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GROWTH INHIBITION OF CLONAL OSTEOSARCOMA CELL-LINES BY LOW CONCENTRATIONS OF GLUCOCORTICOID HORMONES

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Summary: Clonal osteosarcoma cell line, ROS 2/3, showed marked inhibition of [^{3}H]thymidine incorporation in response to low concentrations (10^{-10} M) of triamcinolone acetonide and dexamethasone. Hydrocortisone and corticosterone induced inhibition at somewhat higher concentrations. The osteosarcoma cell line ROS 17/2 responded similarly to triamcinolone acetonide and dexamethasone but at higher concentrations of the hormones. In ROS 2/3 the inhibitory effects of triamcinolone acetonide were accompanied by only slight elevation in the amount of intracellular exchangeable Ca^{2+}. In contrast, in primary cultures of normal rat-calvarian bone cells, [^{3}H]thymidine incorporation was inhibited to a much lesser extent only at higher concentrations of triamcinolone acetonide (10^{-7} M). The difference in the susceptibility of normal and malignant bone cells to the inhibitory effects of glucocorticoids may have potential therapeutic importance.

Osteosarcomas are malignant tumors of bones. Cultured osteosarcoma cells have recently been shown to contain cytosolic receptors for glucocorticoid hormones (1). These hormones were also found to cause some ultrastructural changes in cultured osteosarcoma cells, such as mitochondrial and nuclear enlargements (2, 3). To date, there are no reports concerning the effects of glucocorticoids on DNA synthesis in osteosarcoma cells. Thus the purpose of the present study was to investigate the effect of various glucocorticosteroid hormones upon the DNA synthesis of two established clonal cell lines of osteosarcoma. These cells retain their differentiated osteoblast-like functions such as production of alkaline phosphatase (4) and response to parathyroid hormone and calcitonin by enhanced cyclic AMP production (5). This study provides interesting data regarding the different

Abbreviations: TA, triamcinolone acetonide. Saline Hepes, 150 ml NaCl, 5 mM Hepes pH 7.4, 1 mM $MgCl_2$, 5 mM KCl and 1 mM $CaCl_2$.

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responses of normal and malignant bone cells to glucocorticoids. In comparison, we present data showing the lack of similar effects on primary cultures of normal bone cells prepared from fetal rat calvaria.

MATERIAL AND METHODS

Materials Triamcinolone acetonide, corticosterone, hydrocortisone, dexamethasone and progesterone were purchased from Sigma. [^{3}H]Thymidine (28 Ci/mmole) and [^{45}Ca]Cl_2 (10 mCi/mg) were purchased from Amersham.

Cell Culture. Clonal osteosarcoma cell lines, ROS 17/2 and ROS 2/3 derived from rat tumors, were generously provided by Dr. G. Rodan, University of Connecticut. The cells were cultured in BGJ-medium, Fitton-Jackson modification (Gibco formula) containing 10% fetal calf serum and antibiotics (200 U/ml penicillin, 200 µg/ml streptomycin, and 12 U/ml mycostatin). The cell lines were maintained in culture dishes (5 cm diameter, Falcon) at 37°C, in a humidifier 5% CO_2-air mixture incubator. The medium was changed every 3 days and the cells were subcultured every 6 days. For measurements of [^{3}H]thymidine incorporation and Ca^{2+} content, cells were subcultured on Tissue Culture Clusters of 24 wells (16 mm well diameter, Costar) 3 days before the experiment. The medium was changed to fresh medium with 10% serum 24 hrs before the measurements. Glucocorticoids were added directly to the culture medium 24 hrs before the measurements or as indicated. Primary cultures of bone cells were prepared from 19-20-day old fetal rats calvaria. The calvaria was removed, minced to small pieces and digested for 30 minutes in medium containing 0.25% trypsin (Difco) 8 g/l NaCl, 4 g/l KCl, 1 g/l glucose, 0.35 g/l $NaHCO_3$, 10^5 units/l penicillin, and 0.1 mg/l streptomycin, at 37°C in CO_2 incubator. The large pieces were removed by short centrifugation and the cells were precipitated by centrifugation for 10 min at 3500 rpm. The cells were resuspensed in BGJ medium - Fitton-Jackson modification (Gibco formula), containing 10% fetal calf serum (Gibco) and antibiotics as above. The cells were plated at $5 \cdot 10^4$ cells/cm^2 in Tissue Culture Clusters of 24 wells (16 mm well diameter, Costar) and kept in a humidified 5% CO_2-air mixture incubator at 37°C. The experiments were done on 4-day cultures. 24 hours before the experiment the medium of the cultures was replaced by fresh medium with 10% serum to induce reinitiation of DNA synthesis. Glucocorticoids were added to the culture medium 24 hrs before the measurements.

[^{3}H]Thymidine incorporation. For measuring [^{3}H]thymidine incorporation, cells were pulsed with [^{3}H]thymidine (0.5 µCi/ml) for 30 min. [^{3}H]Thymidine was added directly to the culture medium. The incorporation was terminated by washing the cells in the wells 3 times with cold saline-Hepes and addition of 0.5 ml 10% cold TCA. The wells were scraped with a rubber spatule, and the cells were collected with 3 ml 10% cold TCA and centrifuged at 3000 RPM for 10 min. The pellets were dissolved in 0.1 N NaOH, and the radioactivity determined in a liquid scintillation counter in 10 ml of Hydro-luma (Lumac) scintillation fluid.

Cell number was determined by incubating triplicate wells with 0.25% trypsin in medium (Gibco formula) and counting the cells in a haemocytometer. The variation between the number of the cells in a triplicate seldom exceeded 10%.

Cellular Ca^{2+} content. To determine the effect of hormones on the cellular content of exchangeable Ca^{2+}, cultures were preincubated with ^{45}Ca (0.5 μCi/ml) which was added directly to the culture medium 24 hours before the experiment. In previous work we have shown (6) that after 24 hrs of preincubation, equilibrium was established between the extracellular and intracellular ^{45}Ca. Under these conditions, changes in the cell-associated radioactivity, induced by the hormones, provide a measure of changes in cellular Ca^{2+} content rather than changes in the rates of Ca^{2+} transport. Glucocorticoid hormones were added to the cultures 24 hrs before the termination of the experiments or as indicated. The experiments were terminated by aspirating the medium and washing the cells in the wells 3 times with 2 ml volumes of cold (0 °C) saline Hepes solution. The washing of each well required 6 seconds. The cells were solubilized by 0.1 N NaOH and their radioactivity determined in a liquid scintillation counter after the addition of 10 ml of Hydro-luma (Lumac) scintillation fluid.

RESULTS

Twenty-four hrs of preincubation with triamcinolone acetonide (TA), a synthetic analogue of high potency, inhibited the incorporation of [^{3}H]thymidine into ROS 2/3 osteosarcoma cells. At a concentration as low as 10^{-10} M TA caused inhibition of 70%, whereas at 10^{-8} M the inhibition was 82%. Parallel studies in normal bone-cell cultures revealed that [^{3}H]thymidine incorporation was inhibited only by 30% at a relatively high concentration of the hormone (10^{-6} M).

A significant inhibitory effect of TA (10^{-7} M) on [^{3}H]thymidine incorporation into RSO 2/3 osteosarcoma cells could be observed already after a lag period of 1 hr. The degree of inhibition increased with time reaching a level of 60% at 5 hrs and 80% at 24 hrs (Fig. 2).

The structural-activity relationship of various steroids on [^{3}H]thymidine incorporation into ROS 2/3 cells is shown in Fig. 3. Dexamethasone, another fluorinated synthetic glucocorticoid, was less active than TA. Hydrocortisone was slightly less active than dexamethasone. Corticosterone, the native glucocorticoid of rodents, showed inhibitory effects only at higher concentrations (10^{-7} M), and progesterone, a non-glucocorticoid steroid hormone, had no effect.

For comparison, we examined the effect of the two potent glucocorticoids: TA and dexamethasone, on an additional clonal osteosarcoma

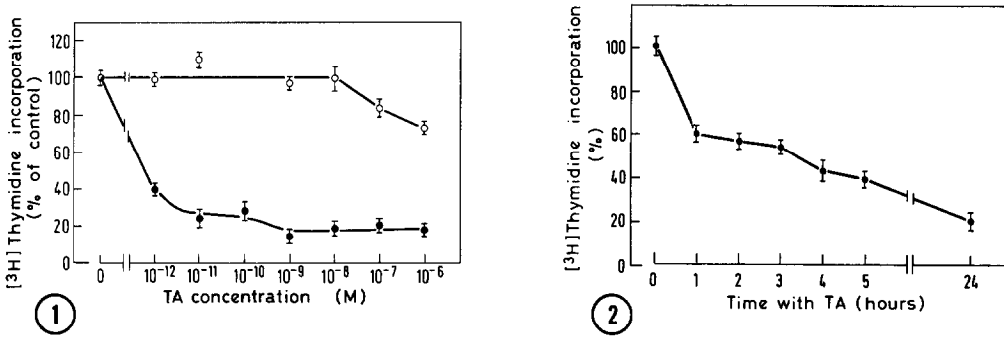

Figure 1. The effect of TA on the inhibition of [^{3}H]thymidine incorporation into ROS 2/3 osteosarcoma cell line and primary cultures of bone cells.

TA was added to the culture medium 24 hrs before the initiation of [^{3}H]thymidine - 30 min. pulse. Results represent mean ± S.E. of 4 replicate measurements.

● - ROS 2/3 osteosarcoma cell cultures

0 - normal bone cell cultures

Figure 2. The time course of the effect of TA on [^{3}H]thymidine incorporation into ROS 2/3 cells.

TA (10^{-7} M) was added to the cultures at the indicated times before the initiation of the [^{3}H]thymidine - 30 min. pulse. Results represent mean ± S.E. of 4 replicate measurements.

cell line - ROS 17/2. The inhibitory effects of the hormones on DNA synthesis were similar to those in ROS 2/3, but the inhibition occurred at higher concentrations of the hormones (Fig. 4). Progesterone was without effect. Of interest may be the finding that at very low concentrations (10^{-11} M) the hormones induced a stimulatory effect (10 - 30%) on DNA synthesis.

Calcium ions have been recently suggested as mediators for the action of a number of hormones, including glucocorticoids (7 - 9). A recent work from our laboratory has shown that TA induced changes in the intracellular content, fluxes, and cellular distribution of Ca^{2+} in primary cultures of bone cells (10). It was, therefore, of interest to elucidate whether the inhibitory effects of glucocorticoids in RSO 2/3 were accompanied by changes in the content of cellular exchangeable Ca^{2+}. Fig. 5a shows that in TA-treated ROS 2/3 cells the amount of exchangeable cellular Ca^{2+} was only slightly higher than that in the controls. The increase became apparent 1 hour after the

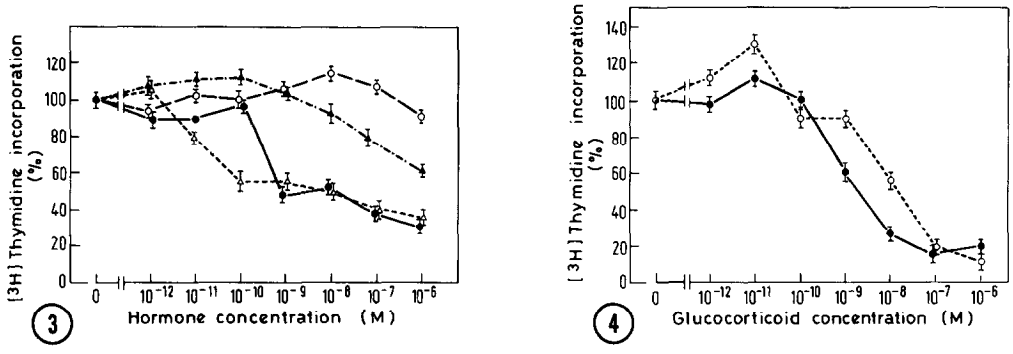

Figure 3. Structure-activity relationship of several steroid hormones on the inhibition of [^{3}H]thymidine incorporation into RSO 2/3 cells. Results represent mean ± S.E. of 4 replicate measurements.
(Δ) - Dexamethasone
(●) - Hydrocortisone
(▲) - Corticosterone
(O) - Progesterone
Hormones were added to the cultures 24 hours before the measurement.

Figure 4. The effect of TA and dexamethasone on [^{3}H]Thymidine incorporation into line ROS 17/2 of osteosarcoma cells.
Hormones were added 24 hours before the measurement. Results represent mean ± S.E. of 4 replicate measurements.
(●) - TA
(O) - Dexamethasone

application of the hormones and persisted for 5 hrs. At the time of maximal inhibition of DNA synthesis (24 hrs) the amounts of exchangeable cellular Ca^{2+} were slightly lower than in the control, and were not affected by the concentration of the hormone.

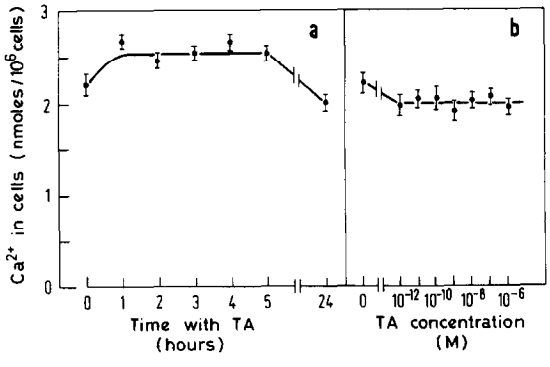

Figure 5. The effect of TA on the amount of intracellular exchangeable Ca^{2+} in ROS 2/3.
a. The time course of the effect. TA(10^{-7} M) was added at the indicated times before the measurement.
b. Dose-response curve. TA was added 24 hrs before the measurement.
Results represent mean ± S.E. of 4 replicate measurements.

DISCUSSION

Glucocorticoids reduce cell viability of normal lymphocytes and certain types of neoplastic lymphocytes (11) and are currently used in the therapy of some human lymphoid malignancies (12, 13). The effect on growth of other mammalian cell types appears to be complex; for example, growth of line-L929 of mouse fibroblasts is inhibited by corticosteroids (14) but the same hormones stimulate cell division in 3T3 cell line of mouse fibroblasts (15). The effect of glucocorticoids on growth of several tumors and normal tissues appears to vary considerably with the type of cells and the degree of differentiation (16). It appears unique, therefore, that osteosarcoma cells, but not normal bone cells, responded by growth inhibition to low concentrations of TA and dexamethasone. Receptors for corticosteroids were found in primary cultures of bone cells (17) as well as in osteosarcoma cell lines (1). Both types of cells responded similarly to the calcium regulating hormones, parathyroid hormone and calcitonin, by elevating the cyclic AMP levels (5, 18, 19). However, the effect of glucocorticoids on the inhibition of DNA synthesis and on cellular content of Ca^{2+} in the two types of cells were found to be different. In normal bone cells TA, at 10^{-7} M, induced an early increase in Ca^{2+} content (by 40-60%), followed by a subsequent decrease at 24 hrs (10) with only a mild effect on DNA synthesis. The absence of DNA synthesis inhibition by hydrocortisone im primary cultures of bone cells was also previously reported (20). In osteosarcoma cells only slight changes in Ca^{2+} content were observed, but the inhibition of DNA synthesis was marked at low concentrations of the hormones. These results suggest that in osteosarcoma, the glucocorticoid-induced growth inhibition is not mediated by changes in cellular Ca^{2+} content. The differences in the response to the growth inhibitory effects of glucocorticoids, between normal and malignant bone cells, may have potential therapeutic importance.

ACKNOWLEDGEMENTS

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