Ultrastructure of Erythrocytes during Calcium-Induced *In Vitro* Aging

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Vesiculation of the plasmalemma followed by fragmentation to microvesicles and formation of microspherocytes are shown to be the main phenomena during aging in rat erythrocytes. In human erythrocytes microvesiculation from the apexes of echinocyte spicules and hemolysis in echinocytes II-IV are predominant. Hemolysis, vesiculation, and microvesiculation are observed in rabbit, and hemolysis and microvesiculation in canine erythrocytes. An increased level of free fatty acids (about 30% per hour) in human and rat erythrocytes is also detected.

Key Words: *ultrastructure of erythrocytes; calcium-induced aging*

Many investigators suppose the main causes of aging of erythrocytes both *in vivo* and *in vitro* to be catabolic changes in their cytoskeleton and plasmalemma induced by a drop of ATP and a rise of intracellular calcium [4,5,7,10]. In modeled aging of human erythrocytes in the presence of calcium ions and calcimycin structural echinocytic transformation of the erythrocytes with the subsequent detachment of 0.2-μ vesicles is noted which is a characteristic feature of irreversible aging [5,6,10].

The aim of the present study was a detailed investigation of morphological transformation and vesiculation during *in vitro* aging of human, rat, rabbit, and canine erythrocytes, and the dynamics of the level of free fatty acids (FFA) as an indicator of the hydrolysis of membrane lipids, since there are several data on the accumulation of lysolecithin [2], diacylglycerides, and phosphatidylinosite [4,10] in erythrocytes during aging.

MATERIALS AND METHODS

Suspensions of human, rat, rabbit, and canine erythrocytes were obtained by three-time centrifugation in isotonic phosphate buffered saline; the hematocrit of the test suspension was 5%. Calcium-induced aging was achieved by incubating the erythrocyte suspension in isotonic phosphate buffered saline containing 2 mM $CaCl_2$ and 5 μM calcimycin at 37°C; samples were taken after 1, 5, 15, 30, and 60 min. The rate of erythrocyte hemolysis was spectrophotometrically assessed by measuring the content of hemoglobin in the supernatant after 10-min centrifugation at 3000 g. The volume of rat erythrocytes was evaluated with an ABX cell blood automatic counter. Ultrastructural changes of the erythrocytes were evaluated with a Hitachi S-500 scanning electron microscope at an accelerating voltage of 20-25 kV. Samples for microscopy were prepared as described earlier [8]. Different forms of erythrocytes were counted both from the display and from electronograms comprising no less than 200 cells and expressed in percents. Four groups of cells were distinguished among echinocytes. Erythrocytes which had lost the disk shape and had several tubercular processes were considered as echinocytes I. The cells with 25-30 regular conic processes were echinocytes II. In echinocytes III these processes were sharp-ended with some signs of microvesiculation. Echinocytes IV, or spheroechinocytes, represented globular cells with sparse thin processes, or spicules, on their

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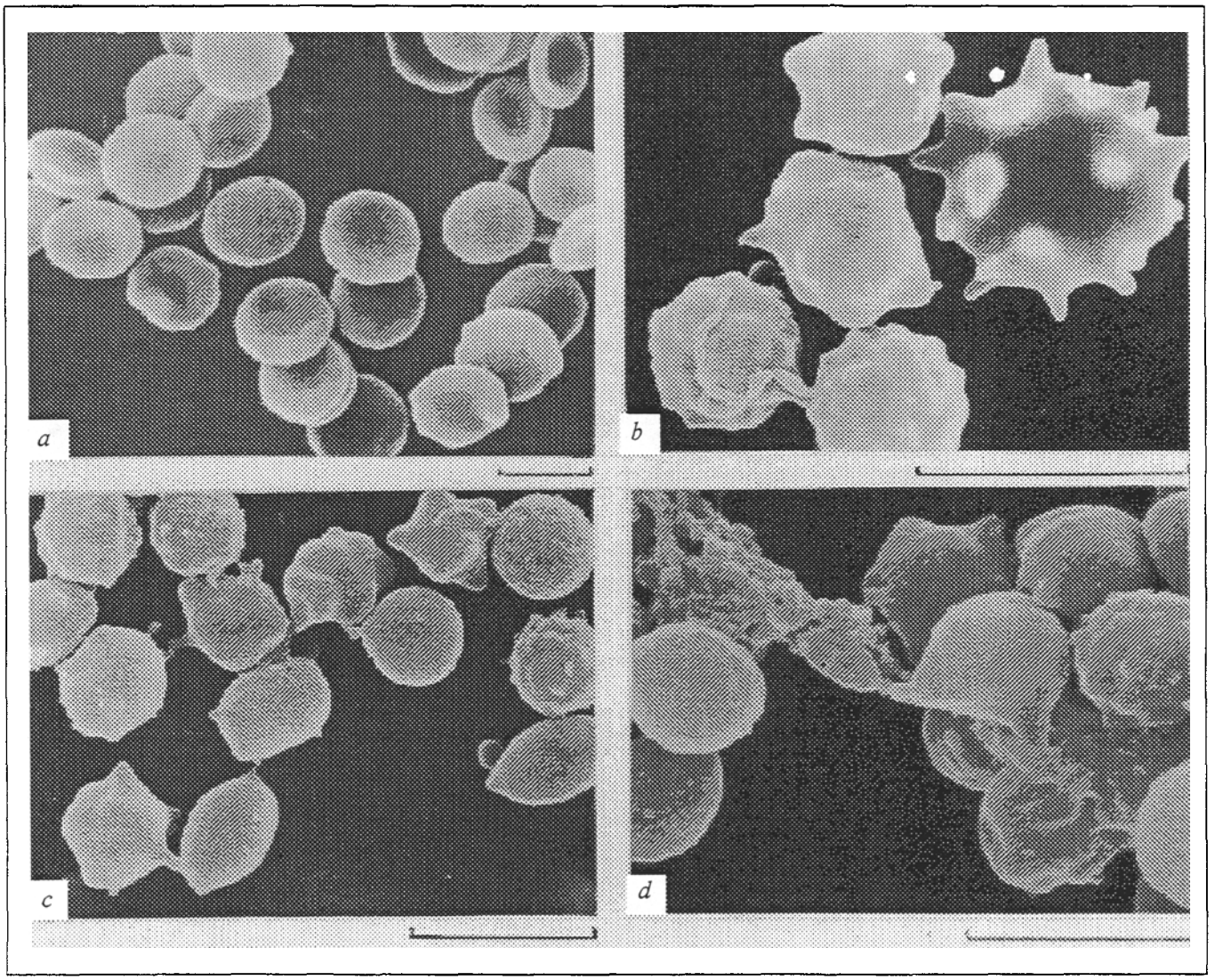

Fig. 1. Rat erythrocytes during *in vitro* aging. *a*) normal rat erythrocytes; *b*) echinocytes II and III with signs of vesiculation, microvesiculation, and fragmentation of vesicles (15-min incubation); *c*) echinocytes II and IV with signs of vesiculation, fragmentation of vesicles, and microvesiculation (30-min incubation); *d*) microspherocytes, hemolysis (30-min incubation). SEM, scale 5 μ.

surface. The content of FFA in the erythrocyte plasmalemma was determined after Anderson with modifications proposed by Nikitina *et al.* [1] . Lipolytic activity of the plasmalemma was assayed by the increase in the content of FFA after 1-hour incubation with 2 mM $CaCl_2$ at 37°C. The content of protein in the plasmalemma was measured using the biuret reaction.

RESULTS

Maximal variation in morphological phenomena during aging was observed in rat erythrocytes (Fig. 1).

As soon as after 1-min incubation of freshly isolated rat erythrocytes the number of echinocytes attained 35-40% (10-12% in the norm). The 5-min incubation led to a virtually complete transformation of erythrocytes to echinocytes I and II, and to the appearance of echinocytes III, while echinocytes II and IV were noted after 15-min incubation. Even at this stage and especially during further incubation an intense vesiculation and microvesiculation from the entire surface of the plasmalemma of echinocytes III and IV were observed. The detached vesicles varied considerably in both shape and size. The loss of membrane material by erythrocytes occurred either in the form of single spheres up to 0.6 μ or in the form of 0.1-0.2-μ-long processes with a diameter of 1-2 μ. Thereafter different variants of fragmentation of these processes to spherical vesicles and cylindrical formations undergoing subsequent fragmentation to 0.1-0.2-μ microspheres were observed (Fig. 1, *b, c*). Due to this vesiculation the mean volume of erythrocytes decreased from 55 to 41 μ^3, which corresponded to a 30-35% loss of erythrocyte sur-

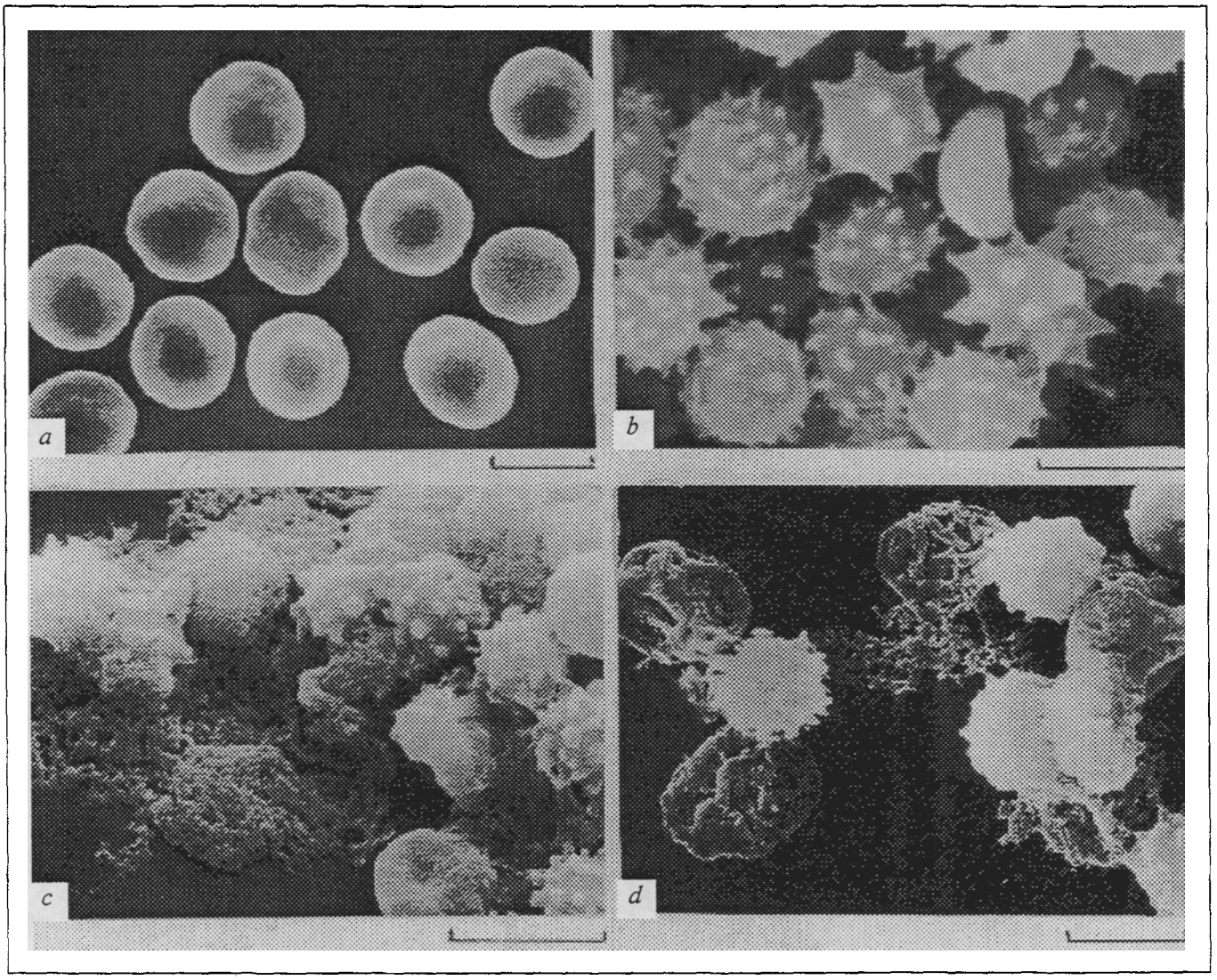

Fig. 2. Human erythrocytes during *in vitro* aging. a) normal human erythrocytes; *b*) echinocytes II–IV with signs of microvesiculation (15-min incubation); *c*) echinocytes II–IV (hemolysis); *d*) echinocytes II–IV (hemolysis, ghosts). SEM, scale 5 μ.

face and resulted in the transformation of erythrocytes to microspherocytes with a diameter of 3-4 μ; thereafter a discharge of hemoglobin occurred most commonly through a single defect of the plasmalemma (Fig. 1, *d*). In some cases hemolysis of spheroechinocytes also occurred.

In analogous experiments with human erythrocytes the transition from diskocytes to echinocytes during aging also occurred rapidly, but the succeeding picture was different (Fig. 2). At the stage of echinocytes III and IV vesiculation occurred just from the apexes of echinocyte spicules (Fig. 2, *b*). Detached microvesicles were up to 0.2 μ in diameter. A characteristic feature of aging of human erythrocytes was pronounced hemolysis (Fig. 2, *c*) in echinocytes II, III, and IV, which resulted in the formation of erythrocyte ghosts with 50-80-nm defects of the membrane (Fig. 2, *d*).

Phenomena of hemolysis, vesiculation, and microvesiculation during aging were also observed in rabbit, and hemolysis and microvesiculation in canine erythrocytes.

The rate of hemolysis in human and animal erythrocytes was assessed by spectrophotometric measurement of hemoglobin leaking from the erythrocytes. The maximal rate of hemolysis was observed in rabbit, and the minimal (5-fold lower) in human erythrocytes.

In these experiments the level of FFA in rat erythrocytes was shown to increase from 9 to 12 nmol FFA/mg protein per hour, and in other experiments (incubation of rat erythrocyte ghosts in the presence of 2 mM $CaCl_2$) this rise was 3.2 nmol FFA/mg protein per hour). Special experiments showed that the process of Ca-induced aging was not accompanied by marked

accumulation of thiobarbituric acid-reactive substances.

Thus, by comparing our results with the data of other investigators we can determine two key events in the process of aging in erythrocytes. The first is an accumulation of calcium in the cytosol, manifesting itself in the transformation of diskocyte to echinocyte I-II, and the second is a destabilization of the plasmalemma bilayer due to activation of phospholipases A and C, resulting in an accumulation of FFA, lysophospholipids, and diacylglycerides. This leads to a physical separation of the membrane cytoskeleton and plasmalemma [9], followed by a detachment of the membrane vesicles without reticular structure (echinocytes III-IV). These membrane vesicles and microvesicles have been previously shown [3,9] to be enriched with proteins of the 3, 4.1, and 7 bands of the erythrocyte membrane, to contain minor amount of hemoglobin, and to have no proteins of the cytoskeleton (spectrin, actin). This results in the conversion of erythrocytes to microspherocytes, hemoglobin-containing ghosts, and microvesicles. The ration between these products of aging varies in different biological species. These differences may be attributed to the various activity of phospholipases A and C, differences in cytoskeleton, and differences in the systems of active ion transport.

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