Angiotensin II Type I Antagonist Prevents Pulmonary Metastasis of Murine Renal Cancer by Inhibiting Tumor Angiogenesis

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Abstract

Angiotensin II (AII) is a potent vasoconstrictor peptide from the renin-angiotensin system in the kidney. The AT1 receptor (AT1R) is reportedly expressed in several tumors including renal cell carcinoma, and all is involved in tumor angiogenesis. We p.o. administered the long-acting AT1R antagonist, candesartan (10 mg/kg), to the 16 days mouse renal cancer lung metastasis model to test the preventive effects in tumor metastasis. Pulmonary metastases of renal cancer showed prominent AT1R expression in both mice and humans, and candesartan treatment dramatically prevented lung metastatic nodules (14.9 ± 1.8; P < 0.0001; n = 12) in mice along with the inhibition of neovascularization and vascular endothelial growth factor expression, compared with control metastatic mice (123.5 ± 8.6; n = 13). Candesartan is widely used clinically, so it seems to be a reasonable therapy for patients with lung metastases of renal cell carcinoma.

Introduction

Between 20% and 30% of patients with localized RCC2 relapse after radical nephrectomy, although surgery is still considered the standard therapy for this tumor (1). Immunotherapy with IFNs and interleukins has also been studied, because metastatic or relapsed RCC is refractory to adjuvant chemotherapy. However, the overall response rate achieved by immunotherapy remains ~20% (2). The lung is the most common site of distant recurrence with lung metastases being found in 50–60% of patients (3), but there is no known therapy that can reduce the rate of relapse. AII is a key biological peptide in the renin-angiotensin system that regulates blood pressure and renal hemodynamics. There are two major subtypes of the AII receptor, which are AT1R and AT2R. The AT1R belongs to the seven transmembrane domain superfamily, and its stimulation activates classical second messenger systems that lead to the rapid production of diacylglycerol and inositol 1, 4, 5-triphosphate as well as the activation of protein kinase C (4). A potential role of AII in promoting tumor growth has been suspected based on its known hormonal actions in addition to its vasoconstrictor effect (5). Lever et al. (6) reported the first clinical evidence that long-term AII blockade may have a protective effect against cancer and suggested that it could prevent carcinogenesis. There have been several reports that AII can induce neovascularization in experimental systems (7) via the AT1R (8). The AT1R is also expressed frequently in various human tumors (9, 10). A recent in vitro study showed that treatment with an AT1R antagonist could inhibit the growth of a pancreatic cancer cell line, suggesting that AT1R blockade may be a possible treatment for cancer (11). In addition to the potential superiority of AT1R antagonists over angiotensin-converting enzyme inhibitors, the expectation has been raised that these AT1R antagonists may be useful in the prevention of cancer. In rats, the angiogenic action of AII on the cremaster muscle is mediated via the AT1R, whereas stimulation of the AT2R inhibits angiogenesis. Accordingly, specific AT1R blockade might be expected to inhibit carcinogenesis or angiogenesis more efficiently than blockade of both the AT1R and AT2R by an angiotensin-converting enzyme inhibitor. Candesartan was developed as a long-acting specific AT1R antagonist, and it has been used to treat patients with hypertension. We hypothesized that candesartan might be able to specifically delay tumor progression, and we administered it to a mouse renal cancer lung metastasis model to test its effect.

Materials and Methods

Experimental Lung Metastasis Model. Inbred male BALB/c mice were inoculated with 2 × 105 Renca cells via the tail vein on day 0 and were randomly assigned to various treatment groups. Candesartan was generously provided from an affiliate of Takeda Chemical Industries (Osaka, Japan). Candesartan (10 mg/kg) in 0.5 g/ml carboxymethyl cellulose was administered p.o. every day from day 1. Carboxymethyl cellulose solution was administered as a control. On day 16, the mice were euthanized for evaluation of pulmonary metastases. When the lungs were harvested, the lungs were inflated endotracheally with 15% India Black Ink solution and bleached in Fekete’s solution. Total metastatic nodules on the lung surface were counted, as described (12). Animal treatment adhered to approved institutional guidelines.

Lung Histopathology. Lung tissues were fixed in 10% formalin and embedded in paraffin. Paraffin sections (5 μm) were stained with hematoxylin.

TGFB1 Bioassay. Whole lung tissue TGF-β1 was extracted, and MLEC cells were used as described previously (13). MLEC cells (104/well) were plated in 96-multiwell plates, and they were allowed to attach at 37°C in a humidified atmosphere of 5% CO2/95% O2. After 24 h, the medium was replaced with the test sample and incubated overnight at 37°C. For the bioassay, the TGF-β1 in the conditioned medium was activated as follows. Samples were acidified to pH 1.5 with 1N HCl (1 h at 4°C) followed by equilibration at neutral pH with 1N NaOH. At the end of the incubation, [3H]thymidine incorporation assay was performed to determine the effect of samples on MLEC growth. A TGF-β1 standard curve was derived in the range of 0.01–2.0 ng/ml. All of the assays were performed in triplicate. Results are expressed as active form of pg/mg lung tissue TGF-β1.

RCC Sample. Ten lung specimens were randomly selected from patients who had died of RCC (Table 1). There were 7 men and 3 women, with a mean (±SD) age of 56.0 ± 9.3 years. Histological type of 10 samples was all of a conventional type (clear cell).

Immunostaining of the AT1R, CD34, and VEGF. Paraffin sections (5 μm) were deparaffinized, rehydrated, and washed in PBS. Endogenous peroxidase was quenched. A blocking step was included using 1% BSA (or 5% goat serum) in conjunction with avidin and biotin blocking solutions. Primary antibody (anti-AT1R polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA; anti-CD34 monoclonal antibody; Hy Cult Biotechnology, Uden, Netherlands; anti-VEGF polyclonal antibody; NeoMarkers, Fremont, CA) was

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conventional type (clear cell).
applied at 4°C overnight. Biotinylated secondary antibody was applied, then incubated with avidin-biotin peroxidase complex, and developed with diaminobenzene. After washing slides, counterstaining was done with 10% hematoxylin for 1–2 min. CD34-positive neovessels were counted in 10 high-power fields (×400) by two independent investigators who operated in a blinded fashion. The intensity of VEGF staining in tumor lesions was graded on a scale of 0 to +3, with 0 indicating no detectable staining and +3 indicating the strongest staining. Immunopositivity of tumor lesions for VEGF was assessed in all of the lesions at a high power (×400) by two independent investigators operating in a blinded fashion, and mean value was determined. AT1R-positive cells in human lung metastasis were counted in 10 high power fields by two independent investigators working in a blinded fashion. Then the percentage of AT1R cells was graded into one of three categories: +1 (<35%), +2 (35–75%), or +3 (>75%).

Statistical Analysis. All of the results were analyzed for significance by the ANOVA. \( P < 0.05 \) was considered significant. Statistical analysis was performed using Statview 5.0 software.

Results and Discussion

In the present study, immunostaining clearly demonstrated prominent expression of AT1R in the metastatic lung tumors of mice (Fig. 1A). We also observed that 9 of 10 patients showed AT1R expression in lung metastasis (Fig. 1B; Table 1), which was consistent with our results in mice. The AT1R is expressed in various normal organs, including the blood vessels, brain, kidney, lung, adrenal gland, and pituitary gland (14). In situ hybridization of angiotensin receptor mRNA and ligand-binding assays have shown that the main AII receptor subtype in the pulmonary vessels is type 1 (15). The present study showed that not only endothelial cells, but also tumor cells, expressed the AT1R in the lungs of mice (Fig. 1A). Goldfarb et al. (10) used autoradiography to demonstrate that tumor cells express the AT1R in human RCC tissues. It has been suggested that the cytoplasmic localization of AT1R in tumor cells may be attributable to its internalization (11). Our findings regarding AT1R localization seem to be consistent with previous observations.

We found that injection of Renca cells into the tail vein induced multiple lung metastases in mice after 16 days. Mice without candesartan treatment had numerous nodules (Fig. 2A), whereas oral administration of candesartan caused a striking decrease of lung metastases (Table 2). Histopathological examination of hematoxylin-stained sections demonstrated that control mice had prominent lung tumor formation (Fig. 2B), whereas these changes were ameliorated by candesartan administration (Fig. 2C).

In the development of renal fibrosis, there is growing evidence that AII induces TGF-β and that AII blockade reduces TGF-β overexpression, suggesting that AII blockade may be of therapeutic value for fibrotic diseases (16). TGF-β has also been reported to play an important role in inducing angiogenesis and metastasis during cancer progression (17). These results suggest that specific AII blockade may possibly prevent TGF-β-induced cancer progression in the lungs. In our mouse model, we determined lung tissue TGF-β levels by bioassay and showed that metastatic lungs had a significantly higher tissue TGF-β content than normal lungs (Table 2). On the other hand, candesartan treatment significantly decreased the lung tissue TGF-β content (Table 2), suggesting that AT1R blockade decreases tissue TGF-β in metastatic lungs. However, the inhibition of TGF-β by candesartan was not as marked as the decrease in metastatic lung nodules, so other factors may be involved. We also investigated the effect of AT1R blockade on neovascularization in the present mouse model. To evaluate neovascularization, immunostaining for CD34 was carried out. Lung metastases contained multiple CD34-positive neovessels (Fig. 3A). Candesartan treatment significantly decreased the CD34-positive tumor neovessels when compared with control lungs (Fig. 3B; Table 2). Investigation of tumor expression of VEGF in lungs of mice showed that control metastatic lung tumors had significantly stronger VEGF expression (Fig. 4A) than tumors treated with candesartan (Fig. 4B; Table 2). This suggests that AT1R blockade may be able to prevent renal cancer progression by inhibiting angiogenesis.

Candesartan cilexetil has shown a potent and long-lasting antihypertensive effect in clinical trials (18) and in several animal models (19). The irreversible inhibition of vasoconstriction by candesartan is the result of its tight binding to AT1R and slow dissociation from the AT1R (20). After oral administration, candesartan was ~50 times more potent than losartan (a conventional AT1R antagonist) in rats (21). These characteristics may be related to the higher potency and long action of candesartan demonstrated in the present study. A 4-week oral toxicity study of candesartan showed that doses of up to 300 mg/kg/day were not toxic in rats (22) and dogs (23). In addition, a 26-week oral administration study revealed that the nontoxic dose of
candesartan was 10 mg/kg/day in rats (24), so we chose 10 mg/kg/day for this study. Inada et al. (19) reported that 1 mg/kg of candesartan had no hypotensive effect in rats, and that even higher dose (10 and 100 mg/kg) reduced the blood pressure slightly (by ~10 mm Hg) and had no effect on the heart rate in normotensive rats. It was also demonstrated that oral administration of candesartan (10 mg/kg) for 2 weeks did not have an excessive hypotensive effect in rats (25). However, this dose has not been studied clinically, although doses of up to 96 mg/day (~2 mg/kg/day) have been achieved clinically (26).

We also observed that 1 mg/kg of candesartan could significantly reduce metastatic lung nodules (55.4 ± 8.2; P < 0.001; n = 10) when compared with control metastatic mice. Because administration of candesartan at 1 mg/kg is clinically feasible, this inhibitory effect on tumor growth could be achievable and warrants additional investigation.

Only 40% of patients have RCC confined to the kidney at diagnosis, and unfortunately, 25–30% of the patients present with metastatic disease. If the tumor cannot be completely resected, the usual course is relentlessly progressive, and 85% of relapses occur during the first 3 years. Even if the tumor can be completely resected, 20–30% of patients suffer a relapse. Accordingly, the 5-year survival rate of patients with metastasis is <10% (1). Identification of the factors that mediate relapse would help to predict the prognosis clinically. Al-

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**Table 2** Results of lung metastatic nodules, TGF-β content, CD34-positive cells, and VEGF-positive intensity (mean ± SE)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average number of nodules per lung</th>
<th>Active TGF-β content (pg/mg lung tissue)</th>
<th>CD34-positive cells in the tumor (HPIF)</th>
<th>VEGF intensity in the tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>123.3 ± 8.6 (n = 12)</td>
<td>32.8 ± 3.1 (n = 11)a</td>
<td>22.3 ± 2.9 (n = 10)</td>
<td>2.6 ± 0.2 (n = 10)</td>
</tr>
<tr>
<td>Candesartan (10 mg/kg)</td>
<td>14.9 ± 1.8 (n = 13)b</td>
<td>21.5 ± 3.3 (n = 10)c</td>
<td>7.3 ± 1.6 (n = 10)d</td>
<td>1.2 ± 0.2 (n = 10)d</td>
</tr>
<tr>
<td>Negative control</td>
<td>Not detectable</td>
<td>15.1 ± 1.7 (n = 10)</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

*a* P < 0.01, compared with negative control.

*b* P < 0.0001, compared with control.

*c* P < 0.01, compared with control.

*d* P < 0.001, compared with control.
References


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