MEMBRANE PROTEIN SEGREGATION DURING RELEASE OF MICROVESICLES FROM HUMAN ERYTHROCYTES

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1. Introduction

In previous studies of the release of cytoplasm-filled microvesicles from human red blood cells either during storage [1,2] or following treatment with Ca^{2+} and the ionophore A23187 [3] we have frequently observed slender protuberances ('tails') on many of the vesicles, but we have been uncertain whether they represented a genuine feature of microvesicle structure or an artefact of preparation for electron microscopy. The reality of these structures has now been confirmed using a variety of preparative techniques for electron microscopy and they have been isolated and their lipid and polypeptide composition determined. The results indicate that during microvesicle formation there is segregation of membrane proteins, and that this segregation occurs in two stages.

2. Experimental

2.1. *Isolation of microvesicles and 'tails'*

Microvesicles were isolated from blood aged by storage [2] for 8–9 weeks and from 4–5 days old erythrocytes treated with Ca^{2+} + ionophore A23187 [3]. The microvesicles from 500 ml packed red blood cells were suspended in 20–30 ml Hepes–saline solution (150 mM NaCl, 1.5 mM Hepes, pH 7.0) and sonicated in an MSE sonicator set at 245 V, for 10–15 s at 1.5 A at 4°C. The sonicated suspension was centrifuged at 76 000 × *g* for 30 min at 4°C and the resulting supernatant was centrifuged at 99 000 × *g* for 30 min. The 'tails' released from the vesicles by sonication were then sedimented from the supernatant at 157 000 × *g* for 90 min, and the pellet was collected in a minimum volume of the Hepes–saline.

2.2. *Analytical methods*

Acetylcholinesterase activity was assayed by the method in [4].

Protein was estimated by the method in [5].

Polypeptides were analysed on polyacrylamide gels by the method in [6].

Extraction, analysis and estimation of phospholipids were as in [7].

2.3. *Electron microscopy*

Pellets were treated successively with 6.25% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.4) and 1% osmium tetroxide, then dehydrated and embedded in Epon resin. Thin sections were stained with methanolic uranyl acetate and lead citrate. For negative staining, sample suspensions were fixed with glutaraldehyde, post-fixed with 1% osmium tetroxide (fig.1c,2a only) and then treated on EM grid with 2% phosphotungstic acid, pH 7.4.

3. Results and discussion

The electron micrographs in fig.1 demonstrate that narrow protuberances ('tails') are seen on membrane microvesicles released from human erythrocytes during either aging or treatment with Ca^{2+} plus the ionophore A23187. 'Tails' are seen on a large proportion of the vesicles and they are apparent both in fixed and sectioned specimens (fig.1, a,b,d) and after negative staining of fixed specimens (fig.1c,2a).

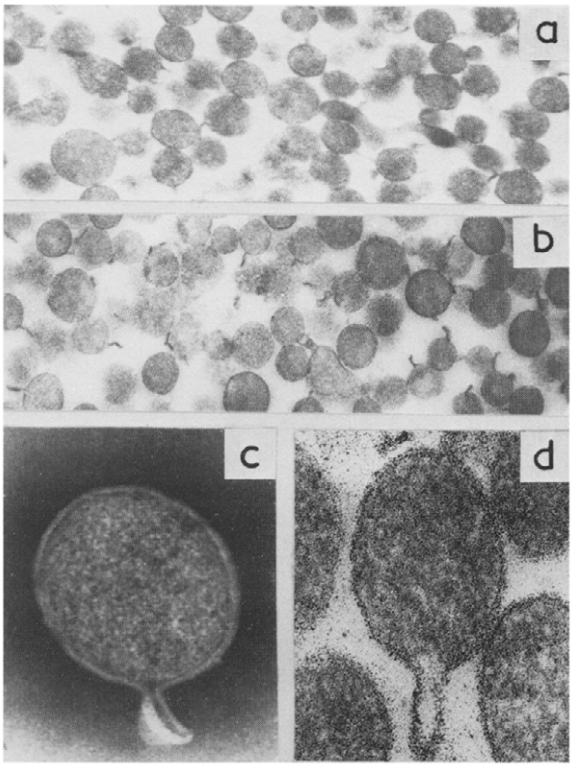

Fig.1. Electron micrographs of microvesicles showing 'tails'. (a) Section of microvesicles from aged erythrocytes (× 42 000). (b) Section of microvesicles from erythrocytes treated with Ca^{2+} + A23187 (× 42 000). (c) Enlarged micrograph of a negatively-stained microvesicle (× 200 000). (d) Enlarged micrograph of sectioned microvesicle (× 160 000).

Mild sonication of the vesicle preparations removed the 'tails' (fig.2b), and these were recovered as a relatively homogeneous pellet by high-speed centrifugation (fig.2c,3). Approximate calculations, based upon a vesicle radius of 50 nm, a 'tail' radius of 10 nm and a 'tail' length of 50 nm, suggest that about 5% of the total membrane material (surface area) of the vesicle may be in the 'tails'. In several experiments, a litre of packed red blood cells (approx. 4 mmol phospholipid) yielded around 2 μmol 'tail' phospholipid. Since about 10–15% total phospholipid of the erythrocytes was obtained in our vesicle fractions, the yield of 'tails' represents about 0.3–0.5% of the total vesicle phospholipid.

The lipid:protein ratio of the 'tails' (table 1) was

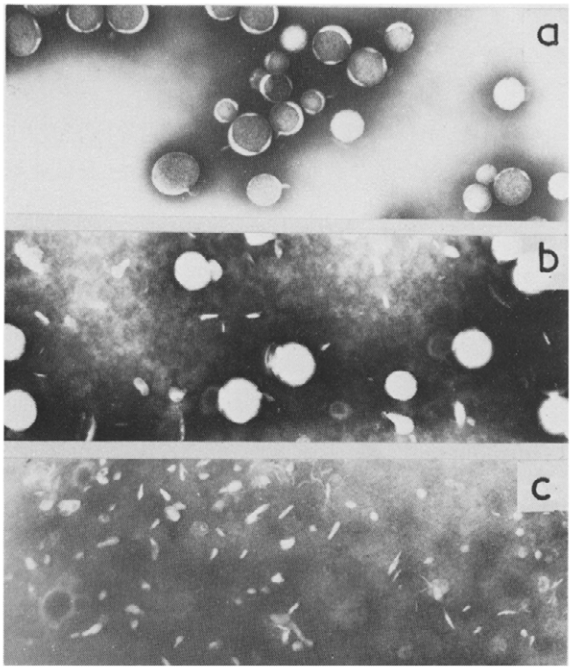

Fig.2. Electron micrographs (negatively-stained preparations) showing isolation of 'tails' from microvesicles (× 42 000). (a) Intact microvesicle with 'tails'. (b) Sonicated microvesicles showing separated 'tails'. (c) Isolated 'tails'.

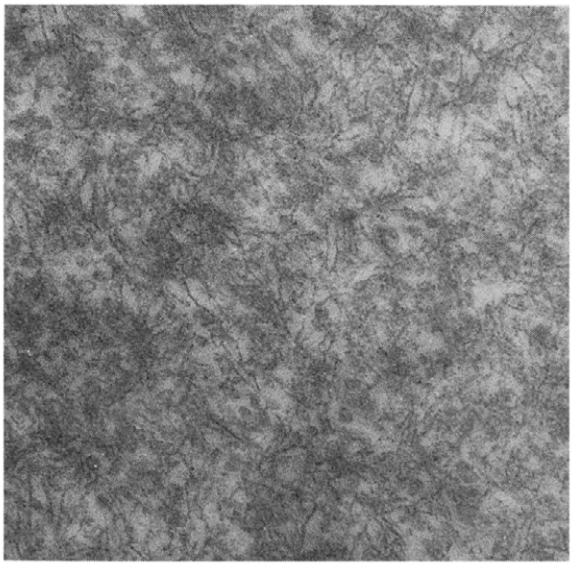

Fig.3. Electron micrograph of section of a 'tails' preparation (× 60 000).

Table 1
Acetylcholinesterase activities and lipid:protein ratios of membranes

Sample	Acetylcholinesterase activity (μmol/min/μmol phospholipid)	Phospholipid/protein ratio (μmol/mg)
Intact cells	3.0 ± 0.2 (3)	n.d.
Isoionic ghosts	1.8 ± 0.2 (3)	0.71 [3]
Microvesicles (stored cells)	6.0 ± 0.6 (4)	n.d.
Microvesicles (ionophore treatment)	4.8 ± 0.8 (3)	1.9 [3]
'Tails' (from microvesicles of stored cells)	10.7 ± 1.8 (3)	2.5 ± 0.5 (3)
'Tails' (from microvesicles of ionophore treated cells)	9.4 ± 0.7 (3)	2.4 ± 0.3 (3)

n.d., not determined

Methods were as described in section 2. Results are reported as means ± SEM (no. expts.)

much higher than that of the erythrocyte membrane, but similar to that of the microvesicle membranes. Polypeptide analysis of the 'tails' by SDS–gel electrophoresis showed marked depletion of bands 1 and 2 (spectrin) as in the case of microvesicles. Band 3, the dominant component of the microvesicle membrane, was virtually absent from the 'tails'. The dominant polypeptide in both types of 'tail' preparation stained both with Coomassie blue and with periodic acid–Schiff reagent and migrated in the 4.1/4.2 region during gel electrophoresis (fig.4). Additional minor components of lower molecular weight were also detected, particularly in the case of 'tails' obtained using Ca^{2+} plus ionophore A23187. The activity of acetylcholinesterase, a component of the outer surface of the erythrocyte membrane, was higher in 'tails' than in microvesicles (table 1). The phospholipid profile was similar to that of erythrocytes and of microvesicles (table 2). The major non-polar lipid was cholesterol, with traces of other components including unesterified fatty acids and diacylglycerol, but insufficient material was available for quantitative analysis.

The electron microscopic images of the 'tails' show a trilamellar membrane unit similar to that of the vesicle itself, but the chemical analyses of the isolated 'tails' have clearly established that their polypeptide content is considerably simplified as compared with the vesicle membranes. This implies segregation of membrane proteins during the formation and release of microvesicles. It seems likely that this occurs in 2 stages:

1. A depletion with respect to extrinsic proteins, particularly spectrin, which is associated with the 'blebbing' of the cell surface.
2. A further segregation process, probably in a narrow neck which forms before the vesicle can pinch off from the residual cell.

This achieves the accumulation of a glycopeptide

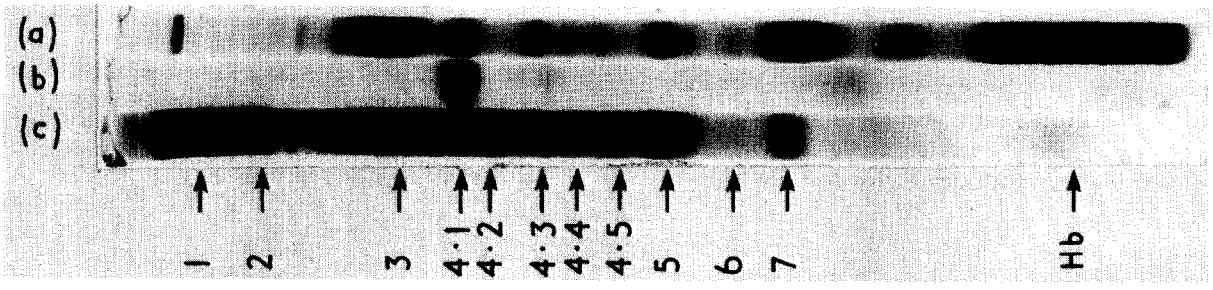

Fig.4. SDS–polyacrylamide gel electrophoresis, gels stained with Coomassie blue. (a) Microvesicles obtained from aged cells. (b) 'Tails' isolated from microvesicles obtained from aged cells. (c) Haemoglobin-free erythrocyte ghosts.

Table 2
Phospholipid composition of 'tails'

Phospholipid	Percentage of total phospholipid		
	Erythrocytes[a]	'Tails' from microvesicles obtained from A23187	'Tails'from microvesicles obtained from stored cells
Sphingomyelin	26 ± 2	27 ± 3 (4)	28 ± 0.2 (2)
Phosphatidylcholine	31 ± 3	33 ± 2 (4)	30 ± 0.1 (2)
Phosphatidylserine + phosphatidylinositol	15 ± 3	12 ± 1.5 (4)	15 ± 0.5 (2)
Phosphatidylethanolamine	27 ± 3	27 ± 2.5 (4)	27 ± 1 (2)

[a] Values taken from [9]

Extraction, analysis and estimation of phospholipids were as described in section 2. Values represent means ± SEM (no. expts.)

component (possibly PAS-1) of the 4.1/4.2 region, and the exclusion of most other polypeptides, particularly band 3, from the small proportion of the membrane destined to become the 'tails'.

It has been suggested that the initial blebbing of the erythrocyte membrane which precedes microvesicle release is driven by the accumulation of diacylglycerol produced by a Ca^{2+}-stimulated polyphosphoinositide phosphodiesterase at the inner surface of the membrane [3,8]. From the composition of the isolated 'tails', it now seems that the final membrane fusion event which leads to vesicle release may also require the elimination of some membrane-spanning polypeptides, particularly band 3, from the region of fusion, as envisaged in the general model of fusion developed in [10]. The accumulation of a particular glycopeptide component in 'tails' is intriguing, but there is not yet any indication what role this might play in the fusion event. Similarly, our present information gives no indication why 'tails' persist on the vesicles after they have separated from the parent erythrocytes. If particular polypeptides can segregate into and out of a region of membrane when it is preparing to undergo fusion then one might expect that after fusion they would also diffuse back, thus restoring uniformity of composition and of curvature to the membrane and eliminating the 'tails': clearly this does not happen.

Acknowledgement

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