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European Journal of Cancer 37 (2001) 1703–1708

European Journal of Cancer

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In vivo inhibition of tumour growth by dexamethasone in murine osteosarcomas

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Received 17 November 2000; received in revised form 27 March 2001; accepted 16 May 2001

Abstract

This study was performed to determine whether glucocorticoid (GC) is an effective inhibitor of tumour growth in murine osteosarcoma (OS) *in vivo*. The effects of dexamethasone (DEX) on the growth of this tumour were studied in male C3H/He mice. The animals received a dose of 1.25 or 5 μg/g of DEX in 0.1 ml of steroid solution daily intraperitoneally (i.p.) for 14 days. In each DEX-treated group, significant inhibition of the tumour growth curve was seen in a dose- dependent manner compared with the control group ($P<0.0001$). The percentage of proliferative cell nuclear antigen (PCNA)-positive cells was 22.7% in the 5 μg/g DEX treatment group compared with 67.6% in the control group ($P=0.009$). Furthermore, mifepristone, a GC receptor antagonist, blocked the inhibition of tumour growth induced by DEX. In the control group, tumour cells showed positive reactivity for nuclear glucocorticoid receptors (GR) by immunohistochemistry. The results of this study indicate that tumour growth inhibition by DEX in murine osteosarcoma may be via GR.

Keywords: Osteosarcoma; Dexamethasone; Glucocorticoid; Mifepristone; Glucocorticoid receptor

1. Introduction

The role of glucocorticoid (GC) therapy is well established in the treatment of haematopoietic malignancies such as leukaemia and lymphoma in combination with antineoplastic agents. It is well known that the effects of GC on these tumour cells occur through the apoptotic and differentiation pathways [1–4]. In contrast, although GC has been widely investigated in solid tumours, little information is available on the effects of GC on the proliferation of solid tumours, especially sarcomas. There have been only two reports on the inhibitory effect of GC on fibrosarcomas [5,6], one report on human leiomyosarcomas [7] and one report on human osteosarcomas (OS) [8].

OS is the most common primary malignant bone tumour, and mainly occurs in children and adolescents. The current treatments for OS are multi-agent chemotherapy and limb salvage surgery of the primary tumour. The prognosis of OS patients has improved with the development of adjuvant chemotherapy. A survival rate of approximately 60–70% at 5 years for non-metastatic OS patients has been described in the latest reports [9,10]. However, new modalities are essential to further improve the prognosis of patients with OS. There are no reports of experimental or clinical GC therapies for osteosarcoma, thus standard chemotherapeutic regimens have not included any steroid hormone in the treatment of OS. We document here the interesting results of glucocorticoid therapy for murine OS.

2. Materials and methods

2.1. Tumours and animals

Dunn OS is a murine OS cell line established by Dunn and colleagues [11]. Dunn OS cells (1×10^7 cells/mouse)

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0959-8049/01/$ - see front matter
PII: S0959-8049(01)00185-X

were injected into the subcutis in the backs of male 4-week old C3H/He mice. One-cm^3 tumours were observed within 5–6 weeks following inoculation. Tumours were transplanted by mincing fourth to sixth generation sources, and implanting 3-mm fragments in the subcutis of the right backs of 4-week-old C3H/He male mice. For all experiments, male C3H/He mice, 18–20 g in body weight (BW) were housed 3–4/cage.

2.2. Dexamethasone (DEX) therapy

Animals were injected daily intraperitoneally (i.p.) with 0.1 ml of steroid solution containing either 1.25 μg/g BW (group A) or 5 μg/g BW of DEX (group B). The control mice received 0.1 ml injections of the vehicle only (group C). The steroid solution consisted of 4.0 ml of polysorbate 80, 5.0 g of carboxy-methylcellulose, 9.0 ml of benzylalcohol, 9.0 g of NaCl and 1000 ml of distilled water. Treatment began when the tumours were implanted (day 0) and continued for 14 days. The number of animals in each group was 12.

2.3. Effect of GC receptor antagonist on tumour growth with DEX

One mg of mifepristone (MP) (Biomol research Lab., Inc.), a GC receptor antagonist, in 1 ml of steroid solution 5 minutes after the 5 μg/g of DEX was injected daily for 14 consecutive days (group D). Three other groups were set up. They were mice given 5 μg/g BW of DEX (group E), MP only (group F) and vehicle only (group G).

2.4. Evaluation of tumour growth

After implantation (day 0), tumour volume was estimated on days 8, 10, 12 and 14. This was done by measuring three orthogonal diameters of the tumour with calipers and multiplying the product by $\pi/6$ (mm^3). On day 14, the BW of the mice were measured, then tumours were removed and tumour weights (TW) and were measured. TW divided by BW reflected the final tumour weight (FTW).

2.5. Histological examination

Tumours arising from the backs of the mice were fixed in 20% formalin, embedded in paraffin blocks and 4-μm sections were cut and stained with haematoxylin and eosin. Immunohistochemical staining with a monoclonal antibody against proliferative cell nuclear antigen (PCNA) (Bio genex) at a 1:100 dilution, was performed to evaluate tumour cell proliferation.

PCNA-positive cells were counted on the slides at a magnification of 400. Five fields of non-necrotic areas were selected randomly across each tumour section and in each field. The % PCNA was scored as a percentage of PCNA-positive cells based on the scoring of 1000 tumour cells.

Immunohistochemical staining with a polyclonal antibody (M-20) (Santa Cruz Biotechnology) directed against glucocorticoid receptors (GR)-alpha and GR-beta of the mouse was also performed. The avidin-biotin-peroxidase complex procedure was carried out on 4-μm thick paraffin-embedded sections.

2.6. Statistics

Tumour growth curves, FTW and BW on day 14 were evaluated by repeated measure ANOVA, two-factor factorial ANOVA, and Student's *t*-test (Stat View J 4.5, Macintosh), respectively. Evaluation of the effect of MP was performed using Student's *t*-test in FTW. The difference in the % PCNA staining between the two groups was evaluated by the Mann–Whitney test.

3. Results

3.1. Inhibitory effect of DEX on tumour growth

In both groups A and B, significant inhibition of tumour growth was seen in a dose-dependent manner compared with the vehicle only group (group C) ($P<0.0001$ in groups A and B) (Fig. 1).

Means of FTW on day 14 for groups A, B and C were 14.6×10^{-3}, 6.6×10^{-3} and 43.3×10^{-3}, respectively. FTW in both DEX-treated groups were significantly lower

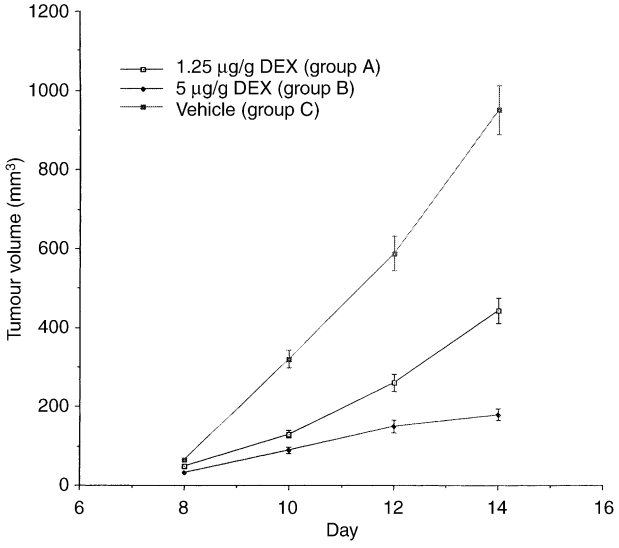

Fig. 1. Growth inhibition in Dunn osteosarcoma (OS) of two different doses of dexamethasone (DEX). The mean tumour volume at each day of treatment is indicated in the graph, and bars for the standard errors (S.E.) are also indicated. A significant decrease in tumour volume in the DEX-treatment groups was observed. This effect was dose-dependent.

Table 1
Effect of dexamethasone (DEX) on tumour growth

Group	Treatment	No.	FTW (TW/BW)	BW (g)
A	1.25 μg/g DEX	12	$14.6\pm3.7\times10^{-3}$[a,b]	15.8 ± 0.2[a,c]
B	5 μg/g DEX	12	$6.6\pm1.7\times10^{-3}$	15.9 ± 0.3
C	Vehicle	12	$43.3\pm9.7\times10^{-3}$	17.9 ± 0.3

FTW, final tumour weight; TW, final tumour weight; BW, body weight.
[a] Mean ± standard error (S.E.).
[b] Analysis by two factorial ANOVA. A differs from C ($P=0.015$) and B differs from C ($P=0.001$).
[c] Analysis by Student's *t*-test. A and B differ from C ($P<0.0001$ and $P=0.0001$, respectively).

than those in the vehicle only group ($P=0.015$ in Group A, $P=0.001$ in group B) (Table 1).

3.2. Blockage of DEX tumour growth inhibition by GR antagonist (mifepristone)

The tumour growth curve for group D was elevated compared with group E ($P<0.001$) (Fig. 2). Means of the FTW on day 14 for groups D, E, F and G were 51.9×10^{-3}, 14.1×10^{-3}, 67.3×10^{-3} and 47.7×10^{-3}, respectively. MP significantly blocked the growth inhibition of the tumour induced by treatment with 5 μg/g BW of DEX ($P=0.0014$) (Table 2).

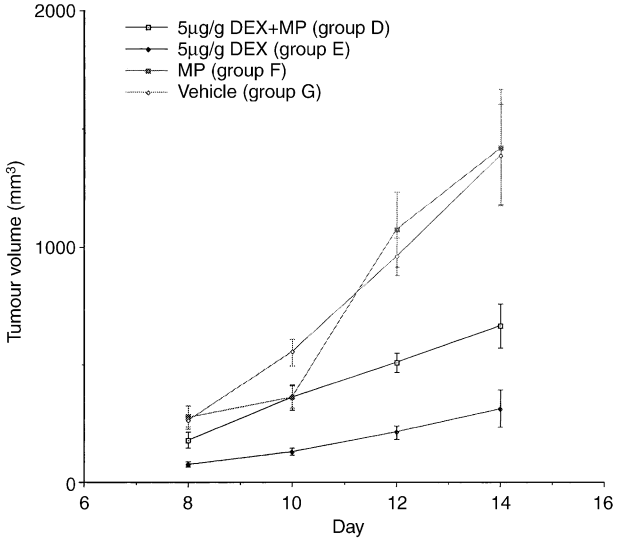

Fig. 2. Blockage by the glucocorticoid (GC) antagonist, (mifepristone) (MP) of the growth inhibition of the tumour induced by dexamethasone (DEX) therapy. The mean tumour volume for each day of treatment is indicated in the graph, and bars for the standard errors (S.E.) are also indicated. The growth curve for the MP- and DEX-treated group (group D) is elevated compared with that in the DEX-treated group (group E). The growth curves for the two different control groups (groups F and G) are similar, whereas the final tumour volume in the MP group (group F) is slightly larger than that in vehicle only group (group G).

Table 2
Blockage by mifepristone (MP) of the tumour growth inhibition induced by dexamethasone (DEX)

Group	Treatment	No.	FTW (TW/BW)	BW (g)
D	5 μg/g DEX + MP	7	$51.9\pm8.7\times10^{-3}$[a,b]	17.5 ± 0.3[a]
E	5 μg/g DEX	7	$14.1\pm2.8\times10^{-3}$	16.6 ± 0.4
F	MP	7	$67.3\pm15.0\times10^{-3}$	19.5 ± 0.4
G	Vehicle	6	$47.7\pm7.8\times10^{-3}$	17.9 ± 0.6

FTW, final tumour weight; TW, final tumour weight; BW, body weight.
[a] Mean ± standard error (S.E.).
[b] Analysis by Student's *t*-test. D differs from E ($P=0.0014$).

3.3. Histological examination

The tumours demonstrated solid spindle cell proliferation with hyperchromatic nuclear atypism. This finding was consistent with Dunn OS. Immunohistochemical staining with M-20 for the GR of tumours showed positive staining of nuclei (Fig. 3). The positive stain demonstrated the presence of proteins with the same antigenicity as GR in the Dunn OS cells.

3.4. Cell proliferation activity

In the 5 μg/g of DEX treatment group the %PCNA staining was 22.7% compared with 67.6% in the control group (Figs. 4 and 5). This difference was statistically significant ($P=0.009$).

3.5. Toxicity of DEX

Means of BW on day 14 for groups A, B and C were 15.8, 15.9 and 17.9 g, respectively. Body weight loss was seen to a greater degree in the DEX treated groups compared with group C ($P<0.0001$ in group A,

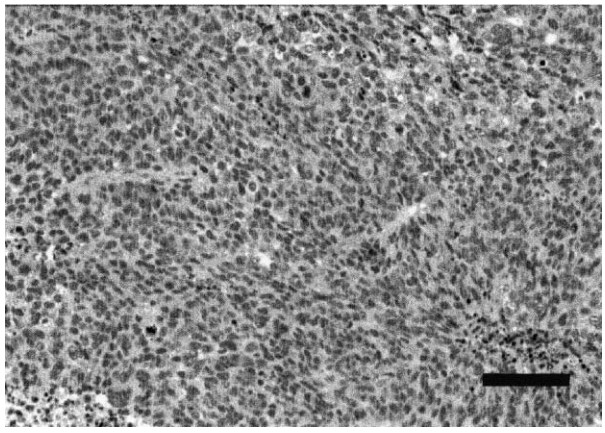

Fig. 3. Immunohistochemistry for glucocorticoid (GC) receptor (M-20) in Dunn osteosarcoma (OS) cells. Many nuclei were positive. Scale bars: 100 μm.

$P = 0.0001$ in group B) (Table 1). None of the mice died during these experimental periods. No other physical complications due to DEX were seen in any of the experiments apart from body weight loss. Thus, these doses of DEX were tolerated without any adverse effects.

4. Discussion

The inhibitory effects of GC on haematopoietic malignancies such as leukaemia and lymphoma are well known and it is included in standard therapeutic regimens. However, in solid tumours, although the anti-tumour effect of GC is widely investigated in experimental studies, it is not generally used in the treatment of human malignant solid tumours. This is because the clinical inhibitory effects of GC on solid tumours are uncertain. Previous authors have described a GC-induced inhibition of proliferation in various kinds of solid tumours such as lung cancer [12], murine mammary tumour [13,14], breast cancer [15,16], colon tumour [17], melanoma [18], fibrosarcoma [5,6] and rat adenocarcinoma [19]. Only one report has described a GR-mediated growth inhibition by GC in human OS cultured cells [8]. However, until now, no clinical evidence that this steroid hormone is effective in inhibiting OS proliferation has been found, thus GC has never been included in the chemotherapeutic regimens for OS. In the current treatments for OS, systemic chemotherapy and aggressive surgery are essential. The prognosis of OS patients has been dramatically improved by the use of several antineoplastic agents to which OS is sensitive for example doxorubicin, cisplatin, methotrexate and ifosfamide. Chemotherapy has resulted in a survival rate of 60–70% at 5 years in patients without metastases at first presentation [9,10]. However, it is still difficult to treat patients with recurrence or distant metastases of the lung, bone and/or brain [20]. Therefore, a new modality in the treatment for OS is necessary to further improve its prognosis.

Although there have been several reports of the effects of GC on OS cells in *in vitro* studies [8,21,22], there have been no *in vivo* studies. This is thus the first report describing the effect of GC on OS *in vivo*. Dunn OS used in this study is a good experimental model for the therapeutic study of OS, since most of the mice die from their tumours within 3 months of implantation because of rapid tumour growth *in vivo*. Inhibition of tumour growth by DEX in Dunn OS was observed in a dose-dependent manner in this study. Inhibition of tumour growth was not using a daily dose less than 0.5 μg/g of DEX (data not shown). However, a daily dose of 1.25 μg/g or more was able to inhibit tumour growth. Other studies have also indicated that the anti-tumour effect of GC is dose-dependent in experimental solid tumours [5,12,14,15]. The clinically equivalent dose to that used in this study is unclear, because the pharmacokinetics of DEX in mice is unknown. The doses of DEX used in this study seem high, and BW loss was seen in the DEX-treated groups, probably due to the growth-inhibitory effects of the steroid hormone. However, high doses of

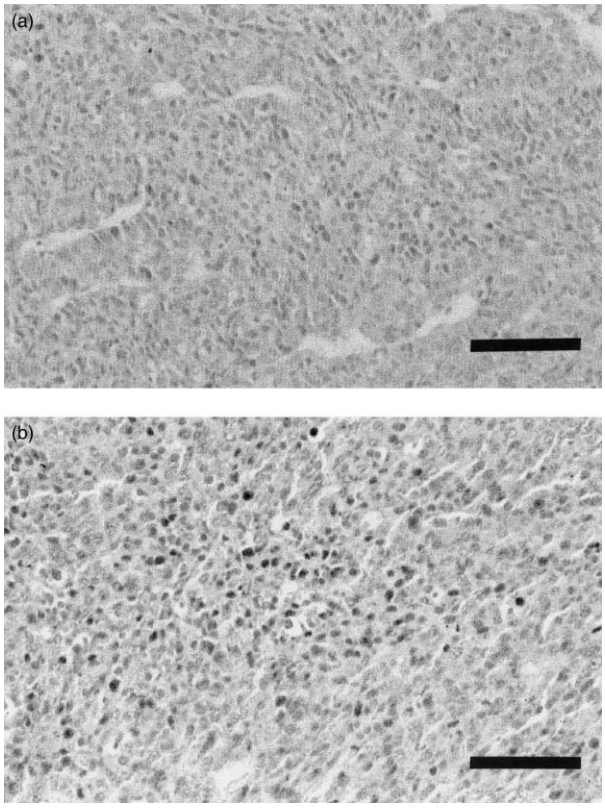

Fig. 4. Immunostaining with antibody against proliferative cell nuclear antigen (PCNA). Scale bars. 100 μm. (a) Low incidence of PCNA-positive cells in the 5 μg dexamethasone (DEX) treatment group; (b) high incidence of PCNA-positive cells in the control group.

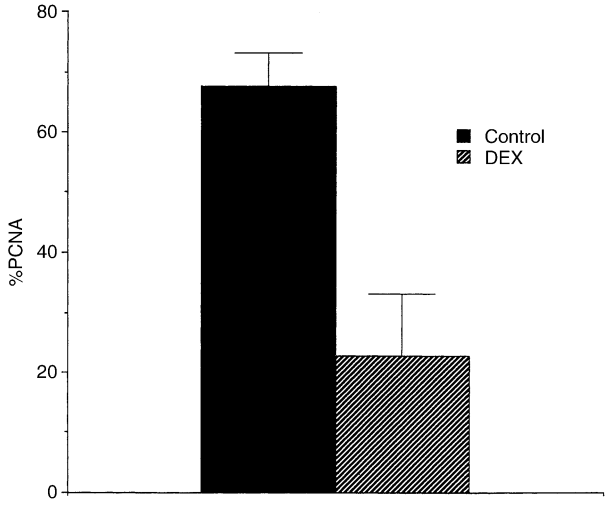

Fig. 5. % Proliferative cell nuclear antigen (PCNA) staining in the 5 μg DEX-treated group and the control group. Analysis by the Mann–Whitney test ($P = 0.009$).

DEX were well tolerated without any unacceptable complications. These results suggest that a relatively high-dose GC therapy may potentially be used for the treatment for human OS.

Although we have documented the fact that GC has obvious inhibitory effects on the local tumour growth of OS, the most important clinical problems are the micrometastases at the first presentation, and distant metastases to the lung, bone and/or brain. The murine model used in this study does not have the potential to develop distant metastases, therefore we could not evaluate the effect of GC against metastatic lesions. Further investigation in a metastatic model is therefore needed to demonstrate this effect.

The results from the PCNA analysis showed that the proliferative activity of the tumour cells was decreased by DEX treatment. The presence of GR in Dunn OS was shown by immunohistochemistry, as well as by western blotting (data not shown). GR is present in many human and animal malignant tumours [23,24], however, there is little information available on the mechanism of action of GC in most of these malignant tumours. The induction of apoptosis and differentiation are well-known antitumour mechanisms of GC via the GR in lymphomas and leukaemias. A recent investigation indicates that GR-mediated growth inhibition by DEX results in apoptosis and the downregulation of anti-apoptotic proteins, including Bcl 2, in a human OS cell line [8]. In the present study, apoptosis was also seen in both DEX-treated and the control groups. However, there was no difference in the percentage of apoptosis in the non-necrotic areas between the two groups (data not shown). Moreover, differentiation of Dunn OS cells by GC was not recognised histologically. Since the administration of the GR antagonist with DEX resulted in a blockage of the tumour growth inhibition induced by DEX, the inhibitory effect of DEX on tumour growth in Dunn OS would seem to be due to an unknown mechanism via the GR rather than apoptosis or differentiation. Another study [25] also described the blockage of the tumour growth inhibition of GC by GC antagonist (RU486) in lung cancer cell lines *in vitro*. However, there are no reports of an abrogation of GC-induced inhibitory effect in solid tumours *in vivo*. The slightly increased tumour growth in the GR antagonist group compared with the control group may be caused by the inhibition of endogenous GC.

However, steroid hormones have some adverse side-effects; that is, immune suppression, peptic ulcer, hypertension, diabetes mellitus and osteoporosis [26,27]. In particular, GC-induced immunosuppression, including inhibition of natural killer (NK) activity and suppression of interleukin I and II production, causes problems in the treatment of malignancies. Many previous reports have indicated that NK activity in human peripheral blood is inhibited *in vitro* by GC [28–30].

Under the conditions used in the present animal experiments, the only adverse side-effect of GC was BW loss.

As mentioned above chemotherapy with GC in the treatment of OS has not been reported. On the contrary, in the treatment of malignant lymphproliferative or myeloproliferative disorders, combination chemotherapy with GC has been used widely and its effectiveness is well understood, although the molecular mechanism of GC-induced apoptosis in lymphoid cells is not fully understood. Moreover, high doses of DEX increase the frequency of response to chemotherapy and prolong survival in multiple myeloma patients [31]. Therefore, there is, at present, no evidence that GC counteracts the cytotoxic effects of antineoplastic agents such as cyclophosphamide and doxorubicin, which are usually used in the treatment of OS. However, clinical trials of standard chemotherapeutic regimen with GC are necessary to determine the effectiveness of GC in the treatment of human OS. In conclusion, the present study indicates the possibility of using high-dose GC therapy for the treatment of OS.

Acknowledgements

The authors thank Dr Masayuki Mano for his immunohistochemistry analysis.

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