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TRANSLOCATION OF HYALURONIC ACID IN CELL SURFACE OF CULTURED MAMMALIAN CELLS AFTER X-IRRADIATION AND ITS RECOVERY BY ADDED ADENOSINE TRIPHOSPHATE

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Summary

To investigate the mechanism of radiation-induced decrease in cell electrophoretic mobility and its recovery by added adenosine triphosphate, specific enzymes and buffer solutions of different ionic strength were utilized. Decrease in the mobility of irradiated cells was detected only with the buffer solution of ionic strengths higher than 0.100. In this range of ionic strengths, removal of hyaluronic acid from cell surface by hyaluronidase had no effect on the electrophoretic mobility of irradiated cells, while the enzyme treatment resulted in 27% mobility reduction in non-irradiated cells. The removal of sialic acid and chondroitin sulfate by their specific enzymes resulted in the similar decrease in mobility either in irradiated and non-irradiated cells. These results suggest that the X-ray induced translocation of hyaluronic acid from the peripheral zone of 0—7.5 Å into the deeper zone of about 10—17 Å, if we use the Debye-Hückel's thickness of ion atmosphere for an approximate estimate of effective depth of electrokinetic plane of shear. Hyaluronic acid reappeared to the peripheral zone by the subsequent incubation after small dose irradiation, or by the addition of 1 mM adenosine triphosphate with Ca^{2+}.

Introduction

In previous papers [1—4], we reported the dose-dependent decrease in electrophoretic mobility of cells and isolated nuclei after X-irradiation. As the mechanism of the X-ray induced mobility reduction, we have tentatively proposed a conformation change in the cell surface, based upon the following findings.

(1) Electrophoretic mobility was mainly dependent on the negative charge of sialic acid, chondroitin sulfates, and hyaluronic acid of the cell surface [5]. The

amount of sialic acid and radioactive labels previously incorporated into these acidic sugars were not affected by X-irradiation at the time when the electrophoretic mobility showed minimum value [6].

(2) Sulfhydryl-blocking agents (*p*-chloromercuribenzoic acid, *N*-ethylmaleimide and iodoacetamide) and disulfide-bridging agent (Fluorescein mercuric acetate) in a concentration range 10^{-8} to 10^{-6} M completely prevented the mobility change [6]. These agents were supposed to restrict a conformation change of membrane protein.

(3) A small amount of Concanavalin A or succinyl-Concanavalin A modified the mobility change depending on its binding condition [7].

(4) The decrease in mobility after irradiation was temperature-dependent similar to the fluidity of membrane lipid, which probably determines the facility in the rearrangement of membrane components.

(5) The electrophoretic mobility of irradiated cells was reversibly altered by exogenous adenosine triphosphate [8].

In the present investigations, we utilized combined methods of specific enzymes and various ionic strength to clarify the nature of "conformation change" of the membrane. The ionic strength of buffer solution for measuring electrophoretic mobility determines the effective depth of electrokinetic plane of shear. The new method revealed the X-ray-induced translocation of hyaluronic acid into a deeper zone, and its recovery by added adenosine triphosphate.

Materials and Methods

A cultured cell line FM3A derived from mouse mammary carcinoma [9] was used. The cells were cultured in suspension in Eagle's minimum essential medium + 10% calf serum, and used at logarithmic growing phase ($2—5 \cdot 10^5$ cells/ml).

To estimate the contributions of each glycosaminoglycans to the mobility [5], the enzymatic treatment of cells was performed with neuraminidase from *Vibrio cholerae* (General Biochemicals, Inc., U.S.A.) 20 units/ml, or chondroitinase-ABC from *Proteus vulgaris* [10] (Seikagaku Kogyo Co., Tokyo) 1 unit/ml, or hyaluronidase from *Streptomyces hyalurolyticus* [11] (Seikagaku Kogyo Co., Tokyo) 10 Turbidity Reducing Units/ml. About 10^7 cells were suspended in 1 ml of buffer solution and incubated at 37° C for 30 min with each enzyme. The buffer solutions used were Hanks' balanced salt solution (pH 7.0) for neuraminidase, and 0.05 M Veronal-buffered saline (pH 8.0) containing 0.1% albumin for chondroitinase-ABC, and 0.02 M Veronal-buffered saline (pH 6.0) for hyaluronidase. The enzyme-treated cells were washed twice with the electrophoretic medium, and their electrophoretic mobility was then compared with that of control cells similarly treated in the corresponding buffers, but without enzyme. These enzymes had no glycosidase activities as far as tested.

The electrophoretic mobility of individual cells was measured at 25 ± 0.5° C with a Zeiss cytopherometer [12]. Each cell was allowed to move 16 μm alternatively in both directions following reversal of current in phosphate buffer supplemented with 5.4% glucose (pH 7.3). The mobility was determined from separate experiments on 30—70 cells for each set of conditions and calculated

as $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$. For the measurement of mobility at different ionic strengths of solution, phosphate buffer (67 mM, pH 7.3, ionic strength 0.167) was diluted with water and then supplemented with 5.4% glucose to maintain isotonicity. The conductance (μv/cm) of each buffer solution was measured with a conductometer, and resistance (Ω cm) determined. The mobility was calculated according to the following equation:

$$\text{Mobility} = \frac{\text{distance of traverse } (\mu m) \times 0.9228 \times \text{height (cm)} \times \text{depth (cm)}}{\text{time (s)} \times \text{electric current } (A) \times \text{resistance } (\Omega \text{ cm})}$$

The correction factor 0.9282 is for the position of the first stationary plane of no liquid circulation due to electroendosmosis in the measuring vessel. Distance and current were chosen to give about 10 s of traverse time for each cell.

X-irradiation of the cells suspended in culture medium in a 9 cm diameter plastic petri dish was carried out at 3° C. The physical factors of exposure were: 200 kVp, 20 mA, 0.5 mm Al + 0.5 mm Cu filter added, half value layer 1.13 mm Cu, and exposure rate in air 105 R/min.

Results

Time-course change in electrophoretic mobility

The electrophoretic mobility of FM3A cells (measured with the buffer of ionic strength 0.167) changed with time after irradiation with 50, 100, 500 or 3000 R as shown in Fig. 1. The mobility diminished progressively with culture time at 37° C and reached a minimum 4 h after exposure. Irradiation with smaller doses produced a slighter decrease and an earlier recovery in the mobility. The addition of 1 mM adenosine triphosphate to the cells irradiated with 3000 R for 24 h resulted in a rapid recovery within 10 min at 37° C. Ca^{2+} (more than 10^{-4} M) was required for the recovery by added adenosine triphosphate.

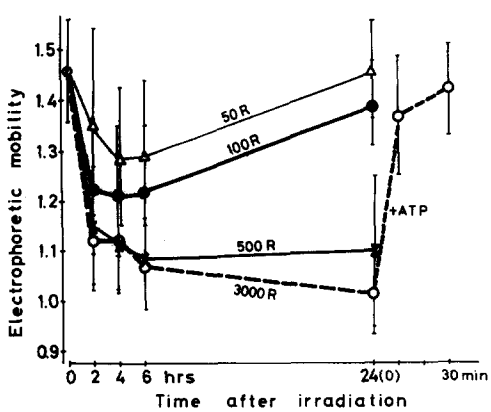

Fig. 1. Change in the electrophoretic mobility (—$\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) with time after X-irradiation with 50 R (△), 100 R (●), 500 R (X) or 3000 R (○). Adenosine triphosphate was added in the final concentration of 1 mM to the cells irradiated with 3000 R 24 h previously. The vertical lines represent one standard deviation for 30—70 measurements.

Effects of enzyme treatment on the mobility (at ionic strength 0.167)

Table I shows the electrophoretic mobilities of cells treated with each enzyme for 30 min at 37°C. Control cells or irradiated cells with 3000 R for 4 h were pooled and then equally divided into six groups for the treatment with each enzyme or corresponding buffer solution. The mobility of 30 cells was measured for each sample, and the complete series of experiments was performed 3 times. The mobility reduction by neuraminidase treatment was 23.1% in control cells and 27.8% in irradiated cells. Treatment with chondroitinase-ABC resulted in 30.2% reduction in control cells and 29.0% reduction in irradiated cells. Standard deviations are not small, but the mean values of three separate experiments did not differ more than 0.05 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$ (less than 5% of the mean). The frequency distribution of electrophoretic mobilities showed normal distribution. The difference in the mobility reduction by neuraminidase or chondroitinase-ABC between control and irradiated cells was not statistically significant ($t = 0.077$, $n = 30$, $p > 0.05$ for neuraminidase, and $t = 2.033$, $n = 30$, $p > 0.05$ for chondroitinase-ABC). With regard to hyaluronidase, however, the mobility reduction was 22.9% in control cells but was not observed in irradiated cells: the difference between the two groups was highly significant ($t = 11.524$, $n = 30$, $p < 0.01$). The electrophoretic mobility of control cells treated with hyaluronidase was almost the same with the mobility of irradiated cells with or without the hyaluronidase treatment.

Table II indicates that the mobility reduction by hyaluronidase treatment was evident in control cells and recovered cells, but not observed in cells irradiated 4 h previously. The electrophoretic mobility recovered to control value by 24 h incubation after irradiation with 100 R, or by the addition of adenosine triphosphate to irradiated cells with 3000 R.

Effects of ionic strength on the mobility

Fig. 2 shows the relationship between the ionic strength of measuring buffer solution and the electrophoretic mobility in control cells, cells irradiated with 3000 R 4 h previously and non-irradiated cells treated with hyaluronidase.

TABLE I

DECREASE IN THE CELL ELECTROPHORETIC MOBILITY ($-\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) BY THE REMOVAL OF ACIDIC SUGARS WITH THEIR SPECIFIC ENZYMES IN IRRADIATED OR NON-IRRADIATED CELLS

Ionic strength: 0.167, mean ± S.D.

Treatment	3000 R 4 h	0 R
Neuraminidase		
—	−1.234 ± 0.075	−1.484 ± 0.137
+	−0.891 ± 0.104 (−27.8%)	−1.139 ± 0.076 (−23.2%)
Chondroitinase-ABC		
—	−1.232 ± 0.100	−1.422 ± 0.174
+	−0.875 ± 0.092 (−29.0%)	−0.993 ± 0.086 (−30.2%)
Hyaluronidase		
—	−1.154 ± 0.110	−1.485 ± 0.096
+	−1.137 ± 0.132 (− 1.5%)	−1.145 ± 0.095 (−22.9%)

TABLE II

CHANGE IN THE CELL ELECTROPHORETIC MOBILITY (−μm · sec^{-1} · V^{-1} · cm) BY TREATMENT WITH HYALURONIDASE

Ionic strength: 0.167, mean ± S.D.

Cells	Hyaluronidase (−)	Hyaluronidase (+)	%change
0 R	1.485 ± 0.097	1.145 ± 0.095	−22.9
100 R 4 h	1.213 ± 0.091	1.232 ± 0.078	+ 1.4
100 R 24 h	1.376 ± 0.082	1.050 ± 0.079	−23.7
3000 R 4 h	1.154 ± 0.110	1.137 ± 0.132	− 1.5
3000 R 24 h	1.011 ± 0.080	1.061 ± 0.082	+ 4.9
3000 R 4 h ± ATP 10^{-3} M 30 min	1.424 ± 0.090	1.081 ± 0.092	−24.1

Each buffer contained 5.4% glucose to maintain isotonicity. With decreasing ionic strength, the mobility increased. The difference in the mobility between irradiated and control cells was detected at ionic strengths of 0.067 and greater. At ionic strengths lower than 0.033, however, the mobility was the same in irradiated and control cells. The mobility values were the same in irradiated cells and hyaluronidase-treated unirradiated cells at ionic strengths higher than 0.1.

With decreasing ionic strength, the thickness of ion atmosphere increases according to Debye-Hückel's equation, as follows for water solution at 25°C.

Thickness of ion atmosphere = 3.06 × (ionic strength)$^{-1/2}$ Å

Table III shows the influence of ionic strength and thickness of atmosphere on the contribution of hyaluronic acid to the mobility. Treatment of control cells with hyaluronidase resulted in similar mobility reduction with every ionic strength tested. The treatment of irradiated cells, however, produced the mobility reduction only with ionic strengths 0.067 and lower. After incubation with

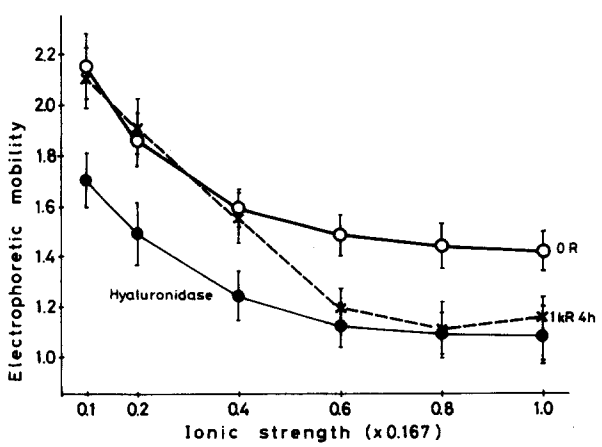

Fig. 2. Relationship between the electrophoretic mobility and the ionic strength of measuring phosphate buffer solution in non-irradiated cells (○), cells irradiated with 1000 R 4 h previously (×) and non-irradiated cells treated with hyaluronidase 10 Turbidity Reducing Units/ml for 30 min (●). The vertical lines represent one standard deviation for 30—70 measurements.

TABLE III

INFLUENCE OF IONIC STRENGTH OF THE MEASURING BUFFER SOLUTION ON THE EFFECT OF HYALURONIDASE ON ELECTROPHORETIC MOBILITY ($-\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) OF IRRADIATED, CONTROL OR RECOVERED CELLS

Mean of 3 separate experiments.

Ionic strength	Thickness of ion atmosphere (Å)	Mobility reduction by hyaluronidase		
		1000 R 4 h	0 R	1000 R 4 h + ATP
0.017	24	0.372	0.381	0.364
0.033	17	0.385	0.343	0.357
0.067	12	0.218	0.337	0.369
0.100	9.7	0.008	0.354	0.352
0.134	8.4	−0.003	0.337	0.340
0.167	7.5	−0.001	0.340	0.343

1 mM adenosine triphosphate and 1 mM Ca^{2+} for 30 min, irradiated cells regained the similar mobility of unirradiated cells at each ionic strength and similar mobility reduction by hyaluronidase treatment.

Discussion

The increase in cell electrophoretic mobility with decreasing ionic strength of measuring buffer solution has been generally observed in erythrocytes and tumor cells. As the decay of potential with distance is less rapid in low ionic strength solution, the internal ionized groups exert their greater influence at lower ionic strength [13—15]. The effective thickness of the ionic double layer at 25°C is given by $3.06 \times (\text{ionic strength})^{-1/2}$ (Å). It is difficult, however, to determine the exact depth of the electrokinetic plane of shear from the value of ionic strength because the cell surface is not smooth and the architecture of cell surface is poorly understood and might change with ionic strengths. If we assume a free penetration of ions into the hydrophilic layer, the thickness of the ion atmosphere could give an approximate estimation of the electrokinetic plane of shear. Negatively charged sugars are present in the hydrophilic layer outside the lipid bilayer, and could be removed by their specific enzymes. The concentration of enzymes and time of treatment we used was the condition for the maximal release of each sugar. The reduction in the electrophoretic mobility by the treatment with hyaluronidase represents the relative amount of hyaluronic acid at the position where it can influence the zeta potential. If we use the thickness of the ion atmosphere as an approximate estimate of the electrokinetic plane of shear, loss of the effect of hyaluronidase at ionic strengths higher than 0.100 after X-irradiation suggest a dislocation of hyaluronic acid from the peripheral zone of 0—7.5 Å into a deeper zone of 9.7—17 Å. While sialic acid and chondroitin sulfates seem to remain at the same position. Recovery in the effect of hyaluronidase on the mobility by the incubation subsequent to small dose irradiation, or by added adenosine-triphosphate, suggests the reappearance of hyaluronic acid to the peripheral layer. Only a few methods such as surface-label method [16]) are available for the investigation of the organization of cell surface coat. The combined method of

specific enzymes and electrophoresis at different ionic strengths utilized in this experiment might be another useful technique.

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