J Cancer Res Clin Oncol (2004) 130: 567–573
DOI 10.1007/s00432-004-0582-7

ORIGINAL PAPER

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Effects of the angiotensin-I converting enzyme inhibitor perindopril on tumor growth and angiogenesis in head and neck squamous cell carcinoma cells

Received: 5 February 2004 / Accepted: 19 April 2004 / Published online: 27 July 2004

Abstract *Purpose*: Recently, it has been reported that angiotensin-I converting enzyme (ACE) inhibitors have anticancer activity. In particular, the ACE inhibitor, perindopril, significantly inhibits tumor growth and angiogenesis in hepatocellular carcinoma cells along with suppression of the VEGF level. However, the mechanisms of suppression of the VEGF level are still unclear, and there are no previous reports on this subject related to head and neck squamous cell carcinoma (HNSCC). In some previous studies, angiotensin II, which is produced from angiotensin I by ACE, directly stimulates VEGF expression. *Methods*: In the present study, we focused upon angiotensin II, and investigated the effect of perindopril on VEGF expression, angiogenesis, and tumor development of HNSCC with in vitro and in vivo studies. *Results*: In the in vitro cell proliferation assays, there was no significant difference between the perindopril-treated group and the control group. However, the perindoprilat-treated group showed a significant reduction in mRNA expression of VEGF and inhibited the induction activity of the VEGF promoter in comparison to the control group. Perindoprilat treatment also significantly suppressed angiotensin II production in vitro. In the in vivo studies, perindopril had a significant inhibitory effect on tumor growth, and reduced blood vessel formation surrounding the tumors. *Conclusions*: Our findings suggest that perindopril has no direct cytotoxicity against tumor cells, but has a potential to inhibit tumor growth due to suppression of VEGF-induced angiogenesis in vivo. Angiotensin II might have an important role in carcinogenesis, and the antiangiogenic activity of perindopril is at least partly mediated by angiotensin II inhibition. The ACE inhibitor perindopril has clinical potential as a useful antitumor agent.

Keywords ACE inhibitor · Perindopril · VEGF · Angiotensin II · Head and neck squamous cell carcinoma

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Introduction

Angiotensin-I converting enzyme (ACE) inhibitor is widely used in the treatment of several cardiovascular diseases and hypertensive diseases, due to its ability to improve left ventricular function and to reduce blood pressure (Burris 1995; Garg and Yusuf 1995). Recently, it has been reported that ACE inhibitors have anticancer activity (Chen et al. 1991; Volpert et al. 1996). Mechanistic studies indicate that ACE inhibitors have various anticancer effects, such as inhibition of angiogenesis and extracellular-matrix degradation (Prontera et al. 1999; Yoshiji et al. 2001). In particular, it was reported that the ACE inhibitor, perindopril, significantly inhibits tumor growth and angiogenesis in hepatocellular carcinoma cells along with suppression of vascular endothelial growth factor (VEGF) level (Yoshiji et al. 2001).

Angiogenesis, which is the formation of new blood vessels, is essential for growth and metastasis of solid tumors, including head and neck squamous cell carcinoma (HNSCC) (Folkman 1990; Folkman and Shing 1992). The obliteration of the feeding vessels to a tumor can cause its shrinkage to death (Kuiper et al. 1998). HNSCCs are characterized by their rapid invasion into bone and muscle, and a high degree of neovascularity (Shpitzer et al. 1996; Shemirani and Crowe 2000; Mat-

sumoto et al. 2001). VEGF, also known as vascular permeability factor, is a 34–50 kDa protein initially identified as an endothelial cell specific mitogen and a significant mediator of angiogenesis during a variety of nonpathological and pathological processes. VEGF acts directly on the endothelial cells, promoting their proliferation and permeability, and it induces angiogenesis in pathological and physiological situations (Moriyama et al. 1997). Therefore, angiogenesis can be reflected by the expression of VEGF and its receptors (Mustonen and Alitalo 1995; Risau 1997). Increased expression of VEGF and its receptors have been reported in invasive HNSCC (Shemirani and Crowe 2000; Matsumoto et al. 2001), which suggests that the growth of HNSCC is dependent in part on angiogenic activity. In fact, the concentrations of VEGF mRNA and the expression of VEGF protein in human solid tumors correlate positively with malignant progression, including HNSCC (Denhart et al. 1997). However, the mechanisms of the inhibition of VEGF expression by perindopril are still unclear and, to the best of our knowledge, there are no studies on this subject related to HNSCC. Studies in this area may potentially lead to new approaches in the prevention and treatment of this important group of human cancers.

In some previous studies, angiotensin II, which is a key regulator of blood pressure homeostasis in humans and is produced from angiotensin I by ACE, directly stimulates VEGF expression in vitro (Fujita et al. 2002; Shemirani and Crowe 2002). In the present study, we focused upon angiotensin II, and investigated the effect of perindopril on VEGF expression, angiogenesis, and tumor development of HNSCC with in vitro and in vivo studies.

Materials and methods

Chemicals and cell lines

Perindopril and perindoprilat, the active derivative of perindopril, were obtained from SERVIER Japan (Tokyo, Japan). Since perindopril is a pro-drug, perindoprilat was dissolved in the cell culture medium and was used for in vitro studies, whereas perindopril was used for in vivo studies. The murine squamous cell carcinoma cell line, SCC-VII, which was kindly donated by Dr. G. Matsumoto (Kanagawa Dental Collage), and the human oral squamous cell carcinoma cell line, KB, were obtained. The SCC-VII cell was cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), trypsin-EDTA (0.05% Trypsin, 0.53 mM EDTA.4Na) and penicillin/streptomycin 1,000 IU/ml. The KB cell was cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), trypsin-EDTA (0.05% Trypsin, 0.53 mM EDTA.4Na) and penicillin/streptomycin 1,000 IU/ml. Cells were maintained in a humidified incubator at 37 °C under 5% CO_2 in air.

In vitro proliferation assays

Five thousand cells of each cell line were seeded into 35-mm dishes in RPMI 1640 plus 10% FCS or DMEM plus 10% FBS. Twenty-four hours later, the indicated concentrations of perindoprilat were added. The number of cells was then determined and counted every 48 h for 10 subsequent days, in triplicate assays, using a Coulter Counter (Beckman Coulter, Fullerton, Calif., USA). The mean values were used to generate growth curves.

Quantification of VEGF mRNA by real-time quantitative RT-PCR

1×10^4 KB cells were seeded into 100-mm dishes. Twenty-four hours later, the indicated concentrations of perindoprilat were added. After incubation for 48 h, total RNA from cells was isolated with TRIZOL reagent (Gibco BRL, Gaithersburg, Md., USA) following the manufacturer's instruction. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL) with 0.5 mg oligo (dT) 12–16 (Amersham Pharmacia Biotech, Piscataway, N.J., USA). The reaction mixture was incubated at 42 °C for 50 min followed by incubation at 72 °C for 15 min. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized. The RNA from KB cell line was used as a positive control (standard curve). All PCR reactions were performed using LightCycler-FirstStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). The sequences of PCR primers were as follows: human VEGF forward primer, 5′-CGAAACCATGAACTTTCTGC-3′, reverse primer, 5′-CCTCAGTGGGCACACACTCC-3′; human GAPDH forward primer, 5′-CACCCATGGCAAATTCCATGGC-3′, reverse primer, 5′-GCATTGCTGATGATCTTGAGGCT-3′. The cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles at 95 °C for 1 s, 55 °C for 5 s, and 65 °C for 5 s. Quantitative analysis was performed using the LightCycler Software (Roche Diagnostics) using a real-time fluorogenic detection system for a kinetic rather than end point approach as on conventional agarose or polyacrylamide gel. The sizes of the amplicons for VEGF and GAPDH were 302 bp and 297 bp, respectively. VEGF mRNA levels were normalized by GAPDH mRNA levels.

Luciferase reporter assays

The effect of perindoprilat on the transcriptional regulation of VEGF in KB cells was examined using transient transfection with a VEGF promoter (luciferase)-reporter construct. The VEGF promoter-

luciferase reporter plasmid phVEGF1, which contains the 5'-flanking region (−2279 to +54) of the VEGF promoter, was originally constructed by Dr. Minchenko (Minchenko et al. 1994) and provided by Dr. M. Esumi (Kimura et al. 2000). The method we used for transient transfection luciferase reporter assays were described previously (Masuda et al. 2002). Triplicate samples of 1×10^5 cells in 35-mm plates were transfected using lipofectin (Life Technologies, Rockville, Md., USA). One microgram of DNA of indicated luciferase reporter plasmid and 10 ng of the control pCMV-β-gal plasmid were cotransfected in opti-MEM I medium (Life Technologies). After 16 h, the medium was changed to serum-free DMEM containing the indicated concentration of perindoprilat. For growth factor stimulation assays, 50 ng/ml of TGF-α were added 30 min after adding perindoprilat. The cells were then incubated for 24 h, and luciferase activity was determined with the luciferase assay system (Promega, Madison, Wis., USA). β-gal activities were determined using the β-gal enzyme assay system (Promega). Luciferase activities were then normalized with respect to β-gal activities.

Measurement of angiotensin II level by enzyme immunoassay (EIA)

KB cells (1×10^5) cells were plated in triplicate in six-well plates. Some cultures were treated with 1 μM perindoprilat. Serum-free DMEM was added for up to 48 h to produce conditioned medium. Angiotensin II concentrations in conditioned media samples were determined using a commercially available kit (SPI bio, France) in accordance with the supplier's instructions.

Tumor growth in animals

To evaluate the effect of perindopril on tumor development, 1×10^7 SCC-VII cells were injected into the flank of 6–8-week-old female BALB/c nude mice. Experimental animals received the indicated dose of perindopril, which is a clinically comparable dose (Yoshiji et al. 2001; Yoshiji et al. 2002; Noguchi et al. 2003), by gavage once a day from 3 days after tumor implantation. In brief, perindopril was given orally through a 22G micro-catheter. Each group consisted of five or six mice. Implanted tumors were measured using calipers on the days indicated until 2 weeks after implantation. Tumor volume was estimated using the formula: tumor volume (mm^3) = length (mm)×width $(mm)^2\times1/2$. Animal studies were in accordance with the guidelines of the Kyushu University.

Immunohistochemical studies

Two weeks after SCC-VII tumor cells implantation, the mice were killed and the implanted tumors were then surgically removed. Implanted tumor specimens were fixed in formalin and embedded by paraffin for immunohistochemical staining. Briefly, tissue sections were deparaffinized and dehydrated in a graded series of alcohol. They were then digested in 0.05% protease XXIV for 10 min, and blocked by endogenous peroxidase using 0.3% hydrogen peroxidase. The slides were incubated with rat-anti mouse CD31 monoclonal antibody (clone MEC13.3, Pharmingen) overnight at 4 °C, rinsed three times in PBS and incubated with biotinylated goat anti-rat Ig G for 10 min at room temperature. The streptavidin-biotin peroxidase method (Histofine SAB-PO kit, Nichirei, Japan) was used for detection, employing 3, 3'-diaminobenzidine (DAB) as the chromogen. The sections were counterstained slightly with hematoxylin. The microvessel density was assessed using the method described by Bosari (Bosari et al. 1992). Briefly, the sections were scanned and areas of the highest vascularization were chosen at low power (×100). The microvessels were then counted by two investigators in three chosen ×250 fields with the highest density. The microvessel density was the mean of the vessel counts obtained. Microvessels adjacent to necrosis areas were excluded from the appraisal.

Statistical analysis

Statistical analyses were performed using the Mann Whitney U-test. Differences with a P value < 0.05 were considered to be significant.

Results

Effect of perindoprilat on in vitro cell proliferation

To verify whether perindoprilat has direct cytotoxicity or an inhibitory effect against each cell line, a cell proliferation assay was performed. Perindoprilat-treated groups grew similarly to the control group. There were no significant differences in the cell proliferation among them (data not shown).

Effect of perindoprilat on VEGF mRNA expression in vitro

As is shown in Fig. 1A, the PCR products of VEGF and GAPDH were visualized at the expected size on agarose gel. Normalized and calibrated comparisons among the groups indicated. Treatment with either 1 μM or 2 μM perindoprilat showed a significant reduction of VEGF mRNA expression ($P = 0.012$, $P = 0.008$) (Fig. 1B).

Effect of perindoprilat on VEGF promoter activity in HNSCC cells

We examined the effects of perindoprilat on the transcriptional activity of the promoter of the VEGF

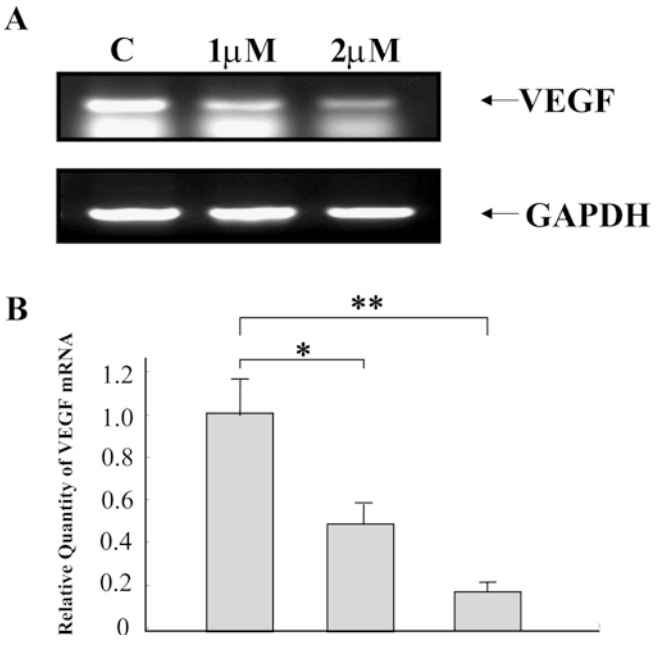


Fig. 1A,B Effect of perindoprilat on VEGF mRNA expression in KB cells in vitro. **A** mRNA expressions of VEGF and GAPDH are shown at the expected location in the gel. *C* control group; *1* μM 1 μM perindoprilat-treated group; *2* μM 2 μM perindoprilat-treated group; **B** Relative quantity of mRNA expression of VEGF. Statistically significant difference between control group and perindoprilat-treated groups. * $P = 0.012$, ** $P = 0.008$

gene in KB cells. Treatment with 1 μM perindoprilat significantly inhibited induction in the activity of the VEGF promoter, both in the absence and presence of TGF-α (Fig. 2).

Effect of perindoprilat on angiotensin II level in HNSCC cells

As shown in Fig. 3, 1 μM perindoprilat treatment significantly suppressed the angiotensin II production in KB cells. ($P = 0.028$)

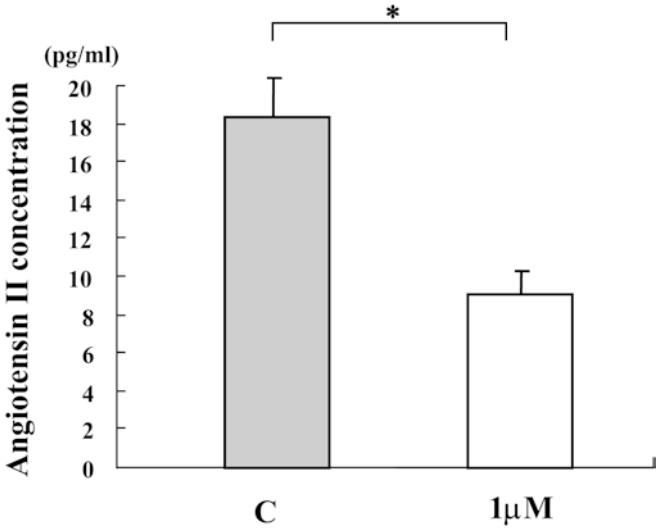


Fig. 3 Effect of perindoprilat on angiotensin II level in HNSCC cells. *C* control group; *1* μM 1 μM perindoprilat-treated group; * $P = 0.028$

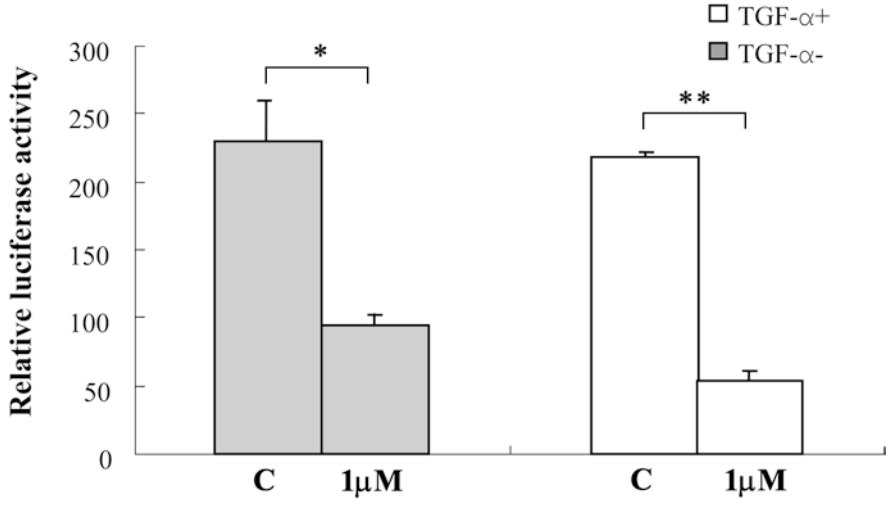
Effect of perindopril on tumorigenicity in vivo

The changes in the body weight of the mice used in the control group and in the experimental group during the course of the experiment were not significant (data not shown). The mean tumor volume of SCC-VII cells in the control group increased during the experiment, as is illustrated in Fig. 4. Perindopril had significantly inhibited the growth of SCC-VII cells in vivo during the experiment ($P = 0.051$, $P = 0.029$).

Pathological and immunohistochemical descriptions

SCC-VII tumor cells grew with features of poorly differentiated SCCs. Subsequent local invasion of the tumor cells were also observed. Tumor stromal angiogenesis including the invasive zone was assessed by

Fig. 2 Effect of perindoprilat on VEGF promoter activity in vitro. *C* control group; *1* μM 1 μM perindoprilat-treated group; * $P = 0.031$ ** $P = 0.024$



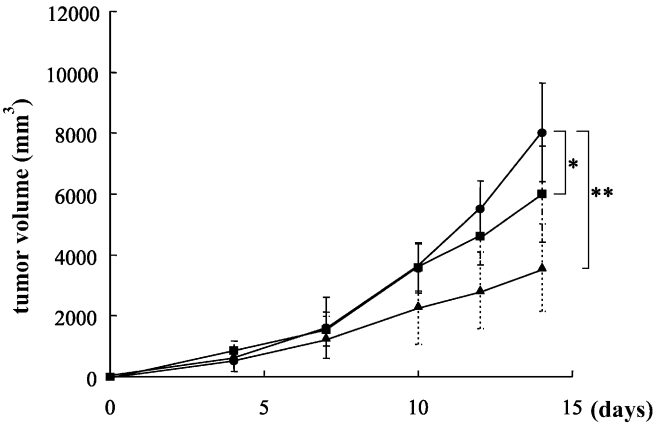


Fig. 4 Effect of perindopril on tumor growth in murine SCC-VII cells in vivo. Mean tumor volume after administration of drug. Mean tumor volume was significantly smaller in perindopril-treated group than in the control group. ○ control group; □ 1 mg/kg perindopril-treated group; △ 2 mg/kg perindopril-treated group; $*P = 0.051$, $**P = 0.029$; Each *point* represents the mean ± 1 standard deviation (SD)

CD31-immunostaining of tumor tissues. CD31 is specifically expressed on the surface of endothelial cells of blood vessels and is weakly expressed on peripheral lymphoid cells and platelets. The control group appeared more vascularized than the experimental groups ($P = 0.03$, $P = 0.019$). These results indicated that perindopril reduced stromal vascularization in the tumor within the experimental group in comparison to the control group in SCC-VII tumor tissues (Fig. 5A–C).

Discussion

Antiangiogenic therapy is one of the promising treatment modalities for cancer. 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 (Ferrara and Alitalo 1999; Lau and Bicknell 1999). These agents show less toxicity and cause less drug resistance compared with conventional chemotherapeutic agents. However, the effectiveness of this antiangiogenic agent for the treatment of HNSCC has not been investigated. Recently, it was reported that the long-term administration of ACE inhibitors reduces morbidity and mortality in cancer patients (Lever et al. 1998). In particular, Yoshiji et al. reported that the ACE inhibitor, perindopril, significantly inhibits tumor growth and angiogenesis in hepatocellular carcinoma cells along with suppression of the vascular endothelial growth factor (VEGF) level (Yoshiji et al. 2001).

In our present study, we also demonstrated that 1 μM perindoprilat greatly inhibited VEGF expression, although it did not suppress the proliferation of KB cells. In addition, this inhibition occurred at the level of

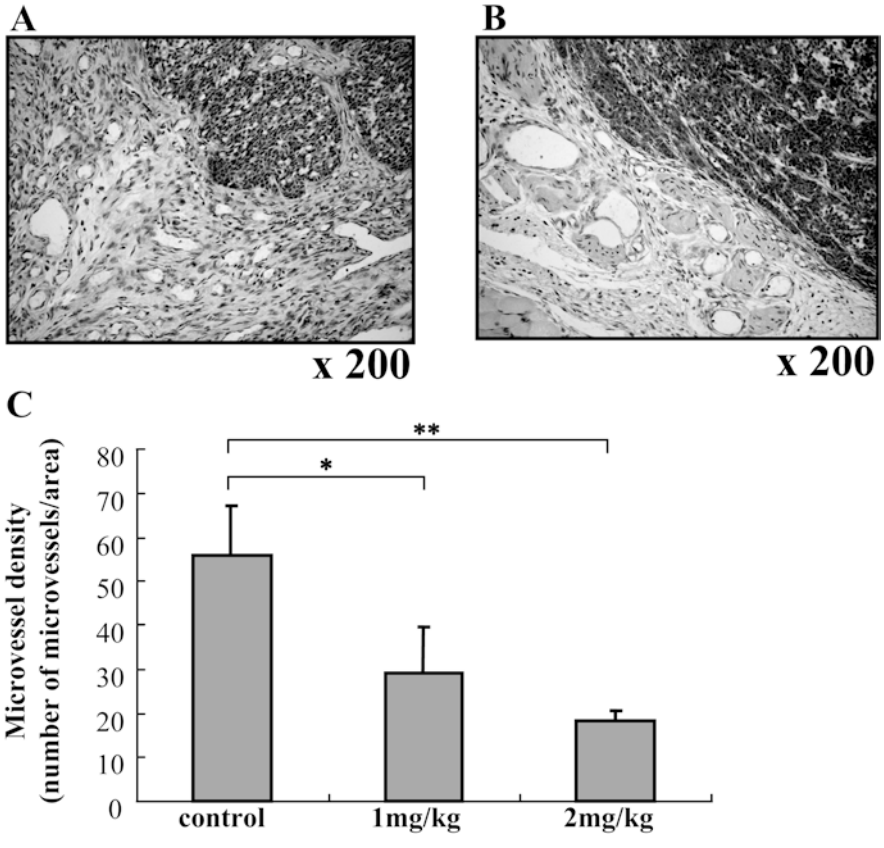


Fig. 5A–C Immunohistochemical features of CD31 and microvessel density on a section of SCC-VII tumor (original magnification, ×200). **A** control group; **B** 1 mg/kg perindopril-treated group; **C** Microvessel density. The number of CD31-positive blood vessels was counted as described in "Materials and methods". The final quantification was carried out in terms of the number of microvessels/area obtained from three fields in the same section at a magnification of ×250. Microvessel density was significantly lower in the perindopril-treated group than in the control group. *Control* control group, *1 mg/kg* 1 mg/kg perindopril-treated group, *2 mg/kg* 2 mg/kg perindopril-treated group; $*P = 0.03$, $**P = 0.019$

transcriptional regulation, which was manifested by a decrease in transcription levels due to the decrease in VEGF promoter activity. On the other hand, our in vivo study also showed that perindopril treatment significantly reduces new vessel formation and suppressed the growth of tumor compared to control at a clinically comparable dose. These results suggest that perindopril has no direct cytotoxicity against tumor cells, but has a potential to inhibit tumor growth due to suppression of VEGF-induced angiogenesis in vivo. We proved that perindopril is at least a potent antitumor agent for HNSCC via an antiangiogenic effect. Since perindopril was given to the mice starting on the day of cancer cell inoculation, our model may only reflect the early stage of HNSCC progression and a further study may be necessary to demonstrate the effect against the entire HNSCC progression.

Recently, the presence of the intracellular renin-angiotensin system has been established in several organ systems including cancer cells (Chen et al. 1991). Angiotensin II, the effecter peptide of this system, is one of the most potent regulators of blood pressure, and it exerts a variety of physiological effects. It regulates circulating blood volume and stimulates neovascularization and cell proliferation (Fernandez et al. 1985; Daemen et al. 1991). It has been shown that angiotensin II directly stimulates the secretion of VEGF and neovascularization in some experimental models (Chen et al. 1991; Daemen et al. 1991).

The VEGF promoter region contains several potential binding sites. Page et al. showed that angiotensin II increases the expression of VEGF, following activation of the hypoxia-inducible factor-1 which regulates the transcription of VEGF (Page et al. 2002). The ACE inhibitor plays a key role in the control of the renin-angiotensin system, since it inhibits the ACE that converts angiotensin I into the vasopressor angiotensin II and inactivates vasodilatory bradykinin. We also found that 1 μM perindoprilat treatment significantly suppressed angiotensin II production in KB cells. These data indicate that angiotensin II might have an important role in carcinogenesis and that the antiangiogenic activity of perindopril is at least partly mediated by angiotensin II inhibition. However, Yoshiji et al. reported that they could not find an inhibitory effect of tumor growth in angiotensin II receptor antagonists, which also suppress physiological effects of angiotensin II (Yoshiji et al. 2001). It has been reported that other ACE inhibitors suppress some angiogenic molecules, such as matrix metalloproteinases (Prontera et al. 1999). The inhibition of cancer angiogenesis by ACE inhibitors probably involves multiple pathways other than VEGF transcription. Further studies would be needed to define these mechanisms.

In conclusion, this study provides further evidence that the ACE inhibitor, perindopril, inhibits HNSCC angiogenesis. This observation is very important clinically. Currently, much effort is being directed at developing antiangiogenic drugs to treat cancer. A major shortcoming of the vast majority of the antiangiogenic drugs is that they require intravenous or subcutaneous administration. This is particularly problematic because antiangiogenic drugs have to be given on a long-term basis to control cancer growth. Furthermore, many of these compounds are complex peptides that are difficult and expensive to produce in the quantities and purities required for human use. Thus, perindopril, which can be administered orally and inexpensively, may be a clinically useful antitumor agent.

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