Eur J Cancer Clin Oncol, Vol. 20, No. 5, pp. 673–677, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Discrimination between Human Melanoma Cell Lines by Fluorescence Anisotropy

A. WEINREB*† and P. TRAVO‡§

*Laboratoire de Physique, ERA 551 CNRS and ‡Laboratoire de Pharmacodynamie, ERA 787 CNRS, U. 243 INSERM, Faculté de Pharmacie, 74, Route du Rhin, 67400 Illkirch, France

Abstract—*The fluorescence polarization of diphenylhexatriene (DPH) and trimethylammonium diphenylhexatriene (TMA-DPH) was measured when these markers were imbedded in cells of the human melanoma cell lines IGR37, IGR39, IGR3 and IGR4, as well as in cells of the mouse melanoma cell lines B16 F1 and B16 F10. These measurements were performed on cell cultures which were grown on quartz plates as well as on cell suspensions. Considerable differences are found between the polarization values of the human cell lines that are related to their different origins. Differences for the plated cells are considerably greater than those for the suspensions. No differences in the polarization values were found for the two mouse melanoma lines. It is concluded that differences in lipid structural order can be found between cell types endowed with different metastasizing capabilities.*

INTRODUCTION

CANCER is a malignant disease primarily because of the ability of tumour cells to form metastases. Among all known cancers, melanoma is one of the most actively and unpredictably metastasizing. The metastasizing process often begins a long time after the primary tumour has been found and treated. Once it has begun, it cannot efficiently be stopped or retarded by any known therapy. The understanding of the metastasizing process is thus of great importance to melanoma therapy. It is well established that cells endowed with a metastasizing capability are different in many respects from those of the tumour from which they originate. In effect, in order to form a metastasis they must leave the tumour, enter the blood stream, then leave the blood stream and enter and settle in a new tissue. Their capacity to pass the vessel wall, in either direction, and to creep inside a healthy tissue implies that they are capable of high mobility, active deformability and substratum recognition [1].

Cells capable of forming metastases represent only a fraction of the tumour cell population, and the study of their biological properties *in situ* is virtually impossible. Nonetheless, several cell lines from human and murine melanoma were established which display various degrees of metastasizing capability. Since metastasizing cells may differ from other tumour cells by their higher mobility and active deformability, one would expect their lipid structural order to be lower than that of non-metastasizing cells. One approach to the study of cell lipid structural order is the measurement of fluorescence anisotropy of a marker molecule which has been introduced into those cells.

Fluorescence anisotropy is considered to be a physical parameter which gauges the lipid structural order of the environment. Recently much work has been published which demands a qualification of this concept. We tend to agree with those authors [2, 3] who claim that it is the structural features of the lipid membranes rather than the dynamic viscosity which is represented by the fluorescence anisotropy of the marker molecule. So far fluorescence studies have been conducted almost exclusively on cell suspensions and any physical theory that has been used for the interpretation of the results assumed a macroscopically isotropic medium. However, cells like melanocytes, which normally grow in tissue, may, and almost certainly will, exhibit altered structural properties when they are in suspension, specifically those related to mobility and active

Accepted 26 October 1983.

†On sabbatical leave from the Racah Institute of Physics, Hebrew University, Jerusalem, Israel.

§To whom correspondence and requests for reprints should be addressed at: Laboratoire de Pharmacodynamie, B.P. 10, F 67048 Strasbourg Cédex, France.

deformability. One indication of such gross changes is that tissue cells do not divide when in suspension. Obviously the ideal arrangement would be to study the fluorescence anisotropy *in situ*. Since present techniques, however, do not yet permit such studies, we deemed it worthwhile to endeavour this study of fluorescence anisotropy using different human and murine melanoma cell lines as an experimental model under the limitations indicated by Tveit and Pihl [4]. Cell lines were chosen according to their histological origin and metastatic potential.

MATERIALS AND METHODS

Stock cultures

IGR3, IGR4, IGR37 and IGR39 are human melanoma cell lines established at the Institut Gustave Roussy, Villejuif, France [5]. They were kindly supplied to us by Dr. D. Aubert. IGR3 and IGR4 originated from the same nodular melanoma. IGR3 was obtained from level IV and IGR4 from level II of the tumor. IGR39 was obtained from a superficially spreading melanoma (S.S.M.). IGR37 was obtained from a lymph node metastasis of the same patient. They were grown in minimum essential medium with Earle's Salts (E. MEM, Biomerieux) supplemented with 2 mM glutamine, 10% foetal calf serum (F.C.S.) and 100 U/ml penicillin G. B16 F1 and B16 F10 are mouse melanoma cloned cell lines [6]. They were kindly supplied to us by Dr. I. Vlodavsky, Hadassah University Hospital, Jerusalem. They were grown in E. MEM (Biomérieux), supplemented with 2 mM glutamine, non-essential amino acids and vitamins (Flow Labs), 10% F.C.S. and 100 U/ml penicillin G.

Cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2 in air. Media were renewed twice a week. Cultures were replated once a week at an average initial density of 20,000 living cell/cm^2 (trypan blue exclusion).

Fluorescence anisotropy measurements

Plated cells. For fluorescence studies, cells were grown on quartz slides (1.2 cm × 4 cm × 0.2 cm) in 60-mm Petri dishes. They were plated at an average initial density of 50,000 living cells/cm^2. IGR3, IGR4, IGR37, IGR39, B16 F1 and B16 F10 cultures were used for fluorescence studies from day 2 to day 5 after setting. Prior to use, slides were taken out of the medium and put separately into 60-mm Petri dishes containing 10 ml Hank's balanced salt solution (H.B.S.S.) without phenol red for 1 min at room temperature. H.B.S.S. was then renewed twice. The last rinsing took 10 min. Then H.B.S.S. was renewed (10 ml) and the fluorescent probe was added. Incubation was carried out at 37°C for 90–150 min. We used a control to ensure that cultures were not damaged by this treatment.

Cell suspensions. Cell suspensions were obtained from cultures on quartz plates. Prior to resuspension, plates were processed as above. Then H.B.S.S. in which cultures were incubated with the fluorescent probe was replaced by 5 ml of fresh H.B.S.S. Cells were resuspended with a rubber policeman. The absence of a significant number of clumps was controlled for both with a haemocytometer and a calibrated ZBIc C1000 Channelyzer (Coultronics). When necessary, suspensions were successively diluted until the optical density of the suspension for the excitation wavelength reached a value close to or below 0.1. At this optical density the effect of light scattering by cells on the polarization values is negligible.

Fluorescent markers. Two types of marker molecules were used: diphenylhexatriene (DPH) [7] and trimethylammonium diphenylhexatriene (TMA-DPH) [8]. It is now well established that DPH penetrates into the cell and is distributed over all membranes, in fact over all lipidic structures [9]. It is believed that the hydrophobic hydrocarbon part (the DPH) of TMA-DPH enters the membrane while the polar head (the trimethylammonium) is anchored at the lipid–water interface, which prevents the molecule from penetrating any further into the lipid bilayer. Hence the fluorescence polarization of this molecule most probably represents the lipid structural order of the peripheral zone of the lipid bilayer of the membranes.

DPH was administered by injecting 5 μl of a solution of 2×10^{-3} mol/l of DPH in tetrahydrofuran; TMA-DPH was administered by injecting 3 μl of a solution of 10^{-3} mol/l of TMA-DPH sulphonate in the same solvent. We did not find any evident influence of incubation time on the measured polarization values. Preliminary experiments, however, indicated that the temperature of incubation may influence the results.

Experimental set-up. The experimental set-up was similar to those described by de Laat *et al.* [10] and Yuli *et al.* [11] for glass plates. The computerized monitoring equipment of the SLM apparatus gives the calculated values of r (fluorescence anisotropy). Precautions were taken to minimize the photochemical effects of the exciting radiation (350 nm). The influence of reflected light on the measurements was negligible.

RESULTS

The results are summarized in Table 1. It can be seen that the fluorescence anisotropy of DPH and TMA-DPH is considerably affected by the cell type and experimental conditions.

Table 1. *Fluorescence anisotropy* (r) *of DPH and TMA-DPH in IGR37, IGR39, IGR3, IGR4, B16 F1 and B16 F10, at* 37°C *in Hank's balanced salt solution*

	DPH			TMA — DPH		
	Plated cells (*r*)		Suspensions (*r*)	Plated cells (*r*)		Suspensions (*r*)
IGR37	0.128 ± 0.012 (25)	n.s.	0.139 ± 0.017 (10)	0.298 ± 0.006 (6)	s	0.266 ± 0.009 (4)
	s		n.s.	s		n.s.
IGR39	0.174 ± 0.017 (30)	s	0.157 ± 0.010 (10)	0.334 ± 0.012 (6)	s	0.268 ± 0.005 (4)
IGR3	0.126 ± 0.004 (6)	n.s.	0.128 ± 0.002 (4)	0.266 ± 0.002 (4)		0.252 (a)
	s		s	n.s.		0.252
IGR4	0.186 ± 0.013 (6)	s	0.152 ± 0.003 (4)	P.270 ± 0.008 (4)		0.252 (a)
						0.250
B16 F1	0.182 ± 0.007 (11)		n.d.	0.355 (a)		0.256 (a)
	n.s.			0.354		0.260
B16 F10	0.182 ± 0-007 (11)		n.d.	0.354 (a)		0.264 (a)
				0.358		0.260

Results are means ± S.E.M.; ( ) number of determination. Each determination is the mean of at least six measurements; n.d.: not determined; (a): two determinations only. Each determination is the mean of six measurements. Statistical significance was determined (*t* test) for $P < 0.05$; n.s.: non-significant; s: significant.

In plated human melanoma cells the fluorescence anisotropy of DPH enabled the distinction between two groups of values: low values for IGR37 and IGR3 and high values for IGR39 and IGR4, indicating differing lipid structural orders. The differences between the two groups were greatly diminished when the cells were in suspension, mainly because of a marked decrease of fluorescence anisotropy of DPH in IGR4 and IGR39. The fluorescence anisotropy of TMA-DPH was much higher than that of DPH. This is due to the much shorter decay time of this compound when compared with DPH. With this marker the fluorescence anisotropy was very different in plated IGR37 and IGR39, while there was no difference between IGR3 and IGR4.

No differences were found with either marker between the murine melanoma cell lines, even with plated cells. However, as with human melanoma cell lines, the fluorescence anisotropy for TMA-DPH was higher for the plated cells than for the cells in suspension.

DISCUSSION

The characteristic features of these results are: (1) the differences in the fluorescence anisotropy of plated cells and cells in suspensions for a given marker; (2) the difference in the fluorescence anisotropy of human melanoma cell lines from the same donor; and (3) the absence of such differences for the murine melanoma cell lines.

It is not surprising that the fluorescence anisotropy of both markers is different for plated cells and for cells in suspension. This observation is further evidence that the lipid structural order of plated cells is profoundly changed by placing the cells in suspension. As a consequence, differences in fluorescence anisotropy between cell lines which are clearly seen for plated cells may be reduced or obliterated by performing the measurement on cells in suspension.

Among the human melanoma cell lines which we studied, IGR39 and IGR4 displayed a higher lipid structural order with DPH than did IGR37 and IGR3. With TMA-DPH as the marker, fluorescence anisotropy could distinguish IGR37 from IGR39 but not IGR3 from IGR4. This seems to indicate that the lipid structural orders of the peripheral zone of the membrane in IGR3 and IGR4 are identical, whereas the overall lipid structural order of the membranes is different. In IGR37 and IGR39 both the peripheral zone and the overall lipid structural order are different.

With regard to these results, we wish to underline the following facts. IGR37 cells which were established from a metastatic lymph node tumour have a much lower fluorescence anisotropy value than IGR39 cells which were established from a primary tumour of the same patient. Also, while it is true that IGR4 cells are more tumorigenic than IGR3 cells when injected into nude mice, IGR4 cells which show the higher fluorescence anisotropy originate from level II of invasion, while IGR3 cells which have a much lower polarization value were taken from level IV of invasion of the same nodular tumour. These latter cells lie much deeper in the skin and could be more apt to invade the lymphatic or vascular system. The much lower polarization value of TMA-DPH for IGR4 cells when compared to

IGR39 cells is perhaps related to the fact that they originate from a nodular type tumour whereas IGR39 cells originate from an SSM tumour. In future it may thus perhaps be possible to resolve the basic controversy [12] as to the definition of horizontal vs vertical growth phase, and whether nodular melanoma represents a pure vertical growth phase, by extended experiments of the kind reported in this work.

No difference in fluorescence anisotropy was found between the murine melanoma cell lines with both markers, even for plated cells. For DPH as marker and cells in suspension the similarity of the anisotropy values has already been pointed out by Raz *et al.* [13]. These results seem at first surprising, considering the fact that F10 cells have a much higher metastatic potential than F1 cells. It has, however, been demonstrated by Weiss *et al.* [14] that this difference is due primarily to the higher retention of F10 cells in the lungs rather than to their higher intravasation potential. Our results conform with these findings.

In spite of the dedifferentiation, which is a general feature of established cell lines when compared to the tissue from which they originate, our results show as a whole that in the human melanoma cell lines studied a relationship may exist between their overall structuredness and both their histological origin and their metastatic potential. The generalization of this observation requires a similar study to be carried out with short-term primary cultures of human melanoma cells obtained from different melanomas. More generally, our results show that, whenever possible, the study of fluorescence anisotropy of marker molecules in relation to biological properties should be carried out under conditions in which the cells are allowed to maintain their physiological three-dimensional organization.

Acknowledgements—The authors wish to thank Dr C. Aubert, Head of the Melanoma Research Group, Unité 119 de l'INSERM, Marseille, for the gift of the IGR3, IGR4, IGR37 and IGR39 cell lines and for his continued interest in this work; to Dr I. Vlodavsky of the Department of Oncology, Hadassah University Hospital, Jerusalem, for the gift of the B16 F1 and B16 F10 mouse melanoma cell lines; and to Professor E. Grosshans, Head, Department of Dermatology, Hôpital Civil, Strasbourg, for providing tumors and for his continued interest and encouragement. A.W. received much help from the members of the Melanoma Research Group during his visit there and expresses his thanks. He also expresses his gratefulness to Professor G. Laustriat, Head of the Physics Laboratory of the Faculty of Pharmacy, Strasbourg, for making his stay at the Laboratory possible.

REFERENCES

1. KRAMER RH, NICOLSON GL. Interaction of tumor cells with vascular endothelial cell monolayers: a model for metastatic invasion. *Proc Natl Acad Sci USA* 1979, **76**, 5704–5708.
2. KINOSITA K, KAWATO S, IKEGAMI A. A theory of fluorescence polarization. *Biophys J* 1977, **20**, 289–305.
3. VAN BLITTERSWIJK WJ, VAN HOEVEN RP, VAN DERMEER BW. Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence measurements. *Biochim Biophys Acta* 1981, **644**, 323–332.
4. TVEIT KM, PIHL A. Do cell lines *in vitro* reflect the properties of the tumors of origin? A study of lines derived from human melanoma xenografts. *Br J Cancer* 1981, **44**, 775–786.
5. AUBERT C, ROUGE F, GALINDO JR. Tumorigenicity of human melanocytes in nude mice in relation to their differentiation *in vitro*. *JNCI* 1980, **64**, 1029–1040.
6. POSTE G, DOLL J, HART IJ, FIDLER IJ. *In vitro* selection of murine B16 melanoma variants with enhanced tissue-invasive properties. *Cancer Res* 1980, **40**, 1636–1644.
7. SHINITZKY M, BARENHOLZ Y. Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing diethylphosphate. *J Biol Chem* 1974, **249**, 2652–2657.
8. PRENDERGAST FG, HAUGLAND RP, CALLAHAN PJ. 1-[4-(Trimethylamino) phenyl]-6-phenylhexa-1,3,5-triene: synthesis, fluorescence properties, and use as a fluorescence probe of lipid bilayers. *Biochemistry* 1981, **20**, 7333–7338.
9. VAN HOEVEN RP, VAN BLITTERSWIJK WJ, EMMELOT P. Fluorescence polarization measurements on normal and tumor cells and their corresponding plasma membranes. *Biochim Biophys Acta* 1979, **551**, 44–54.
10. DE LAAT SW, VAN DER SAAG PT, SHINITZKY M. Microviscosity modulation during the cell cycle of neuroblastoma cells. *Proc Natl Acad Sci USA* 1977, **74**, 4458–4461.
11. YULI I, WILBRANDT W, SHINITZKY M. Glucose transport through cell membranes of modified lipid fluidity. *Biochemistry* 1981, **20**, 4250–4256.
12. LIEBLICH LM. Classification of malignant melanomas. A view of the current controversy. *Am J Dermatopathol* 1982, **4**, 435–441.

13. RAZ A, MCLELLAN WL, HART IR *et al.* Cell surface properties of B16 melanoma variants with differing metastatic potential. *Cancer Res* 1980, **40**, 1645–1651.
14. WEISS L, MAYHEW E, RAPP DG, HOLMES JC. Metastatic inefficiency in mice bearing B16 melanomas. *Br J Cancer* 1982, **45**, 44–53.