Combination of Interferon-β and the Angiotensin-Converting Enzyme Inhibitor, Perindopril, Attenuates Murine Hepatocellular Carcinoma Development and Angiogenesis

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ABSTRACT

Purpose: **Angiogenesis is now recognized as a crucial step in the development of tumors, including hepatocellular carcinoma (HCC). The aim of this study was to elucidate the combined effect of the clinically used angiotensin I-converting enzyme (ACE) inhibitor, perindopril (PE), and IFN-β on the development and angiogenesis of murine HCC at clinically comparable low doses.**

Experimental Design: **PE and IFN were administered at doses of 2 mg/kg/day and 1 × 10^4 IU/twice a week, respectively.**

Results: **Both PE and IFN significantly suppressed HCC development and inhibited neovascularization in the tumor, although the effect of low-dose IFN was weaker than that of PE. A combination regimen of PE plus IFN was effective; IFN significantly augmented the tumoricidal effect of PE. These inhibitory effects of PE plus IFN could be detected even on established tumors. The potent angiogenic factor, vascular endothelial growth factor, was markedly suppressed by combined treatment with PE and IFN, whereas these agents produced a marked increase of apoptosis in the tumor. The *in vitro* studies exhibited that PE and IFN inhibited endothelial cell tubular formation. IFN also suppressed endothelial cell proliferation, whereas neither IFN nor PE showed any inhibitory effect on proliferation of HCC cells.**

Conclusion: **The combination treatment of PE and IFN at clinically comparable low doses could inhibit HCC development and angiogenesis and suppress vascular endothelial growth factor as well. Because both agents are widely used in clinical practice, this combination regimen may represent a potential new strategy for HCC therapy in the future.**

INTRODUCTION

Therapies aiming at destruction of 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 (1–3). It has been documented that antiangiogenic therapy shows less drug resistance than conventional chemotherapies. With regard to conventional chemotherapies, drug resistance is encountered in ~30% of all cancer patients. Tumor cells have been shown to readily acquire drug resistance because of their genetic instability, heterogeneity, and high mutation rate, whereas ECs[3] are genetically stable and acquire much less drug resistance (4, 5). Accordingly, antiangiogenic therapies are under investigation around the world, including the use of gene therapies, antiangiogenic recombinant proteins, monoclonal antibodies, and various drugs. Although some of these agents are now in use in Phase I, II, and III clinical trials at certain institutes, no agent is widely available at this time in clinical practice (6). In of the concept of antiangiogenesis therapy, long-term administration is required to examine the compound toxicity. One potential alternative strategy may be the use of drugs with antiangiogenic activity that are available in an oral formulation and are at present being administered to patients for treatment of different diseases. Some clinically available compounds, such as thalidomide and penicillamine, have been shown to possess antiangiogenic activity and are being used in clinical trials (6). Long-term administration of these agents, however, sometimes leads to severe side effects, such as bone marrow suppression.

Recently, a retrospective cohort study of 5207 patients receiving ACE inhibitors or other antihypertensive drugs with a 10-year follow-up has showed that an ACE inhibitor decreased the incidence of cancer (Glasgow study; Ref. 7). AT-II is an

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[3] The abbreviations used are: EC, endothelial cell; ACE, angiotensin-converting enzyme; AT-I and II, angiotensin-I and II; HCC, hepatocellular carcinoma; PE, perindopril; VEGF, vascular endothelial growth factor; TUNEl, terminal deoxynucleotidyltransferase (Tdt)-mediated nick end labeling; MTT, 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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octapeptide produced mainly by proteolytic cleavage of its precursor AT-I by ACE (8). It has been shown that AT-II selectively increases blood vessel flow and that an ACE inhibitor decreased intratumoral blood flow without affecting the blood flow in the healthy organs (9). AT-II induces angiogenesis in several types of cells, including HCC, and the activity of ACE has been used as a tumor marker in HCC patients (10–12). We previously reported that the clinically used ACE inhibitor PE possesses a strong antiangiogenic activity and that it inhibited the growth of murine HCC at clinically comparable low doses. PE also suppressed expression of VEGF, which is one of the most potent angiogenic factors, in tumors (12).

IFNs are a family of natural glycoproteins initially discovered on the basis of their antiviral activity (13). It is now known that IFNs are multifaceted agents with the ability to influence cell proliferation and differentiation as well as the immune system (14). In addition, IFNs also show antiangiogenic activity both *in vitro* and *in vivo* (15, 16). Frequent systemic administration or gene delivery of IFN exerted a therapeutic effect in some experimental models (17–19). In HCC, it has been shown that high-dose and long-term therapy with IFN-α inhibited experimental HCC development and that IFN-β prevented recurrence of HCC in humans (20, 21). Most patients receiving high doses of IFNs, however, experience some degree of acute toxicity. The most common side effects are flu-like symptoms, including chills, fever, myalgia, and headache. Chronic exposure results in several additional symptoms, including fatigue, anorexia, weight loss, dizziness, and some hematological disorders (21, 22). These side effects make long-term administration of high dose of IFNs unlikely to be acceptable.

It has been reported that combination treatments with different antiangiogenic agents exert a more potent inhibitory effect on tumor development than single-agent treatments (6). In the present study to evaluate the feasibility of future clinical applications, we examined the combination effects of PE and IFN-β at clinically comparable low doses on murine HCC tumor development and angiogenesis and attempted to investigate the possible mechanisms involved.

MATERIALS AND METHODS

Compounds and Cell Lines. PE and IFN-β were generously supplied by Daiichi Pharmaceutical Co. (Tokyo, Japan) and TORAY Industries, Inc. (Tokyo, Japan), respectively. The murine HCC cell line BNL.1 ME A.7R.1 (BNL-HCC), and human umbilical vascular ECs were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and KURABO (Osaka, Japan), respectively. BNL-HCC cells are an adherent chemically transformed mouse liver cell line derived from the normal BALB/c embryonic liver cell line BNL CL2 (TIB 73; American Tissue Culture Collection, Manassas, VA) as described previously (23). Cells were grown in the media recommended by the respective suppliers.

Animal Treatment. A total of 40 male 6-week-old BALB/c mice, purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan), were housed under controlled temperature conditions and relative humidity, with 10–15 air changes/h and light for 12 h a day. To create the allograft model, we injected 1×10^6 of BNL-HCC cells into the flanks of BALB/c mice. The mice were randomly divided into four groups ($n = 10$ in each group). Group 1 consisted of untreated mice and served as a control group. Mice in groups 2 and 3 received 1×10^4 IU of IFN twice a week by s.c. injection on the contralateral side of the tumor and 2 mg/kg/day of PE by daily gavage, respectively. The doses of these agents that we used are almost comparable to those used in clinical practice (12, 20). The mice receiving combination treatment with PE and IFN group was designated as group 4. The animals were allowed free access to food and water throughout the acclimation and experiment protocols. Tumor volume was measured twice a week, and the mice were killed at 32 days after tumor cell implantation.

The next experiment was conducted to examine the effects of PE and IFN on the growth of fully established tumors. In this experiment, either IFN or PE administration was started on day 14, when mean tumor volume was ~200 mm^3). The mice were killed 35 days after tumor cell implantation. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

Immunohistochemistry. For determination of *in vivo* angiogenesis, we used immunohistochemical detection of platelet/EC adhesion molecule (CD31), which is widely used as a marker of neovascularization, in frozen sections of tumors of the same size to avoid the necrotic effect of hypoxia as described previously (24). The immunostained microvessel length was assessed under ×200 magnification. In each tumor sample, five areas showing the highest density of staining were selected for counting. In counting, large vessels with a thick muscular wall or with a lumen >50 μm in diameter were excluded. These immunopositive vessels were evaluated with Adobe Photoshop and NIH image software as described previously (25). Apoptosis was detected with DNA fragmentation products that were stained by *in situ* 3′-end labeling (TUNEL) with paraffin-embedded sections. The TUNEL-positive cells were counted in the PE-treated, IFN-treated, and control groups by a light microscope. In each tumor, the positive cells in 10 high-power fields at a magnification of ×400 were examined as described previously (25).

Measurement of VEGF Expression in Tumors. We measured the VEGF protein expression level in the tumor. Because a different size of tumor may cause different hypoxic conditions, which strongly induced VEGF (26), five tumors having the same size were chosen from each group. The tumor samples were prepared as described previously (27). 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.

***In Vitro* Proliferation and Angiogenesis Assay.** Because PE is a prodrug, the active form, perindoprilat, was used for the *in vitro* studies. *In vitro* proliferation was determined by MTT assay as described elsewhere (27). The cell proliferation was quantified by conversion of MTT by cells cultured in 12-well plates. MTT was added to each well at a final concentration of 5 mg/ml in the presence or absence of perindoprilat (100 μM) and/or IFN (10, 10^2, or 10^3 IU/ml). After a 4-h incubation at 37°C with MTT, the untreated MTT and medium were removed and 2 ml of DMSO were added to solubilize the MTT formazan. After gentle agitation for 10 min, the absorb-

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ance of each well, which is directly proportional to the number of living cells, was measured with a 540 nm filter. The absorbance was read with an ELISA plate reader ($n = 6$/group). For the *in vitro* assessment of angiogenesis, we used the EC tubule formation assay as described previously (12). Briefly, Matrigel (Becton Dickinson Labware, Bedford, MA) was placed in 6-well tissue plates (2 ml/well) and allowed to set at 37°C for 30 min. We then added 1.5×10^5 human umbilical vascular ECs to each well and incubated them in the presence or absence of perindoprilat (1 μM) and/or IFN (10 IU/ml) at 37°C for 20 h under a 5% CO_2 atmosphere. Semiquantitation of tubule formation was performed in the same way as for the *in vivo* assay.

Statistical Analysis. To assess the statistical significance of intergroup differences in the quantitative data, Bonferroni's multiple comparison test was performed after one-way ANOVA. This was followed by Barlett's test to determine the homology of variance.

RESULTS

Effects of IFN and PE on Tumor Development. We first examined the effect of clinically comparable doses of IFN and PE on HCC development. As shown in Fig. 1*A*, single agent treatment with either IFN (group 2) or PE (group 3) showed a marked inhibitory effect on HCC development compared with the control group (group 1; $P < 0.05$ and 0.01, respectively). The inhibitory effect in group 2 was weaker than that in group 3 ($P < 0.05$). Mice treated with IFN plus PE (group 4) showed a significant decrease in the tumor volume compared with those in the control group and groups 2 and 3. We next examined whether the inhibitory effects of PE and IFN could be found even after the tumor was established. When the tumor volume reached ~200 mm^3, either PE or IFN treatment was started. In this experiment, IFN alone did not show a significant inhibitory effect. However, IFN significantly augmented the tumoricidal effect of PE, and the combination effect of IFN plus PE was more potent than the additive effect of both compounds. Neither the single-agent treatment nor the combination treatment at the doses used affected health status, such as body weights, during the experiment (data not shown).

Tumor Neovascularization and Apoptosis. To determine whether the combined inhibitory effect of IFN and PE on the tumor development was accompanied by suppression of neovascularization, we examined the tumor expression of CD31. As shown in Fig. 2, and similar to the results for suppression of HCC development, the CD31-positive vessels in the tumors of either group 2 or group 3 were significantly fewer than those in the control group. The combination treatment of IFN and PE mostly attenuated the CD31-positive vessels in the tumor. To perform semiquantitative analysis of neovascularization in the tumor, we used computer-assisted image analysis techniques as described previously (12). Semiquantitative analysis of the CD31-positive vessels in group 2 or 3 revealed a statistically significant suppression compared with the control group ($P < 0.05$ and < 0.01, respectively). Combination treatment with IFN and PE exerted a much stronger inhibition on CD31-positive vessels in the tumor compared with group 3 ($P <$ 0.05; Fig. 3). On the contrary, the number of TUNEL-positive cells was significantly increased by treatment with IFN or PE

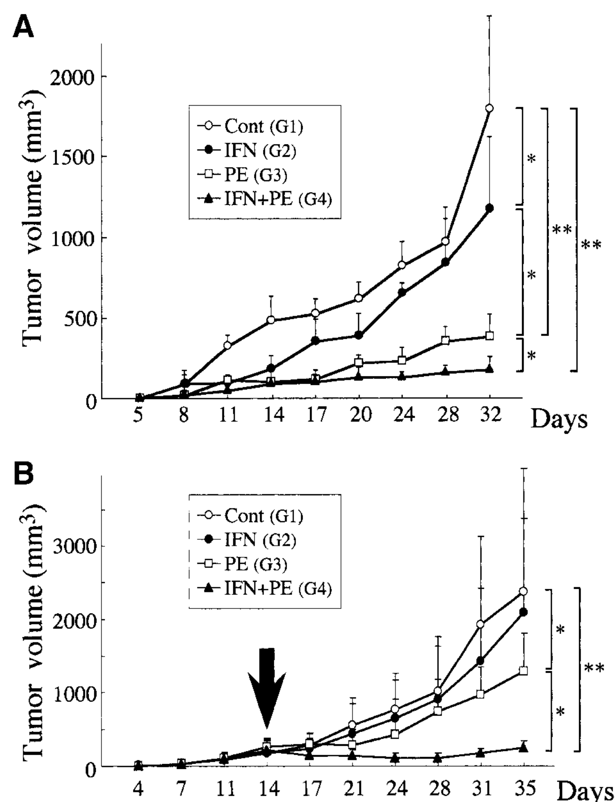

Fig. 1 Effects of IFN and PE on BNL-HCC development. Group1 (*G1*) is the untreated control group (○). Animals in groups 2 (*G2*; ●) and 3 (*G3*; □) received IFN and PE at doses of 1×10^4 IU/twice a week and 2 mg/kg/day, respectively. The animals receiving combination treatment of IFN and PE were designed as group 4 (G4; ▲). IFN and PE were administered from the beginning of the experiment (*A*) and from day 14 (*B*; mean tumor volume ~200 mm^3), respectively. The tumor volume was determined by calipers at the indicated time points. Each point represents the mean ± SD (*bars*; $n = 10$). * and ** indicate statistically significant differences between the indicated experimental groups ($P < 0.05$ and 0.01, respectively). The *arrow* indicates the time point at which PE and IFN treatment was started (day 14).

($P < 0.05$ and < 0.01, respectively). Moreover, combination treatment with IFN and PE revealed many more TUNEL-positive cells in the tumor than in group 3 ($P < 0.05$; Figs. 4 and 5). The incidence of apoptosis in the tumor almost corresponded to the effect of tumor development inhibition. Histological examination of H&E-stained sections did not exhibit a numerical increase in inflammatory cells, mainly macrophages, and extensive necrosis in groups 2 and 4, suggesting that suppressive effect of IFN was not attributable to the immunoresponse alterations at the currently used low doses (data not shown).

VEGF Expression in Tumors. Because we previously observed that PE treatment suppressed VEGF expression in the experimental HCC model (12), we also examined the combined effect of IFN and PE on VEGF expression in the tumor in the present study. As shown in Fig. 6, VEGF expression in the tumor was significantly suppressed by treatment with IFN or PE ($P < 0.05$). Combination treatment with IFN and PE exerted a much stronger inhibition of VEGF expression in the tumor than in group 3 ($P < 0.05$). We also examined expression of VEGF

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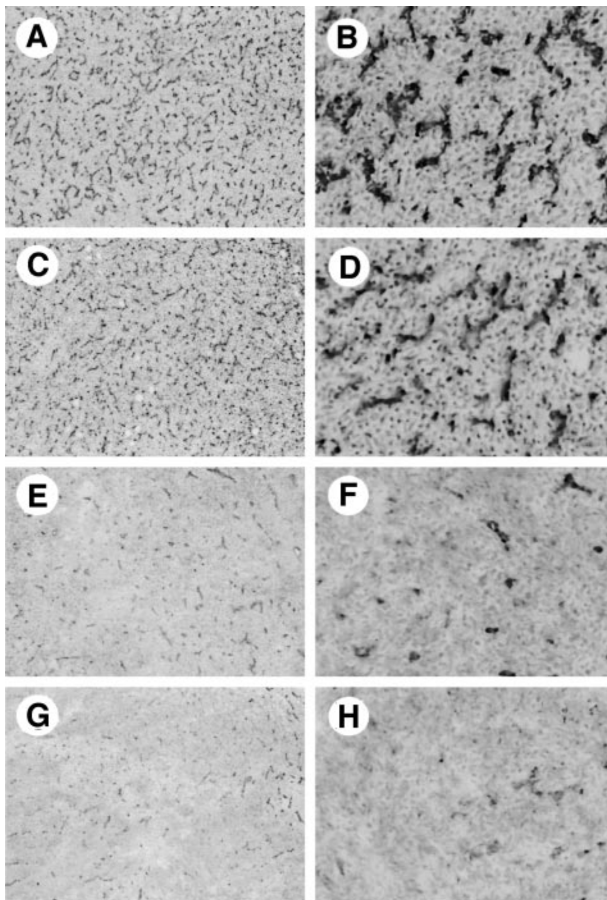

Fig. 2 Immunohistochemical analysis of CD31 expression in the tumor. Tumor vascularization was visualized by immunostaining of the CD31 vascular endothelial adhesion protein. *A* and *B*, control untreated group; *C* and *D*, IFN-treated group (1×10^4 IU). *E* and *F*, PE-treated group (2 mg/kg/day). *G* and *H*, group treated with combination of IFN and PE. Original magnifications: *A*, *C*, *E*, and *G*, ×40; *B*, *D*, *F*, and *H*, ×200.

mRNA in the tumor by reverse transcription-PCR and found a similar inhibitory effect of IFN and PE (data not shown).

Effects of IFN and PE on HCC and EC Proliferation *in Vitro*. To elucidate the possible mechanism of the inhibitory effect of IFN and PE, we examined whether the inhibitory effects of IFN and PE were related to cytotoxicity. As shown in Fig. 7*A*, we found that neither IFN (1×10^3 IU/ml) nor PE (100 μM) influenced the *in vitro* proliferation of HCC tumor cells *in vitro*. Even combination treatment with IFN and PE showed no inhibition of *in vitro* HCC cell proliferation. At a dose of 100 μM, PE also did not show any inhibitory effect on EC. On the other hand, IFN had a marked inhibitory effect on EC proliferation even at a low dose (10 IU/ml; $P < 0.01$) compared with the untreated control group. IFN treatment markedly inhibited EC proliferation in a dose-dependent manner (Fig. 7*B*). PE did not show any additional inhibitory effect to that of IFN on EC proliferation (data not shown).

Effects of IFN and PE on *in Vitro* Angiogenesis. We also investigated the *in vitro* EC tubule formation in the presence or absence of IFN and PE. We found that IFN or PE alone significantly inhibited EC tubule formation in Matrigel and that combination treatment with IFN and PE almost completely attenuated EC tubule formation at low doses (10 IU/ml and 1 μM, respectively; Fig. 8). The inhibitory effect of PE seemed to be stronger than that of IFN. Our semiquantitative analysis showed that the total length of tubules formed in the IFN- or PE-treated cultures were significantly less than in the untreated control culture ($P < 0.05$ and < 0.01, respectively). Combination treatment with PE and IFN resulted in a further inhibition of EC tubular formation than PE alone ($P < 0.05$; Fig. 9).

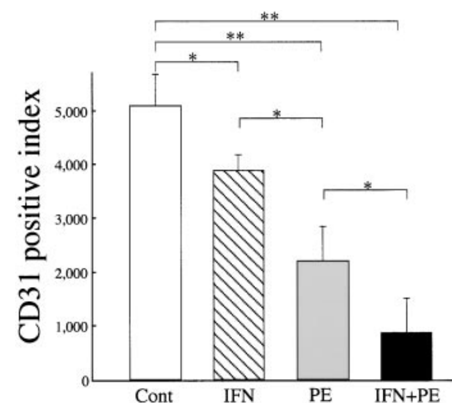

Fig. 3 Semiquantitative analysis of CD31-immunopositive vessels. The length of CD31-positive vessels in each tumor was measured by an image analysis system as described in the "Materials and Methods." The data represent the mean ± SD (*bars*; $n = 5$). * and ** indicate statistically significant difference between the indicated experimental groups ($P < 0.05$ and 0.01, respectively). *Cont*, untreated control group. *IFN* and *PE*, IFN- (1×10^4 IU/twice a week) and PE-treated (2 mg/kg/day) groups, respectively. *IFN+PE*, group treated with combination of IFN and PE.

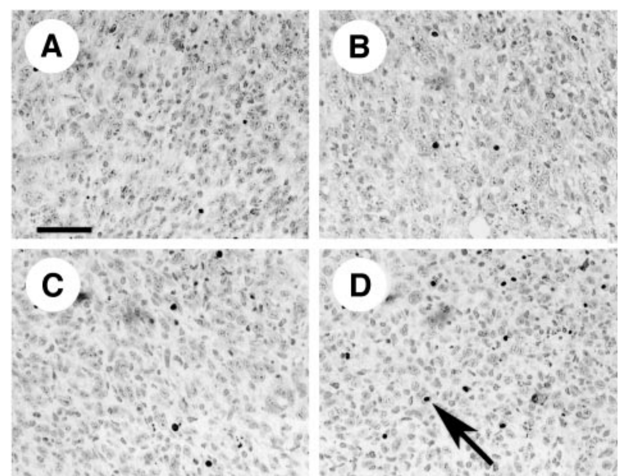

Fig. 4 Immunohistochemical analysis of apoptosis in tumors. The apoptotic cells were visualized by immunostaining with TUNEL assay. *A*, untreated control group. *B* and *C*, IFN- (1×10^4 IU/twice a week) and PE-treated (2 mg/kg/day) groups, respectively. *D*, group treated with combination of IFN and PE. *Arrow* indicates the apoptotic cell in the tumor. The original magnifications were ×200. *Scale bar*, 100 μm.

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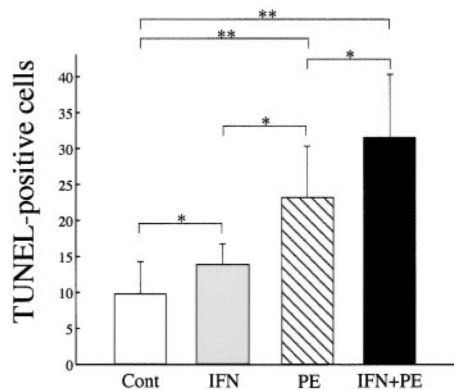

Fig. 5 Semiquantitative analysis of TUNEL-immunopositive apoptotic cells. The data represent the mean ± SD (*bars*; $n = 5$). * and ** indicate statistically significant differences between the indicated experimental groups ($P < 0.05$ and 0.01, respectively). *Cont*, untreated control group. *IFN* and *PE*, IFN- (1×10^4 IU/twice a week) and PE-treated (2 mg/kg/day) groups, respectively. *IFN+PE*, group treated with combination of IFN and PE.

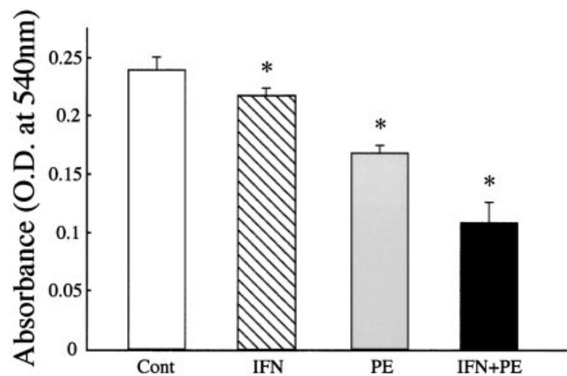

Fig. 6 Effects of IFN and PE on VEGF expression in tumors. The VEGF protein level was measured by ELISA as described in the "Materials and Methods." The data represent the mean ± SD (*bars*; $n = 5$). *, statistically significant difference compared with the control group ($P < 0.05$). *Cont*, untreated control group. *IFN* and *PE*, IFN- (1×10^4 IU/twice a week) and PE-treated (2 mg/kg/day) groups, respectively. *IFN+PE*, group treated with combination of IFN and PE.

DISCUSSION

HCC is the most frequent primary malignancy of the liver, and its incidence appears to be rising in the United States and other developed countries, although the United States and Western Europe still have a low incidence of HCC (28, 29). The prognosis of HCC is still poor because most cases are found in conjunction with chronic liver diseases, such as liver cirrhosis. Radical operation is the only curative modality for HCC, but it is appropriate only in the minority of patients because of the limited hepatic reserves. Therefore, various palliative therapeutic modalities, such as transarterial embolization, percutaneous intratumoral ethanol injection, and orthotopic transplantation, have been used, but no satisfactory treatment for HCC is as yet available (28, 29). A novel approach is required if the overall survival rate of patients with HCC is to be significantly improved.

It is now widely recognized that any solid tumor cannot grow beyond a few millimeters in size without angiogenesis (1, 2). One of the characteristic features of HCC in clinical practice is hypervascularity. As expected, it has been shown that several angiogenic factors, such as VEGF, are significantly up-regulated in human HCC tissues than in the surrounding noncancerous lesions, and angiogenesis plays a pivotal role in the experimental HCC development (26, 30, 31). Accordingly, it is likely that antiangiogenic therapy would be a promising approach against HCC.

In this study, we found that IFN or PE exerted a significantly inhibitory effect of HCC development associated with suppression of angiogenesis and that combination treatment with IFN and PE showed a more inhibitory effect than single-agent treatments at clinically comparable low doses. These inhibitory effects were also detected even after the tumor was fully established.

The pivotal role of VEGF in tumor angiogenesis has been demonstrated in various experimental systems, including HCC (26, 32, 33). Inhibition of VEGF function, *e.g.,* by neutralizing the monoclonal antibodies to VEGF or VEGF receptor-2, by antisense gene or ribozyme transfer, or by specific inhibitors of

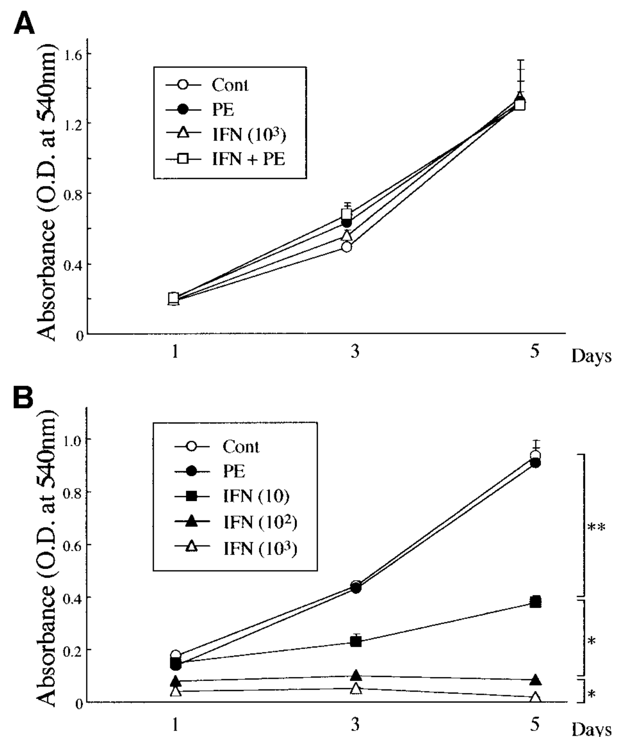

Fig. 7 Effects of IFN and PE on HCC cell (*A*) and EC (*B*) proliferation *in vitro*. Cell proliferation was measured by MTT assay after harvest from day 1 to day 5 as described in the "Materials and Methods." Each plot represents the mean ± SD (*bars*; $n = 5$). * and ** indicate statistically significant differences between the indicated experimental groups ($P < 0.05$ and 0.01, respectively). *Cont* (○), untreated control group; *PE* (●), perindoprilat (the active form of PE; 100 μM)-treated group; *IFN* (*10*, *10*^{2}, *10*^{3}), IFN-treated groups at doses of 10, 10^2, 10^3 IU/ml (■, ▲, and △, respectively). *IFN+PE* (□), group treated with combination of IFN (10^3 IU/ml) and perindoprilat (100 μM).

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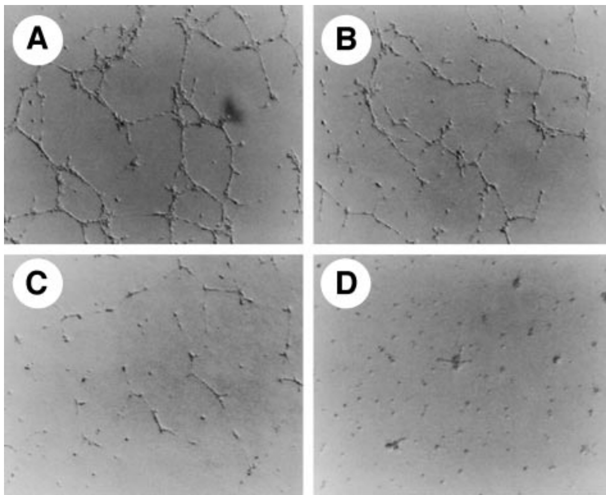

Fig. 8 Effects of IFN and PE on *in vitro* EC tubular formation on Matrigel. *A*, untreated control group. *B* and *C*, IFN- (10 IU/ml) and PE (perindoprilat; the active form of PE; 1 μM)-treated groups, respectively. *D*, group treated with combination of IFN and PE.

the VEGF-signaling cascade, has unequivocally demonstrated the requirement for VEGF in tumor angiogenesis and, consequently, tumor development (26, 33). In HCC, we reported previously that VEGF tightly regulated HCC development and that suppression of the VEGF–VEGF receptor interaction or VEGF-mediated signaling pathway significantly attenuated HCC development and angiogenesis (23, 24, 32). VEGF gene expression is regulated several factors, including AT-II (26, 34). AT-II is a product of the renin–angiotensin system, which has been reportedly activated in patients with chronic liver diseases, such as cirrhosis (35, 36). We have shown previously that inhibition of AT-II by PE significantly suppressed VEGF expression in tumors (12, 37). It has been reported that IFN can also down-regulate several angiogenic factors, such as basal fibroblast growth factor, interleukin-8, matrix metalloproteinase-2, matrix metalloproteinase-9, and VEGF (38–41). We observed in the present study that treatment with IFN and PE markedly suppressed VEGF expression in HCC. These results suggest that the inhibitory effect of IFN and PE was at least partly mediated by suppression of VEGF in HCC.

It has been shown that treatment with antiangiogenic agents induces a marked increase in apoptosis in tumors, whereas it does not alter tumor cell proliferation (1, 6, 25). In this study, immunohistochemical analysis by TUNEL assay revealed that IFN and PE significantly increased apoptosis in the tumor. On the other hand, tumor cell proliferation was not altered by treatment with IFN and PE (data not shown). It was important to determine whether apoptosis was observed mainly in the EC or in the tumor cells. We do not have an exact answer at this time. Although we performed a double immunohistochemical analysis with CD31 and TUNEL a few times, we failed to obtain a good result. The background was very intense, and the interpretation was very difficult (data not shown). In the present study, we found that IFN significantly inhibited EC proliferation *in vitro*, whereas neither IFN nor PE affected HCC cell proliferation. It has been reported that IFN-α and IFN-β exert different activities on EC proliferation. IFN-β exerts a 100-1000-fold stronger inhibitory effect on EC proliferation than IFN-α (42). Both IFN and PE exert a significant inhibitory effect on EC tubular formation *in vitro*. Furthermore, IFN and PE attenuate the expression of VEGF in the tumor, which is known as a survival factor for ECs. Taken together, these findings suggest that IFN and PE first induce EC apoptosis and that this might induce secondary apoptosis of the tumor cells.

The use of antiangiogenic agents as monotherapies in treating patients with advanced cancer has not yet shown significant efficacy (1, 6). The limitations of antiangiogenic monotherapy in this setting were in fact predicted by preclinical studies with the angiogenesis inhibitors endostatin and angiostatin. It has been reported that combination treatments with antiangiogenic agents, such as endostatin and angiostatin, revealed a synergistic inhibitory effect on tumor development and angiogenesis (6, 43). It has also been reported that the combination of TNP-470, which is one of the antiangiogenic agents under clinical trials, and IFN inhibited angiogenesis synergistically (44). We used IFN and PE in the present study and found that combination treatment with these agents had a significant tumor inhibitory effect associated with suppression of angiogenesis. PE is widely used at present without serious side effects in >100 countries, and its safety for use in patients with liver cirrhosis has been reported (45, 46). It has also been shown that long-term treatment with low-dose IFN is tolerable to patients with chronic liver disease (21, 22). A noteworthy finding in this study was that the combined inhibitory effect of IFN and PE could be observed at clinically comparable low doses of both agents, as described previously (12, 20). PE had no effect on EC proliferation, whereas it significantly suppressed EC tubular formation *in vitro*. On the other hand, IFN exerted a potent inhibitory effect on EC proliferation, but its inhibitory effect on EC tubular formation was not strong. Furthermore, we previously found

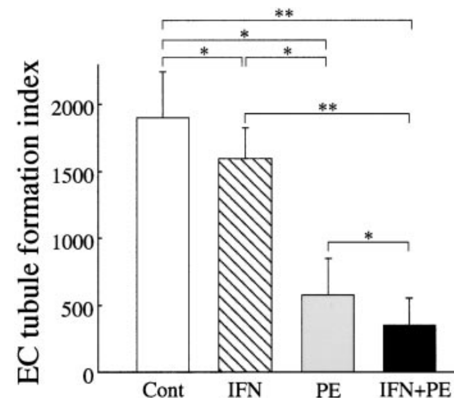

Fig. 9 Semiquantitative analysis of EC tubular formation on Matrigel. The total tubule length was measured by an image analysis system as described in the "Materials and Methods." The data represent the mean ± SD (*bars*; $n = 5$). * and ** indicate statistically significant differences between the indicated experimental groups ($P < 0.05$ and 0.01, respectively). *Cont*, untreated control group. *IFN* and *PE*, IFN- (10 IU/ml) and PE-treated (perindoprilat, 1 μM) group, respectively. *IFN+PE*, group treated with combination of IFN and PE.

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that PE markedly inhibited VEGF in tumor cells both *in vitro* and *in vivo* (12) and that PE also inhibited VEGF-induced EC migration (37). It could be possible that the coordination of these different biological activities produced the *in vivo* combination tumoricidal effect of PE and IFN. Further studies are required to elucidate the exact mechanism in the future.

In summary, we have shown here that combination treatment with the ACE inhibitor PE and IFN significantly inhibited HCC development and angiogenesis as well as suppressing VEGF. Because both agents are widely used in clinical practice at present, this combination regimen may represent a potential new strategy for HCC therapy in the future.

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