Human placental lipid induces mitogenesis and melanogenesis in B16F10 melanoma cells

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A hydroalcoholic extract of fresh term human placenta was found to be mitogenic as well as melanogenic on B16F10 mouse melanoma in an *in vitro* culture. The extract, a reservoir of a large number of bioactive molecules, was resolved to get the lipid fraction. Its activity was evaluated on B16F10 mouse melanoma by assessing the change in cellular morphology, growth and melanin induction. The lipid fraction, placental total lipid fraction (PTLF) tested in the study employed doses of 0·01 to 200 μg/ml; optimum growth and melanization accompanied by morphological changes were recorded at 10 and 100 μg/ml respectively. At intermediate doses growth and melanization were found to show a pattern of change over between growth and melanization and finally reached at an inverse relation at the respective optimal dose of response. Compared with defined sphingolipids, C_2 ceramide and sphingosine-1-phosphate, the results were mostly corroborative. The duality of biological response of sphingolipids as reported in numerous studies was comparable for the PTLF suggesting that its active component is a sphingolipid and showing its use for pigment recovery in vitiligo.

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1. Introduction

Visible pigmentation in human results from the synthesis and distribution of melanin in organ such as the skin, hair bulbs and eyes. The pigment, melanin plays a crucial role on the absorption of free radical generated in the cytoplasm and shielding the host from various types of ionizing radiation, including the UV-light. Cellular melanization is a process which has been described as a fine stress regulatory mechanism (Eller *et al* 1996; Gilchrest *et al* 1996) linked to growth arrest involving a signalling cascade of stress activated protein kinase (Coroneos *et al* 1996). Human placenta is an enriched reservoir of many vital bioactive molecules that include keratinocyte growth factor (O'keffe 1985; Chiu and O'Keefe 1989), endothelial cell growth stimulator (Presta *et al* 1985), endothelin-1 (Wilkes *et al* 1993), *a*-melanocyte stimulating hormone, adrenocorticotrophic hormone (Shibasaki *et al* 1982; Liotta 1997) and bioactive lipids interestingly sphingolipids (Levery 1989; Strasberg *et al* 1989). Metabolites of sphingolipids have been implicated in stress response regulation and are being emerging as crucial second messenger molecules (Spiegel and Milstien 1995) with an opposing role in mammalian cell growth arrest and survival; their relative cellular level has been proposed to be a rheostat that determines the fate of cells (Mandala *et al* 1998).

One prototype hydroalcoholic extract containing numerous lipids, peptides, vitamins and neucleotides (Pal *et al* 1995) prepared from term human placenta of HIV and Hbs antigen negative mother (Bhadra *et al* 1997)

Keywords. Melanogenesis; melanoma; mitogenesis; morphogenesis; placental lipid

Abbreviations used: C_2-cer., N-acetyl-D-sphingosine; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; MTT, [3-(4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide; PTLF, placental total lipid fraction; SPP, sphingosine-1-phosphate.

showed pigment inducing activity in mammals (unpublished results).

In this study, we have investigated the effect of placental total lipid fraction on cytotoxicity, morphological changes, growth and pigmentation of B16F10 melanoma *in vitro*.

2. Materials and methods

2.1 *Materials*

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), 100X PSN antibiotic and 10X Trypsin/EDTA solution and other medium supplements were obtained from GIBCO.BRL. Synthetic Melanin, [3-(4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sphingosine-1-phosphate (SPP), N-acetyl-D-sphingosine (C_2-cer.) were from Sigma Chem. Co., St. Louis, USA; [methyl-^{3}H]thymidine from New England Nuclear Inc, USA. Tissue culture plastic wares were obtained from NUNC, Denmark. All other chemicals used were of highest purity.

2.2 *Preparation of the PTLF from the whole extract*

Lipids present in hydroalcoholic human placental extract were separated according to the method described previously (Osborne 1986). Briefly, the total solid constituents of the hydroalcoholic placental extract was treated with diethyl-ether at 4°C. Following centrifugation (2500 *g*, 10 min at 4°C), the pelleted matter was treated identically thrice. The process was repeated three times more with ether : ethanol (3 : 1 v/v) to get the total lipid in the organic phase. All the organic fractions were pooled and evaporated to dryness yielding the placental total lipid fraction or PTLF.

2.3 *Cell culture*

B16F10 mouse melanotic cell line procured from the National Centre for Cell Science, Pune, was used in this study. Cells were grown in DMEM supplemented with 10% FBS and 1% PSN antibiotic at 37°C in a humidified incubator with 5% CO_2. Cells were grown to semiconfluence, harvested with 0·025% trypsin and 0·52 mM EDTA in phosphate buffered saline. All experiments were conducted in DMEM containing 2% heat inactivated FBS and 1% PSN antibiotic.

2.4 *Treatment of lipids*

The lipids specially sphingolipids/ceramides are not soluble in aqueous medium and these are usually emulsified with a protein (like a bovine serum albumin) and then used to study their biological response (Zhang *et al* 1990). In this study the PTLF, SPP, C_2-cer. were dissolved in $CHCl_3$: CH_3OH (2 : 1 v/v). The required amount of lipid solution was taken in a sterile vial, the solvent evaporated and sonicated with DMEM containing 2% FBS and then used in subsequent experiments.

2.5 *Assessment of morphological change*

B16F10 mouse melanoma was seeded in a six well plate at a density of 3×10^4 cells/well in DMEM supplemented with 2% FBS for 24 h. The cells were then treated with or without PTLF, SPP, C_2-cer. and grown at 37°C in a 5% CO_2 incubator. After this incubation the morphological changes were seen under a light microscope.

2.6 *Assay of DNA synthesis by [^{3}H]thymidine incorporation*

DNA synthesis was evaluated by [^{3}H]thymidine incorporation (Komori *et al* 1999) in B16F10 mouse melanoma cells *in vitro*. Briefly the cells were seeded in a 96 well TC-plates at a density of $1{\cdot}2 \times 10^4$ cells/well in DMEM supplemented with 2% FBS for 24 h. The cells were treated with various concentrations of PTLF, SPP and C_2-cer. for 18 h at 37°C in a 5% CO_2 incubator. The cells were then pulsed with 1 μCi/ml [^{3}H]thymidine (sp. activity 6·70 Ci/nmol) for 6 h prior to harvesting. Finally the cells were harvested using Nunc-cell harvester and the incorporation of radioactivity into the cell was measured by liquid scintillation counter (model: LKB Wallac 1209 RACK BETA). Values were the means of triplicate determinations.

2.7 *Estimation of melanin content in the cells*

B16F10 cells were seeded in 6-well TC-plates in DMEM supplemented with 2% FBS at a density of 10^5 cells/well and cultured for 24 h. Following treatment with various concentrations of PTLF, SPP C_2-cer. and without it (control) for stipulated period the cells were washed with PBS, treated with 1 ml of 0·4 mM EDTA and 0·25% trypsin and incubated for 5 min at 37°C (Naeyaert 1991). Subsequently, 1 ml PBS was added to each well and the total cell suspension was divided into two parts. Five hundred microlitre was used for cell counting and the rest was centrifuged for 5 min to get the cell pellet. The pellet was dissolved in 1 ml of 1 M NaOH and melanin concentration was calculated by determining the absorbance value of the NaOH solution at 475 nm (Naeyaert 1991).

The melanin content was calculated from a standard curve synthetic melanin (Sigma).

2.8 *Cytotoxicity test by MTT assay*

MTT cytotoxicity assays were performed as described earlier (Mossman 1983). Briefly, 10^4 cells/well were plated in 96-well TC-plates. After the required incubation with the stimulants for 24 h and 48 h, MTT solutions were added and the insoluble derivative formed by cellular dehydrogenase enzymes was solubilized with acidic isopropanol and absorbance was measured at 620 nm by ELISA reader (model: Labsystem Multiscan MS).

3. Results

3.1 *Effect of the PTLF, SPP and C_2-cer. on B16F10 mouse melanoma cell morphology*

B16F10 melanoma cells maintained in the DMEM medium contain 10% FBS, attach to the culture flask, grew with large dendrites and formed a confluent monolayer within two to three days in culture. When cells were cultured with 2% heat inactivated FBS, the cells maintained their viability with very slow proliferation. These melanoma cells exhibited less dendritic extensions with clear cytoplasm (figure 1A). Under these culture condition, the morphological changes of cells were monitored after treatment with PTLF. Multipolar highly branched dendritic network and also dense pigmented granules appeared in the cytoplasm of the treated cells, clusters like growing cell assembly were also visible (found viable by Trypan blue dye exclusion test) in monolayer cultures (figure 1B). On the other hand the standard bioactive sphingolipids, SPP acts as a mitogen and C_2-cer. did not show marked morphological changes (figure 1C,D).

3.2 *Effect of PTLF, SPP and C_2-cer. on [^{3}H]thymidine incorporation of B16F10 melanoma cells*

The results of DNA synthesis induced by PTLF for B16F10 cells have been shown in figure 2. PTLF stimulated distinct proliferative response of B16F10 cells as evidenced from higher extent of [^{3}H]thymidine incorporation. The mitogenic response was just initiated at a

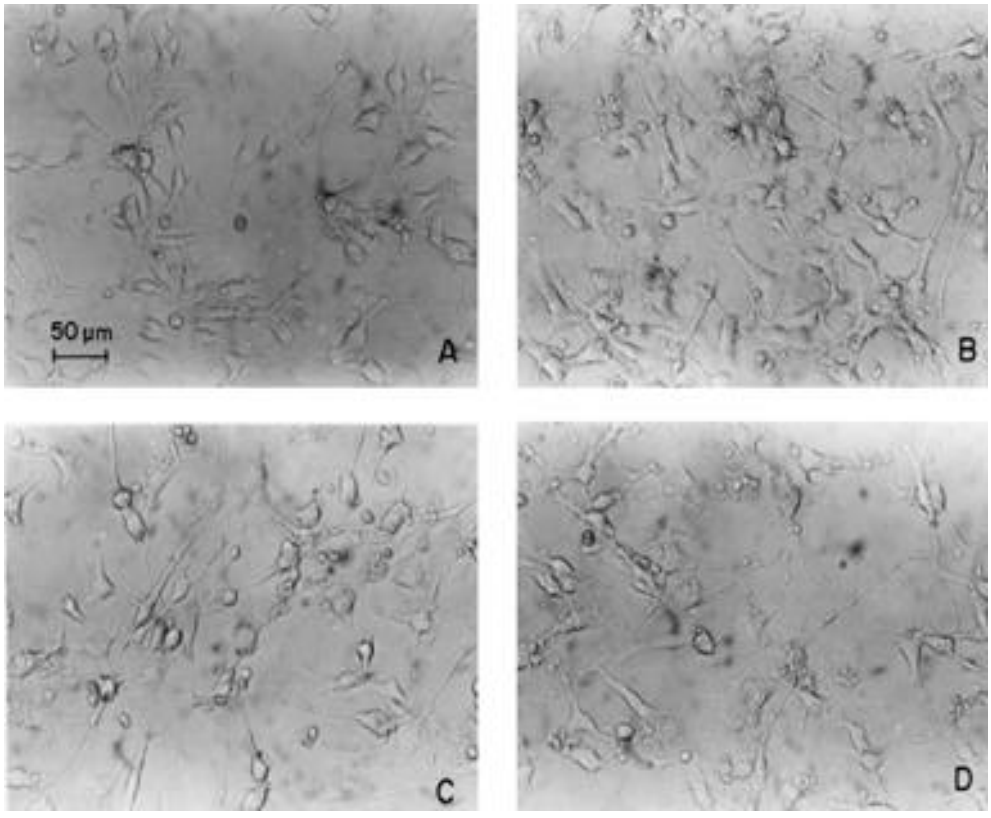

Figure 1. Morphological change for 2 days old culture of B16F10 mouse melanoma cells. Untreated cells (**A**), cells treated with 10 µg/ml of PTLF (**B**), 5 µM of SPP (**C**), and 1 µM of C_2-cer. (**D**). All photographs are under equal magnifications.

concentration as low as 0·01 μg/ml of PTLF (101·6%) and reached its maximum (152%) ($P < 0·01$) at 10 μg/ml as compared to the control (100%) (figure 2A). However, at concentrations higher than 10 μg/ml, growth stimulation started to decline and nearly 95% ($P < 0·10$) of the control growth was observed at 200 μg/ml. The decline in growth was not due to cell death since there was no loss of cell viability (evidenced by MTT assay). The standard bioactive sphingolipid such as SPP – a known mitogen (Zhang *et al* 1991) induced cell growth and this was maximum ($P < 0·02$) at a dose of 5 μM (136%) (figure 2B). While C_2-cer. also induced cellular proliferation of B16F10 very marginally ($P < 0·1$) at 1 μM concentration, however at higher concentration it inhibited the cellular growth (figure 2C).

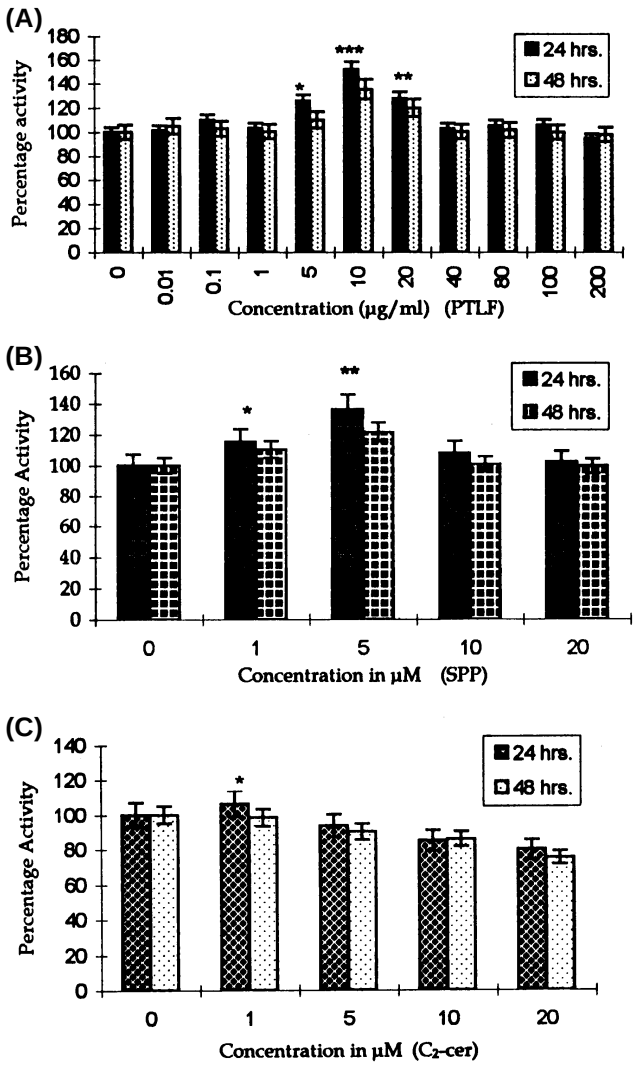

Figure 2. Effect of 24 h treatment of PTLF (**A**), SPP (**B**) and C_2-cer. (**C**) on [^{3}H]thymidine incorporation in B16F10 mouse melanoma cells. Values are the mean ± SE from triplicate determinations (1 μM SPP = 0·38 μg/ml, C_2-cer. = 0·34 μg/ml). The results refereed to control vs treated cells after analysis by Student's *t*-test showed highly significant mitogenic effect for PTLF. *$P < 0·1$; **$P < 0·02$; ***$P < 0·01$.

3.3 *Effect of PTLF, SPP and C_2-cer. on melanin synthesis of B16F10 melanoma cells*

Melanin synthesis occurred as a late event following the growth phase. B16F10 mouse melanotic melanoma cells has an inherent capacity to synthesize the pigment melanin. The melanogenic activity of PTLF was studied over a wide range of concentration from 1 μg to 200 μg/ml. Optimal melanization 191% (5·74 ± 0·12 μg melanin/10^5 cells) ($P < 0·001$), compared to untreated (control) cells (3·0 ± 0·10 μg melanin/10^5 cells) was observed at 100 μg/ml of PTLF, a dose which was not growth stimulatory but slightly suppressive (found non-cytotoxic by MTT assay). The bioactive sphingolipids SPP and C_2-cer., unlike PTLF at concentrations suppressive for growth were found to reduce the synthesis of melanin though marginally ($P < 0·1$). The response of C_2-cer. mediated decrease was however more pronounced (table 1).

3.4 *Cell viability assay by MTT on B16F10 mouse melanoma cells* in vitro

The results of cell cytotoxicity study on B16F10 cells were given in figure 3A. At a growth dose of 10 μg/ml of

Table 1. Comparison of melanin biosynthesis by PTLF, SPP and C_2-cer. on B16F10 mouse melanoma cells.

Stimulant	Melanin (μg) per 1 × 10^5 cells
Control	3·00 ± 0·10
1 μg/ml PTLF	3·51 ± 0·11
10 μg/ml PTLF	3·53 ± 0·09
50 μg/ml PTLF	3·68 ± 0·13
100 μg/ml PTLF**	5·74 ± 0·12***
200 μg/ml PTLF	5·60 ± 0·15
1 μM SPP*	3·09 ± 0·08
5 μM SPP	3·08 ± 0·11
10 μM SPP	3·14 ± 0·09
20 μM SPP	3·03 ± 0·13
1 μM C_2-cer.*	3·07 ± 0·12
5 μM C_2-cer.	2·89 ± 0·10
10 μM C_2-cer.	2·90 ± 0·11
20 μM C_2-cer.	2·81 ± 0·14

*1 μM SPP = 0·38 μg/ml, 1 μM C_2-cer. = 0·34 μg/ml.
**Optimal melanogenic dose.
***$P < 0·001$ for experimental as compared to control (analysed by Student's *t*-test).
Values are mean ± SE from triplicate determinations.

PTLF, cell viability ($P < 0{\cdot}1$) was 110 and 111·7% for 24 h and 48 h treatment respectively. At the optimal dose of melanization, 100 μg/ml the cell viability was 149 and 114·6% at 24 h and 48 h of treatment respectively, taking the viability ($P < 0{\cdot}01$) of control untreated cell as 100%. At this condition growth of PTLF untreated and treated cells measured in terms of [^{3}H]thymidine incorporation was not very comparable (figure 2A 106·2 and 100·1% at 24 h and 48 h respectively). The higher dose of PTLF, such as 200 μg/ml showed cell viability ($P < 0{\cdot}1$) as 110 and 100% at 24 h and 48 h treatment respectively. Bioactive sphingolipid SPP at a dose of 20 μM, the highest one used in this study, showed growth suppression but was devoid of cytotoxicity ($P < 0{\cdot}1$) by MTT assay (figure 3B). On the other hand C_2-cer. also at a growth suppressive dose of 5 μM did not have cytotoxicity ($P < 0{\cdot}1$) on B16F10 cell (figure 3C).

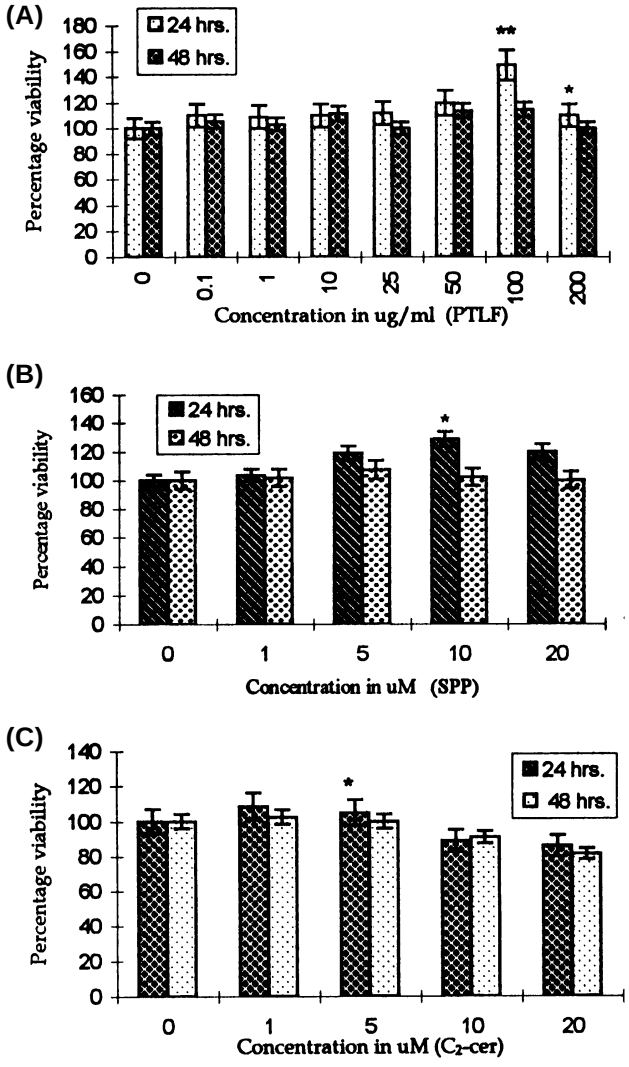

Figure 3. Cell viability assays as measured by MTT method by PTLF (**A**), SPP (**B**) and C_2-cer. (**C**) on B16F10 mouse melanoma cells. Values are the mean ± SE from triplicate determinations (1 μM SPP = 0·38 μg/ml, 1 μM C_2-cer. = 0·34 μg/ml). The results refereed to control vs treated cells after analysis by Student's *t*-test showed significant cell viability effect for PTLF. *$P < 0{\cdot}1$; **$P < 0{\cdot}01$.

4. Discussion

In this report, we have demonstrated the efficacy of the PTLF from a prototype human placental extract to induce growth and melanization of B16F10 mouse melanoma *in vitro*. The morphological features of melanocytes was highly coupled with melanogenesis and infact pigmentation was reported to be associated with increase in length and number of dendritic processes (Nakazawa *et al* 1998). PTLF was found to be active in regulating cellular dendritic processes on B16F10 cells in a similar manner favourable to melanogenesis and thus for the pigment recovery expected in vitiligo. Because growth and melanization were already reported to be associated with morphological changes (Mandal *et al* 2000) and also with the pigment recovery in vitiligo (Cui *et al* 1991). Pigmentation again related to the stress specific physiological event. The ceramide produced during the metabolism of sphingolipid have been implicated in stress management (Hannun 1994). The effect of ceramide was reported to occur at concentrations as low as 1–5 μM resulting in internucleosome DNA fragmentation (Obeid *et al* 1993). Such a change is known to occur with psoraline and thus qualitative relation might be indicated here. One of the sphingolipid metabolites such as sphingosine-1-phosphate was reported to be mitogenic in mammalian cells (Spiegel and Milstien 1995; Zhang *et al* 1991). In earlier studies four sphingolipids were detected in the lipid fractions of the currently used placental extract which showed pigment induction in animal model (Pal *et al* 1995). It is very likely that some or all of these sphingolipids might be contributing to the melanogenesis of B16F10 cells. This study has also showed growth induction of B16F10 melanoma cells in the presence of SPP as well as PTLF. However, B16F10 melanoma cell responded to cellular proliferation only at low concentration and this was like growth induction of Swiss 3T3 fibroblast by sphingosine (Zhang *et al* 1990). But the higher concentration of PTLF induced melanization with the decline of growth. This type of correlation has already been documented in melanocyte system where pigment induction and growth response was found to be inversely related. This duality of response of melanocyte to PTLF was therefore an unique feature. This study has also indicated an interesting feature of B16F10 melanoma cell activity with respect to the exposure of placental lipid. The results of MTT assay clearly indicated higher cell

viability, even when there was no growth or growth was inhibited partially by PTLF compared to untreated (control) cells. Here it implied that melanin synthesis was possibly linked to energy metabolism, since MTT assay provided the status of mitochondrial dehydrogenase activity. Higher viability meant higher dehydrogenase activity which possibly had a regulatory role on melanin synthesis here, though this remains to be established.

UV-induced DNA damage resulting in cellular stress reportedly switched on melanocyte melanization through induction of tyrosinase (Gilchrest *et al* 1996). There was possibly a crucial physiological state, where cellular stress regulated growth retardation and the minute level of melanogenesis co-exist and the level of sphingolipids or its metabolites might play a significant role. As was evident in this study the PTLF at low (10 μg/ml) and high concentrations (100 μg/ml) promoted growth and melanization respectively. While at an intermediate concentration (40 μg/ml) growth and pigmentation of B16F10 might be in a compromized state. Again stress possibly due to high concentration of PTLF did not lead to cell cytotoxicity as was evident by the MTT assay. The decline in growth and melanization might occur in concurrence, accompanied with cell viability. This was supported by recovery of full growth and morphology, when spent media was replaced with fresh growth medium.

This study thus demonstrated that the mitogenic and melanogenic responses of B16F10 melanoma might occur in the presence of PTLF (placental total lipid fraction) accompanied with morphological changes. Similar results with respect to growth were also observed in response to defined bioactive sphingolipids SPP and C_2-cer. But C_2-cer. resulted in marginal growth stimulation at very low concentration while it has virtually no influence on melanin production even at high (5 μM) concentration (Kim *et al* 2001). As it was clear that sphingolipids and its metabolites behave differently in the crucial regulation of cell growth, it is very important to get the pure components of bioactive sphingolipid/ceramide present in PTLF. Work in this direction is in progress and HPTLC single spot preparation found to modulate B16F10 cell in respect of growth and melanization (data not shown).

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