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MICROVISCOSITY CHANGES DURING DIFFERENTIATION OF NEUROBLASTOMA CELLS

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Summary

Microviscosity ($\overline{\eta}$) of the plasma-membrane lipid matrix was measured in exponentially growing and differentiating C1300 mouse neuroblastoma cells, attached to a glass substratum, by fluorescence polarisation of 1,6-diphenyl-1,3,5-hexatriene. Upon differentiation $\overline{\eta}$ decreases progressively, reaching values below those observed in the growth phase. Treatment of the cells with dipalmitoyl phosphatidylcholine vesicles reversibly inhibits morphological differentiation. The results show that a high membrane fluidity is a prerequisite for differentiation.

Neuroblastomas are highly malignant tumours that originate from the anlage of the sympathetic nervous system, hence the tumour cells strongly resemble embryonic neuroblasts. In vivo, this type of tumour has a high probability of spontaneous regression during which tumour cells may differentiate into nerve cells [1].

In recent years several neuroblastoma cell lines have been established which retain the capacity to differentiate in vitro. Upon differentiation, these lines express a variety of neuronal properties such as the formation of neurites and excitable membranes and the production of neuro-specific enzymes, receptors and transmitters. Furthermore, the cells stop proliferating and lose their malignant characteristics [2]. Neuroblastoma cell lines thus offer unique systems for studying the regulation of neuronal differentiation in vitro.

In the following we report that differentiation of neuroblastoma cells is accompanied by a progressive increase in plasma-membrane lipid fluidity ex-

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ceeding the changes which were observed during the normal cell cycle [3].

C1300 mouse neuroblastoma cells, clone Neuro-2A, were obtained from the American Type Culture Collection, Rockville, Md. The cells were grown in Dulbecco's modified Eagle's medium without bicarbonate, but with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer, supplemented with 10% fetal calf serum (Flow Laboratories Ltd., Irvine, U.K.). Differentiation was induced 24 h after plating by adding 200 μg 3-isobutyl-1-methylxanthine per ml plus 1 mM dibutyryl cyclic AMP or, alternatively, 200 μg 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724) plus 10 μg prostaglandin E1 per ml [2]. Prostaglandin E1 was a gift of the Unilever Research Laboratories, Vlaardingen, The Netherlands; R020-1724 was a gift of Hoffman-La Roche, Mijdrecht, The Netherlands. No significant differences between these two methods were detected. More than 75% of the cells expressed morphological differentiation as assessed by the fraction of cells showing cell processes longer than twice the diameter of the cell body.

The fluorescence hydrocarbon 1,6-diphenyl-1,3,5-hexatriene was used as a probe for monitoring the fluidity properties of the plasma-membrane lipid matrix [3—5]. For fluorescence polarisation measurements cells were grown on glass cover slips and processed as described earlier [3]. We have previously shown that the fluorescence of diphenylhexatriene-labelled neuroblastoma cells originates almost exclusively from the plasma membrane [3]. The degree of fluorescence polarisation and fluorescence intensity of labelled cells in situ, attached to a glass substratum, were measured with a specially designed instrument [3]. The fluorescence intensities polarised parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excitation beam were corrected for background signal [3] and the corresponding fluorescence anisotropy (r) and intensity (F) were calculated according to Eqn. 1:

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

Microviscosities ($\bar{\eta}$) were derived as previously described [3, 5] by a method based on the Perrin equation (Eqn. 2) for rotational depolarisation of non-spherical fluorophores:

$$\frac{r_0}{r} = 1 + C(r)\frac{T\tau}{\bar{\eta}} \tag{2}$$

where r_0 and r are the limiting and measured fluorescence anisotropies, T is the absolute temperature, τ is the excited-state lifetime, and $C(r)$ is a molecular shape parameter. Microviscosity is an operational term which translates the resistance of the micro-environment to rotation of the probe-molecule into macroscopic viscosity units (poise). It does not take into account the anisotropy of the membrane structure nor the possible microheterogeneity of solvation sites for the fluorescent probe-molecule [6]. Thus, the obtained $\bar{\eta}$-value should be considered as a weight average of possible subpopulations.

Fig. 1 shows micrographs of Neuro-2A cells at different times after the induction of differentiation. In exponentially growing cultures cells showing neurite-like cell processes are absent (Fig. 1a). Within a few hours after the in-

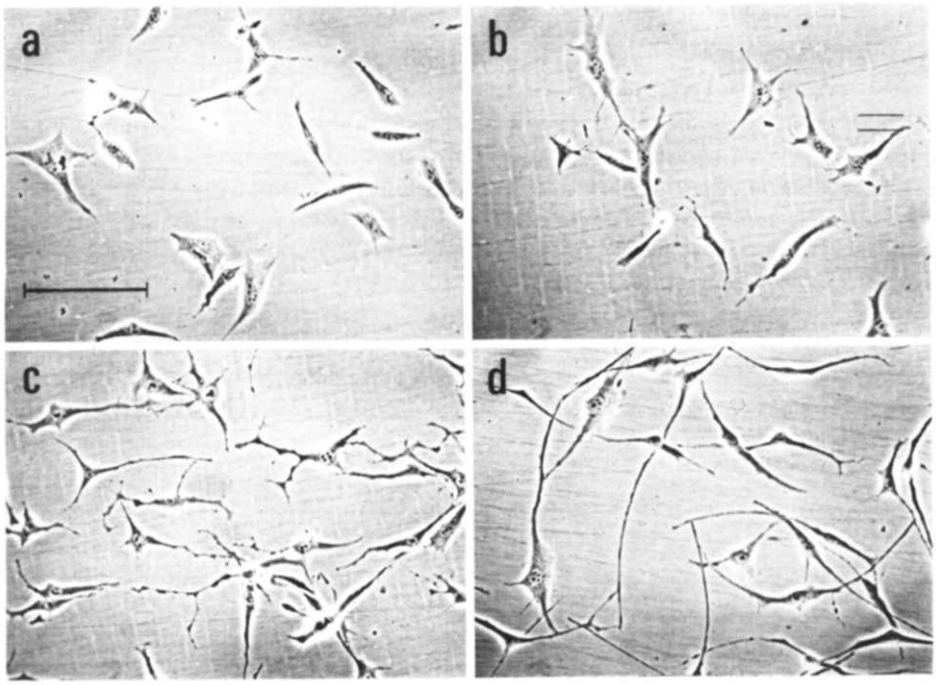

Fig. 1. Phase contrast micrographs of differentiating Neuro-2A cells. a, exponentially growing cells (t = 0 h); b, c and d, 2, 6 and 24 h, respectively, after induction of differentiation. Bar represents 60 μm.

duction of differentiation the cells start to form relatively thin processes, probably partially due to cell body retraction (Fig. 1, b and c). During further differentiation the processes elongate drastically and become thicker. After 24 h most cells exhibit neurite-like extensions longer than 100 μm (Fig. 1d).

Five experiments were carried out to determine the changes in microviscosity during differentiation. In each experiment triple samples were measured at each time. The results are summarised in Fig. 2. As shown, within an hour of the initiation of differentiation the measured fluorescence anisotropy, which correlates qualitatively with $\bar{\eta}$ (see Eqn. 2), decreased markedly. A stable value is reached after six hours. Fig. 3 gives examples of plots of log $\bar{\eta}$ versus $1/T$ for exponentially growing cells, and after 24 h of differentiation. A linear regression analysis was carried out on such plots to determine $\bar{\eta}$ values at various temperatures and to derive the flow activation energy, ΔE, from its slope. Table I summarizes the calculated mean values. No detectable phase transitions can be inferred from the linearity of the plots. The decrease in $\bar{\eta}$ was associated with an increase in ΔE, indicating a reduction in the degree of order in the membrane lipid domain [5].

By comparing the $\bar{\eta}$ values for differentiating cells with those for synchronised proliferating cells (see ref. 3), it is evident that during differentiation $\bar{\eta}$ reaches values 25% lower that the observed minimum during a normal cell cycle (S-phase). Hence, the observed decrease in $\bar{\eta}$ is not simply due to an arrest of the cells in a particular phase of the cell cycle [3]. This comparison further shows that the growing, malignant neuroblastoma cells exhibit higher

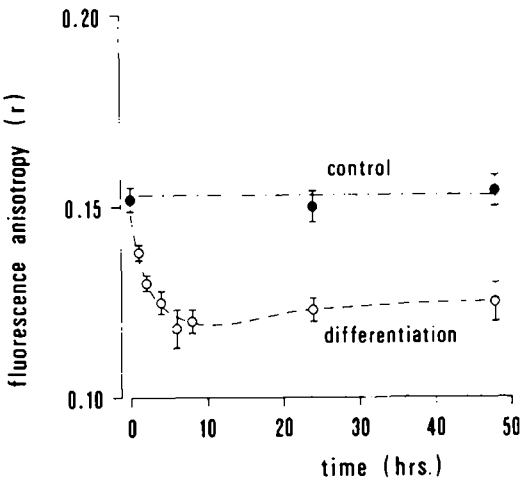

Fig. 2. Fluorescence anisotropy at 37°C of Neuro-2A cells during exponential growth (control), and during differentiation as a function of time elapsed after its induction. Mean values ± S.E. are given.

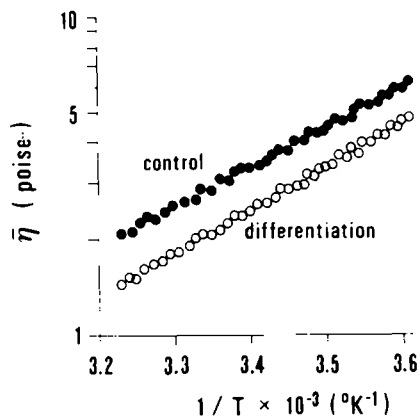

Fig. 3. Temperature dependence of microviscosity ($\bar{\eta}$) in Neuro-2A cells growing exponentially (control) and after 24 h of differentiation, plotted as log $\bar{\eta}$ vs. $1/T$.

membrane microviscosities than the reverted, non-growing cells, as was also found for virus-transformed 3T3 cells [7].

The decrease in $\bar{\eta}$ is one of the first molecular changes observed upon induction of morphological differentiation in neuroblastoma cells. From the time course of the changes in $\bar{\eta}$ it can be concluded that the decrease in $\bar{\eta}$ precedes the overt gross morphological alterations (Fig. 1). This indicates that the changes in membrane fluidity are not the result of neurite formation but are associated with the initiation of morphological differentiation.

To test the significance of the observed increase in membrane fluidity during differentiation, cells were cultured for 24—48 h in the presence of 200 μg 3-isobutyl-1-methylxanthine/ml plus 1 mM dibutyryl cyclic AMP and various concentrations of lipid vesicles. The vesicles were prepared from either

TABLE I

MICROVISCOSITIES AND FLOW ACTIVATION ENERGIES (ΔE) OF THE MEMBRANE-LIPID LAYER OF EXPONENTIALLY GROWING (CONTROL) AND DIFFERENTIATING NEURO-2A CELLS

	Microviscosity (poise)			ΔE (kcal/mol)
	37°C	25°C	4°C	
Control	2.1	3.1	6.2	5.3
24 h differentiation	1.4	2.2	4.9	6.1

dipalmitoyl phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl-choline) or dioleoyl phosphatidylcholine (1,2-dioleoyl-*sn*-glycero-3-phosphoryl-choline). Incubation with dipalmitoyl or dioleoyl phosphatidylcholine vesicles increased and decreased, respectively, the measured r value reversibly (details to be published elsewhere). Incubation with dioleoyl phosphatidylcholine vesicles at concentrations up to 2.5 μmol dioleoyl phosphatidylcholine/ml had no effect on morphological differentiation (Fig. 4a). In contrast, incubation with dipalmitoyl phosphatidylcholine vesicles at a concentration of 0.6 μmol dipalmitoyl phosphatidylcholine/ml completely blocked neurite formation (Fig. 4b). This inhibition of differentiation was overcome upon removal of the dipalmitoyl phosphatidylcholine vesicles (Fig. 4c). These results strongly suggest that an increased membrane fluidity is a prerequisite for morphological differentiation of neuroblastoma cells.

Further evidence for a change in the fluidity properties of the plasma membrane during differentiation comes from fluorescence photobleaching recovery measurements [8] of the lateral mobilities of both membrane lipids and membrane proteins. The lateral mobilities of both classes of probe molecules show an increase upon differentiation, more so in the outgrowing neurites than in membrane regions of the perikaryon (de Laat et al., in prepara-

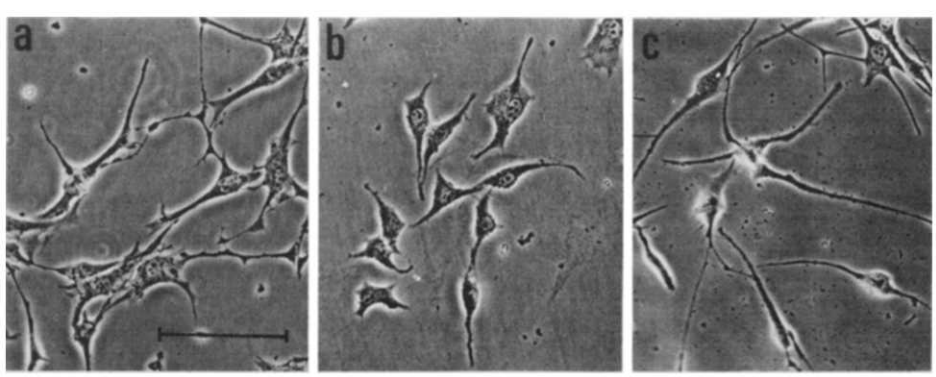

Fig. 4. Phase contrast micrographs of Neuro-2A cells showing the effect of lipid vesicles on morphological differentiation. Bar represents 60 μm. a, cells after 48 h differentiation in the presence of 0.6 μmol dioleoyl phosphatidylcholine vesicles per ml. Differentiation has proceeded normally. Fluorescence anisotropy at 37°C: r = 0.110. b, cells after 24 h differentiation in the presence of 0.6 μmol dipalmitoyl phosphatidylcholine vesicles per ml. Differentiation is completely inhibited. At 37°C: r = 0.175. c, cells cultured initially for 24 h as the cells in b. The micrographs were taken 24 h after removal of the dipalmitoyl phosphatidylcholine vesicles from the medium and show the reversibility of the inhibition of neurite formation by dipalmitoyl phosphatidylcholine vesicles. At 37°C: r = 0.135.

tion). Taking this into account, it seems likely that the more fluid membrane regions become localised in the extending neurites.

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References

1 Pochedly, C. (1977) Neuroblastoma, pp. 155—203, Edward Arnold, London
2 Prasad, K.N. (1975) Biol. Rev. 50, 129—165
3 De Laat, S.W., van der Saag, P.T. and Shinitzky, M. (1977) Proc. Natl. Acad. Sci. U.S. 74, 4458—4461
4 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652—2657
5 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133—147
6 Chen, L.A., Dale, R.E., Roth, S. and Brand, L. (1977) J. Biol. Chem. 252, 2163—2169
7 Fuchs, P., Parola, A., Robbins, P.W. and Blout, E.R. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3351—3354
8 Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E. and Webb, W.W. (1976) Biophys. J. 16, 1055—1069