two SK-Hep-1 clones, of which HDGF expressions were stably knock-downed 64% and 40%, their proliferation was significantly suppressed, but partially at about 35% and 11%, in clone 1 (HDGF-shRNA1) and clone 3 (HDGF-shRNA3), respectively (Fig. 2c). Thus, HDGF is partly involved in the proliferation of HCC cells.

Effect of vitamin K_2 on HDGF protein expression in HCC cells

HDGF protein in the HuH-7 cells decreased after vitamin K_2 treatment by a Western blot analysis (data not shown). By the use of an ELISA for HDGF, we measured intracellular levels of HDGF protein in three HCC cell lines after vitamin K_2 treatment for 96 h. The vitamin K_2 treatment significantly suppressed the HDGF protein expression on three HCC cell lines (Fig. 3).

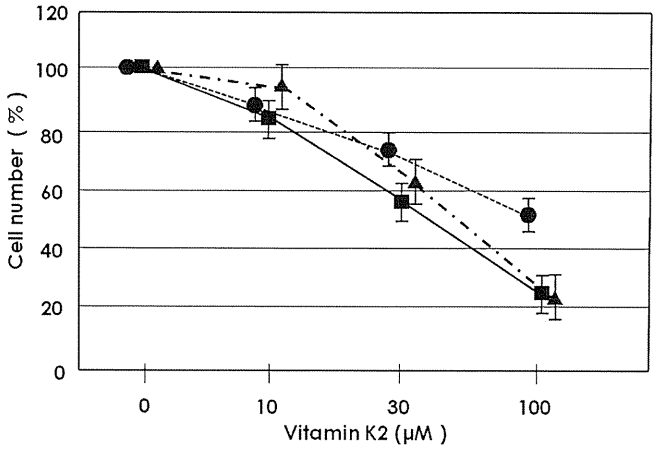

Fig. 1. Vitamin K_2 suppressed the proliferation of human hepatocellular cells dose-dependently. HepG2 (●), HuH-7 (▲), and SK-Hep-1 (■) were treated with various concentrations of vitamin K_2. After vitamin K_2 treatment for 96 h, the cell numbers were determined by the MTT method. Data are mean ± SE of three independent experiments

Recovery of vitamin K_2-induced suppression of HCC proliferation by overexpression of HDGF

Next, we investigated the restorative effect of HDGF on the suppression of HCC cell proliferation by vitamin K_2 by use of HDGF-overexpressing HepG2 cells. The overexpression of HDGF significantly recovered the vitamin K_2-induced suppression of HepG2 cell proliferation, but partially, about 50% (Fig. 4). Thus, these findings suggest that the suppression of HDGF expression is one pathway of vitamin K_2-mediated growth inhibitory mechanisms in HCC cells.

Effect of vitamin K_2 on HDGF mRNA expression

HDGF mRNA expression was measured by a quantitative real-time PCR method. In the HepG2, HuH-7, and SK-Hep-1 cells, HDGF mRNA expression was suppressed 36.5%, 39.5%, and 22.5%, respectively, after vitamin K_2 treatment for 96 h at the dose of 30 μM. The HDGF mRNA expression was suppressed by vitamin K_2 at 51.1%, 63.3%, and 66.2% in the HepG2, HuH-7, and SK-Hep-1 cells, respectively, at the dose of 100 μM (Fig. 5). Next, we investigated whether vitamin K_2 suppressed the promoter activity of HDGF by a dual luciferase assay. It is difficult to transfect plasmids to HuH-7 cells, and we examined this reporter assay in the other two HCC cell lines. In the HepG2 and SK-Hep-1 cells, vitamin K_2 significantly suppressed the luciferase

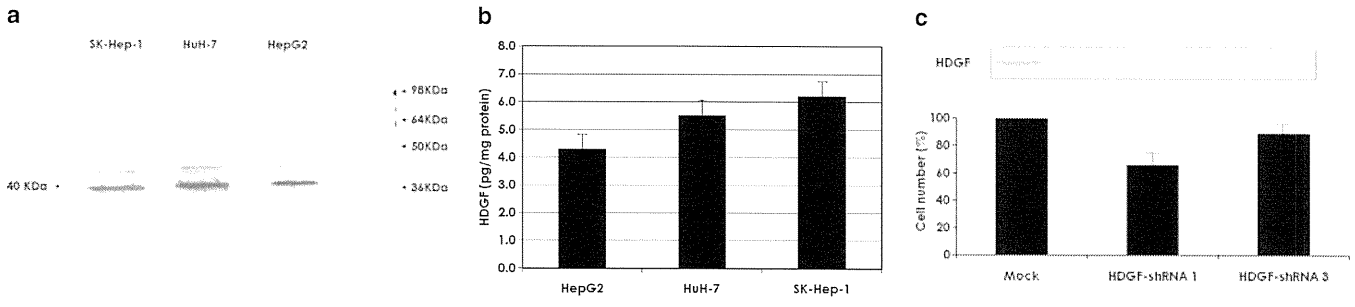

Fig. 2. Expression of hepatoma-derived growth factor (HDGF) protein in human HCC cell lines. Three HCC cell lines were lysed and vortexed with lysis buffer after 48 h culture. After centrifugation, the supernatants of each cell line were used for analysis. **a** Western blot analysis. The cell lysate with 5 μg protein from each cell line was loaded and electrophoresed. After electroblotting, the membrane was blotted with anti-HDGF antibody (C-terminus) at a dilution of 1:10000. **b** Intracellular HDGF protein by an enzyme-linked immunosorbent assay (ELISA). The cell lysates after centrifugation were analyzed in an ELISA kit for HDGF. Data are mean ± SE of three independent experiments. **c** Knock-down of HDGF expression suppressed the proliferation of SK-Hep-1 cells. The stably HDGF-knock-down SK-Hep-1 clones (HDGF-shRNA1 and -3) and mock cells were cultured for 96 h, and then cell numbers were measured by MTT assay. HDGF protein expression in each clone was shown by Western blot

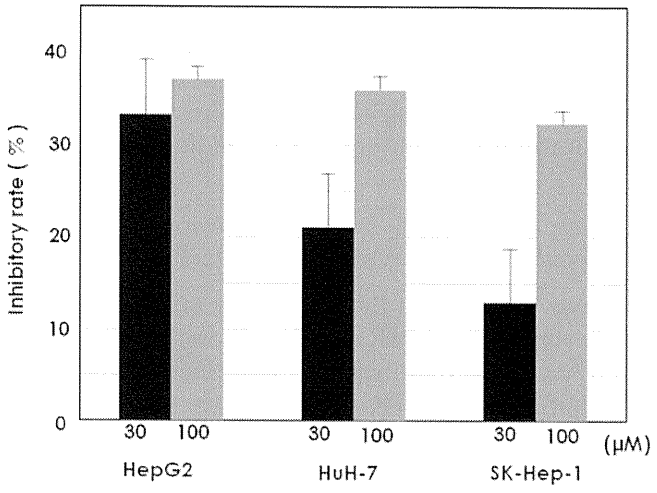

Fig. 3. HDGF protein expression in HCC cells was suppressed by vitamin K_2. Inhibitory rate of HDGF protein expression by vitamin K_2 is shown. Three HCC cell lines were treated with vitamin K_2 at the dose of 30 μM or 100 μM for 96 h. The HCC cells were lysed and vortexed with lysis buffer, and the cell lysates after centrifugation were analyzed by an ELISA kit for HDGF. The data are shown as the mean ± SE of three independent experiments

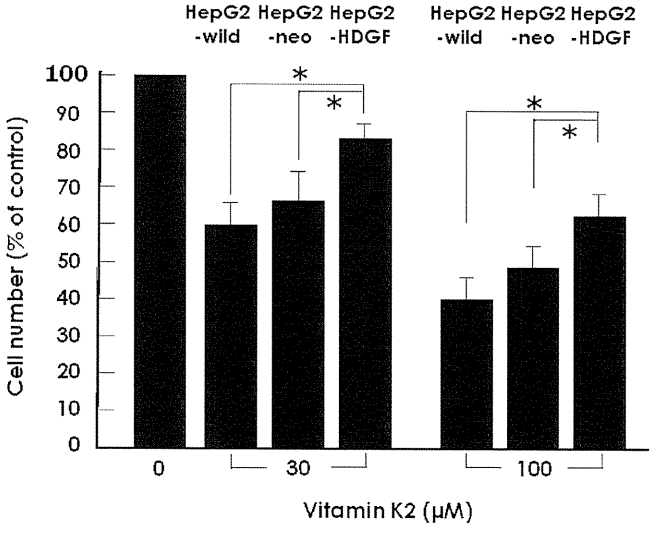

Fig. 4. HDGF overexpression recovered the vitamin K_2-induced suppression of cell proliferation. HDGF-overexpressing HepG2 (HepG2-HDGF), mock (HepG2-neo), and parent HepG2 (HepG2-wild) cells were treated with 30 or 100 μM vitamin K_2. Ninety-six hours later, cell numbers of each well were measured by MTT assay. $^*P < 0.05$

activity by 47.7% and 86.9%, respectively, at 30 μM vitamin K_2 after transfection of the H2 promoter (Fig. 6). The luciferase activity was suppressed 78.2% and 97.0% in the HepG2 and SK-Hep-1 cells at the dose of 100 μM, respectively. Therefore, vitamin K_2 significantly suppressed the gene expression of HDGF in the HCC cells.

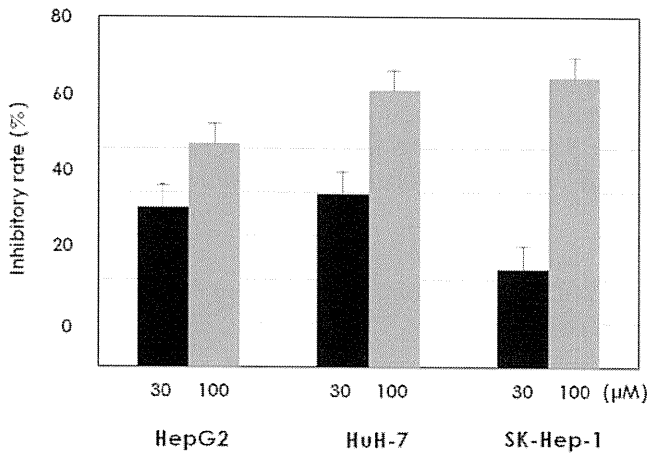

Fig. 5. HDGF mRNA expression was suppressed by vitamin K_2. Three HCC cell lines were treated with vitamin K_2 at 30 μM or 100 μM for 96 h. After RNA extraction, HDGF mRNA expression was measured by the quantitative real-time PCR method. The data are shown as the mean ± SE of three independent experiments

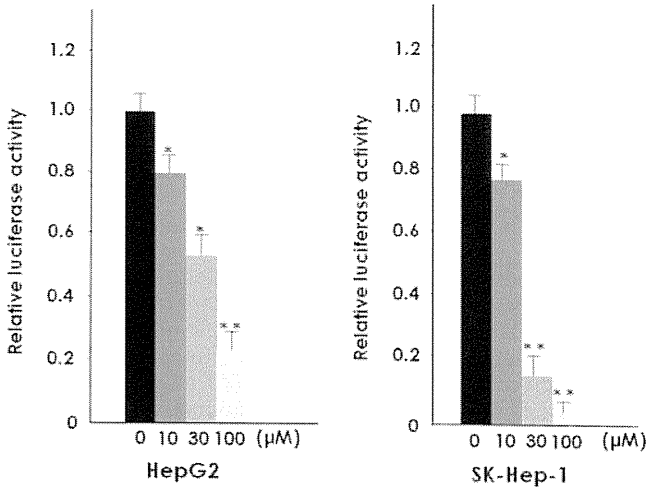

Fig. 6. Vitamin K_2 suppressed the transcription of HDGF. HepG2 and SK-Hep-1 were transfected with 1 μg promoter vector (pLuc-H2). After incubation with the indicated concentrations (μM) of vitamin K_2 for 24 h, the cells were harvested and the relative luciferase activities were measured. The data are shown as the mean ± SE of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ vs. control

Possible interaction site of vitamin K_2 in the promoter of the HDGF gene

Next, we constructed the luciferase-reporter plasmids including a truncated promoter region (Fig. 7a). The luciferase activity of the H2 promoter was significantly suppressed in the SK-Hep-1 cells, but that of H12 or -13 was not (Fig. 7b). Therefore, the interaction site of vitamin K_2 seems to reside in the region –1 to –150 bp of the HDGF gene. These findings suggest that the sup-

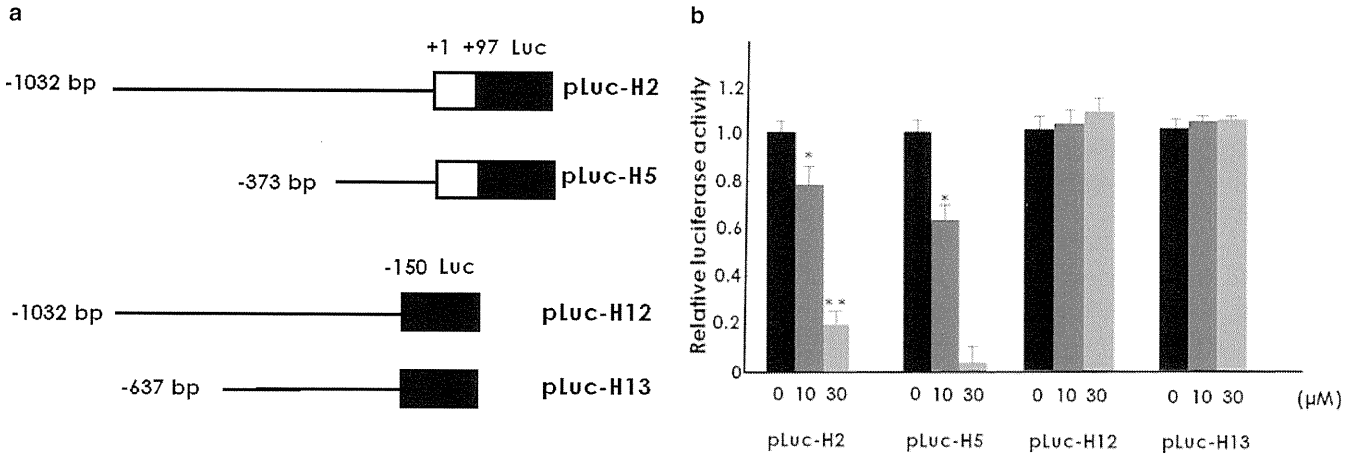

Fig. 7. Possible action site of vitamin K_2 in the promoter of the HDGF gene. **a** The construct of the luciferase-reporter assay plasmids for the HDGF promoter region. **b** SK-Hep-1 cell was transfected with 1 μg each promoter vector (pLuc-H2, -H5, -H12, -H13). After incubation with the indicated concentrations (μM) of vitamin K_2 for 24 h, the cells were harvested, and the relative luciferase activities were measured. Data are shown as mean ± SE of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ vs. control

pression of HDGF is one of the pathways to inhibit cell growth by vitamin K_2 treatment, through its interaction with the promoter region of the HDGF gene.

Discussion

The inhibitory mechanisms of vitamin K_2 in cancer cell proliferation have not yet been clarified. Some possible pathways of vitamin K_2 action have been reported, specifically protein kinase A activation, the induction of cell-cycle regulatory proteins, and the suppression of the cyclin-dependent kinases.[26–28] Another pathway has been reported to suppress the cyclin D1 expression through the inhibition of nuclear factor (NF) kappa B activation.[29] Recently, vitamin K_2 is reported to inhibit the phosphorylation of the retinoid X receptor (RXR) alpha protein, which is a critical factor for hepatocarcinogenesis.[30] We previously reported that p21 induction is a significantly important pathway for the growth inhibitory action of vitamin K_2 by the use of HepG2 cells.[28] However, vitamin K_2 suppressed the growth of the HuH-7 cells more strongly than the HepG2 cells, although the HuH-7 cells are deficient in the p21WAF1/CIP1 protein. Therefore, other mechanisms for the vitamin K_2 growth inhibition remained to be clarified.

In the present study, vitamin K_2 significantly suppressed HDGF mRNA and protein expression in HCC cells. Few data have been reported about the inhibition of the expression of growth factor and/or growth factor receptor genes in HCC cells. Acyclic retinoid suppressed fibroblast growth factor (FGF) receptor 3 gene expression in an HCC cell line.[31] Therefore the downregulation of HDGF by vitamin K_2 should play an important role in the suppression of HCC cell growth by vitamin K_2.

HDGF is one of the critical growth factors that play important roles in the proliferation of HCC cells. Enhanced expression of HDGF showed malignant potential of tumor cells and a poorer prognosis in patients with HCC as well as gastric cancer, lung cancer, and pancreatic cancer.[12,13,15,16,18,19,32] Downregulation of HDGF may induce cancer growth inhibition and improve the prognosis for cancer patients. Indeed, the downregulation of HDGF by either antisense oligonucleotides or antisense viral treatment and gene silencing by siRNA inhibit the cell growth both in vitro and in vivo.[8,14,17] In the present study, the knock-down expression of HDGF by shRNA partially suppressed the proliferation of SK-Hep-1 cells. Thus, HDGF is apparently one of the growth factors involved in the proliferation of HCC cells. On the other hand, other growth factors, including hepatocyte growth factor (HGF), FGF, epidermal growth factor (EGF), HB-EGF, and transforming growth factor-alpha (TGF-α), should be related to the proliferation of HCC; however, until now, no evidence has been reported that vitamin K_2 suppressed these growth factors and their receptors. In the present study, we showed a significant suppression of the HDGF gene expression by vitamin K_2. This is the first report that vitamin K_2 regulates the expression of growth factor genes. The regulation of the gene expression of one growth factor, HDGF, by vitamin K_2 suggests to us an important approach to investigate the mechanisms of vitamin K action.

By a luciferase assay using the promoter region of HDGF, vitamin K_2 significantly downregulated the

HDGF expression. Vitamin K_2 must act directly or indirectly on the promoter region of HDGF and regulate the expression of HDGF. Recently, vitamin K_2 suppressed cyclin D1 expression through inhibition of nuclear factor (NF)-kappaB activation with inhibition of phosphorylation and degradation of I-kappaB alpha and I-kappaB kinase activity.[29] In the promoter region of –150 to 0 in the HDGF gene, no NF-kappaB binding site could be detected. Other transcriptional factors, including cAMP response element-binding protein (CREB), upstream transcription factor (USF), and activating enhancer-binding protein (AP)-2, are reported to mediate the vitamin K_2 effects; however, their binding motifs are absent in this promoter region of HDGF (–1 to –150). Therefore, another pathway should be critical for the suppression of the HDGF gene expression by vitamin K_2. It remains to be clarified whether vitamin K_2 directly reacts to the DNA sequence or indirectly via other factors, including transcriptional regulatory factors or binding cofactors. Vitamin K_2 may possibly downregulate some of other growth factor genes, too. It is very important to clarify the mechanism whereby vitamin K_2 reacts on and suppresses the promoter activity of the HDGF gene. These findings suggested that the regulation of growth factor gene expression is one of the crucial mechanisms of the vitamin K_2-induced cell growth inhibition.

In conclusion, the downregulation of the HDGF expression in the promoter region is one of the growth inhibitory mechanisms of vitamin K_2. To elucidate the suppressive mechanism of the HDGF promoter region by vitamin K_2 will possibly lead to the development of a novel growth inhibitory mechanism, thus resulting in a new drug design.

Acknowledgments. This study was supported by a Grant-in-aid of Hyogo Science and Technology Association, Grant-in-aid for researchers, Hyogo College of Medicine, and Health and Labour Sciences Research Grants (Research on Hepatitis).

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Original Article

Vitamin K2 downregulates the expression of fibroblast growth factor receptor 3 in human hepatocellular carcinoma cells

Ke Cao,[1,2]* Weidong Liu,[1]* Hideji Nakamura,[1] Hirayuki Enomoto,[1] Teruhisa Yamamoto,[1] Masaki Saito,[1] Hiroyasu Imanishi,[1] Soji Shimomura,[1] Peiguo Cao[2] and Shuhei Nishiguchi[1]

[1]*Division of Hepatobiliary and Pancreatic Medicine, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan, and* [2]*Department of Oncology, the Third Xiang-Ya Hospital of Central Southern University, Changsha, Hunan, China*

Aim: Vitamin K2 exerts an antitumor activity on human hepatocellular carcinoma (HCC), however, its inhibitory mechanism has not yet been clarified. This study was designed to identify the attractive target molecule of vitamin K2 and shed some light on its effects on fibroblast growth factor receptor (FGFR)3 in HCC cells.

Methods: The changes in the gene expression of HuH-7 after vitamin K2 treatment were evaluated by a DNA chip analysis. The mRNA and protein levels of FGFR were evaluated by semiquantitative reverse transcription polymerase chain reaction (RT–PCR), real-time PCR and western blot analysis. The promoter activity of the *FGFR3* gene was measured by a dual-luciferase assay.

Results: The DNA chip analysis revealed different inhibitory rates of gene expression of *FGFR3* (60.6%) and *FGFR1* (19.4%) after vitamin K2 treatment. Vitamin K2 suppresses the proliferation of HuH-7 in a dose-dependent manner and its inhibitory rate reached approximately 61.8% at the dose of 30 μM. FGFR3 mRNA was significantly reduced based on semiquantitative RT-PCR and decreased 61.5% by a real-time PCR method after vitamin K2 treatment, but FGFR1 mRNA was not. The level of FGFR3 protein was also reduced by vitamin K2 treatment. The luciferase assay demonstrated that vitamin K2 significantly suppressed the promoter activity of *FGFR3*. Furthermore, the FGFR3–ERK1/2 signaling pathway was suppressed by vitamin K2 treatment.

Conclusion: These findings suggest that vitamin K2 may suppress the proliferation of HCC cells through the downregulation of the FGFR3 expression. The transcriptional suppression of FGFR3 may be a novel mechanism of the vitamin K2 action for HCC cells.

Key words: fibroblast growth factor receptor 3, hepatocellular carcinoma, luciferase assay, vitamin K2

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most aggressive cancers with an increasing incidence and high mortality rate, though intensive efforts have been made to improve the treatment for this cancer. Further development of effective therapeutic agents is therefore still needed urgently.

Correspondence: Dr Hideji Nakamura, Division of Hepatobiliary and Pancreatic Medicine, Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. Email:nakamura@hyo-med.ac.jp

*These authors contributed equally to this work.

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Recent studies have revealed that vitamin K2 (menatetrenone, MK-4) possesses antitumor activity on a number of cancer cell types including HCC.[1–3] It has been reported to play an important role in the prevention of HCC in patients with hepatitis C virus-related cirrhosis,[4] while also showing a suppressive effect on the recurrence of HCC and a beneficial effect on survival.[5] *In vitro*, vitamin K2 inhibits the growth and invasiveness of HCC cells and causes cell-cycle arrest and apoptosis of HCC cells via different pathways such as upregulating the transcription of the *p21* gene, while also suppressing cyclin D1 expression and protein kinase A activation.[6–8] In addition, it exerts a synergistic inhibitory effect against liver carcinogenesis and on the proliferation of HCC cells when combined with other drugs.[9,10]

However, the mechanisms of such inhibitory action have not yet been fully elucidated.

On the other hand, fibroblast growth factor receptor (FGFR)3 is reported to be overexpressed in HCC.[11] The treatment of anti-FGFR3 antibodies, small interfering RNA (siRNA) of *FGFR3* and an ERK inhibitor suppressed the growth of HCC cells.[12,13] Moreover, acyclic retinoid inhibits HCC cell growth by suppressing FGF-mediated signaling pathways.[12] The association of FGFR3 expression with HCC and the therapeutic potential of this receptor tyrosine kinase (RTK) make it a particularly attractive target for facilitating the development of more effective therapies. However, the role of the FGF signal pathway in growth suppression of HCC cells induced by vitamin K2 has not yet been clarified. This report showed that vitamin K2 inhibits the FGFR3 protein expression by suppressing its gene promoter in HCC cells.

METHODS

Materials

VITAMIN K2 (MK-4) was supplied by Eisai (Tokyo, Japan).

Cell culture

Human HCC cell lines, HuH-7 and HepG2, were purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle's essential medium (DMEM; Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO_2.

Cell growth assays

The cells were seeded onto 96-well plates at a density of 2.5×10^3 cells. After 24-h culture, 100 μL of fresh medium containing different concentrations of vitamin K2 (30, 10, 1, 0.1, 0.01 and 0 μM) was added to each well. Vitamin K2 was dissolved in 99.9% ethanol at the concentration of 10 mM then diluted with DMEM to the appropriate concentrations for the experiments. Control cells were cultured in DMEM containing the corresponding concentration of ethanol to each dose of vitamin K2. After 2, 3 or 4 days culture with vitamin K2 treatment, the number of viable cells in each well was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche, Nutley, NJ, USA) according to the manufacturer's instructions. All experiments were carried out four times concurrently then were repeated three times.

RNA isolation and mRNA preparation

The total RNA was extracted from the cultured HuH-7 cells using ISOGEN (Wako, Osaka, Japan) and Poly(A)+RNA was purified from total RNA with an Oligotex-Dt30 mRNA purification kit (Takara Otsu, Shiga, Japan).

DNA chip analysis

The expression of each gene was simultaneously analyzed through hybridization of the targets which were prepared by using Poly(A)+RNA as template. A DNA chip spotted with 886 cDNA from identified human genes (IntelliGene Human CHIP 1K Set I, ver. 1.0) was from Takara Shuzo, (Kyoto, Japan). The results were also analyzed by normalizing fluorescence intensities between experiments using a subset of cDNA clones.

Reverse transcription polymerase chain reaction analysis

Reverse transcription polymerase chain reaction (RT–PCR) was done using an RNA LA PCR Kit (Takara-bio, Otsu, Japan) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was transcribed with reverse transcriptase using random 9mers. Subsequently, each RT reaction mixture was subjected to PCR amplification using Takara LA Taq polymerase with cycle numbers varying from 15 to 40. Each cycle consisted of heat denaturation (94°C for 0.5 min), annealing (55°C for 0.5 min) and extension (72°C for 1.5 min). The PCR products were size fractionated on a 2% agarose gel and visualized under ultraviolet light. The sequences of primers used for RT–PCR to determine the expression of the target gene were: *FGFR3* (241 bp) sense 5′-ACGTTACCGTGCTCAAGACGGC-3′, antisense 5′-AGGAAGAAGCCCACCCCG-3′; *FGFR1* (210 bp) sense 5′-CCTCCCAGATGTTGGACC-3′, antisense 5′-CG AAGCACTGACCTCCCTA-3′; and *β-actin* (661 bp) sense 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, antisense 5′-CTAGAAGCATTTGCGGTGGACGATGA AGGG-3′.

Quantitative real-time PCR analysis

Real-time PCR quantification was used to verify the RT–PCR data. Total RNA was reverse transcribed to cDNA using an ABI High-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The expression of mRNA for genes of interest was measured by the Taqman real-time PCR method using an ABI PRISM

7900HT Sequence Detection System (Applied Biosystems). The probe and primer set for each gene was derived from Taqman Gene Expression Assays (Applied Biosystems). The codes of *FGFR1*, *FGFR2* and *FGFR3* were Hs00915137_ml, Hs01552916_ml and Hs00997396_gl, respectively. The real-time reactions were set up in triplicate for each gene in 384-well plates and run at the default PCR thermal cycling conditions: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 15 s; and 60°C, 1 min. As an endogenous control, *β-actin* (Hs99999903_ml) cDNA was quantified in a similar manner.

Western blotting

After 96-h culture with vitamin K2, HuH-7 cells were washed twice with ice-cold phosphate buffered saline (PBS), lysed and sonicated in RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 100 μg/mL phenylmethylsulfonyl fluoride, 45 μg/mL aprotinin, 100 μM sodium orthovanadate). The supernatant of the homogenate was used for protein determination with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and electrophoresis. Samples with equal amounts of total protein were separated by electrophoresis on a 5% sodium dodecylsulfate polyacrylamide gel under reducing conditions and blotted to a polyvinylidine difluoride (PVDF) membrane by electroblotting. Actin was also used to confirm equal loading. The membranes were blotted with Anti-FGFR3 (B-9)-Mouse Monoclonal IgG. Signals were developed with an ABC kit (Vector, Burlingame, CA, USA) and diaminobenzidine. The densities of the immunoreactive bands of FGFR3 protein were estimated using NIH Image software program.

RNA interference

FGFR3 Silencer Select siRNA (s5168) and Silencer Negative Control siRNA (s14544) were purchased from Ambion (Austin, TX, USA). HuH-7 cells were seeded at a density of 2.5×10^3 cells in 96-well plates and oligonucleotides (0, 10 and 50 nM) were transfected by siPORT NeoFX Transfection Agent (Ambion), according to the manufacture's instructions. MTT assays were performed at 72 h after oligonucleotide transfection.

Phospho-FGFR3, ERK1/2 and AKT assay

DuoSet IC Human Phospho-FGF R3 ELISA Kit and Cell-Based ELISA Kit for phospho-ERK1/2 and phospho-Akt were purchased from R&D Systems (Minneapolis, MN, USA). For phospho-FGFR3 detection, HuH-7 cells were seeded at a density of 1×10^5 cells in six-well culture dishes with 2 mL of DMEM, and for phospho-ERK1/2 and phospho-Akt detection, cells were seeded at a density of 2.5×10^3 cells in 96-well plates with 100 μL of DMEM. After overnight incubation, the media were changed to fresh media containing 0, 10, 30 and 100 μM of vitamin K2. Forty-eight hours later, the media were changed with vitamin K2 and incubated for 48 h again. The phosphorylated FGFR3, ERK1/2 and Akt were detected according to the manufacturer's recommendations.

Construction of luciferase expression plasmids for the human FGFR3 gene promoter

Human genomic clone RZPDB737H082155D, which includes the *FGFR3* gene, was purchased from ImaGenes Company (Berlin, Germany). The promoter sequence of *FGFR3* was acquired by PCR using a sense primer (5′-ATTAGGTACCAGCTGCGCGCTATGCAAA CA-3′) and an antisense primer (5′-AGACTACAGG AGGAGGAAGGCA-3′). The 850-bp PCR product was confirmed by DNA sequencing, then polished, purified and digested by Kpn I and ligated to pGL3 basic firefly luciferase expression plasmid (digested by Kpn I and Sma I). The pFGFR3-luc plasmid contains the 5′-flanking region of the *FGFR3* gene, spanning the region from base pair −838 to base pair +2. Transfection efficiency was monitored by the co-transfection of pRL-TK renilla luciferase vector (Promega, Madison, WI, USA).

Luciferase reporter assay

We used HCC cell line, HepG2, for the luciferase reporter assay, because HuH-7 cells are difficult to transfect of plasmids. Hep G2 cells (7.5×10^4 cells/well) were seeded in a 12-well culture dish in 10% FBS-DMEM. On the next day, the cells were co-transfected with 0.5 μg of pFGFR3-luc and 0.0125 μg of pRL-TK vectors by 1.5 μL of FuGene 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA), under serum-free DMEM for 6 h, and then were changed to 10% FBS-DMEM overnight. Culture medium was changed to 0.8 mL of phenol red-free DMEM containing 10% charcoal-dextran-stripped fetal bovine serum (FBS-CCS) and several concentrations (0, 10, 30 and 100 μM) of vitamin K2. After incubation for 24 h, the cells were harvested and lysed with Passive Luciferase Lysis Buffer (Promega). Luciferase activity of each sample was measured using a Dual-luciferase Assay Kit (Promega). The level of inhibition was calculated by dividing the mean luciferase activity of samples treated with vitamin K2 by

the mean activity of untreated control samples. The results are shown as the mean ± standard deviation of at least three experiments.

Statistical analysis

The results are expressed as the means ± standard error. At least three separate experiments were performed for each data point. Statistical analyses were done using an unpaired Student's *t*-test (two-tailed). Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Vitamin K2 suppresses the proliferation of HuH-7 in dose-dependent manner

VITAMIN K2 SHOWED a dose-dependent inhibition on the proliferation of HuH-7 cells (Fig. 1). The significant differences of cell numbers were observed after 72 and 96 h in the 10- and 30-μM vitamin K2 treatment ($P < 0.01$). The inhibitory rates by vitamin K2 treatment after 96 h reached approximately 61.8% and 36.3% at 30 μM and 10 μM, respectively. No significant differences in the cell numbers after 72 and 96 h were observed in the 0.01-, 0.1- and 1-μM vitamin K2 treatment ($P > 0.05$).

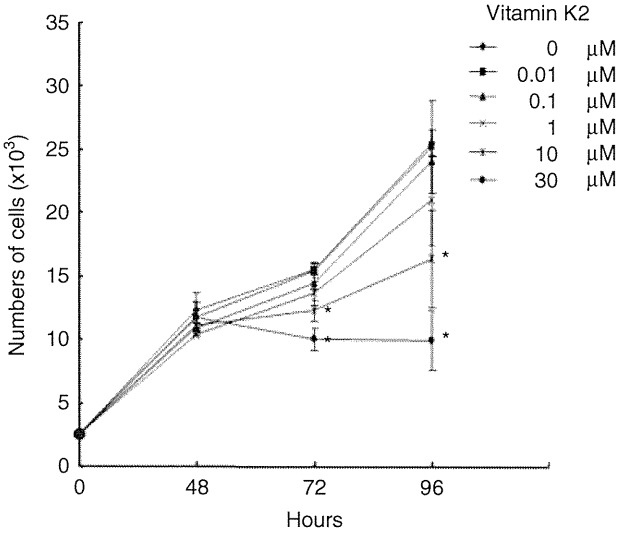

Figure 1 Vitamin K2 suppressed the proliferation of HuH-7 cells in a dose-dependent manner. HuH-7 cells were treated with various doses of vitamin K2 or the medium containing ethanol corresponding to 30 μM of vitamin K2 as the control. Proliferation curves of HuH-7 cells at various doses of vitamin K2 for 48–96 h. Data are shown as the mean ± standard error of three independent experiments. *$P < 0.01$ vs control.

Different inhibitory effects of vitamin K2 on the FGFR mRNA

DNA chip analysis revealed the genes which were differently expressed in HuH-7 cells after vitamin K2 treatment for 96 h. The inhibitory rates of gene expression of *FGFR1*, *FGFR3* and *FGFR4* were 19.4%, 60.6% and 43.0%, respectively. RT–PCR showed that vitamin K2 inhibited the expression of FGFR3 mRNA in a dose-dependent manner. However, no remarkable inhibitory effect of vitamin K2 on FGFR1 mRNA was shown (Fig. 2a). Real-time PCR revealed significant reductions in the mRNA levels of FGFR3 in the 10- and 30-μM vitamin K2 treatment in comparison to the controls (0.692 ± 0.087 [$P = 0.0124$] at 10 μM and 0.385 ± 0.053 [$P < 0.01$] at 30 μM) after 96 h treatment. A significant difference was demonstrated between 10- and 30-μM vitamin K2 treatment ($P < 0.05$), as well. No significant reductions of the mRNA level of FGFR1 were detected in the 10- or 30-μM vitamin K2 treatment in comparison to the control (0.938 ± 0.051 [$P > 0.05$] at 10 μM and 0.933 ± 0.096 [$P > 0.05$] at 30 μM) (Fig. 2b). The *FGFR2* gene was not included in the DNA chip. Therefore, we detected the expression of FGFR2 mRNA in HuH-7 cells after vitamin K2 treatment by real-time PCR. FGFR2 gene expression was not suppressed by vitamin K2 treatment at the dose of 30 μM (data not shown). *FGFR3* gene expression was significantly downregulated by vitamin K2 treatment.

Vitamin K2 downregulates the protein expression of FGFR3

The expression of FGFR3 was evaluated by western blotting. There were two bands (125 and 135 KDa) and the relative densities of immunoreactive bands were measured using NIH Image software. After 96-h treatments, significant suppression of the FGFR3 protein expression was revealed in the 10- and 30-μM vitamin K2 treatment in comparison to the control (125 KDa: 0.915 ± 0.025 [$P < 0.01$] at 10 μM and 0.530 ± 0.030 at 30 μM [$P < 0.0001$]; 135 KDa: 0.869 ± 0.019 [$P < 0.0001$] at 10 μM and 0.655 ± 0.024 at 30 μM [$P < 0.0001$]). A significant difference between 10- and 30-μM vitamin K2 groups was also demonstrated ($P < 0.0001$; Fig. 3).

Knockdown of FGFR3 expression suppresses the proliferation of HuH-7 cells

The proliferation of HuH-7 cells was significantly suppressed, but partially at approximately 33.0% and 44.3% after *FGFR3* siRNA treatment at the dose of 10 and 50 nM, respectively (Fig. 4). The negative control

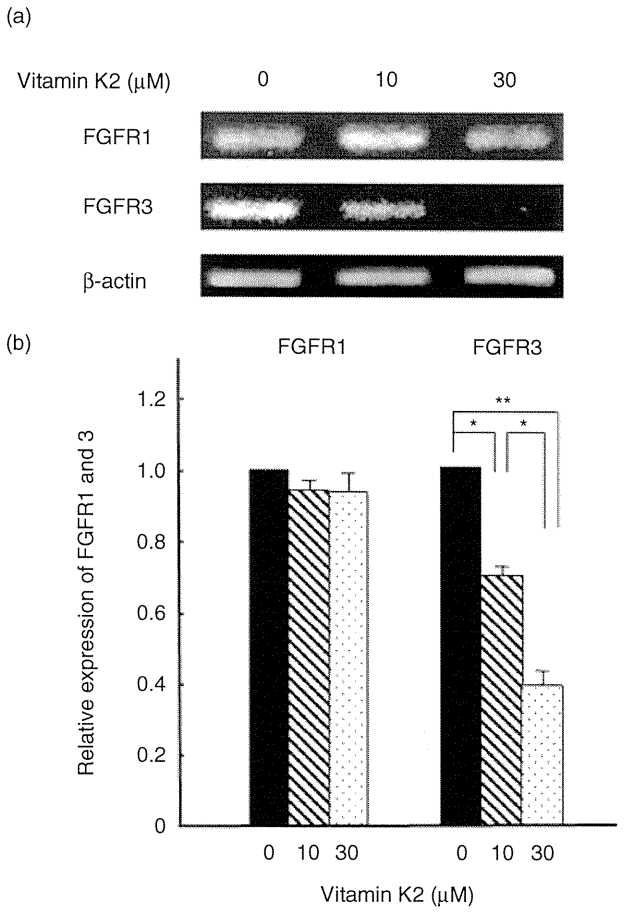

Figure 2 Vitamin K2 suppressed the expression of fibroblast growth factor receptor (FGFR)3 mRNA. The inhibition of the FGFR3 mRNA expression by vitamin K2 occurred in a dose-dependent manner; however, no remarkable inhibition of FGFR1 mRNA was shown. HuH-7 cells were treated with vitamin K2 for 96 h and subjected to reverse transcription polymerase chain reaction (RT–PCR) and quantitative real-time PCR analysis. (a) RT–PCR analysis. PCR amplification was done with 25 cycles. (b) Quantitative real-time PCR analysis. The results are expressed by taking the relative amount of FGFR3 expression of the control group as 1. Data represent the mean ± standard error of three experiments. $*P < 0.05$; $**P < 0.01$.

siRNA treatment has no effect on the proliferation of HuH-7 cells. Therefore, the proliferation of HuH-7 cells is partly dependent on FGFR3-mediated signal pathways.

Vitamin K2 suppresses the transcription of FGFR3

Hep G2 cells were transiently transfected with pFGFR3-luc reporter plasmids and luciferase activities were examined. In Hep G2 cells transfected with pFGFR3-luc, the relative luciferase activity decreased 1.27-, 1.49- and 2.11-fold more than the control after vitamin K2 treatment of 10, 30 and 100 μM, respectively (Fig. 5). Therefore, vitamin K2 downregulated the activity of the FGFR3 promoter in a dose-dependent manner.

Vitamin K2 suppresses the FGFR3 signal pathway

Next, the phosphorylation levels of FGFR3, ERK1/2 and Akt were investigated after vitamin K2 treatment

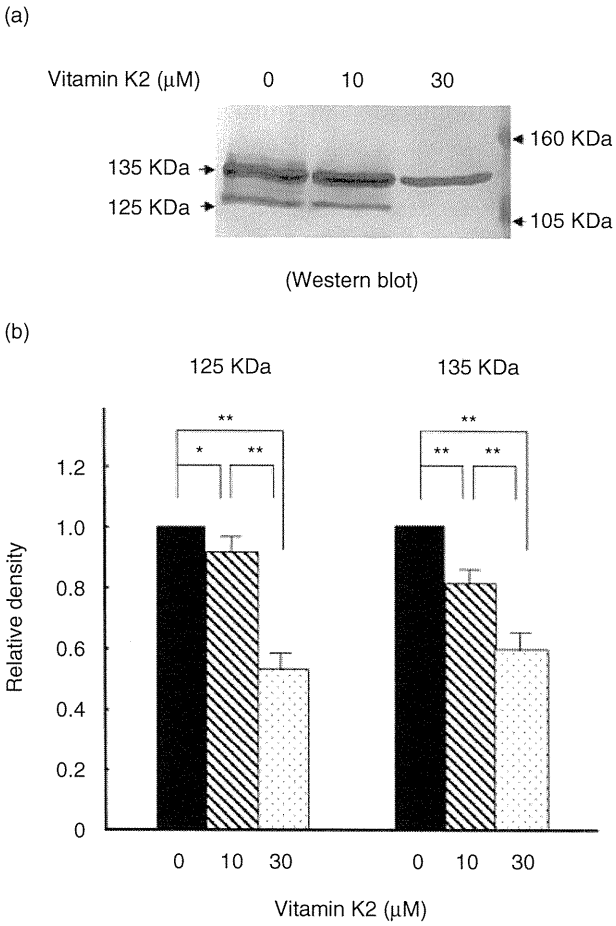

Figure 3 Vitamin K2 suppressed the protein expression of fibroblast growth factor receptor (FGFR)3. HuH-7 cells were treated with vitamin K2 for 96 h and western blot analysis was done. (a) Expression of FGFR3 protein. There were two bands (125 and 135 KDa) for FGFR3 proteins. (b) The densities of immunoreactive bands were measured by NIH Image software. The results are expressed by taking the relative amount of FGFR3 expression of the control group as 1. Data represent the mean ± standard error. $n = 6$; $*P < 0.01$; $**P < 0.0001$.

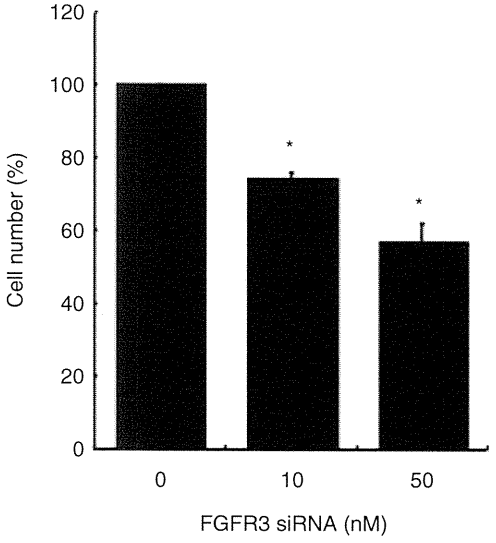

Figure 4 Knockdown of fibroblast growth factor receptor (FGFR)3 by small interfering RNA (siRNA) suppressed the proliferation of HuH-7 cells. The *FGFR3* siRNA was transfected as described in Methods. Seventy-two hours later, the cell numbers were determined by MTT assay. The results are expressed by taking the relative cell number of the control group as 100%. Data represent the mean ± standard error. $n = 3$; $^*P < 0.01$.

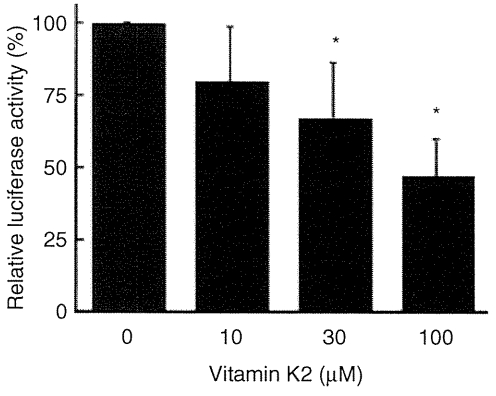

Figure 5 Vitamin K2 suppressed the transcription of the fibroblast growth factor receptor (FGFR)3 gene promoter. pFGFR3-luc was constructed and used as luciferase reporter plasmid for the *FGFR3* gene promoter. HepG2 cells were transiently transfected with pFGFR3-luc and pRL-TK vectors. After incubation with the indicated concentrations of vitamin K2 for 24 h, cells were harvested and luciferase activities were measured. Data are shown as the means (bars, standard error) of three independent experiments, $^*P < 0.05$.

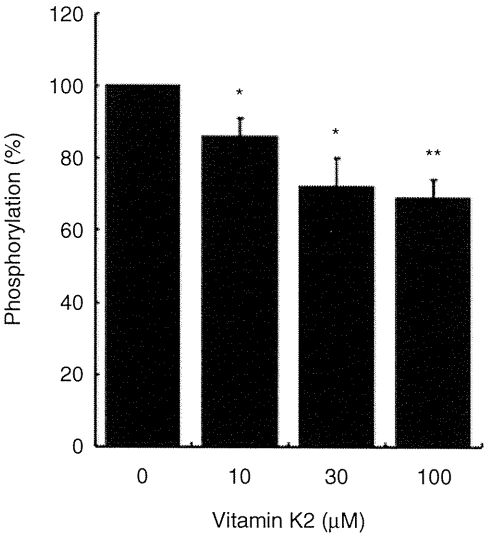

Figure 6 Vitamin K2 suppressed the phosphorylation of fibroblast growth factor receptor (FGFR)3. HuH-7 cells were treated with vitamin K2 for 96 h and the cell lysates were extracted. Phospho-FGFR3 level in the 50 μg of total protein from each sample was measured by enzyme-linked immunosorbent assay. The results are expressed by taking the relative amount of phospho-FGFR3 of the control group as 100%. Data represent the mean ± standard error. $n = 3$; $^*P < 0.05$; $^{**}P < 0.01$.

for 96 h by enzyme-linked immunosorbent assay. The phosphorylation levels of FGFR3 decreased by 0.857 ± 0.029-, 0.773 ± 0.050- and 0.693 ± 0.022-fold more than the control after vitamin K2 treatment of 10, 30 and 100 μM, respectively (Fig. 6). The phosphorylation rates (phospho-protein/total protein) of ERK1/2 also decreased by 0.867 ± 0.124-, 0.697 ± 0.066- and 0.573 ± 0.050-fold more than the control after vitamin K2 treatments of 10, 30 and 100 μM, respectively; however, those of Akt were not changed (Fig. 7). The phosphorylation of FGFR3 and ERK1/2 was significantly suppressed by vitamin K2, but Akt was not. Vitamin K2 significantly inhibited the FGFR3-ERK1/2, but not the FGFR3-Akt signal pathway in HuH-7 cells.

DISCUSSION

THE 23 MEMBERS of the FGF family and their four receptor tyrosine kinases (RTK) designated FGFR1–4, mediate the intracellular signaling pathways that control cell proliferation, differentiation and migration. FGFR3 is associated with normal angiogenesis and embryonic development, as well as with a variety of human malignancies.[14,15] For instance, it is associated

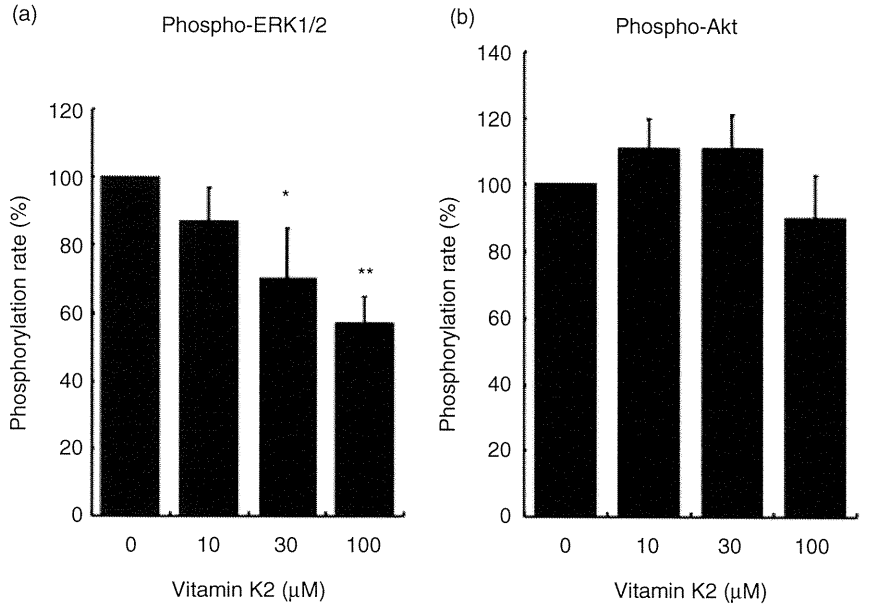

Figure 7 Vitamin K2 inhibited the ERK1/2, but not Akt phosphorylation. HuH-7 cells were seeded and treated with vitamin K2 for 96 h. Then the total and phosphorylated proteins of ERK1/2 and Akt in each well were directly measured by enzyme-linked immunosorbent assay. The phosphorylation rate of ERK1/2 or Akt was acquired after dividing the amount of phosphorylated protein by total protein. The results are expressed by taking the relative rate of phospho-ERK1/2 (a) or phospho-Akt (b) of the control group as 100%. Data represent the mean ± standard error. $n = 3$; $^*P < 0.05$; $^{**}P < 0.01$.

with multiple myeloma cell growth and survival but also to bladder carcinogenesis.[16,17] FGFR3 is overexpressed in urinary tract carcinomas and modulates the neoplastic cell growth.[18] Interestingly, the expression of FGFR3 increases significantly in HCC and correlates with pathological factors including HCC differentiation and nuclear/cytoplasmic ratio.[11] Exogenously-supplied FGF stimulates the growth of HCC cells, and anti-FGFR3 antibody can suppress the proliferation of HCC cells.[12] The RNA interference targeted to the FGFR3 gene effectively reduces the growth of HepG2 cells *in vitro*.[12] In the present study, knockdown of FGFR3 expression by siRNA inhibited the proliferation of HuH-7 cells. These findings suggest that FGFR3-mediated signaling pathways are involved in the proliferation of HCC cells.

In the present study, both phosphorylated FGFR3 and ERK1/2 decreased after vitamin K2 treatment, but phospho-Akt did not. The levels of phosphorylated FGFR3 were inhibited by the same degree as those of total FGFR3 protein after vitamin K2 treatment. Therefore, vitamin K2 suppresses the proliferation of HuH-7 cells through downregulating the expression of FGFR3 protein, but not through directly suppressing the phosphorylation of FGFR3. Vitamin K2 significantly inhibited the phosphorylation rates of ERK1/2 in HCC cells, which was coincident with the previous report.[19] These findings suggest that vitamin K2 inhibits the proliferation of HuH-7 cells through the FGFR3–ERK1/2, but not FGFR3–Akt, signaling pathway.

Fibroblast growth factor receptor 1 and FGFR3 are expressed in most well-differentiated types of thyroid carcinoma cell lines and both may be associated with cholesteatomas.[20,21] The siRNA mediated knockdown of FGFR1 or FGFR3 inhibits FGF-induced anchorage-independent clonogenicity in the SW-13 human adrenal adenocarcinoma cell line.[22] FGFR1 participates in mediating the proliferation mechanism of HCC cells and its ectopic expression, observed commonly in hepatocarcinoma cells, can accelerate hepatocarcinogenesis by driving cell proliferation at early stages and promoting neoangiogenesis at late stages of progression.[23,24] In view of the previous results of DNA chip analyses of *FGFR1* and *FGFR3*, their consanguineous relationships are involved in all kinds of tumors, especially in HCC, and so FGFR1 was compared with FGFR3. This study showed that vitamin K2 suppresses the proliferation of HuH-7 in a dose- and time-dependent manner and downregulates the expression of FGFR3, but there were no remarkable reductions of the mRNA level of FGFR1 and FGFR2 in the 10- or 30-μM vitamin K2 groups. The suppressive effect of vitamin K2 should be mainly on FGFR3, not FGFR1 and 2.

For further evaluation of the inhibitory function of vitamin K2 on the *FGFR3* gene, a luciferase expression plasmid of the human *FGFR3* gene promoter was thus constructed. We successfully amplified a 838-bp sequence of the human *FGFR3* gene promoter from a human genomic clone, but failed to acquire longer DNA

sequence of *FGFR3* promoter region directly from human genomic DNA, which was prepared from human leukocytes, HuH-7 and HepG2 cells. Thus, we constructed a luciferase reporter vector using this sequence. The promoter activity of *FGFR3* was suppressed dose-dependently by vitamin K2 treatment. We searched the transcription factors' binding sites in the 838-bp *FGFR3* promoter sequence by Web Promoter Scan Service.[25] A CpG island, but no CAAT and TATA box, was found in the region near the transcription initiation site. These characteristics are shown in mouse *FGFR3* promoter.[26] Five Sp1 binding sites are located in the 100 bp of the sequence 5′ to the initiation site of the mouse *FGFR3* gene and four in the human gene.[26] Because Sp1 binding sites have been reported to be responsible for the transcription activity in mouse *FGFR3* promoter, we consider that they are also responsible for the transcription activity of the human *FGFR3* promoter. Mouse FGFR3 transcription is also considered to be regulated by cyclic adenosine monophosphate response element-binding protein (CREB) and serum response factor (SRF), the binding sites of which are located between nucleotides −2311 and −1537.[27,28] However, neither CREB nor SRF binding site is found in the DNA sequence of the human *FGFR3* promoter between nucleotides −3061 and −1. Therefore, vitamin K2 may downregulate the transcription of human FGFR3 through the Sp1 binding sites.

Receptor tyrosine kinases serve as the key players in the oncogenic pathways, thereby leading to a variety of human malignancies. Several tyrosine kinase inhibitors recently developed, including Sorafenib as a prominent example for HCC, can remarkably inhibit tumor survival and induce cell apoptosis.[29] It is not surprising that RTK have been in great demand for targeted therapy in the oncology field. As far as FGFR3 is concerned, the anti-FGFR3 scFv antibodies may have potential applications as antitumor agents in bladder cancer.[30] The novel and highly specific anti-FGFR3-neutralizing antibody (PRO-001), ribozyme-targeted downregulation, short hairpin RNA (shRNA) and several small molecule inhibitors such as CHIR-258 and PKC412 might be ideal and novel therapeutic interventions in multiple myeloma.[31–36] These studies, taken together with the current results, suggest the potential therapeutic efficacy of FGFR3 inhibitors for HCC, because of the significant role of FGFR3 on the proliferation of HCC cells. Patients with a very poor prognosis would be most likely to benefit from FGFR3 inhibitor strategies.

Previous evidence suggests that *FGFR3* germ line mutations cause skeletal dysplasia and craniosynostosis syndromes and FGFR3 mutations are restricted to a few tumor types.[37–40] Its mutations characterize a subgroup of bladder cancers with good prognosis and the shRNA treatment to knockdown the mutated FGFR3 (S249C) can markedly decrease cell proliferation of the bladder cancer cell line, 97-7.[41,42] Further investigations are ongoing to identify the somatic FGFR3 mutations in HCC cells and contribute to our understanding of the role of vitamin K2 on them.

In summary, the current results indicate that vitamin K2 downregulates the expression of FGFR3, through the transcriptional suppression. These results provide novel evidence for the inhibitory mechanism of vitamin K2 on the FGFR3-mediated signaling pathway. Although future study is still needed to investigate the details of the effect of vitamin K2 on FGFR3 functions, these findings suggest that vitamin K2 might therefore be a useful treatment for HCC.

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Original Article

Hepatoma-derived growth factor is induced in liver regeneration

Hirayuki Enomoto,[1] Hideji Nakamura,[1] Weidong Liu,[1] Kenya Yoshida,[2] Yorihide Okuda,[2] Hiroyasu Imanishi,[1] Masaki Saito,[1] Soji Shimomura,[1] Toshikazu Hada[3] and Shuhei Nishiguchi[1]

[1]*Division of Hepatobiliary and Pancreatic Medicine, Department of Internal Medicine,* [3]*Hyogo College of Medicine, Hyogo, and* [2]*Department of Molecular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan*

Aim: Hepatoma-derived growth factor (HDGF) is a heparin-binding protein, which has been suggested to be involved in the development of kidneys, the cardiovascular system and the liver. We have shown that HDGF is highly expressed in parenchymal hepatocytes in the developing liver and promotes fetal hepatocyte proliferation. In the present study, we asked whether HDGF expression was related to liver regeneration.

Methods: We examined the mRNA and protein expressions of HDGF in two liver regeneration models. In addition, cellular distribution of HDGF in the regenerating liver was investigated by immunohistochemistry.

Results: In the carbon tetrachloride (CCl_4)-treated liver, HDGF expression was induced and the peak was detected at 24 h after the CCl_4 injection. HDGF expression was also enhanced in the hepatectomy model and the peak was detected at 12 h after surgery. The increased expression of HDGF protein was also confirmed by western blotting. Expression of the *HDGF* gene in the regenerating liver was dominantly detected in parenchymal hepatocytes.

Conclusion: These findings showed that *HDGF* expression was induced in parenchymal hepatocytes before the DNA synthesis in the regenerating liver, suggesting the possible involvement of *HDGF* in liver regeneration as an autocrine factor.

Key words: hepatoma-derived growth factor, hepatocyte, liver, regeneration

INTRODUCTION

HEPATOMA-DERIVED GROWTH FACTOR (HDGF) is a heparin-binding protein, which has been purified from the conditioned media of HuH-7 hepatoma cells.[1,2] HDGF promotes the growth of various types of cells, including fibroblasts, endothelial cells, vascular smooth muscle cells and some hepatoma cells such as HuH-7 and HepG2.[1–5] The primary sequence of HDGF protein contains two putative nuclear localization signals and HDGF can traffic to the nucleus, indicating that HDGF is a unique nuclear/growth factor.[2] Recently, several proteins (HDGF-related proteins), which share highly homologous N-terminal amino acid sequences to mouse HDGF, have been reported, suggesting that HDGF and its related proteins form a new gene family.[6–10] Although HDGF was initially identified in human hepatoma-derived cells, *HDGF* mRNA is ubiquitously expressed in adult normal tissues, including testis, kidney, liver, heart, brain and skeletal muscle,[2] and recent studies strongly suggest the important roles of *HDGF* in the development of kidneys and the cardiovascular system.[3,4]

Previous studies in cellular biology have shown that *HDGF* has a wide range of biological functions, including mitogenic activity and vascular development.[1–5,11] Overexpression of *HDGF* induces tumorigenesis *in vivo* through the combination of intrinsic angiogenic activity by the induction of vascular endothelial growth factor.[11] As for neoplasm, accumulating findings suggest that *HDGF* is closely related to the aggressive biological

Correspondence: Dr Hideji Nakamura, Division of Hepatobiliary and Pancreatic Medicine, Department of Internal Medicine, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo 663-8501, Japan. Email: nakamura@hyo-med.ac.jp
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potential of cancer cells.[12,13] Indeed, it has been reported that *HDGF* expression significantly correlated with the prognosis of various kinds of cancer disease.[14–20] In the experimental and clinical studies, we have shown the possible involvement of *HDGF* in the proliferation of hepatoma cells.

In addition, we have shown that *HDGF* is highly expressed in immature fetal hepatocytes and promotes their proliferation, suggesting that *HDGF* helps regulate the proliferation of fetal hepatocytes.[21] Because several growth-related genes are involved in the cellular proliferation of both liver development and regeneration, we investigated the expression of *HDGF* in regenerating liver. In the present study, we show that *HDGF* expression is induced in hepatocytes of the regenerating liver, suggesting the possible involvement of *HDGF* in liver regeneration.

MATERIALS AND METHODS

Mice

C57BL/6CRSLC MICE (Nihon SLC, Shizuoka, Japan) were used in all experiments in the present study. All animal experiments were carried out according to the guidelines of Osaka University Medical School and Hyogo College of Medicine.

Hybridization probes

The probes used for northern blot analysis were as follows: a 0.4 kb fragment of mouse *HDGF* cDNA[6] and mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA.

RNA extraction and northern blot analysis

Total RNA was extracted from liver tissue samples using ISOGEN (Nippon Gene, Tokyo), denatured with formamide and blotted onto nylon membranes (Amersham Life Science, Tokyo, Japan) according to the manufacturer's instructions. The mouse cDNA described above were labeled with [α-^{32}P] dCTP using a Megaprime DNA labeling kit (Amersham Life Science) and used for hybridization.[21] The intensities of mRNA bands were quantitated by densitometry using NIH image software.

Reverse transcription polymerase chain reaction

Total RNA was isolated from the liver, and first-strand cDNA was generated from 1 μg of total RNA with a random hexamer (Perkin Elmer Cetus, Norwalk, CT, USA) using Superscript II reverse transcriptase (Invitrogen GIBCO, Tokyo, Japan). Mouse cDNA fragments were amplified with Amp Taq DNA polymerase (Perkin Elmer Cetus) and a Gene Amp polymerase chain reaction (PCR) system 2400 (Perkin Elmer Cetus) (30 s at 94°C, 30 s at 60°C or 50°C, and 30 s at 72°C) using the following primers: heparin-binding epidermal growth factor-like growth factor (*HB-EGF*; GenBank: NM010415.1); 5′-ATG AAG CTG CTG CCG TCG-3′ (262–279) and 5′-TCA GTG GGA GCT AGC CAC-3′ (888–279): transforming growth factor-α (*TGF-α*; GenBank: NM031199.2); 5′-CGA CCG GAC AGC TCG CTC TG-3′ (101–120) and 5′-TGG GTG TAC TGA GCG AGC CC-3′ (779–760): hypoxanthine guanine phosphoribosyl transferase (*HPRT*; GenBank: NW013556.2); 5′-GCT GGT GAA AAG GAC CTC T-3′ (635–653) and 5′-CAC GGA CTA GAA CAC CTG C-3′ (879–864). The PCR product was confirmed as expected fragments by the multiple digestions of restriction enzymes. The intensities of PCR bands were quantitated by densitometry using NIH image software (National Institutes of Health, Bethesda, MD, USA).

Histochemical and immunochemical procedures

To detect HDGF protein, removed liver tissues were fixed in 4% paraformaldehyde in PBS overnight at 4°C, embedded in OCT compound and then frozen. Then, 6 μm sections were cut with a cryostat and mounted on APS coated glass slides. Specimens were rehydrated and subsequently endogenous peroxidase activity was removed by dipping tissue sections into 0.3% H_2O_2 in methanol for 30 min. Thereafter, nonspecific binding was blocked with normal goat serum and sections were incubated with a polyclonal antibody raised against C-terminal amino acids (aa 231–240) of human *HDGF* sequence.[2,21] Localization of the antibody was visualized with DAB by the ABC method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. To detect TGF-α and HB-EGF, we prepared paraffin-embedded specimens and carried out the immunostaining by the standard techniques. Briefly, after the inactivation of the endogenous peroxidase activity, non-specific binding was blocked with the blocking solution (Vector Laboratories), and then subjected to the ABC method described above, using the antibodies raised against TGF-α (Santa Cruz Biotechnology, CA, USA) and HB-EGF (Santa Cruz Biotechnology).

Western blot analysis

An equal amount of proteins (10 or 15 μg) were separated on a 10.0% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore Japan, Tokyo, Japan). After blocking the membrane with 5% skimmed milk, the membrane was incubated with polyclonal rabbit antibody raised against C-terminal amino acids (aa 231–240) of the human *HDGF* sequence at a 1:1000 dilution and then with peroxidase-conjugated anti-rabbit IgG goat antibody (Amersham Life Science). Bound antibody was visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Life Science). The intensity of the signals was quantitated by densitometry using NIH image software.

Partial hepatectomy and carbon tetrachloride treatment

Eight- to 12-week-old mice were subjected to a 70% partial hepatectomy using the method of Higgins and Anderson.[22] The remaining liver tissues were harvested for RNA extraction after the indicated periods. In studies of acute drug-induced liver injury, 8- to 12-week-old mice were intraperitoneally injected with 1 mg/kg body-weight carbon tetrachloride (CCl_4; 5% solution in olive oil). CCl_4 treatment resulted in marked increases of serum alanine aminotransferase activity. The maximum level was observed about 12 h after the injection and reached an approximately 100-fold increase compared with controls (olive oil only treated animals; data not shown). Parenchymal hepatocytes and non-parenchymal liver cells from adult mice were isolated by *in situ* perfusion of the liver with collagenase. The liver cells were separated into two fractions by differential centrifugation as previously reported.[23]

RESULTS

Expression of HDGF mRNA in the regenerating liver

WE INVESTIGATED THE expression profile of *HDGF* mRNA in the regenerating liver by northern blotting. We used two different models of the liver regeneration, which occur after CCl_4-treated liver damage and partial hepatectomy. In the CCl_4-treated liver, *HDGF* mRNA was induced prominently and the peak was detected at 24 h after the injection (Fig. 1a,b). The peak of DNA synthesis was reported to be induced 36–48 h after the injection of CCl_4.[24–26] Thus, the induction of *HDGF* mRNA was before the induction peak of DNA synthesis in the regenerating liver. *HDGF* mRNA expression was also enhanced in the hepatectomized liver, and its peak was detected at 12 h after surgery, although the level of induction was not so strong as that observed in CCl_4-treated liver (Fig. 1c,d). The peak of *HDGF* mRNA induction was also before the induction peak of DNA synthesis (24–36 h after surgery) in hepatectomized liver.

Expression of HDGF protein in the regenerating liver

Next, we examined the expression of *HDGF* at the protein level in the regenerating liver by western blotting. The expressed level of HDGF protein increased at 24 h after CCl_4 treatment (Fig. 2a,b). After partial hepatectomy, the level of HDGF protein increased at 12 h in the remnant liver (Fig. 2c,d). *HDGF* was confirmed to be induced by its expression in the liver during regeneration at the protein level as well as at the mRNA level in two mouse models.

Cellular distribution of HDGF in the regenerating liver

We investigated whether HDGF was induced in parenchymal hepatocytes or non-parenchymal cells in the regenerating liver. We examined the cellular distribution of *HDGF* expression in the CCl_4-treated liver, because of the extremely high induction of *HDGF*. We isolated parenchymal and non-parenchymal cells from both normal and CCl_4-treated livers. *HDGF* mRNA expression was stronger in the fraction of parenchymal cells than that of non-parenchymal cells by northern blotting (Fig. 3a,b). In non-parenchymal cells, *HDGF* mRNA was detected very weakly in the normal liver, but was barely induced in the regenerating liver after CCl_4 treatment. Immunohistochemical analysis using anti-C-terminus of HDGF antibody confirmed that hepatocytes in the CCl_4-treated liver showed a strong *HDGF* signal. These findings suggested that *HDGF* was induced predominantly in parenchymal hepatocytes in the regenerating liver. Therefore, *HDGF* was induced mainly in hepatocytes, but not in non-parenchymal cells during liver regeneration. As shown in Figure 3c, HDGF expression was detected mainly in the nucleus of hepatocytes in whole hepatic lobes. Because we have shown that the nuclear translocation is important for the growth promoting activity of *HDGF*,[27] the nuclear localization of *HDGF* in hepatocytes suggests the involvement in the hepatocyte proliferation during liver regeneration.

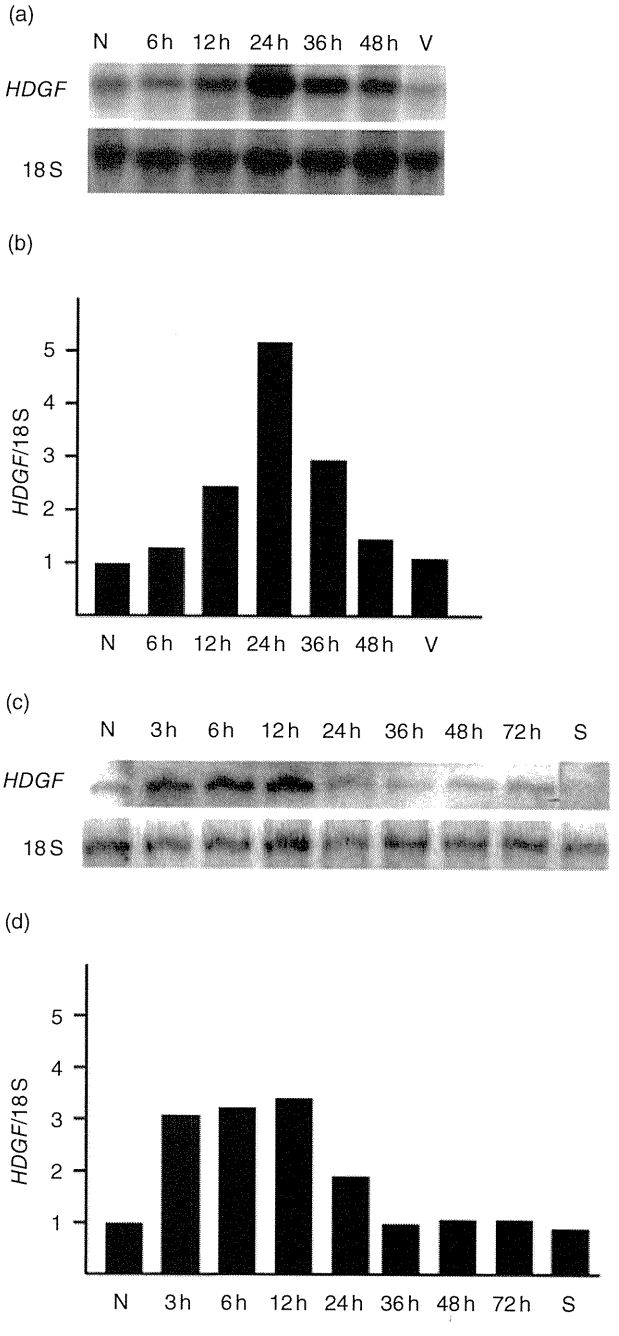

Figure 1 Induction of hepatoma-derived growth factor (*HDGF*) mRNA in the regenerating liver. (a,b) Northern blot analysis of *HDGF* mRNA in the livers of carbon tetrachloride (CCl_4)-treated mice. Total RNA was isolated at 0 (no treatment), 6, 12, 24, 36, 48 h after the injection (V, vehicle injected). Twenty micrograms of total RNA was loaded and hybridized with mouse cDNA of the *HDGF* specific sequence (a). *HDGF* expression/control (18S) ratio was shown (b). (c,d) Northern blot analysis of *HDGF* mRNA in the livers after 70% partial hepatectomy. Total RNA was isolated at 0 (no treatment), 3, 6, 12, 24, 36, 48, 72 h after surgery (S, sham operated). Twenty micrograms of total RNA was loaded and hybridized with the *HDGF* specific probe (c). *HDGF* expression/control (18S) ratio was shown (d).

Expression of TGF-α and HB-EGF during mouse liver regeneration induced by carbon tetrachloride

Previous studies have shown the increased expression of several growth factors such as *TGF-α*, *HB-EGF* and *HGF*, during liver regeneration.[24] However, most researches have been carried out with rat models, and the expression patterns of the growth factors in the CCl_4-treated mouse liver have not been well investigated, although the present results described above were mainly obtained from mouse livers given CCl_4.

TGF-α is an autocrine stimulator of hepatocyte proliferation, which is induced transiently in replicating hepatocytes.[28,29] HB-EGF is produced in non-parenchymal cells in liver regeneration, suggesting a possible role in hepatocyte growth in a paracrine manner.[30] In contrast, reports suggested the importance of extra-hepatic production of HGF in liver regeneration as well as its induction in the liver.[23,31] Therefore, we focused on the expression patterns of TGF-α and HB-EGF in the CCl_4-treated livers.

As shown in Figure 4, reverse transcription (RT)–PCR showed that the *TGF-α* mRNA increased by 6 h, showing a major peak at 12–24 h after the administration. In addition, *TGF-α* mRNA expression decreased at 36 h, and increased again at 48 h. *HB-EGF* mRNA was induced by CCl_4 treatment, showing the two peaks at 6–12 h and 36 h after the administration. Although our PCR products did not completely show the quantification of mRNA, the two different cycles of RT–PCR (25 and 30 cycles) showed similar patterns (Fig. 4b,c). Therefore, the time courses of the mRNA expression should be definitive, and these expression patterns resemble the previous findings observed in CCl_4-treated rat livers.[29,32]

Finally, we examined the expression of TGF-α and HB-EGF in liver tissues 24 h after CCl_4 treatment using immunohistochemistry. TGF-α expression was detected in the cytoplasm of hepatocytes, but not in non-parenchymal cells (Fig. 5a, arrowheads). HB-EGF signals were located in parenchymal cells and were predominantly detected in the peripheral region of the liver

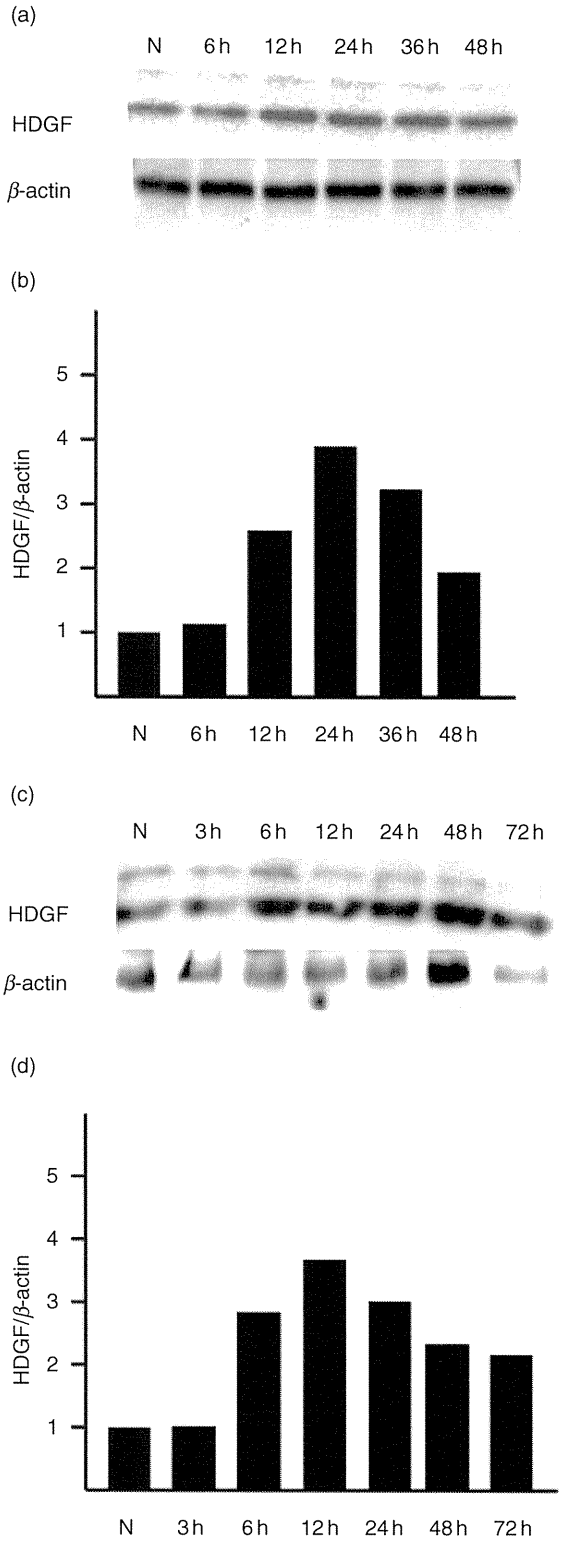

Figure 2 Induction of hepatoma-derived growth factor (HDGF) protein in the regenerating liver. HDGF protein was induced in the regenerating livers after carbon tetrachloride (CCl_4)-injection (a) or after 70% partial hepatectomy (c). Protein samples were obtained at the indicated times after the treatments. Then, 10 or 15 μg of whole lysates were loaded and the expression of HDGF protein was detected by western blotting. HDGF expression/control (β-actin) ratio was shown (b,d).

lobe (Fig. 5b, arrowheads). In addition, positive signals of HB-EGF were also detected in non-parenchymal cells (Fig. 5b, arrows). No immunoreactivity of TGF-α and HB-EGF was observed in control livers (Fig. 5c,d).

DISCUSSION

MANY GROWTH FACTORS have been suggested to be involved in liver regeneration. EGF is a paracrine or endocrine growth factor that has growth stimulating effects on primary cultured hepatocytes. Because only a slight increase in plasma EGF concentration has been observed during liver regeneration,[33] the role of EGF in liver regeneration is unclear. TGF-α is a mitogen for cultured hepatocytes and promotes their growth through binding the EGF receptor. Expression of TGF-α is induced in hepatocytes within several hours after partial hepatectomy and the elevated expression continues over 48 h. TGF-α is produced by hepatocytes and promotes their growth in an autocrine manner.[28,29] HB-EGF is a paracrine growth factor, which stimulates the DNA synthesis of primary cultured adult rat hepatocytes. The expression of *HB-EGF* mRNA in a regenerating rat liver increased after both partial hepatectomy and CCl_4 injection.[30,32] HGF is the most potent mitogen for mature hepatocytes,[34,35] and HGF stimulates hepatocyte replication by a paracrine or endocrine mechanism.[23,31] Besides the growth factors described above, it has been suggested that aFGF,[36] bFGF[37] and VEGF[38] also stimulate the proliferation of mature hepatocytes and appear to participate in liver regeneration, but their roles in liver regeneration are still unclear.

In the present study, we showed that two growth factors (TGF-α and HB-EGF) were induced in mouse regenerating livers as well as rat models (Fig. 4). Although many studies about liver regeneration have been carried out with rat models, experiments using mouse models are not abundant. In particular, a smaller number of studies has been reported regarding CCl_4-induced mouse liver regeneration. In rat livers given