INCREASE IN FLUIDITY OF MEMBRANE LIPIDS IN LYMPHOCYTES, FIBROBLASTS AND LIVER CELLS STIMULATED FOR GROWTH

J. G. COLLARD, A. DE WILDT, E. P. M. OOMEN-MEULEMANS, J. SMEEKENS and P. EMMELOT
The Netherlands Cancer Institute, Division of Cell Biology, Sarphatistraat 108, Amsterdam, The Netherlands

and

M. INBAR
The Weizmann Institute of Science, Department of Membrane Research, Rehovot, Israel

Received 4 March 1977

1. Introduction

The cell surface has been found to participate or even to play a leading role in growth control of cells [1,2]. When normal fibroblasts in vitro make extensive contact with each other they stop growing [3]. Mechanical disruption of a confluent monolayer by scratching allows quiescent cells to move into the cell-free scratch. This results in loss of growth controlling contact and induction of cell division [4]. Mitogenic stimulation of lymphocytes into blasts cells is caused by binding of a mitogen (e.g., concanavalin A) to the cell surface. Concanavalin A (Con A) coupled to acrylic polymer particles, which cannot enter the cells, causes the same stimulation as uncoupled Con A, indicating that only the interaction of the mitogen with the cell surface is the trigger for blast formation [5].

Recent studies have indicated that fluorescence polarization analysis of 1,6-diphenyl-1,3,5-hexatriene (DPH) embedded in membranes of intact cells and isolated plasma membranes of these cells is a valuable tool to study dynamic parameters of lipid regions in cellular membranes [6–9]. To investigate the possible role of membrane lipid fluidity in growth control, we measured membrane lipid fluidity as determined by the fluorescence polarization technique in resting and stimulated cells as well as in plasma membranes isolated from these cells.

The effects of three different growth stimuli were investigated in three different cell systems in order to avoid the possibility that our results were due to peculiarities of a specific model system. The results indicate that the proliferating cells in all three systems, show a lower degree of fluorescence polarization, indicating a higher membrane lipid fluidity as compared with the corresponding unstimulated control cells.

2. Materials and methods

2.1. *The lymphocyte system*

Lymphocytes were prepared from spleens of 3 month-old (C57BI X DBA)F_1 mice [5]. The mitogenic activity of Con A and succinyl–Con A was determined by measuring [^{3}H]thymidine incorporation into acid precipitable material [5]. Succinyl–Con A was prepared from Con A (Calbiochem) according to Günther et al. 1973 [10] and purified by affinity chromatography on Sephadex G-50. At 51 h after stimulation, stimulated and unstimulated cultures were washed 2 times with phosphate buffered saline (PBS) and prepared for fluorescence polarization analysis.

2.2. *The fibroblast system*

Swiss albino 3T3 mouse fibroblasts were obtained commercially from Flow laboratories (res. grade) [11].

Cells were seeded at a density of 10 000–20 000

cells/cm^2 in Petri dishes (diam. 8 cm) containing 10 ml Dulbecco's modified Eagle medium supplemented with 3% foetal calf serum and antibiotics and allowed to grow to confluency (40 000–50 000 cells/cm^2). Serum stimulation was initiated by replacing the original medium by a medium containing 25% serum resulting in 60–80% cycling cells. Cell cultures, suspended (0.05% trypsin) at different times after serum stimulation, were washed twice with cold PBS containing egg-white trypsin inhibitor (Calbiochem, 0.01%) and used for fluorescence polarization analysis and pulse cytophotometry.

2.3. *The liver system*

Regenerating livers were obtained by dissecting 70% of livers of 3 month old male (R × U)F_1 rats. Cell suspensions of normal and regenerating livers were prepared 24 h after partial hepatectomy by perfusion as described [12]. The isolation procedure of plasma membranes from livers has been extensively reported previously [13]. Total lipids of isolated plasma membranes were extracted with chloroform/methanol, 2 : 1 (v/v), followed by partition according to Folch [14]. Cholesterol/phospholipid molar ratios of total lipids in isolated plasma membranes were estimated according to Van Hoeven and Emmelot [15]. Cholesterol was quantitatively determined as trimethylsilyl derivative by gas–liquid chromatography using cholestane as internal standard [15].

2.4. *Fluorescence labeling of cells, membranes and liposomes*

Cell suspensions of lymphocytes (10×10^6 cells/ml), fibroblasts (2×10^6 cells/ml), liver (1×10^6 cells/ml) and isolated plasma membranes (100 μg protein/ml) were labeled with an equal volume of stable, aqueous dispersion (2×10^{-6}M) of the fluorescent hydrocarbon 1,6-diphenyl 1,3,5 hexatriene (DPH, Koch Light Laboratories Ltd) which was practically void of fluorescence. After incubation during 30 min at 37°C, cells were used for fluorescence polarization analysis (P) at 25°C. The variation in fluorescence polarization values of two identical samples or of repeated measurements of the same samples was maximally 0.003 units. Total lipids (200 μg) in isolated plasma membranes were evaporated to dryness under nitrogen and dispersed in 2 ml PBS containing 8×10^{-6}M DPH. Next, liposomes were prepared by sonication (Branson Sonifier, B30) under nitrogen at 0°C incubated during 45 min at 37°C and subsequently used for fluorescence polarization (P) analysis at 25°C. For analysis of the fluorescence polarization on a single cell level, cells were labeled with 10×10^{-6}M DPH in PBS during 30 min at 37°C.

2.5. *Fluorescence polarization analysis*

The degree of fluorescence polarization (P) was measured in the Elscint microviscosimeter model MV-1A (Elscint Ltd, Haifa, Israel) as described [6,9]. Individual cells were measured in the Elscint Single Cell Microviscosimeter at room temperature [16].

The method employed for the evaluation of membrane microviscosity, $\bar{\eta}$, has been outlined previously [6] and is based on the fluorescence polarization properties of a fluorescent probe as described by the Perrin equation. The excited state lifetime of DPH at 25°C is put at 10 ns ± 10% [9,17]. High P-values correspond to high microviscosity ($\bar{\eta}$) values and represent low lipid fluidity and vice versa.

2.6. *Pulse cytophotometry*

After fluorescence polarization analysis, samples of cell suspensions were fixed in 96% ethanol and prepared for pulse cytophotometry to measure the amount of DNA/cell and to calculate the percentages of G_1, S, G_2, and mitotic cells in the cultures from the DNA distributions as described earlier [18].

3. Results and discussion

3.1. *Changes in membrane lipid fluidity after mitogenic stimulation of lymphocytes*

The dimeric succinyl–Con A is mitogenically active in a much wider concentration range (2–150 μg/ml) than tetravalent Con A (1–5 μg/ml). This is probably due to the reduced cytotoxicity of the dimeric derivative as reported before [10]. For that reason we used in our stimulation experiments succinyl–Con A. Table 1 shows that the degree of fluorescence polarization (P) in stimulated cultures is lower than in unstimulated controls at 51 h after addition of the mitogen when the DNA synthesis is maximal [19]. Comparable observations were made in cultures measured after 48 h or 71 h. This result agrees with earlier observations in which a similar change in

Table 1
Difference in degree of fluorescence polarization (P) and microviscosity ($\bar{\eta}$, poise) of lymphocytes at 51 h after stimulation

	P, at 25°C	$\bar{\eta}$, at 25°C	[^{3}H]Thymidine incorporation[a]
Unstimulated cultures	0.307	4.70	2800 dpm/10^6 cells
Stimulated cultures (25 μg Succinyl–Con A/ml)	0. 289	3.89	50 600 dpm/10^6 cells

[a] [^{3}H]Thymidine incorporation measured during 44–70 h after addition of the mitogen

membrane lipid fluidity was observed using tetravalent concanavalin A [7]. By measuring the degree of fluorescence polarization of individual cells by the use of a single-cell microviscometer [16], we could demonstrate that the stimulated cultures contained more cells with low P-values than did the unstimulated cultures, probably reflecting the blast formation after stimulation (fig.1).

3.2. *Changes in membrane lipid fluidity of contact inhibited 3T3 fibroblasts after serum stimulation*

The addition of serum to a contact inhibited 3T3 fibroblast culture leads to a rise in RNA synthesis followed after a few hours by protein synthesis and eventually by DNA synthesis [3,4]. This type of stimulation has the advantage that it results in a partial synchronization of the cycling cells in the different phases of the cell cycle. Table 2 shows the differences in the degree of fluorescence polarization between unstimulated cultures at various times after serum stimulation. The distribution of the cells over

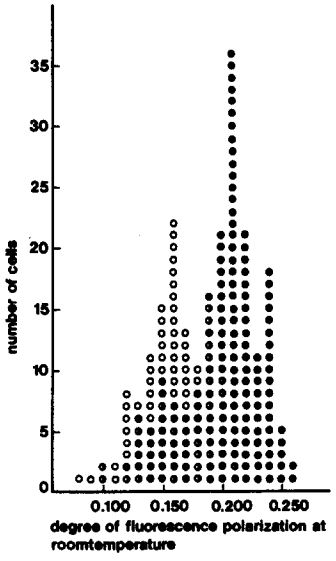

Fig.1. Frequency distribution of the fluorescence polarization values of single cells from unstimulated (•) and stimulated (○) (25 μg succinyl–Con A/ml) spleen lymphocytes at 51 h.

Table 2
Changes in degree of fluorescence polarization (P) and microviscosity ($\bar{\eta}$, poise) in contact inhibited 3T3 fibroblasts after serum stimulation

Time after serum stimulation (3→25%)			Distribution of cells over the cell cycle phases (%)		
(h)	P, 25°C	$\bar{\eta}$, 25°C	G_1	S	G_2/M
0	0.260	2.90	90	5	5
12	0.235	2.26	91	6	3
20	0.212	1.79	37	54	9
24	0.216	1.86	35	29	36
30	0.243	2.45	65	15	20
24[a] (control)	0.262	2.96	90	4	6

[a] Kept in the original 3% serum medium

the cell cycle was estimated from pulse cytophotometric measurements of the DNA/cell distributions. Serum stimulation of contact inhibited 3T3 cultures causes a decrease in *P*- and microviscosity values indicating an increase in membrane lipid fluidity during the first hours after stimulation. Most of the changes in microviscosity probably occurred during transition of G_1, before the cells had entered the *S*-phase. The highest membrane lipid fluidity was observed in cultures around mitosis, while *P*-values in stimulated cultures after mitosis returned again to the level of unstimulated cultures. The results suggest also a difference in membrane lipid fluidity between cells in the various phases of the cell cycle. This aspect is presently being further investigated.

3.3. *Changes in membrane lipid fluidity of liver cells after partial hepatectomy.*

Partial hepatectomy causes synchronous growth in the remaining liver tissue with a maximal DNA synthetic activity after about 24 h. As shown in table 3, fluorescence polarization analysis of regenerating liver cells, 24 h after partial hepatectomy, results in lower *P*-values, indicating higher membrane lipid fluidity when compared with control liver cells. The degree of stimulation has been determined by pulse cytophotometry as shown in fig.2. Approximately 60% of both diploid and tetraploid liver cells were stimulated for growth and were found mainly in *S*-phase at 24 h. Because of the high number of cells available in this system we were able to study changes in fluorescence polarization on isolated plasma membranes. The isolation procedure [13] and chemical characterization [20] of the liver plasma membrane have been extensively reported by us before. Isolated plasma membranes from both normal and regenerating liver cells as well as liposomes prepared from the total lipid extracts of these plasma membranes show similar differences in *P*-values as observed between intact resting and regenerating liver cells (table 3). In the lymphoid cell system, it has been suggested that membrane lipid fluidity is determined by the

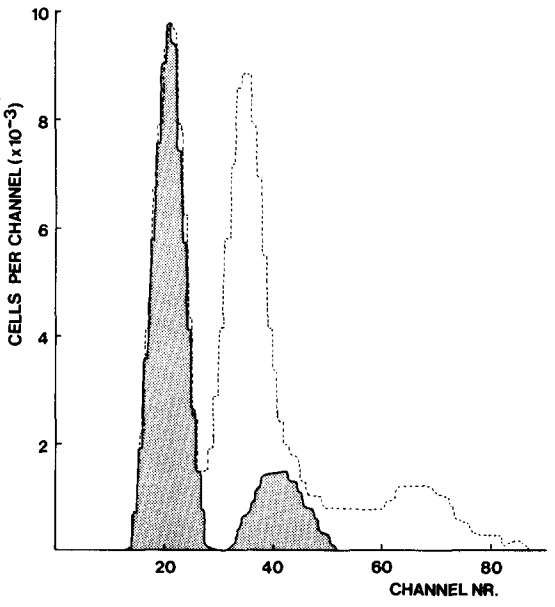

Fig.2. Pulse cytophotometric recordings of the distribution of liver cells in the cell cycle. Channel numbers are proportional to the increasing amount of DNA/cell. The solid line (shaded area) represents the diploid (channel No. 20) and tetraploid (channel No. 40) cells in resting liver, whereas the dotted line represents the diploid and tetraploid regenerating cells in the different phases (mainly *S*-phase) of the cell cycle, 24 h after partial hepatectomy.

Table 3

Comparison of the degree of fluorescence polarization (*P*) and microviscosity ($\bar{\eta}$, poise) at 25°C between resting and regenerating liver cells, isolated plasma membranes and liposomes of total lipid extracts from plasma membranes

Exp. No.	Intact cells		Isolated plasma membranes		Liposomes of total lipids in plasma membranes		Molar ratio cholesterol/phospholipids in plasma membranes
	P	$\bar{\eta}$	*P*	$\bar{\eta}$	*P*	$\bar{\eta}$	
I Control	0.194	1.50	0.310	4.86	0.278	3.48	0.60
Regen. liver	0.171	1.18	0.278	3.48	0.253	2.71	0.47
II Control	0.185	1.36	0.318	5.28	0.286	3.77	0.71
Regen. liver	0.158	1.03	0.297	4.22	0.254	2.73	0.38

cholesterol–phospholipid molar ratio in plasma membranes [6,9]. Our results agree with these observations since the cholesterol/phospholipid ratio in plasma membranes isolated from regenerating liver was about 60% of that of resting liver.

The authors are well aware of several open questions regarding the fluorescence polarization technique used to measure membrane lipid fluidity. Especially, the location of the probe DPH in intact cells is important. It has been argued that DPH monitors mainly the cell surface of intact cells [7]. However it is quite possible that in various cell systems endomembranes may also contribute to *P*-values measured on intact cells. Therefore, we included measurements on isolated plasma membranes of the liver cell system. The results obtained from isolated plasma membranes demonstrated the same tendency as those obtained from intact cells (table 3). However, comparison of intact cells and plasma membranes isolated from these cells shows lower *P*-values for intact cells. This can be due either to contribution of more fluid endomembranes in *P*-analysis of intact cells and/or to selection of more rigid parts of plasma membranes during the plasma membrane isolation procedure as suggested recently for *L*-cells [8].

Comparative fluorescence polarization analysis of intact leukemic cells and thymocytes with plasma membranes isolated from these cells [9] shows the same discrepancies as observed here in the liver cell system. However, in all cases differences in membranes fluidity measured on intact cells are always also exhibited by the plasma membranes isolated from these cells and the liposomes prepared from total lipid extracts of these plasma membranes. Thus possible effects of contribution of endomembranes to the *P*-analysis of intact cells, or selection of particular parts of plasma membranes for the *P*-analysis of isolated plasma membranes will only affect the absolute *P*- and microviscosity values but not the observed differences between resting and proliferating cells. Apart from the uncertainties inherent in this new technique, the data obtained from these three cell systems, each stimulated in a different way, indicate that cycling cells show a lower degree of fluorescence polarization, indicating a higher membrane lipid fluidity than non-cycling cells. It remains to be established whether the increase in fluidity of membrane lipids (exo- and possibly also endomembranes) is a necessary link with processes that trigger resting cells into proliferation or whether it is rather a general accompaniment of the altered metabolic state of cycling cells. In any case, fluorescence polarization analysis may be used to discriminate between resting and cycling cells and may give information regarding the physiological state of cells.

Acknowledgements

Thanks are due to Dr E. Scherer for the performance of the partial hepatectomy, Dr A. Tulp and Miss J. Welagen for preparing the liver cell suspensions and Dr C. A. Feltkamp for electron microscopic control of the isolated plasma membranes. We gratefully acknowledge the critical comments of Drs R. P. van Hoeven, L. A. Smets and J. H. M. Temmink during the preparation of this manuscript. We thank the Elscint company (especially Dr A. Bruck) for the opportunity to make use of their test model of the Single Cell Microviscosimeter.

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