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Reducing Effect of Steroids on Fragility of Human Erythrocytes Detected by a Prompt Hemolysis Test

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ABSTRACT. Steroids reduced the fragility of human eythrocytes. Progesterone was so far the most effective (1.25×10^{-5}～1.0×10^{-4}M). This can be detected by a prompt hemolysis test, but not by the currently-used hemolysis test (Sanford method).

(^{14}C) progesterone can be easily uptaken and released by the erythrocyte membrane. Discocytes were transformed into stomatocytes and spherocytes by the addition of progesterone (5×10^{-5} M～2×10^{-4}M).

Key words : Steroids — Human erythrocytes — Fragility and shape

Currently-used hypotonic hemolysis tests consist of Sanford method,[1] Parpart method,[2] and coil planet centrifuge system method,[3] in which erythrocytes were exposed to a plain hypotonic saline solution after being preincubated with the test drugs. Those methods were suitable for testing such drugs as saponins and bile acids which can break the membrane structures or bind tightly to the membrane components, but failed to show any effect of 4-pregnene-3, 20-dione (progesterone). However a prompt hemolysis test described here has proved an inhibitory effect of progesterone on the hemolysis of human erythrocytes suggesting that the action mechanism of progesterone is different from those of saponins and bile acids. In this study, the effect of progesterone on erythrocytes was shown by the uptake and release experiments with (^{14}C) progesterone and the erythrocyte morphology. Moreover, structure-activity relationship of steroids with regard to reducing effect on the fragility of erythrocytes was studied by the prompt hemolysis test.

MATERIALS AND METHODS

Freshly drawn heparinized human blood from healthy adults was centrifuged at 2,500 rpm for 5 min to remove the plasma and buffy coat. The sedimented erythrocytes were rinsed three times with isotonic saline solution.

In the prompt hemolysis test, 50 μl of progesterone (Sigma Chemical Co.) dissolved in ethanol (the final concentration 1 (v/v)% was added to 5 ml of hypotonic sodium chloride solution (140 mOsm/kg) to give the final concentration

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of progesterone from 6.25×10^{-6} to 1.0×10^{-4} M. 50 μl of the erythrocyte suspension made up to about 50 % hematocrit value was added to this solution, gently mixed and allowed to stand for 5 min at room temperature. Then the tube was centrifuged at 2,000 rpm for 5 min, and the optical density of the supernatant was measured in a HITACHI spectrophotometer 100-60 at 540 nm. The same procedure without progesterone was performed as a control. Relative hemolysis is expressed as a ratio of absorbance of the released hemoglobin in the supernatant with progesterone to that of the control. Comparison of various steroids was carried out by the prompt hemolysis test mentioned above with 2.0×10^{-4} M steroids (Sigma Chemical Co.).

In the hemolysis test of Sanford, one volume of the washed erythrocytes was added to 100-fold of the isotonic saline solution containing progesterone ($6.25\times10^{-6}\sim1.0\times10^{-4}$ M), and then centrifuged to remove the supernatant after incubation for 1 hr at 37°C. 50 μl of the sedimented erythrocytes was transferred into 5 ml of the hypotonic sodium chloride solution (140 mOsm/kg). After 5 min standing, the optical density of the released hemoglobin was measured as described in the prompt test. Uptake of (^{14}C) progesterone (57.2 mCi/mMol, New England Nuclear Co.) into erythrocytes was estimated by the decrease of the radioactivity in the extracellular solution. 2 ml of the packed washed erythrocytes was added to 30 ml of the isotonic saline solution (150 mM NaCl, 0.3 (w/v)% d-glucose, 1 (v/v)% ethanol) containing 8.0×10^{-5}M (^{14}C) progesterone. The suspension was centrifuged at 2,500 rpm for 5 min after incubation for 0, 2, 6, 20 and 30 hr at 37°C, and the radioactivity of the supernatant was measured. Release of progesterone from the erythrocytes preincubated with (^{14}C)progesterone was determined by the radioactivity of the washing solution of the erythrocytes. The erythrocytes incubated with (^{14}C)-progesterone for 0 and 20 hr at 37°C were centrifuged at 2,500 rpm for 5 min, and the supernatant was completely removed. This erythrocyte layer was washed four times with the same volume of isotonic saline solution as removed, and the radioactivity of each washing was measured.

The radioactivity was determined by an ALOKA scintillation spectrometer (model LSC-700). 0.5 ml of each sample was mixed with 5 ml of scintillation solution containing 5.5 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis(2-(5-phenyl-oxazolyl)) benzene, 667 ml of toluene, and 333 ml of Triton X-100.

Morphological study on the effect of progesterone on erythrocytes was done by a scanning electron microscopy. 50 μl of washed erythrocytes was gently mixed with 5 ml of isotonic saline solution (145 mM NACl, 10 mM Tris-HCl : pH 7.4, 0.3 (w/v)% d-glucose, 0.5 (v/v)% ethanol) containing each 0, 5.0×10^{-5}, 1.0×10^{-4} and 2.0×10^{-4}M progesterone, and incubated for 12 hr at 37°C. A small amount of each suspension was dropped on coverslips which were dipped in 1% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 50-60 min at 4°C. After three times washing in the isotonic PBS, the samples were fixed in 1% osmium tetroxide in PBS for 50-60 min at 4°C, dehydrated in a graded series of ethanol and dried in a Hitachi HCP-1 critical point drying apparatus after two times dipping in iso-amyl acetate for 10 min. They were coated with gold-palladium in a Hitachi IB-2 ioncoater and examined with a Hitachi HHS-2R scanning electron microscope at accelerating voltage of 20 KV. 800 to 1,000 erythrocytes were observed in each sample at a mag-

nification of 2,000 and classified into discocyte, echinocyte I, II and III, spherechinocyte, stomatocyte and spherocyte according to Bessis' criteria.[4]

RESULTS AND DISCUSSION

Progesterone showed no effect on the fragility of the human erythrocyte membrane by the hemolysis test of Sanford. However, progesterone significantly inhibited the hypotonic hemolysis at the concentration higher than 1.25×10^{-5} M by a prompt hemolysis test (Fig. 1). As a comparison several steroids were tested by the prompt hemolysis test at the concentration of $2.0\%\times10^{-4}$ M (Table 1). Progesterone was the most effective to protect hemolysis (relative hemolysis

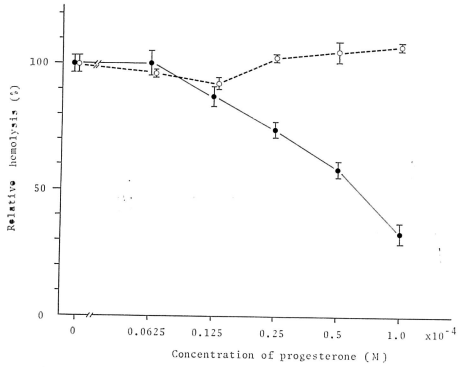

Fig. 1. Inhibitory effect of progesterone on hemolysis by the prompt hemolysis test compared with the currently-used hemolysis test (Sanfod method). Progesterone significantly decreased the fragility of erythrocyte membrane by the prompt hemolysis test (●——●), however, this effect was scarcely shown by Sanford's test (○---○). Each point represents the mean ± S.D. of five measurements.

TABLE 1. Effect of steroids on the hemolysis of human erythrocytes.

steroid	relative hemolysis (%)
4-pregnene-3, 20-dione (progesterone)	11.9 ± 0.5*
4-pregnene-17α-ol-3, 20-dione (17α-hydroxyprogesterone)	85.4 ± 6.2
4-pregnene-11α-ol-3, 20-dione (11α-hydroxyprogesterone)	88.3 ± 5.6
4-pregnene-17α, 21-diol-3, 11, 20-trione (cortisone)	88.9 ± 5.4
4-pregnene-11β, 2l-diol-3, 20-dione (corticosterone)	80.4 ± 1.4
4-pregnene-11β, 17α, 21-triol-3, 20-dione (cortisol)	88.1 ± 5.2
5α-androstane-3, 17-dione	31.1 ± 4.3*
5α-androstane-17β-ol-3-one	78.4 ± 6.8
5α-androstane-3α-ol-17-one	81.6 ± 4.9
5α-androstane-3α, 17β-diol	105.9 ± 6.8
4-androstane-3, 17-dione	95.4 ± 2.9
4-androstane-17β-ol-3-one (testosterone)	90.3 ± 7.4
1, 3, 5-estratriene-3, 17β-diol (estradiol-17β)	86.9 ± 2.8
1, 3, 5-estratriene-3, 16α, 17β-triol (estriol)	88.0 ± 7.6

Each steroid was added in the hypotonic saline solution at the final concentration of 2.0×10^{-4}M.
*$p<0.01$

11.9±0.5%). However, the introduction of a hydroxyl group at 17α- or 11α-position greatly reduced the protective effect of progesterone (relative hemolysis 85.4±6.2, 88.3±5.6%, respectively). Glucocorticoids having the hydroxyl groups at 21-position and 11- or 17-positions were scarcely protective from the erythrocyte lysis. 5α-androstane-3,17-dione had a definite effect (relative hemolysis 31.1±4.3%), but either 3α- or 17β-hydroxyl derivatives and 3α, 17β-dihydroxyl derivatives of this compound had almost no effect. Uptake of (^{14}C) progesterone by erythrocytes was estimated by the reduction of radioactivity in the suspending saline solution after the addition of erythrocytes (Fig. 2). The radioactivity of the suspending solution was immediately reduced from 1.41×10^4 to 1.05×10^4 dpm after the addition of erythrocytes, but thereafter it remained constant throughout the 30 hr incubation. (^{14}C)progesterone taken

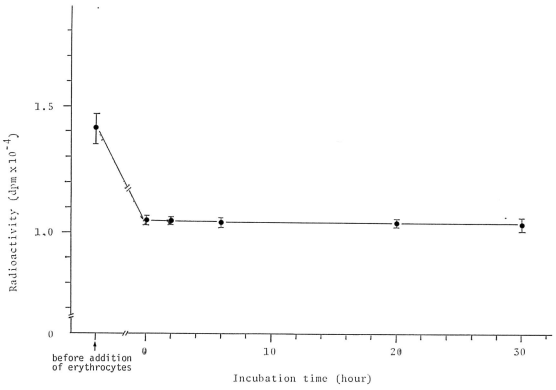

Fig. 2. Uptake of (^{14}C)progesterone by erythrocytes. The radioactivity of the suspending solution after the addition of erythrocytes was reduced immediately but thereafter it remained constant during 30 hr incubation. Each point represents the mean ± S.D. of five measurements.

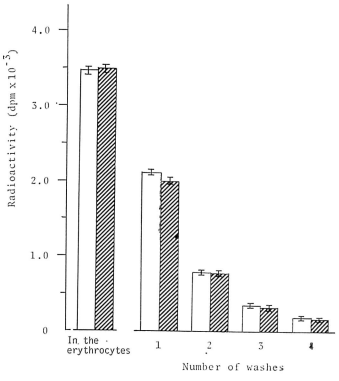

Fig. 3. Release of (^{14}C)progesterone from erythrocytes after washing. Each column shows the mean of five measurements of radioactivity of the supernatant obtained from the erythrocytes suspension preincubated wiih (^{14}C)progesterone for 0 hr (□) and 20 hr (▨). The left two columns show the radioactivity in the erythrocytes before washing. After four washes the greater part of the radioactivity flew out of erythrocytes.

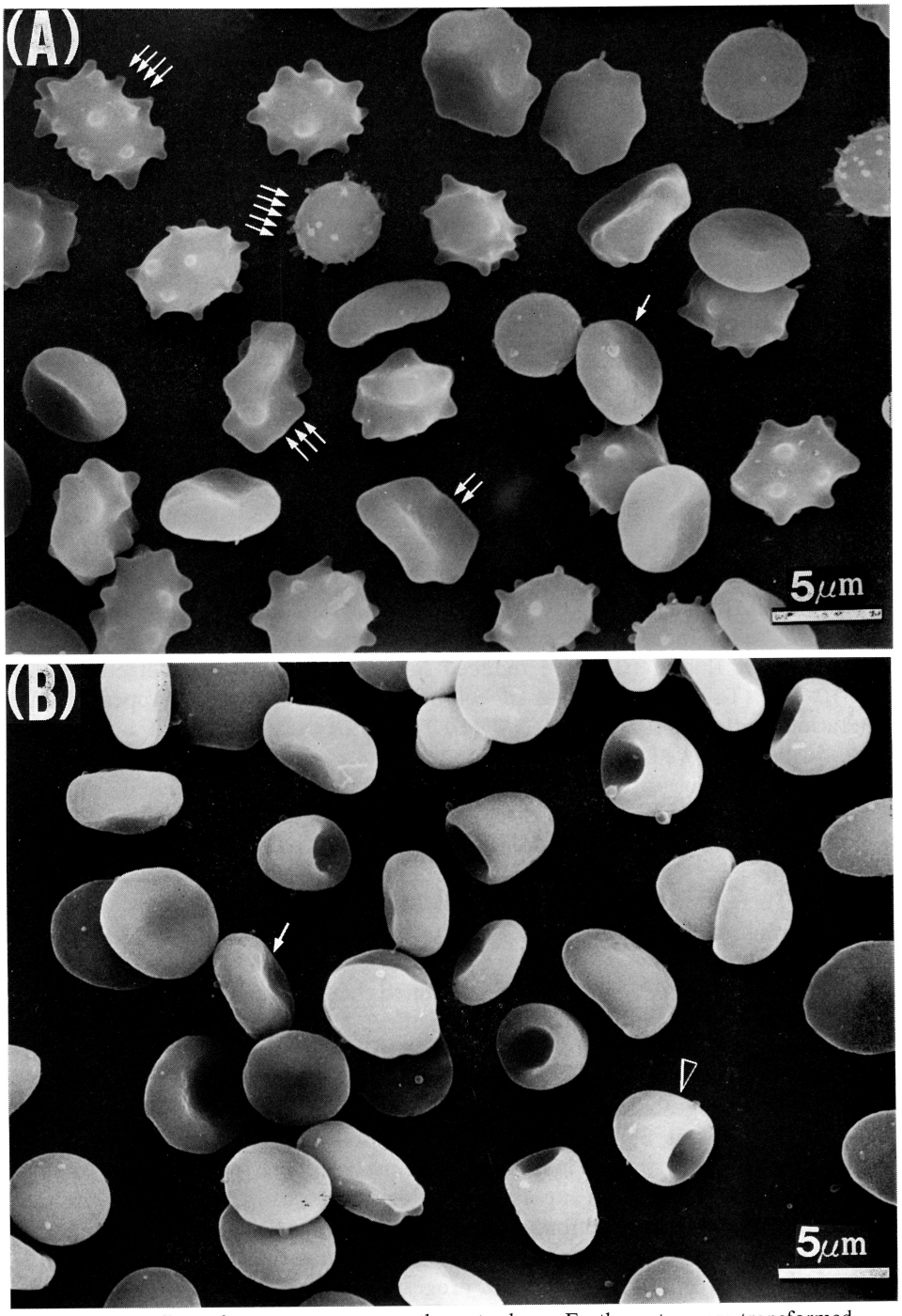

Fig. 4. Effect of progesterone on erythrocyte shape. Erythrocytes were transformed from the original discocyte (↓) into echinocyte I (↓↓), II (↓↓↓) and III (↓↓↓↓) and sphero-echinocyte (↓↓↓↓↓) after incubation without progesterone (A) for 12 hr at 37 °C. However, progesterone (1.0×10 M) (B) inhibited this transformation and, in addition, induced stomatocyte (▼).

into the erythrocytes during 0 or 20 hr incubation can be easily released into isotonic saline solution (Fig. 3). After four washes of erythrocytes 98 percent of the radioactivity flew out of the erythrocytes. These results show that progesterone can easily enter into and go out of erythrocytes. While progesterone stays in the erythrocytes, it can protect their membrane from the hypotonic lysis. It suggests that progesterone may interact with the membrane structure. On this point the effect of progesterone on the shape of erythrocytes provides the further evidence. When the washed erythrocytes were incubated for 12 hr at 37°C in isotonic saline solution, discocytes became the echinocytes (77.9%) and spherо-echinocytes (3.3%) (Fig. 4A, Table 2). After incubation with progesterone

TABLE 2. Effect of progesterone on erythrocyte shape.

erythrocyte shape (%) / concentration of progesterone (M)	discocyte	echinocyte			sphero-echinocyte	stomatocyte	spherocyte
		I	II	III			
0	18.8	36.4	20.1	21.4	3.3	0	0
5.0×10^{-5}	43.9	48.9	4.2	2.1	0.3	0.7	0
1.0×10^{-4}	69.9	3.0	0.4	0.2	0.1	26.3	0
2.0×10^{-4}	6.3	0.5	0	0	0	11.5	81.8

at the concentration of 5.0×10^{-5} and 1.0×10^{-4} (Fig. 4B) M, a greater number of erythrocytes remained as discocytes (43.9% and 69.9%, respectively), and the echinocytes (I, II and III) were reduced in number and hardly detectable with 2.0×10^{-4} M (Table 2). Instead of echinocytes the stomatocytes appeared after incubation with 1.0×10^{-4} and 2.0×10^{-4} M progesterone (26.3% and 11.5%, respectively), followed by the appearance of spherocytes (81.8% with 2.0×10^{-4} progesterone). Those transformation of erythrocyte shapes provides the evidence that progesterone did not pass through the erythrocyte membrane but it was trapped by the membrane components. Considering the formation of stomatocyte, progesterone might be inserted into the inner half of the erythrocyte membrane.[5] This morphological effect of progesterone was abolished by a minor structural change such as 17α- or 11α-hydroxylation. 17α-hydroxyprogesterone did not produce the stomatocytes nor spherocytes even at a higher concentration (2.0×10^{-4}M) and merely inhibited the transformation of echinocytes (data not shown). Steroids have distinct effect on the membrane stabilization and membrane-associated functions in erythrocytes,[6-8] nerve cells,[6,7,9,10] uterine cells[11] and Xenopus oocyte.[12] In addition, progesterone extends the life of erythrocytes during storage in the blood bank even in lower concentration.[13] Those effect can not be mediated by the cytosolic steroid receptors but progesterone interacts with the membranes themselves. The present study showed that progesterone could be uptaken and released easily by the erythrocyte membrane. Within the membrane progesterone protected the erythrocytes from the hypotonic lysis and it induced the transformation from discocytes into stomatocytes. It is conceivable that progesterone could be trapped into the inner half of the membrane bilayer and stabilize it. Relative hemolysis values of several steroids (Table 1) show there is no direct relationship between the polarity of the steroids and the protection of hemolysis, suggesting that steroids can be bound to the lipid components of the membrane with structural

specificity.

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