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A CHANGE IN MEMBRANE MICROVISCOSITY OF MOUSE NEUROBLASTOMA CELLS IN ASSOCIATION WITH MORPHOLOGICAL DIFFERENTIATION

YUKISHIGE KAWASAKI, NOBOYUKI WAKAYAMA, TATSURO KOIKE *, MASAO KAWAI and TAKEHIKO AMANO

Mtisubishi-Kasei Institute of Life Sciences, Minamiooya 11, Machida, Tokyo 194 (Japan)

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Summary

Changes in membrane microviscosity as well as in membrane constituents of mouse neuroblastoma clone N-18 were studied in association with neurite formation. The membrane microviscosity studied by fluorescence technique increased with the formation of neurites. The concomitant increase in the ratio of cholesterol to phospholipids was also observed.

Introduction

It is well established that certain clones of mouse neuroblastoma cells grown on substrates tend to develop neurites; in contrast, these cells remain round under conditions where they do not attach to substrates [1—4]. Morphological observations on these neurites confirmed the involvement of microtubules and neurofilaments characteristic of neurites of nerve cells [5,6]. Therefore, this system provides an opportunity for analyzing the neuronal cell differentiation at molecular and cellular levels. This morphological differentiation is known to be associated with changes in the composition of membrane proteins [7,8], glycopeptides [9,10], and cell-surface antigens [11,12]. Possible electrophysiological and enzymatic correlations have also been described [13,16]. Recently, the concept of membrane microviscosity has been advanced [17,18], and the functional organization of the surface membranes has been suggested to be linked to dynamic properties of the membrane components in certain biological phenomena [11—24]. However, no information is available on this

* Present address: Department of Microbiology, The University of Connecticut Health Center, Farmington, Connecticut 06032, U.S.A.

Abbreviations: 12-PS, 12-[4-(1-pyrenyl)butyryloxy]staeric acid; ANS, 1-anilinonaphthalene 8-sulfonic acid.

aspect in neuroblastoma cells, particularly in relation to morphological differentiation. It should be noted in this regard that the neurite formation of neuroblastoma cells was enhanced by adding liposomes to the culture medium, suggesting a possible crucial role of phospholipids in the process of differentiation [25]. In an attempt to study the relation between neurite formation and membrane microviscosity we measured the fluorescence anisotropy and the fluorescence life time of lipophilic fluorescent probes embedded in the surface membranes of neuroblastoma cells. We observed an increase in the membrane microviscosity of these cells accompanied with the formation of neurites.

Methods and Materials

Cell culture

Mouse neuroblastoma cells (N-18 clone) were grown for 5—6 days at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum (Nakashibetsu Serum Preparation Center), in humidified atmosphere of 90% air/10% CO_2 [26,27]. Monolayer culture was carried out either on glass surface, or on quartz plates which can be directly inserted into the cuvette for fluorescence measurements. Petri dishes (60 mm) coated with 0.8% agar (Difco) were used for suspension culture in order to prevent cell attachment on substrates [28]. Medium was changed every other day. The doubling time for both cultures were identical in our conditions (17—20 h). Initial outgrowth of neurites was followed using cells cultured in the medium containing 10% fetal calf serum (Gibco) instead of newborn serum [3]. Cultures were checked to be free of micoplasma contamination [29].

Fluorescent dyes

Perylene was a gift from Professor Inokuchi of the Institute for Molecular Science. ANS was purchased from a commercial source. 12-PS was synthesized as follows. A benzene solution of 400 mg of 12-hydroxystearic acid and 1 mol of 4-(1-pyrenyl)butyryl chloride, prepared from 4-(1-pyrenyl)butyric acid and oxalyl chloride [30], was refluxed for 8 h. After the precipitate was filtered off, the solvent was evaporated and the residue was chromatographed over silica gel (18 g). Elution with benzene/ethyl acetate (20 : 1) gave crude 12-PS. The crude 12-PS was subjected to silica gel layer chromatography (Merck 5717, benzene/ethylacetate (3 : 1)) and the main band was eluted with ethyl acetate, washed with 0.5 M HCl and aqueous NaCl and dried over Na_2SO_4. Evaporation of the solvent gave colorless oil (100 mg) which solidified on standing. Melting point was 35—37°C. Elementary analysis of the final product gave, C, 79.63%, H, 9.01%. These values were in good agreement with the expected ones of C, 79.96%, H, 8.83%.

Labeling of the cells with fluorescent dyes

Aliquots of 12-PS and perylene dissolved in ethanol and in acetone, respectively, were mixed by shaking vigorously in Dulbecco's modified Eagle's medium in final concentration of 1—15 μM. Labeling was initiated by replacing the medium with Dulbecco's modified Eagle's medium containing the dye at 37°C. When fetal calf serum was used, labeling was performed in Dulbecco's

modified Eagle's medium containing 10% fetal calf serum at 37°C. The time course of the incorporation of the dye into the cells was studied as follows: at appropriate time intervals the cells in 60 mm petri dishes were washed with phosphate-buffered saline (NaCl 8.0 (g/l)/KCl 0.20 (g/l)/$CaCl_2$ (0.10 g/l)/$MgCl_2 \cdot 6\,H_2O$ (0.10 g/l)/$Na_2HPO_4 \cdot 2\,H_2O$ (1.15 g/l)/KH_2PO_4 (0.20 g/l)) incubated in 0.25% trypsin (Difco) in phosphate-buffered saline for 5 min, and scraped off from the glass surface. The cells were finally suspended in 2.5 ml of phosphate-buffered saline: four fifths of the cell suspension was used for fluorescence measurements after sonication and the remainder was used for cell counting. The viable cells were counted by a hemocytometer by the extrusion of 0.2% nigrosin. For the routine fluorescence anisotropy measurements, the cells were labeled for 13—15 h, and then washed three times with phosphate-buffered saline, pH 7.0, before measurements. The amount of dye labeled was less than $1 \cdot 10^{-8}$ mol/mg protein. Labeling with ANS was initiated by mixing ANS into cuvette in which cells were placed in phosphate-buffered saline. Fluorescence measurements were performed within 1 h after labeling with ANS.

Fluorescence microscopy

Fluorescence microscopic observations were made using a Zeiss universal Microscope equipped with HBO 100 W lamp, excitation filter UG1 for 12-PS, and UG5 and BG3 for perylene. Appropriate combination of reflectors and cut filters were employed in both cases. The method of mounting was described previously [27]. Kodak recording films were used for photographs.

Isolation of membrane fractions and labeling with the probes

Surface membranes were isolated by the procedure of Brunette and Till [31] with a slight modification as described elsewhere (Koike, T. and Kobayashi, J., in preparation). The membrane fractions dissolved in 3.0 ml of phosphate-buffered saline were labeled with the dye at room temperature for 1 h, then subjected to the fluorescence measurements.

Analysis of lipids

The cells in monolayer culture were scraped off from the glass surface by the incubation in $5 \cdot 10^{-4}$ M EDTA in phosphate-buffered saline for 10 min, and finally suspended in 0.15 M NaCl. Aliquots of the cell suspensions were used for protein assay according to Lowry et al. [32]. The remainder of the suspension was subjected to the isolation procedure for lipids [33]. The lipid extract was finally dissolved in chloroform and stored at −20°C. Cholesterol and cholesterol esters were assayed by the color reaction using ferric chloride-sulfuric acid [34]. Total phospholipids were estimated from the amount of inorganic phosphates in the lipid fraction after ashing [35]. Molecular weight of average phospholipids was assumed to be 775.

Fluorescence measurements

Stational fluorescence measurements were performed with a Shimadzu RF502 fluorescence spectrophotometer. A quartz plate on which cells were cultured in monolayer was mounted diagonally in quartz cuvette filled with

phosphate-buffered saline. Cells grown in suspension culture and membrane fractions were suspended in phosphate-buffered saline and were gently stirred during measurements. Time-dependent fluorescence intensity and anisotropy were measured by a single photon counting technique and analysis was performed by the method of Grinvald and Steinberg [36]. Membrane microviscosity was estimated by two methods; fluorescence anisotropy measurements and excimer formation measurements.

Time-averaged fluorescence anisotropy, $\bar{r}$, is defined as

$$\bar{r} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of vertical and horizontal components being excited by vertically polarized light.

Microviscosity around the dye is related to $\bar{r}$ as [37,38]

$$\frac{r_0}{\bar{r}} - 1 = \frac{3_\tau}{\rho} = \frac{kT_\tau}{V_\eta} \tag{2}$$

where r_0 is the limiting value of $\bar{r}$ at 0 K, τ is the fluorescence life time, ρ is the rotational relaxation time of the rotating unit, k is the Boltzman constant, T is the absolute temperature and V is the effective volume of the rotating unit. Absolute value of η was estimated by the use of r_0 and V of the dye in glycerol.

The degree of excimer formation of 12-PS can be another clue in estimating membrane microviscosity. The fluorescence intensities of excimer, I_E, and of monomer, I_M, are related to η as [39]

$$\frac{I_E}{I_M} = \frac{C[P]T_\tau}{V_\eta} \tag{3}$$

$$\eta = \frac{C[P]T_\tau}{V}\left(\frac{I_M}{I_E}\right) \tag{4}$$

where C is the proportional constant, $[P]$ is the concentration of dye, T is the absolute temperature, V is the volume of translating unit and τ is the fluorescence life time of monomer. 12-PS emits monomer fluorescence at 375 nm as well as at 396 nm, while excimer fluorescence is at 480 nm. In the present study, the ratio of excimer fluorescence at 480 nm to monomer fluorescence at 396 nm was employed for the determination of membrane microviscosity. $[P]$ was estimated from total dye bound to cells divided by the amount of total protein.

Relative value of excitation energy transfer from aromatic amino acids in proteins to 12-PS and ANS, I_P/I_D, was used for the estimation of the localization of the dyes [40]. For 12-PS, I_P and I_D are the fluorescence intensities at 396 nm being excited at 280 nm and 343 nm, respectively. For ANS, I_P and I_D are the fluorescence intensities at 480 nm being excited at 290 nm and 380 nm, respectively. I_P/I_D of monolayer culture was taken as 1.00.

The fluorescence measurements were performed at an absorbance of less than 0.1 in order to minimize the scattering interference. A correction for the fluorescence measurements to remove a small amount of scattered light and of stray light was always performed by inserting a plate of non-labeled cells into

the cuvette instead of labeled cells. Fluorescence measurements were performed at 37°C.

Concentration of dye

The concentration of dye was determined by the absorption measurements. $\epsilon_{343nm} = 3.4 \cdot 10^4$ was used for 12-PS, $\epsilon_{350nm} = 4.95 \cdot 10^3$ was used for ANS and $\epsilon_{436nm} = 4.2 \cdot 10^4$ was used for perylene [38]. Absorption was measured with a Cary 17 spectrophotometer equipped with scattered transmission accessory to reduce scattering interference. The labeled cells were sonicated for 2 min with a Branson ultrasonicator prior to the absorption measurements.

Results

Incorporation of fluorescent dyes to cells

The time course of the incorporation of dyes into cells are shown in Fig. 1. Both 12-PS and perylene are incorporated into cells linearly with time until saturated after 30 h at 37°C. Viability of the cells remains constant (80—85%) for 30 h under our conditions. Therefore, 12-PS and perylene are proved not to damage cells and can be used for the study of neuroblastoma cells. Incorporation of ANS into cells is saturated within 1 h at 37°C in phosphate-buffered saline.

Optical microscopy measurements

In Fig. 2 are shown the phase-contrasted microscopic images of the cells in monolayer culture as well as in suspension culture (A, B).

In suspension culture, cells are round and have no neurite. In monolayer, however, cells assume several elongated neurites.

Also shown in Fig. 2 are the fluorescence microscopic images of cells labeled

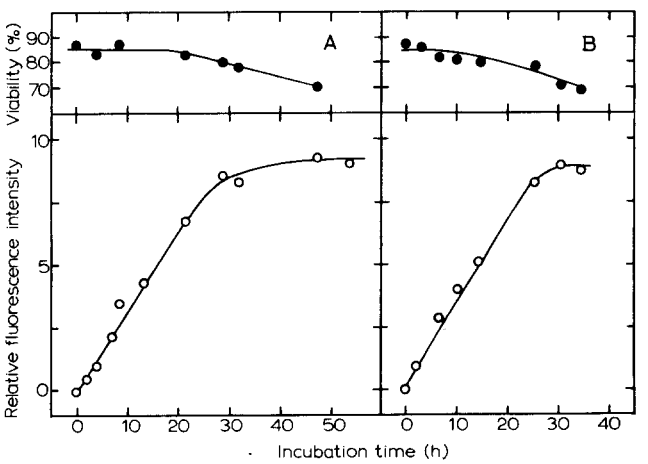

Fig. 1. The incorporation of fluorescent dyes into the cells and the viability of the cells. A, 12-PS; B, perylene. At around 14 h of incubation, the concentrations of dyes incorporated were $2 \cdot 10^{-9}$ mol/mg protein for 12-PS and less than 10^{-9} mol/mg protein for perylene.

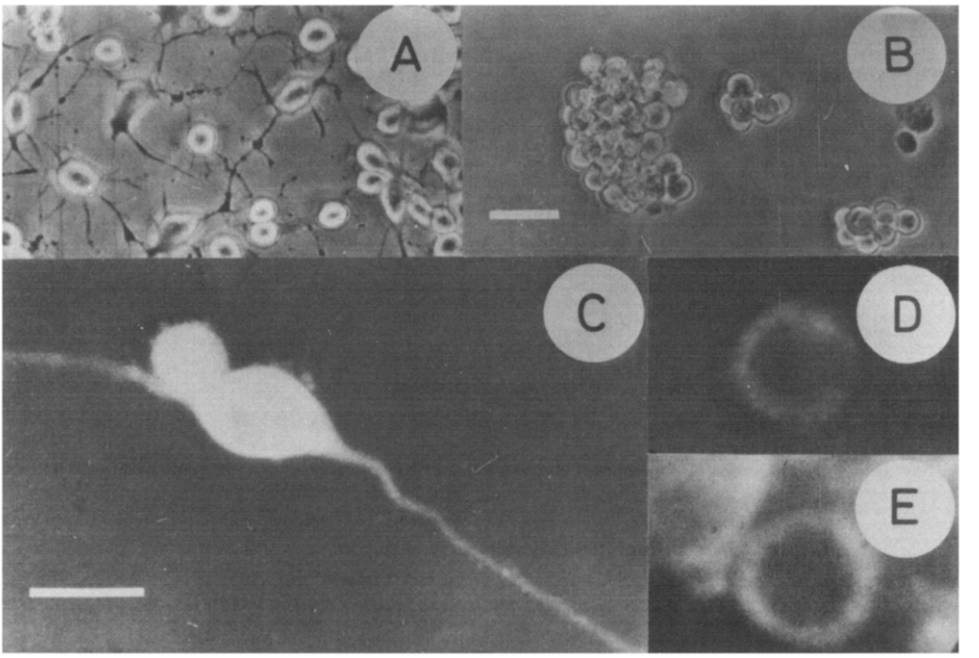

Fig. 2. The phase contrasted and the fluorescence microscopic images of neuroblastoma cells. A, the phase contrasted image of the cells in monolayer culture; B, the phase contrasted image of the cells in suspension culture; C, the fluorescence image of the cells in monolayer culture labeled with 12-PS; D, the fluorescence image of the cells in suspension culture labeled with 12-PS; E, the fluorescence image of the cells in suspension culture labeled with ANS. Bars inserted in the photographs represent 50 μm.

with 12-PS and ANS (C, D, E). When 12-PS or ANS is labeled, the peripheral region of the cells in suspension culture emits fluorescence intensely, though weak fluorescence is always observed in the remaining region. It can be concluded, therefore, that 12-PS and ANS are located mainly at the plasma membrane of the cells. In perylene, on the other hand, fluorescence is observed almost homogenously over the cells indicating the significant internalization.

Fluorescence anisotropy and fluorescence life time measurements

In Table I, the fluorescence anisotropy, the fluorescence lifetime and the membrane microviscosity estimated are summarized. Here, the degree of excitation energy transfer from aromatic amino acids in proteins to dyes is also listed. When 12-PS or perylene is used, membrane microviscosity, η, is higher in monolayer than in suspension culture. This finding is also true in membrane fractions. On the other hand, no difference is found in r when ANS is used. Time-dependent fluorescence measurements of ANS show constant $\bar{r}$ in the range of 100 ns in monolayer culture as well as in suspension culture. However, the interpretation of $\bar{r}$ of ANS is complicated because of the difference in I_P/I_D between monolayer culture and suspension culture and of the multiple fluorescence lifetimes of ANS. This is not the case with 12-PS.

The most striking morphological difference between monolayer culture and suspension culture is the presence of neurites. One difficulty in investigating

TABLE I

THE FLUORESCENCE ANISOTROPY, $\bar{r}$, THE FLUORESCENCE LIFE TIME, τ, THE MICROVISCOSITY, η, AND THE DEGREE OF EXCITATION ENERGY TRANSFER FROM AROMATIC AMINO ACIDS OF PROTEINS TO 12-PS, I_P/I_D

R_0 and V used to estimate η are 0.050 and $980 \cdot 10^{-24}$ cm^3, respectively.

		Culture	$\bar{r}$	τ (ns)	η (poise)	I_P/I_D †
Membrane fraction	12-PS	Monolayer	0.015 ± 0.005 **	120 ± 1 ***	3.5—1.3	1.00
		Suspension	0.008 ± 0.003	116 ± 1	1.4—0.5	0.99
Intact cells	12-PS	Monolayer	0.017 ± 0.002	108 ± 1	2.9—2.2	
		Monolayer *	0.012 ± 0.004	108 ± 1	2.2 —0.8	
		Suspension	0.007 ± 0.003	82 ± 1	0.9—0.3	
Membrane fraction	perylene	Monolayer	0.070 ± 0.003	4.7 ± 0.20	2.3—1.8	
		Suspension	0.053 ± 0.003	4.7 ± 0.20	1.6—1.3	
Intact cells	perylene	Monolayer	0.052 ± 0.002	5.5 ± 0.25	1.8—1.5	
		Suspension	0.038 ± 0.003	5.2 ± 0.25	1.2—0.9	
Intact cells	ANS	Monolayer	0.158 ± 0.002	3.7 ± 0.10 (70%) 13.1 ± 0.20 (30%)		1.00
	ANS	Suspension	0.156 ± 0.011	2.2 ± 0.10 (70%) 12.9 ± 0.20 (30%)		1.18

* Cells were scraped from quartz plate and suspended in phosphate-buffered saline.
** Errors are S.E. of 5 measurements.
*** Errors are the time widths of the analysis.
† The value of monolayer culture is taken as 1.00.

the correlation between neurite formation and the change in $\bar{r}$ is fact that the cells used for results shown in Table I were cultured separately (see Methods and Materials). Therefore, cells in monolayer culture and in suspension culture may differ not only in the presence of neurites but also in many other respects. To confirm that such a change in $\bar{r}$ is closely related to neruite formation, it is more decisive to measure the fluorescence just before and after the formation of neurites using identical cells. In 10% fetal calf serum, formation of neurites is blocked [3] (less than 10% cells contain neurites). Upon replacement into phosphate-buffered saline at 37°C, cells begin to form neurites [3] and after 2 h, 80% cells contain neurites. $\bar{r}$ of 12-PS labeled to cells is 0.014 ± 0.003 (S.D.) just after the replacement into phosphate-buffered saline, and is 0.026 ± 0.002 (S.D.) after 2 h. This finding strongly suggests that the change in membrane microviscosity is closely related to the neurite formation.

Excimer formation measurements

For intact cells, the degrees of the excimer formation of 12-PS labeled to cells, $[P]$ (I_M/I_E) are $1.68 \cdot 10^{-15}$ and $0.74 \cdot 10^{-15}$ mol/s per mg protein for monolayer culture and suspension culture, respectively. This tendency is also true in membrane fractions. These facts also support the foregoing conclusion that the membrane microviscosity is higher in monolayer culture.

Analysis of membrane constituents

Does the change in membrane microviscosity originate from changes in membrane constituents accompanying the cell differentiation? In this respect, the relative amounts of proteins to phospholipids and of cholesterol to phospholipids were analyzed. The weight ratios of cholesterol to phospholipids are

0.17 ± 0.01 (S.D.) and 0.13 ± 0.01 (S.D.) for monolayer culture and suspension culture, respectively. On the other hand, no changes are obtained in the weight ratios of proteins to phospholipids. Taking into account the preceding results, correlation between membrane microviscosity and the change in membrane constituents are obtained; the higher content of cholesterol leads to the higher membrane microviscosity in monolayer culture.

Discussion

Recently, the application of [19—24] fluorescence anisotropy measurements to biological membranes has been used to obtain information on viscosity. The application of Eqn. 2 to such a complicated system as neuroblastoma cells is based on many assumptions and leads to oversimplification. Although the absolute values of η shown in Table I are in good agreement with those reported in many other cell membranes [41], they should not be emphasized. At least, a relative change of η, however, can give information on membrane properties accompanying cell differentiation, and so can the degree of excimer formation.

When intact cells are used, the possible internalization of dye may make the analysis of the results complicated [42]. In the present study, however, the change in $\bar{r}$ is obtained in membrane fractions as well as in intact cells (Table I). Besides, according to the fluorescence microscopy observation, 12-PS and ANS are predominantly located in the peripheral region of the cell body (Fig. 2). These findings confirm the conclusion that the membrane microviscosity is higher in monolayer culture than in suspension culture. Optical interference of quartz plate inserted into cuvette is ruled out because the membrane fractions suspended in phosphate-buffered saline exhibit the change in $\bar{r}$, and also the monolayer culture scraped off from the quartz plate shows this change.

The interpretation of the degree of excimer formation has a difficulty that the local concentration of dye cannot be known. However, as far as the spacially averaged concentration of dye is used as $[P]$ in Eqn. 4, it can be said that the results obtained by the excimer formation measurements also support the above conclusion.

Referring to the degree of excitation energy transfer in Table I, the simple adhesion of 12-PS to membrane proteins is not the origin of high membrane microviscosity in monolayer culture. On the other hand, the environments around ANS in cells seem to be different between monolayer culture and suspension culture. Therefore, it is difficult to estimate the membrane microviscosity using ANS in neuroblastoma cells.

Constitutional and organizational changes are also reported to take place in association with the neurite formation [5—16]. The increase in the ratio of cholesterol to phospholipids is just one of them. It was reported that the increase in cholesterol content in cell membranes resulted in the increase in membrane viscosity [43]. The same is reported in liposomes of phospholipids above the phase transition temperature [44]. These findings are consistent with the present results. Not only the change in membrane fraction, but also the change in intracellular organization such as the formation and assembly of neurofilaments [6], and of microtubules [5], may also be responsible for the change in membrane microviscosity.

The physiological significance of the change in membrane microviscosity of neuroblastoma cells defined in the present study remains unclear. Recently, evidence has been offered as to possible involvements of lipids in the acetylcholine esterase [45], function of acetylcholine receptor [46], and presynaptic membrane events [47]. Therefore, it is worth considering that the functional aspects of the cell membranes may be regulated by the lipid environments and in turn by the membrane microviscosity during the process of cell maturation of nerve cells.

Recently, two articles focused on the membrane properties in association with the morphological differentiation of neuroblastoma cells were published [48,49]. Erkell [48] used ANS as a membrane probe and obtained $\bar{r}$ of 0.157 and 0.073 for suspension culture and monolayer culture, respectively. These results are not consistent with ours (Table I). This apparent inconsistency is probably due to the different cell line of neuroblastoma cells and also to the different cultural system they employed. Zagyanski et al. [49] measured the lateral diffusion of concanavalin A attached to neuroblastoma cells. The lateral diffusion coefficient of concanavalin A attached to round cells was smaller than in differentiated cells. This finding is also apparently contradictory to the present results. However, such a negative correlation between lipid viscosity and the mobility of concanavalin A attached to cells was reported in lymphocytes and lymphoma cells [50].

In the present study, we are concerned with the macroscopic measurements which involve a number of cells. In order to study the more precise mechanisms of cell differentiation, measurements of a single cell or even of a localized region within a single cell are necessary.

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