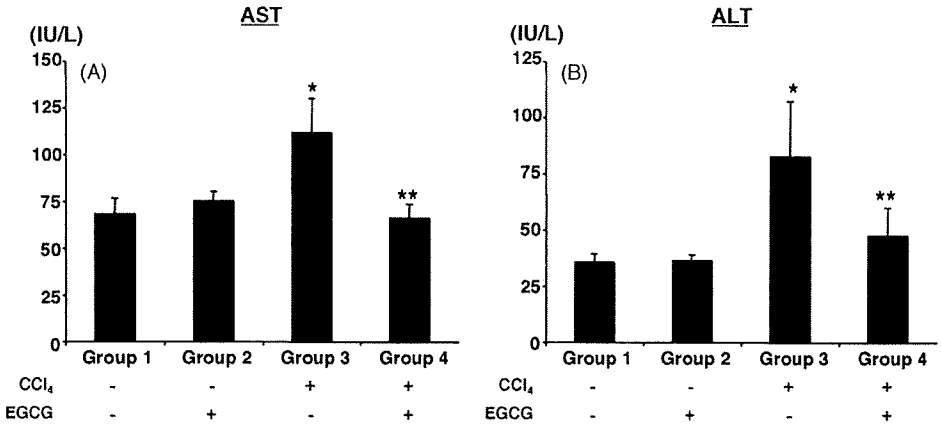

Fig. 1. Effects of EGCG on the serum levels of AST and ALT in the experimental rats. At sacrifice, blood samples were collected and the serum levels of AST (A) and ALT (B) were then assayed. Values are the means ± SE ($n = 5$). *$p < 0.01$, compared with control group (Group 1, olive oil-injected group); **$p < 0.01$, compared with CCl_4-injected group (Group 3).

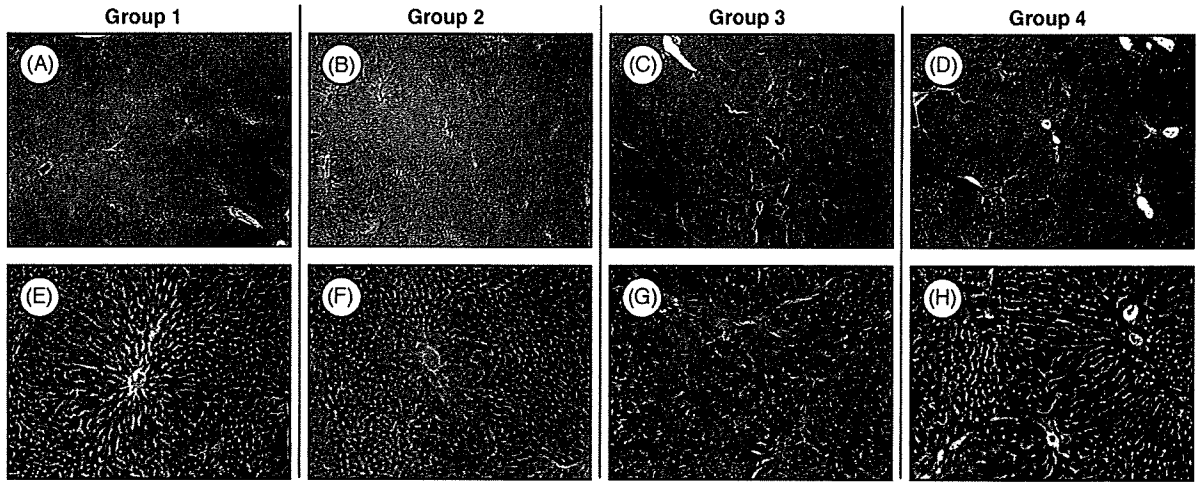

Fig. 2. Photomicrographs of liver sections from the rats in control group (Group 1, A and E), olive oil-injected and EGCG drinking group (Group 2, B and F), CCl_4-injected group (Group 3, C and G) and CCl_4-injected and EGCG drinking group (Group 4, D and H). Paraffin-embedded sections were stained with Azan stain to show fibrosis. Original magnification: ×40 (A–D) and ×100 (E–H).

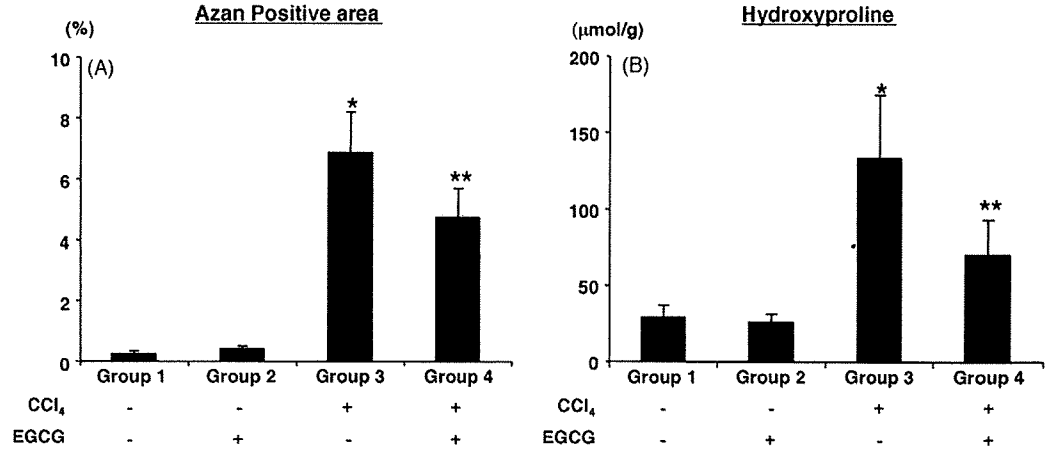

Fig. 3. Effects of EGCG on hepatic fibrosis area and hydroxyproline content in the experimental rats. (A) The fibrosis area was evaluated by Azan stain (Fig. 2) using an image analyzer. (B) The hepatic hydroxyproline contents were quantified colorimetrically, as described in Section 2. Values are the means ± SE ($n = 5$). *$p < 0.01$, compared with control group (Group 1); **$p < 0.01$, compared with CCl_4-injected group (Group 3).

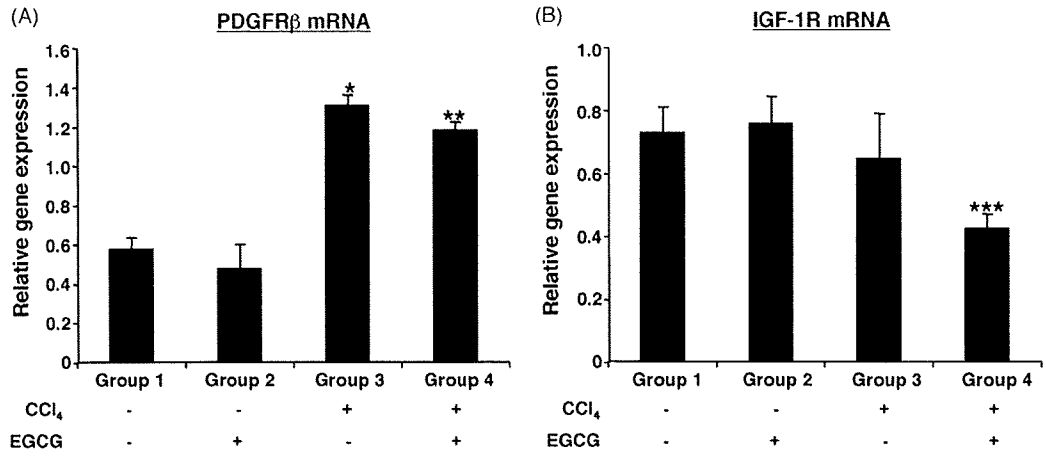

Fig. 4. Effects of EGCG on the expression levels of PDGFRβ and IGF-1R mRNAs in the experimental rats. cDNA was synthesized from the livers of experimental rats and real-time PCR was performed using PDGFRβ (A) and IGF-1R (B) specific primers. The expression levels of these genes were normalized to the level of *GAPDH* gene. Values are the means ± SE ($n = 5$). *$p < 0.01$, compared with control group (Group 1); **$p < 0.05$, compared with CCl_4-injected group (Group 3); ***$p < 0.01$, compared with CCl_4-injected group (Group 3).

in the olive oil-injected group either with (Fig. 2B and F) or without EGCG (Fig. 2A and E). A densitometric analysis showed the fibrosis areas to be markedly suppressed in the EGCG-treated rats (Fig. 3A, $p < 0.01$). Similar findings were also observed in measurements of the liver hydroxyproline contents; in the CCl_4-injected rats, drinking water with 0.1% EGCG caused a significant decrease in the amounts of hydroxyproline observed in the liver (Fig. 3B).

3.3. *Effects of EGCG on the expression of PDGFRβ and IGF-1R mRNAs in the liver of the CCl_4-injected rats*

To elucidate the possible mechanisms in regard to how EGCG attenuates liver fibrosis (Figs. 2 and 3), the effects of this agent on the expression levels of PDGFRβ and IGF-1R mRNAs in the experimental liver were then examined because these RTKs play a critical role in the development of liver fibrosis [1,5,13]. The expression level of PDGFRβ mRNA was elevated in the liver of CCl_4-injected rats and drinking EGCG significantly lowered the level of this mRNA raised by CCl_4 (Fig. 4A). No significant increase was observed in the level of IGF-1R mRNA by CCl_4 injection, whereas treatment with EGCG remarkably decreased the expression of this mRNA (Fig. 4B).

3.4. *Effects of EGCG on the expression of PDGFRβ and α-SMA proteins in the liver of the CCl_4-injected rats*

Next, the effects of EGCG on the expression levels of PDGFRβ and α-SMA, an indicator of HSC activation, in the rat liver were examined using a Western blot analysis. As shown in Fig. 5A, the intraperitoneal injection of CCl_4 markedly increased the levels of both PDGFRβ and α-SMA proteins in the experimental rat liver. On the other hand, drinking EGCG significantly decreased the expression of PDGFRβ as well as α-SMA proteins raised by CCl_4 (Fig. 5A). Immunohistochemical analysis also indicated that the α-SMA-immunoreactive areas remarkably increased in the liver of the CCl_4-injected group when compared to the olive oil-injected group. In addition, drinking EGCG significantly reduced the expression area of this protein, thus indicating the inhibition of HSC activation (Fig. 5B).

4. Discussion

The activation of HSCs, which is induced by PDGF/PDGFR interaction, plays a pivotal role in the development of liver fibrosis [1,5]. Therefore, targeting the PDGF/PDGFR axis is considered to be an effective strategy to inhibit the progress of hepatic fibrosis. Yoshiji et al. [14] reported that, imatinib mesylate, a clinically used PDGFR tyrosine kinase inhibitor, markedly attenuated liver fibrosis in rats by inhibiting the PDGF-induced proliferation and migration of activated HSCs. The present study demonstrates that drinking EGCG significantly suppressed the liver injury caused by CCl_4 (Fig. 1). Moreover, the results of the present study clearly indicate that EGCG effectively prevented the development of liver fibrosis (Figs. 2 and 3) and this finding was associated with the inhibition of the expression of PDGFRβ and α-SMA (Figs. 4A and 5). These findings are consistent with those of a previous *in vitro* report which showed EGCG to inhibit both HSC proliferation and *PDGFRβ* gene expression by blocking the activation of AP-1 and NF-κB [20]. This report seems to be interesting because these transcription factors are regarded as effective targets of EGCG to exert its anticancer properties [9,21].

Several studies have pointed the interactions between the IGF-1R and PDGF/PDGFR axis in the development of liver fibrosis. For instance, PDGF stimulated the IGF-1R mRNA expression through the activation of the *IGF-1R* gene promoter [22]. Functional IGF-1R was required for the mitogenic activity of the PDGFR in liver myofibroblasts [13]. The cooperative activation of the intracellular signaling pathways, including extracellular signal-regulated kinase (ERK) and Akt, by PDGF and IGF-1 played an important role in perpetuating the activated state of HSC during liver fibrogenesis [23]. Recent studies have revealed that EGCG in drinking water suppressed obesity-related colonic carcinogenesis by inhibiting the activation of the IGF/IGF-1R axis in the colonic mucosa [10]. Treatment of HepG2 human HCC cells with EGCG also decreased the production of IGF-1 from these cancer cells, thus inhibiting the phosphorylation of IGF-1R and its downstream ERK and Akt proteins [11]. These reports, together with our present finding that drinking EGCG significantly reduced the expression of IGF-1R mRNA in the fibrotic liver (Fig. 4B), may suggest that EGCG, which targets both the PDGF/PDGFR and IGF/IGF-1R axes, might also be useful for inhibiting liver fibrosis.

Moreover, in addition to the effects of EGCG on specific RTKs, recent studies have indicated that green tea polyphenols may also possess other anti-fibrotic properties, such as antioxidant properties. EGCG has also been shown to arrest the progression of hepatic fibrosis in the rat model by inhibiting oxidative damage [24]. Supplementation with green tea extract inhibited a progression of cirrhosis in a rat model of steatohepatitis and this was associated with its antioxidant and radical scavenging activities [25]. These

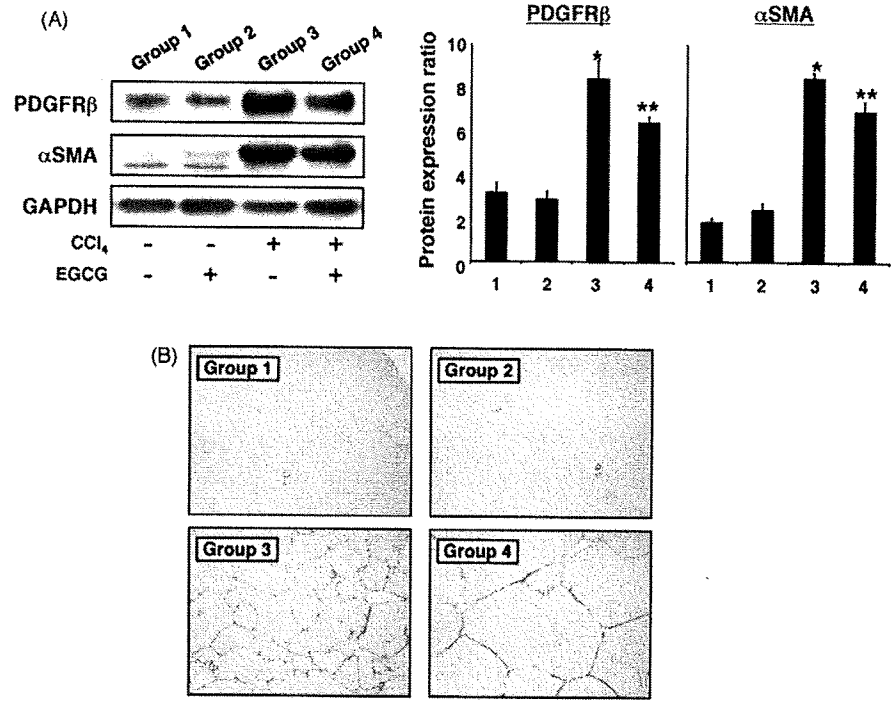

Fig. 5. Effects of EGCG on the expression levels of PDGFRβ and α-SMA in the experimental rats. (A) Total protein was extracted from the liver of experimental rats and equivalent amounts of protein were examined by a Western blot analysis using the respective antibodies. An antibody to GAPDH served as a loading control. Repeat Western blots gave similar results. The results obtained from Western blot analysis were quantitated by densitometry and are displayed in the right panels. Values are the means ± SE ($n = 5$). $^{*}p < 0.01$, compared with control group (Group 1); $^{**}p < 0.01$, compared with CCl_4-injected group (Group 3). (B) Immunohistochemical expression of α-SMA in the liver of control group (Group 1), olive oil-injected and EGCG drinking group (Group 2), CCl_4-injected group (Group 3) and CCl_4-injected and EGCG drinking group (Group 4). Original magnification: ×40.

reports also support the possibility that the administration of EGCG is useful for preventing the progression of hepatic fibrosis. In conclusion, the ability of EGCG to target PDGFR and IGF-1R, both of which play critical roles in the progression of liver fibrosis, is considered to provide evidence that this naturally occurring agent may be effective in both the prevention and therapy of liver fibrosis.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Extracellular matrix is required for the survival and differentiation of transplanted hepatic progenitor cells

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ABSTRACT

Engelbreth–Holm–Swarm (EHS) gel has been reported to maintain the mature hepatocyte phenotypes in primary cultured hepatocytes. We investigated the effect of EHS gel on the differentiation of fetal liver cells, which contain stem/progenitor cells. The isolated fetal liver cells cultured on EHS gel formed a spherical shape and increased liver-specific gene expressions compared with cells cultured on collagen. The hepatic progenitor cells that were transplanted subcutaneously to BALB/c nude mice could survive and express hepatocyte marker alpha-fetoprotein when the cells were suspended with EHS gel. These findings demonstrate that EHS gel supports cytodifferentiation from immature progenitor cells to hepatocytes and maintain its differentiated phenotypes *in vitro* and *in vivo*.

Extracellular matrix (ECM) plays an important role in cell survival, proliferation, and differentiation [1]. Adhesive interactions between hepatocyte and ECM retain its differentiated phenotypes and maintain liver-specific functions, accompanied with up-regulation of the liver-enriched transcription factors including hepatocyte nuclear factor (HNF)s [2,3]. Lamining-rich ECM, Engelbreth–Holm–Swarm sarcoma (EHS) gel has been reported to keep a high expression of liver-specific products such as albumin, and normal cell polarity and structure for prolonged periods in primary cultured hepatocytes [4]. On the other hand, culture on dried type 1 collagen leads the cells to dedifferentiated phenotypes such as flattened monolayer and low expression of liver-specific proteins [2,5].

Liver transplantation is the primary treatment for severe liver diseases. However, the therapy is limited because of insufficient organ availability, and cell transplantation is believed to become alternative therapy. Various types of cells are reported as candidates for cell transplantation for liver diseases. THLE-5b cells, an immortalized non-tumorigenic human cell line, were well localized in the peritoneal cavity of BALB/c nude mice for 3 weeks after cell transplantation [6]. Immortalized human hepatocytes provide life saving metabolic supports in rats when they are transplanted into spleen [7].

Recently, stem cells have been thought to be suitable source of cell transplantation for liver diseases. Bone marrow derived mesenchymal stem cells rescued experimental mouse liver failure when they were engrafted to the liver [8]. Human embryonic stem cells [9], oval cells [10], and cord blood cells [11] are also reported to have the potential to develop into viable hepatocytes. However, these cells might be too immature to be directed to mature differentiated hepatocytes. Fetal liver cells contain stem/progenitor cells, which are able to differentiate bipotentially into hepatocytes and cholangiocytes, and represent differentiated property of hepatocyte by transduction of HNF-4 gene [12] or culture on ECM [13]. However, it is still not known if the cells cultured on EHS gel survive and function as the mature hepatocytes. In this study, we investigated if the hepatic stem/progenitor cells obtain and retain the differentiated hepatocyte phenotypes under such condition *in vitro* and *in vivo*.

Materials and methods

Fetal liver cell isolation and culture. The beta-actin promoter-driven GFP-transgenic mice (GFP mice) were bred for studies. Pregnant female C57BL/6J mice were purchased from Nippon SLC (Sizuoka, Japan). Fetal mouse liver parenchymal cells were harvested as previously reported [12]. Briefly, the cells were isolated from the embryonic day 14.5 livers by mechanical pipetting. Hematopoietic cells were removed by magnetically activated cell sorter using a Lineage cell depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Then the separated fetal liver cells were plated on either normal plastic, EHS gel coated (BD Bioscience,

Abbreviations: EHS gel, Engelbreth–Holm–Swarm gel; AFP, alpha-fetoprotein; ECM, extracellular matrix; HNF, hepatocyte nuclear factor; GFP, green fluorescent protein; CK-19, cytokeratin-19; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; C/EBP, CCAAT/enhancer binding protein.

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Bedford, MA, USA), or type 1 collagen coated dishes (Iwaki, Tokyo, Japan) (5×10^6 cells/100 mm dish) in DMEM/F12 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10^{-6} M insulin (Wako, Osaka, Japan), 10^{-7} M dexamethasone (Wako), 10% fetal bovine serum (ThermoTrace, Melbourne, Australia), 10 mM nicotinamide (Wako), 50 μM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), 5 mM HEPES (Wako), and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). The medium was replaced every other day. Animal use and experimentation was performed under the strict guidelines of the Institutional Animal Use and Care Committee at the Gifu University Graduate School of Medicine.

Immunohistochemistry. The cells were fixed by methanol at −20 °C for 10 min, and washed in PBS including 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) (Wako). Nonspecific binding was blocked with 10% nonimmune rabbit serum. Fixed cells were incubated with the rabbit primary antibodies, fluorescein isothiocyanate-conjugated anti-mouse albumin (BETHYL, Montgomery, TX, USA) and anti-mouse cytokeratin 19 (CK19, gift from Dr. N. Tanimizu, Kanagawa Academy of Science and Technology, Japan), in a moist chamber at 4 °C for 16 h. After washing in PBS-Tween 20, cells were incubated with Texas Red-conjugated goat anti-rabbit IgG (Invitrogen, CA, USA) at 4 °C for 3 h to detect CK19. Nuclear counterstain was performed with 4′,6-diamidino-2-phenylindole. The signal was detected using a fluorescence microscope (Olympus, Tokyo, Japan).

Quantitative real-time RT-PCR. Fetal liver cells were cultured for 4 days and detached by cell scraper. Total RNA was isolated from the cells using ISOGEN (Wako). For each sample, 2.0 μg of total RNA was reverse-transcribed using a high-capacity complementary DNA reverse transcription kit as described by the manufacturer (Applied Biosystems, Foster City, CA, USA). The mRNA quantification was performed using a 2-step real-time RT-PCR (Light Cycler, Roche Diagnostics, IN, USA) with Light cycler TaqMan Master and Universal ProbeLibrary Probes (Roche). The PCR primers and probes were as follows; albumin (sense, 5′-CCAAAGT CAACAAGGAGTGCT-3′; antisense 5′-TCGCCTGGTTTTCACACAT-3′; probe #62), HNF-4 (sense, 5′-CCGAGGGACGATGTAGTCAT-3′; antisense 5′-CAAGAGGTCCATGGTGTTCA-3′; probe #68), CK19 (sense, 5′-CCTCAGGGCAGTAATTTCCTC-3′; antisense 5′-TGACCTGGAGATG CAGATTG-3′; probe #17). Target complementary DNAs were normalized to the endogenous mRNA levels of 18S ribosomal RNA (sense, 5′-GCTCTTAGAATTACCACAGTTATCC-3′; antisense, 5′-AATCAGTTATGGTTCCTTTGTCG-3′; probe #55).

Western blot analysis. For the preparation of total cell proteins, the cells cultured for 4 days on the dishes were homogenized in the radioimmunoprecipitation lysis buffer (Santa Cruz, CA, USA). The protein concentration was measured using a DC-protein assay (Bio-Rad). The extracted proteins (10 μg) were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were first incubated with the primary antibodies against albumin (Santa Cruz, sc-46293), HNF-4 (Santa Cruz, sc-6556), HNF-1 (Santa Cruz, sc-8986), CK19 (Santa Cruz, sc-33119), alpha-fetoprotein (AFP) (Santa Cruz, sc-8108) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling, #2118) and then incubated with the HRP-coupled secondary antibodies (Santa Cruz). Detection was performed using ECL system (Amersham Biosciences, NJ, USA).

Cell transplantation to nude mice. To determine the effect of EHS gel on cell survival and cytodifferentiation *in vivo*, the fetal liver cells (1×10^5 cells) isolated from GFP mice were suspended in EHS gel, collagen gel (0.3% Cellmatrix type 1-A, Nitta-Gelatin), or DMEM/F12 medium, and then injected subcutaneously to 6-week-old male BALB/c nude mice (Nippon SLC). The grafts were removed with skin tissue at 3 weeks after transplantation, and observed with fluorescent microscope to detect GFP. The sections were stained with hematoxylin and eosin (HE). Immunohistochemical staining for AFP were performed with anti-AFP antibody (Santa Cruz, sc-8108) using avidin–biotin–peroxidase complex technique (Vector, Burlingame, CA, USA).

Results

Morphology and hepatic gene expression of fetal liver cells on different culture substrate

We have previously shown that the fetal liver contains hepatic stem/progenitor cells [12]. When the fetal liver cells were cultured on EHS gel coated dishes, cells formed clusters like a spherical shape 4 days after inoculation. In contrast, the cells showed a flattened and extended shape on type 1 collagen coated or normal plastic dish (Fig. 1). The spherical cells on EHS gel and flattened, extended cells on collagen or plastic dishes expressed albumin, a hepatocyte marker, and CK19, a cholangiocyte marker. In cells on collagen and plastic, cells located in the center of colonies were stained by albumin antibody, whereas the peripheral cells were mainly stained by CK19 antibody. These results indicate that fetal liver cells have bipotent differentiation ability.

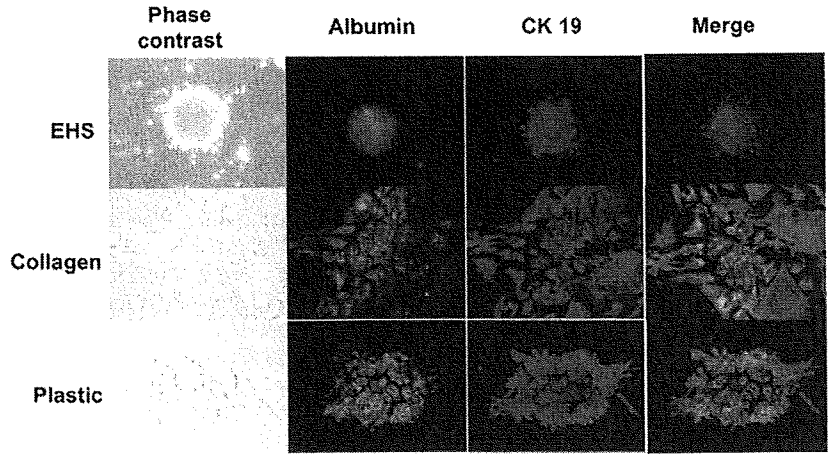

Fig. 1. Morphological changes of the fetal liver cells cultured on ECMs. The isolated fetal liver cells were cultured on each indicated ECMs for 4 days. Expression of albumin (green) and CK19 (red) were examined by double immunofluorescent staining with anti-albumin and CK 19 antibodies. The fluorescence was visualized with fluorescent conforcal microscope. (Original magnification ×200.)

Effects of ECMs on the liver-specific genes and proteins

As observed in immunohistochemistry, Western blot analysis also showed that the protein level of albumin was higher in the cells cultured on EHS gel than in the cells on type 1 collagen or plastic dish 4 days after culture. Reversely, CK19 was higher in the cells on type 1 collagen or plastic dish (Fig. 2A). Another hepatocyte maker AFP was also higher in the cells on EHS gel (A). Moreover, HNF-4 and -1 proteins were also higher in the cells on EHS gel. In parallel with the protein expressions, mRNA of albumin was higher in the cells cultured on EHS gel and CK19 mRNA was higher in the cells on type 1 collagen (Fig. 2B). These results demonstrate that EHS gel supports cytodifferentiation of hepatic stem/progenitor cells to hepatocytes.

Cell transplantation to nude mice

On the basis of these findings, we performed a further experiment to examine the cytodifferentiation ability of EHS gel *in vivo*. The fetal liver cells were isolated from GFP mice, were suspended with EHS gel, collagen gel, or DMEM medium, and were transplanted into subcutaneous tissues of BALB/c nude mice. The

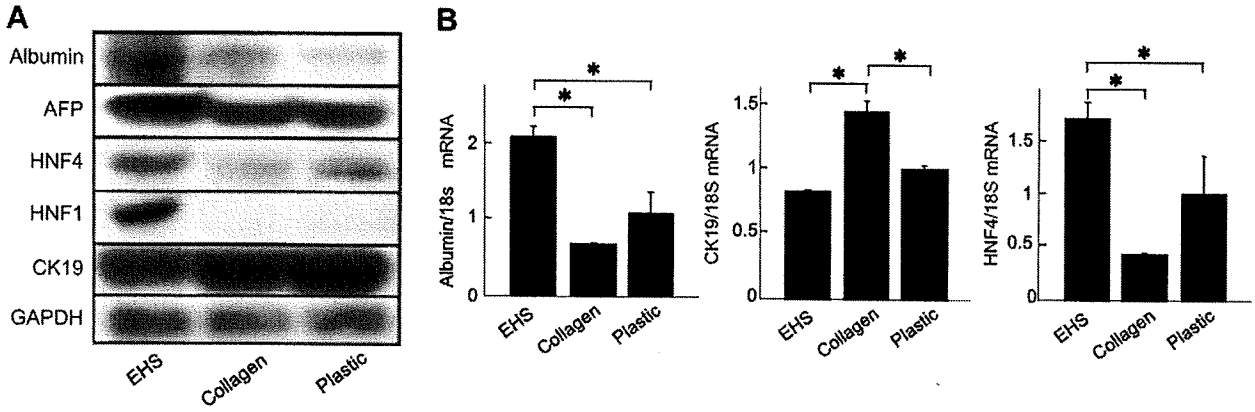

Fig. 2. Liver-specific protein and gene expressions of the fetal liver cells cultured on ECMs. The isolated fetal liver cells were cultured on each indicated ECMs for 4 days. (A) Extracted proteins were subjected to SDS–PAGE, and immunoblotting was performed with anti-albumin, AFP, HNF-4, HNF-1, CK19, and GAPDH antibodies. (B) mRNA levels of albumin, CK19, and HNF-4 in the cultured cells were determined by quantitative real-time RT-PCR. Data are means ± SD from at least three independent experiments. *, $P < 0.05$ using Student's *t*-test.

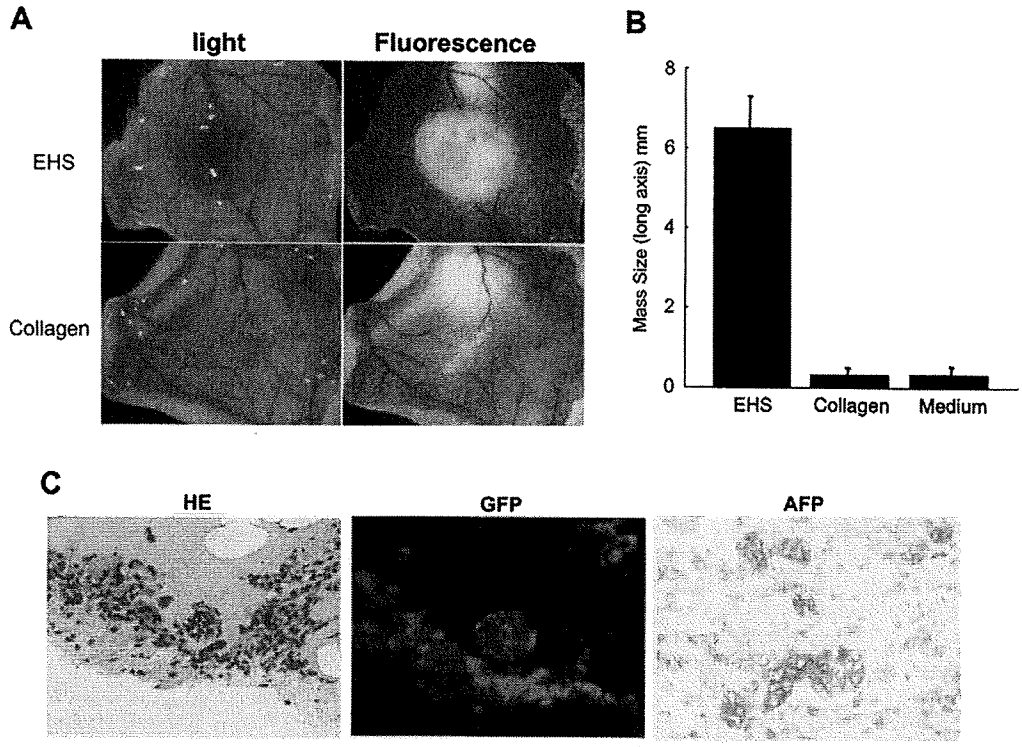

Fig. 3. Transplantation of the fetal liver cells with ECMs into BALB/c nude mice. (A) The fetal liver cells were isolated from GFP mice. The cells were suspended with EHS gel, collagen gel, or DMEM medium, and then transplanted subcutaneously into BALB/c nude mice. The animals were killed at 3 weeks after transplantation, and subcutaneous tissue was excised and photographed with light (left panels) or fluorescence (right panels). (B) The mass size (long axis) of the GFP positive area was measured. Data are means ± SD from at least three independent experiments. (C) The grafts of fetal liver cells with EHS gel were excised and stained with HE (left panel). GFP fluorescence was detected with fluorescence confocal microscope (middle panel). Expression of AFP was examined 12 days after transplantation by immunohistochemistry with anti-AFP antibody (right panel). (Original magnification ×200.)

grafted fetal liver cells with EHS gel remained even 3 weeks after transplantation, whereas cells with collagen gel or DMEM medium showed faint GFP signals (Fig. 3A and B). Moreover, the transplanted cells with EHS gel expressed AFP (Fig. 3C). These results suggest that EHS gel is able to maintain the cell survival and supports cytodifferentiation to hepatocyte *in vivo*.

Discussion

Cell transplantation has been investigated for an alternative therapy of liver organ transplantation. Differentiated hepatoma cell lines or primary cultured hepatocytes from adult liver have been thought as potential candidates [9]. However, hepatoma cells provide low levels of liver functions, and primary cultured hepatocytes rapidly lose their differentiated phenotypes and show a reduction of liver-specific gene expression once they are plated on plastic dishes [14]. Several ECMs are reported to maintain differentiated hepatocyte phenotypes. EHS gel modulates the shapes of cultured rat hepatocytes [14] and rat small hepatocytes [15]. EHS gel keeps hepatocyte-phenotypic gene expression through high expression of liver-enriched transcription factors, such as HNF-4 [16], and enhances many liver-specific functions [17]. EHS gel or sandwich culture with collagen gel maintains features of mature hepatocytes such as albumin expression, and they are attempted to use for development of bioartificial liver system [3]. However, insufficient availability of donor organ is still a problem because primary cultured hepatocytes have less regenerative ability. Thus, it would be greatly beneficial for cell transplantation if functional hepatocytes could be generated from other sources. Recently, stem cells, which have abilities of self-renewal and multilineage differentiation, are highlighted as candidates for cell transplantation. Liver stem cells can be isolated from the fetal liver, and these cells have extensive replication ability and maintain the expression of liver-specific genes, such as albumin and CK19, for prolonged cultured period [12].

In this study, we demonstrated that EHS gel promoted cytodifferentiation of hepatic progenitor cells to hepatocytes and maintained their efficiency as hepatocytes consistent with primary cultured adult hepatocytes. Interaction with ECM influenced the cell morphology and maturation of fetal liver cells. In matured hepatocytes, cells in spheroids retain the *in vivo* levels of albumin and glucokinase, whereas cells in monolayer lose the differentiated phenotypes [18]. Organization of the cytoskeleton by cell–cell and cell–ECM interaction associates with liver-specific gene expression [19]. Indeed, actin depolymerization in hepatocytes by EHS gel increases HNF-4 and albumin expression [20]. In small hepatocytes, morphological changes of the cells induce specific liver-enriched transcription factors such as HNF-4α, HNF-6, C/EBPα, and C/EBPβ [21]. HNF-4 is a key molecule for fetal liver development [22]. Overexpression of HNF-4 in hepatic progenitor cells increases liver-specific gene expression such as albumin and ApoA1 [12]. Reversely, blockage of HNF-4 expression by siRNA inhibits the up-regulation of the liver-specific gene expression in primary cultured hepatocytes cultured on EHS gel [20]. These findings and our results indicate that EHS gel induces the morphological change of hepatic progenitor cells to a spherical shape, and that HNF-4 expression via the organization of the cytoskeleton might be required for the cytodifferentiation of hepatic progenitor cells.

The differentiation potential of fetal liver cells to hepatocytes was also supported by EHS gel *in vivo*. The fetal liver cells suspended with EHS gels kept their viability and AFP expression, suggesting that cell–ECM interaction is also required for cell survival and cytodifferentiation to the hepatocyte *in vivo*. Hepatocyte transplantation into liver has been performed via portal vain for various liver diseases [23,24]. The transplanted cells might interact with ECM of the liver. Consistent with our results, it has been reported that ECM components are the predominant factor to maintain hepatocytes at heterotopic sites [25]. Ectopic subcutaneous hepatocyte transplantation may have advantages as follows; (i) surgical operation is not required, (ii) risk of embolisms is low, and (iii) the transplanted cells are removable or replaceable. Of note, the ectopic cells require additional ECMs such as EHS gel to maintain hepatocyte functions.

In conclusions, our results indicate that EHS gel promotes the differentiation of hepatic progenitor cells to functional hepatocytes. We also demonstrated that EHS gel supports cytodifferentiation and maintains the cell survival and functions *in vivo*. Our findings may therefore have relevance to the clinical application of ectopic liver stem/progenitor cell transplantation as an option to treat liver diseases.

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Synergistic effects of acyclic retinoid and gemcitabine on growth inhibition in pancreatic cancer cells ☆

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ABSTRACT

Pancreatic cancer is a serious healthcare problem worldwide because of its high mortality. Gemcitabine, a DNA synthesis inhibitor, is the standard first-line treatment for advanced pancreatic cancer and is also expected as a key drug for the combination therapy of this malignancy. Retinoids, which are derivatives of vitamin A, exert anti-tumor effects in various types of human malignancies, including pancreatic cancer. This study examined whether combination therapy with gemcitabine and acyclic retinoid (ACR), a new synthetic retinoid, had enhanced anti-tumor efficacy in pancreatic cancer. ACR, 9-*cis*-retinoic acid and gemcitabine preferentially inhibited the growth of human pancreatic cancer cells (Panc-1 and KP-2) in comparison to PE normal human pancreatic epithelial cells. The combination of ACR plus gemcitabine synergistically inhibited the growth of Panc-1 cells. The combined treatment with these two agents also acted synergistically to induce apoptosis and to inhibit Ras activation in these cancer cells. *In vivo*, the combination therapy augmented tumor growth inhibition through the induction of apoptosis and inhibition of cell proliferation in tumor tissue. These results suggest that the combination of ACR plus gemcitabine may therefore be an effective regimen for the chemotherapy of pancreatic cancer.

1. Introduction

Pancreatic cancer is a significant healthcare problem worldwide because of its high mortality. The diagnosis of this malignancy is usually established at a local spreading or metastatic stage and the lack of effective treatments and resistance to conventional chemotherapy contribute to an extremely poor prognosis [1,2]. The deoxycitidine analogue gemcitabine is the standard first-line treatment for advanced pancreatic cancer since it has been shown to improve the clinical benefit regarding the response and survival in comparison to 5-fluorouracil, but the efficacy of gemcitabine as a single-agent remains low, with a median survival of 5.65 months and a 1-year survival of 18% [3]. Therefore, the combination of gemcitabine with other chemotherapeutic agents has recently received increasing attention. For instance, in a recent randomized phase III trial, the combination of gemcitabine plus the EGFR-targeted agent elrotinib modestly improved survival in advanced pancreatic cancer patients [4]. However, most clinical trials that tested gemcitabine-based chemotherapy have failed to demonstrate the superiority of the combination over single-agent gemcitabine, in terms of the survival [1,2].

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Abbreviations: ATRA, all-*trans*-retinoic acid; 9cRA, 9-*cis*-retinoic acid; ACR, acyclic retinoid; RXR, retinoid X receptor; RAR, retinoic acid receptor; RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor-2; FGFR, fibroblast growth factor receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; CI, combination index; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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Retinoids, a group of structural and functional analogues of vitamin A, exert fundamental effects on the regulation of epithelial cell growth, differentiation and development [5]. Retinoids also have proven anti-tumor activity and can cause growth inhibition, accompanied by induction of differentiation and/or apoptosis in various types of cancer cells, including pancreatic cancer cells [6–9]. In addition, Pettersson et al. [10] reported that the conventional retinoids, such as all-*trans*-retinoic acid (ATRA) and 9-*cis*-retinoic acid (9cRA), increased the cytotoxic effects of gemcitabine and cisplatin in pancreatic cancer cells. On the other hand, a pilot phase II trial showed that the activity of the combination of gemcitabine plus 13-*cis*-retinoic acid in patients with advanced pancreatic cancer is limited. Thus, the rates of response, as well as the median survival, were equivalent to the single-agent gemcitabine treatment [11]. Therefore, further studies with the combination of gemcitabine plus other retinoids seems to be of interest and might be beneficial to the patients with advanced pancreatic cancer.

We previously reported that the administration of acyclic retinoid (ACR), a novel synthetic retinoid, reduces the incidence of a post-therapeutic recurrence of liver cancer and improved the survival rate of patients, without causing significant adverse effects [12,13]. ACR inhibits cell growth and induces apoptosis in various types of malignant cells, including human liver cancer, colon cancer and squamous cell carcinoma cell lines [14–16]. In addition, ACR acts synergistically with various anti-tumor agents, such as interferon, OSI-461, vitamin K_2 and trastuzumab in suppressing growth and inducing apoptosis in human liver cancer cells [17–20]. These findings suggest that ACR may be a valuable agent in the chemotherapy of certain types of human malignancies and the efficacy may be enhanced by its combination with agents that target other signaling pathway in cancer cells. The aim of this study is to investigate whether the combination of ACR plus gemcitabine exerts synergistic growth inhibitory effects on human pancreatic cancer cells and to examine possible mechanisms for such synergy, especially focusing on the induction of apoptosis by the combination of these agents.

2. Materials and methods

2.1. Materials

ACR (NIK-333) was supplied by Kowa Pharmaceutical Co., Ltd. (Tokyo, Japan). 9-*cis*-RA was purchased from SIGMA (St. Louis, MO). Gemcitabine was from Eli Lilly Japan (Kobe, Japan). RPMI-1640 and fetal calf serum (FCS) were from Invitrogen (Carlsbad, CA, USA). CS-C complete medium was from Cell Systems Biotechnologie Vertrieb GmbH (St. Katharinen, Germany).

2.2. Cell lines and cell culture

The human pancreatic cancer cell lines Panc-1 and KP-2 were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan) and were maintained in RPMI-1640 medium supplemented with 10% FCS. PE human normal pancreatic epithelial cell line was purchased from ACBRI (Kirkland, WA, USA) and was maintained in CS-C complete medium. The cells were cultured in an incubator with humidified air with 5% CO_2 at 37 °C.

2.3. Cell proliferation assays

Five thousand Panc-1, KP-2 and PE cells were seeded into 96-well plates. The following day, the indicated concentrations of gemcitabine, ACR or 9cRA were added to each well and the cells were incubated for additional 48 h. The number of viable cells in replica plates were then counted using Trypan Blue dye exclusion method, as previously described [17,20]. To determine whether the combined effects of ACR plus gemcitabine were synergistic, Panc-1 cells were treated with the combination of the indicated concentrations of ACR and gemcitabine for 48 h and the combination index (CI)-isobologram was calculated as described previously [18,20].

2.4. TUNEL assays

Ten thousand Panc-1 cells were treated with 10 μM ACR alone, 1.0 μg/ml gemcitabine alone, or the combination of these agents for 48 h on cover slips. The cells were then fixed with 3.7% formaldehyde at room temperature for 10 min, permeabilized with 0.3% Triton X-100 in TBS (pH 7.4), and stained with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany), as described previously [17,20].

2.5. Ras activation assays

The Ras activities were determined using a Ras activation assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacture's instructions. Ras was precipitated in equivalent amounts of Panc-1 cell extract (30 μg) using Raf-1/Ras-binding domain-immobilized agarose and was then subjected to Western blot analysis using an anti-Ras antibody, as described previously [19].

2.6. Animal protocol

Twenty-four male BALB/c nude (5 weeks of age) mice were obtained from Japan SLC, Inc., (Shizuoka, Japan). All mice were maintained at Gifu University Life Science Research Center, according to the Institutional Animal Care Guidelines, and were housed in plastic cages with free access to drinking tap water and a basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan). Xenograft tumors were made by subcutaneous injection of Panc-1 cells, at a concentration of 1×10^6 cells per 200 μl, into the flanks of these mice. Starting 2 weeks after the tumor cell injection, the mice were randomly divided into 4 groups (6 mice per each group). The mice in Group 2 (gemcitabine alone group) received an intraperitoneal injection of gemcitabine (100 mg/kg body weight) twice a week for 6 weeks. Group 3 (ACR alone group) were given the basal diet containing

0.03% ACR, with free access to feeding for 6 weeks. Group 4 (combination group) was treated with both ACR and gemcitabine, as described above. Group 1 served as an untreated control. The tumor size and body weight were measured once a week and the tumor volume was calculated using the formula: largest diameter × (smallest diameter)^2 × 0.5.

2.7. Immunohistochemistry

At the termination of the *in vivo* experiment, the mice were sacrificed by CO_2 asphyxiation and the tumors were then excised. Immunohistochemical analysis for PCNA, a G1-to-S phase marker, was performed as described previously [21], employing a polyclonal antibody to PCNA (sc-7907; Santa Cruz Biotechnology, Santa Cruz, CA; 1:40 final dilution). PCNA-positive cells in xenograft tumor were counted and expressed as a percentage of the total number of tumor cells. The PCNA-labeling index was determined by counting at least 500 cancer cells in each tumor (total of 2,000 cancer cells per group). Detection of apoptosis was performed using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (S7100; Chemicon, Billerica, MA), which is based on the TUNEL assay, according to the manufacturer's instructions. The apoptotic index was defined as number of apoptotic nuclei per 10 random microscopic fields at 200× magnification, as described previously [10].

2.8. Statistical analysis

The data are expressed as the means ± SD. The statistical significance of the difference in mean values was tested using a one-way analysis of variance (ANOVA) and unpaired *t*-test. Significance was defined as a *P* value less than 0.05. All analyses were performed using the StatView ver. 5.0 software program (SAS Institute, Cary, NC).

3. Results

3.1. Retinoids and gemcitabine cause preferential inhibition of growth in human pancreatic cancer cells in comparison to PE normal human pancreatic epithelial cells

The growth inhibitory effect of gemcitabine, ACR and 9cRA were initially examined on Panc-1 and KP-2 human pancreatic cancer cell lines and PE normal human pancreatic epithelial cells. As shown in Fig. 1, ACR and 9cRA similarly inhibited the growth of Panc-1 cells with an IC_{50} value of about 31.9 and 31.4 μM, respectively. Likewise, ACR and 9cRA also caused a growth inhibition in KP-2 cells, but the IC_{50} values (52.4 and 51.9 μM, respectively) were slightly higher when compared to those of Panc-1 cells. Gemcitabine inhibited the growth of these pancreatic cancer cells with an IC_{50} value of about 10 and 1.6 μg/ml, respectively. In contrast, PE cells were more resistant to these agents since the

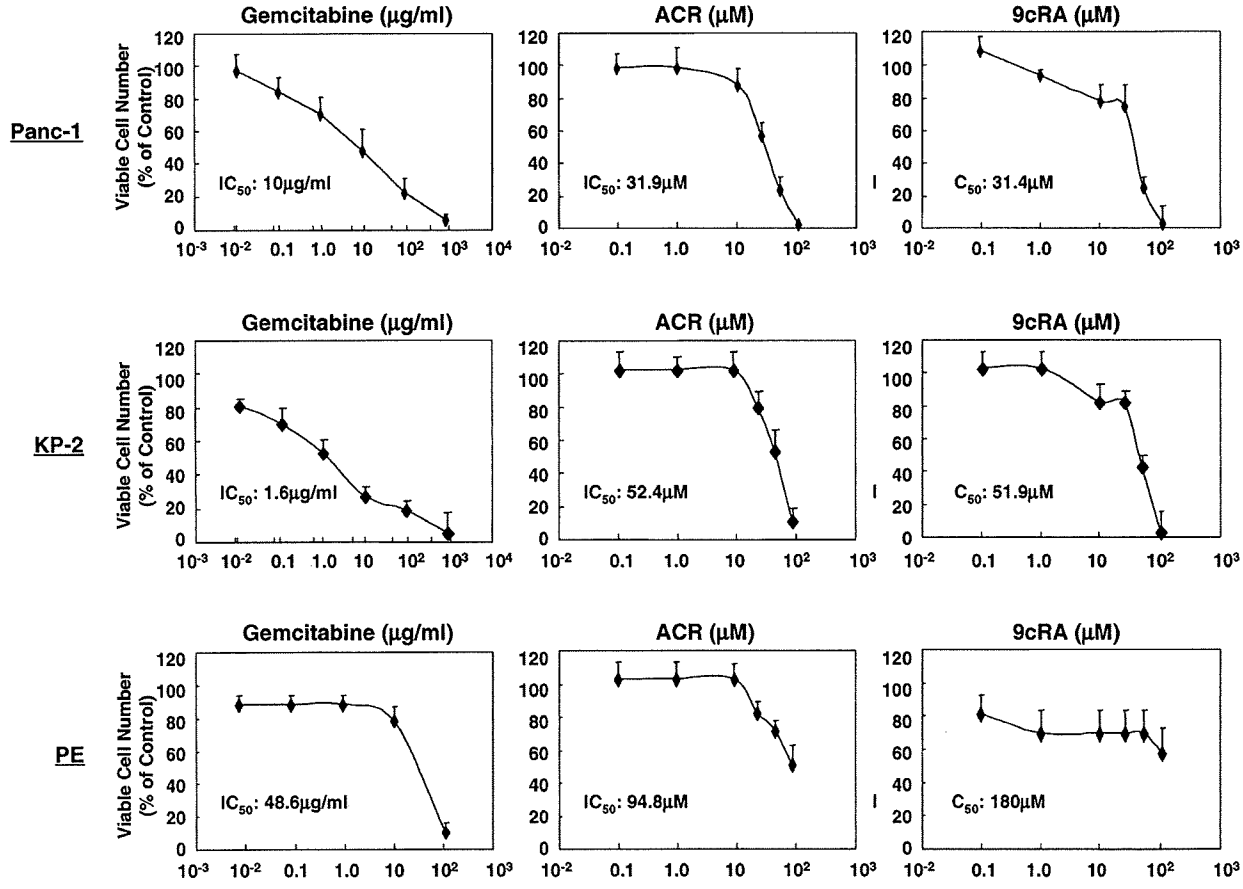

Fig. 1. Inhibition of cell growth by gemcitabine, ACR, or 9cRA in Panc-1 and KP-2 human pancreatic cancer cell lines and in PE human normal pancreatic epithelial cells. The results are expressed as the percentage of growth with 100% representing control cells treated with 0.05% ethanol. Bars, SD of triplicate assays.

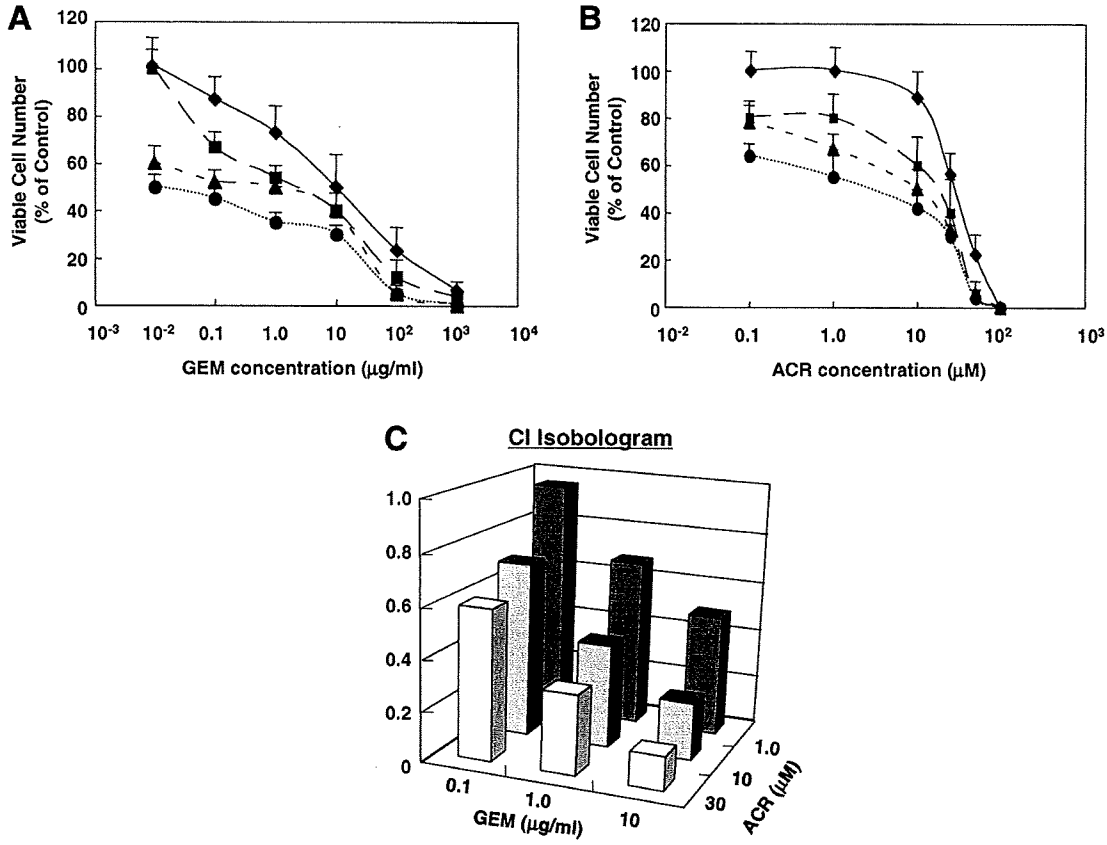

Fig. 2. Inhibition of cell growth by ACR alone, gemcitabine alone and various combinations of these agents in Panc-1 cells. (A and B) Panc-1 cells were treated with the indicated concentrations of ACR alone, gemcitabine alone and various combination of these agents for 48 h. The numbers of viable cells in replica plates were then counted using the Trypan Blue dye exclusion method and expressed as the percentage of the control value. (A) Gemcitabine alone (♦, solid line); gemcitabine + 1 μM ACR (■, long dashed line); gemcitabine + 10 μM ACR (▲, short dashed line); gemcitabine + 30 μM ACR (●, dotted line). (B) ACR alone (♦, solid line); ACR + 0.1 μg/ml gemcitabine (■, long dashed line); ACR + 1.0 μg/ml gemcitabine (▲, short dashed line); ACR + 10 μg/ml gemcitabine (●, dotted line). Bars, SD of triplicate assays. (C) The data obtained in A and B was used to calculate the combination index, as described in Section 2.

Table 1
Combined effects of gemcitabine and ACR on Panc-1 cells

ACR concentration (μM)	Gemcitabine concentration (μg/ml)		
	0.1 μg/ml	1.0 μg/ml	10 μg/ml
1 μM	±	++	+++
10 μM	++	+++	++++
30 μM	+++	++++	++++

Abbreviations: CI, combination index; "±", CI 0.9–1.1 additive effect; "+", CI 0.8–0.9 slight synergism; "++", CI 0.6–0.8 moderate synergism; "+++", CI 0.4–0.6 synergism; "++++", CI < 0.4 strong synergism.

IC_{50} values with ACR, 9cRA and gemcitabine were about 94.8, 180, and 48.6 μg/ml, respectively. These findings suggest that retinoids and gemcitabine preferentially inhibit the growth of pancreatic cancer cells. In addition, ACR can exert anti-proliferative effect in pancreatic cancer cells to almost the same extent as 9cRA (Fig. 1).

3.2. ACR plus gemcitabine cause synergistic inhibition of growth in Panc-1 cells

Next, the effects of combined treatment were examined with a range of concentrations of ACR plus gemcitabine to synergistically inhibit the growth of Panc-1 cells. We found that the combination of as little as 10 μM ACR (about IC_{10} value) and 1.0 μg/ml gemcitabine (about IC_{30} value) exerted synergistic growth inhibition (Figs. 2A and B). Thus, when analyzed by the isobologram method [18,20], the CI index for this combination was 3+ and thus indicating "synergism" (Fig. 2C and Table 1). These findings suggest that ACR plus gemcitabine might be an effective combination for the inhibition of pancreatic cancer cell growth due to their synergistic efficacy.

3.3. ACR plus gemcitabine synergistically induce apoptosis in Panc-1 cells

We then examined whether the synergistic growth inhibition by ACR plus gemcitabine (Fig. 2C and Table 1) was associated with induction of apoptosis because both of these agents can induce apoptosis in cancer cells [15,16,22,23]. The treatment of Panc-1 cells with either 10 μM ACR alone or 1.0 μg/ml gemcitabine alone induced TUNEL-positive cells in approximately 15% of the total remaining cells (Fig. 3A, columns 2 and 3). Moreover, when the cells were treated with combination of these agents, TUNEL-positive cells increased to 25% of the total remaining cells (Fig. 3A, column 4). These results suggest the induction of apoptosis to be enhanced by the combination of ACR plus gemcitabine and that might explain the strong growth inhibition caused by this combined treatment (Fig. 2C and Table 1).

3.4. ACR and gemcitabine inhibit activation of Ras in Panc-1 cells

We then examined whether the combined treatment of ACR plus gemcitabine inhibits the activation of Ras because this oncogene is aberrantly activated in up to 90% of pancreatic cancers and thus play a role in the development of this malignancy [27]. As shown in Fig. 3B, the Ras activities were significantly inhibited when the Panc-1 cells were treated with 10 μM ACR alone and with the combination of ACR plus 1.0 μg/ml gemcitabine.

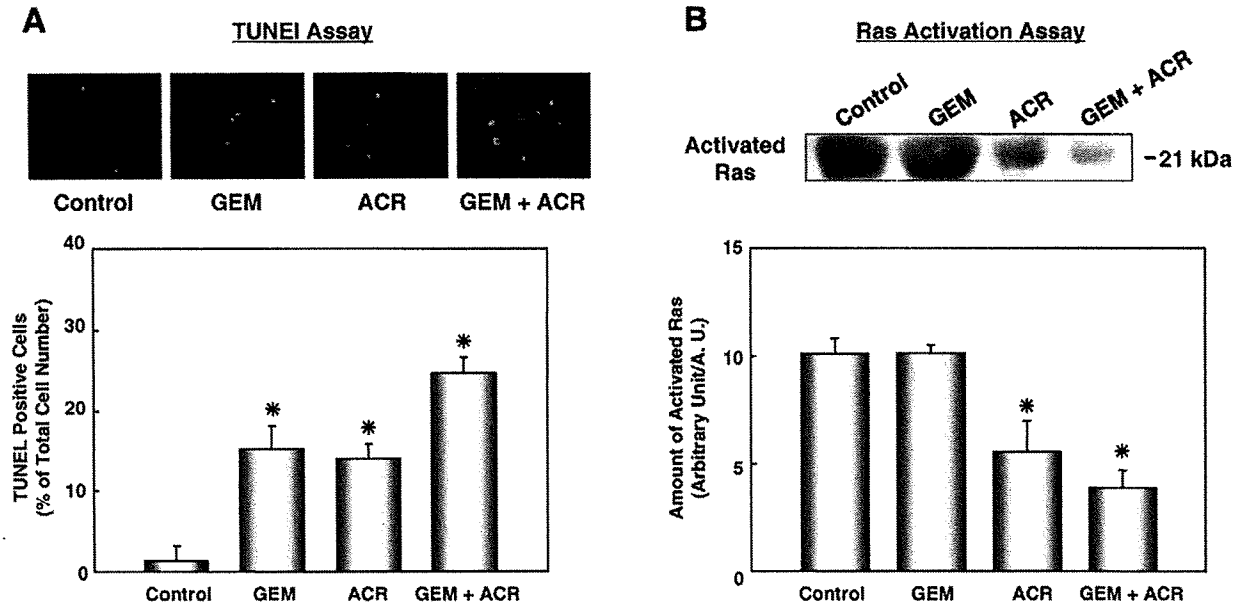

Fig. 3. Effects of the combination of ACR plus gemcitabine on the induction of apoptosis and inhibition of the Ras activity in Panc-1 cells. The cells were treated with vehicle (control, column 1), 1.0 μg/ml gemcitabine alone (GEM; column 2), 10 μM ACR alone (ACR; column 3), or the combination of 1.0 μg/ml gemcitabine plus 10 μM ACR (GEM + ACR; column 4) for 48 h. (A) The apoptotic cells were detected using the TUNEL method. TUNEL-positive cells were counted and expressed as the percentage of the total cell number (500 cells were counted in each flask). (B) The Ras activities were determined using a Ras activation assay kit. Relative intensity of the bands was quantitated by densitometry and is displayed in the lower panel. Values are the means ± SD. *$p < 0.01$: in comparison to vehicle-treated cells (column 1). Representative results from three independent experiments with similar results are shown.

3.5. ACR plus gemcitabine synergistically inhibit growth of pancreatic tumor xenografts in nude mice

We next examined whether the synergistic growth inhibition and apoptosis induction by the combined treatment with ACR plus gemcitabine in pancreatic cancer cells were also observed *in vivo* using a nude mice xenograft model. Fig. 4A shows that both ACR and gemcitabine exerted anti-tumor activity as single agent in this model, thus causing decreased growth rates until 5 weeks after the cancer cell injection. However, at 6 weeks after the injection, the Panc-1 tumors which developed in either ACR alone- or gemcitabine alone-treated mice demonstrated an augmenting tendency. On the other hand, the combined treatment of ACR and gemcitabine strongly inhibited the growth of the pancreatic cancer xenograft during this experiment (Fig. 4A). In addition, the PCNA-labeling index in the tumors was significantly lower in both the ACR alone- and gemcitabine alone-treated mice when compared to the control mice, but the combined treatment of these agents caused a marked decrease in this index (Fig. 4B). Furthermore, although apoptosis occurred at a low level in the control and ACR-treated mice, there was a significant increase in the induction of apoptosis by the treatment with gemcitabine and, to a greater degree, by the combination of ACR plus gemcitabine in these tumors (Fig. 4C). All of the treatments were well tolerated and the body weights remained stable in all groups during the experiment (data not shown).

4. Discussion

Pancreatic cancer is still one of the most lethal cancers of all human malignancies because monotherapy with gemcitabine, the standard first-line treatment for this cancer, results in only a marginal survival benefit [3]. Therefore, improved therapeutic regimens are needed and gemcitabine-based combination chemotherapy may be a promising strategy for this purpose [4]. The present study demonstrated that the combination of ACR plus gemcitabine synergistically inhibited the growth of Panc-1 human pancreatic cancer cells both *in vitro* (Fig. 2 and Table 1) and *in vivo* (Fig. 4A) and that this was associated with the induction of apoptosis (Figs. 3A and 4C) and inhibition of cell proliferation (Fig. 4B). ACR prevented the development of second primary liver cancer without causing significant adverse effects. In this clinical trial, the plasma concentration of ACR ranged from 1 to 5 μM [12,13]. Therefore, the dosage of ACR that we used in the present combination studies (10 μM) is near the practical level. These findings suggest the possibility that the combination of these agents may have benefits in the treatment of pancreatic cancer due to their synergism. The findings are also consistent with the previous report that ATRA or 9cRA enhanced the cytotoxic effects of gemcitabine in pancreatic cancer cells [10]. However, in comparison to these conventional retinoids, ACR might be a more preferable partner for gemcitabine because its safety and cancer chemopreventive efficacy have been proven in previous clinical trial [12,13].

How can ACR inhibit growth of pancreatic cancer cells? This might be explained by the effect of ACR to inhibit the activation of Ras (Fig. 3*B*) because constitutive activation of this oncogene confers uncontrolled stimulatory signals to downstream cascades, including Raf-MAPK and PI3K/Akt, in pancreatic cancer cells [28,29]. ACR inhibits Raf-1-bound Ras activity and its downstream signaling pathways, thereby inhibiting the growth of liver cancer cells [19,20,30]. Moreover, recent studies have indicated that the inhibition of Ras activity enhances the chemotherapeutic effect of gemcitabine and thereby reducing pancreatic tumor growth [31,32]. Therefore, inhibition of Ras activity by ACR (Fig. 3B) may contribute to exert a synergistic growth inhibition when combined with gemcitabine (Fig. 2, Table 1 and Fig. 4).

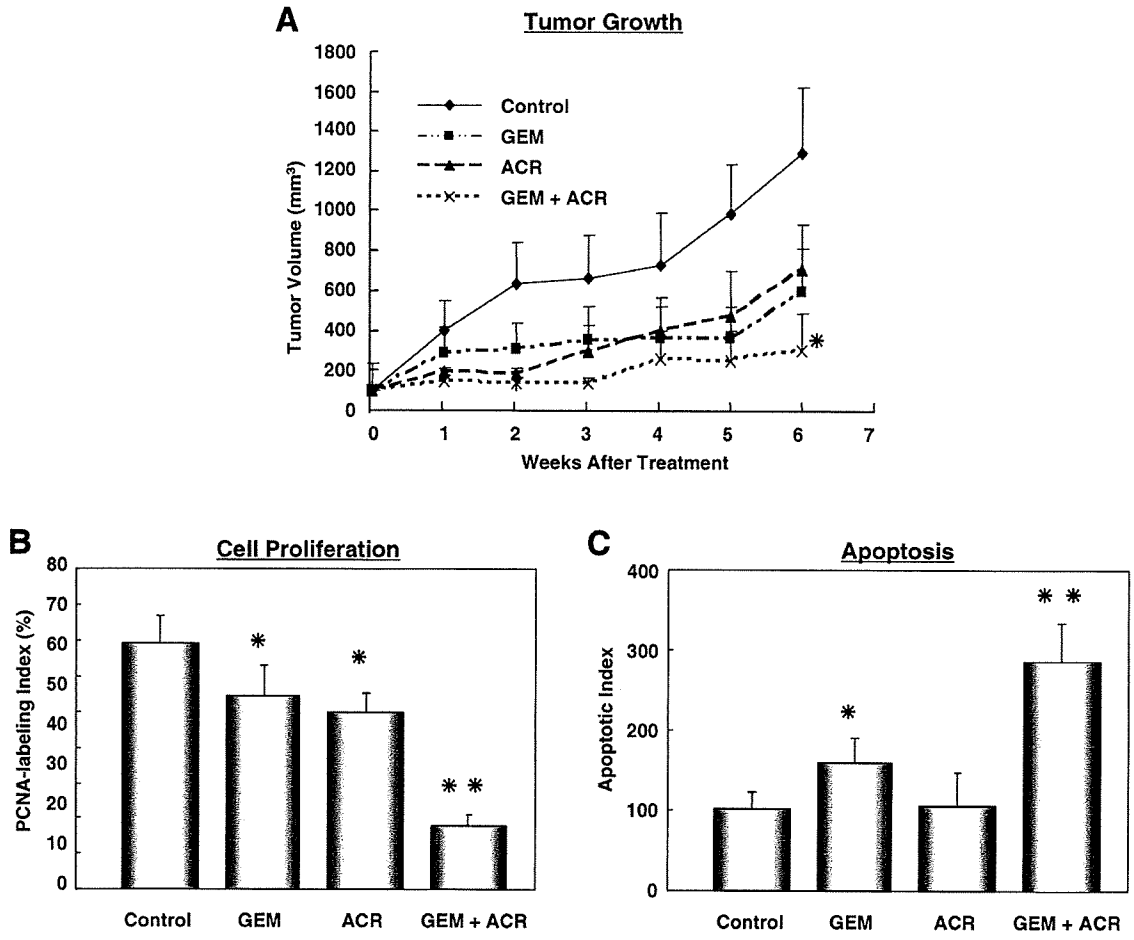

Fig. 4. Effects of the combination of ACR plus gemcitabine on the growth of Panc-1 tumors in nude mice. Male BALB/c nude mice were injected subcutaneously with 1×10^6 Panc-1 cells. Two week after the injection, the mice were divided into 4 groups and were treated with following conditions for 6 weeks. Group 1, control (untreated group). Group 2 received an intraperitoneal injection of gemcitabine (100 mg/kg body weight) twice a week (GEM, gemcitabine alone group). Group 3 was given the basal diet containing 0.03% ACR (ACR, ACR alone group). Group 4 was treated with the combination of ACR plus gemcitabine (GEM + ACR, combination group). (A) Growth curve of Panc-1 tumors in each group are represented. (B) PCNA-labeling index and (C) Apoptotic index. The Panc-1 tumors were excised from each animal at the termination of the experiment, and the number of proliferative cells (B) and apoptotic cells (C) were detected using PCNA immunohistochemistry and an ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, respectively, as described in the "Materials and Methods". Values are the means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$: in comparison to the untreated group (column 1).

In addition to the inhibition of Ras activity, ACR is considered to possess other beneficial effects to inhibit growth of pancreatic cancer cells. For instance, ACR, the ligand for RXR [24], suppresses pancreatic cancer cell proliferation via binding to RXR because retinoids mediate their biological effects, including anti-cancer effect, by binding to nuclear, ligand-activated receptors [5,6]. These receptors are divided into RAR and RXR families, both composed of three subtypes (α, β, and γ) and most pancreatic cancer cell lines, including Panc-1, express both RXRα and RXRβ [25,26]. This presumption may be supported by the previous findings that high-affinity RXR-selective ligands can efficiently suppress pancreatic cancer cell proliferation [9]. ACR may also inhibit the pancreatic cancer cell growth by inhibiting the activation of some members of receptor tyrosine kinases (RTKs), such as EGFR, HER2, and FGFR, which are targets of ACR [16,20,33]. This hypothesis seems plausible because these RTKs are frequently overexpressed in pancreatic cancers and are associated with the development of this malignancy [34]. It also should be noted that the overall survival in patients with advanced pancreatic cancer is significantly improved with the combination of erlotinib, an EGFR tyrosine kinase inhibitor, and gemcitabine compared with placebo plus gemcitabine in a recent phase III trial [4].

In the present study ACR enhanced the induction of apoptosis caused by gemcitabine (Figs. 3A and 4C). This finding is consistent with previous reports that ACR exerts synergistic anti-cancer effects, including induction of apoptosis, in human liver cancer cells when it is combined with other agents [17–20]. The inhibition of Akt activation by ACR is one of the underlying mechanisms in this synergism [20]. This is significant because the reduction of phosphorylated (i.e., activated) Akt is correlated with the enhancement of gemcitabine-induced apoptosis and antitumor activity in pancreatic cancer cells, thus suggesting that the PI3K/Akt pathway plays a significant role in mediating gemcitabine resistance in these cancer cells [35,36]. A previous study also reported that bexarotene, a selective RXR agonist, enhances the growth inhibition of gemcita-

bine in non-small cell lung cancer cells by preventing and reversing gemcitabine resistance [37]. Therefore, ACR may help to either prevent or overcome gemcitabine resistance, presumably, by inhibiting the Akt activation. In conclusion, the results of this experiment support the possibility that the combined treatment of ACR plus gemcitabine may be a potentially effective and critical strategy for pancreatic cancer chemotherapy.

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Alimentary Pharmacology & Therapeutics

Evolution of prognostic factors in hepatocellular carcinoma in Japan

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SUMMARY

Background
The surveillance of hepatocellular carcinoma (HCC) has become prevalent, and the modalities for its treatment have improved.

Aim
To understand the changes that occur in the characteristics and prognostic factors of HCC with time.

Methods
Newly diagnosed HCC patients were divided into two groups; patients treated before 31 December 2000 (n = 504), and after 1 January 2001 (n = 746), and their clinical backgrounds and prognostic factors were analysed.

Results
The number of patients negative for both Hepatitis B surface antigen (HBsAg) and Hepatitis C virus antibody (HCVAb) increased with time (NBNC-HCC). The size of HCC decreased in patients who were positive for HBsAg (B-HCC) or HCVAb (C-HCC), whereas no difference was observed in NBNC-HCC. The patient survival of C-HCC improved; however, no difference was detected for NBNC-HCC. In multivariate analysis, low albumin, high aspartate aminotransferase (AST), ascites, large tumour size, multiple tumour number and high alpha-fetoprotein were risk factors for survival before 2000, whereas the presence of HBsAg was additionally selected as a good prognostic factor and AST was excluded after 2001.

Conclusions
The prognostic factors as well as clinical background of HCC changed with time, and the presence of HBsAg was found to be an additional good prognostic factor after 2001.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death in the world.[1] Globally, more than 80% of HCC cases develop in patients suffering from long-lasting viral hepatitis. Among these patients, imaging studies such as ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI) are regularly performed to detect HCC at an early stage.[2–4] As a result, the proportion of HCC that can be treated by local ablation therapies or surgical resection has increased.

The effectiveness of the treatment has also increased. The mortality rates resulting from surgery have decreased,[5] and the outcomes of these patients have improved during the last few decades. Percutaneous ethanol injection therapy (PEIT), microwave coagulation therapy (MCT) and radiofrequency ablation therapy (RFA) have also been used for the treatment of small HCC, and have become more popular because they are safe and the damage they cause to the liver is minimal. Moreover, evidence-based treatment algorithms are presented by several groups and so the selection of treatment has been conducted more appropriately.[6–8]

Interferon and nucleotide analogues are drugs used to eradicate hepatitis virus infection. Recent studies have demonstrated that interferon can reduce the incidence of HCC in patients with hepatitis C virus infection and even improve the prognosis of HCC.[9, 10] Nucleotide analogues are now frequently used in patients with hepatitis B virus infection. They decrease the inflammation caused by hepatitis B virus, normalize transaminase in about 90% of the patients treated with the drugs and prolong the survival of these patients.[11] This effect was observed even in patients with HCC.[12, 13]

Although the circumstances of patients with HCC have dramatically changed as demonstrated above, few studies have been conducted to analyse the changes in the prognostic factors of HCC. In this study, we analysed the trends in HCC patients and tried to elucidate the changes that have occurred in the prognostic factors with time.

PATIENTS AND METHODS

A total of 1267 consecutive, newly diagnosed HCC patients who were admitted to Okayama University Hospital for treatment between January 1991 and February 2009 were followed up. Among these patients, 17 were excluded because they had received a liver transplant during the follow-up, so the remaining 1250 patients were enrolled in this study. The patients were divided into two groups; patients treated before 31 December 2000 (n = 504), and those treated after 1 January 2001 (n = 746), and analysed. Informed consent was obtained from all patients for use of their clinical data. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki, and was approved by the Ethical Committee of our institute.

Diagnosis

All patients were diagnosed as having HCC by using imaging modalities such as angiography, computed tomography and magnetic resonance imaging, or by tumour biopsy. The diagnostic criteria for HCC via imaging was based on previous reports of hyperattenuation at the arterial phase, hypoattenuation at the portal phase in dynamic CT or MRI, and tumour staining on angiography.[14]

Treatments and follow-up

The selection of the therapies was performed according to the evidence-based clinical practice guidelines for HCC in Japan.[8] The rate of observance of the guidelines was 74.3% and 78.0% before 2000 and after 2001 respectively. Biochemical liver function tests and US, dynamic CT or MRI were performed at least every 3 months after the initial treatment. Diagnosis of recurrence was made with the same diagnostic criteria used for the initial diagnosis. Re-treatment was performed depending on the condition of the recurrence and background liver function.

Statistical analysis

The Wilcoxon test was used to compare continuous data, and the chi-squared test was used to compare categorical data. Survival was compared using the Kaplan–Meier method, and the difference was evaluated using the log-rank test. For the analysis of prognostic factors, 15 parameters were collected: age, gender, tumour size, tumour number, alpha-fetoprotein (AFP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelet count, prothrombin time (PT),

total bilirubin (T. Bil), serum albumin, hepatitis B virus surface antigen (HBsAg), hepatitis C virus antibody (HCVAb), the presence of ascites and alcohol consumption. Continuous scales and ordinal scales were categorized into two groups using the cut-off levels indicated in Tables 2 and 3. In cases before 2000, the patients who survived at the end of 2000 were no longer followed for the study from 1 January 2001 (censored at the end of 2000). The Cox proportional hazard model was used to calculate risk ratios for survival. We did not include treatment factors (e.g. nucleotide analogues, interferon and treatment modalities of HCC) because they are confounding factors in the analysis. All statistical analyses were performed using JMP software (Ver. 8.0 SAS institute, Cary, NC, USA).

RESULTS

Changes in patients' background

The clinical backgrounds of the HCC patients changed with time (Table 1). The median age at diagnosis after 2001 was greater than that before 2001 (63 vs. 67 years old, $P < 0.01$). From 2000 to 2001, the percentage of viral hepatitis decreased, and the ratio of

Table 2. The changes in patients' profiles with time in different hepatitis virus statuses

	~Dec 2000	Jan 2001~	*P*-value
Total bilirubin (mg/dL)			
B-HCC	0.90 (0.64–1.31)	0.87 (0.66–1.24)	N.S.
C-HCC	0.99 (0.75–1.37)	0.84 (0.64–1.14)	$P < 0.01$
NBNC-HCC	1.08 (0.65–1.46)	0.87 (0.61–1.23)	N.S.
Albumin (g/dL)			
B-HCC	3.69 (3.33–3.96)	3.87 (3.40–4.25)	N.S.
C-HCC	3.55 (3.22–3.90)	3.60 (3.30–3.90)	N.S.
NBNC-HCC	3.82 (3.31–4.20)	3.77 (3.42–4.10)	N.S.
Tumour size (cm)			
B-HCC	3.2 (2.1–4.9)	2.5 (1.7–3.8)	$P = 0.04$
C-HCC	2.7 (1.8–4.2)	2.1 (1.5–3.2)	$P < 0.01$
NBNC-HCC	3.2 (2.2–5.5)	3.0 (1.7–5.5)	N.S.
Tumour number (single, %)			
B-HCC	42.7	51.0	N.S.
C-HCC	54.9	56.4	N.S.
NBNC-HCC	57.6	51.6	N.S.

All numbers are medians (inter-quartile range) unless otherwise noted. B-HCC, hepatocellular carcinoma positive for hepatitis B virus surface antigen; C-HCC, hepatocellular carcinoma positive for hepatitis C virus antibody; NBNC-HCC, hepatocellular carcinoma negative for both hepatitis B virus surface antigen and hepatitis C virus antibody; N.S., not significant.

Table 1. Clinical background of 1250 patients

	~Dec 2000	Jan 2001~	
Patient number	504	746	*P*-value
Age (years)	63 (58–68)	67 (60–73)	<0.001
Gender (male)	366 (72.6%)	530 (71.1%)	0.544
HCVAb (positive)	391 (77.6%)	546 (73.2%)	<0.001*
HBsAg (positive)	93(18.5%)	108(14.5%)	
HCVAb and HBsAg negative	37(7.3%)	106(14.2%)	
Total bilirubin (mg/dL)	0.97 (0.73–1.38)	0.85 (0.64–1.17)	<0.001
Albumin (g/dL)	3.6 (3.2–3.9)	3.7 (3.3–4.0)	0.100
AST (IU/L)	63 (46–89)	54 (39–77)	<0.001
ALT (IU/L)	57(38–79)	46(31–69)	<0.001
Platelet ($\times 10^4/mm^3$)	10.1(6.8–13.8)	11.7(7.8–16.4)	<0.001
Prothrombin time (%)	82(66–97)	92(82–102)	<0.001
Ascites (present)	75(14.9%)	123(16.5%)	0.444
Alcohol (>90 g/day)	62(12.4%)	80 (10.9%)	0.438
Tumour size (mm)	28 (19–45)	22 (15–35)	<0.001
Tumour number (single)	258(53.4%)	393(55.1%)	0.561
AFP (ng/mL)	38.2 (12.4–240.9)	18.9 (6.8–86.9)	<0.001

All numbers are medians (inter-quartile range) unless otherwise noted. *P-value among three viral statuses.
HCVAb, hepatitis C virus antibody; HBsAg, hepatitis B virus surface antigen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, alpha-fetoprotein.

Table 3. Univariate analysis for the prognostic factors of HCC

	~Dec 2000 (n = 504)			Jan 2001~ (n = 746)		
	RR	95%CI	P-value	RR	95%CI	P-value
Age (>65 years)	1.25	0.97–1.61	0.08	1.11	0.84–1.49	0.44
Gender (male)	1.08	0.81–1.44	0.59	1.18	0.86–1.65	0.28
HCVAb (positive)	1.28	0.93–1.18	0.12	0.91	0.66–1.26	0.56
HBsAg (positive)	0.95	0.66–1.32	0.77	0.86	0.56–1.27	0.47
Total bilirubin (>2 mg/dL)	1.92	1.19–2.94	<0.01	2.72	1.59–4.37	<0.01
Albumin (<3.5 g/dL)	2.01	1.56–2.60	<0.01	2.65	1.95–3.60	<0.01
AST (>40 IU/L)	2.29	1.57–3.48	<0.01	1.74	1.20–2.57	<0.01
ALT (>40 IU/L)	1.17	0.88–1.57	0.25	1.09	0.80–1.51	0.56
Platelet (<10 × 10^4/mm^3)	1.29	1.00–1.66	0.04	1.12	0.82–1.52	0.44
Prothrombin time (<80%)	1.40	1.08–1.81	0.01	1.84	1.33–2.51	<0.01
Ascites (present)	1.93	1.38–2.64	<0.01	3.00	2.17–4.10	<0.01
Alcohol (>90 g/day)	0.95	0.64–1.37	0.81	0.92	0.56–1.41	0.72
Tumour size (>3 cm)	2.64	2.05–3.41	<0.01	4.00	2.99–5.37	<0.01
Tumour (multiple)	2.81	2.17–3.65	<0. 01	2.03	1.52–2.72	<0.01
AFP (>200 ng/mL)	2.20	1.67–2.87	<0.01	2.51	1.77–3.49	<0.01

RR, risk ratio; 95% CI, 95% confidence interval. Other abbreviations are the same as listed in Table 1.

hepatitis virus negative patients increased from 7.3% to 14.2% (P < 0. 01). In addition, tumour size at diagnosis became smaller, and liver functions such as bilirubin and prothrombin time were improved. Table 2 demonstrates the clinical backgrounds of the patients with different viral infection statuses. Total bilirubin of the patients who were positive for HCVAb (C-HCC) declined; however, no difference in albumin was observed in any group. The detected HCCs were smaller after 2001 in patients who were positive for HBsAg (B-HCC) or C-HCC, whereas no difference was observed in the patients without these viral markers (NBNC-HCC). The percentages of tumours over 5 cm in diameter were 23.6% and 17.8% in B-HCC (P = 0.52), 17.3% and 8.7% in C-HCC (P < 0.01) and 27.2% and 28.8% in NBNC-HCC (P = 0.86), before 2000 and after 2001 respectively.

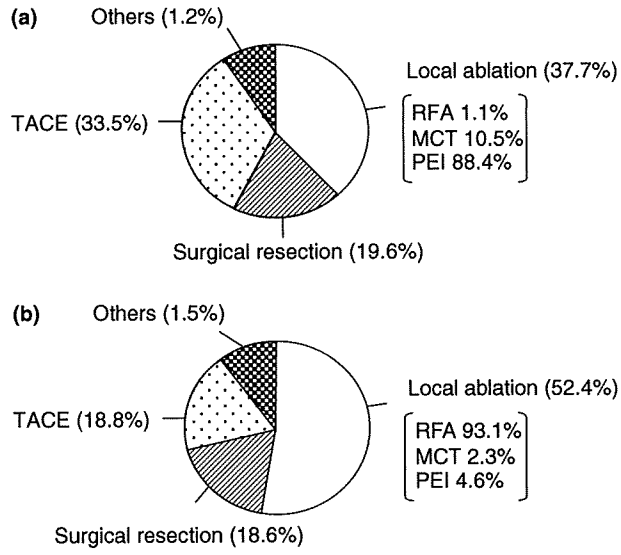

Figure 1. Changes in treatment modalities with time. The percentage of local ablation was 37.7% before December 2000 (a) and increased to 52.4% after January 2001 (b). PEI was popular before 2000; however, RFA was chosen as the standard therapy after 2001. Abbreviations: RFA, radiofrequency ablation; MCT, microwave coagulation therapy; PEI, percutaneous ethanol injection; TACE, transcatheter arterial chemoembolization.

Nucleotide analogues were used in 1.1% and 64.8% of B-HCC before 2000 and after 2001 respectively. Interferon treatment was performed in 15.5% and 19.8% of the patients who were treated before 2000 and after 2001 respectively. In all of the patients, except 22 (7 peg-interferon, 15 peg-interferon + ribavirin), treated after 2001, the treatment was carried out using conventional interferon.

Changes in treatment modalities

The treatment methods changed with time (Figure 1). The percentage of patients who received local ablation therapy increased from 37.7% (n = 190) to 52.4%