Dose-responsive Growth Inhibition by Glucocorticoid and Its Receptors in Mouse Teratocarcinoma OTT6050 in Vivo

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ABSTRACT

The effects of glucocorticoid treatment against mouse teratocarcinoma OTT6050 were studied in vivo. Tumors transplanted s.c. on the backs of syngeneic male and female 129/Sv-CP + SI J mice were measured, and tumor growth curves were constructed using the products of three principal tumor diameters designated as "tumor volume." The day when tumor volume exceeded 100 cu mm was designated as Day 0. The growth curves from Day 0 to death were drawn. A calibration curve between tumor volume (V) and the actual excised tumor volume (Va) was constructed using the following equation

\[ \ln V_a = -98.8 + 1731.5 \ln V (r = 0.9628; n = 70; p < 0.001) \]

In the first experiment, we observed the effects of glucocorticoid on the growth of the tumor. Four groups of seven mice each received daily i.p. injections of hydrocortisone (sodium succinate) in doses of 1.5, 3.0, 6.25, or 12.5 mg/mouse. Our results showed significant tumor growth inhibition. The exponential regression of tumor volume on Day 30 (V30) due to the injections (D; mg/day) can be expressed as

\[ \ln V_{30} = \ln 14,297 - 0.1200D (r = 0.9908; n = 42; p < 0.001 \text{ for males}) \]

\[ \ln V_{30} = \ln 10,893 - 0.0985D (r = 0.9488; n = 47; p < 0.001 \text{ for females}) \]

All the mice receiving hydrocortisone treatment, except the male mice treated with 12.5 mg, survived as long or longer than the control. The mean survival times of male and female mice receiving 1.5 mg of hydrocortisone were significantly longer than the control (p < 0.05), but the survival times of the male mice receiving 12.5 mg of hydrocortisone per day were significantly shorter than the control (p < 0.05, one-way analysis of variance test).

Histologically, no specific types of differentiated tissues different from the control tumor were observed.

In the second experiment, we observed the presence of glucocorticoid receptors in five groups of tumors. The glucocorticoid receptors were measured in embryoid bodies in ascitic form, tumors treated with 12.5 mg of hydrocortisone and control tumors using [1H]hydrocortisone. The amount of receptors in the tumor cytosol obtained using Sephadex G-25 chromatography was compared to that obtained using the dextran-coated charcoal adsorption technique, dextran-coated charcoal concentration was determined to be 4.6%.

The mean level of receptors was 26.6 fmol/mg cytoplasmic protein, and the corresponding dissociation constant was 13.7 nM in embryoid bodies, 129.3 fmol and 37.9 nM in the tumors treated with 12.5 mg of hydrocortisone for 30 days, and 296.4 fmol and 35.1 nM in the volume-matched control tumors. As the tumor grew bigger, the amount of receptors increased not only in the control tumors but also in the treated tumors.

These results indicate that the growth inhibition of mouse teratocarcinoma OTT6050 is mediated by glucocorticoid receptors. The amount of receptors is thought to correlate with the degree of differentiation in teratocarcinoma.

INTRODUCTION

Teratocarcinomas are malignant tumors which are characterized by the presence of a distinctive cell type known as embryonal carcinoma, as well as a variety of differentiated cell types (29). Mouse teratocarcinoma OTT6050 originated from a six-day-old 129/Sv strain mouse embryo that was grafted into the testis of a F1 hybrid mouse (28). In ascitic fluid of mice, these tumor cells form aggregates resembling the first 5-day stages of early mouse embryogenesis and are therefore termed embryoid bodies. The solid tumors, produced by s.c. injections of embryoid bodies into mice, can differentiate to a wide range of tissue types (19). In addition, unlike most other tumors which are characterized only by their ability to proliferate, the major characteristics of teratocarcinoma are its ability to differentiate and its sensitivity to its environment in its host (18).

So far, various factors controlling teratocarcinoma growth and differentiation have been studied. Insulin, transferrin and epidermal growth factors, and fibroblast growth factors were found to be necessary for the serum-free culture of teratocarcinoma, on which receptors for these factors were also found (22).

Epidermal growth factor receptors are known to increase during the differentiation of embryonal carcinoma cells (20). Retinoic acid has been shown to differentiate teratocarcinoma cells into endoderm cells not only in vitro but also in vivo. Dietary administration of retinoic acid has also been shown to significantly retard tumor growth and lengthen the survival time of the host mice (30). Although some metabolic deviations of steroid hormones in teratocarcinoma were studied in vitro (1), the effects of glucocorticoid on teratocarcinoma are still unclear.

In this study, we observed the presence of glucocorticoid receptors in mouse teratocarcinoma OTT6050 and studied the effects of glucocorticoid on the growth of the tumor in vivo. The receptor-mediated mechanism of glucocorticoid treatment against teratocarcinoma and the receptor distribution in the tumor are also discussed.
MATERIALS AND METHODS

Tumor and Mice. Mouse teratocarcinoma OTT6050 and syngeneic inbred 129/Sv-Cp + Sf mice (28) were maintained in our laboratory and used in these studies. 129/Sv-Cp + Sf mice were housed 2 or 3 per cage in our facilities. They were provided with standard laboratory food and water ad libitum under a diurnal light cycle (light from 8 a.m. to 8 p.m.). The constant room temperature of 23°C was maintained. A suspension of the embryoid bodies, teratocarcinoma in ascitic form, was prepared as described previously (13), and 0.1 ml of suspension (8 x 10^6 cells/ml) was inoculated on the backs of the 129/Sv-Cp + Sf mice.

Measurement of Tumor Growth. Solid tumors produced on the backs of the mice were measured with vernier calipers. The product of 3 principal diameters of the tumor was designated as "tumor volume," i.e., if the diameters were 5, 5, and 4 mm, then tumor volume was 100 cu mm. The day when tumor volume exceeded 100 cu mm was designated as Day 0. Tumor growth curves from Day 0 to the death of the mice were drawn.

Administration of Drug. In the first experiment, we observed the effects of glucocorticoid on the growth of the tumor. Ten-week-old male and female mice bearing solid tumors on their backs were given i.p. injections of hydrocortisone (sodium succinate) [Pregn-4-ene-3,20-dione-21 -(3-carboxy-1-oxopropoxy)-11,17-dihydroxymonosodium salt (11β)-] (Japan Upjohn Ltd., Tokyo, Japan). Injections of 1.5, 3.0, 6.25, or 12.5 mg of hydrocortisone (sodium succinate) per mouse were given daily to groups of 7 mice each starting from Day 0 until death, and tumor volumes were measured 6 times per week. A group of 7 untreated mice was used as the control. A dose-response curve was drawn from each tumor volume on Day 30.

The survival period of the mice from Day 0 was also studied. Autopsies were performed, and the excised tumors were fixed, sectioned, and stained with hematoxylin and eosine for histological examination.

Glucocorticoid Receptor Assay. Tris was purchased from Nakarai Chemicals Ltd. (Tokyo, Japan); EDTA, dithiothreitol, glycercin, and Norit A were from Wako Pure Chemical Industries Ltd. (Osaka, Japan); Dextran T-70 and Sephadex G-25 were from Pharmacia Fine Chemicals AB (Uppsala, Sweden); and PCS (Phase Combining System) was from Amersham Corporation (Arlington Heights, Ill.). [1,2,6,7-3H]Hydrocortisone (114.5 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.), and unlabeled hydrocortisone was from Sigma Chemical Co. (St. Louis, Mo.).

The DCC used in our experiments consisted of a suspension of 4.6% activated charcoal and 0.46% dextran. DCC concentration was determined from the results of gel chromatography on a Sephadex G-25 column that appears hereafter in a TEDG buffer at 4°C.

In the second experiment, we observed the presence of glucocorticoid receptors in 5 groups of tumors. Tumors treated with 12.5 mg of hydrocortisone excised on Days 9 - 13 and Days 28 - 33, and control tumors of the same sizes, and embryoid bodies from peritoneal cavities were used for receptor studies. These tumor groups consisted of 7 samples each.

Cytosol Preparation and Incubation. Cytosol was prepared by the method of Bhakoo et al. (5). The binding of glucocorticoid to teratocarcinoma cytosol was measured by incubating 200 µl of cytosol with 100 µl of [3H]hydrocortisone or 100 µl of [3H]hydrocortisone containing 1000-fold molar excess of unlabeled hydrocortisone for 4 hr at 0°C in 12- x 83-mm tubes. Incubation was carried out using 5 different concentrations of [3H]hydrocortisone varying from 5 to 100 nM.

Column Chromatography. Two glass columns (1.1 x 30 cm) were packed with Sephadex G-25 equilibrated in TEDG buffer (length, 20.2 cm and 24.0 cm). The elution rate was set at 0.2 ml/min at 4°C, and the eluate was collected in 1-ml fractions. Each incubation mixture was applied to both columns. One hundred µl of each fraction were counted in 10 ml of PCS in a Beckman LS150 liquid scintillation counter at 40 to 42% efficiency. Some loss of receptor complex occurring during gel filtration could be corrected by the extrapolation of gel length to 0 cm from the 2 different columns (9).

The incubation mixtures used in the gel filtration were also added to various concentrations of DCC in TEDG buffer in order to assay the amount of hydrocortisone receptors by the method of DCC that appears hereafter (21). Because of the time-consuming nature of column chromatography and the fact that the DCC concentrations of 4.6% matched our column chromatography results exactly, DCC was used throughout the rest of this study.

DCC Method. The method of Bhakoo et al. (4) was followed to measure the amount of hydrocortisone receptors using 4.6% DCC in TEDG at 0°C. Specific binding was determined by the difference between total binding and binding in the presence of a 1000-fold molar excess of unlabeled hydrocortisone. Total binding capacity and dissociation constants (Kd) were calculated according to the method of Scatchard (24).

RESULTS

Calibration Curve. Chart 1 showed that there was a linear correlation between the estimated tumor volume, which was a product of 3 tumor diameters in vivo, and the actual excised tumor volume. Therefore, we thought that there was no systematic error in using the product of 3 diameters as the tumor volume, and these products were plotted to construct the tumor growth curve. In this experiment, the estimated tumor volume (V) and the actual excised tumor volume (Va) were calculated using

Va = -98.8 + 1731.5 V (r = 0.9628; n = 70; p < 0.001)

Growth Curves. Calculated tumor volumes for all male mice

![Chart 1. Tumor volume calibration curve. Abscissa, actual excised tumor volume (cu cm) (Va) by measuring cylinder filled with 0.9% NaCl solution; ordinate, estimated tumor volume (cu cm) (V) as the product of 3 principal diameters of the tumor on the backs of mice measured with vernier calipers. Their regression line is expressed as: Va = -98.8 + 1731.5 V (r = 0.9628; n = 70; p < 0.001)
receiving daily injections of 12.5 mg of hydrocortisone were plotted on a log-linear graph (Chart 2). Daily tumor volume measurement graphs were obtained for every treated group. Graphs for all groups showed smooth and steady tumor growth (data not shown). To determine the effect of treatment and the dose-effect relationship, tumor volumes for every 5 days were averaged in each group, and the tumor growth curves were compared [Chart 3, A (male) and B (female)]. Daily hydrocortisone injections begun after the tumors were 100 cu mm caused a marked reduction on tumor volume but no complete regression. Tumor growth curves suggested that the hydrocortisone injections resulted in dose-response growth inhibition. Chart 4 shows the relationship for each sex between tumor volume on Day 30 and the amount of hydrocortisone administered daily (mg). These dose-response curves were approximately straight lines in the log-linear graph, implying that in our experimental dose range, tumor growth inhibition was an exponential function of the injection dose. Using the least-squares method, the regression curves of tumor volume on Day 30 \((V_{30})\) and injection dose \((D; \text{mg/day})\) can be expressed as

\[
\ln V_{30} = \ln 14,297 - 0.1200 D \quad (r = 0.9908; \ n = 42; \ p < 0.001 \text{ for males})
\]

\[
\ln V_{10} = \ln 10,893 - 0.0985 D \quad (r = 0.9488; \ n = 47; \ p < 0.001 \text{ for females})
\]

As shown in the regression curves, growth inhibition of teratocarcinoma was stronger in males than in females in response to hydrocortisone treatment.

**Effects of Hydrocortisone on Survival.** The mean survival times of 4 treated and control groups of mice from Day 0 are shown in Table 1. The mean survival times of all treated groups, except the male mice treated with 12.5 mg of hydrocortisone, were equal to or longer than that of the control. In our experimental dose range, the mean survival times of males and females were significantly prolonged at the low dose of 1.5 mg.
After the glucocorticoid treatment, no specific types of differentiated tissues different from those found in control tumors were observed, but larger masses of differentiated tissues were seen and the area of undifferentiated embryonal carcinoma cells had decreased.

From these findings, glucocorticoid treatment was not thought to inhibit tumor growth through cytotoxicity and specific differentiation induction.

Results of Histological Examination. Solid teratocarcinoma excised from the mice consisted of both well-differentiated and undifferentiated tissues (13, 27). When the tumors were small, tumor volume less than 500 cu mm, the tumor tissues consisted mainly of undifferentiated carcinoma cells, and there were only a few clumps of nervous tissue, recognized by the presence of primitive neural tubules. Larger tumors contained necrotic areas, and the areas of undifferentiated carcinoma cells were relatively small, but there were large masses of various kinds of well-differentiated tissues around the necrosis. These included: nervous tissues; ciliated, nonciliated, or keratinized epithelial tissues with which cysts and tubules were often lined; and also keratin pearls. Fatty tissues, hyaline cartilage, bone, and skeletal muscle were also found.

Results of Receptor Assay. Chart 7 shows the saturation curve of the specific binding of [3H]hydrocortisone to teratocarcinoma cytosol protein. Scatchard analysis of the binding data shown in Chart 8 indicated a single class of high-affinity binding. Table 2 represents the concentrations of hydrocortisone receptors and Kd obtained from Scatchard analysis of 7 samples from each experimental group as well as the average of the actual excised tumor weights. The 5 experimental groups included ascites cells, tumors treated with 12.5 mg of hydrocortisone and excised on Days 9 ~ 13 and Days 28 ~ 33, and volume-matched control tumors of the latter 2 groups.

We observed glucocorticoid receptors in every tumor ex-
Chart 6. Comparison of receptor values from Sephadex G-25 chromatography and from various concentrations of DCC methods. Percentages showed charcoal concentrations in TEDG, and the amount of dextran was one-tenth of each charcoal concentration. DCC concentration was determined as 4.6% in TEDG in this study.

Chart 7. A representative plot of specific binding of \([^{3}H]\text{hydrocortisone}\) to teratocarcinoma cytosol receptors as a function of \([^{3}H]\text{hydrocortisone}\) concentration (saturation curve).

Chart 8. Scatchard plot of the binding data. In this tumor, the receptor site concentration was 411 fmol/mg protein, and the \(K_d\) was calculated to be 42.4 nM.

tiated cells would result in higher receptor concentrations.

Hydrocortisone-treated tumors also contained glucocorticoid receptors. When the tumors were small, the receptor concentrations in the treated tumors were equivalent to those in the volume-matched control tumors. Although the amount of receptors increased as the treated tumors grew, the final amount of receptors was always less than the volume-matched control tumors. Since glucocorticoid receptors were found in the hydrocortisone-treated tumors and since the treated tumors exhibited growth inhibition, we propose that glucocorticoid receptors were responsible for growth inhibition during the 30-day treatment period.

DISCUSSION

Glucocorticoid has been reported to inhibit the growth of mammary tumors (7), other murine experimental tumors (10), and Lewis lung carcinoma (25) and to promote the remission of certain lymphoid cancers.

In our study, hydrocortisone inhibited the growth of teratocarcinoma OTT6050, and its growth inhibition showed a clear logarithmic dose-response relationship, although the precise mechanisms by which this compound acts are still unknown.

Since glucocorticoid receptors were present in not only the control tumors but also the tumors treated with hydrocortisone for 30 days, teratocarcinoma growth inhibition was considered to be induced by the direct effect of hydrocortisone. We believe the growth inhibition was directly induced by the receptor-mediated mechanism rather than indirectly via the pituitary or immune system.

Although i.p. injection of rather high doses of glucocorticoid may induce some endocrinological disorder and immune suppression in mice, these secondary effects were not evaluated in this experiment.

It is very difficult to obtain dose-response curves of the effect of tumor treatment \(in vivo\), since the direct measurement of tumor volume \(in vivo\) is a difficult procedure. Because most s.c. solid tumors are an irregular oval shape and because growth rates differ from shape to shape, single-diameter measurements of tumors are not considered to be accurate (26).
Therefore, the products of 3 principal diameters were used in our experiment, since they could trace the irregular oval shape and the growth of tumors more accurately. As shown in Chart 1, these measurements had a clear linear relationship to the excised tumor volume data, demonstrating the reliability of our experimental growth curve system. The accuracy of the dose-response curve in vivo derived from the growth curve data is also supported by this calibration study. We finally concluded that the effect of hydrocortisone on teratocarcinoma growth inhibition was exponentially dependent on the treatment dose.

Our study confirmed that male mice were more sensitive to hydrocortisone than were females. This sensitivity resulted in not only greater dose-response growth inhibition in the male mice but also a shorter survival time for males treated with 12.5 mg. Side effects of hydrocortisone, such as immune suppression and susceptibility to infection, endocrinological disorder, or gastric ulcer, seemed to be the cause of early death in the males treated with 12.5 mg.

In our experiment, lymph node metastasis and liver metastasis were more often observed in the treated group than in the control (data not shown). These enhancing effects of hydrocortisone on tumor metastasis were reported earlier (12).

Glucocorticoid receptors in teratocarcinoma were present in both the embryoid bodies, ascitic form of teratocarcinoma, and the solid tumor. As tumors grew bigger, the amount of glucocorticoid receptor increased, although at different rates. These results suggested that some differentiated tissues in solid teratocarcinoma had more glucocorticoid receptors than the undifferentiated embryonal carcinoma cells and the endodermal cells which make up the embryoid bodies, and the receptor concentration varied according to the amount of the differentiated cells.

Hydrocortisone-treated tumors also included glucocorticoid receptors. The receptor concentrations in the treated tumors were less than in the control tumors. Histologically, however, the masses of differentiated tissues which appeared in the hydrocortisone-treated tumors were greater than in the control tumors. These findings suggest that the receptor content in each differentiated tissue was reduced by the hydrocortisone treatment or that glucocorticoid induced the undifferentiated carcinoic cells to differentiate into tissues without receptors.

The latter hypothesis is not in accordance with the histological finding that the types of differentiated tissues in the treated tumors were identical to the control.

The former hypothesis is not only compatible with a previous report that glucocorticoid receptor concentration in lymphoma varied during the glucocorticoid treatment and that sensitivity to glucocorticoid therapy changed (14), but it is also compatible with the results of our receptor assay. Therefore, it is plausible that the hydrocortisone treatment reduced the receptor content in the differentiated tissues and finally reduced the overall receptor concentration in the tumor.

Recent studies have shown the importance of the interaction between steroid hormones and specific cytoplasmic receptor proteins as the first step in the expression of biological reaction by steroid hormones. Studies on breast cancer have shown that the measurement of specific estrogen receptors is useful for the prediction of response to estrogen therapy (16). Similarly, determination of glucocorticoid receptor levels in lymphoma (6) and leukemia (14) has shown to be a necessary part in predicting the response of a patient to glucocorticoid therapy.

Dexamethasone is known to induce morphological differentiation and growth inhibition in murine neuroblastoma cells in culture (23). Mouse myeloid leukemia cells were induced to differentiate into normal functional macrophages and granulocytes by glucocorticoid treatment (11). Not only malignant cells, but also normal embryonal tissues were shown to have glucocorticoid receptors (2). Glucocorticoid and its receptors are known to play important roles in the normal development of fetal tissues (31). In our previous paper, we suggested that teratocarcinoma growth is affected by the changes of hormone levels in older mice (13). Although the endocrinological nature of mouse teratocarcinoma has not been well elucidated, it is not surprising that glucocorticoid receptors are present in teratocarcinomas and that tumor growth was inhibited by glucocorticoid treatment.

In this paper, we are the first to describe teratocarcinoma growth inhibition by hydrocortisone and the presence of its receptors. Our studies suggested the possibility of combination chemotherapy including glucocorticoid against human teratocarcinoma and embryonal carcinoma. Whether the receptors are located in embryonal carcinoma cells, endodermal cells, or both and the mechanism of growth inhibition and its relation to teratocarcinoma differentiation remain to be studied. For instance, cell biological analysis, such as how much receptor protein is present in each constituent cell of teratocarcinoma, why the cells stop proliferating and die, and why they differentiate after glucocorticoid treatment remain to be described.

Although several histological methods for revealing steroid hormone receptors have been proposed (17), none is presently considered valid (8).
Further studies about these mechanisms and the relationship between this experimental teratocarcinoma model and human teratocarcinoma are now under way.

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