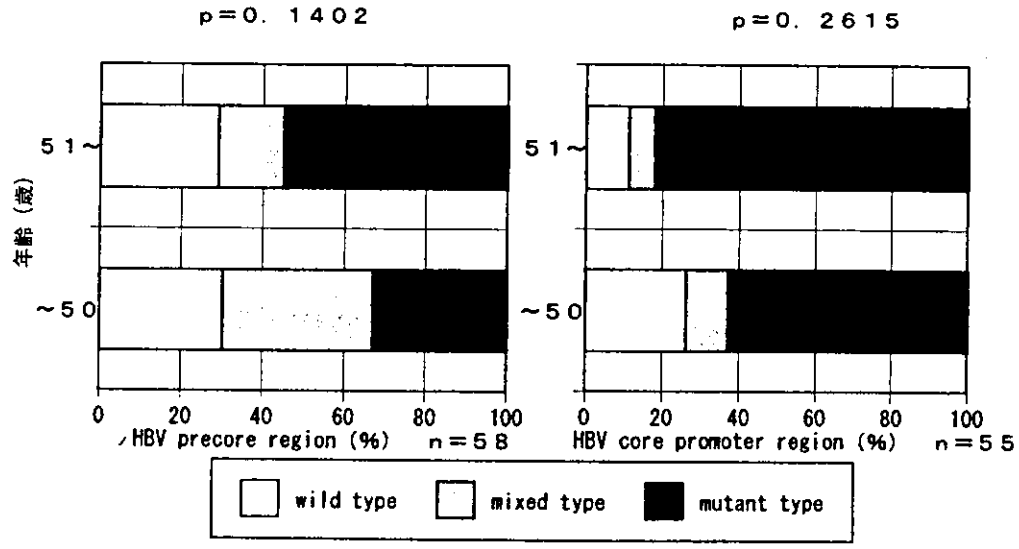

図1　年齢別のHBVプレコア／コアプロモーター変異の頻度

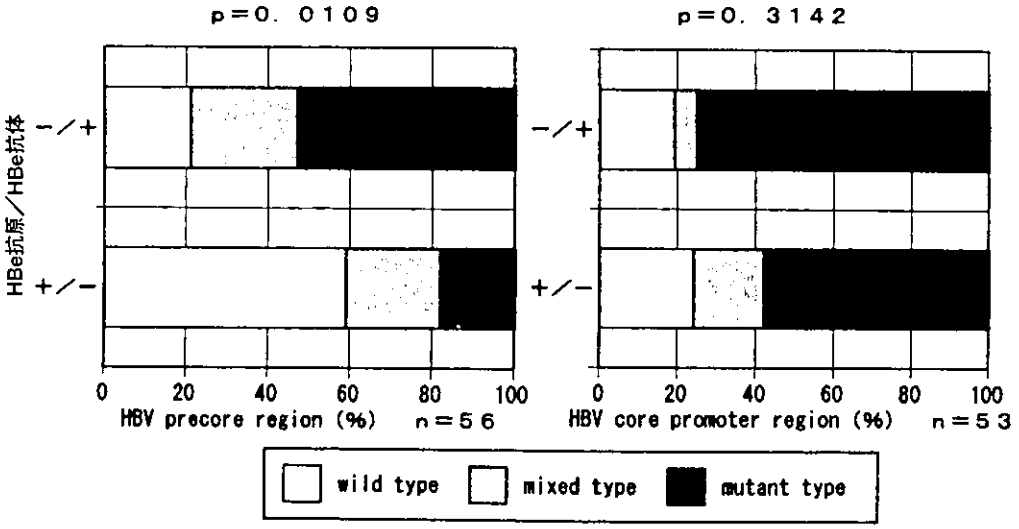

図2　HBe抗原／抗体状態でみたHBVプレコア／コアプロモーター変異の頻度

合型4/35（11.4％），変異株26/35（74.3％）であり，HBV DNA値5.0 LC/mL以上で野生株の占める割合が高かった（p＝0.3159）（図3）。HBVプレコア／コアプロモーター変異を慢性HBV感染患者の肝疾患の進行の程度で分けて検討した。HBVプレコア領域の変異は慢性肝炎で野生型12/28（42.9％），混合型6/28（21.4％），変異株10/28（35.7％），肝硬変で野生株1/17（5.9％），混合型6/17（35.3％），変異株10/17（58.8％），肝細胞癌で野生株4/13（30.8％），混合型3/13（23.1％），変異株6/13（46.2％）であり，慢性肝炎に比べて，肝硬変，肝細胞癌で変異株の占める割合が高かった（p＝0.1333）。HBVコアプロモーター領域の変異は慢性肝炎で野生株6/27（22.2％），混合型4/27（14.8％），変異株17/27（63.0％），肝硬変で野生株4/17（23.5％），混合型0/17（0.0％），変異株13/17（76.5％），肝細胞癌で野生株1/11（9.1％），混合型1/11（9.1％），変異株9/11（81.8％）であり，慢性肝疾患の進行に伴って変異株の占める割合が高かった（p＝0.4274）（図4）。

Ⅳ．考　　察

HBVゲノタイプBとCは日本を含めたアジアの代表的なHBVゲノタイプである[7]。HBVゲノタイプCはゲノタイプBに比べて，HBe抗原seroconversion率が低く，程度の高い肝疾患と関連することが示唆され[8]，また，HBVゲノタイプCはゲノタイプBに比べて，

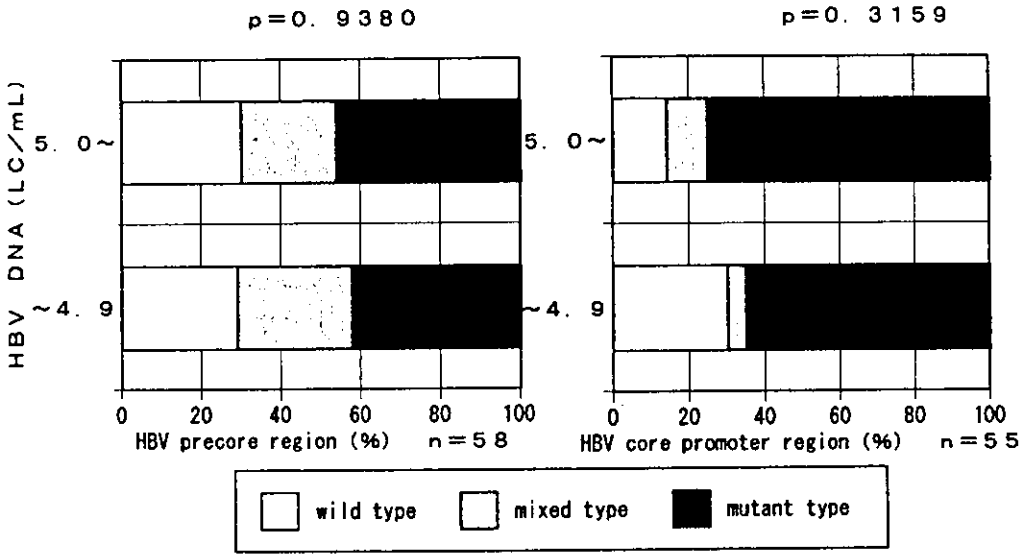

図3　HBV DNA値でみたHBVプレコア／コアプロモーター変異の頻度

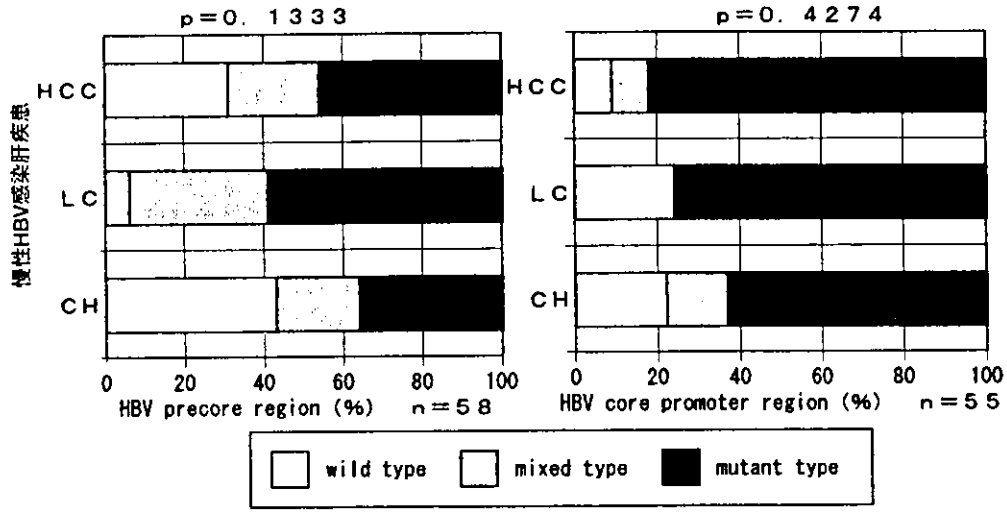

図4　慢性肝疾患の進行でみたHBVプレコア／コアプロモーター変異の頻度
（CH；慢性肝炎，LC；肝硬変，HCC；肝細胞癌）

コアプロモーター変異（T1762/A1764変異）の頻度が高いことが示されている[9]。

今回，われわれは国立病院呉医療センターで加療中の患者を対象にして，HBVプレコア／コアプロモーター変異を検討した。HBVゲノタイプは58例中11例で測定されているが，全例ゲノタイプCであった。肝細胞癌とHBVコアプロモーター変異との関連を示したKaoらはHBVコアプロモーター変異の存在が肝細胞癌と有意に相関し，これはHBVゲノタイプBあるいはCに関係なく，若年者肝細胞癌患者のHBVコアプロモーター変異の頻度が，年齢が一致する非活動性キャリアーに比べて有意に高く，高齢者肝細胞癌患者との間に差がなかったことを報告している[5]。これが事実とすると，若年者でHBVコアプロモーター変異を有する患者は肝細胞癌発生の危険性が高い群として，注意深い観察が必要であることを意味している。われわれの対象患者は平均年齢が50.6歳であり，肝細胞癌13例の中に若年者肝細胞癌患者が含まれていなかったが，HBVコアプロモーター変異の頻度は加齢と平行して増加する傾向を示した。したがって，肝細胞癌患者に有意に高い頻度のHBVコアプロモーターは認めることができなかった。

一方，Chuらはアメリカの HBV キャリアーを対象に，HBVコアプロモーター変異の存在が重篤な慢性肝

疾患と関連している可能性があり，HBe 抗原陰性患者では，血清 HBV DNA 高値と関連していることを示している[6]。われわれの HBe 抗原／抗体（−／+）患者は（+／−）患者に比べて，HBV プレコア変異の頻度が有意に高く，コアプロモーター変異の頻度も高い傾向を示したが，血清 HBV DNA 値に関しては，HBV プレコア変異の存在の関与は認められず，コアプロモーター変異を有する患者で高値となる傾向が認められた。

以上を考え合わせると，HBV プレコア／コアプロモーター変異が加齢に伴って頻度が増加し，変異が肝疾患の進行に加担していることが示唆されるが，今回の対象患者の症例数および年齢分布に限界があり，結論を導くまでには至らなかった。今後，特に若年者肝細胞癌患者を含めて，多数例を対象にした検討が必要であると考えられる。

V. 結　　語

慢性 HBV 感染患者 58 例を対象にして，HBV プレコア／コアプロモーター変異を検討した。HBV プレコア／コアプロモーター変異が加齢と関連していることが示唆されたが，コアプロモーター変異が慢性肝疾患の進行に加担している可能性があり，若年者での HBV コアプロモーター変異と肝細胞癌発生の間の関連を検討する必要性が認められた。

文　　献

1) Summers J, Mason WS: Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29: 403–415, 1982.
2) Carmen WF, Jacyna MR et al: Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 2: 588–591, 1989.
3) Buckwold VE, Xu Z et al: Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 70: 5845–5851, 1996.
4) Funk ML, Rosenberg DM et al: World-wide epidemiology of HbeAg-negative chronic hepatitis B and associated with precore and core promoter variants. J Viral Hepat 9: 52–61, 2002.
5) Kao JH, Chen PJ et al: Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterol 124: 327–334, 2003.
6) Chu CJ, Keeffe EB et al: Prevalence of HBV precore/core promoter variants in the United States. Hepatol 38: 619–628, 2003.
7) Kao JH, Chen PJ et al: Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterol 118: 554–559, 2001.
8) Ding X, Mizokami M et al: Different hepatitis B virus genotype distributions among asymptomatic carriers and patients with liver diseases in Nanning, southern China. Hepatol Res 22: 37–44, 2002.
9) Yuen MF, Sablon E et al: Significance of hepatitis B genotype in acute exacerbation, HBeAg seroconversion, cirrhosis-related complications, and hepatocellular carcinoma. Hepatol 37: 562–567, 2003.

（受付　2004－2－18）

Antiangiogenic Property of Pigment Epithelium-Derived Factor in Hepatocellular Carcinoma

Kojiro Matsumoto,[1] Hiroki Ishikawa,[1] Daisuke Nishimura,[1] Keisuke Hamasaki,[1] Kazuhiko Nakao,[2] and Katsumi Eguchi[1]

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.

From the [1]First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan, and [2]Health Research Center, Nagasaki University, Nagasaki, Japan.

Received October 23, 2003; accepted March 29, 2004.

Address reprint requests to: Katsumi Eguchi, First Department of Internal Medicine, Nagasaki University of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: koujirou708@yahoo.co.jp; fax: +81-95-849-7270.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20259

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.

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).

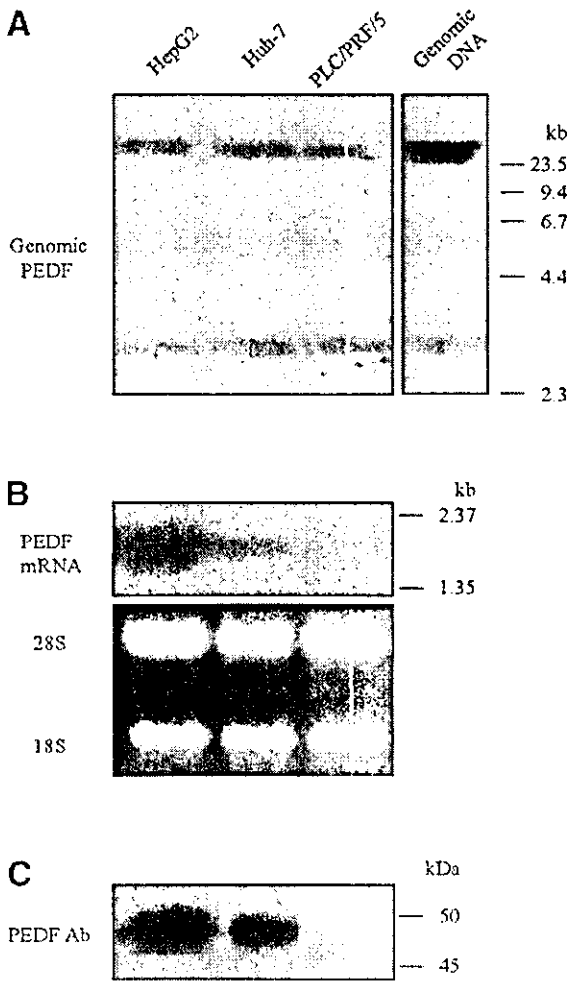

Fig. 1. Expression of pigment epithelium-derived factor (PEDF) in cell lines. (A) Southern blot analysis of genomic DNA derived from hepatocellular carcinoma cell lines and human genomic DNA. (B) PEDF messenger RNA (mRNA) as determined by Northern blot analysis. The 1.5-kb band indicates PEDF gene mRNA. 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.

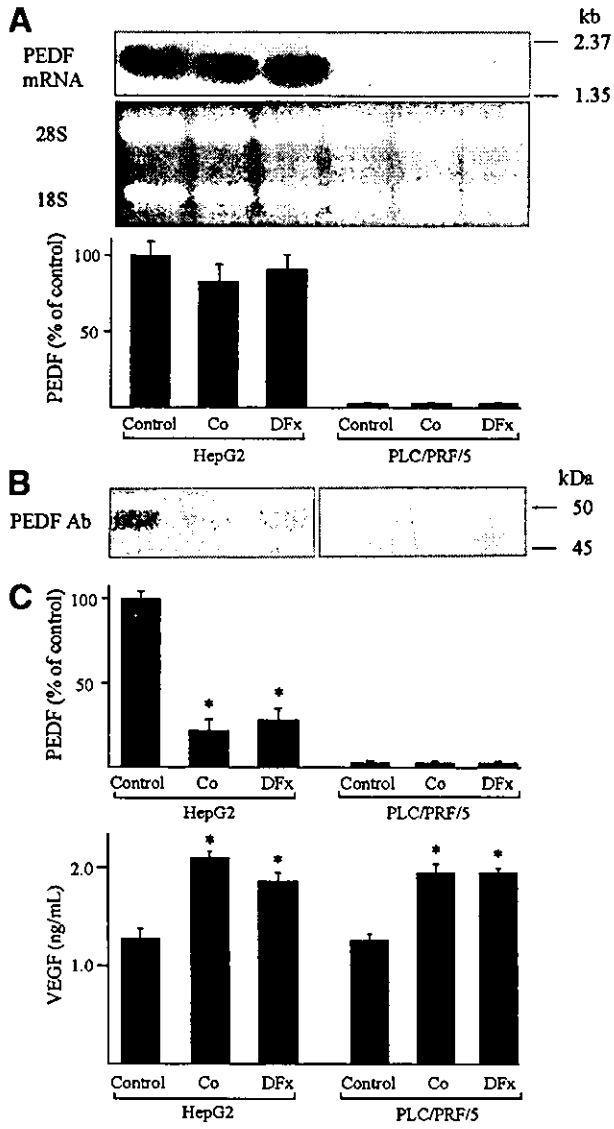

Fig. 2. Effect of chemically induced hypoxia on the expression of pigment epithelium-derived factor (PEDF) or vascular endothelial growth factor (VEGF) in hepatocellular carcinoma cell lines. HepG2 or PLC/PRF/5 cells were incubated for 12 hours with 400 μmol/L cobalt chloride (Co) or 260 μmol/L desferrioxamine (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.

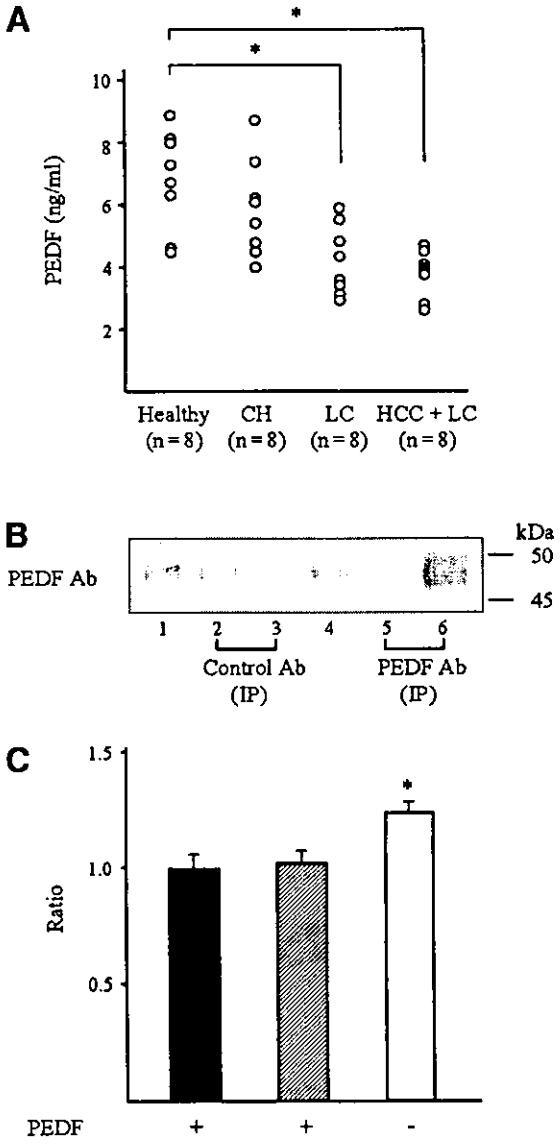

Fig. 3. Expression of pigment epithelium-derived factor (PEDF) in human samples and its inhibitory effect on human umbilical vascular endothelial cell (HUVEC) proliferation. (A) PEDF protein expression in serum of patients with liver diseases. Serum samples were analyzed by enzyme-linked immunosorbent 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

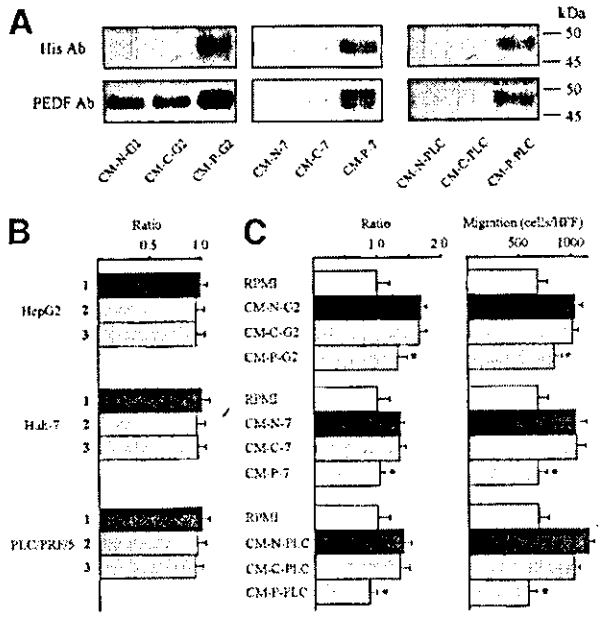

Fig. 4. Inhibition of proliferation and migration of human umbilical vascular endothelial cells (HUVECs) by pigment epithelium-derived factor (PEDF) gene induction. (A) Expression of PEDF protein in conditioned media (CM) derived from each transfected or nontransfected cell line. CM was collected after 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.

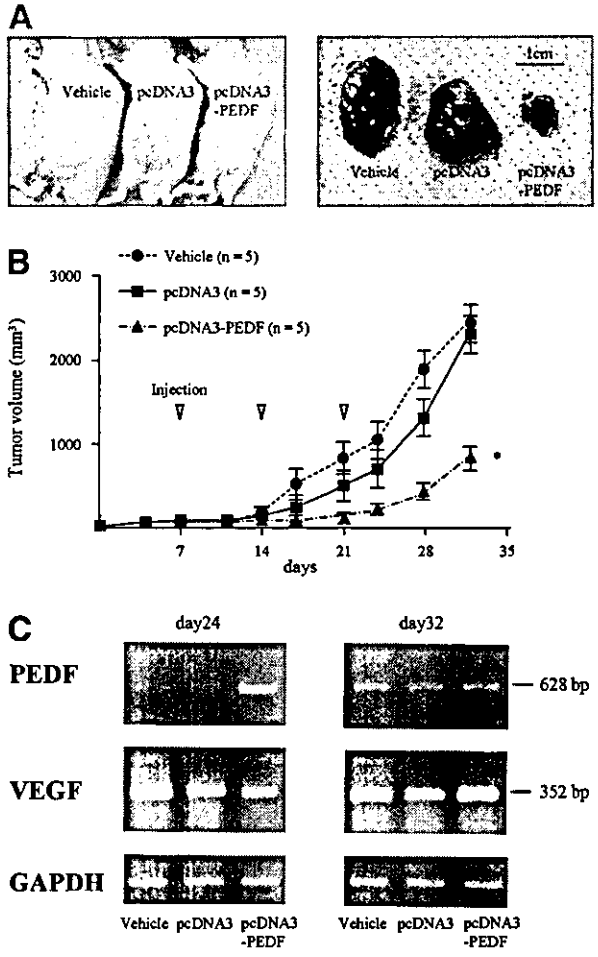

Fig. 5. Inhibition of pre-established tumor growth by injection of pcDNA3-pigment epithelium-derived factor (PEDF) plasmid in athymic mice. Vehicle, pcDNA3, or pcDNA3-PEDF was injected intratumorally into pre-established tumors of Huh-7 cells. Mice were killed on day 24 or day 32 and subcutaneous tumors 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.

References

1. Gimbrone MA Jr, Leapman SB, Cotran RS, Folkman J. Tumor dormancy in vivo by prevention of neovascularization. J Exp Med 1972;136:261–276.
2. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–1186.
3. Eliceiri BP, Cheresh DA. The role of alpha integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J Clin Invest 1999;103:1227–1230.
4. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000;6:389–395.
5. Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat Med 2000;6:41–48.
6. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989; 246:1306–1309.
7. Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267:10931–10934.
8. Tombran-Tink J, Chader GG, Johnson LV. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. Exp Eye Res 1991;53:411–414.
9. Tombran-Tink J, Johnson LV. Neuronal differentiation of retinoblastoma cells induced by medium conditioned by human RPE cells. Invest Ophthalmol Vis Sci 1989;30:1700–1707.
10. Gao G, Li Y, Gee S, Dudley A, Fant J, Crosson C, et al. Down-regulation of vascular endothelial growth factor and up-regulation of pigment epithelium-derived factor: a possible mechanism for the anti-angiogenic activity of plasminogen kringle 5. J Biol Chem 2002;277:9492–9497.
11. Cao W, Tombran-Tink J, Chen W, Mrazek D, Elias R, McGinnis JF. Pigment epithelium-derived factor protects cultured retinal neurons against hydrogen peroxide-induced cell death. J Neurosci Res 1999;57: 789–800.
12. Houenou LJ, D'Costa AP, Li L, Turgeon VL, Enyadike C, Alberdi E, et al. Pigment epithelium-derived factor promotes the survival and differentiation of developing spinal motor neurons. J Comp Neurol 1999;412:506–514.
13. Palmieri D, Watson JM, Rinehart CA. Age-related expression of PEDF/EPC-1 in human endometrial stromal fibroblasts: implications for interactive senescence. Exp Cell Res 1999;247:142–147.
14. Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 1999;285:245–248.
15. Volpert OV, Zaichuk T, Zhou W, Reiher F, Ferguson TA, Stuart PM, et al. Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. Nat Med 2002;8:349–357.
16. Singh VK, Chader GJ, Rodriguez IR. Structural and comparative analysis of the mouse gene for pigment epithelium-derived factor (PEDF). Mol Vis 1998;4:7.
17. Tombran-Tink J, Mazuruk K, Rodriguez IR, Chung D, Linker T, Englander E, et al. Organization, evolutionary conservation, expression and unusual Alu density of the human gene for pigment epithelium-derived factor, a unique neurotrophic serpin. Mol Vis 1996;2:11.
18. Leavesley DI, Schwartz MA, Rosenfeld M, Cheresh DA. Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. J Cell Biol 1993;121:163–170.
19. Goldberg MA, Gaut CC, Bunn HF. Erythropoietin mRNA levels are governed by both the rate of gene transcription and posttranscriptional events. Blood 1991;77:271–277.
20. Pham I, Uchida T, Planes C, Ware LB, Kaner R, Matthay MA, et al. Hypoxia upregulates VEGF expression in alveolar epithelial cells in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol 2002;283:L1133–L1142.
21. Ankoma-Sey V, Wang Y, Dai Z. Hypoxic stimulation of vascular endothelial growth factor expression in activated rat hepatic stellate cells. HEPATOLOGY 2000;31:141–148.
22. Mahtabifard A, Merritt RE, Yamada RE, Crystal RG, Korst RJ. In vivo gene transfer of pigment epithelium-derived factor inhibits tumor growth in syngeneic murine models of thoracic malignancies. J Thorac Cardiovasc Surg 2003;126:28–38.
23. Wang L, Schmitz V, Perez-Mediavilla A, Izal I, Prieto J, Qian C. Suppression of angiogenesis and tumor growth by adenoviral-mediated gene transfer of pigment epithelium-derived factor. Mol Ther 2003;8:72–79.
24. Doll JA, Stellmach VM, Bouck NP, Bergh AR, Lee C, Abramson LP, et al. Pigment epithelium-derived factor regulates the vasculature and mass of the prostate and pancreas. Nat Med 2003;9:774–780.
25. Gasparini G. The rationale and future potential of angiogenesis inhibitors in neoplasia. Drugs 1999;58:17–38.
26. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995;1:27–31.
27. Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat Med 1995;1:149–153.
28. Cao Y, O'Reilly MS, Marshall B, Flynn E, Ji RW, Folkman J. Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. J Clin Invest 1998;101:1055–1063.
29. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994;79:315–328.
30. Petersen SV, Valnickova Z, Enghild JJ. Pigment-epithelium-derived-factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization. Biochem J 2003;374:199–206.
31. Ogata N, Nishikawa M, Nishimura T, Mitsuma Y, Matsumura M. Unbalanced vitreous level of pigment epithelium-derived factor and vascular endothelial growth factor in diabetic retinopathy. Am J Ophthalmol 2002; 134:348–353.
32. Chen QR, Kumar D, Stass SA, Mixson AJ. Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. Cancer Res 1999;59:3308–3312.
33. Sauter BV, Martinet O, Zhang WJ, Mandeli J, Woo SL. Adenovirus-mediated gene transfer of endostatin in vivo results in high level of transgene expression and inhibition of tumor growth and metastases. Proc Natl Acad Sci U S A 2000;97:4802–4807.
34. Ishikawa H, Nakao K, Matsumoto K, Ichikawa T, Hamasaki K, Nakata K, et al. Antiangiogenic gene therapy for hepatocellular carcinoma using angiostatin gene. HEPATOLOGY 2003;37:696–704.
35. Marshall E. Gene therapy death prompts review of adenovirus vector. Science 1999;17:2244–2225.

□ CASE REPORT □

Nonalcoholic Steatohepatitis with Improved Hepatic Fibrosis after Weight Reduction

Keita FUJIKAWA, Kazuyuki OHATA, Takuya HONDA, Seiji MIYAZOE, Tatsuki ICHIKAWA, Hiroki ISHIKAWA, Keisuke HAMASAKI, Kazuhiko NAKAO*, Kan TORIYAMA** and Katsumi EGUCHI

Abstract

A 65-year-old woman was admitted to our hospital for an investigation of liver dysfunction. She had mild obesity with hyperlipidemia, but no history of alcohol abuse. Other known causes of liver dysfunction, such as viruses, autoimmunity and drug effects, were excluded. The liver histology was consistent with nonalcoholic steatohepatitis (NASH). After diagnosis of NASH, the patient started diet and exercise therapy and, in parallel with weight reduction, her liver function improved. One year after the therapy, a liver biopsy showed that steatosis, necroinflammation and even fibrosis were improved. Hence, here we report a case of NASH in which weight reduction was effective in improving both biochemical and histological findings.
(Internal Medicine 43: 289–294, 2004)

Key words: nonalcoholic steatohepatitis, weight reduction, hepatic fibrosis

Introduction

Obesity is an epidemic that is currently recognized as a major public health problem worldwide. In recent years, the number of obese patients has also increased in Japan. Obesity is a risk factor for various diseases such as type II diabetes, hyperlipidemia, hypertension and cardiovascular disease. Furthermore, obesity is a condition that is often reported in association with nonalcoholic fatty liver diseases, including nonalcoholic steatohepatitis (NASH) (1–4).

NASH is a condition characterized by a histologic picture similar to alcoholic liver injury, but without the presence of alcohol abuse (1–4). Many patients with NASH have underlying risk factors such as obesity, diabetes mellitus and hyperlipidemia (1–5). The natural history and the long-term prognosis of NASH are not well understood, but the available data suggest that NASH is a benign disease in most patients. However, it was reported that 43% of patients with NASH had histologic progression and in approximately 8% to 17% of patients with NASH it can lead to cirrhosis with related complications (6–8).

There are a few previous reports on the effect of diet and exercise therapy on the clinical features of NASH (9–12). However, the effect of weight reduction on histologic findings, and especially on fibrosis, is not fully understood. Several drug therapies for NASH have been reported to be potentially useful, but the number of patients in these studies was small. Here, we describe a patient with NASH who was treated using diet and exercise therapy, and consequently showed both biochemical and histologic improvement of the liver.

Case Report

The patient was a 65-year-old woman who had suffered from liver dysfunction from 1998. She had no history of blood transfusion, or alcohol or drug abuse. Although she had received injections of 60 ml of stronger neo-minophagen C (SNMC) three times a week, her elevated transaminase levels were sustained. She was admitted to our hospital on May 9, 2001 for an examination of her liver dysfunction. A physical examination on admission showed mild obesity (body mass index; BMI 25.1 kg/m^2). No hypertension or hepatomegaly were noted. The laboratory data on admission are shown in Table 1.

Blood biochemistry tests showed an aspartate aminotransferase (AST) level of 210 IU/*l*, alanine aminotransferase (ALT) 231 IU/*l*, lactate dehydrogenase (LDH) 293 IU/*l* and alkaline phosphatase (ALP) 385 IU/*l*. The fasting blood glu-

From the First Department of Internal Medicine, *the Health Research Center and **the Department of Pathology, Institute of Tropical Medicine, Nagasaki University, Nagasaki

Received for publication June 23, 2003; Accepted for publication November 19, 2003

Reprint requests should be addressed to Dr. Kazuyuki Ohata, the First Department of Internal Medicine, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501

Table 1. Laboratory Data on Admission

Peripheral blood		Blood Chemistry			
White blood cells	4,900/mm³	Total protein	8.1 g/dl	Iron	125 μg/dl
Neutorophil	57%	Albumin	5.1 g/dl	Ferritin	225.3 ng/dl
Eosinophil	3%	Total bilirubin	1.2 mg/dl	Copper	101 μg/dl
Basophil	0%	Asparate aminotransferase	210 IU/l	Ceruloplasmin	22.4 mg/dl
Lymphocyte	29%	Alanin aminotransferase	231 IU/l	Serology	
Monocyte	11%	Alkaline phosphatase	385 IU/l	Immunoglobulin G	1150 mg/dl
Red blood cells	474×10⁴/mm³	Lactate dehydrogenase	293 IU/l	Immunoglobulin A	64 mg/dl
Hemoglobin	14.8 g/dl	γ-glutamyltranspeptidase	68 IU/l	Immunoglobulin M	642 mg/dl
Hematocrit	42.50%	Blood urea nitrogen	15 mg/dl	HBsAg	0.1 COI
Platelet	16.0×10⁴/mm³	Creatinine	0.7 mg/dl	HBc-Ab	96.2%
Coagulation		Na	145 mEq/l	HBcAb (×200)	16.1%
Prothrombin time	94%	K	4.1 mEq/l	Anti-HCV antibody	(−)
APTT	29.9 sec.	Cl	109 mEq/l	HBV DNA	(−)
		Total cholesterol	231 mg/dl	HEV RNA	(−)
		Triglyceride	155 mg/dl	TTV DNA	(−)
		Blood glucose	86 mg/dl	Antinuclear antibody	(−)
		HbA1c	4.7%	Antimitochondral antibody	(−)
		Fasting insulin	13.2 μU/ml	Antismooth muscle antibody	(−)
		HOMA	2.8		
		ACE	25.9 IU/l		

ATT: activated partial thromboplastine time, HOMA: homeostasis model of assessment, ACE: angiotensin converting enzyme, HBsAg: hepatitis B surface antigen, HBc-Ab: anti-hepatitis B core antibody, HCV: hepatitis C virus, COI: cut off index, HBV: hepatitis B virus, HEV: hepatitis E virus, TTV: TT virus, DNA: deoxyribonucleic acid, RNA: ribonucleic acid.

cose, hemoglobin A1c and fasting insulin levels were all normal. The patient's homeostasis model of assessment (HOMA) score (13), which is commonly used as a surrogate marker for insulin resistance, was elevated to 2.80. Total cholesterol and triglyceride levels were elevated to 231 mg/dl and 155 mg/dl, respectively. The patient tested negative for various viral markers, including hepatitis B surface antigen (HBs-Ag), anti-hepatitis B core antibody (HBc-Ab), antibody to hepatitis C virus (anti-HCV), HBV DNA, TT virus (TTV) DNA and hepatitis E virus (HEV) RNA. HBsAg, and HBc-Ab were assayed using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan). Anti-HCV was determined using a third-generation enzyme-linked immunoadsorbent assay (Ortho Diagnostics Systems, Tokyo, Japan). Serum HBV DNA was tested by using polymerase chain reaction (PCR) reported previously (14), TTV DNA was tested by using PCR kit (Institute of Immunology Co., Ltd. Tokyo, Japan) and HEV RNA was tested by RT-PCR (Mitsubishi Kagaku Bio-Clinical Laboratories, Inc. Tokyo, Japan). Autoantibodies, including anti-nuclear antibody (ANA), anti-mitochondrial antibody (AMA), and anti-smooth muscle antibody (ASMA) were also negative. The autoimmune hepatitis (AIH) score by the revised international criteria (15) for diagnosis of AIH was nine, which was not considered to be AIH. Serum iron, copper, ceruloplasmin and angiotensin-converting enzyme level were normal. Fibrosing markers, hyaluronic acid and procollagen III peptide (P-III-P) were elevated (hyaluronic acid 186 ng/ml, normal range ≤50 ng/ml; P-III-P 1.4 U/ml, normal range ≤1.0 U/ml). An ultrasonography examination suggested that the echogenicity of the liver was diffusely increased, compared to that of the kidneys. A liver biopsy was performed on the fourth hospital day to evaluate liver histology. A specimen evaluated using light microscopy showed the presence of macrovesicular and microvesicular steatosis, spotty necrosis, mild to moderate inflammatory cell infiltration and moderate perivenular, perisinusoidal and portal fibrosis (Fig. 1) with one focus of porto-portal bridging fibrosis [Grade 2 and Stage 3, as categorized by Brunt et al. (16)] . Based on these findings, the patient was diagnosed with nonalcoholic steatohepatitis (NASH).

After diagnosis of NASH, diet therapy (1,280 kcal/day; 25 kcal/kg/day) and exercise therapy (180 kcal/day) was started. The patient made a continuous effort toward achieving weight reduction. Consequently, her body weight decreased from 58 kg to 53 kg (BMI 22.9 kg/m²) and her elevated level of transaminases normalized after 12 months of diet and exercise therapy, in parallel with her weight reduction (Fig. 2). However, the patient's HOMA score did not decrease (HOMA 3.0).

On July 15, 2002, the patient was re-admitted to our hospital for examination of her liver and a second liver biopsy was performed. Compared with the initial biopsy, histologic findings such as steatosis, necroinflammation and even fibrosis of the liver, were improved (Fig. 3: Grade 1 and Stage 2). Based on laboratory data, hyaluronic acid and P-III-P had normalized to 28.2 ng/ml and 0.9 U/ml, respectively.

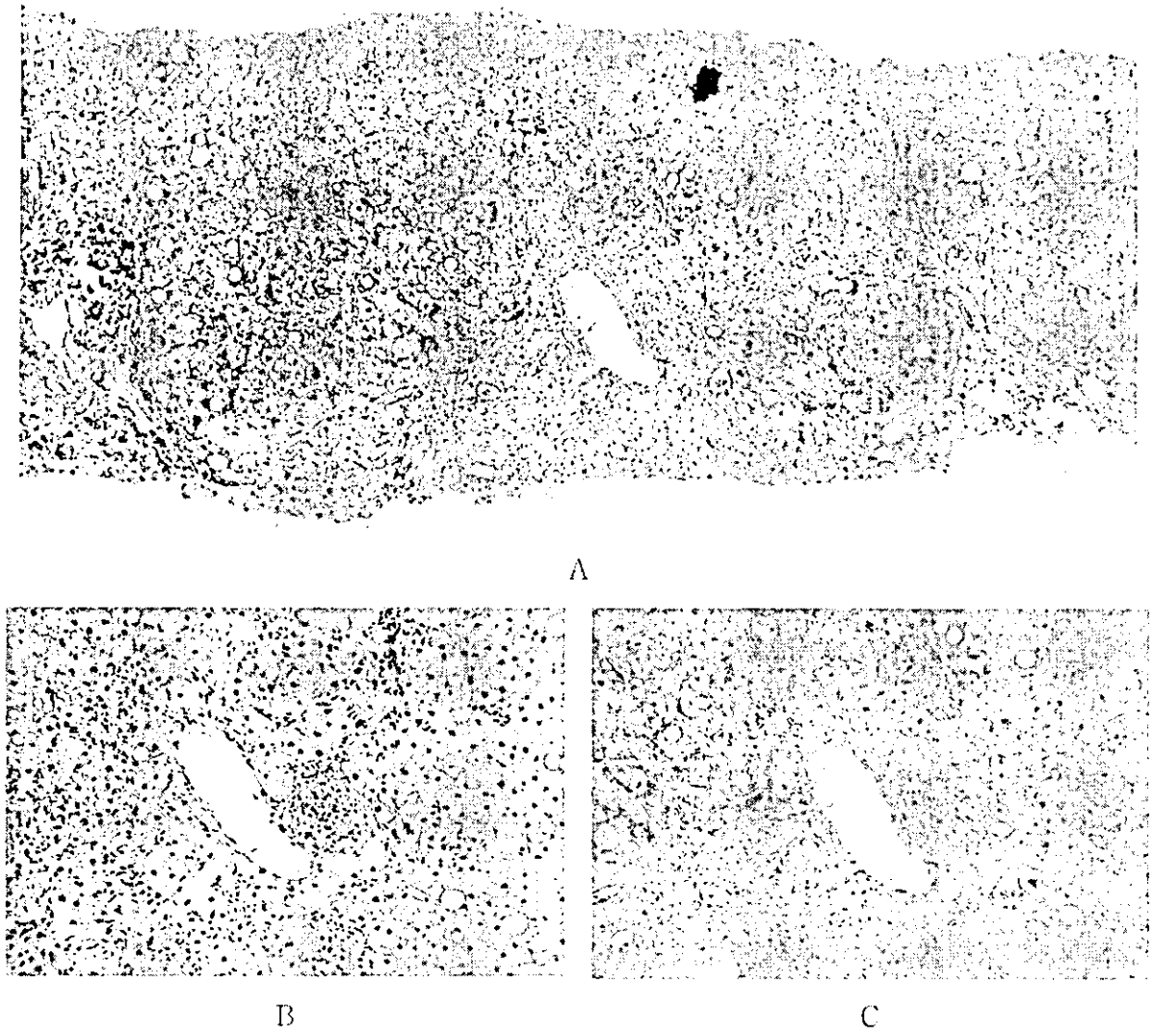

Figure 1. Liver biopsy specimens before diet and exercise therapy. A. Moderate macrovesicular and microvesicular steatosis, and moderate fibrosis in the centrilobular and portal areas (Azan-Mallory, ×40). B. Moderate steatosis, spotty necrosis with lymphocyte and polymorphonuclear leukocyte infiltration and ballooned hepatocytes around the central vein (hematoxylin and eosin, ×100). C. Extensive perivenular and perisinusoidal fibrosis in the centrilobular (zone 3) area (Azan-Mallory, ×100).

Discussion

NASH is defined histologically when a combination of macrovesicular steatosis, hepatocyte injury and necrosis, mixed inflammatory cell infiltration and variable degrees of fibrosis are observed in the absence of chronic abuse of alcohol (1–4). The histologic findings for the patient described above were consistent with NASH, showing 30 to 50% macrovesicular and microvesicular steatosis, porto-portal bridging fibrosis, piecemeal necrosis, and mild-to-moderate inflammatory cell infiltration. NASH is mainly associated with obesity and diabetes mellitus, hypercholesterolemia and hypertriglyceridemia (1–5), and the patient also had some of these risk factors.

Although in most cases fatty liver disease does not progress to more severe liver diseases, approximately 20 to 30% of patients have histologic signs of fibrosis and necroinflammation, indicating the presence of NASH. Furthermore, some cases of NASH are at a higher risk of developing cirrhosis, terminal liver failure, and hepatocellular carcinoma (7, 8, 17, 18). It has been reported that obese persons of relatively advanced age (≥45 years), and those with diabetes mellitus, a greater degree of hepatic steatosis, and higher grades of hepatic inflammation have a risk for progression to

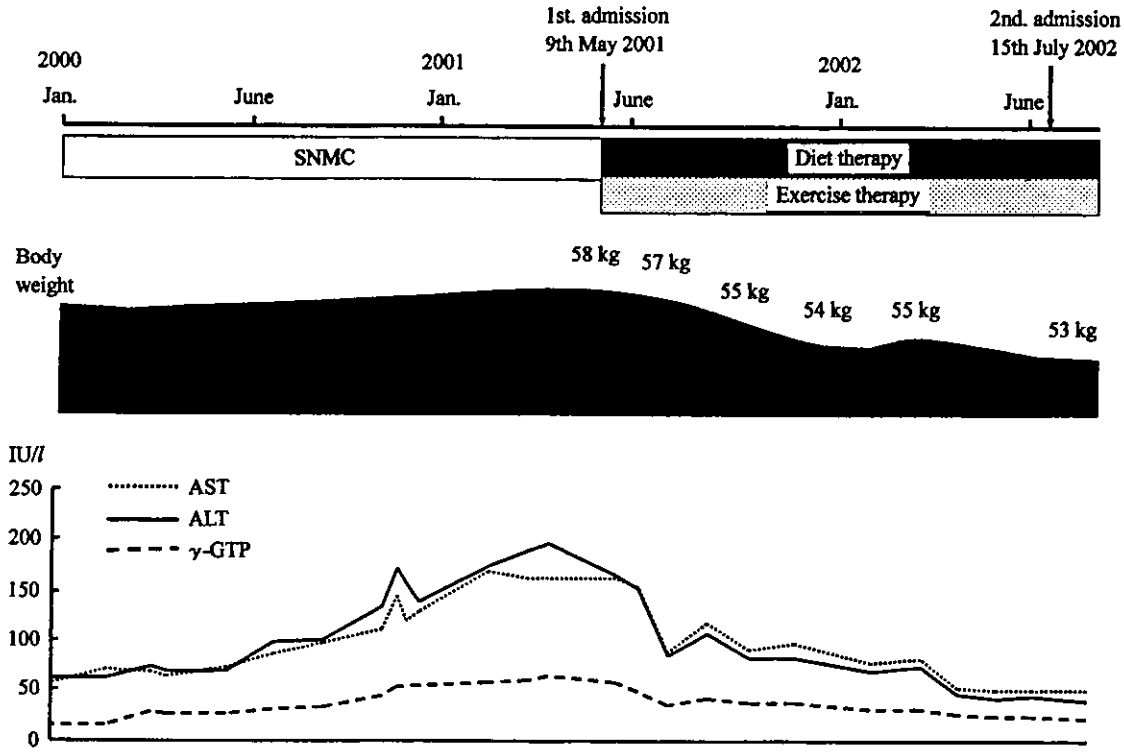

Figure 2. The clinical course of the patient. In parallel with weight reduction, liver dysfunction improved during diet and exercise therapy. AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: γ-glutamyltransferase, P-III-P: procollagen III peptide.

cirrhosis (19).

Current treatments for NASH are unproven. Improvement of liver chemistry, but variable changes in histology, have been reported after weight reduction in a small number of patients with NASH (9–11). The rate of weight reduction is important and may play a critical role in determining whether liver histologic findings improve or worsen. Rapid weight reduction has been associated with exacerbation of steatohepatitis in obese patients, and histologic exacerbation has been observed when the rate of weight reduction exceeded 1,600 g per week (20). Hence, weight reduction should be moderate and should also be monitored carefully. However, the most effective rate of weight reduction still has to be established. In the current case, the patient achieved 5 kg weight reduction during one year of diet and exercise therapy. Originally elevated transaminase levels decreased in parallel with this weight reduction. Furthermore, an improvement in liver histology, including the grade of steatosis, inflammation and the stage of fibrosis, were observed after weight reduction. These findings indicate that weight reduction is a useful therapy for NASH, but large prospective studies are needed to confirm this suggestion.

The mechanisms by which weight reduction improves hepatic inflammation and fibrosis in NASH patients remain unclear. The pathogenesis of NASH is multifactorial. In a model for the development of NASH (21), it is suggested that insulin resistance is an important factor in the accumulation of hepatocellular fat. Other factors, such as genetic mutations, excess carbohydrates, drugs and toxins may also contribute to hepatic steatosis. An excess of fat in the liver predisposes some individuals to hepatocellular injury, caused by the direct cellular toxicity of excess free fatty acids, oxidative stress and lipid peroxidation, or other mechanisms. Although, it is commonly known that diet and exercise therapy alter insulin sensitivity, the current patient showed no significant change in insulin resistance during therapy. These findings might suggest that hepatic steatosis does not depend on insulin resistance only.

Drugs such as gemfibrozil (22), ursodeoxycholic acid (23), vitamin E (α-tocopherol) (24) and metformin (25) have been shown to be promising treatments for NASH. However, studies of these drugs have been limited to only small numbers of patients, and they have also had variation in the definition of NASH and insufficient evaluation of treatment outcomes. In addition, medical therapies for NASH have had other problems, such as costs and side effects. While the es-

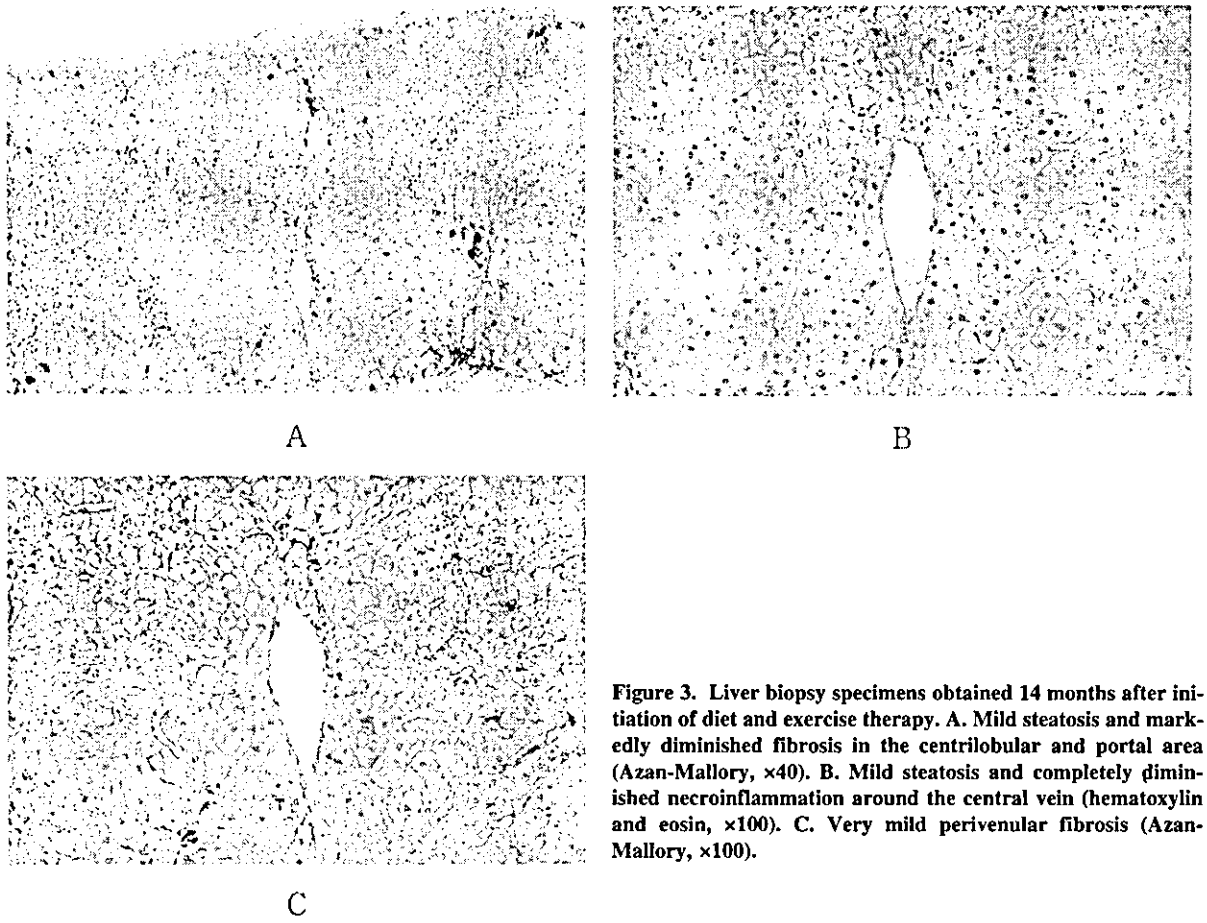

Figure 3. Liver biopsy specimens obtained 14 months after initiation of diet and exercise therapy. A. Mild steatosis and markedly diminished fibrosis in the centrilobular and portal area (Azan-Mallory, ×40). B. Mild steatosis and completely diminished necroinflammation around the central vein (hematoxylin and eosin, ×100). C. Very mild perivenular fibrosis (Azan-Mallory, ×100).

tablishment of an effective therapeutic approach is awaited, we believe that gradual weight reduction might be a useful first step in NASH therapy.

References

1) Sheth SG, Gordon FD, Chopra S. Nonalcoholic steatohepatitis. Ann Intern Med **126**: 137–145, 1997.
2) Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. Hepatology **37**: 1202–1219, 2003.
3) Reid AE. Nonalcoholic steatohepatitis. Gastroenterology **121**: 710–723, 2001.
4) Ludwig J, Viggiano T, McGill D, Oh BJ. Nonalcoholic steatohepatitis. Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc **55**: 434–438, 1980.
5) Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. Hepatology **30**: 1356–1362, 1999.
6) Bacon BR, Farahvash MJ, Janney CG, Neuschwander-Tetri BA. Nonalcoholic steatohepatitis: an expanded clinical entity. Gastroenterology **107**: 1103–1109, 1994.
7) Lee RG. Nonalcoholic steatohepatitis: a study of 49 patients. Hum Pathol **20**: 594–598, 1989.
8) Powell E, Cooksley W, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology **11**: 74–80, 1990.
9) Ueno T, Sugawara H, Sujaku K, et al. Therapeutic effects of restricted diet and exercise in obese patients with fatty liver. J Hepatol **27**: 103–107, 1997.
10) Franzese A, Vajro P, Argenziano A, et al. Liver involvement in obese children. Ultrasonography and liver enzyme levels at diagnosis and during follow-up in an Italian population. Dig Dis Sci **42**: 1428–1432, 1997.
11) Vajro P, Fontanella A, Perna C, Orso G, Tedesco M, De Vincenzo A. Persistent hyperaminotransferasemia resolving after weight reduction in obese children. J Pediatr **125**: 239–241, 1994.
12) Saksena S, Johnson J, Ouiff SP, Elias E. Diet and exercise: important first steps in therapy of NASH. Hepatology **30**: 436A, 1999.
13) Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model of assessment: insulin resistance and β-cell function arising from fasting plasma glucose and insulin concentrations in man. Diabetologia **28**: 412–419, 1985.
14) Kato H, Nakata K, Hamasaki K, et al. Long-term efficacy of immunization against hepatitis B virus in infants at high-risk analyzed by polymerase chain reaction. Vaccine **18**: 581–587, 2000.
15) Alvarez F, Berg PA, Bianchi FB, et al. International Autoimmune Hepatitis Group Report. Review of criteria for diagnosis of autoimmune hepatitis. J Hepatol **31**: 929–938, 1999.
16) Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: A proposal for grading and

staging the histological lesions. Am J Gastroenterol **94**: 2467–2474, 1999.
17) Bugianesi E, Leone N, Vanni E, et al. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology **123**: 134–140, 2002.
18) Shimada M, Hashimoto E, Taniai M, et al. Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. J Hepatol **37**: 154–160, 2002.
19) Garcia-Monzon C, Martin-Perez E, Iacono OL, et al. Characterization of pathogenic and prognostic factors of nonalcoholic steatohepatitis associated with obesity. J Hepatol **33**: 716–724, 2000.
20) Andersen T, Gluud C, Franzmann MB, Christoffersen P. Hepatic effects of dietary weight loss in morbidly obese subjects. J Hepatol **12**: 224–229, 1991.
21) Lin HZ, Yang SQ, Chuckaree C, Kuhajda F, Ronnet G, Diehl AM. Metformin reverses fatty liver disease in obese, leptin-deficient mice. Nat Med **6**: 998–1003, 2000.
22) Basaranoglu M, Acbay O, Sonsuz A. A controlled trial of gemfibrozil in the treatment of patients with nonalcoholic steatohepatitis. J Hepatol **31**: 384, 1999.
23) Guma C, Viola L, Thome M, Galdame O, Alvarez E. Ursodeoxycholic acid in the treatment of nonalcoholic steatohepatitis: results of a prospective clinical controlled trial. Hepatology **26**: 387A, 1997.
24) Lavine JE. Vitamin E treatment of nonalcoholic steatohepatitis in children: a pilot study. J Pediatr **136**: 734–738, 2000.
25) Marchesini G, Brizi M, Bianchi G, Tomassetti S, Zoli M, Melchionda N. Metformin in non-alcoholic steatohepatitis. Lancet **358**: 893–894, 2001.

Journal of Gastroenterology and Hepatology (2004) **19**, 670–675

HEPATOLOGY

High viral load is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis B virus infection

KAZUYUKI OHATA,* KEISUKE HAMASAKI,* KAN TORIYAMA,† HIROKI ISHIKAWA,* KAZUHIKO NAKAO‡ AND KATSUMI EGUCHI*

**First Department of Internal Medicine, Nagasaki University School of Medicine, †Department of Pathology, Institute of Tropical Medicine and ‡Health Research Center, Nagasaki University, Sakamoto, Nagasaki, Japan*

Abstract

Background and Aims: Hepatitis B virus (HBV) is considered a major risk factor for the progression to liver cirrhosis and hepatocellular carcinoma (HCC). The serum level of HBV-DNA is correlated with progression of the disease. The aim of the present study was to determine the relationship between the level of HBV-DNA and hepatocarcinogenesis in patients with chronic HBV infection.

Methods: The authors studied 73 patients who were diagnosed with chronic HBV infection at Nagasaki University Hospital (Nagasaki, Japan) between January 1980 and December 1999. The significance of age, sex, habitual drinking, serum alanine aminotransferase level, HBV viral load, interferon treatment, hepatic fibrosis and hepatic inflammation on the development of HCC were examined using univariate and multivariate analyses.

Results: The cumulative incidence rates of HCC were 14%, 29% and 48% at 5, 10 and 15 years after liver biopsy, respectively. Multivariate analysis identified high viral load, together with age and severe fibrosis, as independent and significant risk factors ($P = 0.045$, 0.047 and 0.013, respectively) for HCC.

Conclusions: The present findings indicate that high viral load is a risk factor for HCC in patients with chronic HBV infection. Patients with a high HBV viral load should be carefully monitored for HCC.

Key words: hepatitis B virus, hepatitis B virus DNA, hepatocellular carcinoma, risk factor.

INTRODUCTION

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV). Complications of chronic hepatitis B, such as cirrhosis, hepatocellular carcinoma (HCC) and end-stage liver disease, account for approximately 1 million deaths each year.[1] Analysis of the risk factors for the development of HCC in patients with chronic HBV has been performed in several studies, and factors such as aging,[2–4] sex,[4] total alcohol intake,[2,4] nucleolar hypertrophy,[5] cirrhosis[6] and the presence of hepatitis B early antigen (HBeAg)[7] have been associated with HCC. A recent study has indicated that interferon (IFN) treatment can reduce the incidence of HCC.[6,8] The identification of additional variables associated with changes in the risk of developing HCC is of particular importance in the optimization of preventive medicine programs.

According to previous studies, seroconversion of HBeAg to antibody to HBeAg (anti-HBe) is believed to result in a decrease in viral load and to indicate a favorable outcome in these patients.[9–13] Furthermore, it has been reported that an increased risk of HCC is associated with HBeAg-positive patients.[7] Recent studies have indicated that the serum level of HBV-DNA is correlated with the progression of the disease.[14] However, it remains unclear if the serum level of HBV-DNA is a risk factor for hepatocarcinogenesis. In the current study, univariate and multivariate analyses of the risk factors, including the serum level of HBV-DNA, were carried out in 73 patients with chronic HBV infection.

Correspondence: Dr Katsumi Eguchi, Professor and Chairman, The First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852–8501, Japan. Email: oohata-gi@umin.ac.jp

Accepted for publication 26 September 2003.

METHODS

Patients

Liver biopsies were obtained from 607 patients at the Nagasaki University Hospital (Nagasaki, Japan) between January 1980 and December 1999. Of these 607 patients, 47 were diagnosed with HCC based on histopathological examination of their tumor tissue, and/or by ultrasonography or computed tomography (CT). Of the 560 patients who had no detectable HCC, 181 were diagnosed with chronic hepatitis or cirrhosis and were confirmed to be negative for the antibody to HCV (anti-HCV), but positive for hepatitis B surface antigen (HBsAg). Of these 181 patients, 73 were followed at the Nagasaki University Hospital for more than 6 months and were enrolled in this study. Patients who had other causes of liver disease, such as primary biliary cirrhosis or autoimmune hepatitis, were excluded from the study. The 73 patients who were enrolled in the study fulfilled the following inclusion criteria: (i) absence of HCC at the time of liver biopsy; (ii) positive serological test for HBsAg; (iii) negative for anti-HCV; (iv) negative for autoantibodies, such as antinuclear antibody and antimitochondrial antibody; and (v) follow up for more than 6 months after the liver biopsy. At the time of liver biopsy, information on alcohol drinking habits was obtained through an interview conducted by the physicians. Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g/day of pure ethanol over a period of more than 5 years. Informed consent was obtained from each patient at the time of liver biopsy.

Serological tests

After informed consent was obtained, a serum sample was taken from each patient at the time of liver biopsy and was stored at –40°C for subsequent analysis of viral markers. The HBsAg, HBeAg and anti-HBe were assayed using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan). Anti-HCV was determined using a second- or third-generation ELISA (Ortho Diagnostics Systems, Tokyo, Japan). Serum HBV-DNA was detected by the transcription-mediated amplification (TMA) method, as described previously[15] and was expressed as the logarithm of the genome equivalent per milliliter (LGE/mL). The detection limit of this method is 3.7 LGE/mL. A value of 0.7 milliequivalents per milliliter (mEq/mL), the detection limit of HBV-DNA using a branched DNA assay, corresponds to that of 5.8 LGE/mL using the TMA method. Analysis of all viral markers, including serum HBV-DNA, was carried out on the serum sample taken from each patient at the time of liver biopsy.

Follow up of patients

Clinical evaluation and biochemical tests were carried out every 1–3 months. Ultrasonography or CT of the liver was carried out at least every 3–6 months. Diagnosis of HCC was based on the histopathological observation of tumor tissue or on characteristic signs in the ultrasonography, CT and hepatic arteriography. The endpoint used in the current study was the appearance of HCC and the reference date used was 31 December 2000. The number of patients who did not develop HCC for 5, 10 and 15 years after liver biopsy were 44, 24 and 10, respectively. A total of 23 patients were followed until the endpoint of the study and the average observation period was 89.6 months (7.4 years).

Histopathological examination of the liver

Liver biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut to a thickness of 4 μm and stained with HE and Azan. All liver tissue specimens were evaluated by one pathologist (KT) who was unaware of the patient's clinical condition. Liver histology was evaluated according to the degree of fibrosis and necroinflammatory activity. The extent of fibrosis (staging) and the degree of necroinflammatory activity (grading) were classified according to Desmet *et al.* as follows: F1 (periportal expansion) and F2 (portoportal septa) were categorized as mild fibrosis, whereas F3 (portocentral linkage or bridging fibrosis) and F4 (cirrhosis) were categorized as severe fibrosis.[16] In terms of necroinflammatory activity, A1 represented mild activity and A2 or A3 represented severe activity.

Statistical analysis

Data were expressed as the mean ± SD for continuous variables and as counts for categorical variables. Continuous and categorical variables were compared using the Student's *t*-test and the χ^2 test, respectively. Cumulative incidence curves were determined using Kaplan–Meier analysis and the differences between groups were assessed using the log-rank test. Univariate and multivariate analyses of the risk ratios for the occurrence of HCC were studied using a Cox proportional-hazards regression analysis. Factors examined included age, sex, habitual drinking, serum ALT level, serum HBV-DNA level, IFN treatment during the follow-up period, histopathological staging (mild fibrosis or severe fibrosis), and histopathological grading (mild activity or severe activity). All *P*-values were two-tailed, and $P < 0.05$ was considered to be significant. The statistical analysis was performed using StatView version 5.0 (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics

Table 1 shows the clinicopathological features of patients upon entry into the study. The study included 58 men and 15 women with a mean age of 39 years (range 16–68 years), and also included five patients (7%) who were habitual drinkers. Measurement of

Table 1 Clinical, laboratory and histological characteristics of the 73 patients

Male/female (%)	58/15 (79/21)
Mean age (years ± SD)	39.4 ± 11.7
Habitual heavy drinking (%)†	5 (7)
Diabetes (%)	7 (10)
Alanine aminotransferase (IU/L; mean ± SD)	170.7 ± 302.3
γ-Glutamyltransferase (IU/L; mean ± SD)	66.3 ± 66.6
Hepatitis B virus DNA (LGE/mL)	6.08 ± 1.68
Hepatitis B early antigen (positive/negative, %)	47/26 (64/36)
Interferon (yes/no)	7/66
Stage of fibrosis (%)	
1	17 (23)
2	13 (18)
3	14 (19)
4	29 (40)
Grade of inflammation (%)	
1	30 (42)
2	39 (53)
3	4 (5)

†Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g/day of pure ethanol over a period of more than 5 years. LGE, logarithm of the genome equivalent.

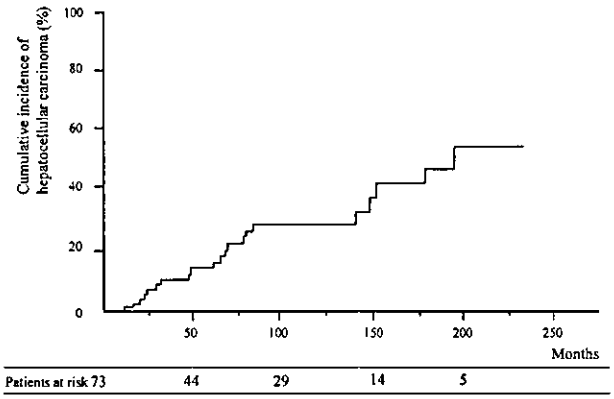

Figure 1 Cumulative incidence of hepatocellular carcinoma (HCC) in 73 patients with chronic hepatitis B.

HBV-DNA was achieved for 63 patients and the mean level of serum HBV-DNA was 6.08 LGE/mL (range <3.7–8.6 LGE/mL). Forty-seven patients (64%) were HBeAg-positive and 26 patients were HBeAg-negative. During the follow-up period, six of the 73 patients received IFN treatment. The numbers of patients with histopathological staging of F1, F2, F3 and F4 were 17 (23%), 13 (18%), 14 (19%) and 29 (40%), respectively. The numbers of patients with histopathological grading of A1, A2 and A3 were 30 (42%), 39 (53%) and four (5%), respectively.

Cumulative incidence of hepatocellular carcinoma

During the observation periods, 21 of the 73 patients (29%) developed HCC. Diagnosis of HCC was based on tumor tissue histopathological findings for eight patients, and on the characteristic appearance of images from ultrasonography, CT and/or hepatic arteriography for 13 patients. As shown in Figure 1, the cumulative incidence rates of HCC were 14%, 29% and 48% at 5, 10 and 15 years after liver biopsy, respectively.

Univariate analysis of clinicopathological factors influencing the development of hepatocellular carcinoma

To determine the factors that could influence the development of HCC, a Cox proportional-hazards regression analysis was carried out. Based on a univariate analysis of eight variables, the following three factors significantly influenced the incidence of HCC: age at the time of liver biopsy (relative risk 12.82; 95% confidence interval [CI], 1.72–100.00; $P = 0.013$); histopathological staging (severe fibrosis: relative risk 6.80; 95% CI, 1.59–3.53; $P = 0.010$) and; HBV-DNA level (high serum HBV-DNA level: relative risk 3.44; 95% CI, 1.07–7.46; $P = 0.035$) (Table 2).

Multivariate analysis of clinicopathological factors influencing the development of hepatocellular carcinoma

A multivariate analysis of the determinants of HCC was also applied using a Cox regression model. Age at the time of liver biopsy, histopathological staging and HBV-DNA level were identified as statistically independent risk factors (relative risk 8.20; 95% CI, 1.03–66.67; $P = 0.047$; relative risk 7.87; 95% CI, 1.54–40.00; $P = 0.013$; relative risk 3.08; 95% CI, 1.03– 9.17; $P = 0.045$, respectively) (Table 2). Figure 2 shows the cumulative incidence of HCC based on the HBV-DNA level. The cumulative incidence rate of HCC in 28 patients with a high HBV-DNA level (≥6.0 LGE/mL) was significantly higher than that in 35 patients with a low HBV-DNA level (<6.0 LGE/mL; $P = 0.0285$).

Clinicopathological findings in patients according to viral load

Table 3 shows the clinicopathological findings in patients according to viral load. Viral load is closely related to serum ALT level and the degree of necroinflammatory activity. Serum levels of HBV-DNA in patients with HCC who were HBeAg-positive were much higher than those in patients who were HBeAg-negative (median 6.4 LGE/mL *vs* 5.5 LGE/mL; $P = 0.032$; Fig. 3).