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FLUORESCENCE STUDIES USING SYNCHROTRON RADIATION ON NORMAL AND DIFFERENTIATED CELLS LABELED WITH PARINARIC ACIDS

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Changes in membrane properties during the differentiation process in K562 cells have been investigated. A decrease of lectin-induced agglutination has been detected. The agglutination assay revealed to be an early and sensitive test to monitor the induced differentiation of the K562 cells. Naturally occurring fluorescent fatty acids (*cis*- and *trans*-parinaric acids) and the recently developed multifrequency phase and modulation technique were used to study cell membrane properties. Changes in fluorescence lifetime and polarization are clearly associated with cell differentiation, suggesting the involvement of the cellular plasma membrane in the differentiation process.

Introduction

The cell differentiation process is generally described as a progressive modification and appearance of new structures and functions. This process has been related to the control of gene expression but the mechanism and the physicochemical changes of the cellular components involved are largely unknown. The cellular plasma membrane is intimately involved in the control and expression of these changes. It has been suggested that cell surface glycoproteins serve as determinants of cell behavior [1,2] and that membrane enzyme activity is strongly related to the physico-chemical state of the phospholipid matrix [3]. Recently, a number of researchers have reported the coexistence of different phospholipid phases in the cellular plasma membrane [4–8]. This observation leads to the hypothesis that phospholipid cluster formation or domain segregation is relevant for the control of physiological membrane function and of the differentiation processes.

Using naturally occurring fluorescent polyenes (*cis*-PNA and *trans*-PNA) and the recently developed multifrequency phase and modulation fluorescence technique [9–12], we studied the plasma membrane modification in K562 proerythroblastic cells after the induction of differentiation. The K562 cell line can be induced to differentiate along the erythroid pathway acquiring some of the characteristics of the mature erythrocytes such as hemoglobin synthesis [13].

trans-PNA and *cis*-PNA show a marked difference in their lifetime values in different pure phospholipid phases [14]. This property of the parinaric acid isomers, their hydrophobicity, their high fluorescence yield in lipids and high limiting polarization values, render these isomers a powerful tool for the investigation of plasma membrane phase segregation.

Abbreviations: *cis*-PNA, *cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid; *trans*-PNA, all-*trans*-9,11,13,15-octadecatetraenoic acid (parinaric acid).

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Multifrequency phase and modulation fluorometry permits the analysis of complex emitting systems. In particular, the quantization of double exponential decay has been demonstrated in a number of interesting biological cases [11]. The simultaneous determination of fluorescence polarization and lifetime on a biological sample allows a better understanding of the emission properties of the probe, and a less ambiguous interpretation of the results [15]. However, preliminary experiments on the *cis*-PNA and *trans*-PNA fluorescence decay in homogeneous solvents showed that these decays are multi-exponential. Consequently, the fractional intensity of each component given by the analysis of *cis*-PNA and *trans*-PNA emission in cellular systems does not represent directly the fraction of different lipid phases in the membrane until an accurate quantization of the intrinsic heterogeneity of the probes is done. In view of these preliminary results, we preferred not to use the reported preferential partitioning property of *trans*-PNA in the gel phase [14]. Instead, we used the known difference of lifetime values of *cis*-PNA and *trans*-PNA in different phospholipid phases to qualitatively determine the state of the cell membrane. Our objective is the determination of the involvement of the cellular membrane in the differentiation process. The use of lifetime values, instead of fluorescence intensity, renders our measurement independent of the amount of scattering of the cell suspension. We used the simplifying assumption that it is possible to define an average lifetime value for the gel and the liquid-crystalline phase. In the gel, the lifetime value is higher than in the liquid-crystalline phase [14]. We analyzed our results in terms of a long and a short lifetime component to more rigid domains of the membrane and the short component of more fluid domains.

Materials

cis-PNA and *trans*-PNA obtained from Molecular Probes Inc. (Oregon) were purified following the method described by Fraley et al. [16]. Stock solution was made in absolute ethanol (spectroscopic grade) previously deoxygenated by N_2 bubbling and in the presence of 0.1% butylated hydroxytoluene (Sigma Chem. Co.). The probes were stored in the dark at 4°C under N_2 atmosphere.

Cell culture, agglutination and differentiation. The K562 cell line was originally isolated and characterized by Lozzio and Lozzio [17] as an outgrowth of a clone from a patient with chronic myelogenous leukemia. This cell line has been shown to produce glycophorin, the major sialoglycoprotein of the erythroid cell surface [18]. Two agents, as hemin and sodium butyrate, can promote hemoglobin accumulation [13]. The K562 subclone, S, originally from Cioe et al. [13], was maintained at 37°C in RPMI 1640 medium, supplemented with 10% fetal calf serum and routinely subcultured every 4 days. For the agglutination test, cells were harvested by centrifugation, washed twice with Dulbecco's phosphate-buffered saline in the absence of calcium and magnesium, and resuspended at a concentration of $1 \cdot 10^8$ cells/ml in the same buffer. 50 μl of the cell suspension were added to wells to which appropriate concentrations of concanavalin A had been previously added. The final volume was 75 μl. After 30 min at room temperature, one drop of suspension was deposited on a microscope slide and the number of clumped and single cells were counted [19]. Control incubations were carried out in the absence of concanavalin A.

For induction of erythroid differentiation, cells were subcultured to a density of $1 \cdot 10^5$ cells/ml before the addition of butyric acid (the final concentration was 2 mM). In the K562 subclone, S, more than 60–70% of the cells were benzidine-positive [13]. Increased hemoglobin synthesis was detected after only 24 h following hemin treatment, whereas 4 days were required for butyric acid-treated cells [13].

For hemoglobin determination, $1 \cdot 10^7$ cells were washed in cold phosphate-buffered saline and lysed in 1 ml of solution containing 0.15 M NaCl/3.5 mM magnesium acetate/0.5% Nonidet P40/10 mM Tris-HCl (pH 7.4). After 15 min, the nuclei were removed by centrifugation and the absorbance of the supernatant was measured at 425, 414, and 403 nm, according to the method reported by Rutherford and Weatherall [20].

Cell labeling and fluorescence measurements. $1.5 \cdot 10^6$ cells were washed twice with phosphate-buffered saline and resuspended in 3 ml of the same medium with 1 mM Ca^{2+} and 0.1% glucose.

The buffer was previously deoxygenated. Aliquots of the *cis*-PNA and *trans*-PNA ethanol solutions were evaporated directly from the cuvette under high vacuum and in the dark to give final probe concentrations of 3 μM. The resuspended cells were incubated in the cuvette for 30–40 min in the dark and under N_2 atmosphere.

Fluorescence polarization experiments were carried out with an SLM 4800 spectrofluorometer with the thermostated cell holder at 37°C. The excitation wavelengths were 324 and 320 nm for *cis*-PNA and *trans*-PNA, respectively (bandwidth of 1 nm). Emission was observed through a Schott cutoff filter, type KV (transmission wavelength greater than 370 nm). Polarization values were corrected for the intrinsic monochromator polarization characteristic response [21]. Cells were maintained in suspension by continuous mild stirring with a small magnetic spinbar.

Corrections for the scattering contribution to the polarization values were small at the cell concentration utilized. A plot of the polarization values vs. cell concentration is shown in Fig. 1. The cell concentration was $5 \cdot 10^5$ cells/ml to be in the concentration range where polarization is almost independent of concentration. From the polarization values, given by $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities parallel and perpendicular, respectively, to the excitation polarization, the anisotropy values, r, were derived by the relation $r = 2P/(3 - P)$.

Fluorescence lifetimes were determined using the multifrequency phase fluorometer at the ADONE storage ring (Frascati, Italy; [22]). This instrument is similar in design to that described by Gratton and Limkeman [10]. The light source is derived from the synchroton radiation at the low energy beam line at the P.U.L.S. Laboratory (Frascati). The excitation wavelength is obtained using an SLM model 380/A holographic grating monochromator. The frequencies set utilized (8.56, 17.13, 25.70, 34.64, 42.84, and 51.4 MHz) permits resolution of double exponential decays [11].

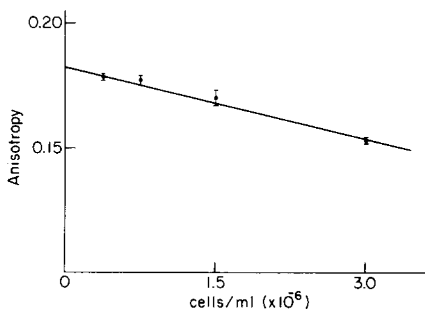

Fig. 1. Fluorescence anisotropy of *trans*-PNA as a function of cell concentration.

Results

The kinetics of differentiation induction of K562 cells were determined following the hemoglobin synthesis as the differentiation marker. After treating K562 cells with 2 mM butyric acid, the presence of hemoglobin was detected only after 4 days and reached the maximum concentration value after 5 days [13]. During the first 3 days after the butyric acid-induction, the hemoglobin concentration varied between 0.022 and 0.056 μg/10^6 cells and at the fifth day increased to 5 μg/10^6 cells. At this time, more than 60% of the cell population was benzidine-positive.

Lectin-induced agglutination was shown to be another test to monitor K562 differentiation. Changes in the composition and physico-chemical properties of erythroid cell plasma membrane are known to occur during differentiation [15]. K562 human cells were highly agglutinable in the absence of differentiation-inducing agents, so that 4 ng/ml concanavalin A produced complete agglutination. 48 h after the butyric acid-induced differentiation, a marked decrease of the agglutination was observed. In this condition, 62 ng/ml concanavalin A were necessary to obtain the full effect. This result suggests that a change occurs in the plasma membrane early during the differentiation process, preceding the onset of globin synthesis.

The degree of probe incorporation must be verified before the fluorescence parameters can be determined. *cis*-PNA and *trans*-PNA labeling were followed, in both cases, by the increase of fluorescence intensity and anisotropy as a function of time. The results are reported in Figs. 2 and 3, respectively. *cis*-PNA fluorescence intensity reached a constant maximum value after 25–30 min, whereas *trans*-PNA fluorescence intensity increased slowly. The corresponding fluorescence intensities for *cis*-PNA and *trans*-PNA in the buffer

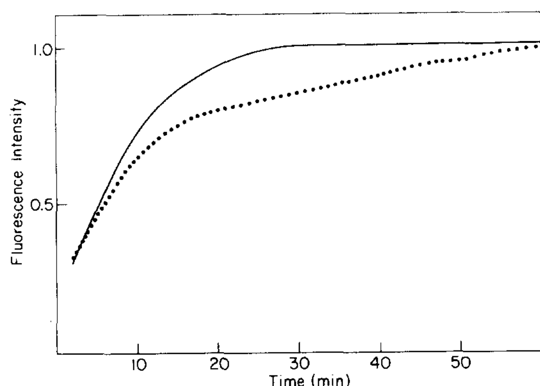

Fig. 2. Time variation of fluorescence intensity during incubation with K562 cells. *cis*-PNA (————), *trans*-PNA (······).

are reported in Fig. 2 as the zero-time values and in Fig. 3 as the polarization values of *trans*-PNA. Since the fluorescence quantum yields of the two probes in water are very small, the contribution to the emission anisotropy of the unbound probes was negligible. The anisotropy reached a constant value after 5–10 min of incubation (Fig. 3), suggesting the absence of diffusion to other inner cellular membranes.

A comparison between *cis*-PNA and *trans*-PNA fluorescence anisotropy values in nondifferentiated and differentiated K562 cells is presented in Table I, together with the values obtained with 1,6-diphenyl-1,3,5-hexatriene-labeled cells. A decrease in the anisotropy value is evident in differentiated cells. *cis*-PNA showed a more relevant

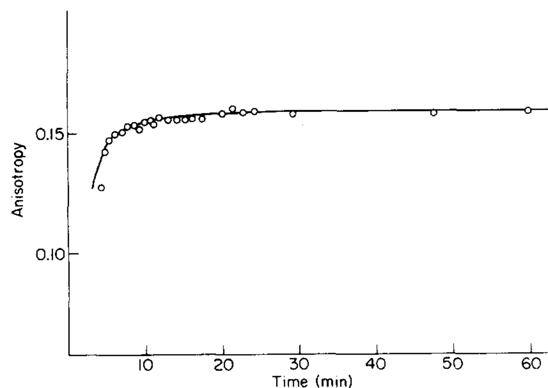

Fig. 3. Time variation of fluorescence anisotropy for *trans*-PNA during incubation with K562 cells.

TABLE I

FLUORESCENCE ANISOTROPY OF LABELED K562 CELLS

Fluorescence anisotropy values (r) for *cis*-PNA and *trans*-PNA in K562 cells, both nondifferentiated and 3 days after induction of differentiation. Also reported is the percent decrease of the fluorescence anisotropy in differentiated cells with respect to nondifferentiated cells. For the diphenylhexatriene (DPH) labeling, the proper aliquot of 2 mM diphenylhexatriene solution in tetrahydrofuran was evaporated from Dulbecco's phosphate-buffered saline with vigorous N_2 bubbling in the dark, to give the final 1.5 μM diphenylhexatriene concentration. $1.5 \cdot 10^6$ cells, twice washed, were resuspended in 3 ml of the diphenylhexatrienephosphate-buffered saline medium and incubated in the dark for 45 min. Diphenylhexatriene excitation was at 360 nm with 1 nm bandwidth and the emission was observed after a Schott cutoff filter at 418 nm.

	Nondifferentiated	Differentiated	Δr (%)
cis-PNA	0.157 ± 0.002	0.143 ± 0.002	-8.9
trans-PNA	0.168 ± 0.002	0.158 ± 0.002	-6.0
DPH	0.155 ± 0.002	0.125 ± 0.002	-19.4

decrease and a lower absolute fluorescence anisotropy value compared to *trans*-PNA. A fluorescence anisotropy decrease was also obtained in diphenylhexatriene-labeled differentiated K562 cells, but to a greater extent.

The fluorescence anisotropy decrease in differentiated cells was accompanied by a decrease in lifetime values as determined by the multifrequency phase-modulation method. *cis*-PNA and *trans*-PNA lifetime values obtained by phase-shift (τ^P) and demodulation (τ^M) at six different modulation frequencies in nondifferentiated cells, and after 3 days from the induction of the differentiation, are reported in Table II. The difference between τ^P and τ^M values for each frequency and their frequency dependence [11] demonstrate the existence of at least two lifetime values which may indicate different environments for the probes. The heterogeneity analysis for the data of Table II using the nonlinear least-square routine described by Jameson and Gratton [23] is reported in Table III.

Averaged lifetime values ($\langle\tau\rangle = \tau_1 \cdot \alpha_1 + \tau_2 \cdot \alpha_2$) decreased in differentiated cells. The calculated average lifetime values indicated that the decrease of the short-component lifetime values from nondifferentiated to differentiated cells was not com-

TABLE II

MULTIFREQUENCY PHASE AND MODULATION LIFETIMES

Lifetime values (ns) for *cis*-PNA and *trans*-PNA as measured by phase-shift (τ^P) and demodulation (τ^M) at various frequencies f(MHz) in differentiated and nondifferentiated cells.

f(MHz)	Nondifferentiated				Differentiated			
	cis-PNA		*trans*-PNA		*cis*-PNA		*trans*-PNA	
	τ^P	τ^M	τ^P	τ^M	τ^P	τ^M	τ^P	τ^M
8.57	4.60	11.32	5.16	8.19	4.28	6.58	4.31	8.40
17.13	3.74	5.29	3.66	6.27	3.90	5.48	3.18	5.33
25.71	3.59	4.99	3.38	5.73	3.54	4.98	3.18	5.17
34.64	3.28	4.40	3.18	4.98	3.23	4.66	2.93	4.04
42.84	3.05	4.18	2.81	5.06	2.81	4.48	2.46	4.67
51.41	2.93	4.05	2.76	4.65	2.93	4.20	2.33	4.35

TABLE III

LEAST-SQUARES ANALYSIS USING TWO EXPONENTIALS

Two-component analysis of *cis*-PNA and *trans*-PNA emission. Short-lifetime component (τ_1), long-lifetime component (τ_2), fractional intensity (f_1, f_2) and preexponential factor (α_1, α_2) for the long and short component, respectively. The average lifetime value was determined using the relation $\langle\tau\rangle = \tau_1\alpha_1 + \tau_2\alpha_2$. The fitted parameters are τ_1, τ_2 and f_1. The errors reported were obtained from the least-squares analysis. The preexponential factors are calculated using $\alpha_i = (f_i/\tau_i)/(\tau_1/\tau_1 + f_2/\tau_2)$ where $i = 1, 2$. χ^2 is the reduced chi-square value.

	τ_1	f_1	α_1	τ_2	f_2	α_2	$\langle\tau\rangle$	χ^2
cis-PNA								
Nondifferentiated	2.89±0.03	0.80±0.03	0.96	19.21±5.59	0.20	0.04	3.54	44.1
Differentiated	2.03±0.20	0.55±0.06	0.82	7.77±0.85	0.45	0.18	3.06	14.0
trans-PNA								
Nondifferentiated	2.19±0.17	0.62±0.04	0.90	11.84±1.61	0.38	0.10	3.16	33.7
Differentiated	1.23±0.90	0.45±0.07	0.82	6.88±0.72	0.55	0.18	2.25	40.4

pletely compensated by the increase in the long-lifetime component fraction (α_2).

Discussion

Several studies have been carried out regarding the appearance of new structures and functions during the differentiation process occurring in some cell lines [1]. In particular, the hemoglobin synthesis increase [13] as well as the appearance of new membrane glycoproteins [12,18] have been reported for K562 cells.

We utilized the lifetime of the two isomers of the fluorescent parinaric acid, which have been shown to discriminate between gel and liquid-crystal phases [14] to qualitatively determine the state of the cell membrane.

3 days after induction of the differentiation process, the long-lifetime component value decreased but its pre-exponential factor increased, for both probes. This result may indicate a rearrangement of the phospholipid matrix of the cellular plasma membrane, the final situation being more liquid-crystal-like in nature, but with a relative increase of the partially 'solid' phospholipid phase.

The fluorescence anisotropy results with the parinaric acids on nondifferentiated and differentiated K562 cells follow the same pattern observed for the lifetime values. Diphenylhexatriene-labeled differentiated K562 cells also confirmed this variation with a 10% decrease of the anisotropy value, although uncertainties on the location of this probe persist [24].

The decrease of fluorescence anisotropy during differentiation cannot be attributed to butyric acid incorporation into the membrane, since this compound is added to the growth medium as a starting pulse and during the following days is metabolized by the cells and disappears from the medium. Fluorescence anisotropy and lifetime variation in differentiated cells cannot be attributed to hemoglobin quenching of PNA fluorescence, since all fluorescence measurements were performed during the third day after induction of differentiation, at which time the hemoglobin concentration is still negligible. This fact suggests that the plasma membrane rearrangement precedes the hemoglobin gene expression activation, a conclusion which is supported by the results on the lectin-induced agglutination. Only 2 days after the induction of the differentiation process, the concanavalin A concentration necessary to produce complete agglutination is increased 16-fold.

From the reported results, we concluded that the cellular plasma membrane is intimately involved in the differentiation process. At present, a more detailed analysis is infeasible due to the complexity of the phenomena that take place in a living cell. Other explanations of our results are possible, such as the production of molecules by the cell, other than hemoglobin, which act as quenchers of the parinaric acid fluorescence and lifetime. However, in line with other experiments on differentiated cells employing different techniques, we tentatively assign the observed variation of the average lifetime value and anisotropy to structural changes of the cell membrane. Finally, we note that the two-state model employed gives a very large value for the chi-square (Table III). This is due in part to the complex decay of the parinaric acid isomers and in part to the heterogeneity of the cellular membrane.

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