The Tumor Suppressive Effect of Angiotensin II Type 1 Receptor Antagonist in a Murine Osteosarcoma Model

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Abstract. *Background: Angiogenesis is involved in the growth and metastasis of most solid tumors. Several reports have demonstrated that angiotensin II stimulates growth and migration of certain cancer cell lines and induces angiogenesis through up-regulation of vascular endothelial growth factor. This study examined whether an angiotensin II type 1 receptor (AT1R) antagonist (CV11974) inhibits osteosarcoma progression and distant metastasis. Materials and Methods: Osteosarcoma (LM8) was transplanted into subcutaneous dorsal tissue of C3H mice. The mice were administered CV11974 daily by intraperitoneal injections at 0.1 mg/kg, 1 mg/kg, or 10 mg/kg, or saline for 28 days. Results: Subcutaneous tumor size was smaller in the CV11974 treatment groups than in the control group. Lung and liver metastases were significantly reduced in the CV11974 treatment groups when compared with the control group. Conclusion: CV11974 is widely used to treat hypertension clinically and therefore may be a novel antiangiogenic therapy for osteosarcoma through blocking AT1R-mediated signaling.*

Osteosarcoma is the most frequently occuring primary malignant bone tumor. Despite recent advances in multimodality treatments, including combined chemotherapy and wide tumor resection, pulmonary metastasis occurs in approximately 50% of patients with osteosarcoma and is a major cause of death (1, 2). Therefore, considerable attention has been focused on the mechanisms responsible for tumor growth and metastasis in order to develop novel strategies for treatment.

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Key Words: Osteosarcoma, metastasis, angiotensin II type 1 receptor antagonist, CV11974.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is involved in the growth, maintenance and metastasis of most solid tumors (3, 4). Several reports have demonstrated that increased angiogenesis correlates with poor prognosis for several types of cancers (5, 6), including osteosarcoma (1, 7), suggesting that inhibition of angiogenesis may be a useful strategy for tumor treatment. Angiotensin II, a multifunctional bioactive octapeptide of the renin–angiotensin system, plays a key role as a vasoconstrictor in controlling cardiovascular function and renal homeostasis. Recent reports have demonstrated that angiotensin II stimulates growth and migration of certain cancer cell lines (8-10) and induces angiogenesis through up-regulation of vascular endothelial growth factor (VEGF) (11-13). A recent large-scale clinical trial for hypertension showed that angiotensin-converting enzyme (ACE) inhibitors reduced not only the mortality rate of cardiovascular disease, but also mortality resulting from malignant tumors (14). ACE inhibitors may exhibit these antitumor effects by reducing angiotensin II-mediated tumor angiogenesis. Angiotensin type 1 receptor (AT1R) antagonists also block angiotensin II signaling but are more selective than ACE inhibitors. Previous reports suggest an AT1R antagonist effectively inhibits proliferation of cancer and sarcoma (15-17).

This study examined the efficacy of an AT1R antagonist in inhibiting growth and pulmonary metastasis of an osteosarcoma and characterized its effects on angiogenesis using a murine osteosarcoma cell line (LM8) with a high rate of pulmonary metastasis.

Materials and Methods

Agents and cell line. CV11974 (candesartan), an AT1R antagonist, was supplied by Takeda Chemical Industries (Osaka, Japan). LM8 murine osteosarcoma cells (18) were maintained in DMEM (Life Technologies, Inc, Grand Island, NY, USA) supplemented with 10% fetal calf serum in an air incubator with 5% CO_2 at 37˚C. After suspending LM8 cells in phosphate-buffered saline (PBS), 100 μl

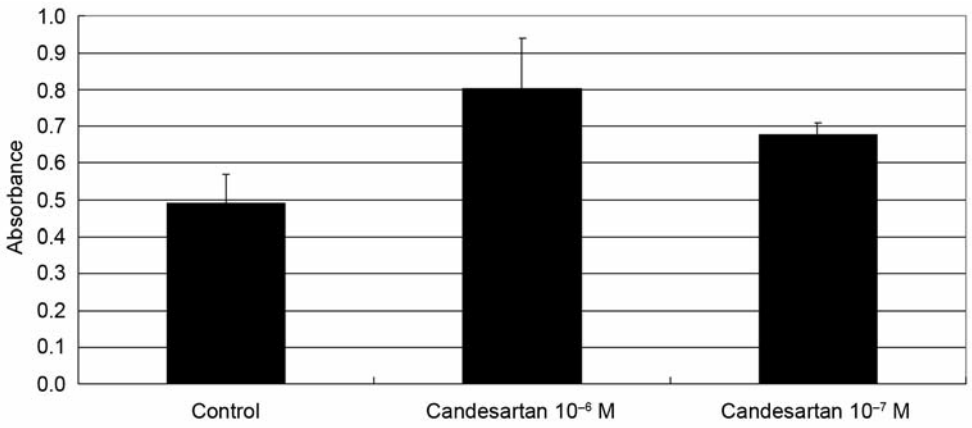

Figure 1. *Proliferation of LM8 cells in response to CV11974 using the MTS assay. Data are expressed as mean±standard deviation of ten animals in each group.*

of the cell suspension (1×10^6 cells) was injected into the dorsal subcutaneous of C3H male mice.

Proliferation assay. Cell proliferation was ascertained using an *in vitro* MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) assay. A total of 1×10^5 LM8 cells were seeded in 96-well plates and cultured in medium alone or medium containing CV11974 at 10^{-6} M or 10^{-7} M for 48 h. Cell proliferation was measured using the CellTiter 96™ AQueous Non-radioactive Cell Proliferation Assay (Promega, Mannheim, Germany).

Grafts of LM8. After suspending LM8 cells in PBS, 100 μl of the cell suspension (1×10^6 cells) were injected into the flank of 5-week-old C3H male mice. The mice were randomly divided into four groups (n=10 mice/group). Treatment was initiated one day after tumor cell injection with daily intraperitoneal injections of CV11974 at 0.1 mg/kg, 1 mg/kg, 10 mg/kg, or PBS (control). Mice were housed in plastic cages on hardwood-chip bedding in an air-conditioned biohazard room with a 12 h light/12 h dark cycle and were allowed free access to food and water. The experimental design was approved by the Animal Care Committee of the Aichi Cancer Center Research Institute, and the animals were cared for in accordance with institutional guidelines. Mice were then sacrificed by an overdose of CO_2 and tumor tissue, the lungs and liver were carefully excised. The total weight of the excised tumors containing tissue was measured immediately after harvesting. Subsequently, the excised lungs and livers were fixed with formalin, embedded in paraffin, sectioned (6 μm thickness) and stained with hematoxylin and eosin for histological observation. The number of lung metastatic colonies was counted under a light microscope with the selected midline section by two investigators in a blinded fashion.

Statsitical analysis. Statistical analysis was performed using the SPSS 12.0 software package (SPSS Inc, Chicago, IL, USA). All data were normally distributed and analyzed with two-way analysis of variance for repeated measurements followed by Student's paired *t*-test. *P*-values ≤0.05 were considered to be statistically significant.

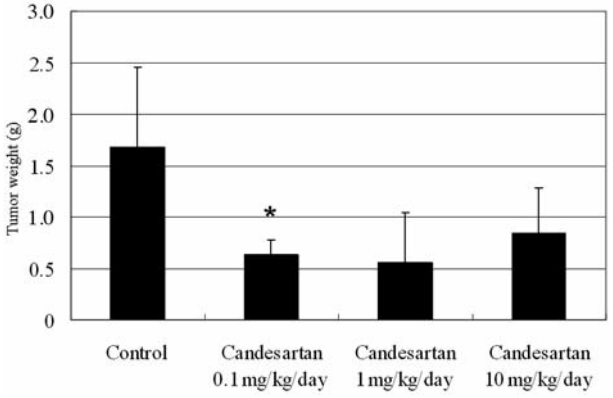

Figure 2. *The weights of grafted LM8 tumors at the time of sacrifice. Data are expressed as the mean±standard deviation of ten animals in each group. *p=0.05 compared with control.*

Results

Effects of CV11974 on cell proliferation of cultured LM8 cells. The effect of CV11974 on *in vitro* cell proliferation was examined. After addition of varying concentrations of CV11974, cell proliferation was examined by the MTS assay and no *in vitro* suppressive effect of CV11974 on LM8 growth was observed (Figure 1).

Inhibition of tumor growth and metastasis by CV11974. The effect of CV11974 treatment on tumor growth and metastasis was investigated. The treatment showed a tendency to suppress the weights of grafted tumors. The weights of grafted tumors were significantly smaller (p=0.05) at the lowest dose of CV11974 (0.1 mg/kg/day) treatment group when compared with the control group but the difference did not reach statistical significance at higher doses (1 or 10 mg/kg/day)

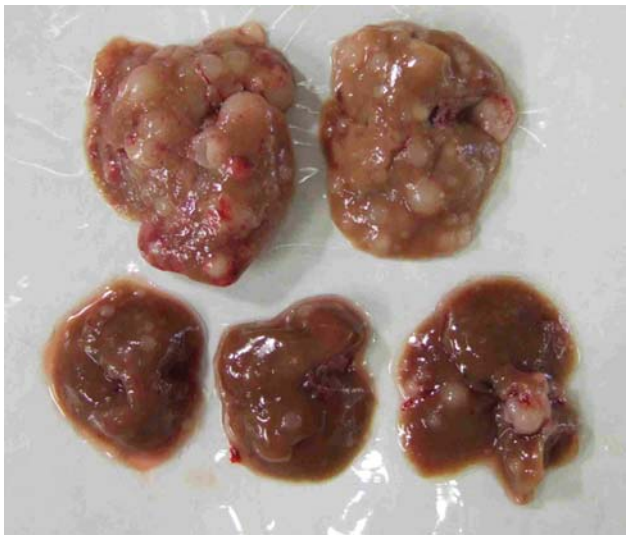

Figure 3. *Macroscopic features of livers. There were many metastatic colonies in the livers of control mice (top) and only a few colonies in the livers of CV11974-treated mice at a dose of 0.1 mg/kg/day (bottom).*

treatment groups (Figure 2). Furthermore, in this study, liver metastases were also reduced in the CV11974 treatment groups compared with the control group (Figure 3), and fewer (p=0.024) lung metastatic colonies were observed in the CV11974 treatment groups compared with the control group (Figures 4 and 5).

Discussion

The data of this study suggest suppressive effects of an AT1R antagonist (CV11974; candesartan) on tumor growth and distant metastasis in a murine osteosarcoma model. Previous studies on other malignant tumors have reported effective inhibition of tumor growth and metastasis through blockade of the renin–angiotensin–androsterone system with AT1R antagonists (16, 17, 19-21). Although the mechanisms involved in antitumor effects of AT1R antagonists have not been elucidated yet, Suganuma *et al*. reported that administration of angiotensin II increases the invasive capacity of tumor cells and VEGF expression (21). Therefore, it is possible that AT1R antagonists inhibit invasive capacity and other factors involved in tumor growth, as well as angiogenesis through blocking AT1R-mediated signaling.

Some studies have suggested that VEGF is expressed in tumor cells themselves, whereas others have indicated that it is expressed in stromal cells surrounding the tumor (22, 23). In either case, interactions within the tumor as well as with the host vascular endothelial cells are crucial. In the present study there was no difference in proliferation of LM8 between *in vitro* cultures with or without adding CV11974,

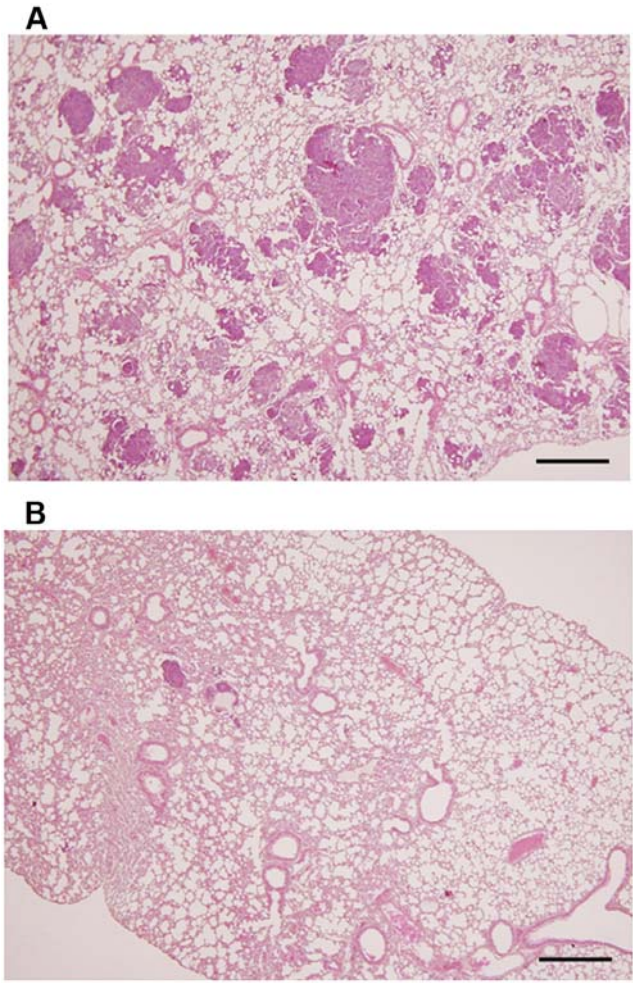

Figure 4. *Lung histology of a control mouse (A) and a mouse treated with CV11974 at a dose of 0.1 mg/kg/day (B). Many metastatic nodules were found in (A) and only few nodules were found in (B). Hematoxylin and eosin staining; Magnification factor, ×40, bars represent 500 μm.*

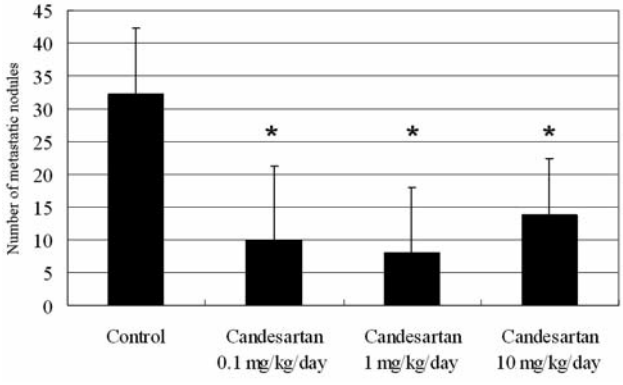

Figure 5. *Number of pulmonary metastatic nodules of LM8. Data are expressed as the mean±standard deviation of ten animals in each group. *p<0.05 compared with control.*

suggesting that AT1R antagonists are unlikely to be directly involved in inhibition of tumor cell proliferation. The immunohistochemical staining sections of some LM8 grafts showed that AT1R and VEGF were expressed primarily in the tumors (data not shown). AT1R antagonists may have suppressive effects during interactions related to angiogenesis such as tumor–host VEGF interaction.

A number of previous studies (3-6) reported that angiogenesis is important for the local growth and distant metastasis of a sarcoma, similar to other types of cancer. Kaya *et al*. suggested that the serum level of VEGF in patients with osteosarcoma proportionally correlates with the recurrence rate (1). In their study of *VEGF* mRNA levels in patients with osteosarcoma, Lee *et al*. reported that expression of the VEGF165 isoform correlated with a higher rate of distant metastasis and a poor prognosis (24). Therefore, a compound that inhibits the action of VEGF is useful as an anticancer drug; based on the data of the present study, CV11974 may act in this fashion.

Advances in anticancer drugs and effective multimodality treatments, consisting of combined surgical resection and chemotherapy, have substantially improved outcomes of osteosarcoma treatments. Nevertheless, pre-existing metastasis at the initial examination or postoperative pulmonary metastasis results in a poor prognosis. As researchers seek new treatment strategies, various experimental studies have reported on antiangiogenesis treatments (18, 25, 26). Unlike experimental antiangiogenesis therapies, this study used an AT1R antagonist, a drug widely used to treat hypertension. Although specific signaling mechanisms related to AT1R-mediated angiogenesis have yet to be elucidated, further investigation is imperative. It will also be necessary to examine the improvement of treatment outcomes by multimodality treatment consisting of chemotherapy and an AT1R antagonist.

Acknowledgements

We thank Sakiko Nishikawa and Akiko Nishioka for technical assistance. This study was supported by a Grant-in-Aid for Scientific Research (c) by the Japanese Society for the Promotion of Science (JSPS) (17591605), Tokyo, Japan.

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Received November 16, 2010
Revised December 16, 2010
Accepted December 17, 2010