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Growth inhibition of the androgen responsive DDT_1MF-2 cell line by glucocorticoids: the role of ornithine decarboxylase

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While testosterone (T) stimulates the growth of DDT_1MF-2 cells, glucocorticoids arrest the growth of these cells in the G_0/G_1 stage of the cycle. Ornithine decarboxylase (ODC), the first and rate-limiting enzyme in the polyamine biosynthetic pathway, is highly sensitive both to growth and inhibitory stimuli. To assess the mechanism of glucocorticoid inhibition of cell growth, the effect of triamcinolone acetonide (TA) on growth and ODC was studied. DDT_1-MF-2 cell growth was inhibited by TA and difluoromethyl ornithine (DFMO), an irreversible inhibitor of ODC. TA (10 nM) inhibited the ODC activity to 10% of the control levels by 12 h and inhibition was maintained at all later intervals studied. Ten μM DFMO inhibited ODC activity to a maximum of 50% of control. The concentration of ODC mRNA was maximally decreased at 15 h after TA administration.

Though TA and DFMO inhibited cell growth and ODC activity in DDT_1-MF2 cells, growth inhibition by DFMO, but not by TA, was overcome by the addition of putrescine, the product of ODC reaction. Thus, inhibition of ODC is one pathway through which glucocorticoids inhibit DDT_1MF-2 cell growth. ODC inhibition, however, is not the only pathway through which glucocorticoids act.

Keywords: glucocorticoid; ornithine decarboxylase; growth control; androgen

Introduction

Tumours of the male reproductive tract frequently exhibit androgen stimulated growth. Castration (either through surgical or chemical modalities) has been used as one therapeutic approach to the inhibition of tumor cell growth. Androgen ablation therapy, although initially effective in the inhibition of androgen-responsive growth, often leads from androgen-responsive tumor growth to a state of hormonal non-responsiveness. Our laboratory has used a steroid-responsive cell line derived from the male reproductive tract to study growth control mechanisms. Our recent studies suggest that, in the absence of androgens, cells may have the plasticity to seek alternative growth stimuli, eventually resulting in the loss of hormone responsiveness (Smith *et al.*, 1991). Therefore, the identification of a central growth inhibitory pathway which blocks the response of the cell to all stimuli would provide a powerful approach to the control of tumor cell growth.

The DDT_1-MF2 smooth muscle cell line was cloned from leiomyosarcoma of the hamster ductus deferens (Norris & Kohler, 1976). These cells contain both glucocorticoid and androgen receptors and their proliferation is differentially regulated by androgens and glucocorticoids (Smith *et al.*, 1984; Syms *et al.*, 1983, 1984). Androgens stimulate while glucocorticoids inhibit DDT_1MF-2 growth (Smith *et al.*, 1984). This cell line has provided an excellent model system for the *in vitro* study of androgen-dependent tumors.

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Glucocorticoid treatment blocks the response of DDT_1-MF-2 cells to a variety of growth stimuli (including androgens, agents which increased intracellular cAMP levels and numerous growth factors) (Smith *et al.*, 1991) and arrests the cells in the G_0/G_1 phase of the cell cycle (Syms *et al.*, 1984). Genes known to be active during this phase of the cell cycle are likely to be regulated by glucocorticoids. The present study evaluates the effect of glucocorticoids on the enzyme ornithine decarboxylase.

The polyamines putrescine (a diamine), spermidine and spermine are present in significant quantities in all cells and are essential for normal cell growth and differentiation. Ornithine decarboxylase (ODC, EC 4.1.1.17), the first and rate-limiting enzyme in polyamine biosynthesis, catalyzes the removal of a carboxyl group from ornithine to yield putrescine. Very small amounts of ODC are present in quiescent cells and its activity is increased many fold by exposure to external growth stimuli. Significantly, ODC is not only sensitive to anabolic stimuli, but also shows a tissue-specific negative response to hormonal stimuli.

Another enzyme involved in polyamine biosynthesis in cells, S-adenosyl-L-methionine decarboxylase (AdoMetDC, EC 4.1.1.50), decarboxylates S-adenosyl-L-methionine which serves as an aminopropyl donor in the conversion of putrescine to spermidine and spermidine to spermine. The actual transfer of the aminopropyl moiety is catalyzed by two distinct enzymes, spermidine synthase and spermine synthase. Because polyamine biosynthesis is required for cell proliferation, inhibition of ODC by specific chemical inhibitors, such as alpha difluoromethyl ornithine (DFMO), suppresses cell growth.

In order to further delineate the mechanism of glucocorticoid action, we have investigated the correlation between inhibition of cell growth by glucocorticoids and ODC activity in the DDT_1MF-2 cell line.

Results

Inhibition of DDT_1MF-2 cell growth by TA

TA inhibited cell growth in serum-free (Figure 1) and serum-containing medium in a dose dependent manner. The TA dose required for significant effect was 0.1 nM with maximum inhibition observed at a dose 1.0 nM in serum-free medium.

ODC activity of DDT_1MF-2 cells is inhibited by TA

ODC activity was measured at different intervals after the cells were treated with 10 nM TA in serum-free medium (Figure 2a). The enzyme activity of the control cells increased following the medium change and reached a plateau by 6 h. The control activity remained at this level for up to 24 h after fresh medium change and declined slightly thereafter. The inhibition of ODC by TA was significant by 8 h after treatment and was almost maximum by 12 h (15% of control). The extent of inhibition remained at that point or slightly lower at all later intervals studied. Interestingly, the ODC activity of TA treated cells was higher than that of control at 4 and 6 h after addition of TA. Because this increase could

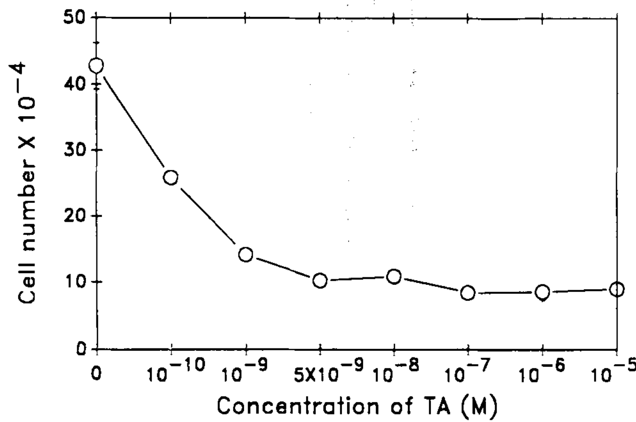

Figure 1 Inhibition of cell growth by TA in serum free medium: The cells were plated in 6 well plates (7000 cells/well) in DME/F12/ITS+, 10 nM T containing 0.1% FBS to aid attachment. Next day they received fresh DME/F12/ITS+ with or without different concentrations of TA ranging from 0.1 nM to 10 μM. The cells were allowed to grow for 7 days. The medium was replaced with fresh medium supplemented with same concentrations of TA every other day. The cells were then trypsinized and counted. Results are expressed as the mean ± standard deviation for triplicate samples. Errors bars for some points were smaller than the symbol size

have been a consequence of fresh medium supplementation to these cells in addition to an effect of TA, 10 nM TA was added to the cells 15 h after a medium change and the ODC activity was measured (Figure 2b). However, at 4–6 h after TA administration, a similar increase in activity of glucocorticoid treated cells was observed. Thus, TA exhibited a biphasic effect on the ODC activity of DDT_1MF-2 cells.

The effect of TA on the enzyme activity was dose-dependent. The TA dose required for an inhibitory effect was 1.0 nM and that required for maximum effect was 10 nM (Figure 2c).

Inhibition of ODC activity in DDT_1MF-2 cells by DFMO

DFMO is a specific inhibitor of ODC. ODC activity was inhibited by DFMO (10 μM) as early as 6 h after addition (50% of the control) and remained at that level at the time points studied (Figure 3a). Like TA, DFMO inhibition of ODC was dose dependent. The inhibition was apparent with 10 μM and maximal at 1 mM (Figure 3b). The inhibition of growth paralleled the inhibition of enzyme activity with an EC_{50} of about 0.1 μM (Figure 3b and c).

Can DFMO mimic the TA effect on cell growth?

If TA regulation of ODC is a central factor in growth inhibition, then inhibition of this pathway by an inhibitor of ODC would be expected to mimic glucocorticoid action *in vitro*. Treatment of DDT_1MF-2 cells with DFMO under serum-free conditions for the prolonged time period (7 days) required for the growth study resulted in toxic effect. Therefore, this experiment was conducted in presence of serum (DME + 2% FBS) (Figure 4). Treatment of the DDT_1MF-2 cells with DFMO resulted in a decrease in cell proliferation. No inhibitory effect was observed at a dose of 10 nM. Cell growth was inhibited at 10 μM concentration and the inhibition was maximum at 0.1 mM concentration. No toxic effect was observed even at a dose as high as 10 mM.

Putrescine can overcome the growth inhibition by DFMO but not by TA

The addition of putrescine, the product of the ODC reaction, to the cultures might be expected to overcome the TA block

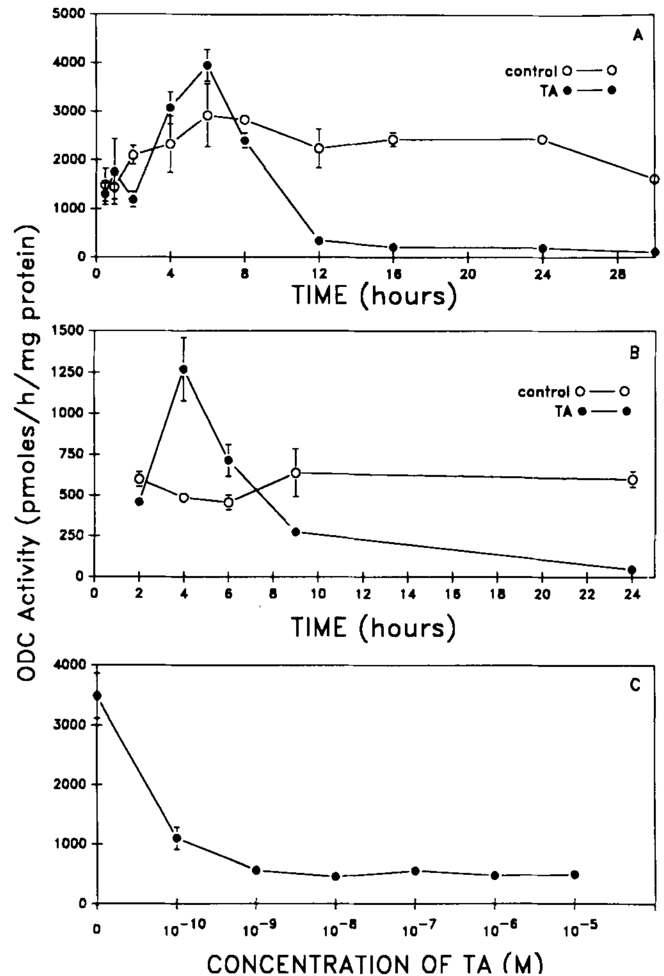

Figure 2 Inhibition of ODC activity by TA and DFMO: (**a**) As described in methods, the cells were plated in DME/F12/ITS+ containing 0.1% FBS. On the third day they received DME/F12/ITS+ without serum with or without 10 nM TA. The cells were collected at various time points and the enzyme activity measured as described in the methods. Results are expressed as the mean ± standard deviation for triplicate samples. Errors bars for some points were smaller than the symbol size. Statistical significance was assessed by a two-way analysis of variance and the Student-Newman-Keuls test. The ODC activity for the TA treated group was different from the control values at 4, 6, 12, 15, 24 and 30 h after treatment ($P \leqslant 0.05$). (**b**) The cells were plated as described in (**a**). On the third day the medium was changed to DME/F12/ITS+ without TA. Half of the dishes received 10 nM TA 15 h later. The cells were collected at different time intervals and the ODC activity measured. Results are expressed as the mean ± standard deviation for triplicate samples. Errors bars for some points were smaller than the symbol size. Statistical significance was assessed by a two-way analysis of variance and the Student-Newman-Keuls test. The ODC activity for the TA treated group was different from the control values at 4, 6, 9, and 24 h after treatment ($P \leqslant 0.05$). (**c**) Dose response of TA: The cells were plated as described in (**a**). On the third day the medium was replaced with DME/F12/ITS+ with different concentrations of TA. The cells were collected 24 h later and the enzyme activity assayed. Results are expressed as the mean ± standard deviation for triplicate samples. Errors bars for some points were smaller than the symbol size

of DDT_1MF-2 cell proliferation. DDT_1MF-2 cells were cultured in the presence of 10 nM TA and increasing concentrations of putrescine. Putrescine did not overcome the growth inhibitory effect of TA (Figure 5). Similarly spermine and spermidine did not overcome the growth inhibition by TA (not shown). In the same experiment, 10 μM concentration of putrescine was sufficient to overcome the inhibitory effect of 0.1 mM DFMO.

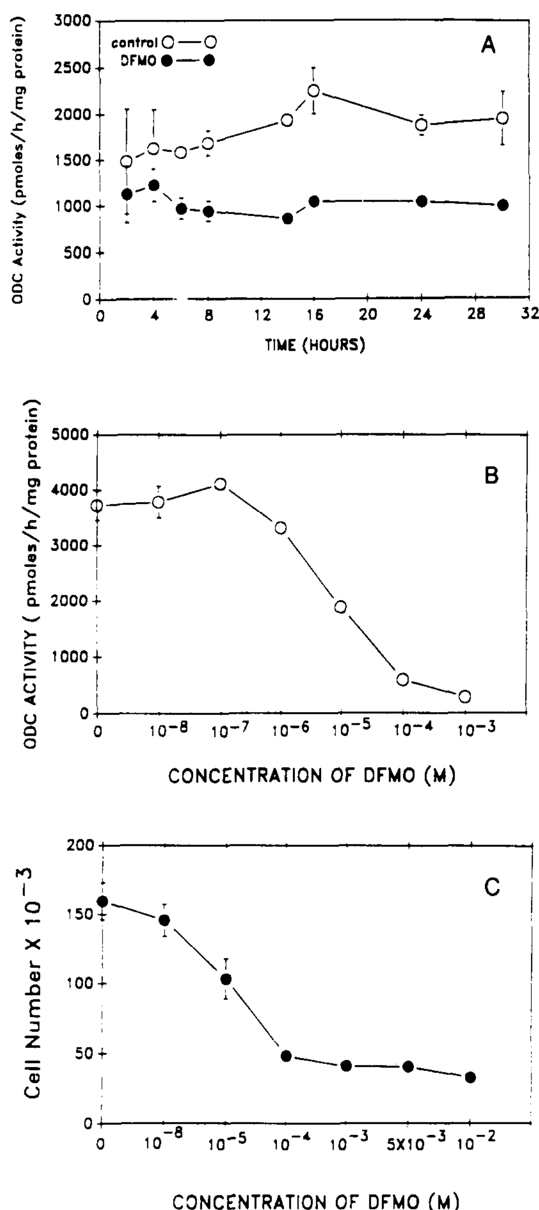

Figure 3 DFMO inhibition of ODC activity: (**a**) Time course: The cells were plated as described in methods. On the third day the medium was replaced with fresh DME/F12/ITS+ with or without 10 nM DFMO and the cells collected at different time points. The ODC activity measured as described in methods. (**b**) Dose response: The experimental conditions were same as described in Figure 2c, except that the cells received fresh medium with different concentrations of DFMO on the third day and were collected 6 h later. (**c**) Cell growth dose response: The experimental conditions were as described in (**a**), except that the concentration of DFMO was tested over a range of 10 nM to 100 mM

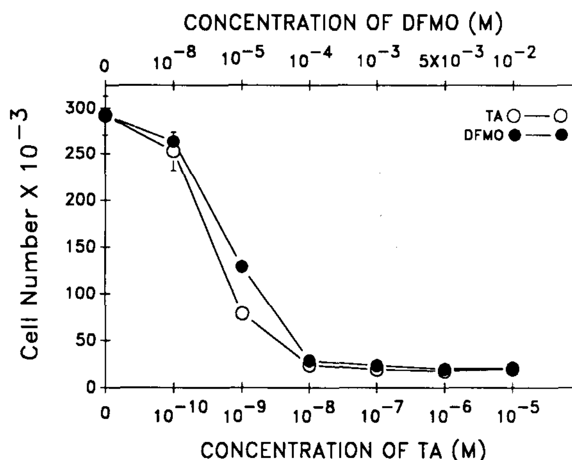

Figure 4 Can DFMO mimic the effect of TA on DDT_1-MF2 cell growth?: The growth study was basically carried out as described in Figure 1. The cells were plated in DME+2% FBS+10 nM T. Next day the medium was replaced with fresh DME+2% FBS+10 nM T containing different concentrations of TA and DFMO. The cells were grown for 7 days with medium change every other day. Results are expressed as the mean ± standard deviation for triplicate samples. Errors bars for some points were smaller than the symbol size

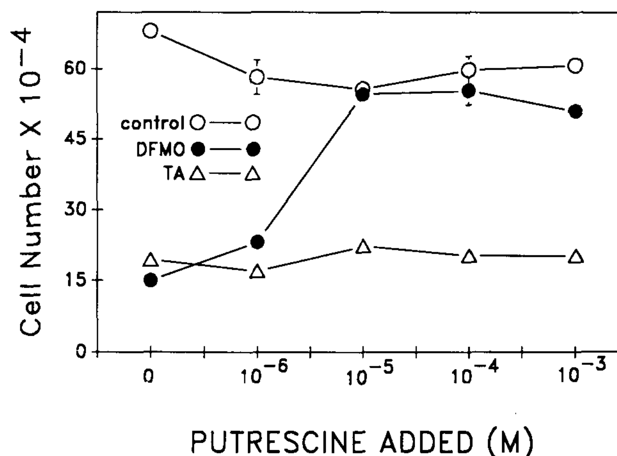

Figure 5 Putrescine can overcome the DDT_1MF-2 cell growth inhibition by DFMO, but not by TA: The cells were plated as described in Figure 5. On the second day they received fresh DME+2% FBS with different concentrations of putrescine ranging from 1 μM to 1 mM in combination with (**a**) nothing (**b**) 0.1 mM DFMO or (**c**) 10 nM TA. The cells were grown for 7 days before counting, with medium change every other day. Results are expressed as the mean ± standard deviation for triplicate samples. Errors bars for some points were smaller than the symbol size

Glucocorticoid receptor dependence of TA inhibition of growth

Syms *et al.* (1987) have isolated a glucocorticoid resistant variant of DDT_1MF-2 cell line (DDT_1MF-2-GR) which has very few glucocorticoid receptors. Treatment of the DDT_1-MF-2-GR cells with TA did not block cell proliferation, and there was little effect on ODC activity. In fact, glucocorticoids stimulated DDT_1MF-2-GR cell growth about two-fold, a phenomenon previously observed by Syms *et al.* (1987). DDT_1MF-2-GR cell growth and ODC activity could be inhibited by treatment with DFMO at concentrations similar to that required by the DDT_1MF-2 cells (Figures 6 and 7). Accordingly, the inhibitory effect of glucocorticoids on cell proliferation and ODC activity was dependent on a functional glucocorticoid receptor.

Effect of TA on the AdoMetDC activity

In contrast to the reduction of ODC activity after treatment with DFMO, AdoMetDC activity is increased (Mamont *et al.*, 1982). Since the effect of TA on the growth of DDT_1-MF2 cells was reflected in the inhibition of ODC activity, we examined the influence of TA on the AdoMetDC activity of DDT_1MF-2 cells (Figure 8). In contrast to the stimulation observed after treatment with DFMO, the enzyme activity was inhibited by 10 nM TA, from 4 h throughout the duration of the study. The AdoMetDC activity of the control was very low compared to its ODC activity and did not increase significantly after medium change.

Discussion

Glucocorticoids act on various cell types to produce anabolic or catabolic effects (Baxter and Ivarie, 1978). Several cells

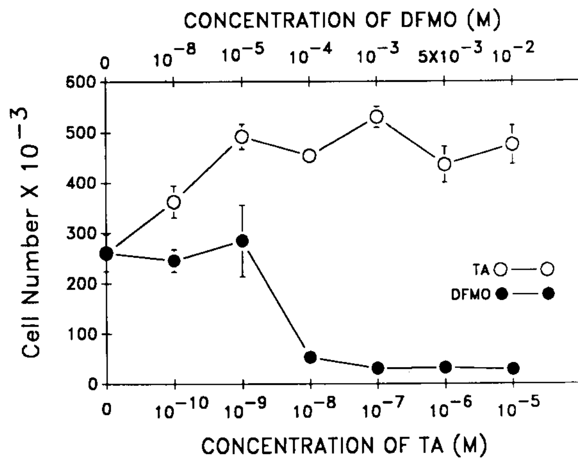

Figure 6 Glucocorticoid receptor dependence of TA inhibition of growth: The DDT_1MF-2-GR cells were plated in DME+2% FBS in 6 well plates (7000 cells/well). The growth study was carried out exactly as described for DDT_1MF-2 cells in Figure 5

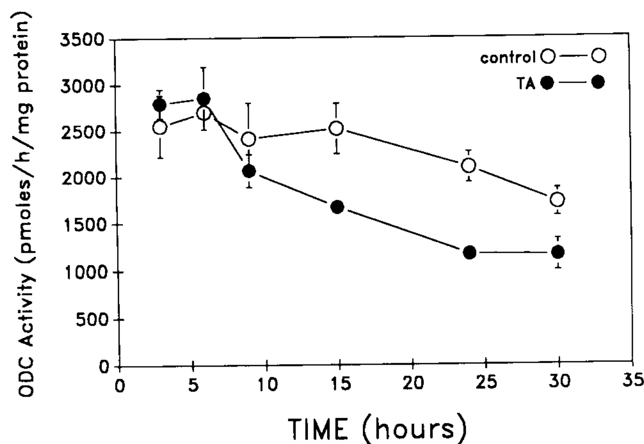

Figure 7 Effect of TA on ODC activity of DDT_1MF-2-GR cells: The experimental conditions were precisely as described in Figure 2a for DDT_1MF-2 cells. Statistical significance was assessed by a two-way analysis of variance and the Student-Newman-Keuls test. The ODC activity for the TA treated group was different from the control values at 15, 24 and 30 h after treatment ($P \leq 0.05$)

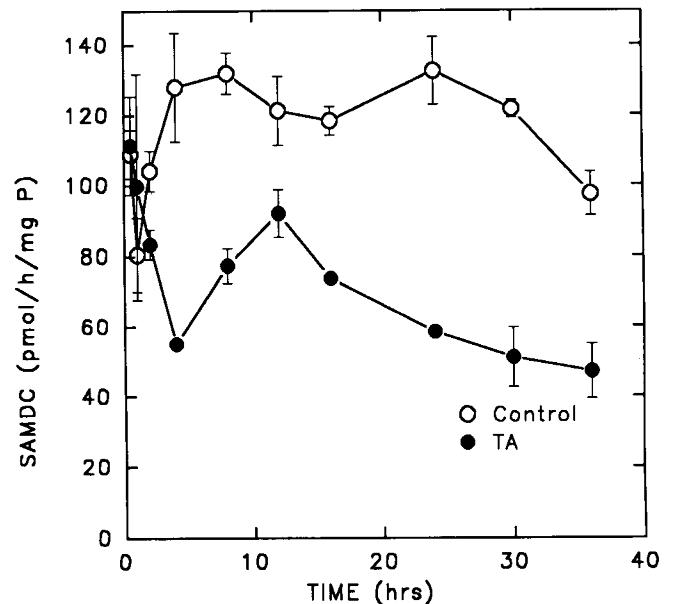

Figure 8 Inhibition of AdoMetDC activity of DDT_1-MF2 cells by TA: The experimental conditions were same as described in Figure 2a. The AdoMetDC (s-adenylmethionine decarboxylase; SAMDC) activity was measured as described in methods. Statistical significance was assessed by two-way analysis of variance and the Student-Newman-Keuls test. The ODC activity for the TA treated group was different from the control values at 2, 4, 8, 12, 16, 24, 30 and 36 h after treatment ($P \leq 0.05$)

exhibit growth inhibition in the presence of glucocorticoids, including DDT_1MF-2 cells, R3327-G8A1 cells, melanoma cells, mammary tumour cells, human salivary gland adenocarcinoma cells, the human T lymphoblastic leukemic cell line CCRF-CEM, rat hepatoma cells, the human fibrosarcoma cell line HT-1080, and mouse epidermal cells (Smith *et al.*, 1985; Walker *et al.*, 1986; Syms *et al.*, 1987; Hatakeyama *et al.*, 1988; Yuh and Thompson, 1989; Gomi *et al.*, 1990; Spydevold *et al.*, 1990; Webster *et al.*, 1990). In general, the cytostatic action of glucocorticoids blocks cell proliferation at the G_1 phase of the cell cycle. However, inhibition is not clearly understood. There are several glucocorticoid sensitive leukemic cell lines (both rodent and human) which, in addition to growth arrest, undergo lymphocytolysis after glucocorticoid administration *in vitro* (Munck *et al.*, 1972; Sibley and Tomkins, 1974). In these cell lines, it has been suggested that the action of glucocorticoids is biphasic; the first action, which is cytostatic, permits the second, cytolytic action (Bitner and Wielckens, 1988).

The synthesis of polyamines, essential for normal cell growth and differentiation, is highly regulated in mammalian cells. Polyamine concentrations are higher in tumor cells than in normal cells. The effect of TA on ODC activity of DDT_1MF-2 cells was biphasic (Figure 2). The ODC activity was increased at 4–6 h after TA treatment and inhibited thereafter. The initial increase in activity was not due to the addition of fresh medium (Figure 2b) and is a phenomenon specific to glucocorticoids. A variety of stimuli result in an increase in ODC mRNA levels, in some cases due to an increase in transcription (Katz and Kahana, 1987), in other cases due to stabilization of the ODC mRNA (Rose-John *et al.*, 1987) resulting in increases in ODC mRNA accumulation (Kontula *et al.*, 1984).

TA markedly inhibited mRNA synthesis of ODC at 15 h (data not shown), although a decline in enzyme activity was observed earlier. Northern analysis of DDT_1MF-2 cells incubated in the presence or absence of TA revealed two ODC transcripts of 2.2 and 2.7 kb which are similar to the sizes reported in mouse kidney (Kontula *et al.*, 1984). In the mouse kidney, the size heterogeneity of the transcripts is due to differences at the 3′ termini and may reflect the synthesis of ODC from two similar ODC genes (Hickok *et al.*, 1986).

The rate of ODC degradation or turnover is another possible target for glucocorticoid regulation through stabilization of the enzyme, a process which requires protein synthesis (reviewed in Persson *et al.*, 1988). The enzyme has the fastest half-life of all known mammalian enzymes (Persson *et al.*, 1988) making it difficult to study the effect of protein synthesis inhibitors. When DDT_1MF-2 cells were treated with inhibitors such as cycloheximide or actinomycin D, there was no measurable ODC activity observed (data not shown).

Interestingly, a natural inhibitor of ODC, antizyme, has been described by Canellakis and colleagues (Fong *et al.*, 1976). It is theorized that antizyme inhibits ODC activity and also accelerates its degradation. The protein has been purified (approximate molecular weight of 26 000), antibodies produced, and the antizyme gene cloned (reviewed in Hayashi *et al.*, 1988). The development of inhibitors that specifically interfere with enzymes of the polyamine biosynthetic pathway has made it possible to deplete polyamines in cells resulting in reduced cell growth and proliferation. DFMO, a specific and irreversible inhibitor of ODC, has been utilized

for cancer treatment. The mechanism of ODC inhibition by DFMO involves inactivation of the enzyme by forming a covalent bond with a residue at the active site (Metcalf *et al.*, 1978). Inhibition of the polyamine biosynthetic pathway by steroid antagonists has also been employed to inhibit tumour cell growth. Treatment of the MCF-7 cell line with tamoxifen inhibited the rise in ODC activity observed with the medium change, an effect which was fully reversible with the addition of high levels of estradiol. Like the glucocorticoid inhibition of growth and ODC activity of the DDT_1MF-2 cell line, addition of putrescine to the steroid treated cells did not overcome the anti-proliferative action of tamoxifen, despite the increase in intracellular polyamine levels (Cohen *et al.*, 1988). *In vitro* treatment of the estrogen responsive MCF-7 cell line with DFMO antagonized the estrogen induced increase in cell proliferation (Shui *et al.*, 1986; Kendra and Katzenellenbogen, 1987).

The induction of ODC expression has been considered to be a molecular marker for competence (the acquisition of the ability to respond to additional growth signals) (Soprano, 1994; Peña *et al.*, 1993). The ODC 5′ regulatory region contains an element that can be regulated by the interaction of the oncogenes, c-Myc and Max (Bello-Fernandez & Cleveland, 1992; Bello-Fernandez *et al.*, 1993; Peña *et al.*, 1993). In quiescent cells, c-Myc/Max regulates ODC gene expression. Of interest, reduction in the levels of c-Myc resulted in a prolongation of the prereplicative phase upon serum stimulation (Soprano, 1994). ODC has also been shown to mediate c-Myc induced apoptosis in myeloid cells after removal of interleukin-3. In this case, withdrawal resulted in a rapid loss of c-Myc and ODC expression, followed by the induction of apoptosis. The death rate could be inhibited by treatment with DFMO (Soprano, 1994). Nevertheless, this effect could not be overcome by the addition of putrescine (Soprano, 1994). This situation is similar to the studies described here and suggest that the inhibition of cell death or in our studies growth is not only due to depletion of the polyamine pools in the cell. Apoptosis could also be induced by constitutive expression of ODC and cytokine withdrawal (Soprano, 1994).

Since DFMO suppressed DDT_1MF-2 cell proliferation and this suppression was completely reversed by putrescine, the results suggest that ODC activity and putrescine are necessary for DDT_1MF-2 proliferation. Glucocorticoid administration inhibited ODC activity, as well as, cell growth; therefore, the ODC pathway participates in the response of these cells to glucocorticoids. Importantly, since putrescine administration did not fully overcome the TA block of cell proliferation, yet did reverse the inhibitory action of DFMO, the data further suggest that the anti-proliferative actions of glucocorticoid administration on the DDT_1MF-2 cell line involves mechanisms in addition to ODC inhibition (Soprano, 1994). The present studies demonstrate that the AdoMetDC activity was also inhibited in the TA treated cells (Figure 8). The inhibition of AdoMetDC preceded the inhibition of ODC activity. Thus, it is probably not surprising that the addition of putrescine could not overcome the inhibition of ODC activity on cell growth. The results do demonstrate that there is significant redundancy in the system. It has previously been observed that stimulation of proliferation by a variety of mechanisms such as phorbol esters, hormone receptors, growth factors, and oncogenes is counteracted by numerous inhibitory pathways (Krujer *et al.*, 1984; Rodriquez-Pena & Rozengurt, 1984).

Materials and methods

Materials

Triamcinolone acetonide (TA), putrescine, L-ornithine, S-adenosyl-L-methionine, dithiotreitol (DTT), pyridoxal phosphate, and Hyamine hydroxide were purchased from Sigma Chemical Company (St Lous, MO). Dulbecco's Minimum Essential Medium (DME), Ham's F-12 (F-12), fetal bovine serum (FBS), antibiotic solution (streptomycin sulphate and penicillin) and trypsin were obtained from Gibco (Grand Island, NY). ITS+ was purchased from Collaborative Research Inc (Bedford, MA). L-[1-^{14}C]-ornithine-hydrochloride and S-adenosyl-L-[carboxyl-^{14}C]methionine were purchased from Amersham (Arlington Heights, IL).

Maintenance of cell line

The DDT_1MF-2 cell line is available from American Type Culture Collection (Rockville, Maryland). The cells were maintained in DME + 2% FBS containing 10 nM testosterone (T). The glucocorticoid resistant DDT_1MF-2-GR cell line was derived from the parent DDT_1MF-2 cell line and maintained in DME + 2% FBS containing 10 nM T and 10 nM TA (Syms *et al.*, 1987).

Growth assays

The DDT_1MF-2 cells were plated in six well plates (7000 cells/well) in DME/F12/ITS+ (equal parts of DME and Ham's F12 containing 1% of ITS+ [5 μg/ml insulin, 5 μg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml BSA, 5.35 μg/ml linoleic acid] containing 0.1% FBS), and allowed to attach overnight. Next day the cells received fresh DME/F12/ITS+ without serum, and with or without different reagents. The cells received fresh medium with the reagents every other day and were cultured for 7 days. They were then trypsinized at different time intervals during the culture and counted using a Coulter counter. In the experiments where DFMO was used, the growth studies were carried out in DME containing 2% FBS, as DFMO was toxic to the cells in serum free medium.

Enzyme assays

For the enzyme assays, the cells were plated in 100 mm dishes (500 000 cells/dish) in DME/F12/ITS+ containing 0.1% FBS. After 48 h, the cells were treated with fresh DME/F12/ITS+ with or without various reagents. The cells were then collected at different time points, washed with phosphate buffered saline and resuspended in 1.0 ml of homogenizing buffer (10 mM Tris, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) pH 7.4). The cells were stored at −70°C for enzyme assays.

The cells were thawed, homogenized at 4°C with a sonicator cell disrupter (Branson Ultrasonic Corporation, Model 250/450 sonifier) using two pulses of 15 s each and centrifuged at 25 000 *g* for 30 min. The supernatant was assayed in duplicate for the enzyme activity. ODC activity was measured by the method of Seely and Pegg (1983). Briefly, the reaction mixture contained 5 μl of 20 mM L-ornithine, 0.4 μCi (10 μl) of L-[1-^{14}C]ornithine, 5 μl of 2 mM pyridoxal phosphate, 12.5 μl of 25 mM dithiothreitol, 2.5 μl of 0.5 M Tris-HCl (pH 7.5), and 215 μl of the cell homogenate. The mixture was incubated at 37°C for 1 h in glass tubes fitted with rubber stoppers and center wells (Kontes, NJ) containing filter paper (Whatman No. 3, 2.5 cm) presoaked in 40 μl of Hyamine hydroxide. The radioactivity absorbed in each filter paper was counted in 10 ml of Scinti Verse (Fisher Scientific, Pittsburgh, PA).

AdoMetDC activity was determined by the radioactivity of [^{14}C]-CO_2 released from S-adenosyl-L-[carboxy-^{14}C]methionine according to the method of Pegg and Poso (1983). The experimental procedure was similar to that of the ODC assay, except that the reaction mixture contained 25 μl of 0.05 M phosphate buffer, pH 7.5, 12.5 μl of 25 mM DTT, 50 μl of 15 mM putrescine dihydrochloride, 12.5 μl of 4 mM unlabeled S-adenosyl-L-methionine, 10 μl of S-adenosyl-L-[carboxy-^{14}C]methionine (0.2 μCi), and 140 μl of cell homogenate in a final volume of 250 μl.

Enzyme activities were expressed as pmoles of $^{14}CO_2$ released per hour per mg protein. The incubation period and the amount of substrate was calibrated for each enzyme. The protein content of the cell homogenate was measured using the method of Bradford (1976).

Statistical analysis

The date were analysed for significance by one way analysis of variance and Duncan's multiple range test. Two way analysis of variance and the Student-Newman-Keuls Test were used to determine significance when multiple variables were assessed.

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