail the morphological spectrum of possible MV variants of platelet origin in our study.

Additional data on the morphology and dimensions of platelet MVs were obtained using the method of negative contrast of isolated microparticles. If MVs isolated from the preparations of resting platelets were in agreement with the classic understanding of MVs in terms of size and shape (Heijnen et al., 1999; van der Pol et al., 2012; Arraud et al., 2014), platelet activation by calcium ionophore, a known inducer of platelet apoptosis, resulted in a portion of large MVs (ectosomes), while thrombin, on the contrary, caused an increase in the number of small and very small vesicles (exosomes). These results confirm the dependence of the MV nature on biochemical stimulus and the relationship of microvesiculation with both activation and apoptosis of platelets, which requires further detailed research.

Thus, morphological heterogeneity of MVs of platelet origin is shown and cell structures, which are the source of their formation, are defined based on ultrastructural studies. The classification of platelet MVs based on their dimensions and the way of formation, the detailed structure, and inclusions and organelles within microparticles are given. These findings are important for understanding the origin of cell MVs and their composition, properties, and diverse roles in physiological and pathological processes.

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