The Angiotensin-I-converting Enzyme Inhibitor Perindopril Suppresses Tumor Growth and Angiogenesis: Possible Role of the Vascular Endothelial Growth Factor

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ABSTRACT

Angiotensin-I converting enzyme (ACE) inhibitor is used widely as an antihypertensive agent, and it has been suggested recently that it decreases the risk of cancer (A. F. Lever et al., Lancet, 352: 179–184, 1998). In this study, we examined the effect of several ACE inhibitors and angiotensin-II type 1 receptor (AT1-R) antagonists on tumor development and angiogenesis in a murine hepatocarcinoma model. Among ACE inhibitors, perindopril appeared to be a potent inhibitor of tumor development and angiogenesis, whereas AT1-R antagonists did not exert such an inhibitory effect. The inhibitory effect of perindopril was achieved even on established tumors. The level of the potent angiogenic factor, vascular endothelial growth factor (VEGF), in the tumor was significantly suppressed by perindopril. In vitro studies showed that perindopril-derived active form, perindoprilat, suppressed the endothelial cell tubule formation. Perindoprilat treatment also significantly inhibited VEGF mRNA expression in BNL-HCC cells in vitro. These results showed that the ACE inhibitor perindopril inhibited tumor development and angiogenesis independent from AT1-R blockage, and that VEGF alternation may be involved in the mechanism of this inhibitory effect. Because perindopril is widely used in clinical practice, it may represent an effective new strategy for anticancer therapy.

INTRODUCTION

It is now widely recognized that the growth of any solid tumor depends on angiogenesis. Without angiogenesis, the tumor ceases to grow even beyond a few millimeters in size (1, 2). Because inhibition of angiogenesis is now considered to be a promising approach for cancer therapy, efforts are being directed at overcoming tumor angiogenesis worldwide. In animal models, antiangiogenic compounds have proven very successful in inhibiting tumor development (3, 4). These agents have exhibited reduced toxicity and a decreased likelihood of causing the development of drug resistance compared with conventional chemotherapy. Several feasible candidates are now undergoing clinical trials at specific institutes, but to date, no compound is widely available in clinical practice.

ACE2 inhibitor is widely used as an antihypertensive agent. Recently, it has been suggested that ACE inhibitors decrease the risk of cancer. A retrospective cohort study of 5207 patients receiving ACE inhibitor or other hypertensive drugs with a 10-year follow-up has shown that ACE inhibitor may decrease the incidence of adult cancer and fetal cancer (5). In vitro, ACE inhibitor retarded growth of cultured cancer cells, and some of ACE inhibitors, such as captopril, inhibited angiogenesis and the growth of induced tumor in rats (6, 7). AT-II, which is an octapeptide produced by the enzymatic cleavage of angiotensin-I by ACE, exerts a large number of physiological effects, including vascular tone, hormone secretion, tissue growth, and neuronal activities (8). AT-II has been also shown to stimulate neovascularization in some animal experimental models (9–12). Because ACE inhibitor causes a decrease in the production of AT-II, it is likely the antiangiogenic activity of ACE inhibitor is at least partly mediated by AT-II inhibition. However, the effect of AT1-R, which is a main receptor of AT-II, antagonists has not been elucidated, and a comparison of the effect of different ACE inhibitors under the same experimental conditions on tumor development and angiogenesis has not yet been made.

To date, many angiogenic factors have been identified. Among these, VEGF is one of the most potent and is known to play a pivotal role in angiogenesis (1, 2). It has been shown that VEGF expression is increased in human surgical specimens in several types of tumors and correlated with aggressive behavior and a poor prognosis of the tumor. In animal models, overexpression of VEGF enhanced tumor growth, whereas suppression of VEGF reduced tumor growth (1, 2). VEGF gene expression has been induced by several types of cytokines, and recent studies have shown that AT-II also induced VEGF in several types of cells, including tumor cells (10, 11). Recently, it has been shown that retinal VEGF mRNA overexpression in dia-

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2 The abbreviations used are: ACE, angiotensin-I converting enzyme; AT-II, angiotensin-II; AT1-R, AT-II type 1 receptor; EC, endothelial cell; HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vascular endothelial cell; RT-PCR, reverse transcription-PCR.
betic rats was significantly suppressed by the ACE inhibitors, ramipril and perindopril (13). The relation, however, between the ACE inhibitor and VEGF expression in the tumor cells has not been examined yet.

In the present study, we examined the effect of several types of ACE inhibitors and AT$_1$-R antagonists on tumor development and angiogenesis in a murine HCC experimental model. We also examined the possible interaction of VEGF and ACE activity in the tumor.

**MATERIALS AND METHODS**

**Compounds and Cell Lines.** Captopril and temocapril were supplied by Sankyo Pharmaceutical Co. (Tokyo, Japan) and perindopril was supplied by Daiichi Pharmaceutical Co. (Tokyo, Japan). The AT$_1$-R antagonist candesartan was supplied by Takeda Pharmaceutical Co. (Tokyo, Japan), and losartan was purchased from Banyu Pharmaceutical Co. (Tokyo, Japan). Murine BNL 1 ME A.7R.1 HCC cells (BNL-HCC cells) and HUVECs were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and Dainippon Pharmaceutical Co. (Osaka, Japan), respectively. BNL-HCC cells are an adherent chemically transformed mouse liver cell line derived from the normal BA/LB/c embryonic liver cell line, BNL CL2 (American Tissue Culture Collection, Manasses, VA; TIB 73; Ref. (14). The cells were grown in the media recommended by the respective suppliers.

**Mouse Tumor Allograft Models.** To create the allograft model, $1 \times 10^6$ BNL-HCC cells were injected into the flank of BALB/c mice. Experimental animals received the test compounds by gavage once a day at the indicated dose from 3 days after tumor implantation. For the experiment on established tumors, the administration of 2 mg/kg of perindopril was started at the time when the mean tumor volume had reached 94 mm$^3$. The tumor volume was calculated twice a week as described previously (14). Each group consisted of five to seven mice.

**Angiogenesis Assay.** To determine angiogenesis in the tumor, we used immunohistochemical detection of platelet/EC adhesion molecule (PECAM/CD31), which is used widely as a marker of neovascularization, in frozen sections as described previously (14). Vascularized area was chosen as described previously (15, 16), and the immunostained microvessel length was calculated using the Fuji BAS 2000 image analyzer (Fuji, Tokyo, Japan) system. For the *in vitro* assessment of angiogenesis, we used the EC tubule formation assay as previously described (17). Because perindopril is a pro-drug, the active form, perindoprilat (1 μM), was used for the *in vitro* assay. Briefly, Matrigel (Collaborative Biochemical Products, Bedford, MA) was placed in 24-well tissue culture plates (150 μl/well) and allowed to set at 37°C for 30 min. Then 2.5 $\times$ 10$^4$ HUVECs were added to each well and incubated in the presence or absence of perindoprilat at 37°C for 20 h under a 5% CO$_2$ atmosphere. Semi-quantitation of tubule formation was performed in the same way as for the *in vivo* assay.

**Measurement of VEGF and ACE in Tumors.** Tumor samples were prepared as described previously (14). After the protein concentration was equalized, the VEGF level was measured with an ELISA kit (R&D Systems, Minneapolis, MN) in accordance with the supplier’s instructions. Because a different size of tumor may cause different hypoxic conditions which strongly induced VEGF (1, 2), six similar sized tumors were chosen from each group. The same sample was also subjected to a determination of ACE activity using an ACE detection kit (Sigma, MI) according to the supplier’s instructions.

**VEGF mRNA Expression in BNL-HCC Cells *In Vitro.*** BNL-HCC cells were cultured with or without perindoprilat (1 μM) at a density of $5 \times 10^3$ cells/cm$^2$ in 60-mm tissue culture plates. After incubation for 48 h, mRNA was extracted as described previously (14). RT-PCR was performed with the mouse VEGF-specific primer as described elsewhere (18). Densitometric analysis was performed by measuring the absorbance with an image analyzer. The level of gene expression was calculated after normalization with glyceraldehyde-3-phosphate dehydrogenase internal control.

**Statistical Analysis.** The statistical significance was analyzed by Tukey’s multiple comparison test.

**RESULTS**

**Effect of ACE Inhibitors and AT$_1$-R Antagonists on Tumor Development.** To elucidate the effect of ACE inhibitors on the tumor development, we first compared the results obtained with perindopril, temocapril, and captopril at the dose of 20 mg/kg/day. As shown in Fig. 1A, all three types of ACE inhibitors showed an inhibitory effect of tumor development compared with the control group. Among these compounds, perindopril revealed a statistically stronger inhibitory effect than...
the other two ACE inhibitors at a dose of 20 mg/kg/day ($P < 0.05$). Therefore, we used this compound for further studies. Next, we conducted a dose range study of perindopril using doses of 2, 10, and 20 mg/kg/day. We found that even the lowest dose (2 mg/kg/day) showed an inhibitory effect of tumor development similar to that of 20 mg/kg/day (Fig. 1B). Furthermore, this inhibitory effect of perindopril at 2 mg/kg/day was detected even after the tumor was established (Fig. 1C). To examine whether this inhibitory effect of ACE inhibitor was mediated by AT1-R inhibition, we then examined the effect of AT1-R antagonists on tumor development. Neither candesartan nor losartan significantly inhibited the tumor development at the dose of 20 mg/kg/day, whereas 2 mg/kg/day of perindopril revealed significant inhibition compared with the control group (Fig. 1D). These results suggested that the ACE inhibitor suppressed the tumor development mainly by an independent mechanism from AT1-R blockage.

**Microvessel Density in the Tumor.** To investigate whether the effect of perindopril on tumor development was accompanied by suppression of tumor microvessel density, we next examined tumor expression of CD31 by immunohistochemistry using tumors of similar size. In perindopril-treated tumors (Fig. 2B), CD31-positive vessels were significantly decreased compared with control tumors (Fig. 2A). Because it has recently been demonstrated that image analysis is more reliable and objective than manual counts of microvessels (16), we used computer-assisted image analysis techniques. A semiquantitative analysis of CD31-positive vessels showed that neovascularization was significantly lower in the perindopril-treated tumors compared with the control tumors ($P < 0.01$; Fig. 2C).

**Effect of ACE Inhibitor on EC Tubule Formation in Vitro.** We investigated the *in vitro* EC tubule formation in the presence or absence of perindopril (1 μM). We found that perindopril inhibited EC tubule formation in Matrigel [Fig. 3, A (control) and B (perindopril treatment)]. Semiquantitative analysis showed that the total length of tubules formed in the perindopril-treated cultures was significantly decreased from that in the control cultures ($P < 0.01$). We also examined whether the inhibitory effect of perindopril was related to cytotoxicity. We found that perindopril did not influence the *in vitro* proliferation of tumor cells and EC (data not shown).

**VEGF Level and ACE Activity in the Tumor.** To elucidate the possible interaction between the ACE inhibitor and the VEGF expression level, we examined the ACE activity and the VEGF level in the tumor of the control and perindopril-treated groups (2 mg/kg/day). The ACE activity in the tumor was significantly suppressed by the treatment of perindopril (Fig. 4A). As shown in Fig. 4B, perindopril treatment also significantly decreased the VEGF level in the tumor compared with the control group ($P < 0.01$).

**Effect of ACE Inhibitor on VEGF mRNA Expression in Vitro.** To examine whether perindopril treatment altered VEGF mRNA expression in BNL-HCC cells, we performed a semiquantitative RT-PCR analysis. As shown in Fig. 5 and Table 1, perindopril (1 μM) treatment significantly suppressed the VEGF mRNA expression in BNL-HCC cells. Densitometric analysis showed that the VEGF gene expression was 3.4-fold less in the perindopril-treated group than in the control group ($P < 0.01$; Table 1).

**DISCUSSION**

The present study revealed that the ACE inhibitor, perindopril, significantly inhibited tumor development and angiogenesis possibly independently from AT1-R blockage, and this inhibitory effect was accompanied by the suppression of VEGF.

It is now widely accepted that angiogenesis plays an es-
ential role in tumor development. Therapies aimed at destroying the tumor vasculature can achieve rapid regression of experimental tumors and it has been shown that tumor cell apoptosis is significantly increased by treatment with antiangiogenic agents (3, 4). These agents show less toxicity and cause less drug resistance compared with conventional chemo-therapeutic agents. Accordingly, antiangiogenic therapy is being investigated around the world, including the use of gene therapy, antiangiogenic recombinant proteins, monoclonal antibodies, and various drugs (3, 4). Although some of these agents such as thalidomide and penicillamine (19) are now used in clinical trials at certain institutions, no method is widely available for clinical use at this time.

ACE inhibitors are currently used in more than 100

Fig. 3 Effect of perindopril on in vitro EC tubule formation. In vitro EC tubule formation on Matrigel is shown in the control group (A) and the perindopril-treated (B) group. The total tubule length was measured by an image analysis system as described in “Materials and Methods.” Cont, control; PE, perindopril-treated (1 μM) group; *, significant differences versus the control group at P < 0.01.

Fig. 4 The VEGF level and ACE activity in the tumor. The ACE activity (A) and VEGF protein level in the tumor (B) were determined as described in “Materials and Methods.” The data represent the mean ± SD (n = 6); Cont, control; PE, perindopril-treated group; *, significant differences versus the control group at P < 0.01.
countries for the treatment of hypertension and congestive heart failure without causing serious side effects, such as myelosuppression. ACE inhibitors decrease the production of AT-II, but also have other functions that might affect the development of cancer. Recently, it has been shown that the ACE inhibitors may protect against cancer (5). Although randomized controlled trials are still required, a retrospective cohort study of 5207 hypertension patients revealed that ACE inhibitors may decrease the incidence of adult cancer and fetal cancer, whereas other antihypertensive drugs, calcium channel blockers, diuretics, and β-blockers did not show such an effect. In experimental models, ACE inhibitor reduced the tumor cell growth rate and modulated gene expression in vitro. (6). In vivo, the ACE inhibitor, captopril, inhibited tumor growth and angiogenesis (7). It has been shown that inhibition of angiogenesis by captopril is not mediated by ACE inhibition but by the suppression of matrix metalloproteinases activities or by the production of angiotatin attributable to the free thiol group, which perindopril does not have (7, 20). Other than cancer, angiogenesis is also an essential factor for the development of diabetic retinopathy (1). The ACE inhibitor, lisinopril, has been shown to slow the progression of diabetic retinopathy in type-1 diabetic patients (21). Perindopril has been reported to decrease the number of small blood vessels, which perhaps represent diabetes-induced angiogenesis, in rats (22). In contrast to the above drugs, the ACE inhibitor, quanaprilot, has been shown to promote angiogenesis in a rabbit model of hind-limb ischemia (23). Taken together, these findings suggest that the influence of ACE inhibitor on angiogenesis occurs in a compound-specific manner.

VEGF is now widely known as one of the most potent angiogenic factors, and as a survival factor of tumor ECs (1, 2). VEGF and its receptor interaction is believed to play a major role in angiogenesis in human tumors. Blocking the VEGF or the VEGF receptor inhibited angiogenesis and suppressed growth of many types of tumors in experimental models (3, 4, 14). VEGF is regulated by several factors, including AT-II (1, 2, 10, 11). The most striking biological difference between ACE inhibitor and AT₁-R antagonist treatment is the AT-II level, which has been shown to stimulate angiogenesis (12). The AT-II level is decreased by ACE inhibitor, whereas the level does not change by AT₁-R antagonist. In the present study, we found that inhibition of ACE by perindopril was accompanied by suppression of VEGF in the tumor, and perindopril decreased the VEGF mRNA expression in BNL-HCC cells in vitro. Other than cancer, it has recently been reported that perindopril reduced the retinal VEGF mRNA overexpression in the diabetic rats (13). Taken together, it is suggested that, unlike captopril, perindopril inhibits tumor development and angiogenesis via AT-II inhibition leading to suppression of VEGF. In the present study, we did not find an inhibitory effect of AT₁-R antagonists that were comparable to that of perindopril. To date, several types of AT-II receptors were identified, although these biological functions are not yet fully understood (24). AT-II, may therefore, use the other types of receptors than AT₁-R for in vivo tumor angiogenesis. Additional studies are required to elucidate these mechanisms.

In summary, we have shown here that the ACE inhibitor perindopril significantly inhibits tumor growth and angiogenesis along with suppression of the VEGF level. Several drugs, such as thalidomide, are now used in the clinical trials as antiangiogenic agents against cancer. Because perindopril is also already used widely in clinical practice without serious side effects, it may also be applicable as an anticancer agent, thus providing a new strategy for cancer therapy.

REFERENCES


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**Table 1** Effect of perindopril on VEGF gene expression in BNL-HCC cells *in vitro*

<table>
<thead>
<tr>
<th>Control</th>
<th>+ perindopril (1 µM)</th>
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<td>VEGF gene expression</td>
<td>0.842*</td>
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*Gene expression presented after normalization with the glyceraldehyde-3-phosphate dehydrogenase internal control.*