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ORIGINAL ARTICLE

Effect of cortisol on cell proliferation and the expression of lipoprotein lipase and vascular endothelial growth factor in a human osteosarcoma cell line

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Abstract
Purpose The aim of this study is to investigate whether cortisol inhibited cell proliferation and the expressions of lipoprotein lipase (LPL), a key enzyme involved in the energy metabolism in tumor cells, and vascular endothelial growth factor (VEGF), a potent angiogenic factor in the tumor, in cultures of OST cells, a human osteosarcoma cell line.
Methods OST cells were treated for 48 h with or without cortisol. To examine the effect of cortisol on cell proliferation, the expression of proliferating cell nuclear antigen (PCNA) was examined by Western blotting, and the amount of ^{3}H-thymidine incorporated into DNA during the last 30 min of the 48-h treatment period was measured. To examine the effect of cortisol on the expression of LPL, the activity and mass of LPL were measured in the extract of acetone/ether powder of cells, and the amount of ^{35}S-methionine incorporated into LPL during the last 2 h of the 48-h treatment period was measured by immunoprecipitation. The expression of VEGF was examined by immunohistochemistry and Western blotting.
Results The amount of ^{3}H-thymidine incorporated into DNA and the level of PCNA were lower in the cortisol-treated cultures than in the untreated cultures, thus indicating that cortisol inhibited the proliferation of OST cells. The synthetic rate and activity of LPL were lower in the cortisol-treated cultures than in the untreated cultures but no difference in the specific activity of LPL between the two cultures was observed, thus indicating that cortisol inhibited LPL synthesis, thereby resulting in a decreased LPL activity. The expression of VEGF was lower in the cortisol-treated cultures than in the untreated cultures.
Conclusion Cortisol not only has the ability to inhibit cell proliferation but also the ability to inhibit the expressions of LPL and VEGF in cultures of OST cells.

Keywords Human osteosarcoma cell line · Cortisol · Cell proliferation · Lipoprotein lipase · Vascular endothelial growth factor

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Introduction

Osteosarcoma is the most common malignant tumor of bone, occurring mainly in the metaphyseal region of the long bones of young people. Osteosarcoma expands the cortex of the bone and erupts through the cortex into the soft tissues. The main treatment of patients with osteosarcoma is to remove the tumor by a wide excision. In most cases, these patients receive pre- and post-operative neoadjuvant chemotherapy. The agents most commonly used in the treatment of patients with osteosarcoma are methotrexate, cisplatin, adriamycin and ifosfamide [1]. The

prognosis of patients with osteosarcoma has been markedly improved by the use of multiple antineoplastic agents, which act on different stages of the cell cycle.

Glucocorticoids have generally been used to treat patients with malignant lymphoma and patients with acute leukemia. However, to date there have been no reports on the use of glucocorticoid as an antineoplastic agent for patients with osteosarcoma. In cultures, dexamethasone has been reported to inhibit the proliferation of osteosarcoma cells [2, 3]. In mice, the intraperitoneal injection of dexamethasone has been reported to inhibit the growth of osteosarcoma implanted into the back by unknown mechanism that did not involve apoptosis and differentiation [4]. This finding raises the question of how dexamethasone inhibited the in vivo growth of osteosarcoma.

Tumor cells utilize fatty acids as fuels for tumor growth [5–10]. The sources for fatty acids are albumin-bound free fatty acids and esterified fatty acids present as triacylglycerols in chylomicrons and very low-density lipoproteins in circulation. These esterified fatty acids are hydrolyzed by lipoprotein lipase (LPL) into free fatty acids and monoacylglycerols before they are incorporated into the tissues. We previously reported that a variety of human sarcomas and carcinomas, including osteosarcoma, malignant fibrous histiocytoma and gastric cancer, contained LPL and that the LPL activity was high in areas containing actively proliferating cells [11]. In culture, OST cells, a cell line established from human osteosarcoma, synthesized and released LPL [8, 11]. The treatment of OST cells with tumor necrosis factor-α (TNF-α) decreased the LPL activity [8]. There have been many reports on the regulation of LPL by glucocorticoids in human and rat adipose tissues [12–18], but the effect of glucocorticoids on tumor LPL has not been reported.

Angiogenesis within the tumor is indispensable for the vascular supply of nutrients and oxygen to the tumor [19]. One mechanism by which tumors induce angiogenesis is the expression of angiogenic factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factors [20–25]. We previously found that OST cells formed osteoids and invaded the cranial bone when inoculated under the periosteum of the ossa cranii of nude mice and that topical treatment of these mice with ketoprofen, one of the nonsteroidal anti-inflammatory drugs (NSAIDs), inhibited the VEGF expression, reduced the development of feeder vessels and suppressed tumor growth [26]. Sawaoka et al. [25] also reported the inhibitory effects of NSAIDs on angiogenesis and tumor growth in nude mice with gastrointestinal tumors. The expression of VEGF in the tumor thus appears to be associated with in vivo tumor growth.

To explore the above question, we examined whether cortisol inhibited the expressions of LPL and VEGF in cultures of OST cells. We also examined the effect of cortisol on the proliferation of OST cells.

Materials and methods

Cell line and materials

OST cells were generously donated by Dr. Katsuro Tomita (Kanazawa University, Japan). A chicken antiserum to bovine LPL was generously donated by Dr. Thomas Olivecrona (Umeå University, Sweden). A mouse monoclonal antibody to VEGF (*sc-7269*) and a rabbit polyclonal antibody to glucocorticoid receptor (GR, *sc-1004*) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A mouse monoclonal antibody to proliferating cell nuclear antigen (PCNA, PC 10) and an immunostaining kit [an ENVISION kit/HRP (DAB)] were from DakoCytomation (Kyoto, Japan). An ECL Plus Western Blotting Starter kit, hybridization buffer, blocking reagent, Hyperfilm™ MP, Hybond-P, [6-^{3}H]thymidine, glycerol tri[9,10(n)-^{3}H]oleate and L-[^{35}S]methionine were obtained from Amersham Biosciences (Tokyo, Japan). An ELISA kit for LPL and ENHANCE® was from Daiichi Pure Chemicals (Tokyo, Japan). Cortisol and RU 486 were from Sigma (St. Louis, MO, USA). A *DC* Protein Assay was from Bio-Rad Laboratories.

Cell culture

OST cells were grown for 5–8 days (60–70% confluence) in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS), 100 Units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B in 60-mm plates. The medium was then replaced with the medium containing cortisol and changed every day. In a preliminary experiment, when OST cells were treated in the medium containing 10^{-9} to 10^{-4} M cortisol, these cells in cultures treated with 10^{-4} M cortisol for more than 48 h began to detach from the bottom of the plates. Therefore, the experiments were performed using cortisol at a concentration of 10^{-5} M, except for the dose-dependent experiments.

DNA replication assay by [^{3}H]thymidine incorporation

OST cells were treated for 47 h with or without 10^{-5} M cortisol. The plates were replenished with a fresh medium containing the appropriate additive and incubated for 30 min. Next, 1.0 μCi of [^{3}H]thymidine was added to each plate. Thirty minutes later, the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) and harvested in 1.8 ml of PBS containing 0.1% SDS and 1 mM EDTA. The measurement of the acid-insoluble radioactivity was performed by trichloroacetic acid (TCA) precipitation, as described by Smulson et al. [27].

Measurements of the LPL activity and mass

OST cells were treated for 24–96 h with or without 10^{-5} M cortisol, harvested into 1.2 ml of 50 mM NH_4Cl/NH_4OH buffer (pH 8.2) containing 2% (v/v) bovine serum albumin (BSA) and 20 μg/ml of heparin, and sonicated briefly at 0°C. An aliquot of the homogenate was used to measure the DNA content. The DNA was measured fluorometrically by the method of Hinegardner [28] using calf thymus DNA as the standard.

Another aliquot of the homogenate was used to prepare acetone/ether powder for LPL assay, as described previously [29]. The extract of acetone/ether powder was made by adding the powder to 1 ml of ice-cold 50 mM NH_4Cl/NH_4OH buffer (pH 8.2) containing 20 μg/ml of heparin, letting the mixture stand at 0°C for 60 min, sonicating briefly at 0°C, centrifuging for 10 min at 4°C and 12,000*g*, and then decanting the supernatant for measurements of LPL activity and mass.

LPL activity in the extract of the powder was measured using phosphatidylcholine-stabilized tri[9,10(n)-^{3}H]oleoylglycerol as the substrate in the presence of heat-inactivated (56°C, 10 min) serum from starved rats, as described previously [29]. One milliunit of lipolytic activity was defined as that releasing 1 nmol of fatty acid/min at 37°C.

The LPL mass in the extract of the powder was measured using an ELISA kit for LPL. Briefly, we used an enzyme-linked immunosorbent assay with a mouse monoclonal antibody to bovine LPL as a coat to capture LPL in the samples. The extracts of the powders were incubated for 2 h and then were detected with a chicken antiserum to bovine LPL followed by a peroxidase-labeled anti-chicken IgG antibody. A solution containing *O*-phenylenediamine and H_2O_2 was used as a substrate solution. The absorbance at 492 nm was measured in a microplate reader.

Synthesis of LPL

OST cells were treated for 46 h with or without 10^{-5} M cortisol, washed twice with PBS and then were incubated for 30 min in methionine-deficient Dulbecco's modified Eagle's medium containing the appropriate additive. Next, 200 μCi of [^{35}S]methionine was added to each plate and the plates were incubated for 2 h at 37°C. The cells were washed once with ice-cold PBS, harvested in a lysis buffer (pH 7.5) containing 0.2 M Tris, 3% Triton X-100, 1% *N*-lauroylsarcosine, 0.15 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF), sonicated briefly at 0°C, and centrifuged for 20 min at 4°C and 12,000*g*. The amount of ^{35}S incorporated into the total protein in an aliquot of the infranatant was determined by TCA precipitation, as described previously [17].

^{35}S-Labeled LPL was immunoprecipitated from another aliquot of the infranatant with a chicken antiserum to bovine LPL and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously [29]. The gels were stained with Coomassie blue, destained, impregnated with ENHANCE®, and dried on a sheet of cellophane under vacuum. Autoradiographs were obtained by exposing Kodak X-Omat film to the dried gels at –80°C for 14 days. The band corresponding to LPL was cut out from the gel and dissolved in 0.75 ml of 30% hydrogen peroxide at 65°C overnight. Radioactivity was determined using a liquid scintillation counter.

Immunohistochemistry for glucocorticoid receptor (GR) and VEGF

Immunohistochemistry for GR and VEGF was performed as follows. OST cells were treated for 48 h with or without 10^{-5} M cortisol, washed twice with ice-cold PBS containing 0.02% sodium azide, fixed in 4% paraformaldehyde (PFA) for 30 min at 4°C, and then were washed 3 times with PBS containing 1% BSA for 30 min. Next, the cells were incubated with either a rabbit polyclonal antibody to the GR or a mouse monoclonal antibody to VEGF overnight and immersed three times in PBS containing 0.05% Tween 20 (PBS-T) for 10 min. The GR-positive cells and VEGF-positive cells were visualized using an immunostaining kit according to the manufacturer's instructions. The nucleus was counterstained with Hematoxylin. The VEGF-labeling score was determined as described previously [26]. Briefly, 4 different microscopic fields per plate were photographed and VEGF-positive cells present in approximately 1,000 cells per photograph were counted. Since the staining intensity varied significantly, the VEGF expression was determined to be ''0'' if negative, ''1+'' for weak intensity, and ''2+'' for either intermediate or strong intensity. The VEGF-labeling score was evaluated as follows:

$$\begin{aligned}&\text{VEGF}-\text{labeling score}\\&\quad=\ [(1\times\text{number of ''1+'' cells}\\&\qquad+2\times\text{number of ''2+'' cells})/\\&\qquad\text{number of total cells}]\times 100.\end{aligned}$$

The number of total cells is the sum of numbers of ''0'', ''1+'' and ''2+'' cells. The results were expressed as the mean ± SD for four determinations.

Regarding immunohistochemistry for the GR, the cells that were incubated with normal rabbit serum, instead of a rabbit polyclonal antibody to the GR, were used as a negative control.

Western blotting

OST cells were treated for 48 h with or without 10^{-5} M cortisol, washed twice with PBS, lysed in a lysis buffer (20 mM HEPES, 3 mM $MgCl_2$, 2 mM EDTA, 100 mM NaF, 1% Triton X-100, 1 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.4) for 20 min at 4°C, and centrifuged to remove any insoluble materials. The protein concentrations in the cell lysates were measured using a *DC* Protein Assay. The same amount (10 μg of protein/lane) of the proteins was denatured by boiling in Laemmli sample buffer containing 10% 2-mercaptoethanol, separated by SDS-PAGE, and transferred electrophoretically to a PVDF membrane (Hybond-P). The blotted membranes were incubated with the indicated primary antibodies (1:500) and horseradish peroxidase-conjugated secondary antibody (1:25,000). Blots were visualized using an ECL Plus Western Blotting Starter kit according to the manufacturer's instructions. The membrane was exposed to a Hyperfilm™ MP with an intensifying screen for approximately 5–15 min. The relative densitometric units were determined using the analysis software program Diversity Database™.

Statistical analysis

Student's *t*-test was used to compare the mean values. For all statistical analyses, the criterion of significance was $P < 0.05$. All data were expressed as the mean ± SD.

Results

Effect of cortisol on the proliferation of OST cells

OST cells were treated for 48 h with cortisol and incubated with [^{3}H]thymidine during the last 30 min of the 48-h treatment period, and the amount of [^{3}H] incorporated into DNA was measured. When the values were expressed as per plate, the amount of ^{3}H in DNA in the cortisol-treated cultures was 45% of that in the untreated cultures (Fig. 1a). As the DNA content of the cortisol-treated cultures was also lower than that of the untreated cultures [the former cultures ($n = 4$), 373 ± 8 μg/plate; the latter cultures ($n = 4$), 400 ± 10 μg/plate; $P < 0.01$], difference in the amount of ^{3}H in DNA was reevaluated after adjustment for the DNA content. The amount of ^{3}H in DNA was 331 cpm/μg DNA in the untreated cultures and 159 cpm/μg DNA in the cortisol-treated cultures. These results indicate that cortisol might inhibit cell proliferation.

To confirm this, we analyzed the expression of PCNA in OST cells by Western blotting, because PCNA appears in the nuclei of proliferating cells [30, 31] and is often used as a marker of proliferating cells [11, 32, 33]. Like the untreated cultures, the cortisol-treated cultures contained PCNA with Mr = 36 kDa. The level of PCNA in the cortisol-treated cultures was 53% of that in the untreated cultures (Fig. 1b). This result indicates that the number of proliferating cells was smaller in the cortisol-treated cultures than in the untreated cultures.

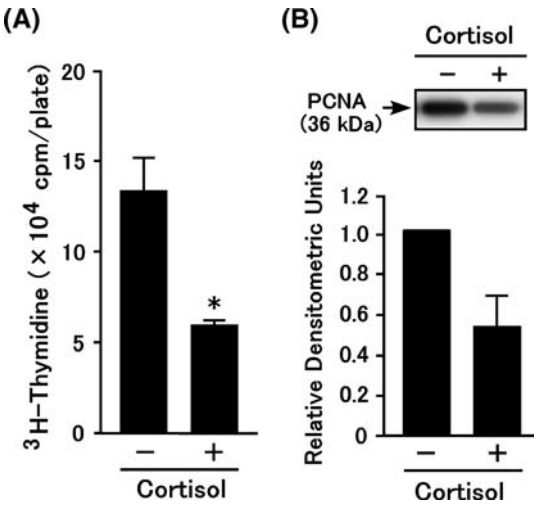
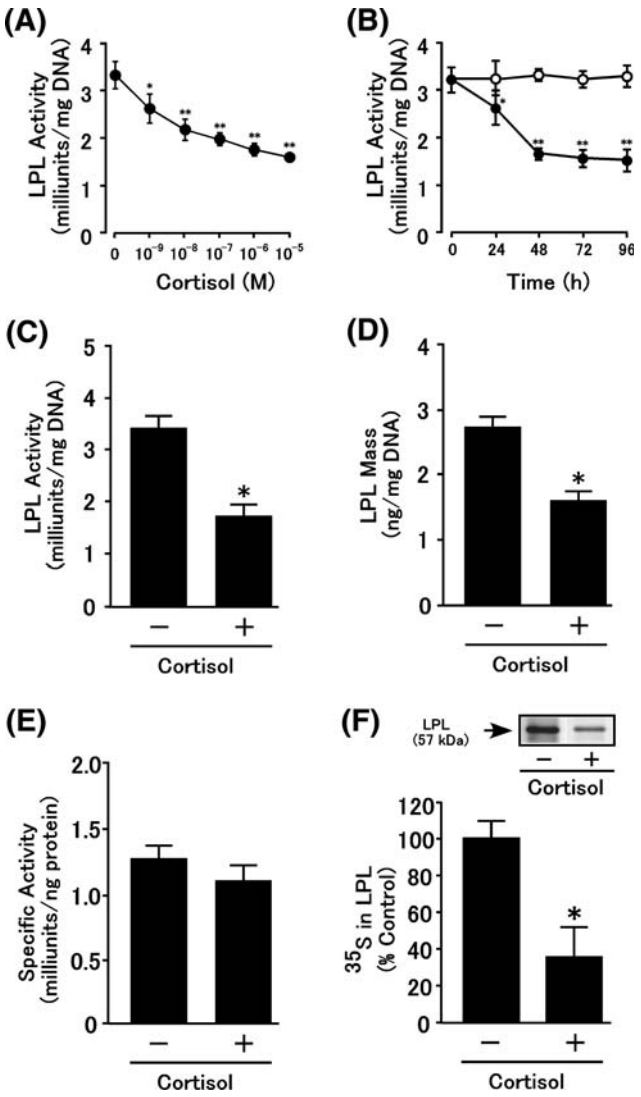


Fig. 1 Effect of cortisol on cell proliferation in cultures of OST cells. **a** Effect of cortisol on the incorporation of [^{3}H]thymidine into DNA. OST cells were treated for 47 h with or without 10^{-5} M cortisol. The plate was replenished with a fresh medium. Thirty minutes later, 1.0 μCi [^{3}H]thymidine was added. The cells were incubated for an additional 30 min and lysed in PBS containing 0.1% SDS and 1 mM EDTA. ^{3}H-Labeled DNA was precipitated with 10% TCA. The precipitate was collected on a membrane filter and washed with 10% TCA. The membrane was dissolved in a liquid scintillation cocktail and the radioactivity was determined in a liquid scintillation counter. Values given are the mean ± SD for four plates. * $P < 0.01$ (compared with the value obtained in the absence of cortisol). **b** Effect of cortisol on PCNA expression. OST cells were treated for 48 h with or without 10^{-5} M cortisol, lysed in a lysis buffer, and centrifuged. The same amount (10 μg of protein/lane) of the proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Western blotting for PCNA was performed using a mouse monoclonal antibody to PCNA and an ECL Plus Western Blotting Detection kit. Relative densitometric units of PCNA were determined using an analysis software program Diversity Database™. Values given are the mean ± SD for three determinants

Effect of cortisol on synthesis, mass and activity of LPL

OST cells were treated for 48 h with cortisol at the indicated concentrations and the LPL activity in the extract of an acetone/ether powder of cells was measured (Fig. 2a). The untreated cultures contained 3.43 milliunits/mg DNA of LPL activity. Cortisol decreased the LPL activity in a dose-dependent manner. Cortisol at 10^{-5} M decreased the LPL activity by 52%. Figure 2b shows the time course of cortisol-induced decrease in the LPL activity. The cortisol



◀ **Fig. 2** Effect of cortisol on LPL activity, LPL mass and LPL synthesis in cultures of OST cells. **a** OST cells were treated for 48 h with cortisol at the indicated concentrations, harvested, and sonicated briefly at 0°C. An aliquot of the homogenate was used to make acetone/ether powder and another aliquot was used to measure the DNA content. The LPL activity was measured in an aqueous extract of an acetone/ether powder. Values given are the mean ± SD for four plates. * $P < 0.05$, ** $P < 0.01$ (compared with the value obtained in the absence of cortisol). **b** OST cells were treated without (○) or with (●) 10^{-5} M cortisol. The cells were harvested at the indicated intervals, and sonicated briefly at 0°C. Values given are the mean ± SD for four plates. * $P < 0.05$, ** $P < 0.01$ (compared with the value obtained in the absence of cortisol). **c–e** OST cells were treated for 48 h with or without 10^{-5} M cortisol, harvested and sonicated briefly at 0°C. An aliquot of the homogenate was used to make an acetone/ether powder. The activity and mass of LPL were measured in an aqueous extract of an acetone/ether powder. The specific activity of LPL was also calculated. Values given are the mean ± SD for four plates. * $P < 0.01$ (compared with the value obtained in the absence of cortisol). **f** OST cells were treated for 46 h with or without 10^{-5} M cortisol. Next, 200 μCi [^{35}S]methionine was added. Two hours later, cells were harvested and sonicated briefly at 0°C. ^{35}S-Labeled LPL was immunoprecipitated with a chicken antiserum to bovine LPL, and separated with SDS-PAGE. The band corresponding to LPL was cut out and dissolved and radioactivity was determined in a liquid scintillation counter. Values given are the mean ± SD for four plates

linearly decreased the LPL activity up to 48 h and thereafter it gradually decreased it.

Next, OST cells were treated for 48 h with 10^{-5} M cortisol and the activity and mass of LPL in the extract of an acetone/ether powder were measured. The untreated cultures contained 3.37 milliunits/mg DNA of LPL activity and 2.71 ng/mg DNA of LPL mass (Fig. 2c, d). The specific activity of LPL of these cultures was 1.24 milliunits/ng enzyme protein (Fig. 2e). Cortisol decreased the activity and mass of LPL by 50 and 42%, respectively. The specific activity of LPL of the cortisol-treated cultures was similar to that of the untreated cultures. This result indicates that the cortisol-induced decrease in LPL activity resulted from the decreased LPL mass.

OST cells were treated for 48 h with 10^{-5} M cortisol and incubated with [^{35}S]methionine during the last 2 h of the 48-h treatment period. Next, ^{35}S-labeled LPL was immunoprecipitated with a chicken antiserum to LPL from the cell extract and resolved by SDS-PAGE. ^{35}S-Labeled LPL of the cortisol-treated cultures migrated on SDS-PAGE with the same mobility (Mr = 57 kDa) as that of the untreated cultures (Fig. 2f), thus indicating that OST cells in the cortisol-treated cultures synthesized normal-sized LPL. The amount of ^{35}S incorporated into LPL in the cortisol-treated cultures was 36% of that in the untreated cultures. There was no difference in the amount of ^{35}S incorporated into the total protein between the untreated cultures and cortisol-treated cultures (data not shown). These results indicate that the cortisol-induced decrease in the LPL mass resulted from a decreased synthesis of LPL.

Involvement of the GR in cortisol-induced actions

Due to the fact that whether OST cells express GR has not yet been elucidated, we examined the expression of the GR in OST cells by immunohistochemistry for GR (Fig. 3). When PFA-fixed cells were treated with non-immune serum, no cells were stained. When the cells were treated with an antibody to the GR, almost all cells were positively immunostained. As a result, OST cells expressed GR.

To determine whether the function of GR was required for the cortisol-induced actions in OST cells, the following experiments were performed using the GR antagonist, RU 486. The simultaneous presence of RU 486 with cortisol reversed the decreasing effect of cortisol on the amount of ^{3}H incorporated into the DNA (Fig. 4a), the level of PCNA (Fig. 4b) and the level of LPL activity (Fig. 4c). The

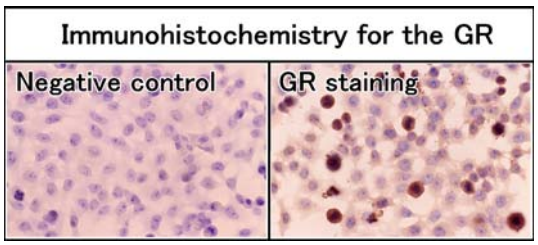


Fig. 3 Immunohistochemical staining for GR. The plate was replenished with a fresh medium. Forty-eight hours later, OST cells were fixed and incubated with either a normal rabbit serum (*left panel*) or a rabbit polyclonal antibody to GR (*right panel*). Immunohistochemical staining for GR was performed by the polymer immunocomplex method as described in the Materials and methods

presence of RU 486 alone did not affect the two parameters for cell proliferation and the LPL activity. These results indicate that cortisol inhibited cell proliferation and decreased the level of LPL activity by the mechanism that involved the GR. Since using cultured human placental trophoblast cells, Sun et al. [34] and van Beek et al. [35] reported that RU 486 by itself did not have the ability to increase 11β-hydroxysteroid dehydrogenase (11β-HSD) activity, which interconverts hormonally active cortisol to inactive cortisone [36], it is unlikely that the reversal of the cortisol-induced actions by RU 486 in OST cells was due to the inactivation of cortisol.

Effect of cortisol on the expression of VEGF

OST cells were treated with cortisol for 48 h and the expression of VEGF in OST cells was analyzed by immunohistochemistry for VEGF (Fig. 5a). Positive immunostaining with VEGF was predominantly observed in the cytoplasm. In the untreated cultures, the majority of cells were intensely immunostained (Fig. 5a, upper panel, left) and the VEGF-labeling score was 155 ± 6 (Fig. 5b), indicating that they expressed a high concentration of VEGF. In the cortisol-treated cultures, immunostaining of VEGF was weak (Fig. 5a, upper panel, right) and the VEGF-labeling score was 74% of that of the untreated cultures. These results indicate that cortisol might thus inhibit the expression of VEGF. To confirm this, the

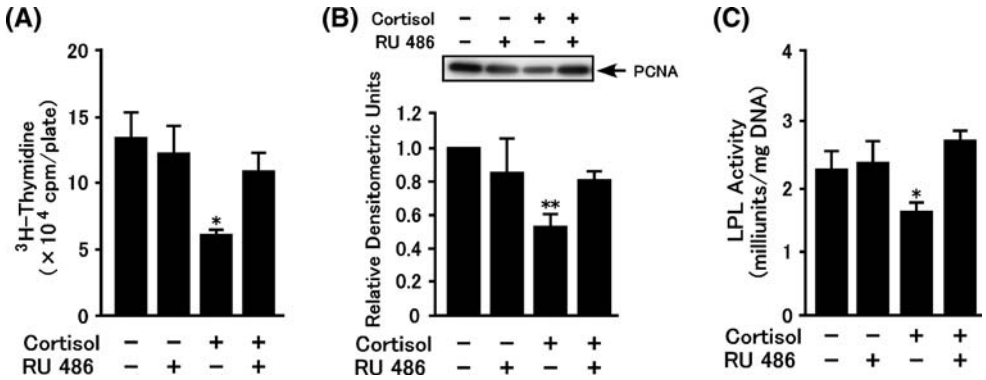


Fig. 4 Effect of RU 486 on cortisol-induced changes in ^{3}H-labeled thymidine incorporation (**a**), PCNA expression (**b**), and LPL activity (**c**). OST cells were treated for 48 h with or without 10^{-5} M cortisol in the absence or presence of 1 μM RU 486. * $P < 0.01$ (compared with the value obtained in the absence of cortisol). ** $P < 0.01$ (compared with the value obtained in the presence of cortisol and RU 482)

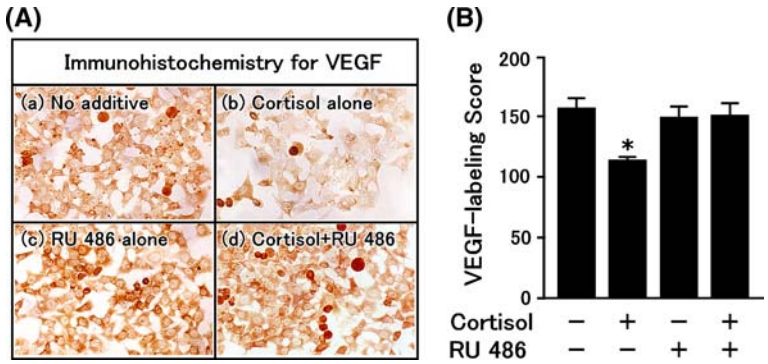


Fig. 5 Immunohistochemical staining for VEGF. **a** OST cells were treated for 48 h with or without 10^{-5} M cortisol in the absence or presence of 1 μM RU 486. Immunohistochemical staining for VEGF was performed by the polymer immunocomplex method as described in the Materials and methods. *a* No additive; *b* cortisol alone; *c* RU 486 alone; *d* cortisol plus RU 486. **b** The VEGF-labeling score in Fig. 5a was determined. Four different microscopic fields per plate were photographed and VEGF-positive cells present in approximately 1,000 cells per photograph were counted. The VEGF-labeling score was evaluated as described in the ''Materials and methods''. Values given are the mean ± SD of four determinations. * $P < 0.01$ (compared with the value obtained in the absence of cortisol)

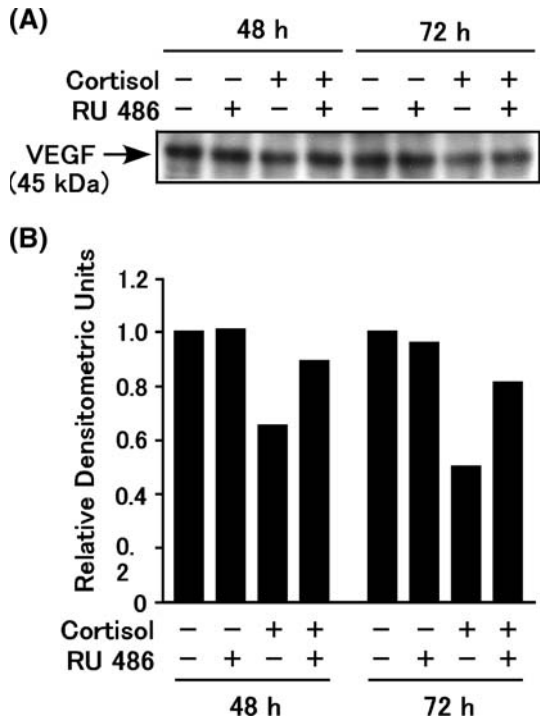


Fig. 6 Western blotting for VEGF. **a** OST cells were treated for either 48 or 72 h with or without 10^{-5} M cortisol in the absence or presence of 1 μM RU 486, lysed in a lysis buffer, and centrifuged. The proteins (10 μg/lane) in the supernatant were separated by SDS-PAGE and transferred to a PVDF membrane. Western blotting for VEGF was performed using a mouse monoclonal antibody to VEGF and an ECL Plus Western Blotting Detection kit. **b** Relative densitometric units of PCNA were determined using an analysis software program Diversity Database™

expression of VEGF in OST cells was analyzed by Western blotting. Similar to the untreated cultures, the cortisol-treated cultures contained VEGF with Mr = 45 kDa (Fig. 6a). The levels of VEGF in the cultures treated with cortisol for 48 and 72 h were 65 and 50%, respectively, of those in the untreated cultures (Fig. 6b).

Although the presence of RU 486 alone did not affect the expression of VEGF (Figs. 5a, lower panel, left and 5b, 6), the simultaneous presence of RU 486 with cortisol reversed the decreasing effect of cortisol on the expression of VEGF (Figs. 5a, lower panel, right and 5b, 6). These results indicate that the cortisol-induced decrease in the expression of VEGF was mediated by GR.

Discussion

Glucocorticoids inhibited cell proliferation in vivo and in vitro in various kinds of experimental solid tumors, including fibrosarcoma [37, 38] and mammary tumors [39]. Moreover, Posner et al. [40] reported that in certain patients with epidural metastatic tumors, glucocorticoids displayed a marked oncolytic effect. In the first set of experiments, we examined whether cortisol had an anti-proliferative activity on OST cells. Since the amount of [^{3}H]thymidine incorporated into DNA was lower in the cortisol-treated cultures than in the untreated cultures, this finding suggested that cortisol had the ability to inhibit DNA replication. This was confirmed by the finding that the level of PCNA in the cortisol-treated cultures was lower than that in the untreated cultures. The expression of PCNA in cells is closely linked to the cell cycle: The level of PCNA in the nucleus begins to increase during the late G1 phase immediately before the onset of DNA synthesis, peaks during the S phase, and then decreases again during the G2 and M phases [30, 31]. Based on these findings, we concluded that cortisol arrested OST cells in the G0/G1 phase, thus resulting in a decreased DNA synthesis and decreased cell proliferation. Song [3] also observed an inhibitory effect of dexamethasone on cell proliferation in cultures of another human osteosarcoma cell line, HOS-8603.

The present study with RU 486 showed that cortisol inhibited the proliferation of OST cells by a mechanism that involved GR. This was consistent with the findings of others that the anti-proliferative activity of glucocorticoids in tumor cell cultures was GR-dependent [2–4]. In mice, Kudawara et al. [4] found dexamethasone to inhibit the in vivo growth of murine osteosarcoma via GR. They described that the inhibitory action of dexamethasone on tumor growth seems to be mediated by an unknown mechanism via GR rather than apoptosis or differentiation [4]. Moreover, using a cell line established from human leiomyosarcoma, called SK-LMS-1, Madsen et al. [41] showed that dexamethasone had an inhibitory effect on in vivo tumor growth and stimulatory effects on in vitro tumor growth. These findings suggest that the inhibitory effect of glucocorticoids on in vivo tumor growth might not merely be due to a direct cytotoxic effect.

Proliferating tumor cells require a large amount of energy for growth. Many studies demonstrated the importance of free fatty acids as a source of energy for tumor growth [5–10]. These fatty acids are in part those synthesized de novo in tumor cells and other large parts were supplied as either albumin-bound free fatty acids or as esterified fatty acids in lipoproteins. Brenneman and Spector [5] reported that triacylglycerol in very low-density lipoproteins present in the ascites fluid of mice with Ehrlich tumor was utilized as the sources of energy for tumor growth and of cellular lipids. In general, triacylglycerol is hydrolysed into free fatty acids and monoacylglycerols by LPL before uptake by the cells. We previously found that OST cells contained LPL and then released it into the culture medium and that the LPL produced by OST

cells played a role in supplying free fatty acids from triacylglycerol as the sources of energy and of synthesis of cellular lipid components for cell growth [8, 11]. In the second set of experiments, we therefore examined the effect of cortisol on the expression of LPL activity. The expression of LPL activity involves the LPL gene transcription, translation and posttranslational modification [42]. The results of the measurements of the activity and mass of LPL showed that cortisol decreased the LPL mass, thus resulting in a decreased LPL activity. A study with [^{35}S]methionine showed that cortisol decreased the synthetic rate of LPL. Moreover, a study with endoglycosidase H, which cleaves the high mannose-type oligosaccharide chain but not complex type chain of glycoproteins [43], showed that no difference in the processing of the oligosaccharide chains of LPL was observed between the untreated and cortisol-treated cultures (data not shown). These findings indicate that cortisol decreased LPL synthesis, thus resulting in a decrease in the expression of LPL activity. As a result, cotisol may be a potent inhibitor of the LPL gene expression in OST cells. This interpretation may be supported by the finding that the blockage of GR by RU 486 completely reversed the decreasing effect of cortisol on the expression of LPL activity.

Tumor growth is preceded by an increased vascular supply of oxygen and nutrients to the tumor. Therefore, angiogenesis within tumors plays a pivotal role in the maintenance, growth and metastasis of many solid tumors [22]. Tumor cells produce potent angiogenic factors, such as VEGF and fibroblast growth factors [20–25]. Kaya et al. [23] reported that 63% of biopsy tumor specimens of 27 patients with osteosarcoma were VEGF-positive while the remaining 27% were VEGF-negative. They also reported that the local microvessel density in osteosarcoma was higher in the VEGF-positive tumors than in the VEGF-negative tumors. Chao et al. [20] reported that 78% of 37 patients with soft tissue sarcomas expressed VEGF highly in the tumor and that the intensity of VEGF expression correlated with their tumor grade. Moreover, blocking the action of tumor-produced VEGF by treating tumor-bearing mice with a monoclonal antibody to VEGF has been reported to decrease the density of the vessels and suppress tumor growth [24]. Some reports have described that NSAIDs, such as ketoprofen and indomethacin, inhibited the expression of VEGF, reduced the density of vessels within the tumor, and suppressed tumor growth [25, 26]. Therefore, VEGF regulates tumor angiogenesis and tumor growth. In the last set of experiments, we examined the effect of cortisol on the expression of VEGF in OST cells. Immunohistochemistry for VEGF showed that the cortisol-treated cultures contained VEGF-positive cells, but their intensity of staining was weaker than that of the untreated cultures. A study with RU 486 showed the function of GR was required for the cortisol-induced inhibition of the expression of VEGF. Similar results were obtained by a Western blotting analysis for VEGF. These findings suggested that treatment with cortisol might inhibit the expression of VEGF through GR.

In conclusion, cortisol not only has the ability to inhibit cell proliferation but also the ability to inhibit the expressions of LPL and VEGF in cultures of OST cells. This suggests that cortisol may inhibit the supply of fatty acids as fuels for osteosarcoma growth and the development of feeder vessels within osteosarcoma. The present study may offer a new strategy against osteosarcoma, though glucocorticoids may cause some adverse side effects such as peptic ulcer, hypertension and diabetes mellitus [44].

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