RELEASE OF CHOLESTEROL-ENRICHED MICROVESICLES FROM HUMAN ERYTHROCYTES CAUSED BY HYPERTONIC SALINE AT LOW TEMPERATURES

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1. Introduction

It has been shown that the treatment of erythrocytes or ghost membranes with hypertonic saline results in a tonicity-dependent liberation of lipids [1–3]. However, the mechanism of lipid liberation from the erythrocyte membrane is still unknown and up to now there is no evidence that small vesicles are released by exposure to hypertonic saline and freeze–thawing. In my studies related to the effect of low temperatures on membrane structures, it has been shown that these treatments result in the release of microvesicles which differ from the membranes in their content of lipids and proteins, and that the ratio of cholesterol:phospholipid (C:P) in these microvesicles increases in an inverse relation with their decreasing content of intrinsic membrane-proteins, as the temperature of the treatment is lowered.

From these results, it is suggested that cholesterol and proteins in the erythrocyte membrane might be partially segregated by exposure to low temperatures, and possibly it may be that the erythrocyte membrane might be fragmented at structurally weak loci by osmotic stress.

2. Materials and methods

2.1. Erythrocytes and resealed ghosts

Erythrocytes were isolated from fresh human blood. Ghosts were prepared from erythrocytes by a modification of the procedure in [4] and resealed by the method in [5].

2.2. Treatment of erythrocytes and resealed ghosts

Suspensions of erythrocytes or ghosts in 0.15 M NaCl were cooled to 0°C and −10°C for 30 min, without freezing, and then they were exposed to 4 M NaCl at the same temperatures for 15 min. Aliquots of the suspensions of erythrocytes or ghosts were slowly frozen to −25°C at a cooling rate of 0.5°C/min, followed by rapid thawing. After completion of the treatment, the suspensions were centrifuged at 105 000 × g for 60 min. The resulting supernatants were centrifuged at 255 000 × g for 120 min and then the pellets were collected and suspended in 0.15 M Tris–HCl buffer (pH 7.4).

2.3. Analytical procedures

Lipid was extracted by the method in [6] and phospholipid was assayed as in [7]. Cholesterol was determined chemically and enzymatically by the methods in [8,9] and expressed relative to the phospholipid content of the extracted lipids.

Membrane proteins were analyzed by SDS–polyacrylamide gel electrophoresis by the method in [10].

2.4. Freeze-fracture and electron microscopy

Droplets of the sample were placed on copper-planchets and frozen rapidly in Freon 22 precooled by liquid nitrogen. Freeze-fractured replicas of the sample were prepared by a modification of the technique in [11].

3. Results

The lipids released from erythrocytes or ghosts

after the above treatments could be quantitatively recovered in the fraction sedimented by the centrifugation at 255 000 X g. The sedimented fraction contained many microvesicles (0.2–0.5 μm diam.) which may be observed in an electron microscope. Figure 1 shows the freeze-fractured surfaces of microvesicles released from either erythrocytes or resealed ghosts. About half the microvesicles from slowly frozen erythrocytes showed a smooth surface without intramembrane particles and the other half showed rough surfaces with relatively few particles and an occasional clusters of particles (fig.1A). The number of particle-free microvesicles from erythrocytes exposed to 4 M NaCl at 0°C was slightly less than from slowly frozen samples. In contrast to the erythrocytes, most of the microvesicles released from slowly frozen ghosts exhibited a smooth surface (fig.1B), while those from ghosts exposed to 4 M NaCl at 0°C almost invariably showed a rough surface (fig.1C).

Table 1 shows the lipid composition of microvesicles, erythrocytes and resealed ghosts. The C:P ratio in microvesicles released by hypertonic saline treatment increased as the temperature of the treatment was lowered. The C:P ratio in microvesicles released from slowly frozen erythrocytes was higher than that of saline treated ones. It was also true that the C:P ratio in all microvesicles was higher than that of untreated erythrocytes. The C:P ratio in microvesicles released from resealed ghosts was higher still than those of microvesicles from erythrocytes.
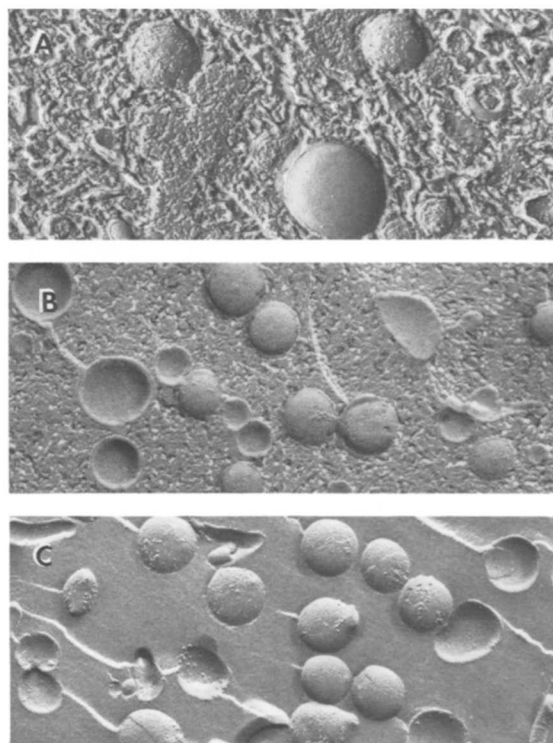

Fig.1. Freeze-fractured surface of microvesicles. (A) Microvesicles released from slowly frozen erythrocytes. (B) Microvesicles obtained from slowly frozen ghosts. (C) Microvesicles obtained from ghosts exposed to 4 M NaCl at 0°C. Magnification, X 33 000.

Table 1
Lipid composition of released microvesicles

Sample	Phospholipid[a] (mg)	Cholesterol[a] (mg)	$\dfrac{\text{Cholesterol}}{\text{Phospholipid}}$	% Released phospholipid
(A) Intact erythrocytes	81.8 ± 3.25 (10)	44.2 ± 2.21	0.54	(100)
Microvesicles (4 M NaCl, 0°C)	11.5 ± 0.55 (5)	6.7 ± 0.27	0.58	14
Microvesicles (4 M NaCl, −10°C)	13.9 ± 0.71 (7)	8.9 ± 0.45	0.64	17
Microvesicles (slow freezing)	3,3 ± 0.13 (10)	2.5 ± 0.11	0.76	4
(B) Resealed ghosts	62.5 ± 2.81 (6)	35.6 ± 1.78	0.57	(100)
Microvesicles (4 M NaCl, 0°C)	9.4 ± 0.38 (3)	5.8 ± 0.22	0.62	15
Microvesicles (4 M NaCl, −10°C)	10.1 ± 0.37 (3)	7.4 ± 0.29	0.74	16
Microvesicles (slow freezing)	3.7 ± 0.15 (6)	3.2 ± 0.12	0.89	5

[a] Values represent means ± SD, σ_n (no. experiments)

% Released phospholipid is calculated on the basis of original erythrocyte (A) and ghost (B) suspensions of 100 ml, respectively
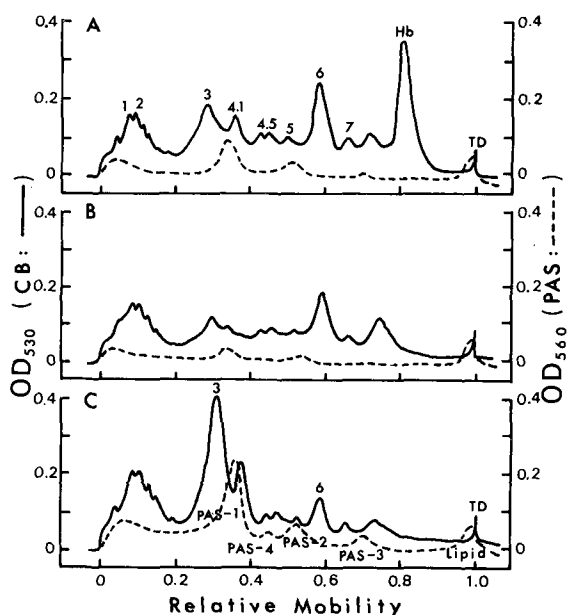
Fig.2. Densitometer tracings of electrophoretic pattern of microvesicle proteins. Microvesicles were obtained from slowly frozen erythrocytes (A), slowly frozen ghosts (B) and ghosts exposed to 4 M NaCl at 0°C (C). Equal amounts of microvesicle phospholipids were used for the electrophoresis. The disc gel (0.1% SDS, 5.6% polyacrylamide) was stained with Coomassie blue (CB: ————) or periodic acid–Schiff reagent (PAS: – – –).

Furthermore, the relative content of various phospholipid classes in microvesicles was similar to that of intact erythrocytes (data not shown).

Figure 2 shows the profiles of microvesicle proteins analyzed by SDS–polyacrylamide gel electrophoresis. The profiles of microvesicle proteins (fig.2) qualitatively corresponded to the morphology of microvesicles (fig.1). Microvesicles with smooth fractured surfaces showed only small peaks of band 3 and PAS-1 (fig.2B). In contrast, these polypeptides were the dominant component in microvesicles with rough fractured surfaces (fig.2C).

4. Discussion

It is shown here that either exposure to hypertonic saline or freeze–thawing results in the release of microvesicles from erythrocyte and resealed ghost

membranes. The facts that these microvesicles contained intrinsic membrane-proteins and the relative content of phospholipid classes in these microvesicles was similar to that of the whole membranes suggest that a fragment in the membrane might be released at structurally weak loci by osmotic stress. It is known that erythrocyte membranes are vesiculated by changing the levels of intracellular Ca^{2+} and ATP [12,13] suggesting that the formation of microvesicles is brought about by the accumulation of diacylglycerol produced by Ca^{2+}-stimulated enzyme reaction in the membranes. However, such an enzymatic microvesicle formation would not be stimulated at low temperatures.

In these experiments, moreover, the cholesterol content in these microvesicles increased in an inverse relation with the decreasing content of intrinsic membrane-proteins, as the temperature of the treatment was lowered. From these results, it is suggested that intrinsic membrane-proteins in the membranes would be dislocated with a concomitant segregation of cholesterol at low temperatures, and consequently cholesterol-enriched and particle-free microvesicles would be released from the membrane by osmotic stress. A major membrane-state transition in erythrocyte membranes was shown [14] to take place at temperatures between 0°C and −20°C. The possibility of cholesterol segregation in the membrane would be supported by the fact that the temperature ranges in which the ratio of C:P in microvesicles increased in the present work are consistent with their results. However, recent experiments suggest that the erythrocyte membrane contains both cholesterol-depleted boundary layers of phospholipid abutting membrane-proteins, relatively low cholesterol domains and cholesterol clusters [15–17]. It, therefore, remains unknown as to whether cholesterol-segregated domains at low temperatures or pre-existing cholesterol clusters are released by osmotic stress. Further evidence is required to explain the temperature-dependent segration of cholesterol in the membranes at low temperatures.

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