

Cytotoxic Effect of Dexamethasone Restricted to Noncycling, Early G1 Phase of Melanoma Cells

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ABSTRACT

This study deals with the inhibitory effects of dexamethasone on the proliferation of a human melanoma cells after separation into different fractions according to their position in the cell cycle using a gravity sedimentation chamber. Fractions with high percentage of G1 cells were more susceptible to the action of dexamethasone than those with a lower percentage of G1 cells. Dexamethasone stimulated the rate of incorporation of radioactive precursors into acid-precipitable materials of melanoma M-5A cells starting 24 hours after treatment for one hour. Moreover, dexamethasone treatment markedly increased the volume of the M-5A cells with increasing the possibility of stimulating the transcriptional/translational activity in the cells. This study may hopefully stimulate the development of new approaches in systemic and/or regional chemotherapy with malignant melanoma using dexamethasone with other alkylating agents to get synergistic interaction.

Keywords: Melanoma Cells, Cell Fractionation, Dexamethasone

1. Introduction

Although malignant melanoma has not generally been regarded a hormonally responsive neoplasm, there are some evidences suggesting that melanoma may respond to the hormonal condition of the host. Pregnancy was reported to be associated with unfavorable prognosis in stage II disease [1]. However, adrenalectomy for melanoma metastatic to the adrenal gland provides good palliation of complete regression of distant metastatic melanoma after bilateral adrenalectomy, suggesting a possible role for adrenal hormones in modifying melanoma progression in certain patients [2]. A few reports have been published about the moderate sensitivity of human as well as animal melanoma to the action of glucocorticoid steroid hormones. Bregman *et al.*, [3] found that dexamethasone at a concentration of 1 μ M inhibited the colony formation in a human melanoma cell strain (C8146c) by 60%. Moreover, Ramirez *et al.*, [4], showed 85% tumor rejection in mice challenged with B16 melanoma after administration of anti glucocorticoid-induced TNF receptor family related protein. Recently, Banciu *et al.*, [5], reported that a glucocorticoid prednisolone phosphate encapsulated in long-circulating liposomes exerts anti-tumor activity through the inhibition of tumor angiogenesis. The marked if not complete reversal of the inhi-

bition of cell proliferation by glucocorticoid [6] raised the question as to what the performance of the cells might be in terms of RNA, protein and melanin synthesis during their arrested or retarded proliferation. Therefore the aim of our study was directed to investigate in what phase of the cell cycle the cells would be the most sensitive to the inhibition of proliferation induced by dexamethasone.

2. Materials and Methods

2.1. Cells and Cell Culture

A human melanoma cell line M-5A was received at unknown passage level from liquid nitrogen store National Cancer Institute, Cairo University. Its characteristics have been described [7]. Cells were grown in modified minimum essential Eagle's medium to which penicillin (100 i.u./ml) and streptomycin (100 μ g/ml) had been added. The medium contained 10% newborn calf serum inactivated by heating at 56°C for 30 min. Cells were cultured at 37°C in humidified 5% CO₂ atmosphere.

2.2. Cell Separation

Cells were separated according to size by velocity sedimentation at unit gravity in a linear density gradient from 2% to 8% BSA on PBS. The chamber as developed by

Tulp *et al.* (1980) [8] (**Figure 1**) contained flow deflectors which permitted undisturbed introduction of the cushion liquid and density gradient and the undisturbed layering of the cells. The chamber was sterilized by filling it completely with 2% formaldehyde in PBS for 24 hours. It was washed thoroughly with sterile bidistilled water followed by sterile PBS. After that the chamber was covered with aluminum foil and kept at 4°C. The gradient mixer and other glassware were dry sterilized at 250°C overnight. At the time of experiment, the chamber was removed from the cold room and filled completely via inlet B with sterile cushion liquid consisting of 10% Ficoll (average molecular weight 400,000, Pharmacia, Sweden) in PBS. All solutions were introduced into the chamber from their reservoir by the force of gravity. A linear density gradient (400 ml), prepared from two different solutions of BSA (Sigma, fraction V) in PBS (2 and 8%, respectively, sterilized by ultrafiltration) was admitted to the chamber via inlet A within 25 min. by letting cushion liquid flow out via B. After that a disposable syringe was filled with 20 ml of a cell suspension in 0.5% BSA in PBS. The plunger was removed and the syringe was connected to A by a piece of sterile silicon tubing. The cells were let to flow into the chamber within 1 min followed by an overlay of 25 ml PBS until the meniscus of the fluid had reached the cylindrical part of the chamber. The cells were then allowed to sediment at unit gravity for about 90 min at 4°C. Routinely, 50×10^6 – 60×10^6 cells were thus processed. After sedimentation the content of the separation chamber were collected via A by allowing cushion liquid to flow into chamber via B. The first 150 ml were discarded and subsequently fractions of 10 ml each were collected. Selected fractions were studied for DNA per cell content by flowcytomter.

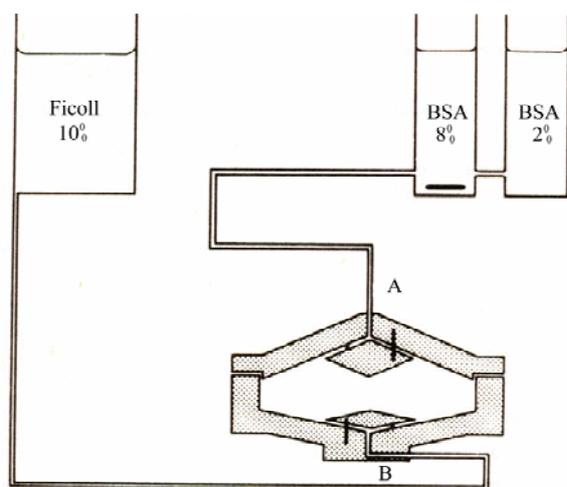


Figure 1. Schematic presentation of the system for separation of cells according to cell size in linear density gradient at unit gravity.

Moreover, their capacity for cell proliferation was determined using colony forming assay procedure described before [9].

2.3. Determination of the Rate of (³H) Uridine and (¹⁴C) Leucine Incorporation

The rate of (³H) uridine and (¹⁴C) leucine incorporations were determined as described before (9). (³H) uridine (0.1 Ci/ml, 23.4 Ci/mmol) and L-(¹⁴C) leucine (0.1 Ci/ml, 340 mCi/mmol) were obtained from Amersham International, Ltd, England.

2.4. Measurement of Cell Volume

Cell volumes were measured using coulter counter (Model ZF) equipped with a multichannel analyzer (Tracor, TN.1705) and an XY-plotter (Tracor, TA-1148) which counts cells in a series of channels. The cell number was automatically plotted against cell size. Exponentially growing cells were inoculated into 75 cm² Falcon flasks and 24 hours later, dexamethasone was supplied at a dose of 12.5 uM for one hour.

2.5. Determination of Relative Melanin Content.

Melanin content was measured by the colorimetric method described by Whittaker [10]. Cell pellets containing 5×10^6 cells placed in duplicate Beckman microfuge polyethylene tubes were suspended, lysed in 0.5 ml of deionized H₂O, and subjected to 2 cycles of freezing and thawing. Perchloric acid was added to a final concentration of 0.5 N, and the suspension was kept on ice for 10 min and then centrifuged for 5 min in a Beckman Model B microfuge. The pellets were extracted twice more with 0.5 N HClO₄ followed by 2 extractions with a cold mixture of ethyl alcohol: ethyl ether (3: 1, v/v) and a final extraction with ethyl ether. The pellets were air dried, 1 ml of 0.85 N KOH was added, and the pellets were dissolved by heating to 60°C for 10 min. After insoluble residue was pelleted the supernatant was cooled to room temperature and the absorbance at 400 nm was measured in a Beckman double-beam spectrophotometer. The relative melanin content is expressed as the absorbance at 400 nm per 5×10^6 cells.

3. Results

3.1. Proliferation Capacity of Separated Cells after Dexamethasone Treatment

The initial fractions obtained from the gravity sedimentation chamber contained exclusively G1-phase cells or at least cells with diploid DNA per cell content as shown by flowcytomter (**Figure 1**). Subsequent fractions were enriched in S and G2/M cells, respectively. Attempts to obtain fractions containing predominantly S and/or G2/M

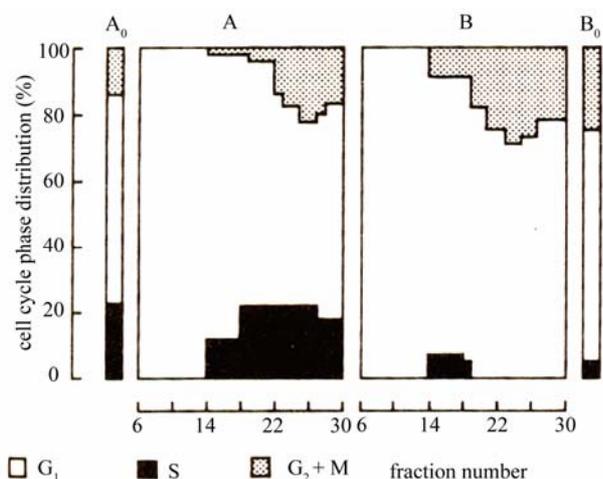


Figure 2. Cell cycle distribution of fractions of M-5A cells obtained by cell separation at unit gravity. A, untreated separated cells; A₀, untreated unseparated control; B, Cells treated with 12.5 μ M dexamethasone for one hour and then separated; B₀, Treated unseparated control.

cells, however, remained unsuccessful. **Figure 2** showed The first 5-6 fractions exhibited a very low capacity for colony formation (**Table 1**). However, this capacity increased sharply in the pure G1 cell fractions with increasing fraction number (149 colonies with cells exclusively with G1 compared to 35 colonies in cells with 50% G1). Moreover, there was 93% inhibition in cell proliferation after dexamethasone treatment in fraction contains 100% G1, while there was only 29% inhibition in fraction contains 50% G1.

3.2. Incorporation of (³H) Uridine and (¹⁴C) Leucine by Dexamethasone Treated Cells

Table 2 showed the effect of dexamethasone (12.5 μ M) treatment on the incorporation of precursors of RNA and protein into M-5A melanoma cells. There was stimulation of (³H) uridine incorporation in M-5A cells starting from 24 hours after treatment which continued till 96 hours (**Table 2**). (¹⁴C) leucine incorporation was also sig-

Table 2. Effect of dexamethasone (12.5 μ M) on the rate of incorporation of (³H) uridine and (¹⁴C) leucine in human melanoma cells.

Time after treatment (hr)	Rate of (³ H) uridine incorporation (dpm/0.25x10 ⁶ cells/60 min) $\times 10^{-3}$			Rate of (¹⁴ C) leucine incorporation (dpm/0.25x10 ⁶ cells/60 min) $\times 10^{-3}$		
	Control	Treated	T/C $\times 100$	Control	Treated	T/C $\times 100$
24	3.7 \pm 0.14	6.3 \pm 0.07 (3)	170	0.35 \pm 0.05	0.53 \pm 0.03 (6)	152 ^b
48	4.18 \pm 0.57	7.20 \pm 0.76 (8)	173 ^c	0.60 \pm 0.06	0.13 \pm 0.04 (6)	187 ^m
72	4.52 \pm 0.49	9.39 \pm 1.14 (3)	208 ^c	0.38 \pm 0.03	0.70 \pm 0.04 (8)	185 ^a
96	3.93 \pm 0.21	8.62 \pm 1.07 (3)	219 ^c	0.50 \pm 0.10	0.91 \pm 0.08 (3)	182 ^d

Cells were treated for one hour with 12.5 μ M dexamethasone, 48 hours after treatment, (³H) uridine and (¹⁴C) leucine were added for 60 min. Cell numbers were counted in a hemocytometer and acid-precipitable radioactivity was determined as described in the materials and methods. Presented are means \pm S.E.M. Number of observation are in parenthesis, *p*-value for the differences between control and treated samples were ^a < 0.001; ^b < 0.002; ^c < 0.02; ^d < 0.05; other differences were not significant.

Table 1. Effect of treatment with dexamethasone on the proliferation of melanoma M-5A cells fractionated according to their position in the cell cycle.

Fraction Number	G1 cell per Fraction	Colony formation per 100 cells ^a		
		Control	Treated ^b	inhibition (%)
10	100 \pm 0	149 \pm 47	11 \pm 2	93
15	81 \pm 18	83 \pm 26	25 \pm 11	70
21	63 \pm 6	88 \pm 8	28 \pm 3	68
27	50 \pm 2	35 \pm 5	25 \pm 1	29

^apresented are mean \pm S.E.M.

^bDexamethasone, 12.5 μ M for one hour

nificantly stimulated (52 and 87% increase compared to control, 24 and 48 hours after treatment, respectively).

3.3. Changes in Cell Volume after Treatment with Dexamethasone

It was noticed microscopically that M-5A cells increased in size after dexamethasone treatment. Therefore, the effect of dexamethasone treatment on the cell volume was investigated in more detail in the cells the frequency distribution curve for control and treated cells obtained after 72 hours is depicted in **Figure 3**.

M-5A cells showed an increased frequency of large-sized cells 72 hours after dexamethasone treatment compared to control cells.

3.4. Melanin Synthesis after Treatment with Dexamethasone.

Table 3 showed the melanin content of M-5A cells, three days after dexamethasone treatment at a dose level of 0.25 to 12.5 μ M for 60 min. Dexamethasone treatment showed an increase in the melanin content in M-5A cells by 14 and 153%, respectively. Moreover, there was a concomitant inhibition of cell proliferation from 49 to 76% at dose level of 0.25 to 12.5 μ M, respectively.

4. Discussion

Malignant melanoma is a relatively uncommon disease comprising 1% - 3% of all cancers [11,12]. Melanoma has received considerable attention from both the clinical and

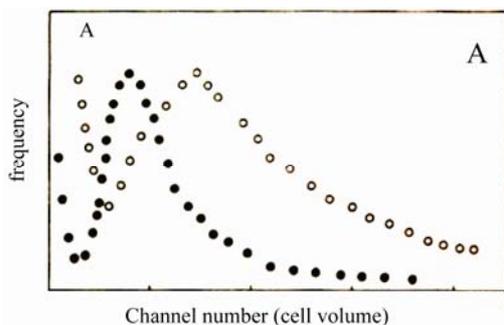


Figure 3. Volume of melanoma M-5A cells after treatment with dexamethasone in comparison with untreated controls. Cells were treated with dexamethasone 12.5 μ M for one hour, cell volume were analyzed 72 hours after treatment. Chart redrawn after the coulter counter plots. A, M-5A cells; • Control; ° Treated.

Table 3. Concentration dependence of the effect of dexamethasone on the cell proliferation and melanin content of M-5A cell^a.

Dose (μ M)	Relative cell number (%) ^b	Relative melanin content (%) ^b
0	100	100
0.25	51	114
0.625	30	197
1.25	25	213
12.5	24	253

^aDexamethasone was supplied for one hour, 72 hours before the measurement of cell number and melanin content. ^bMean of two experiment in duplicate.

investigational point of view due to marked tendency to disseminate and many patients die as result of distant metastases [13,14]. Glucocorticoid (triamcinolone) has shown an inhibitory effect on melanoma B16/F10 cell growth [15]. Moreover, Osman *et al.* [9] showed inhibitory effect of dexamethasone on the proliferation of melanoma M-5A cells by arresting the cells in G1 phase. So this study was directed to gain better insight the sensitivity of cell cycle phases of M-5A cells to the action of dexamethasone. Dexamethasone showed much inhibitory effect on G1 phase of M-5A cells, these results indirectly agreed with that reported by Ramos *et al.* [16] who demonstrated that the steroid-induced expression of C/EBP alpha that is necessary to mediate the glucocorticoid G1 cell cycle arrest of rat hepatoma cells and implicates a role for this transcription factor in the growth control of liver-derived epithelial tumor cells. In addition, the changes in expressions of regulators of cell cycle (p27 (kip1) and cyclin D2) and/or apoptosis (Bak and Bcl-XL) by anti-inflammatory drugs like dexamethasone may contribute to proliferation suppression and apoptosis in human oestoblasts (hOBs) [17]. It seems likely that a cellular response to dexamethasone will in principle be highly selective. During the dexamethasone-induced arrest [9], the cells approximately doubled in size as deter-

mined by coulter counter analysis (Figure 3). This result was not unexpected, because large, nonproliferating cells persisted in the arrested culture as was seen microscopically. These observations suggested a more generalized increase in the synthesis of protein and RNA which have been shown in our study in which there was an increase in both uridine and leucine incorporation (Table 2). Moreover, there was an increase in the melanin content of M-5A cells after dexamethasone treatment which was parallel to inhibitory effect seen on the proliferation of the M-5A cells. This result indirectly agreed with that reported by Koo *et al.*, [18], where they found that in vivo treatment of human melanoma xenograft tumors in athymic nude mice with anthrax lethal toxin (LeTx) resulted in a significant or complete tumor regression without apparent side effects, suggesting that inhibiting the MAPK (mitogen-activated protein kinase) signaling pathway may be a useful strategy for treating melanoma. Additionally, interrupting MAPK signaling with LeTx in both melanoma cells and melanocytes lead to dramatic melanin production, perhaps explaining the inhibitory effect of dexamethasone on the proliferation of M-5A cells which could be due to increase the melanin pig.

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REFERENCES

- [1] M. H. Shiu, D. Schottenfeld, B. Maclean and J. G. Fortner, "Adverse Effect of Pregnancy on Melanoma: A Reappraisal," *Cancer*, Vol. 37, No. 1, 1976, pp. 181-187.
- [2] F. J. Collinson, T. K. Lam, W. M. Bruijn, J. H. M. De Wilt, M. Lamont, J. K. Thompson and R. F. Kefford, "Longterm Survival and Occasional Regression of Distal Melanoma M, Etastases after Adrenal Metastectomy," *Annals of Surgical Oncology*, Vol. 15, No. 6, 2008, pp. 1741-1749. doi:10.1245/s10434-008-9836-y
- [3] M. D. Bregman, E. Peters, D. Sander, F. L. Meyskens Jr., Dexamethasone, "Prostaglandin A and Retinoic Acid Modulation of Murine and Human Melanoma Cells Grown in Soft Agar," *Journal of the National Cancer Institute*, Vol. 71, No. 5, 1983, pp. 927-932.
- [4] M. T. Ramirez, A. Chow, D. Hirschhorn Cymerman, T. H. Terwey, A. A. Hochma, S. Lu, R. C. Miles, S. Sakaguchi, A. N. Houghton and M. R. van den Brink, "Glucocorticoid-Induced TNF Receptor Family Related Gene Activation Overcomes Tolerance/Ignorence to Melanoma Differentiation Antigens and Enhances Antitumor Immunity," *The Journal of Immunology*, Vol. 176, No. 11, 2006, pp. 6434-6442.
- [5] M. Banci, J. M. Metselaar, R. M. Schiffelers and G. Storm, "Antitumor Activity of Liposomal Prednisolone Phosphate Depends on the Presence of Functional Tu-

- mor-Associated Macrophages in Tumor Tissue,” *Neoplasia*, Vol. 10, No. 2, 2008, pp. 108-117. [doi:10.1593/neo.07913](https://doi.org/10.1593/neo.07913)
- [6] L. A. Smets, B. Bout and M. Brouwer, “A Tulp Cytotoxic Effect of Dexamethasone Restricted to Noncycling, Early G1 Phase Cells of L1210 Leukemia,” *Journal of Cellular Physiology*, Vol. 116, No. 3, 1983, pp. 397-403. [doi:10.1002/jcp.1041160318](https://doi.org/10.1002/jcp.1041160318)
- [7] K. Liao, P. B. Dent and P. B. McCulloch, “Characterization of Human Malignant Melanoma Cell Lines. I. Morphology and Growth Characteristics in Culture,” *Journal of the National Cancer Institute*, Vol. 54, No. 5, 1975, pp. 1037-1044.
- [8] A. Tulp, J. G. Collard, A. A. M. Hart and J. A. Aten, “A New Unit Gravity Sedimentation Chamber,” *Analytical Biochemistry*, Vol. 105, No. 1, 1980, pp. 246-256. [doi:10.1016/0003-2697\(80\)90452-2](https://doi.org/10.1016/0003-2697(80)90452-2)
- [9] A. M. Osman, O. A. Nasseir and N. R. Ismail, “Potential Mechanism for the Effects of Dexamethasone on Growth of Human Melanoma Cells *in Vitro*,” *Health*, Vol. 2, No. 8, 2010, pp. 857-886. [doi:10.4236/health.2010.28129](https://doi.org/10.4236/health.2010.28129)
- [10] J. R. Whittak, “Changes in Melanogenesis during the Dedifferentiation of Chick Retinal Pigment Cells in Cell Cultures,” *Developmental Biology*, Vol. 8, No. 1, 1963, pp. 99-127. [doi:10.1016/0012-1606\(63\)90028-9](https://doi.org/10.1016/0012-1606(63)90028-9)
- [11] W. H. McCarthy, A. L. Black and G. W. Milton, “Melanoma in New South Wales: An Epidemiologic Survey, 1970-1976,” *Cancer*, Vol. 46, No. 2, 1980, pp. 427-432. [doi:10.1002/1097-0142\(19800715\)46:2<427::AID-CNCR2820460237>3.0.CO;2-E](https://doi.org/10.1002/1097-0142(19800715)46:2<427::AID-CNCR2820460237>3.0.CO;2-E)
- [12] L. Nathanson, “Epidemiologic and Etiologic Considerations in Malignant Melanoma,” In: J. J. Costanzi, Ed., *Cancer Treatment and Research*, Martinus Nijhoff, the Huige, Vol. 9, 1983, pp. 1-27. [doi:10.1146/annurev.genet.38.072902.092717](https://doi.org/10.1146/annurev.genet.38.072902.092717)
- [13] R. L. Comis, “DTIC (NSC-45388) in Malignant Melanoma: A Perspective,” *Cancer Treat Rep*, Vol. 60, No. 2, 1976, pp. 165-176.
- [14] J. J. Costanzi, “The Chemotherapy of Malignant Melanoma,” In: J. J. Costanzi, Ed., *Cancer Treatment and Research*, Martinus Nijhoff, the Huige, Vol. 9, 1983, pp. 259-274.
- [15] A. Ristic-Fira, M. Vujcic, M. Krstic-Demonacos, D. Kanazir, “Identification and Characterization of Glucocorticoid Receptors in B16 Mouse Melanoma Cells,” *Endocrine Regulations*, Vol. 33, No. 3, 1999, pp. 109-115.
- [16] R. A. Ramos, Y. Nishio, A. C. Maiyar, K. E. Simon, C. C. Ridder, Y. Ge and G. L. Firestone, “Glucocorticoid-Stimulated CCAAT/Enhancer-Binding Protein Alpha Expression is Required for Steroid-Induced G1 Cell Cycle Arrest of Minimal-Deviation Rat Hepatoma Cells,” *Molecular and Cellular Biology*, Vol. 16, No. 10, October 1996, pp. 5288-5301.
- [17] J. K. Chang, C. J. Li, H. J. Liao, C. K. Wang, G. J. Wang and M. L. Ho, “Anti-Inflammatory Drugs Suppress Proliferation and Induce Apoptosis through Altering Expressions of Cell Cycle Regulators and Pro-Apoptotic Factors in Cultured Human Osteoblasts,” *Toxicology*, Vol. 258, No. 2-3, 28 April 2009, pp. 148-156.
- [18] Han-M., Koo, M. VanBrocklin, M. J. McWilliams, S. H. Leppla, N. S. Duesbery and G. F. Vande Woude, “Apoptosis and Melanogenesis in Human Melanoma Cells Induced by Anthrax Lethal Factor Inactivation of Mitogen-Activated Protein Kinase,” *PNAS*, Vol. 99, No. 5, 2002, pp. 3052-3057. [doi:10.1073/pnas.052707699](https://doi.org/10.1073/pnas.052707699)