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# Detection of Reversible and Irreversible Changes of Erythrocyte Osmotic Fragility Induced by Yoshida Tumor Cells in Rats

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**Abstract**—*A coil planet centrifuge (CPC) is an apparatus for the dynamic measurement of erythrocyte osmotic fragility, and it was applied to the observation of altered erythrocyte osmotic fragility induced by Yoshida tumor cells. The osmotic fragility of erythrocytes directly collected from the vein passing through the site of tumor inoculation began to increase from day* 4 *post-inoculation, whereas the osmotic fragility of the erythrocytes collected from the vein through the tumor-free (normal) region of the same animal remained normal until day* 8 *post-inoculation. This discrepancy in osmotic fragility suggests the presence of a plasma factor which recovers the altered erythrocyte membrane, the hypothesis being strongly supported by the results of our* in vitro *experiments. On day* 10 *post-inoculation, osmotically fragile erythrocytes failed to respond to the plasma treatment* in vitro, *suggesting that an irreversible change had occurred on the erythrocyte membranes.*

## INTRODUCTION

COIL PLANET centrifugation is a rapid method for measuring erythrocyte osmotic fragility with a minute sample, which consists of a coil planet centrifuge, a coiled sample tube, a coil incubator, an osmotic gradientor and a scanning photodensitometer [1, 2]. It is particularly suitable for the observation of altered membrane properties of erythocytes in hematologic disorders [3] and hepatobiliary disorders [4].

Altered physiological states observed in tumor-bearing animals that involve a variety of changes in various organ systems are well-known to laboratory investigations [5]. Dumont *et al.* [6], who measured the altered erythrocyte osmotic fragility observed in mice with Ehrlich ascites tumor 15 days after inoculation, reported a uniform pattern of abnormal resistance to hemolysis in hypotonic saline. In this paper, however, we present data of increased osmotic fragility of rat erythrocytes directly obtained from the vein via Yoshida tumor (tumor vein) compared with those from the vein through the tumor-free region of the same animal, and also *in vitro* studies using a short culture system of tumor cells and erythrocytes.

## MATERIALS AND METHODS

*Measurement of erythrocyte osmotic fragility*

A coil planet centrifuge (CPC) is an apparatus having a pair of rotating coil holders carrying coiled tubes filled with samples. The holders are rotated to produce a high centrifugal acceleration acting vertically to the axis of rotation. The coil holders revolve at 1600 rev/min around the main axis, with a self-rotation of 16 rev/min comparable to a planet revolving around the sun with its concomitant self-rotation [2]. Ten $\mu$l of blood is applied to the linear osmotic gradients in the coiled tube and subjected to centrifugation. One is able to observe the membrane properties of erythrocytes in terms of the shift of the hemolysis band appearing in the coiled tube. In this experiment, coil planet centrifugation has been applied to the observation of altered membrane properties of erythrocytes induced by Yoshida tumor cells.

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*Tumor cells*

Yoshida sarcoma cells were kindly offered from the Shionogi Laboratory Research and maintained by the i.p. inoculation of tumor cells in Wistar rats (male, 50 g).

In vivo *experiment*

Tumor was inoculated into the right footpad of Wistar rats (male, 150 g) by the s.c. injection of $1 \times 10^7$ tumor cells suspended in 0.05 ml saline. Tumor growth was estimated by measuring the thickness of the tumor-injected foot with a caliper. The blood samples collected from the right femoral vein (tumor vein), which drained the blood that had passed through the tumor, and from the left femoral vein (non-tumor vein) served for the osmotic fragility test.

In vitro *experiment*

One milliliter of mixture of 10% erythrocytes and 10% Yoshida tumor cells suspended in Eagle's MEM medium (Eagle's Minimum Essential Medium with Earle's Balanced Salt Solution) was incubated at 37°C for 30 min. After incubation supernatant and cells were separated by centrifugation, and then incubated erythrocytes and Yoshida tumor cells were separated on the basis of difference in density by gum acasia solution (specific gravity: 1.066 g/ml) [7, 8]. *In vitro* incubated erythrocytes with tumor cells served for the osmotic fragility test. Supernatant effect on normal erythrocytes and normal plasma effect on incubated erythrocytes with tumor cells were examined by osmotic fragility tests. As a control experiment erythrocytes were incubated with liver parenchymal cells prepared by the method of Berry and Friend [9] and separated by gum acasia solution (specific gravity: 1.077 g/ml).

## RESULTS

In vivo *experiments*

*Tumor growth and hematocrit.* The increase in size of inoculated tumor is presented in Fig. 1. The tumor suddenly began to expand 4–6 days after inoculation of tumor cells and maintained its size 6–12 days after inoculation, whereas the thickness of the footpads of control animals was 0.5 mm throughout the 12 days. By histologic examinations after 4 days of inoculation, tumor cells invaded the normal tissue of the right footpad and some hemorrhages were found there, the extremely massed venous vessels in the tumor representing the most striking difference between tumor and normal tissue microvasculature. Venule

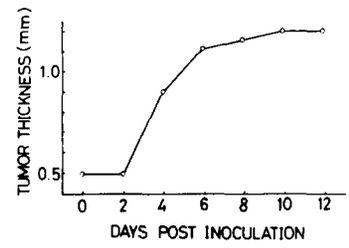


*Fig. 1. Wistar rats received one s.c. injection of Yoshida sarcoma cells ($1 \times 10^7$ cells/0.05 ml saline) into the right footpad. Size of increasing tumor was monitored by measuring the thickness of foot. Each point represents the average of 6 experiments. The thickness of untreated control animals was 0.5 cm for 0–12 days.*

diameter typically increased during tumor growth, and the right femoral vein (tumor vein) dilated more markedly than the left femoral vein (non-tumor vein).

Hematocrit was measured by a micro-hematocrit spinning technique (12,000 rev/min 5 min). The hematocrit value of the normal rat (day 0) was 40.5 ± 1.8% and that of the tumor-bearing rat (day 4) slightly increased to 42.5 ± 2.5%. From day 6 the hematocrit value began to decrease and the value was 34.3 ± 1.9% on day 10 (Fig. 2).

*Measurement of osmotic fragility of erythrocytes from tumor vein and non-tumor vein.* The osmotic fragility of erythrocytes of the tumor vein (right femoral vein) collected from the blood passed through the inoculated tumor was compared with that of the non-tumor vein (left femoral vein), as shown in Fig. 3. The osmotic fragility of erythrocytes of the tumor vein increased suddenly from day 4 and that of the non-tumor vein increased from day 8 post-inoculation. The discrepancy of erythrocyte osmotic fragility between the tumor vein and the non-tumor vein on days 4 and 6 suggests that erythrocytes with increased osmotic fragility passing through the tumor were refreshed by a plasma component in the systemic circulation. Therefore the plasma effect on osmotically fragile erythrocytes was examined following *in vitro* incubation. Osmotically fragile erythrocytes collected from the

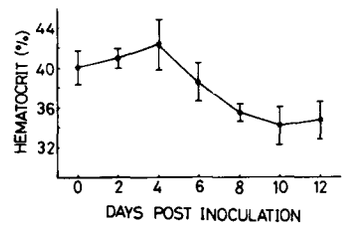


*Fig. 2. Hematocrit of Yoshida tumor rats was measured by micro-hematocrit spinning. Values are means ±S.E.M. of determinations on 6 separate experiments.*

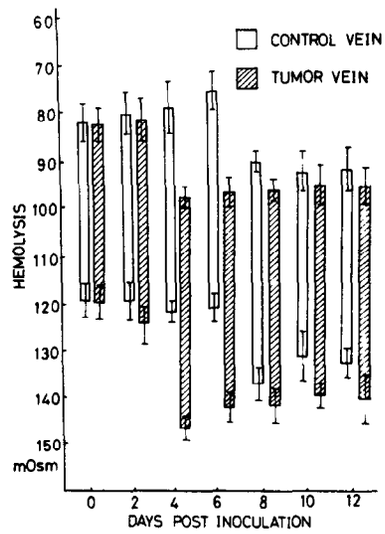


*Fig. 3. Osmotic fragility of erythrocytes from rats with the Yoshida tumor. Erythrocyte samples were collected from the tumor vein (right femoral vein) and from the control vein (left femoral vein) as a non-tumor vein. Value are means ±S.E.M.* (n = 6).

tumor vein on days 4, 6 and 10 (Fig. 4A) were incubated with fresh rat plasma at 37°C for 30 min and the osmotic fragility was measured. The erythrocytes of the tumor vein on days 4 and 6 recovered completely to the normal level of osmotic fragility after incubation with fresh plasma (Fig. 4B). On day 10, however, complete recovery could not be observed. Hemolysis of erythrocytes of the tumor vein on day 10 began at 140.4 ± 2.6 mosmol and ended at 94.5 ± 4.2 mosmol, and after incubation with fresh plasma hemolysis began at 145.0 ± 4.9 mosmol

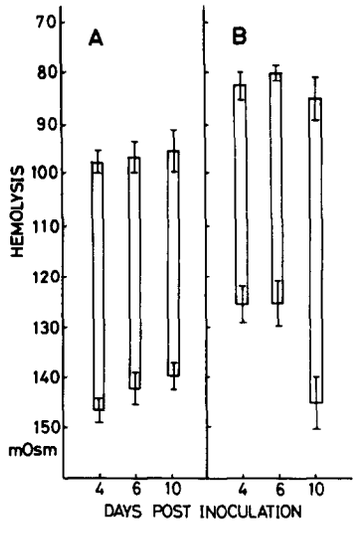


*Fig. 4. Effect of fresh rat plasma on osmotically fragile erythrocytes collected from the tumor vein. (A) Osmotic fragility of erythrocytes from the tumor vein in tumor rats days 4, 6 and 10 post-inoculation; and (B) the same samples after incubation with fresh rat plasma (Ht 50%) at 37°C for 30 min. Values are means ±S.E.M.* (n = 6).

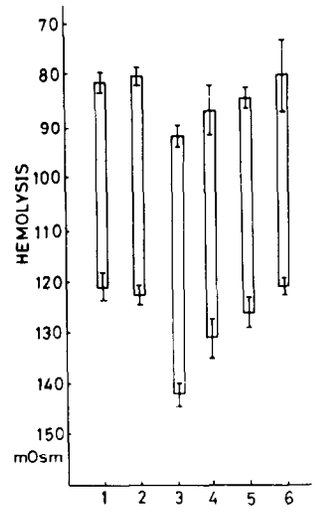


*Fig. 5. Effect of Yoshida tumor cells on erythrocyte osmotic fragility in the* in vitro *experiment.* (1) *Normal rat erythrocytes;* (2) *erythrocytes treated with gum acasia medium;* (3) *erythrocytes separated by gum acasia medium after incubation with Yoshida tumor cells at* 37°C *for* 30 min; (4) *erythrocytes incubated at* 37°C *for* 30 min *with the supernatant obtained from a pre-incubated suspension of erythrocytes and Yoshida tumor cells at* 37°C *for* 30 min; (5) *erythrocytes separated from a pre-incubated suspension of erythrocytes and Yoshida tumor cells at* 37°C *for* 30 min *were incubated with fresh rat plasma at* 37°C *for* 30 min; (6) *erythrocytes separated by gum acasia medium after incubation with liver parenchymal cells at* 37°C *for* 30 min. *Values are means ±S.E.M.* (n = 6).

and ended at 85.1 ± 4.2 mosmol. This result shows that the osmotically fragile sub-population of erythrocytes from the tumor vein on day 10 could not respond to a plasma factor, suggesting that irreversible alteration occurred on the erythrocyte membrane.

In vitro *experiments*

Normal erythrocytes and Yoshida tumor cells were cultured to examine the tumor effect on erythrocytes. Gum acasia solution used as a separating medium had no toxicity against erythrocytes because the osmotic fragility of erythrocytes revealed no changes before and after gum acasia treatment (Fig. 5-1 and 5-2). The hemolysis of normal erythrocytes incubated with Yoshida tumor cells began at 142.0 ± 2.2 mosmol and ended at 91.0 ± 2.2 mosmol (Fig. 5-3), and the supernatant obtained by centrifugation of the incubated mixture of normal erythrocytes and Yoshida tumor cells had an increased effect of osmotic fragility on normal erythrocytes (Fig. 5-4). Osmotically increased erythrocytes induced by Yoshida tumor cells (Fig. 5-3) recovered to the normal fragility level by incubating with fresh plasma (Fig. 5-5). Liver parenchymal cells, used as non-tumor cells, had no effects on erythrocyte osmotic fragility (Fig. 5-6).

## DISCUSSION

The hemolytic response of erythrocytes to hypotonic stress is well-known [10–13]. In the coil planet centrifuge system the erythrocytes set in a coiled tube were forced to pass through a saline solution having a linear osmotic gradient, and were exposed to a gradually changing osmotic pressure down to a point where hemolysis occurs. CPC requires only a minute amount (10 $\mu$l) of blood as a test sample, and slight changes in osmotic fragility can be detected more easily by CPC than by other methods [2]. With the Parpart method for osmotic fragility, rat red cells start hemolysing at 155 mosmol and are completely hemolysed at 125 mosmol. It is not clear why with the coil planet centrifuge method this range is shifted to the low osmolality. However, such a discrepancy may be attributed to differences in the time of exposure of the red cells to the osmotic gradient. This report describes the altered osmotic fragility of erythrocytes measured by CPC in tumor-bearing animals. Other authors reported the altered osmotic fragility of erythrocytes in patients with cancer [14] and in mice with ascites tumor [6], and they prepared the blood samples from the systemic circulation. In our experiment, however, blood was collected from the polyethylene tube inserted into the tumor vein, and increased osmotic fragility of erythrocytes was detected even in an early stage of tumor growth, although systemically circulating erythrocytes was the normal hemolytic pattern in the osmotic fragility test. The results of the *in vivo* studies with the coil planet centrifuge indicate that osmotic fragility of erythrocytes began to increase in the blood of the tumor vein on day 4 (Fig. 3) in accordance with increasing tumor growth rate (Fig. 1), and increased osmotic fragility of the erythrocytes of the non-tumor vein was detectable 4 days later. This sequence suggests that osmotically fragile erythrocytes induced by the Yoshida tumor were restored toward the normal levels on contact with a plasma component in the systemic circulation, and on day 8 restoring action of plasma disappeared and irreversible alteration occurred on the fragile erythrocyte membrane. Support for this view derives from the results of *in vitro* studies (Figs. 4 and 5). Unchanged shape and mean cell volume of osmotically fragile erythrocytes induced by Yoshida tumor cells suggest that reversible and irreversible changes of erythrocyte membrane properties occurred. Although there is a rather impressive amount of literature documenting the multiform relationships between the tumor and the host [15], the chemical natures of the active substances released by tumor cells and the plasma component restoring the fragile erythrocytes are still unknown. In the present study, we found the direct action on erythrocyte membranes of Yoshida tumor cells by measuring the erythrocyte osmotic fragility, and further investigation will need to clarify the nature of the plasma component.

## REFERENCES

1. ITO Y, WEINSTEIN MA, AOKI I, HARADA R, KIMURA E, NUNOGAKI K. The coil planet centrifuge. *Nature* (*Lond*) 1966, **212**, 985–987.
2. NAGASAWA T, SUDO K, NISHI N, SARASHI A, KIMURA E. Graphic analysis of osmotic fragility of erythrocytes. *Tohoku J Exp Med* 1976, **120**, 277–285.
3. SHIBATA S, KOBAYASHI K, SERA Y. Application of coil planet centrifugation to hematology. *J Japan Med Instrum* 1970, **40**, 619–626.
4. KITAZIMA K, SHIBATA S. Coil planet centrifugation and its application to the observation of altered membrane properties of erythrocytes in hepatobiliary disorders. *J Lab Clin Med* 1975, **85**, 855–864.
5. LIEBELT RA, GEHRING G, DELMONTE L, SCHUSTER G, LIEBELT AG. Paraneoplasmic syndromes in experimental animal systems. *Ann NY Acad Sci* 1970, **230**, 547–564.
6. DUMONT AE, NACHBAR MS, MARTELLI AB. Altered erythrocyte osmotic fragility in mice with Ehrlich ascites tumor. *J Natl Cancer Inst* 1977, **59**, 989–991.
7. KIMURA E, SUZUKI T, KINOSHITA Y. Separation of reticulocytes by means of multi-layer centrifugation. *Nature* (*Lond*) 1960, **188**, 1201–1202.
8. KINOSHITA Y, KIMURA S, FUKAMIZU M, NAGASAWA T. Separation of two antigenically different subpopulations of small lymphocytes from rat thymus. *Exp Cell Res* 1974, **86**, 136–142.
9. BERRY MN, FRIEND DS. High-yield preparation of isolated rat liver parenchymal cells. *J Cell Biol* 1969, **43**, 506–520.
10. JACOBS MH. Osmotic properties of the erythrocyte. *Biol Bull* 1930, **58**, 104–122.

11. DACIE JV, VAUGHAN JM. The fragility of the red blood cells: its measurement and significance. *J Pathol Bacteriol* 1938, **43**, 341–356.
12. PARPART AK, LORENZ P, PARPART ER, GREGG JR, CHASE AM. The osmotic resistance of human red cells. *J Clin Invest* 1947, **26**, 636–640.
13. DANON D. A rapid micromethod for recording red cell osmotic fragility by continuous decrease of salt concentration. *J Clin Pathol* 1963, **16**, 377–382.
14. NAHAS GG, HSU J, OSTROWSKI D, POLANVI M, HYMAN G. The definition of erythrocyte osmotic fragility in normal and abnormal blood. *Proc Soc Exp Biol Med* 1974, **145**, 192–195.
15. SHAPOT VS. On the multiform relationships between the tumor and the host. *Adv Cancer Res* 1979, **30**, 89–150.