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Acyclic retinoid synergises with valproic acid to inhibit growth in human hepatocellular carcinoma cells

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ABSTRACT

A malfunction of retinoid X receptor-α (RXRα) due to phosphorylation is associated with the development of hepatocellular carcinoma (HCC) and acyclic retinoid (ACR), which targets RXRα, can prevent the development of second primary HCC. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, induces apoptosis and cell cycle arrest in cancer cells. VPA can also enhance the sensitivity of cancer cells to retinoids. The present study examined the possible combined effects of ACR plus VPA in HepG2 human HCC cell line. The combination of 5 μM ACR and 1 mM VPA, about the IC_{25} value for both compounds, synergistically inhibited the growth of HepG2 cells without affecting the growth of Hc normal human hepatocytes. The combined treatment with ACR plus VPA also acted synergistically to induce apoptosis and G_0–G_1 cell cycle arrest in HepG2 cells. This combination further exerted a synergistic inhibition of the phosphorylation of RXRα, ERK, Akt and GSK-3β proteins and caused an accumulation of acetylated histones H3 and H4 proteins. VPA enhanced the ability of ACR to raise the cellular levels of RARβ and $p21^{CIP1}$. The combination of these agents markedly increased both the RARE and RXRE promoter activities in HepG2 cells. These results suggest that ACR and VPA cooperatively increase the expression of RARβ and $p21^{CIP1}$, while inhibiting the phosphorylation of RXRα, and these effects were associated with induction of apoptosis and the inhibition of cell growth in HepG2 cells. This combination might therefore be an effective regimen for the chemoprevention and chemotherapy of HCC.

1. Introduction

The prognosis for patients with hepatocellular carcinoma (HCC) is poor because of its high incidence and recurrence rate in livers demonstrating chronic inflammation and/or cirrhosis. Therefore, in order to improve the prognosis, there is a critical need to develop more effective strategies for achieving the chemoprevention of HCC and one of the promising agents for this purpose is retinoids [1–3]. Retinoids, a group of structural and functional analogues of vitamin A, exert fundamental effects on the regulation of epithelial cell growth, differentiation and development [4,5]. Retinoids exert their biological functions primarily by regulating gene expression through two distinct nuclear receptors, the retinoic acid receptors

Abbreviations: ACR, acyclic retinoid; CI, combination index; ERK, extracellular signal-regulated kinase; GSK-3β, glycogen synthase kinase-3β; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; RAR, retinoic acid receptor; RARE, retinoic acid receptor responsive element; RT-PCR, reverse transcription PCR; RXR, retinoid X receptor; RXRE, retinoid X receptor responsive element; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; VPA, valproic acid.

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(RARs) and the retinoid X receptors (RXRs), which are both composed of three subtypes (α, β and γ) [4,5]. Among these receptors, RXRα plays an essential role in controlling normal cell proliferation and metabolism, and acts as a master regulator of nuclear receptors [4,5].

Because of its essentiality, abnormality in the function of RXRα is highly associated with the development of various human malignancies, including HCC, and therefore might be regarded as a critical target for cancer chemoprevention and chemotherapy [2,6]. We have previously reported that liver carcinogenesis is accompanied by the accumulation of the phosphorylated (i.e. dysfunctional) form of RXRα (p-RXRα) protein [7–9]. Furthermore, acyclic retinoid (ACR), a synthetic retinoid which targets RXRα, reduced the incidence of post-therapeutic recurrence of HCC and improved survival rate of patients (1). ACR inhibits experimental liver carcinogenesis and induces apoptosis in human HCC-derived cells and this is associated with inhibition of RXRα phosphorylation as well as induction of cellular levels of RARβ [10–12]. Moreover, the effects of ACR in suppressing growth and inducing apoptosis in HCC cells are synergistically enhanced when the agent is combined with specific drugs that target other signaling pathways [13–16]. Therefore, not only used as the sole regimen, the combination chemoprevention using ACR as a key agent might be an effective strategy to prevent the development of HCC.

Recent studies have revealed histone deacetylase (HDAC) inhibitors, including valproic acid (VPA), to inhibit growth and induce apoptosis in human HCC-derived cells [17–19]. In addition, HDAC inhibitors are one of the promising partners of retinoid-based combination chemoprevention and chemotherapy because a greater growth-inhibitory effect was observed with the combination in various types of cancer cells [20–22]. The purpose of this study is to test a synergistic effect of ACR plus VPA on the growth of human HCC cells and to examine the possible mechanisms.

2. Materials and methods

2.1. Materials

ACR (NIK-333) was supplied by Kowa Pharmaceutical Co., Ltd. (Tokyo, Japan). VPA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-RXRα, anti-RARβ and anti-$p21^{CIP1}$ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibodies for ERK, phosphorylated ERK, Akt, phosphorylated Akt, GSK-3β, phosphorylated GSK-3β, acetylated histones H3 and H4 proteins were from Cell Signaling Technology (Beverly, MA). The antibody against GAPDH was from Chemicon International (Temecula, CA, USA).

2.2. Cell lines and cell culture conditions

The HepG2 human HCC cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and was maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS. The Hc human normal hepatocyte cell line was purchased from Applied Cell Biology Research Institute and was maintained in a CS-S complete medium (Cell Systems Biotechnologie Vertrieb GmbH, St. Katharinen, Germany). The cells were cultured in an incubator with humidified air with 5% CO_2 at 37 °C.

2.3. Cell proliferation assays

Three thousand HepG2 or Hc cells were seeded on 96-well plates. The following day, the indicated concentrations of ACR or VPA were added to each well and the cells were then incubated for an additional 48 h. The stock solutions were prepared and test concentrations were set according to reference #15 for ACR and #19 for VPA, respectively. The number of viable cells in replica plates was then counted using the Trypan Blue dye exclusion method, as previously described [16]. To determine whether the combined effects of ACR plus VPA were synergistic, HepG2 cells were treated with the combination of the indicated concentrations of ACR and VPA for 48 h and the combination index (CI)-isobologram was calculated as described previously [15,23].

2.4. Protein extraction and western blot analysis

Total cellular protein and acid soluble proteins were extracted, respectively, and equivalent amounts of protein were examined by a Western blot analysis using specific antibodies, as previously described [16,18]. To detect the expression level of p-RXRα protein, RXRα protein was affinity purified from the total cell extracts using anti-RXRα antibody-immobilized Sepharose beads and then was subjected to Western blot analyses using an anti-phosphoserine antibody [7]. GAPDH expression served as a loading control. The intensities of protein bands were quantified using NIH image software version 1.62.

2.5. RNA extraction and semiquantitative RT-PCR analysis

RNA extraction and a semiquantitative RT-PCR analysis were done as described previously [12,24]. Total RNA was isolated from frozen HepG2 cells using TRIzol reagent as recommended by the manufacturer (Invitrogen). The cDNA was amplified from 1 μg of total RNA using SuperScript one-step RT-PCR with the platinum Taq system (Invitrogen). The primers used for amplification of RARβ and $p21^{CIP1}$ specific gene are described previously [24]. The amplified products obtained with actin-specific primers [12] served as internal control. By using a thermal controller (Programmable Thermal Controller; MJ Research Inc., Watertown, MA), 35-cycle rounds of PCR were chosen for the data analysis of expression of RARβ and $p21^{CIP1}$ mRNAs, respectively, because a semiquantitative assessment indicated that under this condition the reaction had not yet reached a plateau and thus was still in the log phase. The intensities of PCR products stained with ethidium bromide were quantified using NIH image software version 1.62.

2.6. TUNEL assays

HepG2 cells were treated with 5 μM ACR alone, 1 mM VPA 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) methods using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany), as described previously [16].

2.7. Cell cycle assays

HepG2 cells were treated with test drugs for 48 h. The harvested cells were stained with propidium iodide (Sigma, St. Louis, MO), and the samples were then analyzed for DNA histograms and cell cycle phase distribution by flow cytometer using a FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ). The data were analyzed by using the CELL Quest computer program (Becton Dickinson), as described previously [24].

2.8. RARE and RXRE reporter assays

Reporter assays were performed as described previously [16]. HepG2 cells were transfected with RARE or RXRE reporter plasmids (750 ng/35 mm dish), which were provided by the late Dr. K. Umesono (Kyoto University, Kyoto, Japan), along with p*RL*-CMV (*Renilla* luciferase, 100 ng/35 mm dish; Promega, Madison, WI, USA) as an internal standard to normalize the transfection efficiency. Transfections were performed using UniFector reagent (B-Bridge, Sunnyvale, CA, USA) according to the manufacturer's protocol. After exposure of the cells to the transfection mixture for 24 h, the cells were treated with 5 μM ACR alone, 1 mM VPA alone, or the combination of these agents

Table 1
Combined effects of ACR and VPA on HepG2 cells.

ACR concentration (mM)	VPA concentration (mM)			
	0.1	0.5	1	2
1	–	–	++	+++
5	–	±	+++	+++
10	±	++	+++	++++
20	±	+++	++++	++++

Note: "–", CI > 1.3 antagonism; "–", CI 1.1–1.3 moderate antagonism; "±", 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 0.2–0.4 strong synergism.
Abbreviations: CI, combination index; ACR, acyclic retinoid; VPA, valproic acid.

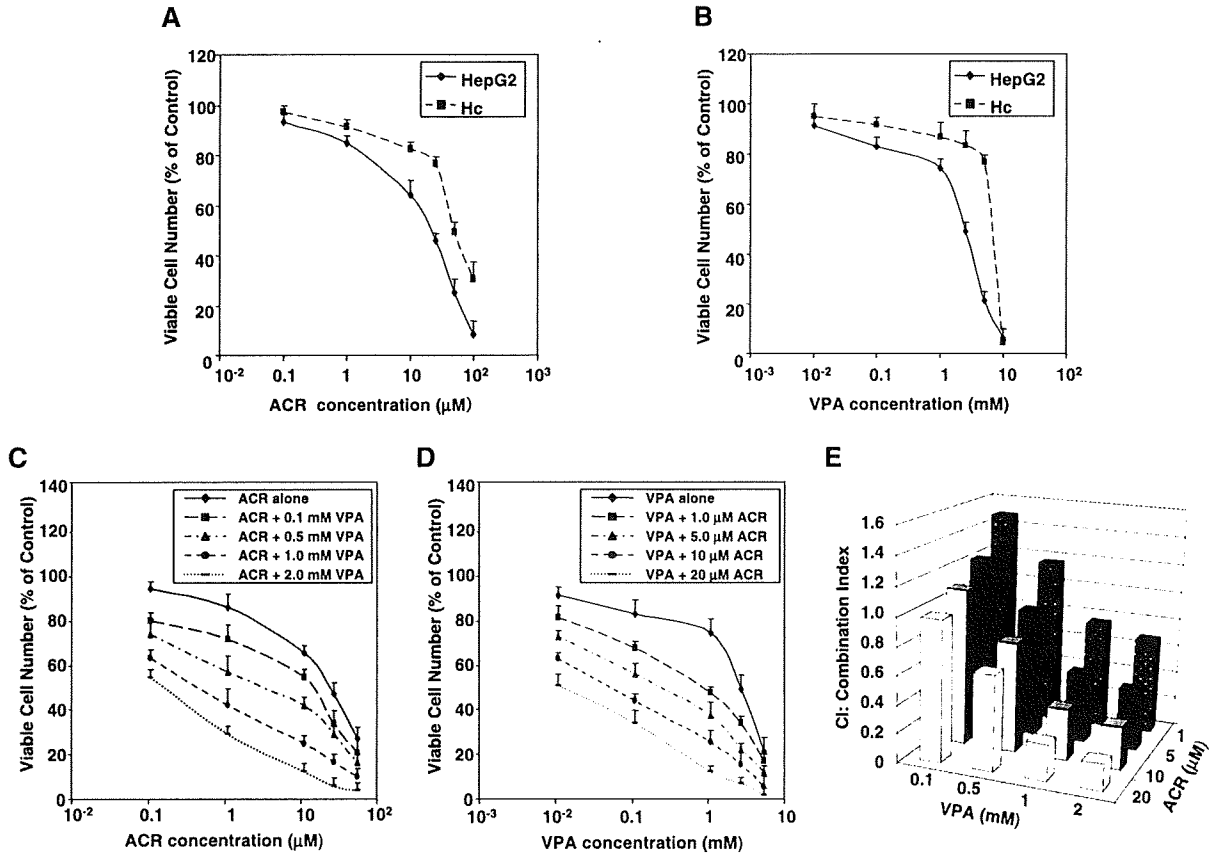

Fig. 1. Inhibition of cell growth by ACR and VPA. (A) and (B), HepG2 human HCC cells and Hc human normal hepatocytes were treated with the indicated concentrations of ACR or VPA for 48 h. (C) and (D), HepG2 cells were treated with the indicated concentrations of ACR alone, VPA alone, and various combinations 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 a percentage of the control value. *Error Bars*, SD of triplicate assays. (E), The data obtained in (C) and (D) were used to calculate the combination index, as described in the "Section 2".

for 24 h. The cell lysates were then prepared and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega), as previously described [16].

2.9. Statistical analysis

The data are expressed as the mean ± SD. Statistical significance of the difference in mean values was assessed with one-way ANOVA, followed by Sheffe's *t*-test.

3. Results

3.1. ACR and VPA causes preferential inhibition of growth in HepG2 human HCC cells in comparison with Hc normal hepatocytes

The initial experiments examined the growth-inhibitory effect of ACR and VPA on HepG2 and Hc cell lines. As shown in Fig. 1A and B, ACR and VPA inhibited the growth of HepG2 cells with an IC_{50} value of about 21.6 μM and 2.4 mM, respectively, and these values were smaller than those which were observed in Hc normal human hepatocytes. These results suggest that ACR and VPA preferentially inhibit the growth of HepG2 cells in comparison to normal human hepatocytes (Fig. 1A and B).

3.2. ACR plus VPA causes synergistic inhibition of growth in HepG2 cells

Next, the effect of combined treatment of ACR plus VPA on the growth of HCC cells was examined (Fig. 1C–E). When the HepG2 cells were treated with a range of concentrations of these agents, the combination of as little as 5 μM ACR and 1 mM VPA, about the IC_{25} value for both compounds, exerted synergistic growth inhibition because the CI-isobologram analysis [15,23] gave the CI index of 3+ to this combination (Fig. 1E and Table 1). These findings suggest that ACR plus VPA might be an effective combination for the inhibition of HCC cell growth due to their synergistic efficacy (Fig. 1E and Table 1).

3.3. ACR plus VPA causes a synergistic increase in the levels of RARβ and $p21^{CIP1}$ and an accumulation of acetylated histones H3 and H4 proteins in HepG2 cells

ACR inhibited the growth of HCC cells by inducing cellular levels of RARβ and $p21^{CIP1}$ [12,15]. $p21^{CIP1}$ is also a target of VPA to inhibit cell proliferation in HCC cells [19]. Therefore, the combined effects of ACR plus VPA in induction of the cellular levels of both RARβ and $p21^{CIP1}$ in HepG2 cells were examined. Western blot and RT-PCR analyses revealed that treatment with 5 μM ACR caused an increase in the cellular levels of RARβ and $p21^{CIP1}$ proteins and mRNAs in these cells (Fig. 2, Group 2). The levels of acetylated histone H3 protein was also increased by ACR in these cells (Fig. 2A, Group 2). In addition, there was a marked increase in the cellular levels of RARβ and $p21^{CIP1}$ and an accumulation in acetylated histones H3 and H4 proteins by the treatment with 1 mM VPA (Fig. 2, Group 3). Moreover, the expression of these molecules was significantly enhanced when the cells were treated with the combination of ACR plus VPA (Fig. 2, Group 4).

3.4. ACR plus VPA synergistically induces apoptosis and G_0–G_1 arrest in HepG2 cells

$p21^{CIP1}$ negatively modulates the cell cycle progression and thus is considered to be an important target for cancer therapeutic strategy [25]. RARβ is an important retinoid receptor with respect to the regulation of apoptosis [26]. Because ACR and VPA cooperatively increased

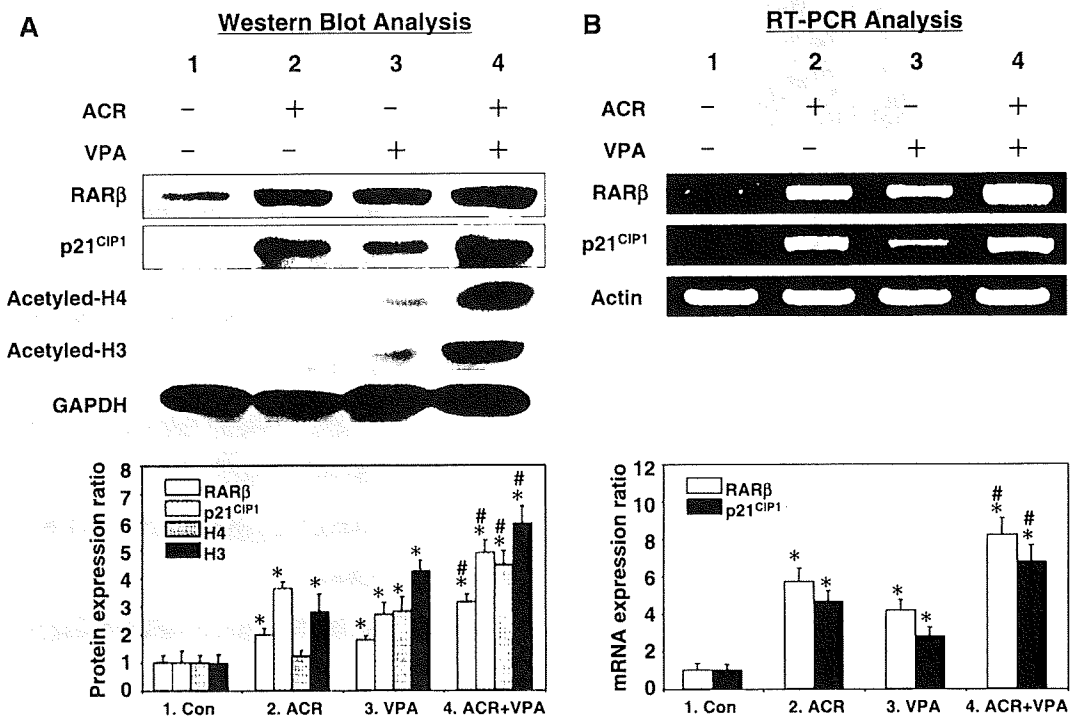

Fig. 2. Effects of the combination of ACR plus VPA on expression of RARβ and $p21^{CIP1}$ and on accumulation of acetylated histones H3 and H4 proteins in HepG2 cells. After the cells were treated with vehicle (Group 1), 5 μM ACR alone (Group 2), 1 mM VPA alone (Group 3), or the combination of 5 μM ACR plus 1 mM VPA (Group 4) for 24 h, the proteins or mRNAs were extracted from these samples. (A), The expression levels of RARβ, $p21^{CIP1}$, acetylated histones H3 and H4 proteins were examined by a Western blot analysis using the respective antibodies. Equal protein loading was verified by the detection of GAPDH. (B), The expression levels of RARβ and $p21^{CIP1}$ mRNAs were examined by semiquantitative RT-PCR analysis using the RARβ or $p21^{CIP1}$ specific primers. Actin primers were used as a control. The results obtained from Western blot analysis (A) and RT-PCR analysis (B) were quantitated by densitometry and are displayed in the lower panels. Columns and lines indicate mean and SD, respectively. *, $p < 0.01$, compared with vehicle-treated cells (Group 1); #, $p < 0.05$, compared with ACR (Group 2) and VPA (Group 3)-treated cells. Repeat Western blots and RT-PCR assays gave similar results.

the expression levels of both $p21^{CIP1}$ and RARβ (Fig. 2), the next series of experiments examined whether the synergistic growth inhibition in HepG2 cells by the combination (Fig. 1E and Table 1) might be associated with the induction of apoptosis and the specific changes in the cell cycle distribution. In TUNEL assays (Fig. 3A), the treatment of HepG2 cells with either 5 μM ACR (Group 2) or 1 mM VPA (Group 3) alone induced TUNEL-positive cells in approximately 10% of the total viable cells, respectively. Moreover, the combination of these agents markedly enhanced the induction of apoptosis in 27.5% of total viable cells (Group 4). Cell cycle analysis by DNA flow cytometry (Fig. 3B) revealed that after 48 h treatment with either ACR or VPA alone, the population of HepG2 cells in G_0–G_1 increased from 27% (Group 1) to 34% (Group 2) or 36% (Group 3), respectively. With the combined treatment, the population of cells in this phase markedly increased to 41% when compared to control untreated cells (Group 4). These results strongly suggest the synergism in inducing apoptosis and in the G_0–G_1 phase cell cycle arrest by the combined treatment of ACR plus VPA.

3.5. ACR plus VPA synergistically suppress the phosphorylation of RXRα, ERK, Akt and GSK-3β proteins in HepG2 cells

RXRα phosphorylation plays a critical role in the development of HCC and thus might be a promising target for HCC chemoprevention [2,7–9]. Therefore, the effect of combination of ACR plus VPA on the phosphorylation of RXRα and related signaling molecules was investigated in HepG2 cells. As shown in Fig. 3C, there was a significant decrease in the expression levels of p-RXRα and p-ERK proteins when the cells were treated with 5 μM ACR (Group 2). Treatment with 1 mM VPA also caused a marked decrease in the expression levels of p-Akt and p-GSK-3β proteins (Group 3). Moreover, the expression levels of p-RXRα, p-ERK, p-Akt and p-GSK-3β proteins were greatly decreased when the cells were treated with the combination of these agents (Group 4).

3.6. VPA enhances the induction of both RARE and RXRE promoter activities by ACR

RARs and RXRs modulate the expression of target genes by interacting with RARE or RXRE elements located in the promoter regions of these genes [4,5]. Therefore, whether VPA might enhance the transcriptional activity of the RARE or RXRE promoters induced by ACR was next examined using transient transfection luciferase reporter assays. As shown in Fig. 4, 5 μM ACR (Group 2) and 1 mM VPA (Group 3) significantly increased both RARE and RXRE reporter activities in comparison to control untreated cells, respectively. Moreover, when VPA was combined with ACR, there was a synergistic increase in the transcriptional activity of these reporter activities (Group 4).

4. Discussion

The present study clearly indicated that the combination of ACR plus VPA caused a synergistic inhibition of growth in HepG2 human HCC cells (Fig. 1E and Table 1) and that this was associated with the induction of apoptosis and arrest of the cell cycle in G_0–G_1 (Fig. 3A and B). The

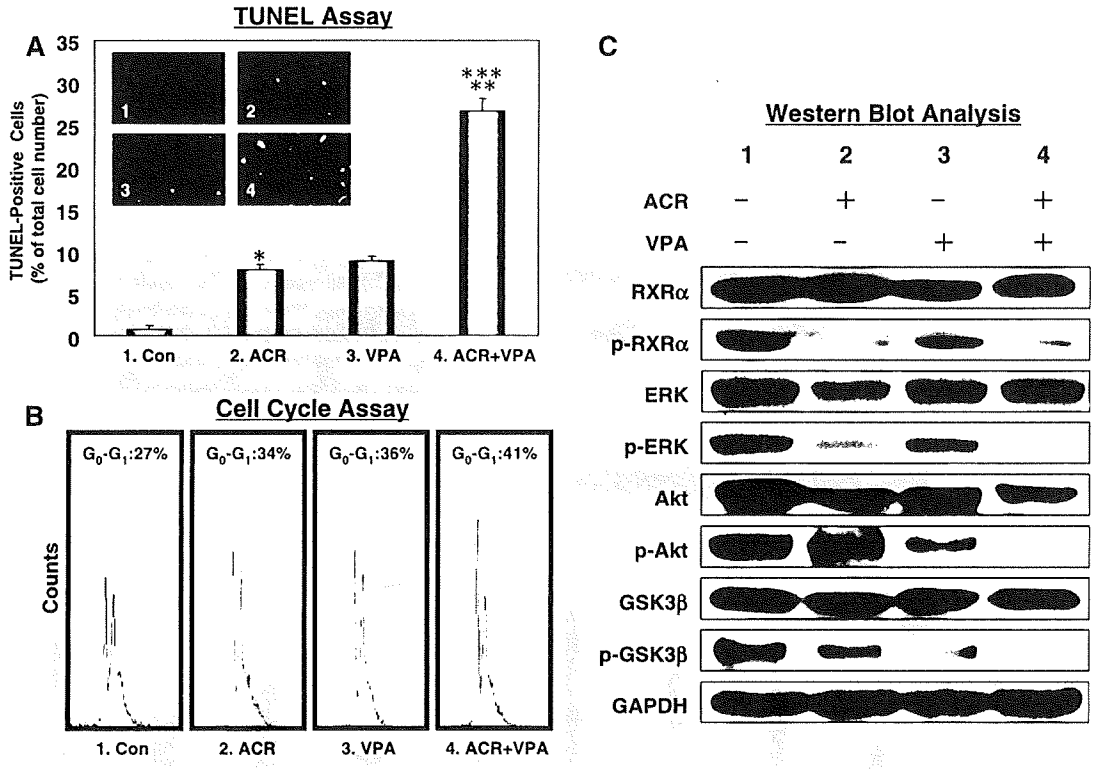

Fig. 3. Effects of the combination of ACR plus VPA on induction of apoptosis (A), progression of cell cycle (B) and phosphorylation of RXRα, ERK, Akt and GSK-3β proteins (C) in HepG2 cells. (A) and (B), HepG2 cells were treated with vehicle (Group 1), 5 μM ACR alone (Group 2), 1 mM VPA alone (Group 3), or the combination of 5 μM ACR plus 1 mM VPA (Group 4) for 48 h. The cells were then stained using the TUNEL method to evaluate induction of apoptosis (A) or were stained with propidium iodide to analyze progression of cell cycle (B). (A), TUNEL-positive cells were counted and expressed as the percentage of the total cell number (500 cells were counted in each flask). (B), The distribution of cells in the G_0–G_1 phase of cell cycle was calculated in each group. Columns and lines indicate mean and SD, respectively. *$p < 0.05$, compared with vehicle-treated cells (Group 1); **, $p < 0.01$, compared with vehicle-treated cells (Group 1); ***, $p < 0.01$, compared with ACR (Group 2) and VPA (Group 3)-treated cells. Representative results from three independent experiments with similar results. (C), HepG2 cells were treated with vehicle (Group 1), 5 μM ACR alone (Group 2), 1 mM VPA alone (Group 3), or the combination of 5 μM ACR plus 1 mM VPA (Group 4) for 24 h and the cell lysates were then prepared. The cell extracts were examined by a Western blot analysis using respective antibodies. Repeat Western blots gave similar results.

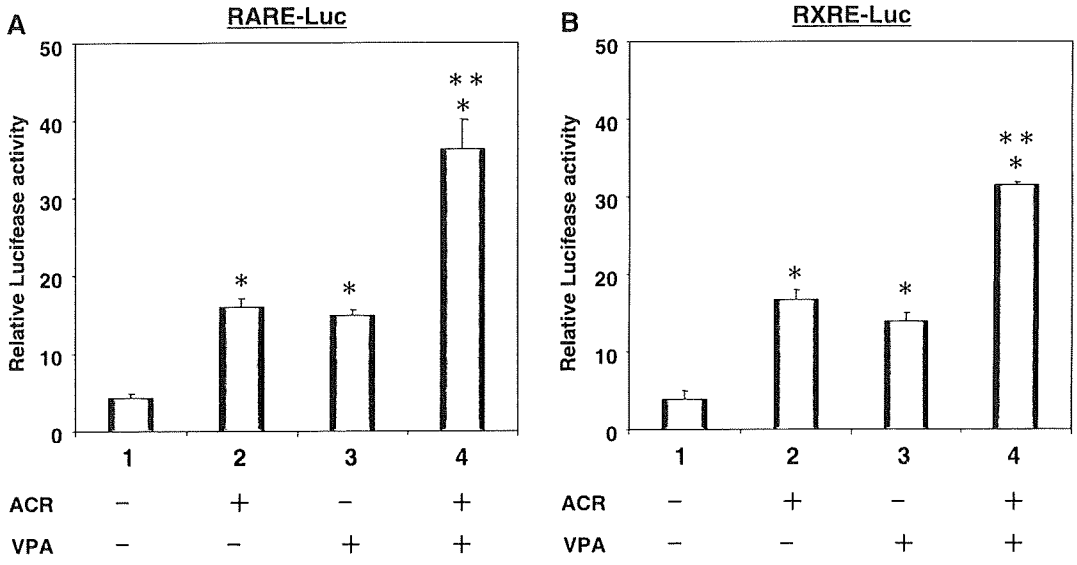

Fig. 4. Effects of the combination of ACR plus VPA on transcriptional activity of RARE (A) and RXRE (B) promoters in HepG2 cells. Transient transfection reporter assays were performed with the indicated luciferase reporter in the presence of vehicle (Group 1), 5 μM ACR alone (Group 2), 1 mM VPA alone (Group 3), or the combination of 5 μM ACR plus 1 mM VPA (Group 4). Relative luciferase activity was determined after 24 h. Columns and lines indicate mean and SD, respectively. *, $p < 0.05$, compared with vehicle-treated cells (Group 1); **, $p < 0.01$, compared with ACR (Group 2) and VPA (Group 3)-treated cells. Representative results from three independent experiments with similar results.

combination of these agents also acts synergistically to induce the cellular levels of $p21^{CIP1}$ and RARβ (Fig. 2). We have previously reported that ACR alone can induce apoptosis and inhibit the growth of human HCC and squamous cell carcinoma cells by arresting the cell cycle in G_0–G_1 and increasing the cellular levels of the RARβ and $p21^{CIP1}$ [12,15,24]. Therefore, VPA might have enhanced the expression of RARβ and $p21^{CIP1}$ caused by ACR in the present study.

A hypothetical scheme which explains the synergism generated by the combination of ACR plus VPA is proposed in Fig. 5. It is reported that the promoter region of both the *$p21^{CIP1}$* and *RARβ* genes contain RARE [27,28], which explains why ACR alone causes an increase in the cellular levels of these molecules (Fig. 2). HDAC inhibitors, including VPA, also strongly activate the expression of the $p21^{CIP1}$ [19,29]. There is an interesting report that introduction of *$p21^{CIP1}$* gene into cells transcriptionally activates the upstream promoter region of the *RARβ* gene [30]. The transcriptional activation by p300 and CREB binding protein (CBP), both of which are histone acetyltransferases and thus controlling the transcription of RARE target genes [3], is stimulated by co-expression of $p21^{CIP1}$ [31]. In addition, treatment of RARβ-overexpressed squamous carcinoma cells with retinoid induced $p21^{CIP1}$, p300/CBP and histone H4 acetylation, while down-regulating the expression of HDAC1 [32]. These reports might explain how VPA alone also increased the cellular levels of both the $p21^{CIP1}$ and RARβ (Fig. 2) and this substantial induction of $p21^{CIP1}$ and RARβ could therefore up-regulate the expression of these molecules in the present study (Fig. 5). The levels of both RARβ and $p21^{CIP1}$ increased by the combination of ACR plus VPA (Fig. 2) therefore induce a synergistic growth inhibition in HepG2 cells (Fig. 1E and Table 1) by activating the RARE promoter activity (Fig. 4A).

Moreover, recent studies revealed that ACR is not only the ligand for retinoid receptors. Indeed, RXRα phosphorylation is highly associated with the development of HCC and ACR dephosphorylates and restores the function of this receptor by inactivating the Ras/MAPK/ERK signaling pathway [2,7–9,11,14]. Therefore, the inhibition of the activation of ERK and phosphorylation of RXRα proteins by ACR (Fig. 3C) is consistent with these previous reports that ACR can inhibit Raf-1-bound Ras activity and phosphorylation of ERK, thereby inhibiting the growth of HCC cells [7,11,14]. In addition, the combinations of ACR plus specific agents, which target RXRα phosphorylation, synergistically inhibited the growth of HCC cells [14,16]. These findings suggest that p-RXRα and its upstream kinases might be useful targets for inhibiting the growth of HCC cells when considering the combination with ACR. In the present study VPA alone inhibited the phosphorylation of Akt and the combination of ACR plus VPA synergistically decreased the expression of p-RXRα protein in HepG2 cells (Fig. 3C). These findings seem to be interesting because unpublished results show that, in addition to the Ras/MAPK/ERK, the PI3 K/Akt signaling pathway also phosphorylates RXRα in HCC cells (Shimizu M. and Sakai H., unpublished data). Therefore, the inhibition of Akt phosphorylation and restoration of the function of RXRα as a master regulator of nuclear receptors by VPA (Fig. 3C) might induce a synergistic effect in the combination with ACR (Fig. 5).

The PI3 K/Akt pathway plays a critical role in liver carcinogenesis and HDAC inhibitors have the ability to inhibit the activation of this signaling pathway [33–35]. The phosphorylation (i.e. inactivation) of serine/threonine protein kinase GSK-3β, which is mediated by PI3 K/Akt, also plays a critical role in cell growth and resistance to apoptosis in HCC cells, and it is thus considered to be one of the

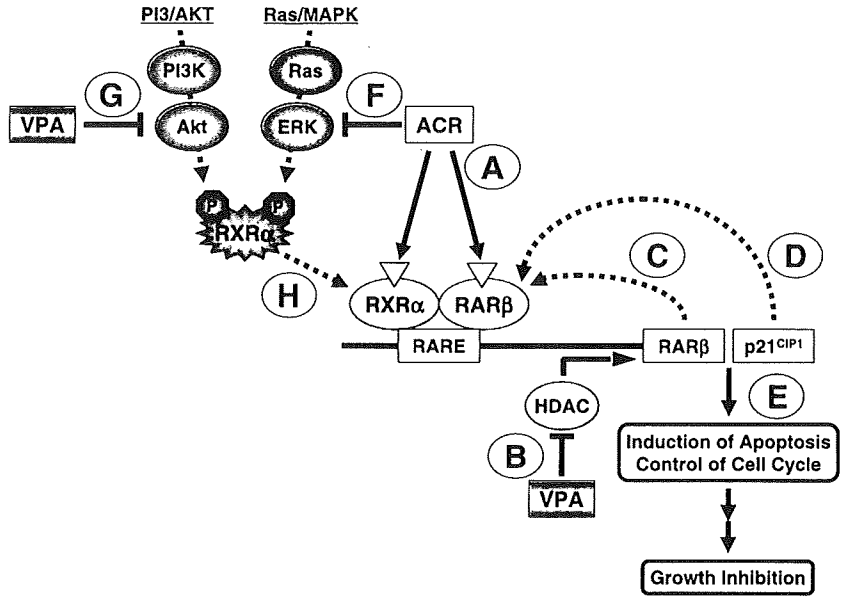

Fig. 5. A hypothetical schematic representation of the effect of the combination of ACR plus VPA on growth inhibition in HCC cells. ACR can bind to both RAR and RXR as a ligand, thus resulting in activation of the RARE promoter activity (A). Because the promoter region of both the *p21*^{CIP1} and *RARβ* genes contain RARE, ACR can cause an increase in cellular levels of these molecules. VPA also increases the expression of p21^{CIP1} and RARβ by remodeling the chromatin template and increasing the histone acetylation by inhibiting the HDAC activity (B). Therefore, the combination of ACR plus VPA cooperatively activates the expression of p21^{CIP1} and RARβ. Thus induced RARβ (C) and its activation by the ligand ACR could further up-regulate the promoter activity of both the p21^{CIP1} and *RARβ* genes. In addition, the up-regulation of p21^{CIP1} itself could also activate the promoter region of the *RARβ* gene (D), thus synergistically inducing apoptosis, controlling the cell cycle and enhancing growth inhibition in HepG2 cells (E). In parallel, ACR inactivates the Ras/MAPK/ERK (F) and VPA inhibits the PI3 K/Akt signaling pathways (G), respectively. Because these signaling pathways phosphorylate RXRα and thus impairing the function of this receptor, cooperative inhibition of RXRα phosphorylation by ACR and VPA might restore the function of this receptor (H) and subsequently activate the RARE promoter activity. These effects also contribute to the growth inhibition of HCC cells. For additional details see Section 4.

promising targets to inhibit HCC cell proliferation [36,37]. Therefore, in addition to the restoration of the function of RXRα, the inhibition of the GSK-3β phosphorylation and thus reactivating this protein by inhibiting the Akt activation (Fig. 3C) also seems to be significant with respect to the inhibitory effects of the combination of ACR plus VPA on HCC cell proliferation.

Finally, it should be noted that a number of clinical trials using retinoids plus VPA are conducted for the treatment of myeloid malignancies and, importantly, a hematologic improvement was observed in these trials [38]. It is also significant that treatment with retinoids plus VPA is well tolerated in these clinical studies [38]. A clinical trial demonstrated that the administration of ACR reduced the incidence of post-therapeutic recurrence of HCC without causing any untoward effects (1). In this trial the plasma concentration of ACR was approximately the same as the dosage used in the present study (1). The dosage of VPA in the present study is also confirmed to be an effective and safe blood concentration [39]. In addition, the observation that the combination of appropriate dosages of ACR and VPA can inhibit the growth of human HCC cells without affecting the growth of normal hepatocytes (Fig. 1A and B) should encourage further clinical studies using these materials to investigate HCC chemoprevention and chemotherapy. In conclusion, the results of our present study suggest that combining ACR with VPA might hold promise as a clinical modality for the prevention and treatment of HCC, due to their synergistic effects.

Conflicts of interest

None declared.

Acknowledgement

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(–)-Epigallocatechin gallate suppresses the growth of human hepatocellular carcinoma cells by inhibiting activation of the vascular endothelial growth factor–vascular endothelial growth factor receptor axis

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The receptor tyrosine kinase vascular endothelial growth factor (VEGF) receptor (VEGFR) plays an important role in tumor angiogenesis of hepatocellular carcinoma (HCC). (–)-Epigallocatechin gallate (EGCG), the major biologically active component of green tea, inhibits growth in a variety of human cancer cells by inhibiting the activation of several types of receptor tyrosine kinases. In this study, we examined the effects of EGCG on the activity of the VEGF–VEGFR axis in human HCC cells. The levels of total and phosphorylated (i.e. activated) form of VEGFR-2 protein (p-VEGFR-2) were observed to increase in a series of human HCC cell lines in comparison to the Hc normal human hepatocytes. EGCG preferentially inhibited the growth of HuH7 HCC cells, which express constitutive activation of the VEGF–VEGFR axis, in comparison to Hc cells. Treatment of HuH7 cells with EGCG caused a time- and dose-dependent decrease in the expression of VEGFR-2 and p-VEGFR-2 proteins. The production of VEGF from HuH7 cells was reduced by treatment with EGCG. Drinking of EGCG significantly inhibited the growth of HuH7 xenografts in nude mice and this was associated with inhibition of the activation of VEGFR-2 and its related downstream signaling molecules, including ERK and Akt. EGCG drinking also decreased the expression of Bcl-x_L protein and VEGF mRNA in the xenografts. These findings suggest that EGCG can exert, at least in part, its growth-inhibitive effect on HCC cells by inhibiting the VEGF–VEGFR axis. EGCG might therefore be useful in the treatment of HCC. (*Cancer Sci* 2009; 100: 1957–1962)

HCC, which commonly arises in the liver with chronic inflammation and cirrhosis, is a major health care problem worldwide.[1] Because the prognosis of patients with HCC is poor, there is a critical need to develop more effective strategies for the therapy and prevention of this malignancy. Recent studies have revealed that the aberrant activation of several RTK and related downstream pathways of signal transduction play a critical role in the development of HCC and thus might be promising targets for the treatment of this cancer.[2–4] For instance, sorafenib, a multikinase inhibitor that targets the serine-threonine kinases Raf-1 and B-Raf and the RTK activity of VEGFR-1, VEGFR-2, and VEGFR-3 and platelet-derived growth factor receptor-β, has shown a survival benefit in patients with HCC.[5] In preclinical experiments, sorafenib exerted antiproliferative effects on the HCC-derived cell lines and reduced tumor growth by inhibiting angiogenesis in a mouse xenograft model of human HCC.[6]

It is widely accepted that neovascularization and angiogenesis play a key role in the growth of solid tumor.[7,8] VEGF, which binds to and activates VEGFR, is important in pathological angiogenesis, and the VEGF–VEGFR axis is therefore closely associated with tumor growth.[7,8] In particular, HCC is a well-known hypervascular tumor and a close relationship has been demonstrated between VEGF expression and either angiogenic activity or tumor progression in HCC.[9,10] Overexpression of VEGFR is also observed in human HCC and this has been shown to correlate with a poor prognosis.[11,12] In addition, several HCC cell lines express VEGFR and VEGF may act as an autocrine growth factor in stimulating the proliferation of these cells.[13] These findings suggest that inhibition of the VEGF–VEGFR axis can theoretically reduce angiogenesis and tumor growth in HCC, and several agents that target this axis have been developed for the treatment of HCC.[2–5] In a recent phase II study, bevacizumab, a humanized anti-VEGF monoclonal antibody, alone or in combination with cytotoxic agents was used as treatment for patients with HCC and they showed a moderate antitumor activity.[14,15] In HCC cells, RTK787, a tyrosine kinase inhibitor of VEGFR, inhibited tumor cell proliferation and induced apoptosis both *in vivo* and *in vitro*.[13]

Numerous epidemiological and experimental studies suggest that green tea catechins have both anticancer and cancer chemopreventive effects at various organ sites.[16–18] One of the anticancer mechanisms of green tea or its constituents is explained by their inhibitory effect on angiogenensis. Namely, EGCG, the major biologically active component of green tea, induces potent inhibition of VEGF-dependent tyrosine phosphorylation of VEGFR-2 and this is associated with the suppression of *in vitro* angiogenesis.[19] The production of VEGF by cancer cells decreases after treatment with EGCG, thus contributing to its potent antiangiogenic activity.[20,21] Green tea extract and EGCG also caused a decrease in VEGF production by HCC cells;[22] however, whether EGCG can inhibit activation of the VEGF–VEGFR axis, thus inducing growth inhibition of HCC tumor, has not yet been examined. In the present study we investigated in detail the effects of EGCG on activation of the VEGF–VEGFR axis and the growth of HCC cells using *in vitro* and *in vivo* models.

Materials and Methods

Chemicals. EGCG was obtained from Mitsui Norin Co. (Tokyo, Japan).

Cell lines and cell culture conditions. Six human HCC cell lines, HLF, PLC/PRF/5, HepG2, HuH7, HLE, and Hep3B, were obtained from the Japanese Cancer Research Resources Bank

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(Tokyo, Japan) and maintained in DF10 medium containing DMEM (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen). The Hc human normal hepatocyte cell line was purchased from Applied Cell Biology Research Institute (Kirkland, WA, USA) and maintained in CS-C complete medium (Cell Systems Biotechnologie Vertrieb, St Katharinen, Germany). The cells were cultured in an incubator with humidified air with 5% CO_2 at 37°C.

Cell viability assays. Cell viability assays were conducted using the MTT cell proliferation kit I (Roche Diagnosis Co., Indianapolis, IN, USA), according to the manufacturer's instructions, as described previously.[23] Three thousand HuH7 or Hc cells were seeded into 96-well plates. Twenty-four hours later, the cells were treated with the indicated concentrations of EGCG (0–100 μg/mL) for 48 h in DF10 medium, and cell viability was examined. All assays were carried out in triplicate.

VEGF production assays. HuH7 cells were plated into six-well plates and grown to 70% confluence. After washing with PBS, the cells were treated with the indicated concentrations of EGCG (0–100 μg/mL) in serum-minus medium for 24 h. The cell-free medium was then collected and the amounts of VEGF secreted by the cells into the medium were measured using a VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Protein extraction and western blot analysis. Total protein was extracted from the cell lines or xenografts of HuH7 cells and equivalent amounts of protein (20 μg/lane) were subjected to a western blot analysis, as described previously.[24,25] The primary antibodies for ERK, p-ERK, Akt, and p-Akt were described previously.[23] The primary antibodies for VEGFR-2 (#2479), p-VEGFR-2 (#2478), Bcl-x_L (#2762), and GAPDH (#2118) were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody to GAPDH served as a loading control.

RNA extraction and semiquantitative RT-PCR analysis. A semiquantitative RT-PCR analysis was carried out, as described previously.[26] Total RNA was isolated from the xenopgrafts of HuH7 cells using ISOGEN reagent (Nippon Gene Co., Tokyo, Japan), according to the manufacturer's instructions. The cDNA was amplified from 1 μg of total RNA using SuperScript one-step RT-PCR with the platinum *Taq* system (Invitrogen). The primers used for amplification of *VEGF* and *GAPDH* specific genes were as follows: *VEGF* forward, 5′-CTA CCT CCA CCA TGC CAA GT-3′; *VEGF* reverse, 5′-AAA TGC TTT CTC CGC TCT GA-3′; *GAPDH* forward, 5′-CGA GAT CCC TCC AAA ATC AA-3′; and *GAPDH* reverse, 5′-TTC AGC TCA GGG ATG ACC TT-3′. Using a thermal controller (Programmable Thermal Controller; MJ Research, Watertown, MA, USA), 35-cycle rounds of PCR were chosen for data analysis of the mRNA expression as a semiquantitative assessment indicated that the reaction had not reached a plateau and was still in log phase. The amplified products obtained with *GAPDH*-specific primers served as an internal control. The intensities of the PCR products stained with ethidium bromide were quantified using the NIH Image software program version 1.62 (URL: http://rsb.info.nih.gov/nih-image/index.html).

***In vivo* experimental protocol.** Twenty-four male BALB/c nude mice (5 weeks of age) were obtained from Charles River Japan (Tokyo, 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 water (tap water supplemented with or without EGCG) and the pelleted basal diet CRF-1 (Oriental Yeast Co., Tokyo, Japan). Xenograft tumors were made by subcutaneous injection of HuH7 cells, at a concentration of 5×10^6 cells per 200 μL, into the flanks of these mice. One week after tumor cell injection, the mice were randomly divided into three groups (eight mice per group) and then treated with (groups 2 and 3) or without (group 1) EGCG for 5 weeks. The mice in groups 2 and 3 were given tap water containing 0.01 or 0.1% EGCG, respectively.

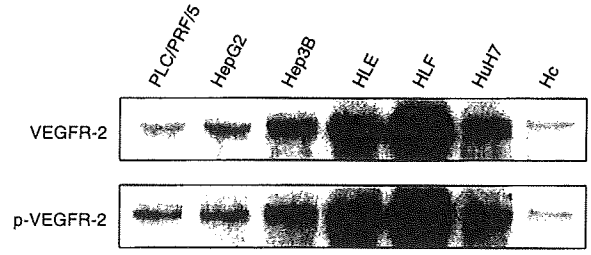

Fig. 1. The expression levels of total vascular endothelial growth factor receptor (VEGFR)-2 and phosphorylated vascular endothelial growth factor receptor (p-VEGFR)-2 proteins in human hepatocellular carcinoma cell lines and Hc normal hepatocytes. Total protein extracts were prepared from 70% confluent cultures of the indicated cell lines and equivalent amounts of protein (20 μg/lane) were examined by western blot analysis using appropriate antibodies. Repeat western blots gave similar results.

The concentrations of EGCG (0.01 and 0.1%) were established according to the findings of previous reports[27,28] because these doses could exert anticancer properties without causing various side effects in any organs. The mice in group 1 were given tap water and served as an untreated control. A freshly prepared solution of EGCG in tap water was supplied to the experimental mice three times a week. The tumor size and bodyweight were measured once a week and the tumor volume was calculated using the formula: largest diameter × (smallest diameter)^2 × 0.5.

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

Results

Expression of VEGFR-2 and p-VEGFR-2 proteins in human HCC cell lines and Hc normal hepatocytes. Among the VEGFR, VEGFR-2 is considered to be the major mediator of the mitogenic and angiogenic effects of VEGF.[7,8] We therefore initially examined whether VEGFR-2 protein is overexpressed and constitutively activated in HLF, PLC/PRF/5, HepG2, HuH7, HLE, and Hep3B human HCC cell lines and in Hc human normal hepatocytes using western blot analysis. Among these HCC cell lines, the level of VEGFR-2 protein was observed to markedly increase in the HLF and HLE cells, whereas it was moderately expressed in Hep3B and HuH7 cells (Fig. 1). The level of phosphorylated (i.e. activated) VEGFR-2 protein (p-VEGFR-2) also increased in these four cell lines, thus indicating the constitutive activation of this receptor (Fig. 1). Moreover, all HCC cell lines that were examined in this experiment significantly expressed VEGFR-2 and p-VEGFR-2 proteins in comparison to the Hc normal human hepatocytes (Fig. 1).

Effects of EGCG on the growth of HuH7 and Hc cells. We then examined the growth-inhibitory effects of EGCG on HuH7 and Hc cell lines using MTT assays. As shown in Figure 2, EGCG inhibited the growth of HuH7 cells with an IC_{50} value of approximately 25 μg/mL. However, the Hc cells were more resistant to EGCG because the IC_{50} value with this agent was approximately 84 μg/mL (Fig. 2). These findings suggest that EGCG preferentially inhibits the growth of HuH7 HCC cells, which express higher levels of the VEGFR-2 and p-VEGFR-2 proteins, when compared with Hc cells that do not express these proteins (Fig. 1).

Effects of EGCG on the expression and activation of VEGFR-2 in HuH7 cells. We next examined whether EGCG alters the expression and activation of VEGFR-2 in HuH7 cells. A time-course study indicated that when the cells were treated with 25 μg/mL EGCG, which is the same as the IC_{50} concentration determined by the

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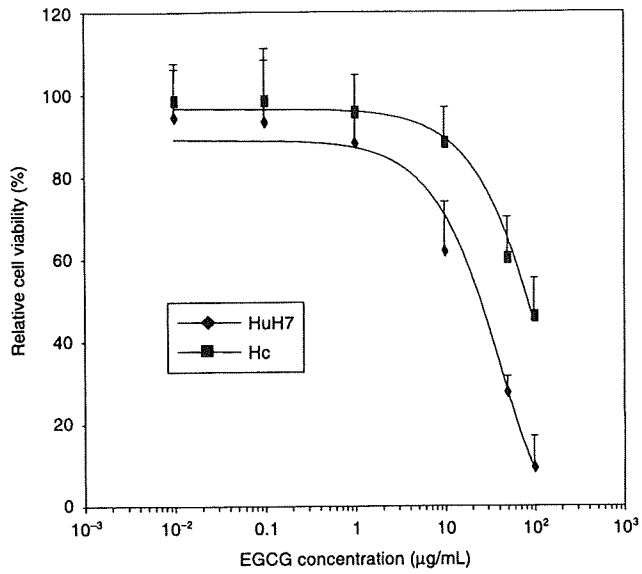

Fig. 2. Inhibition of cell growth by (–)-epigallocatechin gallate (EGCG) in HuH7 human hepatocellular carcinoma cells and Hc normal hepatocytes. These cells were treated with the indicated concentrations of EGCG or DMSO for 48 h and cell viability assays were conducted using the MTT system. Results are expressed as a percentage of growth with 100% representing control cells treated with DMSO alone. Bars, SD of triplicate assays.

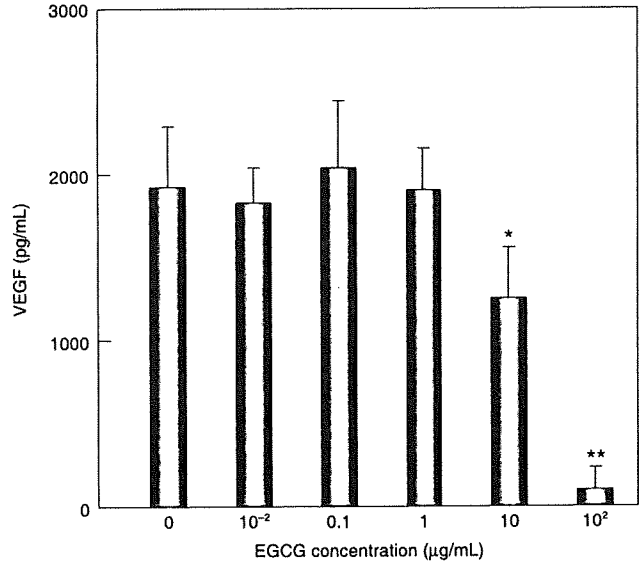

Fig. 4. Effects of (–)-epigallocatechin gallate (EGCG) on production of vascular endothelial growth factor (VEGF) by HuH7 cells. The cells were treated with the indicated concentration of EGCG (0, 0.01, 0.1, 1.0, 10, and 100 μg/mL) in serum-free medium for 24 h. The medium was then collected and assayed for VEGF using an ELISA kit. Bars, SD of triplicate assays. $*P < 0.05$, $**P < 0.01$: significant differences obtained by comparison with EGCG-untreated control group.

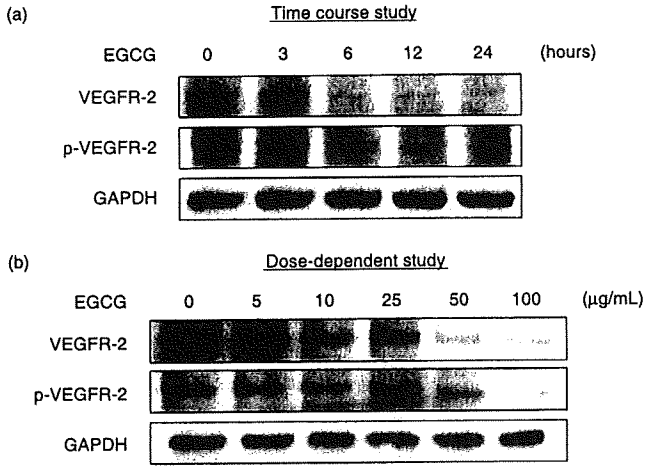

Fig. 3. Effects of (–)-epigallocatechin gallate (EGCG) on expression levels of total vascular endothelial growth factor receptor (VEGFR)-2 and phosphorylated vascular endothelial growth factor receptor (p-VEGFR)-2 proteins in HuH7. The cells were treated with (a) 25 μg/mL EGCG for the indicated times (0, 3, 6, 12, and 24 h, time course study) or (b) the indicated concentration of EGCG (0, 5, 10, 25, 50, and 100 μg/mL, dose-dependence study) for 6 h, and the cell extracts were then examined by western blot analysis using the respective antibodies. An antibody to GAPDH served as a loading control. Similar results were obtained in a repeat experiment.

MTT assays (Fig. 2), a marked decrease was observed in the expression levels of both VEGFR-2 and p-VEGFR-2 proteins within 6 h of the addition of this agent (Fig. 3a). When the cells were treated with the indicated concentrations of EGCG (0–100 μg/mL) for 6 h, the expression levels of VEGFR-2 as well as p-VEGFR-2 proteins were also inhibited in a dose-dependent manner (Fig. 3b).

Effects of EGCG on VEGF production by HuH7 cells. VEGF, which is produced by cancer cells, has been reported to play a critical role in tumor angiogenesis.[7,8] We therefore next examined the effects of EGCG on production of VEGF by HuH7 cells using an ELISA system. As shown in Figure 4, HuH7 cells secreted an abundant amount of VEGF into the growth medium when the cells were cultured in serum-free medium for 24 h and, interestingly, a low (10 μg/mL, less than IC_{50} value) and high (100 μg/mL) concentration of EGCG significantly reduced the production of VEGF from these cancer cells.

Effects of EGCG on the growth of HCC xenografts in nude mice. We next examined whether the growth inhibition of the treatment with EGCG in HuH7 cells was also observed *in vivo* using a nude mouse xenograft model. Figure 5 shows that drinking water with not only a high concentration (0.1%), but also a low concentration (0.01%) of EGCG strongly inhibited the growth of the HuH7 xenograft during treatment with this agent (5 weeks). The tumor volume of the mice treated with 0.1% EGCG was less than that of tumors in 0.01% EGCG-drinking mice, but the difference was not significant (Fig. 5). All of the treatments were well tolerated and the bodyweights remained stable in all groups during the experiment (data not shown). There were no pathological alterations suggesting toxicity of EGCG in the liver, spleen, and kidneys of mice (data not shown).

Effects of EGCG on the activation of VEGFR-2 and its downstream signaling molecules and on the cellular levels of Bcl-x_L in xenografts of HuH7 cells. We next examined whether treatment with EGCG inhibits the activation of VEGFR-2 and its multiple downstream signaling pathways in the HuH7 xenografts. Drinking both 0.01 and 0.1% EGCG decreased the total levels of VEGFR-2 and Akt proteins in these xenografts (Fig. 6a). There was also a marked decrease in the levels of p-VEGFR-2, p-ERK, and p-Akt proteins by treatment with both concentrations of EGCG (Fig. 6a). In addition, EGCG caused a decrease in the levels of Bcl-x_L, an anti-apoptotic Bcl-2 family member, in HuH7 xenogrfts (Fig. 6a).

Effects of EGCG on the expression of VEGF mRNA in xenografts of HuH7 cells. A semiquantitative RT-PCR study showed that there was a significant decrease in the level of VEGF mRNA in the

xenografts of mice treated with 0.1% EGCG compared to that of the tumors in control mice (Fig. 6b). Therefore, the inhibitory effect of EGCG on the production of VEGF was not only observed *in vitro* (Fig. 4) but also *in vivo*.

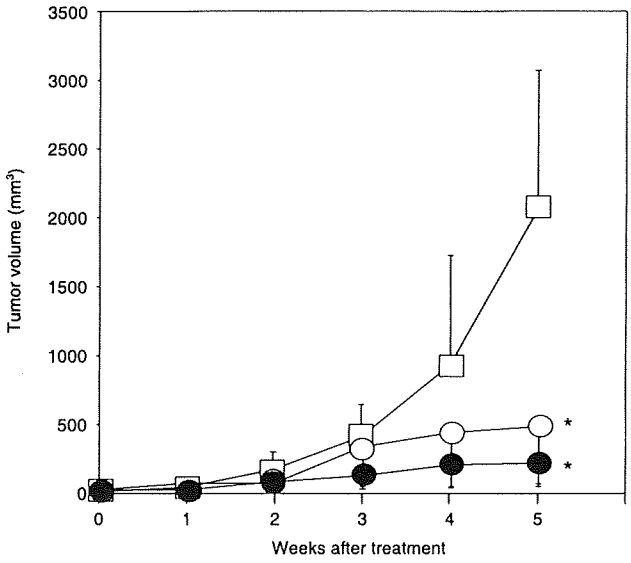

Fig. 5. Effects of (–)-epigallocatechin gallate (EGCG) on the growth of HuH7 xenografts in nude mice. Male BALB/c nude mice were injected subcutaneously with 5×10^6 HuH7 cells. One week after the injection, the mice were divided into three groups and treated with following conditions for 5 weeks: group 1, control group (tap water drinking group, □); group 2, 0.01% EGCG-drinking group (○); and group 3, 0.1% EGCG-drinking group (●). The growth curve of HuH7 tumors in each group are represented. Bars, SD. *$P < 0.05$: significant differences obtained by comparison with EGCG-untreated control group.

Discussion

The results of the present study clearly indicate that EGCG effectively suppresses the growth of HuH7 human HCC cells both *in vitro* (Fig. 2) and *in vivo* (Fig. 5) and this was associated with inhibition of the VEGF–VEGFR axis (Figs 3,6a). It should be particularly emphasized that not only a high concentration (0.1%), but also a low concentration (0.01%) of EGCG similarly inhibited the growth of HCC xenografts by blocking the VEGF–VEGFR axis to the same extent (Figs 5,6a). This is the first report indicating a low dose of EGCG (0.01%) to be sufficient to reduce HCC tumor growth, although the feeding protocol of EGCG at a high dose (0.1%), which mimics an approximate consumption of six cups of green tea per day by an average adult human, has been used in mice in many prior chemopreventive studies.[16,29] These findings, together with the result of a previous study reporting that drinking 0.05% EGCG also significantly repressed the tumor growth of highly angiogenic sarcoma xenografts by inhibiting angiogenesis *in vivo*,[30] might thus be preferable when considering the clinical use of this agent because a lower dose is more acceptable for administration to patients.

One of the main mechanisms of how EGCG can block the VEGF–VEGFR axis in cancer cells is explained by its efficacy in reducing VEGF secretion from these cells.[20–22] The expression of VEGF is regulated by micro-environmental alterations, such as hypoxia, and recent studies have revealed that HIF-1 strongly activates transcription of the *VEGF* gene by phosphorylating the ERK and Akt proteins.[7,31–33] The increased expression of VEGFR and HIF-1α, the regulated subunit of HIF-1, is considered to play a role in the progression of HCC.[34] Hepatitis C virus infection leads to the stabilization of HIF-1α via activation of the MAPK–ERK and PI3K–Akt signaling pathways, thus leading to neovascularization.[35] In the present study we demonstrated that EGCG reduces the expression of VEGF mRNA and production of this growth factor by inhibiting the activation of ERK and Akt proteins in HuH7 cells (Figs 4,6b). These findings are

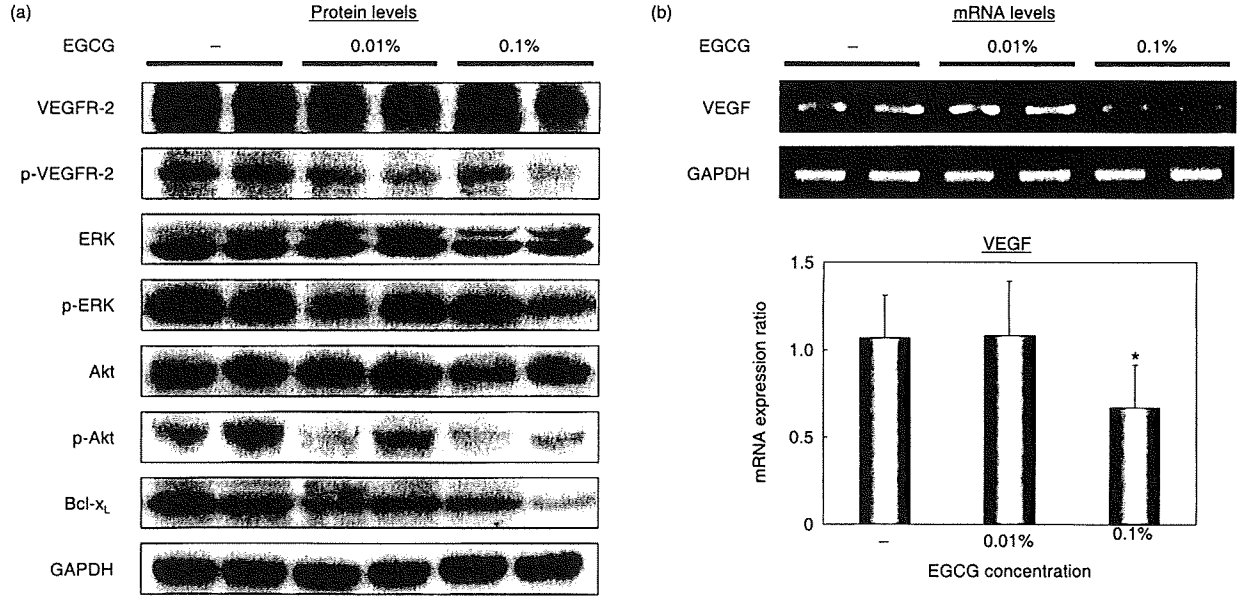

Fig. 6. Effects of (–)-epigallocatechin gallate (EGCG) on activation of vascular endothelial growth factor receptor (VEGFR)-2, its related downstream signaling pathways, and on the cellular levels of Bcl-xL proteins and vascular endothelial growth factor (VEGF) mRNA in HuH7 xenografts. The xenografts were excised from each animal at the termination of the experiment and tumor extracts were examined by (a) western blot analysis using the respective antibodies or (b) a semiquantitative RT-PCR analysis using *VEGF*-specific primers. An antibody to GAPDH served as a loading control (A). Amplified PCR products obtained with *GAPDH*-specific primers served as internal controls. (b) The results obtained from RT-PCR analysis were quantified by densitometry and are displayed in the lower panel. Bars, SD of triplicate assays. *$P < 0.05$: significant differences obtained by comparison with EGCG-untreated control group. p-, phosphorylated.

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consistent with those of a previous report, in which green tea extract and EGCG were observed to cause a drastic decrease in VEGF expression at both the mRNA and protein levels by suppressing the expression of HIF-1α and blocking both the PI3K–Akt and MAPK–ERK signaling pathways in HepG2 human HCC cells.[22] Because several HCC cell lines express the constitutive activation of VEGFR-2 (Fig. 1), our findings, together with those of the previous report,[22] suggest the possibility that EGCG might be able to inhibit cell growth by disrupting the VEGF–VEGFR-related autocrine loop that exists in HCC cells.

The transcription of VEGF mRNA is also induced by the activation of a variety of RTK.[7] For instance, activation of the IGF-1–IGF-1 receptor axis induces expression of the *VEGF* gene via induction of HIF-1α.[31] Both the activation of EGFR and HER2 induce the secretion of VEGF by activating the PI3K–Akt signaling pathway.[32,33] These reports seem to be significant when considering the characteristic effects of EGCG because this agent can inhibit not only VEGFR-2 (Figs 3,6a), but also the activation of several other RTK, including IGF-1R, EGFR, HER2, and HER3, and multiple downstream signaling pathways in human HCC and colon cancer cells.[23–26] One of the possible mechanisms for this remarkable range of effects by EGCG might be associated with its ability to bind directly to all of these receptors, thereby inhibiting their tyrosine kinase activities, perhaps because of sufficient homologies in their kinase domain.[36] This presumption might be supported by the previous report that EGCG can act directly to inhibit the kinase activity of some RTK.[37,38] The recent report describing that EGCG inhibits the binding of VEGF to VEGFR in a concentration-dependent manner[39] also encourages this hypothesis. In addition, the effects of EGCG to decrease the total levels of VEGFR-2 itself contribute to inhibit activation of the VEGF–VEGFR axis in the present study (Figs 3,6a). These findings are consistent with a recent report that green tea extract could decrease the expression of both VEGFR-1 and VEGFR-2 on human umbilical vein endothelial cells.[40]

In addition to the direct effects of EGCG on specific RTK at the cell surface, recent studies revealed that EGCG exerts its effects on RTK indirectly by targeting the lipid organization of the plasma membrane, so-called 'lipid rafts', which are associated with these RTK. Indeed, the inhibitory effect of EGCG on EGF binding to the EGFR and the subsequent dimerization of this receptor is associated with alterations in the lipid rafts of colon cancer cells.[41] EGCG also decreases cell surface-associated EGFR by inducing the internalization of EGFR into endosomal vesicles, thereby inhibiting the activation of this receptor and exerting anticancer effects.[42] Because VEGFR-2 is also localized to lipid rafts,[43] future studies would be required to elucidate whether the inhibitory effect of EGCG on the activation of VEGFR-2 is associated with alterations in membrane lipid order in cancer cells.

Agents that can co-target several different RTK, such as sorafenib, are expected to be promising candidates for the treatment of HCC.[2–6] In conclusion, the ability of EGCG to target both the VEGF–VEGFR axis, as demonstrated in the present study, and other types of RTK that play critical roles in the proliferation of cancer cells,[23–26] is thus considered to provide evidence that this naturally occurring agent may be effective in the chemoprevention and therapy of HCC, and likely of other malignancies as well, that show hypervascularity.

Acknowledgments

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Abbreviations

EGCG	(–)-epigallocatechin gallate
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
HCC	hepatocellular carcinoma
HER	human epidermal growth factor receptor
HIF	hypoxia-inducible factor
IGF	insulin like growth factor
PDGFR	platelet-derived growth factor receptor
RTK	receptor tyrosine kinase
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor.

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(−)-Epigallocatechin gallate prevents carbon tetrachloride-induced rat hepatic fibrosis by inhibiting the expression of the PDGFRβ and IGF-1R☆

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ABSTRACT

Hepatic fibrosis is a major complication of various chronic liver diseases. Activated hepatic stellate cells (HSCs) play a critical role in the development of liver fibrosis and the axis of platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR), a member of receptor tyrosine kinases (RTKs), is closely associated with the activation of HSC. Insulin-like growth factor (IGF)-1 receptor (IGF-1R), which also belongs to RTKs, interacts with the PDGF/PDGFR axis, thereby cooperatively promoting hepatic fibrosis. We herein examined the effects of (−)-epigallocatechin gallate (EGCG), which inhibits the activation of several types of RTKs, on the development of rat liver fibrosis induced by carbon tetrachloride (CCl_4). Drinking water with 0.1% EGCG significantly decreased the serum levels of both aspartate aminotransferase and alanine aminotransferase raised by CCl_4, thus indicating an improvement of liver injury. In CCl_4-injected rats, EGCG markedly attenuated hepatic fibrosis and decreased the amount of hydroxyproline in the experimental liver. The expression of PDGFRβ and IGF-1R mRNAs in the liver was significantly lowered by the treatment with EGCG. EGCG also decreased the expression of PDGFRβ and α-smooth muscle actin proteins, thus indicating the inhibition of HSC activation. These findings suggest that EGCG can exert, at least in part, an anti-fibrotic effect on the liver by targeting PDGFRβ and IGF-1R. EGCG might therefore be useful in both the prevention and treatment of hepatic fibrosis.

1. Introduction

Hepatic fibrosis is a common response to chronic liver injury from a variety of causes, including infection with hepatic viruses, drug-related, alcohol and metabolic disorders [1]. Progressive fibrosis eventually leads to cirrhosis which is often associated with a high risk of liver failure and hepatocellular carcinoma (HCC) [1,2]. Therefore, the inhibition and prevention of the development of fibrosis might be an effective strategy to improve the prognosis of patients with chronic liver disease. Indeed, recent clinical trials have revealed that treatment with interferon prevents or delays the development of liver cirrhosis and HCC in patients with chronic viral hepatitis [3,4].

The activation of hepatic stellate cells (HSCs) plays a key role in the development of liver fibrosis because activated HSCs are major cellular source of collagen in the injured liver [1]. Following liver injury of any etiology, quiescent HSCs transform to activated cells, which are proliferative and fibrogenic [1]. Several types of growth factors, cytokines, chemokines and their cognate receptors are associated with this transition. Among these factors, autocrine signaling by platelet-derived growth factor (PDGF), which binds to and activates PDGF receptor (PDGFR), is regarded as one of the most potent mitogens and chemotactics for HSCs [5]. The expressions of PDGF and the beta isoform of its receptor (PDGFRβ) have been shown to increase in both experimental rat and human models of liver fibrosis [6,7]. These findings suggest that the activated PDGF/PDGFR signaling pathway may therefore be a candidate therapeutic target for antifibrogenic therapy in liver disease.

Numerous *in vivo* and *in vitro* studies suggest that green tea catechins can exert both cancer therapeutic and cancer preventive

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; α-SMA, α-smooth muscle actin; EGCG, (−)-epigallocatechin gallate; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; IGF-1R, insulin-like growth factor-1 receptor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; RTK, receptor tyrosine kinase.

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properties at various organ sites [8]. One of the anticancer mechanisms of green tea or its constituents is explained by their inhibitory effect on the expression and activation of specific receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF)-1 receptor (IGF-1R) and PDGFRβ, and related downstream signaling pathways [9–12]. In the present study we investigated the effects of (−)-epigallocatechin gallate (EGCG), the major biologically active component of green tea, on liver fibrosis and on the expression of PDGFRβ using a rat model of carbon tetrachloride (CCl_4)-induced hepatic fibrosis. We also examined whether EGCG alters the expression of IGF-1R in the fibrotic liver because this RTK is closely associated with the PDGF/PDGFR axis and thus plays an important role in liver fibrosis [13].

2. Materials and methods

2.1. Animals and chemicals

Four-week-old male Wistar rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). CCl_4 was purchased from Sigma Chemical Co. (St. Louis, MO). EGCG was provided by the Mitusi Norin Co., Ltd. (Tokyo, Japan).

2.2. Animal protocol

All rats 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 water (tap water supplemented with or without EGCG) and a pelleted basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan). After 1 week of acclimatization, a total of 26 rats were randomly divided into 4 groups. Groups 1 and 2 (5 rats per group) received an intraperitoneal injection of olive oil (0.5 ml/kg body weight, twice a week) for 8 weeks. Groups 3 and 4 (8 rats per group) received an intraperitoneal injection of CCl_4 (0.5 ml/kg body weight, twice a week) for the same period of time. At the start of the intraperitoneal injections, the rats in Groups 2 and 4 were given tap water containing 0.1% EGCG. The rats in Groups 1 and 3 were given only tap water throughout the experiments. A freshly prepared solution of EGCG in tap water was supplied to the experimental rats three times a week. At the termination of the experiment (13 weeks of age), all rats were sacrificed by CO_2 asphyxiation to determine the development of hepatic fibrosis.

2.3. Histopathological and immunohistochemical examinations

In all experimental groups, 3–4 μm thick sections of 10% buffered formaldehyde-fixed and paraffin-embedded livers were stained with either hematoxylin and eosin (H&E) for histopathology or Azan stain to observe liver fibrosis. Immunohistochemistry of α-smooth muscle actin (α-SMA) was performed using a primary anti-α-SMA antibody (DAKO, Glostrup, Denmark) with paraffin-embedded sections, as previously described [14]. Computer-assisted quantitative analyses of fibrosis development were carried out using the WinROOF image-processing software program (Mitani Corp., Tokyo, Japan) in three low power (×40) fields per specimen, as previously described [14].

2.4. Hepatic hydroxyproline analysis

The hepatic hydroxyproline content (μmol/g wet liver) was quantified colorimetrically in duplicate samples from approximately 200 mg wet-weight of liver tissues, as previously described [15].

2.5. Clinical chemistry

At sacrifice, blood samples were collected from the inferior vena cava and the serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a standard clinical automatic analyzer (type 726, Hitachi, Tokyo, Japan).

2.6. Protein extraction and Western blot analysis

Equivalent amounts of protein lysates (30 μg/lane) from the liver of experimental rats were subjected to a Western blot analysis, as described previously [16]. Anti-PDGFRβ antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-α-smooth muscle actin (α-SMA) antibody was from DAKO. An antibody to GAPDH (Chemicon International, Temecula, CA) served as a loading control.

2.7. RNA extraction and quantitative real-time reverse transcription-PCR analysis

A quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed, as described previously [17]. Total RNA was isolated from the liver of the experimental rats using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX), according to the manufacturer's protocol. The cDNA was synthesized from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen, San Diego, CA). The primers used for the amplification of *PDGFRβ*, *IGF-1R* and *GAPDH* specific genes are described previously [18,19]. Real-time PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN) with SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan). The expression level of both the *PDGFRβ* and *IGF-1R* genes was normalized to the *GAPDH* gene expression level. Each experiment was done in triplicate and the average was then calculated.

2.8. Statistical analysis

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

3. Results

3.1. Effects of EGCG on the serum levels of AST and ALT in CCl_4-injected rats

As shown in Fig. 1, the serum AST and ALT levels significantly increased in the CCl_4-injected group (Group 3, $p < 0.01$) in comparison to the control group (Group 1, olive oil-injected group), but they did not increase in the treatment with EGCG alone (Group 2). When compared to the CCl_4-treated group, drinking water with 0.1% EGCG (Group 4) gave lower serum levels of both AST ($p < 0.01$) and ALT ($p < 0.01$), thus indicating suppression of the liver injury (Fig. 1).

3.2. Effects of EGCG on the liver fibrosis in CCl_4-injected rats

Examinations of the Azan-stained sections indicated that treatment with CCl_4 resulted in the development of marked liver fibrosis (Fig. 2C and G). On the other hand, drinking water with 0.1% EGCG significantly prevented the liver fibrosis in comparison to the CCl_4-injected group (Fig. 2D and H). No evidence of fibrosis was observed

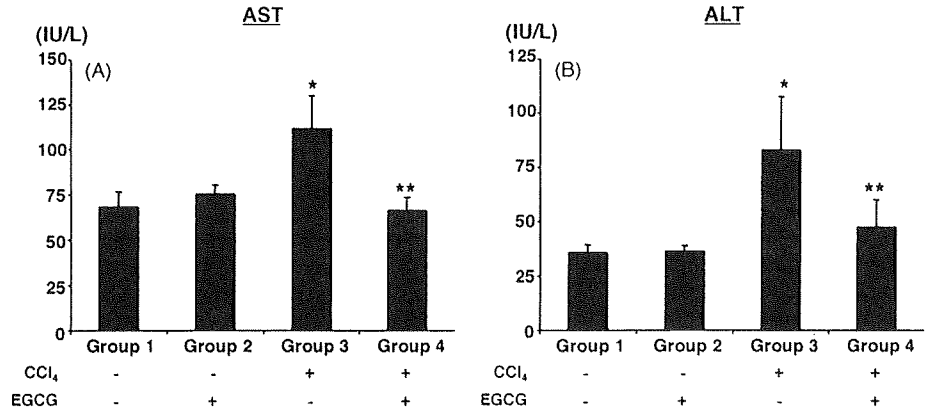

Fig. 1. Effects of EGCG on the serum levels of AST and ALT in the experimental rats. At sacrifice, blood samples were collected and the serum levels of AST (A) and ALT (B) were then assayed. Values are the means ± SE ($n = 5$). $^{*}p < 0.01$, compared with control group (Group 1, olive oil-injected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

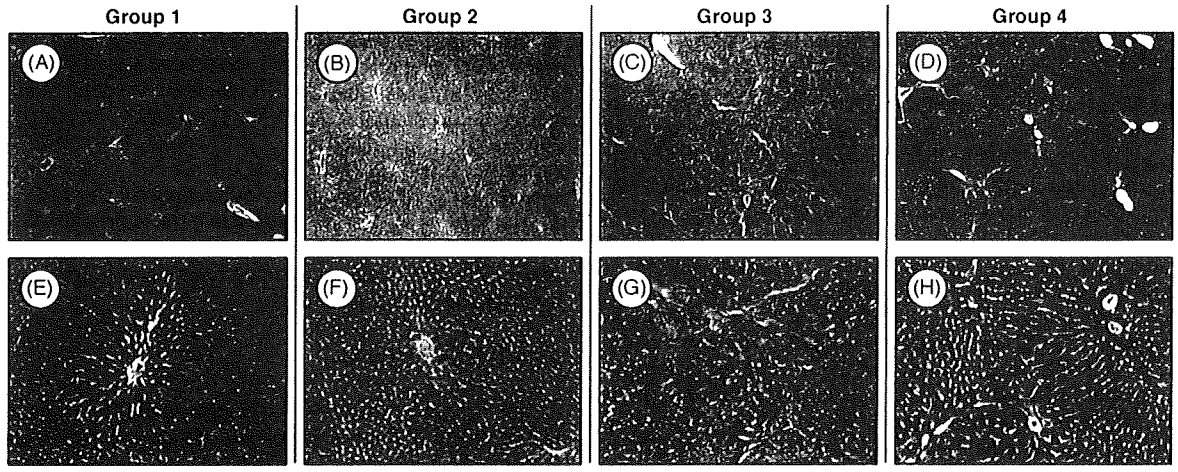

Fig. 2. Photomicrographs of liver sections from the rats in control group (Group 1, A and E), olive oil-injected and EGCG drinking group (Group 2, B and F), CCl_4-injected group (Group 3, C and G) and CCl_4-injected and EGCG drinking group (Group 4, D and H). Paraffin-embedded sections were stained with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

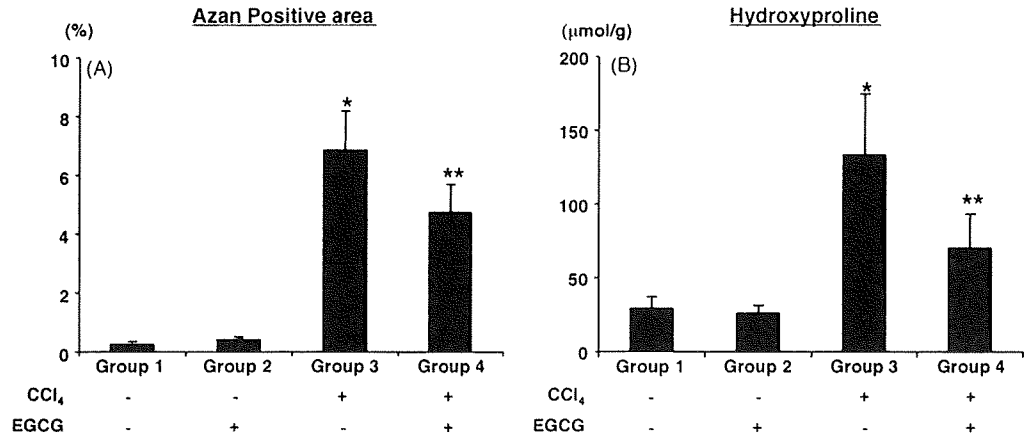

Fig. 3. Effects of EGCG on hepatic fibrosis area and hydroxyproline content in the experimental rats. (A) The fibrosis area was evaluated by Azan stain (Fig. 2) using an image analyzer. (B) The hepatic hydroxyproline contents were quantified colorimetrically, as described in Section 2. Values are the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

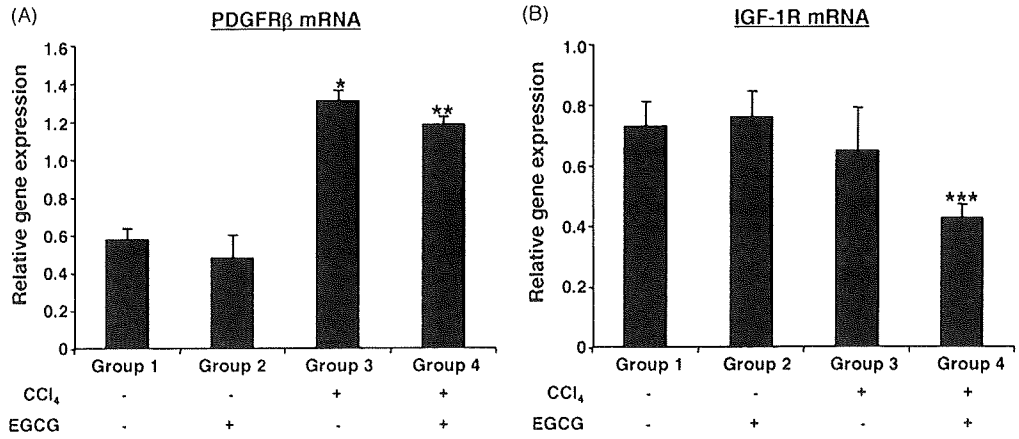

Fig. 4. Effects of EGCG on the expression levels of PDGFRβ and IGF-1R mRNAs in the experimental rats. cDNA was synthesized from the livers of experimental rats and real-time PCR was performed using PDGFRβ (A) and IGF-1R (B) specific primers. The expression levels of these genes were normalized to the 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

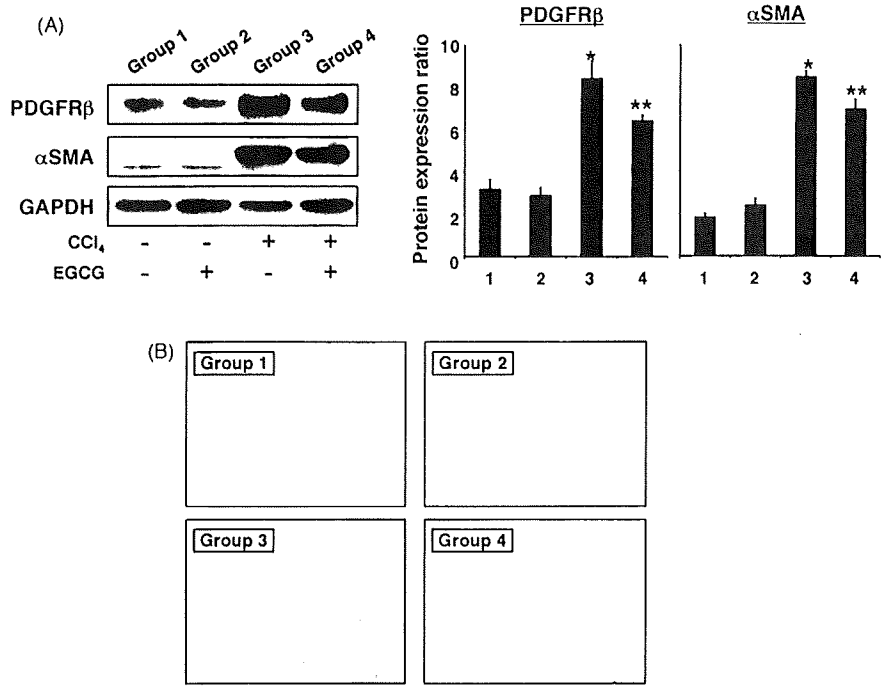

Fig. 5. Effects of EGCG on the expression levels of PDGFRβ and α-SMA in the experimental rats. (A) Total protein was extracted from the liver of experimental rats and equivalent amounts of protein were examined by a Western blot analysis using the respective antibodies. An antibody to GAPDH served as 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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