Antiangiogenic property of pigment epithelium-derived factor in hepatocellular carcinoma

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Antiangiogenic Property of Pigment Epithelium-Derived Factor in Hepatocellular Carcinoma

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Pigment epithelium-derived factor (PEDF) is one of the most powerful endogenous antiangiogenic reagents discovered to date. Its antiangiogenic potential in neoplastic disease remains unclear. In this study, we investigated antiangiogenic property of PEDF in hepatocellular carcinoma (HCC), a typical hypervascular tumor. In HCC cell lines, constitutive messenger RNA and protein expression of PEDF varied. Genomic DNA encoding the PEDF gene was the same in the cell lines examined by Southern blotting. In chemically induced hypoxic conditions, secreted PEDF protein was suppressed in contrast to elevation of vascular endothelial growth factor protein. When PEDF was overexpressed by gene transfer, proliferation and migration of endothelial cells were inhibited in conditioned media derived from all HCC cell lines. However, the serum concentration of PEDF, as measured by enzyme-linked immunosorbent assay, was decreased in patients with cirrhosis or HCC complicated by cirrhosis compared to healthy volunteers and patients with chronic hepatitis. According to the endothelial cell proliferation assay, the serum PEDF of patients with HCC had antiangiogenic activity. Moreover, intratumoral injection of a PEDF-expressing plasmid in athymic mouse models caused significant inhibition of preestablished tumor growth. In conclusion, PEDF plays a role in the angiogenic properties of HCC. Reduction of serum PEDF concentration associated with the development of chronic liver diseases may contribute to the progression of HCC. In addition, gene therapy using PEDF may provide an efficient treatment for HCC. (HEPATOLOGY 2004;40:252–259.)

Neovascularization is essential for the growth of solid malignant tumors larger than 1 to 2 mm in diameter.[1,2] Cancer cells constantly require high oxygen and nutrient concentrations because of their rapid cell division. Therefore, these cells are always exposed to a certain degree of vascular starvation, and blood vessels in the area attempt to sprout from preexisting vessels. This phenomenon is called *angiogenesis*.[2,3] Many secretory agents involved in angiogenesis, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and angiopoietin-1, have been discovered and are well studied in various types of cancer.[4–6] Conversely, several endogenous antiangiogenic factors also have been identified.

Pigment epithelium-derived factor (PEDF) was first discovered in 1989 by Tombran-Tink as a neurotrophic serpin that was secreted by retinal pigment epithelial cells.[7,8] Recently, PEDF was implicated in inhibition of angiogenesis in a dose-dependent manner both *in vitro* and *in vivo*.[9–11] The antiangiogenic efficiency of PEDF is more potent than that of other endogenous angiogenic inhibitors, including angiostatin, thrombospondin-1, and endostatin.[12] PEDF is found throughout the body and is particularly highly expressed in the normal liver.[13,14] In this regard, in hepatocellular carcinoma (HCC), it is speculated that PEDF expression is disadvantageous for tumor progression, but paradoxically, HCC is known to be one of the most hypervascular cancers. PEDF expression has not been well investigated in neoplastic diseases, including HCC. Furthermore, because the clinical nature of premalignant conditions associated with HCC are clearly elucidated, including chronic hepatitis (CH) or liver cirrhosis (LC) resulting from hepatitis B or C virus infection,[15] patterns of PEDF expression in these liver diseases also are of interest.

Abbreviations: VEGF, vascular endothelial growth factor; PEDF, pigment epithelium-derived factor; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis; RPMI, Roswell Park Memorial Institute; HUVEC, human umbilical vascular endothelial cells; CM, conditioned media; ELISA, enzyme-linked immunosorben assay; CM-P, CM from pcDNA3-PEDF-transfected cells; RT-PCR, reverse-transcriptase polymerase chain reaction; mRNA, messenger RNA.

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In the present study, we investigated the antiangiogenic properties of PEDF both *in vitro* and *in vivo* using HCC cell lines and human serum samples from patients with premalignant liver diseases. In addition, we studied the effects of PEDF gene overexpression on angiogenesis *in vitro* and on progression of tumors implanted subcutaneously in nude mice *in vivo*. These issues are extremely relevant to the field of antiangiogenic gene therapy.

Materials and Methods

Cell Culture. Human HCC cell lines were maintained in Roswell Park Memorial Institute (RPMI) supplemented with 10% bovine calf serum (Huh-7, HepG2, or PLC/PRF/5). Human umbilical vascular endothelial cells (HUVECs) were purchased from Sankyo Junyaku (Tokyo, Japan) and were grown in EBM2 medium.

Southern Blotting. DNA samples were digested with *EcoR*I. Ten micrograms of each digested DNA sample were fractionated on a 1% agarose gel, were blotted onto a nylon membrane (Hybond N+; Amersham, Little Chalfont, UK), and were hybridized with a [^{32}P]-labeled PEDF cDNA.

Northern Blotting. Total RNA was isolated using the guanidinium isothiocyanate method. Total RNA (10 μg) was fractionated on a 1% formaldehyde agarose gel, was transferred to a nylon membrane, and was hybridized with [^{32}P]-labeled PEDF or VEGF cDNA probes.

Western Blotting. Conditioned media (CM) or serum containing 10 μg protein was subjected to 4% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was transblotted onto nitrocellulose membrane. Blots were blocked with a solution of 5% nonfat dry milk/Tris-buffered saline containing 0.1% Tween 20 for 1 hour and then incubated overnight at 4°C in the presence of mouse anti-PEDF monoclonal antibody (Chemicon International Inc., Temecula, CA) or rabbit antihexahistidine antibody (ICN, Costa Mesa, CA). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and were incubated with horseradish peroxidase-conjugated antimouse immunoglobulin G. After washing with Tris-buffered saline containing 0.1% Tween 20, immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, UK).

Enzyme-Linked Immunosorbent Assay. VEGF and PEDF concentrations were assayed using the Quantikine HS Human VEGF Immunoassay Kit (R & D Systems, Minneapolis, MN) and the ChemiKine PEDF sandwich enzyme-linked immunosorbent assay (ELISA) kit (Chemicon International Inc.), according to the instructions provided by the manufacturer.

Serum from Healthy Volunteers and Patients With Liver Diseases. We collected sera from healthy volunteers (n = 8), patients with CH (n = 8), patients with LC (n = 8), and patients with HCC complicated with LC (n = 8). HCC patients had undergone selective hepatic angiography or computed tomography, and they showed hypervascularity. Informed consent was obtained from each patient before entering this study according to the guidelines of the Ethics Committee of Nagasaki University.

Immunoprecipitation. Immunoprecipitation was performed using 25 μg of mouse anti-PEDF antibody or normal mouse immunoglobulin G for 500 μL serum with 20 μL packed Protein G/A (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Transfection of Cells. The human hexahistidine-tagged PEDF cDNA was cloned into pcDNA3 (Invitrogen, Carlsbad, CA) from pCEP4-PEDF (kindly provided by Dr. Noel P. Bouck, Northwestern University) to construct pcDNA3-PEDF. Transfection was performed using 10 μg pEGFP (Clontech, Palo Alto, CA), pcDNA3, or pcDNA3-PEDF by the lipofectin (Life Technologies Inc., Gaithersburg, MD).

Preparation of Conditioned Media Derived From HCC Cell Lines. Huh-7, HepG2, or PLC/PRF/5 cells (approximately 2×10^6) were plated on 100-mm cell culture dishes. After 24 hours, transfection was performed and medium was replaced with 10 mL serum-free RPMI. After a further 48 hours of incubation, CM was collected from nontransfected cells, from pcDNA3-PEDF transfected cells (CM-P), or from pCDNA3 transfected cells transfected cells.

Proliferation and Migration Assay of HUVECs. HUVECs were plated onto 96-well culture plates at approximately 5×10^3 cells/well and were incubated for 24 hours. Medium was then replaced with 100 μL of RPMI, CM derived from nontransfected, or CM derived from transfected HCC cells. After 48 hours, proliferation of HUVECs was evaluated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). However, migration was measured using 8.0-μm 24-well Transwells (Corning, Acton, MA), as described previously with some modifications.[16] Briefly, 600 μL of each CM sample was placed in the lower chamber. Subconfluent 16-hour cultures of HUVEC in the growth factor-free medium were harvested, washed, and resuspended in serum-free RPMI. HUVECs (approximately 5×10^4) in 100 μL serum-free RPMI were added to the upper chamber. After 24 hours of incubation, all nonmigrating cells were removed from the upper surface of the membrane with a cotton swab; cells that had migrated to the lower surface were fixed with absolute meth-

anol and were stained with Giemsa. The numbers of migrated cells were counted using a light microscope under a high-power field (magnification, ×200). All experiments were performed in triplicate.

Murine Hepatocellular Carcinoma Tumor Model. Four-week-old male BALB/c nu/nu athymic mice were purchased from Charles River (Yokohama, Japan). Animal experiments were performed in accordance with institutional guidelines, and the study was approved by the Ethics Committee of Nagasaki University. Huh-7 cells (3×10^6) were implanted subcutaneously into the left thigh. Tumor volume was calculated as follows; tumor volume = length (mm) × width^2 (mm) × 1/2. When the tumor volume reached 60 to 110 mm^3, pcDNA3-PEDF or pcDNA3 (75 μg plasmid/100μL of TE [Tris ethylenediamine tetra acetic acid] buffer) with 20 μL of lipofectin was injected into the tumor once weekly for 3 weeks. As a control, 100 μL of vehicle (TE buffer) with lipofectin was injected. Each group consisted of five mice. Tumor volume was measured every 3 or 4 days. Two other mice were killed at day 24 or 32 in each group; tumors were removed and analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR).

RT-PCR. RNA was used after contaminating DNA was completely removed by DNase I treatment. RT-PCR was performed using the instructions provided by the supplier of the OneStep RT-PCR Kit (Qiagen, Valencia, CA), using primers specific for PEDF, VEGF and glyceraldehyde 3-phosphate dehydrogenase. RT-PCR amplification of glyceraldehyde 3-phosphate dehydrogenase was used as a control to assess the integrity of RNA. Ten-microliter samples of the amplification reactions were loaded on 1.2% TAE (Tris Acetic Acid + TE) agarose gels, and the products were visualized by ethidium bromide staining.

Statistical Analysis. All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using Student's *t* test. All reported *P* values are two-tailed, and those less than .01 were considered statistically significant.

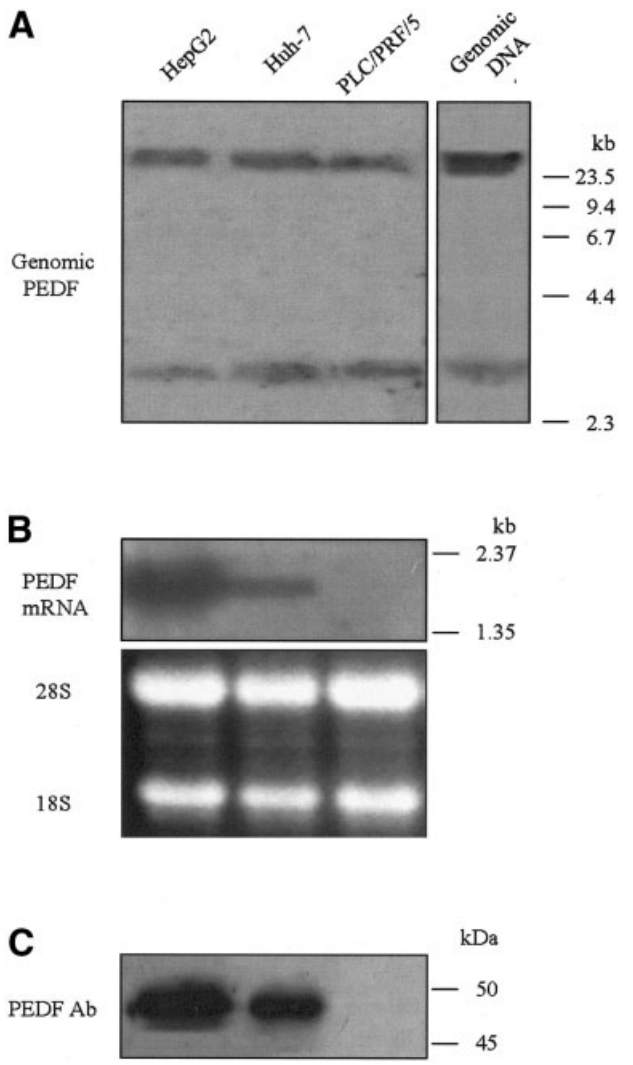

Fig. 1. Expression of pigment epithelium-derived factor (PEDF) in cell lines. (A) Southern blot analysis of genomic DNA derived from hepatocellular carcinoma cell lines and human genomic DNA. (B) PEDF messenger RNA (mRNA) as determined by Northern blot analysis. The 1.5-kb band indicates PEDF gene mRNA. The lower panel shows 28S and 18S ribosomal RNA as internal controls. (C) Expression of PEDF protein in conditioned media derived from each cell line analyzed by Western blotting using anti-PEDF antibody.

Results

Expression and Oxygen Regulation Analysis of PEDF in HCC Cell Lines. We first investigated PEDF expression in HCC cell lines. Northern blot showed that PEDF messenger RNA (mRNA) expression was abundant in HepG2 cells and was detected at considerable levels in Huh-7 cells, whereas it was undetectable in PLC/PRF/5 cells (Fig. 1B). Secreted PEDF protein in CM assessed by Western blotting exhibited a pattern similar to that of mRNA expression (Fig. 1C). In contrast, Southern blot showed that genomic DNA encoding the PEDF gene did not differ among the three cell lines compared with normal human genome (Fig. 1A). For chemical induction of hypoxic conditions,[17–19] HepG2 or PLC/PRF/5 cells were incubated for 12 hours with 400 μmol/L of cobalt chloride or 260 μmol/L of desferrioxamine. After exposure to these compounds, PEDF protein was suppressed in CM derived from HepG2 cells despite no alteration of PEDF mRNA expression (Fig. 2A, B), whereas the VEGF protein level was increased (Fig. 2C). In PLC/PRF/5 cells, both PEDF mRNA and protein remained undetectable, and VEGF protein was increased, similar to that in HepG2 (Fig. 2A–C).

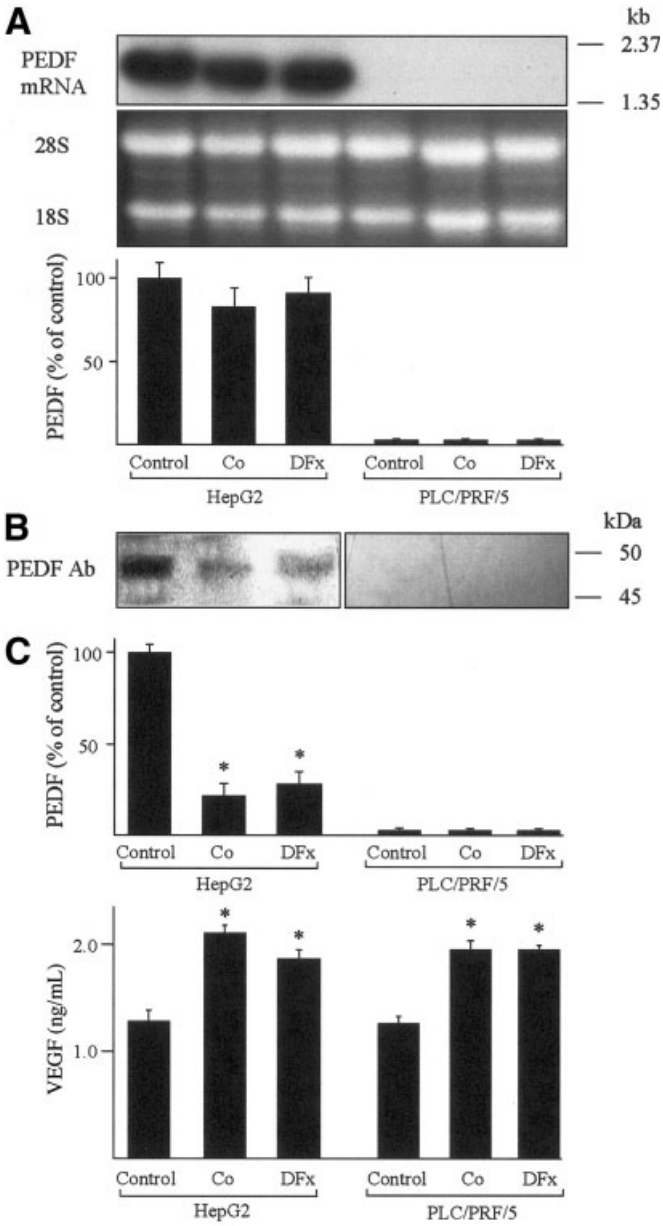

Fig. 2. Effect of chemically induced hypoxia on the expression of pigment epithelium-derived factor (PEDF) or vascular endothelial growth factor (VEGF) in hepatocellular carcinoma cell lines. HepG2 or PLC/PRF/5 cells were incubated for 12 hours with 400 μmol/L cobalt chloride (Co) or 260 μmol/L desferrioxamine (DFx). (A) Northern blot analysis. PEDF messenger RNA (mRNA) expression was quantified by densitometry and was normalized against the density of 28S ribosomal RNA. The PEDF mRNA level is indicated as a percentage of the respective control. (B) PEDF protein analyzed by Western blotting in each conditioned media (CM). The quantification of PEDF protein measured by densitometry is indicated as a percentage of the respective control. (C) VEGF concentrations in the CM derived from HepG2 or PLC/PRF/5 cells measured by enzyme-linked immunosorbent assay. Data are mean ± SD of three separate experiments. *$P < .01$ versus control.

PEDF Expression in Serum and Inhibition of HUVEC Proliferation. ELISA revealed that PEDF protein in both human serum from patients with LC and HCC complicated with LC were lower than that in healthy volunteers and patients with CH (Fig. 3A). Serum PEDF concentrations in patients with LC and in those with HCC complicating LC were almost similar. Next, we investigated the effect of serum PEDF of HCC patients on HUVEC proliferation. PEDF was extracted from the serum of HCC patients by immunoprecipitation, and the effect of this process on HUVEC proliferation was evaluated. Western blotting revealed that PEDF was almost completely removed from the serum by immunoprecipitation using anti-PEDF antibody (Fig. 3B). HUVEC proliferation was significantly higher in PEDF-free serum (1.26 times) than in PEDF-containing serum ($P < .01$; Fig. 3C). In contrast, the serum that was immunoprecipitated with normal mouse immunoglobulin G as a control contained the same amount of PEDF and resulted in the same HUVEC proliferation rate as the PEDF-containing serum. These results suggest that serum PEDF from HCC patients could inhibit angiogenesis.

***Induction of a PEDF Expression Vector in HCC Cell Lines and Its Antiangiogenic Effect* In Vitro.** We constructed a mammalian expression vector for the PEDF gene tagged with hexahistidine (pcDNA3-PEDF). HCC cell lines were transiently transfected with pcDNA3 or pcDNA3-PEDF. To determine the efficiency of transfection, the expression of green fluorescent protein was observed 24 hours after pEGFP transfection in each cell lines. In these experiments, 3%, 7%, and 21% of HepG2, Huh-7, and PLC/PRF/5 cells, respectively, were transfected with the vector. CM derived from 48 hours of incubation of each of the pcDNA3-PEDF-transfected HCC cells (CM-P-G2, CM-P-7, CM-P-PLC) contained more PEDF protein than that derived from nontransfected or pcDNA3-transfected cells. Western blotting with antihexahistidine antibody clearly showed that the increased PEDF protein in CM-P mainly was the result of pcDNA3-PEDF expression (Fig. 4A). Proliferation of each HCC cell line was not influenced by pcDNA3 or pcDNA3-PEDF transfection (Fig. 4B). To determine the proliferation of HUVEC, these cells were incubated in RPMI or CM derived from the transfected or nontransfected cells for 48 hours. We also investigated the migration of HUVECs after 24 hours incubation in RPMI or CM derived from transfected or nontransfected cells as described in Materials and Methods. Quantitative analysis showed that both proliferation and migration of HUVECs were significantly suppressed in CM-P compared with CM collected from nontransfected cells or CM collected from pCDNA3-transfected cells from all three cell lines (Fig. 4C). The average suppressive effect of CM-P on proliferation and migration was 30% and

36.3%, respectively, compared with CM collected from nontransfected cells.

Effect of PEDF Gene Induction on Progression of Preestablished Huh-7 Tumors in an Athymic Mouse Model. Huh-7 cells were subcutaneously implanted and tumors were established in athymic mice because Huh-7 cells were more efficiently transplantable than other cell lines. After reaching an adequate size, the tumor was directly injected with pcDNA3-PEDF, and the effect of treatment on tumor size was determined. Injection of pcDNA3-PEDF resulted in a significant reduction of tumor volume in compared with vehicle- or pcDNA3-injected mice at day 24 after the start of treatment ($P < .01$; Fig. 5B). When the expression levels of PEDF and VEGF mRNAs were analyzed by RT-PCR, PEDF mRNA was increased in pcDNA3-PEDF-injected mice 3 days after pcDNA3-PEDF-injection (Fig. 5C; day 24). However, pcDNA3-PEDF-injected tumors seemed to escape the growth suppression effect at the last time point (Fig. 5B). PEDF overexpression was not seen at day 11 after the third injection (Fig. 5C; day 32). Thus, the period of PEDF expression by pcDNA3-PEDF injection was limited for 24 days. However, expression of VEGF mRNA was not altered at days 3 and 11 after the third injection (Fig. 5C). Therefore, the loss of PEDF expression in the tumor, rather than the increased VEGF expression, seems a better explanation for the escape of growth suppression noted at day 32.

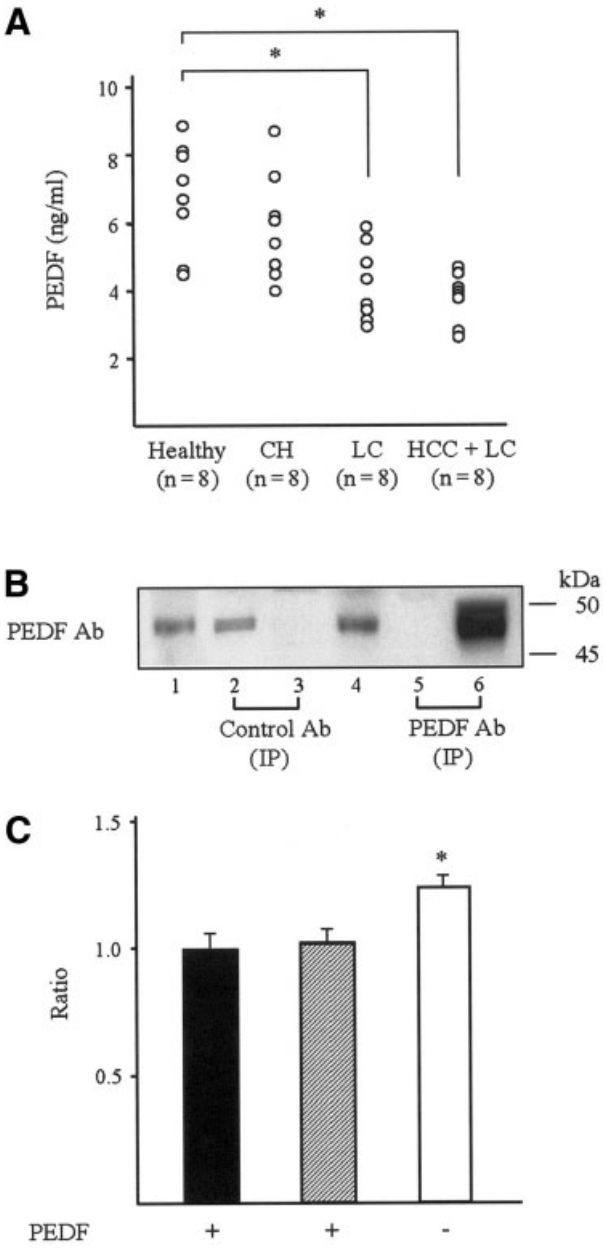

Fig. 3. Expression of pigment epithelium-derived factor (PEDF) in human samples and its inhibitory effect on human umbilical vascular endothelial cell (HUVEC) proliferation. (A) PEDF protein expression in serum of patients with liver diseases. Serum samples were analyzed by enzyme-linked immunosorbent assay. *$P < .01$ versus healthy volunteers. (B) Removal of human PEDF from the serum. Serum samples of hepatocellular carcinoma (HCC) patients were immunoprecipitated using anti-PEDF antibody or anti-mouse immunoglobulin G (IgG) antibody as control. An equal amount of flow-through fraction was analyzed by Western blotting using anti-PEDF antibody. Original serum (**lanes 1 and 4**), flow-through fraction of immunoprecipitation (**lanes 2 and 5**), and elution of immunoprecipitation (**lanes 3 and 6**). (C) Effect of PEDF in serum of HCC patients on HUVEC proliferation. HUVECs were incubated in the conditioned media containing 5% of original serum (**solid bar**), immunoprecipitated serum using normal mouse IgG (**hatched bar**) or anti-PEDF antibody (**open bar**). After 48-hour incubation, HUVEC numbers were determined and expressed as the ratio to their numbers in the original serum (PEDF-containing serum). Data are mean ± SD of all eight HCC patients. *$P < .01$ versus original serum and immunoprecipitated serum using normal mouse IgG. CH, chronic hepatitis; LC, liver cirrhosis; HCC + LC, hepatocellular carcinoma complicated with liver cirrhosis; IP, immunoprecipitation.

Discussion

Human PEDF is expressed in various tissues in the body[13,14] and is involved in retinal angiogenesis, however, there have been only a few specific studies of the antiangiogenic properties of PEDF in neoplastic diseases.[20–24] In the present study, we demonstrated that the mRNA and protein expression of PEDF varied in three HCC cell lines. PEDF mRNA was not detected in PLC/PRF/5, which is consistent with PEDF suppression being advantageous for tumor progression. Southern blotting showed a similar pattern of genomic PEDF in these cell lines. Therefore, PEDF expression seems to be regulated at the transcriptional level or in association with RNA stability. However, PEDF expression was suppressed at the protein level by chemically induced hypoxic conditions in the constitutive PEDF-expressing cell line, HepG2. Although PEDF mRNA was abundantly expressed in

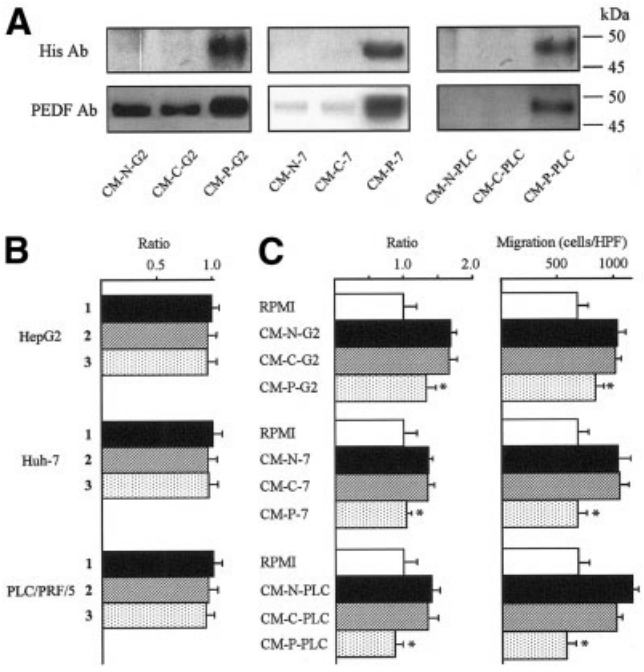

Fig. 4. Inhibition of proliferation and migration of human umbilical vascular endothelial cells (HUVECs) by pigment epithelium-derived factor (PEDF) gene induction. (A) Expression of PEDF protein in conditioned media (CM) derived from each transfected or nontransfected cell line. CM was collected after 48-hour incubation as described in Materials and Methods. Expression of PEDF protein was analyzed by Western blotting using antihexahistidine antibody or anti-PEDF antibody. (B) Growth of transfected or nontransfected HCC cells. HCC cell lines were incubated for 48 hours after transfection, and numbers of viable cells were determined. Data are expressed as the ratio to nontransfected cells (mean ± SD of three separate experiments). **1**, nontransfected; **2**, pcDNA3 transfected; **3**, pcDNA3-PEDF transfected. (C) Endothelial cell proliferation and migration. HUVECs were incubated in CM derived from each cell line. The numbers of proliferated cells after 48-hour incubation were estimated and expressed as the ratio to the number of cells incubated in RPMI. The numbers of migrated cells after 24 hours of incubation was determined under a light microscope with high-power field (magnification, ×200), as described in Materials and Methods. Data represent mean ± SD of three separate experiments. *$P < .01$ versus CM-N or CM-C. His Ab, ; Ab, ; CM-N, CM collected from nontransfected cells; CM-C, CM collected from pCDNA3 transfected cells; CM-P, CM collected from pcDNA3-PEDF transfected cells; CM-G2, CM collected from HepG2; CM-7, CM collected from Huh-7; CM-PLC, CM collected from PLC/PRF/5; RPMI, Roswell Park Memorial Institute; HPF, higher-power field.

HepG2, PEDF protein expression was suppressed in chemically induced hypoxic conditions in contrast to the elevation of VEGF protein expression. Constitutive overexpression of PEDF in HepG2 cells seems to be contradictive because the local environment should shift toward angiogenic conditions in cancer cells for rapid tumor growth. Thus, HepG2 cells may produce enough angiogenic reagents in excess of the level of antiangiogenic reagents such as PEDF. Indeed, VEGF, which is known as a major angiogenic factor, is expressed in adequate amounts in HepG2 cells.[23,24] Moreover, it is possible that PEDF is suppressed in HepG2 cells, as demonstrated in our hypoxic study when the cells were grown *in vivo* and were exposed to more hypoxic conditions than in culture media *in vitro*. Because we did not evaluate angiogenic and antiangiogenic reagents other than VEGF and PEDF and there are no adequate methods for estimating the local angiogenic or antiangiogenic activity directly and separately, precise evaluation of the angiogenic phenotype of specific tumors may be difficult. However, it can be concluded that PEDF must be involved as an antiangiogenic factor in HCC.

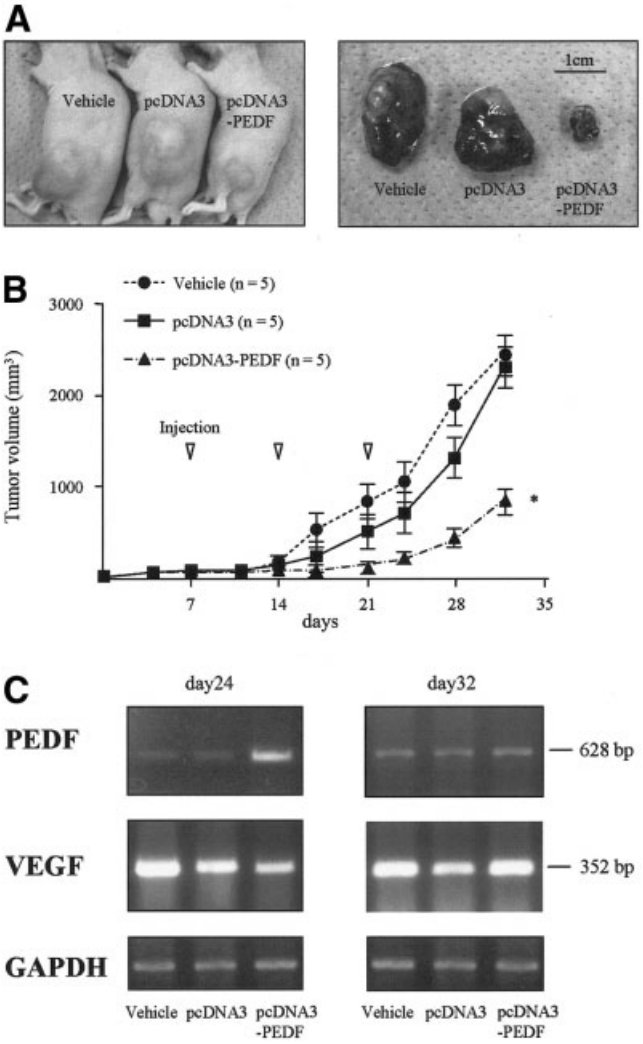

Fig. 5. Inhibition of pre-established tumor growth by injection of pcDNA3-pigment epithelium-derived factor (PEDF) plasmid in athymic mice. Vehicle, pcDNA3, or pcDNA3-PEDF was injected intratumorally into pre-established tumors of Huh-7 cells. Mice were killed on day 24 or day 32 and subcutaneous tumors were extracted. (A) Representative photographs of harvested tumors. (B) Serial changes in tumor volume in the three different groups. Data are mean ± SD of tumor volume. *$P < .01$ versus vehicle or pcDNA3. (C) Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis for PEDF and vascular endothelial growth factor (VEGF) messenger RNA (mRNA) expression, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. Total RNA was extracted from tumor tissues at days 24 and 32. RT-PCR was performed with primers specific for PEDF, VEGF, and GAPDH.

Suppression of malignant metastasis is considered to be partially dependent on the angiogenic phenotype of the primary tumor.[25,26] That is, in the clinical setting, development of multiple metastases sometimes occurs immediately after surgical resection of a primary tumor. This phenomenon has been explained by humoral factors derived from the primary tumor. Removal of the primary tumor may lead to a decrease in circulatory antiangiogenic factors produced by the primary tumor and to progression of metastasis.[27–29] Thus, humoral systemic antiangiogenic agents may be involved in both metastasis and tumor progression. However, chronic liver diseases, especially associated with viral hepatitis B or C, are clearly recognized as premalignant conditions for HCC.[15] In this regard, we investigated the serum PEDF protein concentration in patients with CH, LC, and HCC. In our clinical analysis, PEDF was constitutively and abundantly detected in the sera of healthy volunteers, and PEDF protein concentration was found to be decreased in the sera to levels proportionate with the progression of premalignant liver diseases. However, evaluation of serum samples of patients with compensated LC (Child-Pugh classification A or B; n = 5) and decompensated LC (Child-Pugh classification C; n = 5) showed no differences in serum PEDF concentration between the two groups ($P = 0.293$, data not shown). The number of samples may be too small or PEDF expression may be fully suppressed even in compensated LC before progression to decompensated LC. Next, we evaluated the PEDF mRNA by Northern blot analysis in several liver tissues to confirm the direct causal relationship between PEDF expression and liver disease. There was a tendency for PEDF mRNA expression to decrease to levels proportionate with the progression of liver disease. However, the number of samples that we could obtain from liver surgery in this study was not sufficient for proper statistical analysis (n = 2 for each group). Thus, in this study, we could not provide reliable data on PEDF mRNA expression in liver tissues of patients with various liver diseases. However, previous studies reported a liver-specific high expression of PEDF compared with other organs.[13,14] Hence, serum PEDF concentrations seem to be dependent on hepatic protein production capacity. In this regard, virus protein, hepatic fibrosis, or inflammation itself may affect PEDF production from the liver. Further research is required to clarify the mechanism of the suppressive effect of PEDF in chronic liver disease. In this study, because PEDF protein concentrations in LC and HCC complicating LC were approximately equivalent, development of HCC apparently did not influence the serum PEDF protein concentration. However, reduction of serum PEDF concentration may alter tissue surrounding HCC toward angiogenic conditions and may contribute to the progression of HCC. In other words, PEDF may function as a tumor suppresser in some HCC patients or cell lines. Further investigation, in a larger number of patients with liver diseases or other malignant diseases, may be needed because the sample number of this clinical study was quite small. Moreover, the only other report of circulating PEDF reported 5 μg/mL of this protein in plasma by Western blot analysis using known amounts of purified PEDF.[30] In this study, we used a commercial ELISA kit to measure serum PEDF concentrations. The difference in the two studies may be caused by differences in the method used for measurement, sample type, or both. In this regard, a previous study used the same ELISA kit and reported that PEDF concentration in human vitreous fluid was 1 to 9 ng/mL, although other investigators who used their own ELISA system reported the level to be at 1 to 2.5 μg/mL in the same fluid.[31]

In this study, the CM derived from all PEDF-overexpressing HCC cell lines efficiently inhibited proliferation and migration of HUVECs regardless of the level of PEDF expression, whereas the three HCC cell lines expressed massive levels of VEGF in the CM (data not shown). In addition, overexpression of PEDF in preestablished subcutaneous HCC tumors in nude mice resulted in efficient suppression of tumor progression. When PEDF expression decreased because of the limited period of plasmid expression system, the tumor started to escape growth suppression. Moreover, PEDF protein induced from pcDNA3-PEDF-transfected tumors was not detected in the mouse serum (data not shown). These results indicate that the local concentration of antiangiogenic factors is important for tumor growth inhibition. Indeed, the therapeutic limitation of systemic administration of antiangiogenic compounds has been reported.[32,33] In this regard, antiangiogenic gene therapy may be an attractive strategy, because gene induction may increase the local concentration of the protein product from the therapeutic gene in the tumor. Indeed, we have already reported the enhanced growth inhibition of angiostatin gene-induced PLC/PRF/5.[34] Recently, Wang et al.[23] reported the antitumor effects of systemic or intratumoral administration of adenovirus encoding PEDF in a mouse HCC and lung carcinoma model. However, this model is confounded because the viral vector, including adenovirus, could induce a critical adverse reaction.[35] Although nonviral gene delivery systems are less efficient at inducing transgene expression and have shorter-term expression (compared with viral delivery systems), as shown in the present study, adverse reactions are thought to be less frequent. In the present study, we used a plasmid vector encoding PEDF. Despite the expected low efficiency of gene induction,

marked growth inhibition of preestablished tumors was demonstrated. Therefore, the present study indicates that a sufficient bystander effect was achieved by this strategy, and if the transgene is expressed intratumorally, highly efficient therapeutic gene induction is not necessarily required. HCCs seem to be very sensitive to vascular starvation.

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