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A STUDY ON THE NUCLEAR EXTRUSION OF MAMMALIAN ERYTHROBLAST

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Abstract: A study was carried out to clarify the mechanism of nuclear extrusion of mammalian erythroid cells by observing erythroblasts of rabbit under various conditions *in vitro*. The animals were made anemic by phenylhydrazine injection and erythroblasts were obtained from the peripheral blood and observed morphologically after a certain time of incubation. After two hour incubation at 37°C, about 50% of erythroblasts were denucleated. The nuclear extrusion was remarkably suppressed by the inhibitor for electron transport system or by uncouplers for oxidative phosphorylation. It was also arrested by the inhibitor of cell movement, like cytochalasin B. In contrast, monoiodo-acetic acid, ouabain and colchicine hardly inhibited the nuclear extrusion. The observations indicated that the nuclear extrusion of mammalian erythroblast is an energy-dependent process in connection with the function of contractile microfilamentous system susceptible to cytochalasin B.

The mammalian erythroblast is a peculiar cell that loses its nucleus at the terminal stage of maturation. The denucleation process has been studied by several hematologists. It had been thought by many authors that the denucleation would be made by karyolysis (1, 2, 3) but morphologic studies, especially by electron microscopy, showed that denucleation occurs by extrusion of the nucleus (4, 5, 6), though some may lose the nucleus by karyolysis (6). Finally, AWAI and others (7) clearly showed that denucleation will be made solely by extrusion but not by karyolysis, showing that no DNA nor its degradation substance is found in the denucleated reticulocyte.

But it is not clear by what mechanism the denucleation is brought about. BESSIS *et al* (4), MIYAKE (8), and AWAI *et al* (7) suggested independently that the nucleus of erythroblast may be extruded by a similar mechanism as general cell division. Electron microscopic observation showed that nucleus protruded from the cytoplasm and cleaving furrow divided the erythroblast into the nuclear and cytoplasmic halves. The mitochondria seemed to move to the area of furrow formation, finally migrating into the cytoplasmic half. The protruded nucleus was surrounded by thin cytoplasm and a cytoplasmic membrane (5, 6). SYMPSON and his associates thought that the migration

of mitochondria to the perinuclear area may be the expression for supplying energy to nuclear extrusion (6). Observations by phase contrast microscope revealed a rhythmical contractile movement in erythroblast which seems to squeeze out the nucleus from the cells (8). And this may suggest that some contracting fibrous elements are concerned with denucleation. But no specific structures such as microfilaments or microtubules are seen near the cleaving furrow or at any other cytoplasmic area (5, 6). But there is a possibility that some labile contractile protein may promote the denucleation by consuming energy. With such a view in mind, the author investigated the energy dependency of nuclear extrusion movement and relation to the contractile proteins of erythroblast.

MATERIALS AND METHODS

Blood samples containing erythroblasts: The blood rich in erythroblasts was from male adult rabbits weighing about 2-2.5 kg, made anemic by the subcutaneous injection of phenylhydrazine chloride, 17 mg/kg at a time, once every other day and for three times. Six days after the first injection of phenylhydrazine, about 20 ml of peripheral blood was obtained at a time by drawing from the vein of the ear using a syringe containing a small amount of heparin solution. Thus, the final heparin concentration was 5U/ml. The blood was rich in erythroblasts, 3,000-12,000/mm^3, and served for observation.

Preparation of reagents: Phenylhydrazine chloride was dissolved in distilled water to 2.5%, pH was adjusted to 7.4 with NaOH, and used for injection. Sodium azide (NaN_3), monoiodo-acetic acid (IAA), ouabain and colchicine (Colch.) were dissolved in Hanks' balanced salt solution. Antimycin A (Anti. A), 2, 4,-dinitrophenol (DNP) and carbamylcyanide-m-fluolo phenylhydrazone (FCCP) were dissolved in alcohol and diluted ten-fold with Hanks' balanced salt solution. Cytochalasin B (Cyt. B) was dissolved in dimethyl sulfoxide (DMSO) and diluted ten-fold with Hanks' balanced salt solution. All these solutions were added to incubation media 1/100 in volume. Final concentrations of these reagents were in tables and figures. Phenylhydrazine chloride, NaN_3, IAA, colchicine, DNP, DMSO and cytochalasin B were obtained from Nakarai Chem., Ltd. and FCCP was supplied by Dr. UTSUMI (Department of Biochemistry, Cancer Institute, Okayama University Medical School). Heparin was purchased from Novo Co., Ltd. and ouabain and antimycin A from Merk Co., Ltd..

Observation on nuclear extrusion: Two ml of the blood was incubated at 37°C for two hours, shaking gently with or without the reagents which were added before incubation. Before and after the incubation, the absolute numbers of nucleated cells in the blood were counted by the hemocytometer. And a part of blood was smeared, fixed with methanol and stained with Giemsa. On the smears the number percent of erythroblasts to the 1,000 nucleated cells was calculated and erythroblast counts per mm^3 were taken by multiplying

the total nucleated cell number in mm^3 with number percent of erythroblasts and 1/100 in each sample.

Nuclear extrusion rate was obtained by the difference between erythroblast counts per mm^3 before and after two-hour incubation. The value from the same sample without any denucleation-suppressing agent was taken as 100%.

The process of denucleation was also observed under phase contrast microscope at 37°C. Observations were made at certain time intervals during the incubation and in some samples, the behavier of erythroblasts was recorded by cinematography.

Stages at nuclear extrusion: The processes of nuclear extrusion were recorded by dividing them into four stages on smears according to the classification of MIYAKE (8) (Fig. 1).

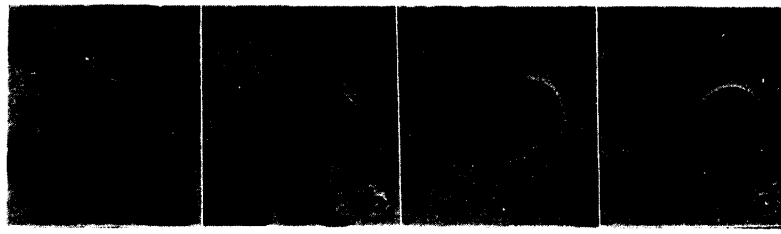

Fig. 1. Demonstration of 4 stages of nuclear extrusion of erythroblast by photos of cells. a, stage 0; b, stage 1; c, stage 2; d, stage 3;

RESULTS

Profile of the nuclear extrusion of erythroblast: Observations made at certain time intervals during two-hour incubation revealed the extrusion of nuclei from erythroblasts *in vitro* and erythroblast counts decreased gradually with lapse of time. After two-hour incubation the number of erythroblasts decreased by about 50% (Fig. 2). Under phase contrast microscope, erythroblasts showed rhythmic amoeboid movements. With the movement the nuclei situated in the central area were transferred to the peripheral zone of the cytoplasm. Then the nuclei protruded from the cytoplasm and were finally squeezed out. Just after extrusion some thread-like cytoplasm was seen connecting the extruded nucleus and the cytoplasm in some cells but finally it was cut off. The mitochondria were seen moving around vigorously gathering at the perinuclear area, but they were finally left in the cytoplasm at denucleation. These nuclear extrusion movements continued for about 10–15 minutes. Thus, before the incubation almost all the erythroblasts were of early stages of denucleation, at stages 0 and 1 according to MIYAKE's classification; that is, nuclei were situated in the central and peripheral areas of cytoplasm and those at later stages, stages 2 and 3 were rarely encountered.

After two-hour incubation those at stage 0 decreased extremely in number, while those at stages 2 and 3 increased. The number of erythroblasts lost by two-hour incubation ("Extruded" in Figures) directly represents the number of denucleated cells in two hours (Fig. 3).

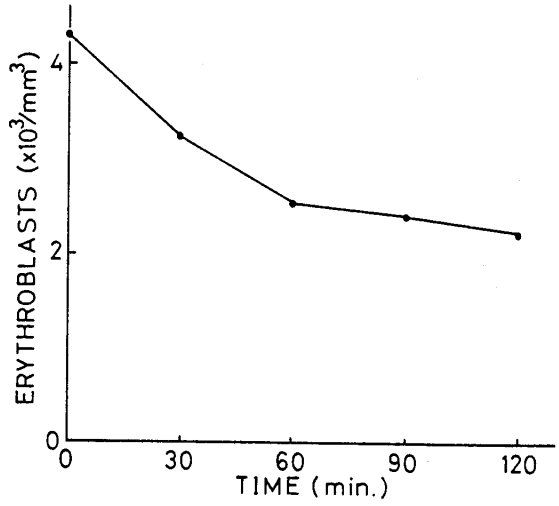

Fig. 2. Decrease of erythroblast count/mm³ during 2-hour incubation *in vitro* at 37°C.

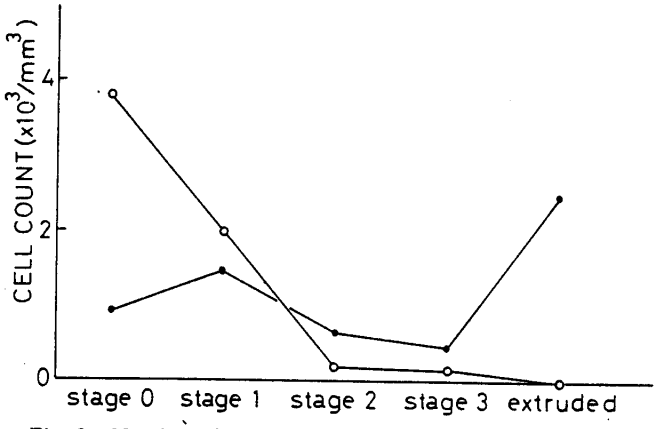

Fig. 3. Number of erythroblasts observed dividing 4 stages of nuclear extrusion before and after 2-hour incubation. Open circles; before incubation, Filled circles; 2 hours after incubation

Inhibition of nuclear extrusion by some agents: Among the inhibitors of energy metabolism, sodium azide inhibited nuclear extrusion moderately by about 50%, and monoiodo-acetic acid slightly by about 15%, but 2, 4,-

dinitrophenol, carbamylcyanide-m-fluolo phenylhydrazone and antimycin A suppressed the denucleation distinctly, by 70% or more. The results indicate that the reagents having inhibitory effect on electron transport or uncoupling effect on oxidative phosphorylation inhibited strongly the denucleation, while those suppressing respiration and glycolysis were less effective in inhibition of nuclear extrusion (Table 1). This suggests that the process of nucelar extrusion is largely dependent on the energy given by oxidative phosphorylation in mitochondria. The inhibitory effect of DNP expressed as 50% denucleation rate was about 40 μM (Fig. 4). Besides the inhibitors of energy metabolism some agents known to have the inhibitory effect on cytokinesis and ion transport have been tested (Table 2). Observations of the effects of these agents revealed that colchicine and ouabain had no effect upon the nuclear extrusion, suggesting that the microtubular structure or Na^+-K^+ ATPase will

TABLE 1 EFFECT OF METABOLIC INHIBITORS UPON NUCLEAR EXTRUSION OF ERYTHROBLAST

Exptl. No.	Percent of nuclear extrusion				
	NaN_3 (0.1 mM)	IAA (0.1 mM)	DNP (0.1 mM)	Anti. A (10 μg/ml)	FCCP (0.1 μM)
1	33	82	15	10	2
2	53	92	24	24	28
3	62	78	24	17	21
4	52	81	7	32	25
5	58	90	31	21	14

IAA and Anti. A, see text.

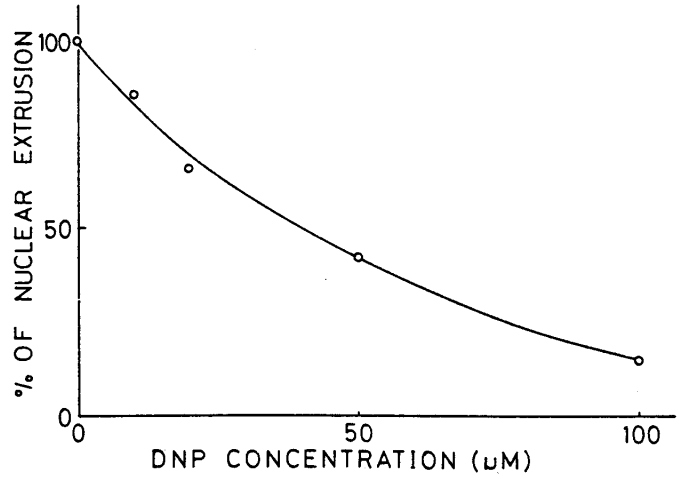

Fig. 4. Nuclear extrusion of erythroblasts after 2-hour incubation in the presence of DNP at varying concentrations.

TABLE 2 EFFECT OF VARIOUS REAGENTS ON NUCLEAR EXTRUSION OF ERYTHROBLAST

Exptl. No.	Percent of nuclear extrusion			
	Ouabain (0.1 mg/ml)	Colch. (25 μM)	Cyt. B (10 μg/ml)	DMSO (0.1%)
1	86	112	27	93
2	108	106	15	78
3	92	90	22	84
4	104	101	4	97
5	76	100	28	83

Colch. and Cyt. B, see text.

not be correlated to the nuclear extrusion. On the smear made after two-hour incubation with colchicine, mitotic figures of erythroblasts were occasionally encountered indicating that the concentration of colchicine used was sufficient to inhibit the mitosis of erythroblasts. In contrast, cytochalasin B inhibited nuclear extrusion prominently, by about 80% inhibition at the concentration of 10 μg/ml. The inhibitory effect of cytochalasin B on the nucelar extrusion decreased with the decrease in concentration, by 50% inhibition at about 5 μg/ml (Fig. 5). Observation upon nuclear extrusion dividing into four stages revealed that in the presence of cytochalasin B, 10 μg/ml, only a slight change was induced in the cell count at each stage even after two-hour incubation, showing a quite similar distribution pattern to that seen before incubation (Fig. 6). The results suggest that some contractile proteins take part in the nuclear extrusion of erythroblast, as cytochalasin B is known to

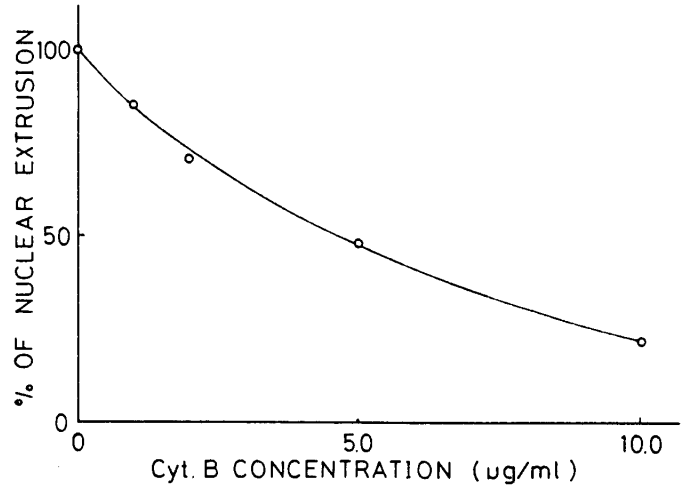

Fig. 5. Nuclear extrusion of erythroblasts after 2-hour incubation in the presence of cytochalasin B at varying concentrations.

affect the contractile fibers in cells (9, 10, 11). Under phase contrast microscope it was seen that the movement of erythroblast ceases in the blood containing cytochalasin B.

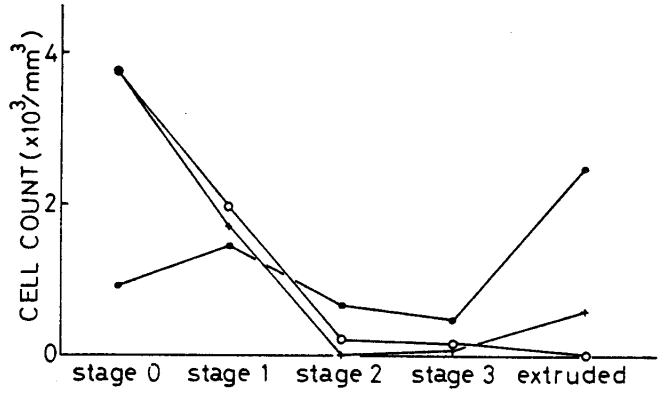

Fig. 6. Effect of cytochalasin B upon nuclear extrusion of erythroblast. Open circles; before incubation, Filled circles; 2 hours after incubation without any reagent, Crosses; 2 hours after incubation with cytochalasin B, 10 μg/ml

DISCUSSION

The results presented in this paper indicate that the nuclear extrusion of erythroblast is an energy requiring process mainly dependent on the oxidative phosphorylation. Observations of living erythroblast under phase contrast microscope show that the denucleation is made by furrow formation which divides the cell into cytoplasmic and nuclear halves, and the extrusion of nucleus is effectively and dramatically suppressed by cytochalasin B. Cytochalasin B is known to bind selectively to a certain contractile microfilamentous system and prevents many cellular events such as cytokinesis and phagocytosis of macrophage (9), morphogenesis of salivary gland (10, 11), cell adhesion of cultured cell (12), and contractility of embryonic heart muscle (13). Therefore, it may reasonably be deduced that the denucleation of erythroblast precedes by the aid of contractile proteins or microfilaments whose contraction consumes energy in the type of ATP. In the case of erythroidc ells it is reported that cytochalasin B inhibits cytokinesis and the separation of cytoplasmic connection at the telophase of dividing cells but does not affect the mitosis at metaphase which is arrested specifically by colchicine (14). The findings indicate clearly that cytochalasin B affects the contractile fibrils or proteins related to cell movement, phagocytosis, cell adhesion and others, while colchicine arrests the microtubules or mitotic

apparatus but not microfilaments. The fact that the denucleation is prevented by cytochalasin B but not by colchicine indicates that the process is rather related to the cell movement, phagocytosis or exocytosis, etc. but not to mitotic cell division. But in erythroblast the treatment with cytochalasin B stimulated the formation of multinucleated erythroblast (14). This means that nuclear division or chromosome separation is of different mechanism from cell division or cytoplasmic division, the former is conducted by microtubules and the latter by microfilaments or contractile proteins. Now it can be said definitely that the nuclear extrusion is brought about by the same mechanism as cytoplasmic division. As far as erythroblast is concerned, spectrin may be a possible candidate for contractile protein. MARCHESI and coworkers reported that the protein obtained from erythrocyte ghost (spectrin) undergoes polymerization to form coiled filament *in vitro* (15). NICOLSON and associates reported that spectrin was exclusively located at the inner surface of the membrane (16) and was related to the topographic distribution of anionic residues on human erythrocytes membranes (17). There is no definite evidence to show that spectrin is directly related to denucleation and more informations should be collected to settle the problem, but the spectrin is one of the most probale candidates at present. In the case of nuclear extrusion *in vivo*, electron microscopic studies showed the passive nuclear extrusion by phagocytosis of erythroblastic nucleus with reticular cell (18, 19) or by "pitting" during passage through the endothelium of marrow sinus (20). So, the results in this paper may not the only factor to explain the nuclear extrusion *in vivo*.

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