Glucocorticoids Suppress Tumor Lymphangiogenesis of Prostate Cancer Cells

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Abstract

Purpose: Glucocorticoids such as prednisone, hydrocortisone, and dexamethasone are known to provide some clinical benefit for patients with hormone-refractory prostate cancer. However, the underlying mechanisms by which glucocorticoids affect hormone-refractory prostate cancer progression are not well established as yet. Our previous study has shown that glucocorticoids inhibit tumor angiogenesis possibly by down-regulation of vascular endothelial growth factor (VEGF) and interleukin 8. Here, we hypothesized that the therapeutic effect of dexamethasone on hormone-refractory prostate cancer can be partly attributed to a direct inhibition of lymphangiogenesis through the glucocorticoid receptor by down-regulating a major lymphangiogenic factor, VEGF-C.

Experimental Design: The effects of dexamethasone on the expression of VEGF-C and its receptor, VEGF receptor-3 (VEGFR-3), were examined using an androgen-independent human prostate cancer cell line, DU145, which expresses glucocorticoid receptor. The effects of dexamethasone on tumor-associated lymphangiogenesis in DU145 xenografts were determined by analyzing VEGF-C gene expression, lymphatic vessel density, and relative lymphatic vessel area.

Results: Dexamethasone significantly down-regulated VEGF-C gene expression and protein production by 48% (P = 0.003) and 44% (P = 0.002), respectively, under normoxic condition. Similarly, hydrocortisone down-regulated VEGF-C gene expression. The effects of dexamethasone were completely reversed by the glucocorticoid receptor antagonist RU486. Even under hypoxia-like conditions, dexamethasone inhibited VEGF-C gene expression. In DU145 xenografts, dexamethasone significantly down-regulated VEGF-C gene expression and decreased lymphangiogenesis. Dexamethasone did not affect VEGF-R-3 gene expression in vitro and in vivo.

Conclusion: Glucocorticoids suppressed tumor-associated lymphangiogenesis by down-regulating VEGF-C through glucocorticoid receptor in androgen-independent prostate cancer cells in vivo.

Prostate cancer is the most common noncutaneous cancer and the second leading cause of cancer-related deaths in men in the United States (1), and the number of prostate cancer patients has been rapidly increasing in Japan (2). Prostate cancer is proposed to change the initial property of androgen-dependent growth to androgen-independent and finally hormone-refractory growth through hormonal therapy (3). Among several treatment options, glucocorticoids (i.e., prednisone, hydrocortisone, or dexamethasone) are known to provide some benefit for hormone-refractory prostate cancer patients. Accumulating evidence of clinical effects of glucocorticoids on hormone-refractory prostate cancer showed a >50% decline in prostate-specific antigen levels in 20% to 79% of patients with hormone-refractory prostate cancer (4–7). Glucocorticoids are therefore recognized as one of a limited number of treatment options for hormone-refractory prostate cancer.

The distinct mechanism of glucocorticoids for hormone-refractory prostate cancer remains ill defined. To date, the effects of glucocorticoids have been proposed to be due to the inhibitory effects on adrenal androgen production (8, 9) and cell proliferation of androgen-independent prostate cancer cells (10). More recently, our group showed that dexamethasone, a synthetic glucocorticoid, suppresses androgen-independent prostate cancer growth in vivo, possibly due to the inhibition of tumor-associated angiogenesis by decreasing vascular endothelial growth factor (VEGF) and interleukin-8 productions directly through glucocorticoid receptor (11), and that in renal cell carcinoma cell lines, dexamethasone suppresses VEGF expression in vitro (12).

Lymphatic spread has been implicated in poor prognosis in various neoplasms, and tumor lymphangiogenesis is a fundamental event in the process of lymphogenic metastasis (13). VEGF-C pathway is well documented as one of the key regulators of tumor lymphangiogenesis and lymph node metastasis (14–16). In prostate cancer, VEGF-C expression was recently shown to be positively associated with high pathologic grade, lymph node metastasis, and poor prognosis (17, 18). In the present study, we hypothesized that the therapeutic effect of dexamethasone on hormone-refractory
prostate cancer can be partly attributed to the direct inhibition of lymphangiogenesis through glucocorticoid receptor by down-regulating a major lymphangiogenic factor, VEGF-C. To elucidate the mechanism by which dexamethasone suppresses lymphangiogenesis is an important initial step for designing strategies and identifying therapeutic targets for hormone-refractory prostate cancer. To confirm this hypothesis, we examined VEGF-C gene expression and protein production by dexamethasone-treated DU145 cells, an androgen-independent prostate cancer cell line known to express functional glucocorticoid receptors (10).

Materials and Methods

Cell culture and drug treatment. Three human prostate cancer cell lines, DU145, PC-3, and LNCaP, were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO2. For treatment, cells were plated at 2 × 104 per well in 96-well assay plates with 100 μl of medium with 10% dextran-coated charcoal-stripped FBS, at 2 × 104 per well in 24-well plates with 500 μl of medium, or at 4 × 103 per dish in 100-mm dishes. After 12 to 24 hours of incubation, cells were treated with 2% dextran-coated charcoal-stripped FBS with or without the test agents. Dexamethasone, hydrocortisone, 5α-dihydrotestosterone, and RU486 were purchased from Sigma (St. Louis, MO). RU486 was used as a glucocorticoid receptor antagonist.

Reverse transcription-PCR. Total RNA was extracted from the prostate cancer cells using ISOGENE according to the instructions of the manufacturer (Wako, Osaka, Japan). cDNA was synthesized using a ThermoScript RT system (Invitrogen Corp., Carlsbad, CA) with 2 μg of total RNA from each of the prostate cancer cell lines (DU145, PC-3, and LNCaP) and produced in final volumes of 20 μl. To evaluate the mRNA expression levels of VEGF-C and its receptor, VEGFR-3 (VEGFR-3), the ratios of VEGF-C and VEGFR-3 to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were measured with real-time quantitative PCR using a LightCycler system as previously reported (19). The PCR primers were designed using the primer3 software and purchased from Bio-Rad Laboratories (Burlingame, CA) according to the protocol of the manufacturer. The sections were visualized by incubating the slides with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hematoxylin (Nichirei Bioscience, Tokyo, Japan) as chromogen and counterstained with Meyer's hematoxylin (MITO Pure Chemicals Co., Ltd., Tokyo, Japan). Negative controls were included by replacement of the primary antibody with PBS and showed no specific staining.

Lymphatic vessel density was assessed by procedures as previously reported (21). Briefly, areas with the highest neovascularization were identified by scanning the tumor sections with light microscopy at low magnification (×40 and ×100). Individual lymphatic vessel counts were made at the areas on a ×200 field (20× objective and 10× ocular, 0.74 mm² per field). Any brown-stained lymphatic endothelial cell or endothelial cluster, clearly separated from adjacent blood vessels, tumor cells, and other connective tissue elements, was considered a single, countable lymphatic vessel. The results were independently reviewed by two blinded authors (Y.F. and A.I.) and two areas of highest lymphatic vessel density identified within any single ×200 field were selected from each sample. Data were expressed as the average of these highest counts.

To confirm the results of lymphatic vessel density, relative lymphatic vessel area was evaluated by computer-assisted quantitative analysis. First, two areas of the highest lymphatic vessel density were identified from each sample. Digital imaging of each hotspot was captured with a Polaroid PDMCle digital camera (Polaroid, Waltham, MA). Relative lymphatic vessel area, which was defined as the percentage of methanesulfonfluoride (Fluka, Buchs, Switzerland). The suspensions were centrifuged at 12,000 × g and the supernatants were stored at −20°C until protein assay. To evaluate VEGF-C protein concentrations, the ratios of VEGF-C to total protein were measured with Human VEGF-C Assay Kit-IBL (IBL Co., Ltd. Takasaki, Japan) and Autokit Micro TP (Wako) according to the instructions of the manufacturers.

In vivo xenograft model. Animal studies complied with the Animal Welfare Regulation of Tokyo Medical and Dental University. To establish a DU145 tumor xenograft model, DU145 cells were detached with trypsin, and a mixture of RPMI 1640 with 10% FBS containing DU145 cells and Matrigel Basement Membrane Matrix (1:1, v/v; BD Biosciences, Bedford, MA) was prepared immediately before inoculation. The cells (1 × 10⁶) were placed in the dorsal s.c. space of 6-week-old male BALB/c (nu/nu) mice. When the average tumor volume reached 200 to 300 mm³, 2 weeks after inoculation, mice were randomly assigned to a control or experimental group (nine mice each). In the experimental group, each mouse was given a s.c. peritumor injection thrice of 1 μg dexamethasone, which had been dissolved in ethanol and diluted 1:2,000 in 100-μl sterile saline, whereas in the control group, ethanol diluted 1:2,000 in 100-μl sterile saline was injected. The tumor volumes were measured weekly and calculated according to the following formula: length × width²/2. The mice were sacrificed 3 weeks after the treatment and the tumors were removed and then either frozen for mRNA extraction or fixed in 10% buffered formalin for immunohistochemical analysis.

Immunohistochemical analysis of lymphatic vessel density and relative lymphatic vessel area. Paraffin-embedded sections (5 μm thick) from DU145 xenograft tumors were placed on slides, deparaffinized, and rehydrated. Subsequently, the sections were microwave thrice at 500 W for 5 minutes to improve antigen retrieval and incubated with 3% (v/v) hydrogen peroxide in PBS for 20 minutes at room temperature to inhibit endogenous peroxidase activity. After the sections were blocked with 10% goat serum albumin for 30 minutes at room temperature, they were incubated for 1 hour at room temperature with a rat monoclonal anti-mouse antibody against the lymphatic endothelial marker podoplanin (AngioBio Co., Del Mar, CA) at a concentration of 10 μg/ml in PBS. The sections were then rinsed thrice with PBS, incubated with goat anti-rat secondary antibody diluted 1:100 in PBS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 minutes at room temperature, and exposed to the avidin-biotin-peroxidase complex (R.T.U. Vectastain ABC Reagent, Vector Laboratories, Inc., Burlingame, CA) according to the protocol of the manufacturer. The sections were visualized by incubating the slides with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hematoxylin (Nichirei Simple Stain DAB solution, Tokyo, Japan) as chromogen and counterstained with Meyer’s hematoxylin (MITO Pure Chemicals Co., Ltd., Tokyo, Japan). Negative controls were included by replacement of the primary antibody with PBS and showed no specific staining.

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positively stained area, was calculated using Adobe Photoshop (Adobe) and Scion Image for Windows (Scion Corporation, Frederick, MD) software.

Statistical analysis. For multiple comparisons, the significant difference was analyzed by one-way ANOVA followed by a multiple comparison Dunnett’s test. For single comparisons, the level of statistical significance was confirmed by Student’s t test. Values were expressed as the mean and SE. P < 0.05 was considered statistically significant. All statistical tests were two sided. Statistical analysis was done with JMP 5.0 software (SAS Institute, Cary, NC).

Results

Effects of dexamethasone on VEGF-C gene expression and production by prostate cancer cells. We and others have previously reported that DU145 and PC-3 express glucocorticoid receptor mRNA whereas LNCaP lacks the glucocorticoid receptor (10, 11). Dexamethasone does not affect proliferation of these prostate cancer cells when incubated with 2% dextrancoated charcoal-stripped FBS with or without dexamethasone for 48 hours (11).

The hypothesis of this study was that the therapeutic effect of dexamethasone on hormone-refractory prostate cancer can be partly attributed to the inhibition of lymphangiogenesis. To confirm this, we first analyzed the gene expression and protein production by dexamethasone-treated prostate cancer cells for VEGF-C. In DU145 cells, the statistically significant down-regulation of VEGF-C mRNA expression by 48% (P = 0.003) and of protein production by 44% (P = 0.002) were observed in dexamethasone-treated group compared with the control group. In PC-3 cells, no significant decrease in both VEGF-C mRNA expression (P = 0.07) and protein production (P = 0.15) was seen compared with the control group (Fig. 1). The DU145 cell line was chosen for subsequent experiments because it expresses the glucocorticoid receptor and dexamethasone exerted an inhibitory effect on VEGF-C in these cells. LNCaP lacked the glucocorticoid receptor and expressed extremely low VEGF-C mRNA levels (data not shown).

We next examined in DU145 cells whether dexamethasone suppressed VEGF-C expression in a concentration-dependent manner at the mRNA and protein levels. DU145 cells were treated with dexamethasone at concentrations of 1 to 1,000 nmol/L for 12 hours for analysis of VEGF-C mRNA expression and protein production analysis, respectively. Relative values of VEGF-C mRNA expression and protein production and VEGFR-3 mRNA expression were quantified by real-time quantitative RT-PCR and ELISA, respectively. VEGF-C protein concentrations were expressed as the ratios of VEGF-C to total protein. Columns, mean of three independent experiments; bars, SE. *, P < 0.01, versus the control (0 nmol/L) group.
Glucocorticoids Suppress Tumor Lymphangiogenesis

We next assessed whether the effects of dexamethasone are also valid under a chemically induced hypoxia-like condition using CoCl2. DU145 cells were treated with 100 nmol/L dexamethasone and/or 100 μmol/L CoCl2 for 12 hours. Even under the hypoxia-like condition, dexamethasone significantly suppressed VEGF-C mRNA expression by 21% (P < 0.001) compared with the control group. The hypoxia-like condition itself resulted in a significant down-regulation of VEGF-C expression regardless of dexamethasone treatment (P < 0.001; Fig. 4).

In vivo effects of dexamethasone on lymphangiogenesis of DU145 xenograft tumors. In the last set of experiments, we examined whether dexamethasone could inhibit the neolymphangiogenesis of androgen-independent DU145 prostate tumors. The low dose of dexamethasone used in this study, 1 μg per mouse thrice a week, was based on a previous report (10, 11) and is widely considered to be clinically beneficial for patients with hormone-refractory prostate cancer. We initiated the treatment ~2 weeks after the inoculation when the mean tumor volume reached 200 to 300 mm³ to confirm the accomplishments of appropriate inoculation. Dexamethasone significantly reduced tumor growth, which is consistent with our previous results (11). The mice were sacrificed 3 weeks after treatment, and we analyzed lymphangiogenesis-related gene expressions in xenograft tumors to confirm the decrease in transcription levels of VEGF-C in vivo. Dexamethasone significantly down-regulated VEGF-C mRNA expression by 15% (P = 0.025) compared with the control group, whereas it did not affect VEGFR-3 expression (Fig. 5A and B).

We counted lymphatic vessel density in the xenograft tumors to determine whether in vivo administration of dexamethasone affects the intratumor neolymphangiogenesis. Lymphatic vessel density identified by podoplanin immunoreactivity in the dexamethasone treatment group was 30 microvessel counts, which was significantly lower (P < 0.001) than that in the control group (41 microvessel counts) per ×200 field (Fig. 6A and C). To confirm the result, we also evaluated relative lympathic vessel area. As shown in Fig. 6B,
dexamethasone significantly decreased relative lymphatic vessel area (3.9% in dexamethasone group versus 8.1% in the control group, \(P < 0.001\)).

**Discussion**

In this study, we have shown that glucocorticoids suppress a major lymphangiogenic factor, VEGF-C, and tumor-associated lymphangiogenesis in androgen-independent prostate cancer cells. Previous reports have shown that VEGF-C expression is positively correlated with tumor lymphangiogenesis, lymph node metastasis, and poor prognosis in various neoplasms including prostate cancer (14–18). Our results therefore support the hypothesis that the therapeutic effect of dexamethasone on hormone-refractory prostate cancer can be partly attributed to the direct inhibition of lymphangiogenesis through down-regulation of VEGF-C. Recently, it was shown that VEGFR-3 expression is closely associated with lymph node metastasis in prostate cancer (24, 25) and that blockade of VEGFR-3 suppresses tumor lymphangiogenesis and lymph node metastasis in lung cancer (22). Interestingly, our results have shown that dexamethasone does not affect VEGFR-3 mRNA expression under current experimental conditions. It is therefore very possible that the direct inhibition of VEGF-C, but not of VEGFR-3, by dexamethasone leads to the reduction of tumor lymphangiogenesis in androgen-independent prostate cancer.

Cancer cells become hypoxic because the new blood vessels that they develop are aberrant and have poor blood flow (26). Importantly, our results revealed that even under hypoxia-like conditions, dexamethasone significantly down-regulates VEGF-C expression. Interestingly, hypoxia-like condition itself induced down-regulation of VEGF-C in DU145 cells as reported in renal cell carcinoma cells (23). However, the mechanism by which hypoxia regulates VEGF-C gene expression is not well established. Although various lines of evidence have shown that hypoxia regulates several angiogenic factors via hypoxia-inducible factor-1 (27), no hypoxia-inducible factor binding motif can be found in the published sequence of the VEGF-C promoter region examined to date (28, 29). Hypoxia-inducible factor sequences outside of the VEGF-C promoter region or other non-hypoxia-inducible factor-related mechanisms may exist (30, 31).

Recently, glucocorticoid receptor–mediated gene regulation has received considerable attention due to the link between cell proliferation and angiogenesis (32). Glucocorticoid receptor activated by glucocorticoids has been shown to interfere with the transcriptional activity of several transcriptional factors, such as nuclear factor-κB (32, 33) and activator protein-1 (32, 34). Nuclear factor-κB binding motif can be found in the published sequence of the VEGF-C promoter region (28). Therefore, our results indicate that glucocorticoids exert inhibitory effect on VEGF-C, at least in part, by the glucocorticoid receptor–mediated suppression of nuclear factor-κB transcriptional activity.

**Fig. 5.** In vivo effects of dexamethasone on VEGF-C and VEGFR-3 mRNA expression of DU145 xenograft tumors. BALB/c (nu/nu) mice were each given an injection in the dorsal area with \(1 \times 10^7\) DU145 cells. Approximately 2 weeks after the inoculations, the mice were each given a peritumor s.c. injection of 1 µg dexamethasone (\(n = 9\)) or 0.05% ethanol (control; \(n = 9\)) thrice a week for 3 weeks. A and B, quantification of VEGF-C and VEGFR-3 mRNA expression in the DU145 xenograft tumors, respectively. mRNA was assessed by real-time quantitative RT-PCR. Columns, mean; bars, SE. *, \(P < 0.05\), versus the control group.

**Fig. 6.** Effects of in vivo treatment of dexamethasone on tumor lymphangiogenesis in the DU145 xenograft tumors. A, quantification of lymphatic vessels was assessed by lymphatic vessel density, which was expressed as the number of vessels per \(\times 200\) field. Columns, mean lymphatic vessel densities; bars, SE. B, relative lymphatic vessel area was evaluated by computer-assisted quantitative analysis of digital image, which was expressed as the percentage of lymphatic vessel areas per \(\times 200\) field. Columns, mean percentages of lymphatic vessel area; bars, SE. C, immunohistochemical analysis of neolymphangiogenesis with the use of an antibody against podoplanin (brown). *, \(P < 0.01\), versus the control group. Original magnification, \(\times 200\).
Glucocorticoids are not experimental agents but have long been clinically used in the treatment of numerous diseases including rheumatoid arthritis, systemic lupus erythematosus, and arteriosclerosis (35). Low-dose dexamethasone therapy was found to be beneficial for patients with hormone-refractory prostate cancer, inducing significant symptomatic improvements and decreasing prostate-specific antigen levels with mild adverse effects (6, 7). In the current study, we observed a maximal inhibition of VEGF-C expression by dexamethasone at concentrations of 10 to 100 nmol/L, achievable in vivo by oral administration of low doses (1-2 mg/d) of dexamethasone. Therefore, low-dose administration seems to provide more favorable therapeutic effects rather than high-dose administration for patients with hormone-refractory prostate cancer, although prostate cancer is known to consist of a heterogeneous population of cancer cells and may possess distinct biological features from DU145 cells.

Glucocorticoids seem to have various effects on hormone-refractory prostate cancer: inhibitions of adrenal production (8, 9) and tumor cell proliferation (10) and other mechanisms we have shown [i.e., inhibitions of tumor angiogenesis (11) and lymphangiogenesis]. Recently, we and others have shown that the inhibitory effects of dexamethasone on DU145 cell growth are more evident in vivo than in vitro (10, 11). This result indicates that the indirect effects by dexamethasone, such as inhibition of tumor vasculogenesis, are more prominent rather than the direct inhibitory effects on prostate cancer cell growth in vivo.

In summary, the current study indicates that glucocorticoids suppress tumor lymphangiogenesis by down-regulation of VEGF-C in androgen-independent prostate cancer cells directly through the glucocorticoid receptor pathway. The clinical use of dexamethasone in combination with antiandrogen, estrogen, or anticancer agents, such as docetaxel, may enhance the therapeutic effect on hormone-refractory prostate cancer.

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