CATECHOLAMINES INCREASE IN VITRO PROLIFERATION OF MURINE B16F10 MELANOMA CELLS

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Abstract

Context. Melanoma is the most aggressive skin cancer, with significant morbidity and mortality, and one factor that may influence the course of disease is stress.

Objective. Our aim was to evaluate the effect of corticosterone, norepinephrine, epinephrine on murine B16F10 melanoma cells in vitro proliferation.

Methods. B16F10 melanoma cells were treated with different concentrations of tested hormones. The proliferative capacity of melanoma cells was quantified by MTS assay and the cell viability was quantified as membrane integrity evaluation measured by lactate dehydrogenase (LDH) release.

Results. B16F10 cells treated with corticosterone showed no significant changes. In contrast, norepinephrine exposure stimulated the cell proliferation (P = 0.0003). Treatment with 1 µM norepinephrine induced the highest increase in cell proliferation (OD 492 = 0.27 ± 0.02) statistically significant to both control (OD 492 = 0.17 ± 0.01; p = 0.0003), 10 nM norepinephrine (OD 492 = 0.16 ± 0.00; p = 0.0004) and 100 nM norepinephrine (OD 492 = 0.19 ± 0.01; p = 0.002). Likewise, treatment with epinephrine increased cell proliferation (p = 0.0004). Exposure to 5 µm epinephrine induced a stimulation of cell proliferation (OD 492 = 0.28 ± 0.02) significantly higher compared to controls (OD 492 = 0.17 ± 0.01; p = 0.0004), 50 nM epinephrine (OD 492 = 0.17 ± 0.00; p = 0.001) and 500 nM epinephrine (OD 492 = 0.173 ± 0.00; p = 0.001).

Conclusions. Our results may open new perspectives concerning the link between stress hormones and melanoma, emphasizing a direct stimulating in vitro effect induced by catecholamines on melanoma B16F10 cells proliferation.

Key words: stress hormones, corticosterone, epinephrine, norepinephrine, melanoma, B16 cells, proliferation.

INTRODUCTION

Melanoma is the most aggressive form of human skin cancer, with significant morbidity and mortality, and an incidence rising faster than any other type of malignant tumour (1-6).
Although melanoma has an established genetic background (7), several environmental factors play an important role in melanoma development and progression (1, 2, 8). Recent evidence suggests that one of the factors that may have an impact on the course of the disease is psychological stress (8-10), this factor being involved in the onset or aggravation of many skin pathophysiological processes (11-14).

Highly stressful occupations appear to increase the risk of melanoma occurrence (8) and studies on melanoma patients suggest the involvement of psycho-emotional factors in the disease evolution and prognosis (10, 15-22).

Experimental studies in C57BL/6 mice bearing B16 melanoma have shown that exposure to restraint stress can induce changes in the pattern of pulmonary metastases regarding both the number and size of metastatic colonies (23). Surgical-stress induced to C57BL/6 mice prior to B16-BL6 melanoma cells inoculation significantly increased the pulmonary metastases number (24). In addition, another study highlighted a correlation between social stress exposure, passive-reactive coping strategy with a high serum corticosterone level and an increased number of murine B16-F10 melanoma lung metastasis (25), while it was reported that B16FIC29 murine melanoma cell line can release catecholamines (26).

Several mechanisms, such as an immune deregulation characterized by decreased number and activity of T and NK cells (8, 23, 25) as well as an increased serum level of TNF alpha and induction of VCAM-1 expression in the endothelium appear to be involved in stress-induced stimulation of melanoma metastases (24).

Other studies suggested that a direct action of stress hormones on melanoma cells activity inducing an increase of cytokines secretion, such as VEGF, IL-8 and IL-6, is involved in stimulation of melanoma progression (27) and moreover could promote a worse clinical outcome of the disease (3).

Stress is associated with activation of the hypothalamic-pituitary-adrenal axis with consequent release of glucocorticoids from the adrenal cortex and increased sympathetic activity, followed by the release of epinephrine from the adrenal medulla, and norepinephrine from sympathetic nerve endings (28, 29). Previous studies have shown that melanoma cells in different stages of the disease have the potential to respond to stress hormones, but this response may be different for different cell populations (27).

The aim of this study is to evaluate the unbiased action of stress hormones on murine B16F10 melanoma cell line which was used due to its cellular characteristic that recommends it as a cell line generating cutaneous melanoma in vivo model as previously published by us (5). Thus we have investigated the in vitro effect of corticosterone, norepinephrine and epinephrine on melanoma B16F10 cells proliferation, by means of two toxicity methods for assessing the percent of intact, viable cells (the extracellular lactate dehydrogenase release test) able to perform metabolic activity under
incubation with stress hormones (MTS reduction test).

**MATERIALS AND METHODS**

**Cells**

Murine B16F10 cells (ATCC® CRL-6475™) were grown in DMEM cell culture medium supplemented with 2mM Glutamine, 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (complete medium) at 37°C and 5% CO₂. For in vitro tests, cells were seeded in 96 well plates at a density of 3000 cells/well and cultured in complete medium. After 24h, in three sets of experiments the B16F10 melanoma cells were treated overnight with different concentrations of the tested stress hormones: corticosterone (C) 100 nM, 1 μM, 10 μM; norepinephrine (NE) 10 nM, 100 nM, 1 μM and epinephrine (E) 50 nM, 500 nM, 5 μM. The results of each experiment were compared with the control group consisting of untreated B16F10 cells. The stock solutions of the compounds were made in sterile water and further dilution in cell culture medium. At the time of treatment, ascorbic acid (30 μM) was added to all wells (control and treated). All chemicals were from Sigma-Aldrich (St. Louis, Missouri, USA), unless otherwise stated.

**Cell membrane integrity**

To assess the cell membrane integrity we have quantified the extracellular lactate dehydrogenase (LDH) release using Cytotox96 Non-Radioactive Cytotoxicity Assay kit (Promega Corporation). Briefly, according to manufacturer’s instructions, 50 μl of supernatant from cells treated overnight with different doses of stress hormones (C, NE or E) as described above, were collected for LDH release test. An equal volume of enzyme substrate was added to each collected sample, incubated at RT in a dark place, and stopped after 30 minutes with 50 μL of Stop Solution. The level of LDH released in cellular supernatant was determined immediately by measuring the optical density recorded at 490 nm (OD 490 nm).

**Cell Proliferation**

The proliferative capacity of murine B16F10 melanoma cells was quantified using the CellTiter 96 AQueous One Solution Cell Proliferation kit (Promega Corporation). The test is based on MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) conversion to a spectrophotometrically quantifiable soluble formazan compound by intracellular dehydrogenases and it is used to estimate the number of metabolically active, proliferating cells. Briefly, 20 μl of MTS reagent ready-to-use was added to each well containing 100 μl suspension of B16F10 melanoma cells treated overnight with different doses of stress hormones (C, NE or E) and incubated for 90 minutes at 37°C in 5% CO₂. The absorbance of each sample was recorded with an ELISA plate reader. Results are expressed as optical density recorded at 490 nm (OD 490 nm).

**Statistical Analysis**

For statistical analysis we used SPSS 15.0 (SPSS, Chicago, IL, USA) and GraphPad Prism (Graphpad Software, Inc., San Diego, CA, USA).
All experiments were triplicated/duplicated and repeated, and for each group, the results were presented as mean ± standard deviation (SD). Differences between experimental groups were evaluated using one-way analysis of variance between groups (ANOVA) followed by Tukey post hoc test. The p values <0.05 were considered significant.

RESULTS

Assessment of stress hormones effect on cell proliferation

The synthetic hormones used by us in the in vitro experimental model induced different effects on the proliferative capacity of tumoral cell line (see Figs 1 and 2). Thus, exposure of B16F10 melanoma cells to corticosterone did not generate significant changes in cell proliferation quantified by MTS assay (P = 0.3349).

In contrast, exposure to norepinephrine induced a marked increase in cell proliferation (P = 0.0003). The 1 µM norepinephrine concentration showed the highest increase in cell proliferation determined by MTS assay (OD 492 = 0.27 ± 0.02) statistically significant to both control (OD 492 = 0.17 ± 0.01; p = 0.0003), 10 nM norepinephrine (OD 492 = 0.16 ± 0.00; p = 0.0004) and 100 nM norepinephrine (OD 492 = 0.19 ± 0.01; p = 0.002).

Figure 1. B16F10 melanoma cells (arrows) - original magnification 200x (A) control; (B) treated with corticosterone 10 µM; (C) treated with norepinephrine 1 µM; (D) treated with epinephrine 5 µM.
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Figure 2. Murine B16F10 cell proliferation quantified by MTS assay. (A) corticosterone (C) exposure did not generate significant changes; (B) norepinephrine (NE) exposure in concentration of 1 µM induced a stimulation of cell proliferation significantly higher compared to control, NE 10 nM and NE 100 nM; (C) epinephrine (E) in concentration of 5 µM induced a significant increase in proliferation compared to controls, E 50 nM and E 500 nM. Error bars represent the SD. ** P <0.01, *** P <0.001, Tukey post hoc test.

Figure 3. Murine B16F10 cell membrane integrity evaluated by LDH release. (A) corticosterone (C) and (B) norepinephrine (NE) exposure induced no significant changes; (C) epinephrine (E) in concentration of 5 µM produced a slight but statistically significant increase of the LDH level, compared to control and E 500 nM. Error bars represent the SD. * P <0.05, ** P <0.01, Tukey post hoc test.
As well, treatment with epinephrine was associated with an important increase of B16F10 cells proliferation (p = 0.0004). Exposure of melanoma cells to 5 µm epinephrine induced a stimulation of cell proliferation determined by MTS assay (OD 492 = 0.28 ±0.02) significantly higher compared to controls (OD 492 = 0.17 ±0.01; p = 0.0004), to 50 nM epinephrine (OD 492 = 0.17 ±0.00; p = 0.001) and 500 nM epinephrine (OD 492 = 0.173 ±0.00; p = 0.001).

Assessment of stress hormones effect on LDH release

For assessing the cellular membrane integrity in our in vitro experiments we have obtained a LDH release with no statistical relevance upon exposure to corticosterone (P = 0.0554 ANOVA) or norepinephrine (P = 0.0595 ANOVA) (see Fig. 3).

Conversely, exposure of B16F10 cells to epinephrine was associated with an increase in LDH release (P = 0.0070 ANOVA). Cells treated with 5 µM epinephrine had a slight but statistically significant increase of the LDH level, compared to control (p = 0.035) and to cells exposed to 500 nM epinephrine (p = 0.005), indicating notable effects induced by this hormone on tumor cells metabolic processes and membrane integrity.

Combining the effect induced upon proliferation and membrane integrity, we can ascertain that epinephrine at a particular (high) dose induces complex cellular alterations that can lead, unfortunately, to an uncontrolled proliferation of melanoma B16F10 cells.

**DISCUSSION**

In our investigation of stress hormones effect on murine B16F10 melanoma cell proliferation, even if cells treated with corticosterone showed no significant changes, exposure to high concentrations of catecholamines, norepinephrine and epinephrine induced an important increase in cell proliferation. Moreover, treatment with epinephrine at high concentrations induced a significant increase in LDH release suggesting a disturbing, toxic effect of epinephrine on metabolic processes and membrane integrity of B16F10 cells. In vitro studies on other cell types (30, 31) have revealed a toxic effect of high concentrations of catecholamines, probably induced by catecholamine autoxidation with formation of oxidation products and subsequent toxicity (32).

There is a distinction that must be highlighted between the in vitro increase of LDH and the elevated serum levels of LDH in melanoma patients which is an independent factor associated with adverse prognosis and a negative predictor of response to therapy (33-38). LDH is not a specific biomarker for melanoma being expressed in all normal or tumor living cells (39). Increased levels of LDH in melanoma patients may occur due to the metabolic and membrane integrity changes induced by the imbalance between the blood supply and the malignant proliferation process creating a hypoxic microenvironment (40).

Previous studies showed that melanoma cells in different stages of the disease have the potential to respond to
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stress hormones but this response may be particular to the differentiation stage of the cells (27).

Assessing the role of catecholamines on melanoma is of particular interest in scientific research. Epinephrine and norepinephrine released during stress reaction can act on adrenergic receptors of melanoma cells which can express both alpha and beta adrenoreceptors (8, 9).

Although previous studies have revealed the presence of alpha1-adrenoceptor in human melanoma cells, they seem to have a reduced affinity for adrenergic agonists and their effect on tumor growth and invasiveness is still unclear (41, 42).

On the other hand, it is suggested a key role played by beta adrenoreceptors in the stimulation of melanoma growth and progression (8, 29). The stimulatory effect of catecholamines on B16F10 melanoma cell proliferation can be induced by direct activation of beta-adrenergic receptors.

The presence of beta 1 and beta 2 adrenoreceptors was highlighted in tissue samples from patients with primary or metastatic melanoma as well as in human melanoma cell lines (27, 43). In vitro studies have shown that norepinephrine and epinephrine, acting through the beta adrenergic receptors of human melanoma cells can activate several mechanisms that favor tumor progression. Thus, norepinephrine increases the expression in melanoma cells of cytokines that have a pro-angiogenic effect and stimulates tumor progression, such as VEGF, IL-8 and IL-6 (27, 43). Also, norepinephrine and epinephrine are able to elicit an invasive behavior of human melanoma cells with increased metalloprotease - dependent motility (43). These effects appear to be mediated by beta 1 and beta 2 adrenergic receptors and are inhibited by non-selective blocking of beta-adrenergic receptors (27, 43).

Recent studies have shown that beta-adrenergic receptor blockade may be associated with the reduction of tumor progression, of the risk of recurrence and mortality in patients with melanoma (44, 45).

Further, study results obtained in animal models of melanoma suggest a link between stress, stimulation of beta-adrenergic receptors and the tumor growth. Such a study in C57BL/6 mice showed that B16 melanoma growth is more noticeable in animals subjected to psychosocial stress, and this effect is inhibited by administration of non-selective beta-adrenergic antagonists (46).

Another study in C57BL/6J mice with experimentally induced melanoma by intraplantar inoculation of murine B16F10.9 melanoma cells showed that preoperative administration of a single dose of a beta-adrenergic antagonist in combination with a cyclooxygenase-2 inhibitor increases the recurrence-free survival after excision of primary tumor (47).

Also, studies on murine melanoma cell lines suggest a link between stress hormones and factors involved in tumor progression. Thus, a recent study showed that both norepinephrine and corticosterone stimulate overexpression of IL-6 in
metastatic B16-F10 cells (48). As regards the effects of catecholamines there is a convergence of the achieved results, the effects of glucocorticoids on cancer being still unclear. If glucocorticoids can promote fast growth and rapid spreading of various neoplasms they also may have pro-apoptotic effects in different cancer cell types (49).

The proliferative effects of epinephrine and norepinephrine highlighted in our study occur in the higher levels of the physiological or pathophysiological relevant concentrations of catecholamines. Different studies in mice have shown a wide spectrum of resting and stress plasma levels of both epinephrine and norepinephrine, depending on mouse strain, gender, type of stress and assessment method. Basal plasma levels may range between 0.5 nM and 15 nM (50-52), up to 200 nM (53) for epinephrine and from 2 to 20 nM (50-52) or even in some conditions over 4 µM (53) for norepinephrine. Stress increases these levels to 10–25 nM (50-52) or even 300 nM (53) for epinephrine and to 25-60 nM (50-52) going in some conditions over 1 µM (53) for norepinephrine.

Moreover, recent results have shown that plasma levels of norepinephrine are significantly higher in mice with B16-F10 melanoma metastases than in non-tumor controls (48).

In skin, in addition to the adrenergic nerve fibers, other important sources of catecholamines, are the keratinocytes (54) which have the capacity to synthesize epinephrine (55-57). Moreover, melanocytes (58) and lymphocytes (59) are able to produce norepinephrine and other studies suggested that rodent melanoma cells hold the capacity for catecholamine synthesis (60).

Furthermore, studies suggested that the levels of catecholamines may reach over 5 µM in certain tumor microenvironment (61-63).

Therefore, in our study, we evaluated the proliferative effects of epinephrine and norepinephrine using concentrations that cover a wide range of possible tissue levels of catecholamines and that are in line with doses that induced similar proliferative effects in other in vitro research. Recent studies show that catecholamines are involved in increasing the proliferation and the invasive behavior of other types of cancer cells.

In one recent research, the effects of the stress hormones on cell proliferation were investigated in SCC9 and SCC15 human oral squamous cell carcinoma cells (64). Treatment with norepinephrine in a concentration considered in the range of the physiological stress levels (10 µM) stimulated cell proliferation in both cell lines, while cortisol induced an increase in cell proliferation only in SCC15 cells. In another study on HKESC-1 esophageal squamous-cell carcinoma cells epinephrine increased cell proliferation with maximal stimulatory effect at a concentration of 10 µM (65).

Epinephrine exposure for 24 h in concentrations of 1 µM to 10 µM enhance the proliferation of HT29 colon cancer cells in a dose dependent manner also decreasing their response to
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cisplatin treatment (66). These results are confirmed by another study that showed a 35% increase of HT29 cell proliferation induced by epinephrine treatment at the concentration of 10 µM (67). Other research on HT29, SW116 and LS174T colorectal carcinoma cell lines showed that the stress hormones epinephrine and norepinephrine at concentrations of 0.1-10 µM markedly increase the cell proliferation with no significant effect induced by corticosterone (53).

Epinephrine and norepinephrine in concentrations of 0.1-10 µM also increase the in vitro invasiveness of EG, SKOV3, and 222 ovarian cancer cells (61).

Catecholamines can also stimulate the invasive behaviour of pancreatic cancer cells. Norepinephrine increases the proliferation, migration and invasion of PANC-1 cells, the maximal proliferative effect being induced by the dose of 10 µM (68).

Although the experiments on cell cultures cannot exactly reproduce the complexity of the stress response that occurs in vivo, the experimental models of cell cultures used by us have the advantage of a controlled environment with a better management of the experimental variables. In addition, the presented methodology is indispensable for the investigation of pathophysiological mechanisms involved at cellular and molecular level in the disease evolution. In accord to previous experimental models attempting to mimic the in vitro stress response by administration of stress hormones into the culture medium (69), a cellular model allows the simplification and shortening of the experimental procedures and also a cost reduction.

Our in vitro study allowed the use of methods with good sensitivity in the evaluation of cell proliferation and changes of membrane integrity.

MTS method is a well-established and extensively used method proving good sensitivity, comparable with the radio-isotopic method (70).

On a 12 well plate, the area of the growing surface is 3.8 cm²; we have cultivated our cells on a 96 well plate flat bottom meaning a growing area of 0.32 cm² /well. (71). Knowing that the published lower linear limit of detection is 10,000 cells on a 12 well plate (72) the system that we have used (3000 cells on a 0.32 cm² surface growing area) puts the linearity of detection right in the middle of the curve. However, each type of cell has its particularities in terms of size and covered surface area when adhering to plates, and hence the actual linearity of the spectrophotometric registered data versus number of cells that display the formazan reaction. Knowing this, the B16 cell line used in our experiments was prior tested in terms of linearity for OD versus adhered cell number in 96 wells and the 3000 cells/well was the optimum one in terms of spectrophotometrically registering a significant increase (due to proliferation of cells) or a significant decrease (due to cell death).

We have chosen LDH assay to measure membrane integrity by monitoring the passage of substances that are normally intracellular. LDH is a commonly measured parameter (73) when assessing cytotoxicity. The sensitivity of this test goes as low as 500
cells, the reason why it is used in NK cytotoxicity where it can depict effector: target ratio as low as 100 effector cells targeting one target. The linearity of this assay goes around 6000 cells slightly depending on the actual cell type. In our experiments the 3000 cells/well covered the exact middle of the linearity curve of the LDH assay (74).

Using both methods we have intended to depict concomitantly from each individual well the effect inflicted upon metabolically active cells and upon cells membrane. By measuring both parameters, the effects of compounds can be depicted on the membrane, intracellular or in both compartments.

Thus the findings exposed herein strengthen the role of stress hormones bringing direct proof of significant tumor cell proliferation in presence of epinephrine and norepinephrine.

Our results represent a step forward from previous in vitro studies which have shown that stress hormones and mainly catecholamines can induce an indirect stimulation of melanoma proliferation and invasiveness (27, 43, 48). Both in vitro and in vivo studies indicate that stimulation of melanoma proliferation induced by catecholamines is mediated by the beta-adrenergic receptors (27, 43, 46, 47). Moreover, the results of clinical research have been in accordance with in vivo and in vitro experimental studies and suggested potential therapeutic implications indicating a possible positive effect of beta-blockers in patients with melanoma (44, 45).

In conclusion, along with previous studies that have demonstrated the role of stress hormones in stimulating the release of factors that promote tumor progression by melanoma cells, our results may open a new perspective on the link between stress hormones and melanoma, emphasizing in vitro a direct stimulating effect on proliferation of murine melanoma cells induced by catecholamines.

Conflict of interest
We declare that there is no conflict of interest.

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