IDENTIFICATION AND CHARACTERIZATION OF GLUCOCORTICOID RECEPTORS IN B16 MOUSE MELANOMA CELLS

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Objective. To gain better insight into the role of glucocorticoids as modulators of cell growth, as well as to investigate the presence and characteristics of glucocorticoid receptors (GR) in mouse melanoma cells.

Methods. In two different B16 mouse melanoma cell clones (B16/F10 and B16/C3) the role of synthetic glucocorticoids (triamcinolone acetonide, TA) as cell growth modulators was investigated.

Results. The inhibitory effect of TA on B16/F10 cell growth after 8 days in culture was observed. The same hormonal treatment applied on B16/C3 melanoma cells also provoked changes in the cell growth. Dot blot analysis, using monoclonal antirodent glucocorticoid receptor antibodies showed the presence of receptor protein in both cell clones. The analysis of glucocorticoid receptors in B16/F10 and B16/C3 cell cytosol by Scatchard assay and ion-exchange chromatography on DEAE-Sephadex A-50 minicolumn indicated that the changes in melanoma cell growth may be mediated by glucocorticoid receptors and may relieve changes in the GR itself.

Conclusions. It was found that B16/C3 melanoma cells exhibited different growth pattern under TA treatment when compared to the results obtained with B16/F10 cells. Such differences may be mediated by glucocorticoid receptors.

Key words: Melanoma cells – Glucocorticoid receptor – Triamcinolone acetonide – Cell growth

The steroid hormone receptor proteins, in particular the glucocorticoid receptor (GR), represent one of the best characterized eucaryotic gene regulating systems known today. It has been reported that the glucocorticoid receptor is a member of the steroid/thyroid receptor superfamily (EVANS 1988; O’MALLEY 1990). The action of glucocorticoid hormones is mediated by their specific binding to the receptors located in the cytoplasm. The activated hormone-receptor complex is then translocated into the nucleus. Binding of this complex to specific hormone-regulatory elements on the DNA and regulation of gene expression are subsequent events that follow the activation of hormone-receptor complex (GUSTAFSSON et al. 1987).

Hormonal treatment is one of accepted form of therapy for some human malignancies. After the initial work indicating the role of glucocorticoids in the treatment of leukemia (SHANBROM and MILLER 1962), the presence of glucocorticoid receptors was demonstrated in lymphosarcoma cells (KIRKPATRICK et al. 1971), in lymphoma cells (BAXTER et al. 1971), as well as in hepatoma cells (BAXTER and TOMKINS 1970).

Several epidemiological studies have already suggested that steroid hormone application may affect the growth of malignant melanoma (CROWLEY et al. 1988; WALKER 1988). It was shown that glucocorticoids have an inhibitory effect on melanomas. Several authors have described the presence of GR in human malignant melanoma specimens (BHAKOO et al. 1981). Although it is not clear what function these receptors perform in general, in some patients, melanomas have been found to respond to glucocorticoids (RISELEY and SHERBET 1983).

Mouse melanoma cells represent a convenient experimental system to investigate molecular mech-
GLUCOCORTICOID RECEPTOR IN MOUSE MELANOMA CELLS

anisms of carcinogenesis. It has been demonstrated that B16 mouse melanoma, like human melanomas, have in vivo growth characteristics that are modulated by hormonal factors (Proctor et al. 1976, 1981; Lopez et al. 1978; Walker 1988).

It has been reported that glucocorticoids inhibit B16 melanoma cell growth in vivo, while such inhibition was not observed in vitro (Crowley et al. 1988). However, the growth inhibition with glucocorticoids has been shown in human melanoma cell lines (Di Sorbo et al. 1983; Osman et al. 1985). A dose-related glucocorticoid induced inhibition has been reported by Benckhuijzen et al. (1987) and in serum free media by Di Sorbo (1986). It was also observed that GR agonist triamcinolone acetonide inhibited the proliferation effect of conditioned media, insulin and transferrin (Di Sorbo 1986).

This study on B16/F10 and B16/C3 mouse melanoma cells was performed in order to gain better insight into the role of glucocorticoids as modulators of cell growth, as well as to investigate the presence and characteristics of GR in mouse melanoma cells. It might be expected that these data might later be extrapolated to human melanoma therapy.

Materials and Methods

Cells. In this study B16/F10 and B16/C3 mouse melanoma cells were used. Cells were maintained in culture with RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin/streptomycin and L-glutamine. 24 hours after plating, cells were treated with triamcinolone acetonide (TA, 0.5 μM). The viable cell number was estimated in a haemocytometer with 0.4% Trypan blue, every day for the next eight days. For the receptor assay cells were cultured in medium supplemented with serum pretreated with charcoal.

The soluble cytosolic protein concentration was determined according to Lowry et al. (1951).

Glucocorticoid receptor (GR) assays. Cells were grown to subconfluence, harvested using rubber-policeman, washed twice in PBS and lysed on ice by homogenisation in glass-teflon homogeniser in EHG buffer (0.1 mM EDTA-Na₂, 20 mM Heps pH 7.4, 10% glycerol, 10 mM DTT and 50 mM NaCl) supplied with 20 mM Na₂MoO₄ (100 µl EHG buffer/10⁶ cells). Cell cytosol corresponding to 4x10⁶ cells, (obtained by centrifugation of homogenate at 12000 rpm for 10 min in Eppendorf centrifuge), was incubated with an increased amount (1-128 nM) of [³H]triamcinolone acetonide ([³H]TA, SA=14.24 Ci/mol, Amersham) in the absence and presence of 100 fold excess of unlabeled TA. Specific binding was demonstrated by the difference between total binding ([³H]TA alone) and binding in the presence of a 100-fold excess of unlabeled TA. The number of binding sites per cell and Kd were calculated according to the method of Scatchard (Scatchard 1949).

Dot blot analysis. B16/F10 and B16/C3 cell extract (approximately 1.4 x 10⁸ cells) diluted 1:1 in the sample buffer according to Laemmli (1970) were applied directly to the nitrocellulose membrane using Manifold filtration apparatus. Dot wells were carefully washed 3 times with TBST buffer (10 mMTris-HCl buffer, pH 7.4, containing 0.9% NaCl and 0.05%Tween 20). Membranes were incubated for 1 h in TBST supplied with 1% BSA, then transferred to TBST buffer containing 1:500 BuGR monoclonal anti-rat GR antibodies and developed for two hours at room temperature under constant shaking. After washing 3 times for 10 min with 20 ml of TBST buffer, the antigen-antibody complex was visualized via anti-IgG-alkaline phosphatase conjugate according to the instruction from ProtoBlot Immunoscreening System, Promega.

Steroid labeling of cytosol and ion exchange chromatography.

The cytosol (1.4-2x10⁶/cell/ml) was labeled in vitro by incubation for 90 min at 4 °C with [³H]triamcinolone acetonide at a final concentration of 50 nM. The labeled cytosol was activated by heating for 30 min at 25 °C. Determination of bound steroid was performed by dextran-coated charcoal assay. The ion exchange chromatography on DEAE-Sephadex A50 minicolumn was performed as described previously (Markovic and Litwack 1980; Vujcic et al. 1995). A column (1.4x7 cm) of DEAE-Sephadex A-50 (including dextran-coated charcoal at the bottom) was equilibrated with 20 mM phosphate buffer (pH 6.8, containing 10mM dithiotreitol, 10 mM Na₂MoO₄₂). Chromatography was performed using KCl gradient for elution. One ml of radioactively labeled cytosol was applied to the column and after passage of 16 ml of buffer (8 fractions, 2 ml each) elution was started with a linear salt gradient formed from 25 ml 20 mM phosphate buffer and 25 ml 1M KCl in the same buffer.
100 fractions of 0.5 ml each were collected at a rate of 1 ml/min, and aliquots of all fractions were analyzed for radioactivity and conductivity.

**Results**

In this study we have investigated the role of glucocorticoids (triamcinolone acetonide, TA) as modulators of mouse melanoma cell growth. We compared untreated control cell cultures (B16/F10 and B16/C3 melanoma cells) to TA (0.5 µM) treated cells. The effect of glucocorticoids on cell growth was observed following the first eight days in culture. The inhibition of the B16/F10 mouse melanoma cell growth obtained with TA showed the same shape as control cultures, reaching maximum values four days after

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**Fig. 1** The effects of triamcinolone acetonide (TA) on B16/F10 (A) and B16/C3 (B) melanoma cell growth. Cells were treated 24 hours after plating.

**Fig. 2** Scatchard plot of [H]triamcinolone acetonide binding to the glucocorticoid receptors of B16/F10 (A) and B16/C3 (B) mouse melanoma cells. The plot is a mean representation of results from six experiments.
plating (Fig. 1A). The same hormonal treatment applied on B16/C3 cells slightly induces cell growth. It was observed that C3 cells treated with TA have reached maximal growth 5 days after plating. Untreated controls have shown maximal growth 48 hours later, 7 days after plating (Fig. 1B). The obtained results clearly show that B16/C3 melanoma cells exhibited different growth dynamics under glucocorticoid treatment in comparison to the B16/F10 clone.

These results suggested that structural characteristics of the glucocorticoid receptor might be different in the B16/F10 and B16/C3 clones. In order to assess receptor status in these cell lines, the presence, number and binding affinity of GR were investigated in both B16 mouse melanoma cell clones. Scatchard analysis (SCATCHARD 1949) of the binding data showed that B16/F10 melanoma has a three times higher number of binding sites (3000 binding sites per cell) compared to the B16/C3 clone (1000 binding sites per cell) (Fig. 2). In order to confirm these data, we used dot blot analysis to investigate the expression levels of receptor protein. The blots were developed using monoclonal antirodent GR antibodies (BuGR; GAMETCHU and HARRISON 1984) and showed the presence of GR in both cell clones with higher intensity detected in B16/F10 cytosol (Fig. 3). These data confirmed the results obtained by the Scatchard method (Fig. 2, Tab. 1).

In order to assess the affinity of glucocorticoid receptor for TA we determined the Kd in both clones. Similar Kd values were observed (1.21 and 1.53 nM in B16/F10 and B16/C3, respectively; Tab. 1). These results showed that melanoma cells have glucocorticoid receptor with similar affinity for hormone in both clones, whereas the F10 clone had a three times higher number of receptors when compared to the C3 clone.

It has been described that many cancer cells have aberrant steroid receptors (KRSTIC 1997). In order to better understand the different effects that glucocorticoid hormones have on the growth of these cell lines, we decided to analyze the biochemical characteristics of GR. To characterize further the glucocorticoid receptor in both clones of mouse melanoma cells, ion exchange chromatography on DEAESephadex A-50 minicolumn was performed. Activated receptor obtained by in vitro incubation at 25°C can be usually eluted, from this column, with 0.2 M KCl, whereas unactivated receptor appears as 0.4 M form (MARKOVIC et al. 1980; VUJIC et al. 1995). The elution profiles of binding [3H]TA in B16/F10 and B16/C3 cell cytosols are shown in Fig. 4. The peak radioactivity was eluted with 0.25 M KCl showing that a specific glucocorticoid receptor in mouse melanoma cells corresponds to an activated form of glucocorticoid receptor even when the receptor was incubated at 4°C. These findings demonstrated that receptor in these two melanoma clones is, perhaps, in already activated form, implying that it is biochemically different from usually observed receptor forms in normal cells.

**Discussion**

The main aim of our experiments was to determine whether glucocorticoid hormone application affects mouse melanoma cell growth in culture. Previous reports (Djordjevic-Markovic 1990; Kanazir 1990) demonstrated the importance of the complex-

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>Kd (nM)</th>
<th>N° binding sites/cell</th>
<th>protein conc. (mg/10⁸ cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16/F10</td>
<td>1.21 ± 0.49</td>
<td>2778 ± 422.5</td>
<td>4.72</td>
</tr>
<tr>
<td>B16/C3</td>
<td>1.53 ± 0.11</td>
<td>1079 ± 96.5</td>
<td>5.96</td>
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GLUCOCORTICOID RECEPTOR IN MOUSE MELANOMA CELLS

...ity of GR structure and its implications to both glucocorticoid and antiglucocorticoid effects. In view of those findings it was of importance to investigate the effects and presence of GR in melanoma cell system.

The controversy in the literature data concerning the role of glucocorticoids in melanoma cell growth is at least partly due to different model systems examined as well as varying experimental conditions (WALKER 1988). In this study the effects of glucocorticoids on B16 mouse melanoma cell growth were studied. B16/F10 mouse melanoma cell growth was inhibited after glucocorticoid (TA) treatments. After glucocorticoid treatment B16/C3 mouse melanoma cells reached a maximal value 24-48 hours earlier compared to the untreated C3 control (Fig. 1B).

It was previously reported that both glucocorticoid and antiglucocorticoid hormones modulate the mouse melanoma cell growth (RISTIC et al. 1995). The effect of steroids on the mouse melanoma cell growth varied in individual melanoma cell clones (WALKER 1988). In this study, we showed that glucocorticoid hormones have different effects on the growth of these cells. The B16/F10 clone is more tumorigenic than the C3 clone and can cause metastasis into the lungs, whereas C3 does not (J.F. DORE, personal communication). The C3 clone produces melanin to a greater extent than the F10 clone (RISTIC et al. 1990; J.F.DORE, personal communication). It is possible that these differences in the growth curves caused by glucocorticoid hormones originate from different number of GR molecules, or different affinities for hormone, or changed characteristics of the receptor in malignant cells. We therefore investigated these parameters.

The presence of GR was studied through its ability to bind tritiated TA according to the method by SCATCHARD (1949) and through recognition by the antiGR specific rodent monoclonal antibodies (BuGR; GAMETCHU and HARRISON 1984). The results obtained by the Scatchard analysis showed that both cell clones contained specific GR but in different numbers. B16/F10 mouse melanoma cells have three times higher number of binding sites – 3200 bd.sites/cell, resembling lymphocyte cells, while the other cell clone (B16/C3) has 1000 binding sites per cell, similar to the GR number reported for different brain structures (GUSTAFSSON et al. 1987). The dot blot analysis, using monoclonal antirodent-GR antibodies BuGR, showed the presence of specific GR in both cell clones, and data obtained confirmed the result of the Scatchard analysis. It is evident that B16/F10 clone has GR and compared to C3 clone the number of receptor binding sites is approximately three times higher. This finding is consistent with the results of a study reporting that experimental melanoma contained significant amounts of GR (BHAKOO et al., 1981).

Similar Kd values were observed in both clones. The range of Kd values was the same as previously reported for various typical GR target tissues like liver, thymus, fibroblasts, lymphocytes etc. (BALLARD et al. 1974).

These results suggest that different effects of glucocorticoid hormones on the growth of F10 and C3...
clones do not originate from different affinities for hormones, since these parameters do not vary significantly between two clones. However, F10 cells have a three times higher number of receptors and that may be the cause of a stronger inhibitory effect of glucocorticoid hormones on these cells. In fact, it is possible that glucocorticoid hormones will trigger programmed cell death in the F10 clone to a greater extent than in the C3 clone, which could be important for potential therapeutic applications in human melanomas. Present experiments which are in progress will test this hypothesis.

In order to study the properties of the GR in greater detail, we analyzed the GR from these two different clones by performing ion exchange chromatography. Cytosol was isolated from these cells and incubated with radiolabeled synthetic glucocorticoid TA at 4 °C and 25 °C. Previous results have shown that unactivated hormone-receptor complex formed at 4 °C under these conditions elutes at 0.4 M salt, whereas activated form obtained by incubation at 25 °C elutes as 0.2 M peak (MARKOVIC and LITWACK 1980). Using potent synthetic glucocorticoid (triamcinolone acetonide, TA), we have shown a specific [3H]TA-receptor complex eluted by 0.25 M KCl from ion exchange chromatography column in B16/F10 mouse melanoma cell cytosol, as well as in B16/C3 mouse melanoma cell cytosol. The receptor complex was formed by incubation on 4 °C. However, the results showed an activated form of receptor-hormone complex corresponding to the elution profile obtained from rat liver and rat kidney cytosol (MARKOVIC et al. 1980). After heat activation the same profiles were obtained (results are not shown) in both cell clones.

Our results suggest that in both clones the receptor is activated even when cytosol is incubated with the hormone at 4 °C and that maybe the sign of receptor aberrant function. In fact, abnormal forms of steroid receptors have been often detected in cancer cells (KRSTIC 1997). For example, GR was found to be already activated before in vitro activation in certain control and cancer tissues in kidney malignancies (DJORDJEVIC-MARKOVIC 1990). Furthermore, estrogen and progesterone receptors with an increased affinity to bind to DNA cellulose were observed in uterine endometrial carcinomas (RIBARAC-STEPIC 1985). Finally, a series of mutant or aberrantly spliced variants of estrogen receptor have been described for the estrogen receptor gene in human breast cancer (CIOCCA and FANELLI 1997). These changes in steroid receptor structures can lead to more or less active receptors or forms that function aberrantly, such as a dominant negative version of these proteins. Our results suggest that in malignant melanoma GR is already activated in both types of cells. It is possible that this change in activation properties of receptor is due to mutation in the receptor gene or in some of the genes encoding receptor associated proteins. Future experiments will determine the precise nature of this molecular change.

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This paper is dedicated to the memory of Professor Radmila Djordjevic-Markovic. The B16/C3 clone was generously provided by Dr. Aleksandar Dujic, Military Medical Academy, Belgrade, Yugoslavia, whereas the B16/F10 clone was provided by Dr. J.F. Dore, Lyon, France.

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BOOK REVIEW

THE IGF SYSTEM, MOLECULAR BIOLOGY, PHYSIOLOGY,
AND CLINICAL APPLICATIONS

Edited by Ron G. Rosenfeld and Charles T. Roberts (Oregon Health Sciences University, Portland), 787 pages, Humana Press

More than 40 yr elapsed from the finding of “sulfation factor” by Salmon and Daughaday which has been later “transformed” into insulin-like growth factors, the purification and sequencing of which has been accomplished about 20 yr later. The book presented by Ron G. Rosenfeld and Charles T. Roberts and edited by Humana Press may be considered a true encyclopaedia on the current status of art in this rapidly developing field.

The book is divided into four main sections: I. Molecular biology of the IGF system, II. Biological actions of the IGFs, III. IGF physiology, IV. Clinical aspects of the IGFs, each sections containing several chapters written by a total of 74 well known experts in this field. Expert editorial work apparently resulted in handy organisation of the text of each chapter which follows the unified hierarchy of titles and subtitles. In addition, each chapter contains the Introduction and Summary and even the appropriate cross-references to the other chapters. The average number of about 150 references included into each of 31 chapters makes a total of about 5000 references which go up to 1997.

Several instructive and carefully selected figures and tables bring convincing evidence on the main facts and findings described in the text.

Undoubtedly, this valuable book will be useful not only for those dealing with various aspects of basic research in this and related fields, but also for those teaching several premedical disciplines. However, several clinicians including endocrinologists, pediatricians, gynecologists will find unique and valid information to enlarge the field of their theoretical basis as well as their diagnostic and therapeutic skills.

Pavel Langer