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BEHAVIOR OF MELANOMA CELLS IN CELL AND ORGAN CULTURES: USE OF BIOMATERIALS TO ACTIVATE CELLS

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Abstract

It is well known that cell behaviors such as adhesion, proliferation and various synthesis are initiated from transmembrane signals. This study uses biomaterials as primary messengers of the cell activation pathways, and we have analyzed the effects of two biomaterials on highly metastatic tumor cells. B16F10 melanoma cells formed heterogeneous populations whose size varied with cell differentiation. In long-term organ cultures grown comparatively on AN 69 and Cuprophan (a biomembrane known to activate cells), we found that Cuprophan increased both adhesion and proliferation of small melanin-rich cells which represented differentiated melanocytes. In dissociated cell cultures, the rate of early cell attachment decreased on Cuprophan compared to AN 69 and control Thermanox® (Nunc Inc., Naperville, IL). Scanning electron microscopy of melanocytes four hours after plating out on Cuprophan revealed only cell aggregates, comparable to the 3T3 fibroblasts aggregates previously described. Nevertheless, the production of the second messenger cyclic adenosine monophosphate (cAMP) was the same on both materials, in contrast to previous results showing more cAMP in cells on Cuprophan. Therefore, biomaterials appear to be useful tools for investigating as well attachment, growth, differentiation as signal transduction pathways of cancerous cells.

Key Words: Organ culture, cell culture, biomaterials, B16F10, melanoma cells, cyclic adenosine monophosphate (cAMP).

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Introduction

In vitro studies on melanoma cells have shown a relationship between differentiation, tumorigenicity and metastatic potential (Kameyama et al., 1990). Drugs, such as retinoic acid (Gruber et al., 1992; Niles, 1994), melanocyte-stimulating hormone (Kameyama et al., 1990), and cyclic adenosine monophosphate (cAMP) elevating drugs (Busca et al., 1996), are all capable of stimulating the differentiation of melanocytes. This results in increased melanin production and slower cell division, together with morphological changes. The cells changed from epithelioid to spindleoid (Xiang et al., 1986; Oh et al., 1997).

Biomaterials are also known to influence the migration, proliferation, and adhesion of cells (Duval et al., 1992) and signal transduction within them. In our laboratory, Faucheux et al. demonstrated a cAMP-dependent rounded and aggregated morphology of mouse NIH and Swiss 3T3 fibroblast cell lines on Cuprophan (Faucheux et al., 1998). As is well known from the literature, these mechanisms appear to be disturbed in cancerous cells, and therefore, we have attempted to use biomaterial substrates as tools to investigate the behavior of highly metastatic tumor cells.

The behavior of pigmented cells derived from mouse melanomas has been the subject of many studies, and the morphology of the cells in culture has been described (Sloboda and Kopac, 1960; Hu and Lesney, 1964). The cell line B16F10 is suitable for studies on metastasis (Fidler, 1973). Cell adhesion is vital for many biological processes, including tumor metastasis (Ruoslahti and Giancotti, 1989; Hynes, 1992), and attempts have been made to modify the adhesive behavior of metastatic melanoma cells (Humphries et al., 1988; Beviglia et al., 1995). The present work was carried out to identify the specific properties of highly metastatic tumor cells derived from melanomas and grown as monolayers or as organotypic cultures (Duval et al., 1988, 1990). Cells were grown on substrates that bring about different cell activations (Schoels et al., 1993; Faucheux et al., 1998): a synthetic polyacrylonitrile
membrane, AN 69 (Herbelin et al., 1990; Martin-Malo et al., 1991), and a cellulose-based membrane, Cuprophan, which activates cells (Mulvihill et al., 1992; Deppisch et al., 1994). The behavior of a melanoma cell line was studied by monitoring the morphology, melanin production, attachment rate and intracellular cAMP production of the cells. A melanoma produced in C57 Black mouse following the intraperitoneal injection of highly metastatic B16F10 cells was grown in organotypic cultures on the two membranes in order to compare cell migration, proliferation and adhesion. The effect of the biomaterials used as substrates on signal transduction in these cancerous cells thus paralleled that of specific drugs.

Material and Methods

Tested materials
- Polyacrylonitrile membrane (Biospal 30005, Hospal Industrie, Lyon, France) (AN 69); thickness = 15 µm.
- Cellulose-based membrane (Lundia IC 6 HF, Gambro Corporation, Lund, Sweden) (Cuprophan); thickness = 15 µm.
- Negative control (Thermanox® Lux Corporation, Nunc Inc., Naperville, IL) (Thx); thickness = 180 µm.

Cell culture

Culture technique: B16F10 murine melanoma cells kindly provided by Institut Curie (Orsay, France) were grown in 25 cm² tissue culture flasks in RPMI 1640 (Gibco BRL, Life Technologies, Egany, France) supplemented with 10% FBS (Fetal Bovine Serum) (Eurobio, Les Ulis, France), 5% L-glutamine 200 mM (Gibco BRL), penicillin (100 UI/ml) and streptomycin (100 mg/ml) (Gibco BRL) in a humidified incubator at 37°C with 5% CO₂ and 95% air and were passaged when they were in the exponential growth phase 2 to 3 days before use. Sterile membranes were cut into 16 mm diameter circles and placed in four-well plates (Nunclon, PolyLabo, Strasbourg, France).

Cell attachment assay: B16F10 cells were harvested from subconfluent cultures by a brief incubation with 0.25% trypsin/0.02% EDTA (EthyleneDiamine-Tetraacetic Acid) followed by gentle pipetting, and suspended in RPMI 1640 containing 10% FBS. The cells were washed twice with serum-free RPMI and resuspended in RPMI. The cells were then added (at 10⁵ cells/cm²) to the membranes in the wells. Cells were trypsinized, harvested and counted in a Coulter® counter (Coulter Electronics, Ltd., Luton, U.K.) at intervals thereafter.

cAMP assay: B16F10 cells on membranes (2 x 10⁵) were incubated at 37°C in a humidified atmosphere for 45 minutes with the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, Sigma, St. Louis, MO; 1 mM) to prevent cAMP breakdown. The medium was then removed and saved, and the cells counted with a hemocytometer. The membranes were gently rinsed with ice-cold phosphate-buffered saline (PBS). cAMP was extracted from the cells by incubating the membranes in 200 µl 1 N HClO₄ for 30 minutes at 4°C. The solution was collected, centrifuged at 200 g and kept frozen until assayed.

B16F10 cells (4 x 10⁵) were suspended in 0.4 ml RPMI 1640 containing 1 mM IBMX, placed in an Eppendorf tube and stored at 4°C for determination of the basal cAMP. The cells were pelleted, washed twice in cold PBS and cAMP was extracted by adding 150 µl 1 N HClO₄, as above.

cAMP was measured by radio immunoassay with 125I-cAMP according to Cailla (Cailla et al., 1973). Samples for cAMP determination were neutralized and acetylated by adding anhydrous acetic acid in 0.1 M citrate buffer pH 6.2. The sample or standard (75 µl) was mixed with 75 µl 125I-cAMP and the mixture was incubated for 20 hours at 4°C with rabbit anti-cAMP antibody. γ-globulin (2.5 mg/ml in citrate buffer pH 6.2), and polyethyleneglycol 6000, (20 mg/100 ml water) were added to each sample tube. Bound ligand was removed by centrifugation. The radioactivity in the pellet was counted in a γ-counter. The concentration of unlabeled cAMP was then determined using a standard curve.

Organ culture

Culture technique: Fragments of melanoma obtained 2 weeks after the intraperitoneal injection of metastatic cells into syngeneic C57Bl/6J mice were layered on the substrate to be tested (Duval et al., 1992), on buffered agar medium containing 37% RPMI 1640 (Gibco BRL), 10% FBS (Eurobio, France), 2% Tricin (Merck, Darmstadt, Germany), 2 mM L-glutamine (Gibco BRL) and 1% Bacto-Agar (Difco, Detroit, MI) in 50% Gey saline solution (Waymouth, 1965). The cells generated by explants grown on the biomaterials and the surface of the cell layers were assessed after incubation for 1, 2 or 3 weeks at 37°C. Control cultures on Thermanox® were assessed in the same way.

Cytocompatibility: The surfaces of the cell layers were stained (neutral red) and measured with a stereo-microscope fitted with a camera lucida and digitizing tablet connected to a microcomputer. The cells were counted with a Coulter® Multisizer (Coulter, France); the explants were dissociated with trypsin, and the number of cells per explant was calculated.

Cell proliferation: Three pools of 3 explants grown on AN 69, Cuprophan or Thermanox® were
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Figure 1. Scanning electron microscopy of B16F10 line melanoma cells. (a) Growth pattern of the subconfluent culture showing the melanocytes and their different shapes. Bar = 100 μm. (b) Bipolar melanocyte. Bar = 10 μm. (c) Multinucleate epithelioid giant cell. Bar = 10 μm. (d) Polydendritic cells. Bar = 10 μm.

transferred at 1 day intervals from the agar medium to Petri dishes (3 explants per dish) containing 1 ml RPMI 1640 medium (Gibco BRL) and 1 μCi/ml [6-3H] thymidine (24 Ci/m mole) (Amersham, Les Ulis, France). The explants were incubated for 18 hours at 37°C, rinsed twice in cold PBS containing thymidine (10^-4 M) and treated with 0.25% trypsin-0.20% EDTA (1:1) at 37°C. The detached explants were discarded using forceps, and the released single cells were pelleted and resuspended in 1 ml of cold PBS. 50 ml was saved for cell counting in a Coulter® Multisizer. Radioactive DNA was precipitated in 10% cold TCA (trichloroacetic acid), filtered on glass filters and allowed to dissolve overnight in 1 ml NCS-water (9:1) (Amersham) in scintillation vials. Twelve milliliters of organic scintillation fluid OCS (Amersham) was added for counting in a Beckman LS 8000 spectrometer (Beckman, Gagny, France).

Cell adhesion: The cells were detached with 0.025% trypsin-EDTA to establish the rate of cell release (Duval et al., 1988). The area included between the curve and the "X" axis was calculated by integration to give cell adhesion behavior. The area was inversely proportional to the cell adhesion to the biomaterial. A comparative results diagram was produced with 3 zones:

1. Area more than 4500 = poor cell adhesion
2. Area between 3000 and 4500 = moderate cell adhesion
3. Area less than 3000 = strong cell adhesion

Scanning electron microscopy: Cells on different biomaterials from both monolayer and organ cultures were rinsed in the PBS, fixed in 3% glutaraldehyde in
Figure 2. Coulter® Multisizer analysis of the cell layers grown in organ culture on the different biomaterials. (a) Cell size distribution of a B16F10 melanoma tumor biopsy before culture. (b) Thermaflow®; (c) AN 69; (d) Cuprophan.

Results

Morphology of B16F10 cells

The B16F10 melanoma cells were melanocytes at various stages of maturation, so that the cell shape varied. Three main groups of cells were identified (Fig. 1a): group 1, small ovoid bipolar cells with little cytoplasm containing melanin granules and with long extensions (Fig. 1b); group 2, multipolar polynucleate epithelioid giant cells with abundant cytoplasm containing melanin granules (Fig. 1c); group 3, bipolar dendritic cells, essentially melanin-free and possibly binucleate (Fig. 1d).

Solid tumors grown in vivo for 2 weeks in mice were dispersed with trypsin. The resulting cells fell into size ranges as measured with a Coulter® Multisizer. Figure 2a shows a major peak of group 2 cells with a diameter of 15 mm, and a smaller peak of group 1 cells (diameter ~ 9 mm). There were medium-sized cells (12 mm diameter), corresponding to group 3 cells, between these two peaks. The peak on the left is cell debris. The Coulter® Multisizer analysis of cell layers grown on materials for 14 days, and dissociated with trypsin-EDTA, revealed different cell populations. The cell layer on Thermaflow® (Fig. 2b) consisted of cells with a maximum diameter of 12 mm (group 3). The cells grown on AN 69 formed a relatively homogeneous population of about 15 mm diameter (group 2) plus a few smaller cells (about 8 mm diameter, group 1) (Fig. 2c). These two cell sizes (groups 1 and 2) were also
Figure 3. Scanning electron micrographs of B16F10 melanoma in organ culture on different biomaterials: Thermanox* (a and b), AN 69 (c and d), and Cuprophan (e and f). Figures 3b, 3d, and 3f are higher magnification micrographs of 3a, 3c, and 3e, respectively. Bars = 100 μm (a, c and e) and 10 μm (b, d and f).
found in cultures grown on Cuprophan (Fig. 2d), but in this case there were many smaller cells (group 1).

Biological and morphological properties of organotypic culture

Scanning electron microscopy of organotypic melanoma cultures on biomaterials showed striking differences. Theranox® favored the growth of group 3 cells (Figs. 3a and 3b). AN 69 (Figs. 3c and 3d) stimulated the growth of large group 2 cells, which formed a continuous layer containing a few smaller group 1 cells. Cuprophan (Figs. 3e and 3f) generated a dense layer of group 1 and 2 cells. A Lillie melanin staining (Fig. 4) of the cell layers clearly demonstrated the presence of differentiated melanin-rich cells on Cuprophan (Fig. 4a), whereas the cells grown on AN 69 and Theranox® were melanin-free (Figs. 4b and 4c).

Migration reached a maximum by day 14 on Theranox® and Cuprophan and by day 21 on AN 69 (Fig. 5a). The migration area was considerably smaller on Cuprophan. Cell proliferation was assessed by following 3H-thymidine incorporation throughout the growth phase. A significant increase in radioactivity incorporation was detected on days 8 and 9 in cultures grown on Cuprophan and AN 69 (Fig. 5b). A smaller radioactive peak was seen by day 6 on Theranox®. The specific radioactivity of cell cultures grown on AN 69 and Cuprophan decreased with incubation time (Fig. 5c).

The adhesion strength of each cell type was studied by identifying the group of cells released by treatment with trypsin for 5 and 60 minutes with the Coulter® Multisizer. The values were plotted against time to assess the adhesion of each cell group. The overall adhesion of the cells was weak. Group 1 cells adhered more strongly to Cuprophan than did group 2 cells (Fig. 5d). Since serum proteins adsorbed onto substrates and matrix proteins secreted by cells were involved in cell adhesion, we attempted to observe extracellular matrix and cellular basal arrangement of cells stripped mechanically from the substrate (Fig. 6). The matrix was disorganized and almost non-existent on Theranox® (Fig. 6a), better organized and thicker on AN 69 (Fig. 6b), whereas basal cells on Cuprophan were embedded in a thick matrix having alveolar structures (Fig. 6c).

Biological and morphological properties of cell cultures

The rate of B16F10 cell attachment to the materials was studied in dispersed cell culture. Adherent cells were counted in a Coulter® Multisizer at various times between 15 minutes and 4 hours after seeding (Fig. 7). The curves for cell attachment to AN 69 and Theranox® reached a plateau at about 80% adherent cells after 45 minutes at 37°C. The plateau was not reached until 4 hours after seeding on Cuprophan and did not exceed

Figure 4. Staining for melanin in cell layers (Lillie). (a) Cuprophan; (b) AN 69; (c) Theranox®. Photo widths = 640 μm.
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Figure 5. Properties of cell layers generated by B16F10 melanoma grown in organ culture on biomaterials. (a) Cell migration on Thermax®, AN 69, and Cuprophan. The areas of cell layers surrounding each explant were measured. Results are means of 60 measurements ± standard deviations. (b and c) Cell proliferation on Thermax®, AN 69, and Cuprophan. 3H-Thymidine incorporated into B16F10 grown on Thermax®, AN 69, and Cuprophan was precipitated with TCA and measured at successive stages of the culture. Results of the total radioactivity incorporated (b) are means of 3 pools of 3 samples ± standard deviations. Results in (c) are expressed as the mean specific radioactivity of 10^6 cells ± standard deviation. (d) Cell adhesion to Thermax®, AN 69 and Cuprophan. Areas under the cell release curves were integrated (1 = group 1 cells, 2 = group 2 cells). Results are means of 60 measurements ± standard deviations.

40% adherent cells.

Scanning electron microscopy showed that the cells spread rapidly 45 minutes after seeding on Thermax (Fig. 8a) and somewhat later on AN 69 (Fig. 8c). The morphology of cells on Thermax® and AN 69 were similar 4 hours after seeding (Figs. 8b and 8d), while cells aggregated and clustered over scattered cells adhering to Cuprophan (Figs. 8e and 8f). The cAMP contents of cells reflect the early biochemical events generated by the biomaterials. Results are expressed with reference to the basal cAMP in the initial cell suspension. All the materials tested gave the same order of stimulation (X 2) (Fig. 9).

Discussion

The size and shape of cells in primary cultures of B16 melanoma cells vary greatly (Hu and Lesney, 1964) due to differences in their growth stage and differentiation. Melanoblasts, melanocytes and mature melanocytes are identified according to their morphology and pigmentation. The small spindle-shaped, pigmented cells (group 1) possess all the morphological characteristics of mature melanocytes, whereas the stellate unpigmented cells are more like undifferentiated melanoblasts. Our organotypic culture method resulted in various degrees of differentiation of the melanoma, depending
Figure 6. Scanning electron micrographs of extracellular matrix secreted by the B16F10 melanoma in organ culture on biomaterials: Thermanox® (a), AN69 (b), and Cuprophan (c). Bars = 10 μm.

Figure 7. Properties of B16F10 melanoma cells in monolayer culture on biomaterials. Cell attachment assay. Cells were seeded at $10^5$ cells/cm$^2$, and the cells released by later trypsinization were counted. The cell numbers were plotted against time. Results are expressed as the means ± standard deviations of three experiments performed in triplicate.

Figure 8. Properties of B16F10 melanoma cells in monolayer culture on biomaterials. Cell migration assay. Cells were seeded at $10^5$ cells/cm$^2$, and the cells released by later trypsinization were counted. The cell numbers were plotted against time. Results are expressed as the means ± standard deviations of three experiments performed in triplicate.

on the biomaterial tested (Figs. 2 and 3). The Thermanox® control support did not favor differentiation, whereas Cuprophan stimulated the growth of the most differentiated melanocytes, unless it gave the strongest stimulation for melanocyte differentiation. Cell migration was more strongly inhibited on Cuprophan than on the other biomaterials. Others have correlated the inhibition of cell growth in vitro with increased cell activation (Ottino and Duncan, 1996) and the induction of morphological differentiation (Prasad, 1989). We therefore analyzed the relationship between these events.

Assessment of cell proliferation demonstrated a delay in cell division on Cuprophan, as shown by the uptake of radioactive thymidine by cultures (Fig. 5d) and the specific cell radioactivity measurements (Fig. 5c) (the day 3 result was uninterpretable because of difficulties in removing the explant for counting the cell layer radioactivity). This experiment also confirmed the heterogeneity of the cell population, with selective cell attachment and growth depending on the biomaterial. Cuprophan enhances adhesion and/or the growth of differentiated cells, which have been reported to have a slower growth rate (Niles, 1994).

Cell adhesion through the extracellular matrix plays a complex role in malignancy. Normal cells usually deposit a fibronectin matrix around themselves, but malignant cells often fail to do so (Albelda, 1993; Ruoslahti, 1994). There are also large changes in the population of adhesion receptors during the transformation of normal melanocytes into benign nevi (Nesbit and Herlyn, 1994). The colonization of marine lungs by
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Figure 8. Scanning electron micrographs of dispersed B16F10 cells grown on biomaterials for 45 minutes and 4 hours. Thermanox® (45 minutes (a) and 4 hours (b)). AN 69 (45 minutes (c) and 4 hours (d)). Cuprophan (45 minutes (e) and 4 hours Cuprophan (f)). Bars = 10 μm.
The behavior of B16F10 cells in monolayer culture was similar to that of cells in organotypic culture. Cuprophan did not cause more cell attachment than AN 69 or the control sample. However, it seems to favor the attachment of tumor cells slightly more than that of NIH 3T3 fibroblasts (Faucheux et al., 1998). Growth on Cuprophan resulted in the same cell morphology as that described by Carracedo et al. (1995).

Our previous results also demonstrated a relationship between the cell second messenger cAMP content and cell aggregation in NIH 3T3 fibroblasts. Some have reported that cAMP-stimulating agents cause the morphological differentiation of melanoma cells, while inhibiting cell division and increasing cell melanin content (Prasad, 1989). Others have demonstrated a cAMP-independent mechanism for the induction of murine melanoma cell differentiation including melanogenesis (Durkacz et al., 1992). Our results show that cell division is inhibited and the morphological differentiation of B16F10 melanoma cells is induced by Cuprophan.

Figure 9. Cell cAMP contents. The cAMP (10^{-15} M/10^6 cells) was referred to the amount of cAMP in the cells in suspension at time 0. Results are means ± standard deviations of three experiments performed in triplicate.

Cuprophan enhanced melanocyte differentiation or stimulated the growth of the most differentiated melanocytes. It also inhibited cell division and stimulated the adhesion of cells which do not usually adhere or adhere only weakly. The mechanisms of cell attachment to Cuprophan seem to be independent of the cAMP pathway, although the shape of the adherent cells is usually correlated with their cAMP content. These results suggest that there is a specific regulation process for melanoma cells. Thus biomaterials may be able to play a part in fundamental studies of the regulation of cancerous cells.

Conclusion

Organotypic cultures have shown that biomaterials have a selective effect on the behavior of tumor cells. Cuprophan enhanced melanocyte differentiation or stimulated the growth of the most differentiated melanocytes. It also inhibited cell division and stimulated the adhesion of cells which do not usually adhere or adhere only weakly. The mechanisms of cell attachment to Cuprophan seem to be independent of the cAMP pathway, although the shape of the adherent cells is usually correlated with their cAMP content. These results suggest that there is a specific regulation process for melanoma cells. Thus biomaterials may be able to play a part in fundamental studies of the regulation of cancerous cells.

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Discussion with Reviewers

J. Schakenraad: Are the differently sized cells on the respective materials subsets of a single cell line, or are they simply the same cells that do not spread as well on one material as compared to another?

Authors: We cannot answer this question. B16F10 cells have generally been described as a heterogeneous cell population (Cobb, 1960; Sloboda and Kopac, 1960; Hu and Lesney, 1964; Fidler, 1973). They contain pigmented and unpigmented cells which vary greatly in size and shape, depending on the stage of growth. Pigmented cells have the morphological feature of melanocytes, whereas unpigmented cells are like the more undifferentiated melanoblasts. Cuprophan may stimulate the division of differentiated melanocytes and/or stimulate their differentiation, as reported in the text. Previous studies showed an increase of cAMP in 3T3 fibroblasts in contact with Cuprophan, linked to morphological changes (Faucheux et al., 1998). We find that Cuprophan caused morphological changes but did not over stimulate melanocytes, and the cAMP in cells adhering to the material was similar whether the cells were on Cuprophan, AN 69, or Thermanox®.

P.B. van Wachem: Why did you test melanoma cells with hemodialysis membranes? If you want to use biomaterials (at all), then biomaterials with extracellular matrix characteristics would have been far more interesting (e.g., collagens). Expression of, e.g., adhesion receptors or production of extracellular matrix, would possibly give more interesting information. The cAMP-levels are the only biochemical markers evaluated, but these give no clues although other results show large differences. Which other cell activating markers would be relevant and could be studied?

Authors: In this study, membranes played the role of primary messenger in activating the transduction pathway. Cuprophan is a hemodialysis membrane that activates blood cells and fibroblasts to a greater extent than does AN 69. The second messenger cAMP, dramatically increases when 3T3 adhere to Cuprophan. This is why we need this material as a tool to obtain a better understanding of the differentiation of cancerous cells.

Additional Reference