Differences in Membrane Order between C3H 10T1/2 Cells and Their Transformed Counterparts as Measured by EPR

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Membrane order of mouse embryo fibroblasts and their ionizing radiation and chemically transformed counterparts was investigated using EPR spectroscopy after labeling the membrane of the cells with the fatty acid spin label, 5-nitroxy stearic acid. The EPR spectra were recorded at temperatures ranging from 18 to 38 °C for both control and transformed cells. The distance between the outer hyperfine splitting ($2T'_{\parallel}$), which is used as an indicator of membrane order, varies in these two cell types. Below 28 °C, $2T'_{\parallel}$ is higher in transformed fibroblasts than in normal cells, whereas above this temperature membrane order is the same. Lipid analysis as carried out by the measurement of the cholesterol/membrane proteins and sphingomyelin/lecithin ratios, showed no difference in the amounts of the main membrane rigidifiers. These findings suggest that cell transformation of mouse fibroblasts induced by radiation or chemicals may produce alterations in the cell membrane, as evidenced by variations in its order at low temperature. These measured differences are presumably not attributable to its fatty acids composition but to its glycoproteins content, since changes in membrane regidifiers were not observed between normal and transformed cells.

Introduction

Currently oncogenes dominate the scene of fundamental cancer research, and the number and nature of molecular events leading to malignancy are beginning to be understood. It seems also evident that changes at the genetic level must become expressed in cellular functions and structures crucial to the control of normal proliferation and cell behavior. In this respect, the plasma membrane is of paramount interest; the structure and the physical state of its components may, in fact, exert a profound influence on cell phenotype and the various functions of the cell because any changes in the composition, orientation, or mobility of membrane components may be expressed as aberrations in intracellular metabolism as well as alterations in membrane dynamic properties.

The potential differences in the mechanisms of radiation *versus* chemical carcinogenesis need yet to be demonstrated. With this in mind, we have compared EPR signals arising from membranes of mouse cells transformed either by ionizing radiation or by the chemical mutagen methylcholanthrene. Changes produced by ionizing radiation or chemical carcinogens on the cellular genome, such as to induce the cells to pass from the normal to the transformed state, may also be reflected in changes in membrane properties. A variety of specific plasma membrane alterations have been described in association with cellular transformation [1, 2]. Although the general structure, or basic organization, of the membrane seems not to be altered, the data in the literature are quite contrasting regarding the changes occurring in its component parts [3]. Nevertheless, it may be deduced from these studies that the differences between the amounts of lipids, proteins and carbohydrates in membranes of normal and transformed cells do not seem to be significant, while alterations in specific membrane structures have been observed [4]. Therefore, measurements by electron paramagnetic resonance (EPR) of lipid motion (flexibility of lipid chains) in intact cells can help elucidate if dif-

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ferences exist in the membrane mobility between normal and transformed cells, and if these differences also reflect structural changes.

Labeling of membranes with the fatty acid spin label 5-nitroxy stearic acid (5-NSA) in EPR experiments provides a measure of the degree of motion around the molecular long axis and the average orientation of the fatty acid chains in the lipid bilayer [5]. The degree of motion of this label compound is sensitive to the flexibility of membrane lipid chains. Although the EPR spin label method is well established as a technique for probing cell membranes, few comparisons of transformed cells with their corresponding normal cells have been made and, to our knowledge, not with cells transformed by ionizing radiation.

Spin label measurements were conducted in mouse embryo C3H 10T1/2 fibroblasts and transformed cell lines derived from these cells. The C3H cell line, developed by Reznikoff *et al.* [6], has been repeatedly demonstrated to undergo morphological and oncogenic transformation in response to a number of chemical and physical carcinogens [7, 8].

Membrane order was calculated from EPR spectra obtained at different temperatures, so as to have a more complete analysis of membrane behavior. In addition, the levels of cholesterol, sphingomyelin, lecithin and membrane proteins, which may be considered the main chemical modulators affecting lipid fluidity [9], have also been measured.

Materials and Methods

Culture and transformation conditions

The C3H mouse embryo fibroblast line 10T1/2 clone 8 developed for transformation assays by Reznikoff *et al.* [6] was used. Control and transformed cultures were grown in monolayer at 37 °C in a humidified 2% atmosphere in Eagle's basal medium prepared in our laboratory with Gibco products and supplemented with 10% fetal calf serum (Flow Laboratories) heat-inactivated for 45 min at 56 °C. The following concentrations of antibiotics were added to the medium: gentamicin, 50 μg/ml; penicillin, 50 u/ml; and streptomycin, 50 μg/ml.

The radiation-induced transformed cells (F6 10T1/2) were kindly provided by Dr. L. Tallone Lombardi and was obtained by exposure of the C3H 10T1/2 cells to 31 MeV protons [10]. Originally F6 10T1/2 cells were picked from a type III focus using the criteria suggested by Reznikoff *et al.* [7], were subcultured and tested for anchorage independent growth in a semisolid agar medium.

The chemically transformed cells (MCA 10T1/2) were generously donated by Dr. J. R. Landolph. They were derived by treatment of the 10T1/2 cells with 3-methylcholanthrene (MCA) for 24 h and produce fibrosarcomas when 10^5 or more cells are inoculated into syngeneic mice [11].

Lipid and total protein extraction and methods of analysis

Growth medium was removed from the subconfluent cell monolayer by aspiration and the cultures were rinsed twice with cold phosphate-buffered saline (PBS). A small volume of cold PBS was then added to each plate, and the cells were scraped off with a polyethylene policeman and transferred to a centrifuge tube. A total number of about 10^7 cells were used for each sample. Cell lysis was performed by thawing in liquid nitrogen and rapid defreezing at 37 °C five times. The broken cell suspension was then centrifuged at 16,000 × *g* for 30 min at 4 °C. The pelleted material was used as the crude membrane preparation. Total membrane proteins (μg/10^6 cells) were determined by the Bradford technique [12]. Lipids were extracted from the pellet by the Downing method [13]. Amounts of the lipid species examined (μg/10^6 cells) were determined following fractionation of the total lipid extract by thin-layer chromatography on small silica 60 gel plates using the system chloroform:methanol:4-N-ammonium hydroxide (17:5:1 v/v). Lipid spots on silica gel plates were visualized after spraying the plates with bromothymol blue reagent. The spots were read with a Helene Laboratories Cliniscan photodensitometer at a wavelength of 610 nm and the areas of different lipid peaks quantified by an integrator analyzer. All solvents were purchased from Carlo Erba (Milan, Italy). Lipid standards were purchased from Sigma-Tau (St. Louis, U.S.A.) and bromothymol blue was supplied by Supelco (Bellefonte, U.S.A.).

Spin labeling procedure and EPR measurements

C3H 10T1/2 cells and their transformed counterparts were grown to subconfluence phase,

washed twice with cold PBS when still in the flask and then trypsinized by standard methods, collected and centrifuged for 10 min at 2,000 × *g* at 4 °C. The pellet was washed twice with PBS to remove the trypsin completely, and recentrifuged. The PBS buffer was removed from the pellet by aspiration, the sides of the tube were cleared of residual buffer. This cell pellet was used directly for EPR measurements. The spin label 5-nitroxy stearic acid (2-(3-carbonoxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidieryloxyl) was purchased from Aldrich (Milwaukee, U.S.A.). A 3.25×10^{-2} M stock solution was made in 100% ethanol and stored at −20 °C until needed. A volume of 1 µl of this solution was added to the cell pellet as reported by Santini *et al.* [14].

Spectra were run on a Varian E-4 X-band Spectrometer at a microwave frequency of 9.12 GHz, at a power of 10 mW and with a field modulation of 100 KHz and modulation amplitude of 1 G. The temperature was monitored with an accuracy of ± 0.1 °C by a digital thermometer with its thermocouple inserted into the capillary tube holder where silicon oil was added to maintain even temperature distribution.

Samples from at least three separate preparations of each cell type were examined and EPR spectra were recorded at temperatures between 18 and 38 °C. For all temperatures values, the distance between the outer hyperfine splitting ($2T'\|$) was measured directly from the spectra and used as an indicator of membrane order.

The order parameter S, which is a measure of the mean angular deviation of the acyl chain of the fatty acid at the position of the nitroxide group from the bilayer normal [5], was also calculated from the already mentioned outer ($2T'\|$) and inner ($2T'^{\perp}$) hyperfine splitting, measured in G, by the relationship:

$$S = \frac{T'\| - (T'^{\perp} + 0.8)}{Tzz - (Txx + Tyy)/2} \qquad (1)$$

where $Txx = 6.3$ G, $Tyy = 5.8$ G and $Tzz = 33.6$ G as reported by Dodd and Kumar [15].

Results

The spin label probe, 5-NSA, inserts (intercalates) in the membranes of cells noncovalently with its longitudinal molecular axis perpendicular to the membrane surface. With 5-NSA, the location of the oxazolidine ring at position 5 of the hydrocarbon chain of the stearic acid allows the polar position of the hydrophobic tail of the lipid bilayer to be examined. For a complete theoretical treatment of the use of spin labels to study membrane order see McConnell and Gaffney McFarland [16]. The EPR spectra obtained are typical of anisotropic motion, partially restricted, as expected in an oriented membrane. Typical spectra of control and transformed C3H 10T1/2 mouse fibroblasts labeled with 5-NSA and the parameters $2T'\|$ and $2T'^{\perp}$ are shown in figure 1.

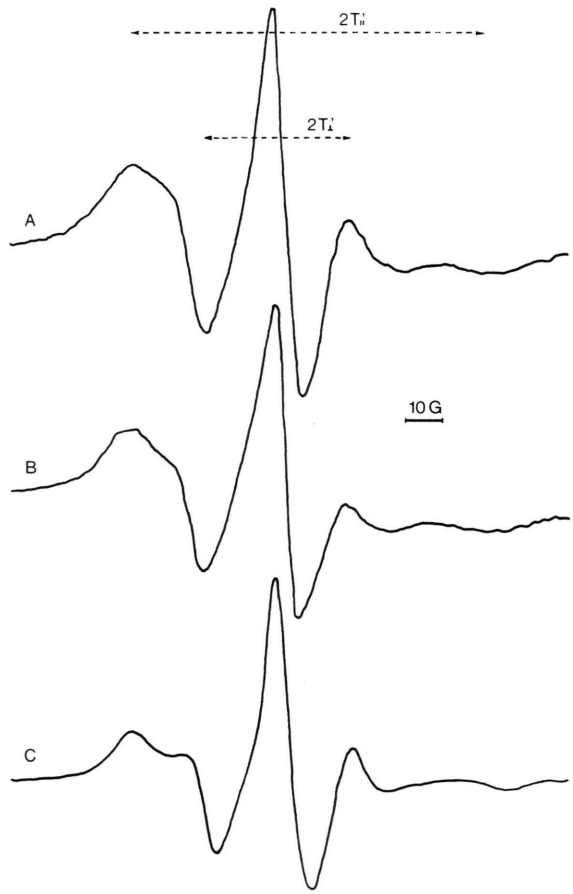

Fig. 1. Representative EPR spectra of control (A), radiation transformed (B) and chemically transformed (C) cells labeled with 5-nitroxy stearic acid. These spectra were recorded at 23 °C as described in "Methods". The outer ($2T'\|$) and inner ($2T'^{\perp}$) hyperfine resonance extrema of the T-tensor used to determine the order parameter S, are indicated. The marked scale indicates 10 G intervals. The shapes of the EPR spectra are identical over the temperature range analyzed both for control and transformed cells.

The parameters which contribute to EPR spectra of cell membranes are not fully understood but they include mobility of phospholipids in the bilayer, local changes in charge across the membrane and molecular ordering. While we have referred to changes in $2T'||$ as changes in membrane order, $2T'||$ should be understood as an empirical measurement of molecular motion in the immediate vicinity of the spin-label probe.

Preliminary measurements of $2T'||$ for 5-NSA spin-labeled C3H 10T1/2 control and transformed cells at room temperature (23 °C), showed a difference of two gauss. It was then decided to make measurements of membrane order in the range between 18–38 °C because spectra of spin-labeled membranes recorded over a range of temperature sometimes reveal more subtle temperature dependent changes in the structure of the lipid phase of the membrane.

The values of $2T'||$ and $2T'^{\perp}$ measured from EPR spectra and the order parameter S, calculated from Eqn. (1), are shown in Table I for various temperatures. The temperature dependence of the outer hyperfine splittings, measured by experiments repeated three times, is shown in Fig. 2. Since the splitting between the inner hyperfine peaks ($2T'^{\perp}$) tends to be obscured at low temperature because of the lowered rate of molecular motion, we have presented $2T'||$ *versus* temperature instead of the usual plot of the order parameter *versus* reciprocal temperature.

Fig. 2 shows that the outer hyperfine splitting decreases linearly with increasing temperature for

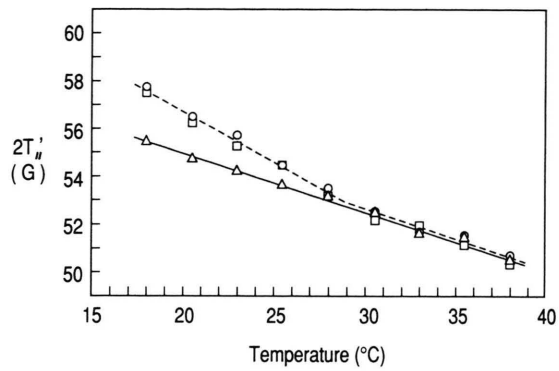

Fig. 2. Temperature-dependence of the outer hyperfine splitting ($2T'||$) for control and transformed cells labeled with 5-nitroxy stearic acid. $2T'||$ was measured over a temperature range of 18–38 °C for control (△) radiation transformed (○) and chemically transformed (□) cells as described under "Methods". The means and standard deviations of three separate experiments are plotted for each point. Graphs have been drawn by eye to facilitate interpretation of the data.

Table I. Observed hyperfine splittings and order parameter for 5-nitroxy stearic acid spin label in control and transformed mouse fibroblasts for various temperatures.

Temperature [°C]	Cell line	Hyperfine splittings $2T'||$ [G]	$2T'^{\perp}$ [G]	Order parameter S
18	C3H 10T1/2	55.50	18.50	0.64
	F6 10T1/2	57.75	18.50	0.68
	MCA 10T1/2	57.50	18.50	0.68
23	C3H 10T1/2	54.25	18.50	0.62
	F6 10T1/2	55.75	18.50	0.65
	MCA 10T1/2	55.25	18.50	0.64
28	C3H 10T1/2	53.25	18.50	0.60
	F6 10T1/2	53.50	19.00	0.60
	MCA 10T1/2	53.25	18.50	0.60
33	C3H 10T1/2	51.75	18.50	0.57
	F6 10T1/2	51.75	19.00	0.57
	MCA 10T1/2	52.00	19.00	0.57
38	C3H 10T1/2	50.25	19.00	0.54
	F6 10T1/2	50.50	19.50	0.53
	MCA 10T1/2	50.00	19.00	0.53

The means of three separate experiments for each point are shown. The standard deviations are 0.25 G for all values of the hyperfine splittings.

Table II. Lipid content of C3H 10T1/2 cells and their transformed counterparts.

	C3H 10T1/2	F6 10T1/2	MCA 10T1/2
Cholesterol/membrane proteins [μg/μg]	0.17	0.17	0.18
Sphingomyelin/lecithin [μg/μg]	0.24	0.24	0.25

The means values were calculated over three experiments. The overall uncertainty was of 10%.

the normal control cells, while for the transformed cells it decreases with an inflection at 28 °C. The hyperfine splitting tensor measured for both types of transformed cell lines is greater than in controls in the temperature range between 18–28 °C. For values greater than the critical temperature of 28 °C, there is no significant difference.

Finally, the levels of two of the main modulators of lipid fluidity, obtained from biochemical assays, are shown in Table II. The cholesterol level is presented as the cholesterol/membrane proteins ratio and the sphingomyelin content is shown as the sphingomyelin/lecithin ratio. There is no difference in the cholesterol, lecithin and sphingomyelin (data not shown) concentrations between control and transformed cells.

Discussion

The main conclusions that can be made from this work are that the dependence of membrane order *versus* temperature in C3H 10T1/2 fibroblasts and their transformed counterparts is not the same, and further that this difference is the same for cells transformed by exposure to either radiation or a chemical. Secondly, membrane order is greater for both transformed cells lines with respect to control cells but the difference is insignificant above 28 °C.

Our results are in agreement with those of other investigators, who have observed smaller rotational mobility of spin label and fluorescent probes in transformed cells [17–24], and inflections at some critical temperature in analogous plots [25]. This study seems to indicate an overall decreased lipid fluidity (increased structural order) in membranes of transformed cells. A lower membrane fluidity was also observed in transformed cells by Barnett *et al.* [26], Nicolau *et al.* [27], and Monti *et al.* [28]. However, there have also been reports that no significant differences exist in membrane fluidity between normal and transformed fibroblasts [25, 29]. These differences in the experimental results might be attributed to the use of different cell types, different transformation agents, or different probes, and, not lastly, different EPR experimental conditions. In an excellent review by Van Blitterswijk [30] several of these inconsistencies and problems have been addressed.

The observed differences in membrane order, seen only at temperatures below 28 °C, might be due to changes in membrane organization and/or composition as a result of cellular transformation, and presumably due to genetic changes.

The results of the lipid analysis presented in this report show that there is no difference in the amounts of cholesterol and sphingomyelin which are both present in the membranes of C3H 10T1/2 cells and their transformed counterparts. Under physiological conditions, cholesterol is one of the main membrane rigidifiers in eukaryotic cells; and its effects are to increase the microviscosity, which simultaneously increases the degree of order in lipid domains. Sphingomyelin, another important rigidifying agent, can also act as a powerful coupler of the two lipid monolayers; it can form separate domains and it seems to have affinity to cholesterol and proteins (see Shinitzky [9] for review). The data presented in the literature concerning the changes in the lipid levels induced by cell transformation are contrasting. The amounts of cholesterol and sphingomyelin, found in transformed fibroblasts compared to normal cells in tissue culture, have been reported to be increased [31, 32], or unchanged [33–35].

The degree of unsaturation of the fatty acid lipid chains must also be considered as an important factor influencing membrane order. In fact, Yau *et al.* [19] have shown by detailed gas-liquid chromatography methods that the degree of unsaturation of fatty acids was lower in transformed than in normal cells. This lowered degree of unsaturation may give rise to higher ordering of the transformed cell membrane. Although data on the de-

gree of unsaturation of the lipid fatty acid chains in control and transformed cells is not presented in this paper, the influence of such a parameter and its consequent effect on membrane order cannot be excluded.

An additional consideration that should be made is the distribution of the spin probe used in different membranes of the cells (plasma, mitochondria, SR, nuclear, etc.). In fact, Nettleton *et al.* [36] have demonstrated that the 5-nitroxystearate spin label distributes throughout the different membranes of cells. Thus, if the probe is located in all of these membranes and if different ratios of these membranes exist in normal and transformed cells, this different distribution of the spin label might also contribute to the differences in membrane order observed.

Under our experimental conditions we found no difference in fatty acid composition between control and transformed mouse fibroblasts. The dependence of lipid composition on cell growth conditions was reduced in our experiments by strict control of experimental conditions and by using cells in the subconfluent phase rather than in exponential growth for all EPR measurements. Apparently, as demonstrated by Nicolau [37], plasma membranes of confluent chick embryo fibroblasts possess a much lower lipid fluidity than at other stages of the cell cycle.

Differences in membrane order observed below 28 °C and not above this temperature are not easily explained. However, it may be postulated that around physiological temperatures membrane differences are less apparent since the transformed cells somehow compensate for some changes in order to carry out proper cellular functions. Below such temperatures, the cells do not need to compensate and, therefore, variations are more evident.

The flexibility of lipid chains is not always determined by chemical composition alone, but may also be affected by the presence of proteins in the intact membrane [29]. As an example, the fluidity of the lipids in the membrane of Sindbis virus is dominated by the presence and the interaction of the viral proteins with the membrane lipids [38]. Similarly, alteration of lipid motion may also be influenced by glycoproteins that occur abundantly on the surface of cell membranes and that decrease, disappear, or are no longer completely glycosilated in the membrane of transformed cells [39, 40]. Therefore, changes in membrane order measurable as lipid motion may also be correlated with differences in protein mobility and/or quality.

Recent results for carcinogenesis studies in mouse skin cells suggest that the target gene(s) for oncogenic activation is different for chemical carcinogens and ionizing radiation [41]. If this proves to be correct, the effects observed by us seem not be distinctive of the inducing agent (radiation or chemical) but perhaps rather a consequence of the subsequent oncogenic processes since the membranes appear to be structurally alike for the two cell lines transformed either by radiation or a chemical.

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