Immunohistochemical analysis by anti-CD31 antibody showed prominent new vessel formation induced by HDGF.In human NSCLC tissues, HDGF expression correlated with intratumoral microvessel density analysed by CD31 staining (38).

Furthermore, in tumors developed by HDGF-over-expressing NIH3T3 cells, a potent angiogenic factor VEGF, was strongly detected immunohistochemically (19). Western blotting using anti-VEGF antibody showed a significant induction of VEGF in HDGF-over-expressing NIH3T3 fibroblasts, and reporter assay using VEGF promoter revealed that VEGF was significantly induced in NIH3T3 fibroblasts by HDGF (19). Interestingly, in pulmonary microvascular endothelial cells, VEGF treatment suppressed HDGF expression (22). HDGF is suggested to stimulate the proliferation of endothelial cells by mechanisms distinct from VEGF. HDGF shows potent angiogenic activity via its own direct stimulation of the proliferation of endothelial cells and vascular smooth muscle cells, and by its VEGF induction. Thus, HDGF works as an angiogenic factor by its own endothelial growth promoting activity and through the induction of VEGF in the nucleus.

HDGF is a usefully prognostic factor for patients with HCC.

In patients with chronic hepatitis and cirrhosis, HDGF was more highly expressed in HCC than in the adjacent liver as shown by Northern blotting. Immunohistochemical analysis by use of specific anti-C terminus of HDGF antibody revealed that HDGF was more strongly and frequently expressed in the nucleus and cytoplasm of HCC cells than in the adjacent normal hepatocytes (32,34). Statistical analysis of the relation between HDGF expression and other clinicopathological features in HCC showed that the HDGF expression level by immunohistochemistry was significantly correlated only to the differentiation of HCC. HDGF expression was higher in well-differentiated HCC than in poorly-differentiated HCC in our study (34). Conversely, another report showed that HDGF was higher in poorly-differentiated HCC than in well-differentiated HCC (39). One possible explanation for the discrepancy between the two groups may be due to the specificity of the anti-HDGF antibody used for immunostaining. However, interestingly, in both our and their studies, the patients with higher HDGF expression in HCC showed an earlier recurrence and a poorer overall survival rate than those with lower expression after hepatectomy for HCC (34,39). By multivariate analysis HDGF expression was an independent prognostic factor for

disease-free and overall survival in patients who underwent a hepatectomy for HCC. In our study analyzing the patients with well-differentiated and moderately differentiated HCC except for poorly differentiated ones, HDGF is a significantly prognostic factor for HCC recurrence and overall survivals, too. Interestingly, in other types of human cancers, including gastric cancer, esophageal cancer, pancreatic cancer and non-small cell lung cancer, HDGF expression is significantly related in over-all survivals, recurrence-free survivals, vascular and lymphatic invasion, infiltrative growth, intratumoral microvessel development or distant metastasis (38,40-43). These phenomena show that HDGF plays important roles on the acquisition of malignant characteristics of HCC cells or the carcinogenic induction of pre-malignant cells. These findings suggest that HDGF is a candidate for use as a prognostic factor for disease-free and overall survival of patients with HCC.

HDGF is a unique nuclear targeted growth factor, which is expressed abundantly in HCC cells and stimulates their proliferation. HDGF generated tumors and promoted their growth *in vivo* via its mitogenic and angiogenic activity deriving from both its own direct angiogenic activity and the induction of VEGF. Multivariate analysis of the relationship of HDGF expression and recurrence-free and overall survival in patients with HCC suggest that HDGF become a significantly efficacious prognostic marker for HCC patients. We can expect that any tool regulating HDGF expression or HDGF signal pathways may be a useful candidate for the suppression of hepato-carcinogenesis and HCC progression in the future.

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Involvement of hepatoma-derived growth factor in the growth inhibition of hepatocellular carcinoma cells by vitamin K_2

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Background. Vitamin K_2 has been reported to suppress the growth of human hepatocellular carcinoma (HCC) in vitro and hepatocarcinogenesis in hepatitis C virus (HCV)-related cirrhosis in vivo. Hepatoma-derived growth factor (HDGF) is a unique nuclear targeting growth factor that is highly expressed in HCC cells and is a possible prognostic factor for patients with HCC. We investigated the regulation of HDGF expression by vitamin K_2. ***Methods.*** Three HCC-derived cell lines, HepG2, HuH-7, and SK-Hep-1, were used. Cell number was determined with the MTT assay. The expression levels of HDGF mRNA and protein were measured by the real-time reverse transcriptase-polymerase chain reaction (PCR) method and ELISA and Western blot analysis, respectively. The HDGF promoter activity was measured by a dual luciferase-reporter assay. ***Results.*** Vitamin K_2 suppressed the growth of the three HCC cell lines in a dose-dependent manner. Vitamin K_2 significantly suppressed the expression of the HDGF protein and mRNA in three cell lines. By a luciferase assay, vitamin K_2 significantly suppressed the promoter activity of the HDGF protein. Based on some luciferase-reporter plasmids containing truncated promoter regions, the possible responsive site of vitamin K_2 seems to reside in the region −1 to −150 bp of the HDGF gene. ***Conclusions.*** These findings suggested that regulation of the HDGF gene expression is one of the crucial mechanisms of vitamin K_2-induced cell growth suppression for HCC.

Key words: HDGF, HCC, vitamin K_2, luciferase assay

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Introduction

Hepatoma-derived growth factor (HDGF) is a unique nuclear targeting growth factor with heparin affinity that was purified and cloned from a human hepatocellular carcinoma (HCC) cell line.[1–4] HDGF has both oncogenic and angiogenic activity.[5,6] HDGF stimulates the proliferation of HCC cells, in addition to fibroblasts, endothelial cells, vascular smooth muscle cells, and fetal hepatocytes, after translocation to the nucleus by use of the bipartite nuclear localization signals.[1–9] HDGF is highly expressed in several cancers including HCC and is closely related to the aggressive biological potential of cancer cells.[10–16] A downregulation of HDGF by antisense oligonucleotides or siRNA treatment suppresses the proliferation of cancer cells that express HDGF endogenously.[14,17] Recently, a significant correlation has been shown between HDGF expression and the prognosis for the recurrence-free and overall survival in patients with HCC.[18,19] HDGF is considered to play an important role in both hepatocarcinogenesis and cancer progression. If HDGF expression is suppressed by drugs or chemical agents, then the growth of HCC cells should be regulated efficiently. However, the regulation mechanism of HDGF expression has not yet been clarified.

Vitamin K, an essential hydrophobic vitamin, and its derivatives have been shown to inhibit the proliferation of cancer cells including HCC.[20–23] However, the precise mechanism of their growth inhibitory action has not yet been clarified. Vitamin K consists of different forms, vitamin K_1–K_5. Vitamin K_2 (menaquinone) is produced by the intestinal flora and is used as an oral medication for patients with osteoporosis. The in vivo preventive effect of vitamin K_2 on the development of HCC, or the recurrence after treatment of HCC, in patients with HCV-related cirrhosis has been reported.[24,25] Recent in vitro studies have advocated some molecular mechanisms for the growth inhibition by vitamin K_2: a pathway

via protein kinase A activation, induction of the cell cycle-regulating proteins including p21, and reduced expression of the cyclin-dependent kinases.[26–29] However, these mechanisms cannot explain the entire suppressive effects of vitamin K_2 on the proliferation of HCC cells. Other unknown mechanisms have been suggested for the cell proliferation inhibitory effects of vitamin K. The transcriptional regulation of the growth factor genes or growth factor receptor genes by vitamin K_2 has not yet been reported.

In the present study, we investigated the regulation of HDGF expression by vitamin K_2 in HCC cells.

Materials and methods

Materials

Vitamin K_2 (menatetrenone, MK-4) was supplied from Eisai Co. (Tokyo, Japan). The human HCC cell lines HepG2, HuH-7, and SK-Hep-1 were purchased from American Type Culture Collection (ATCC). These cell lines 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 proliferation assays

The cells were seeded onto 96-well plates at a density of 2.5×10^3 cells. After a 24-h culture, 100 µl fresh medium containing different concentrations of vitamin K_2 (10, 30, and 100 µM) was added in each well. Vitamin K_2 was dissolved in 99% ethanol at the concentration of 10 mM and then diluted with DMEM to the appropriate concentrations for the experiments. Forty-eight hours later, the culture medium was replaced with fresh medium containing different concentrations of vitamin K_2. The control cells were cultured in DMEM containing the corresponding concentration of ethanol to each dose of the vitamin K_2. After a 4-day culture with vitamin K_2 treatment, the number of viable cells in each well was determined with the 3-(4,5-dimethyl-2-thiazolyl-2,5-diophenyl-2*H*-tetrazolium bromide) (MTT) assay (Roche, Nutley, NJ, USA) according to the manufacturer's instructions.

All experiments were carried out in four wells concurrently, and then were repeated three times.

Western blotting

After a 96-h culture with vitamin K_2, the 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 dodecyl sulfate (SDS), 100 µg/ml phenylmethylsulfonyl fluoride, 45 µg/ml aprotinin, 100 mM sodium orthovanadate]. The supernatant of the homogenate was used for protein determination with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and electrophoresis. The samples with 5 µg total protein were electrophoresed on a 12.5% SDS-polyacrylamide gel under reducing conditions and blotted to a polyvinylidene difluoride (PVDF) membrane by electroblotting. The membranes were blotted with the anti-C terminus of the HDGF polyclonal antibody at a dilution of 1:10000, which was generated by the New Zealand White rabbit.[3] The signals were developed with an ABC kit (Vector, Burlingame, CA, USA) and diaminobenzidine.

HDGF-overexpressing HepG2 cells

We constructed *myc*-tagged human HDGF in pEF-BOS plasmids and selected and cloned stable transfectants after transfection to HepG2 as described previously.[3]

HDGF-knock-down SK-Hep-1 cells by shRNA

SuperSilencing shRNA plasmid for human HDGF was purchased from SuperArray Bioscience Corporation (catalog number: KH10419N). SK-Hep-1 was seeded at 1×10^5 cells in 6-well plates with 2 ml 10% FBS-DMEM medium. Next day, 2 µg HDGF-shRNA and the negative control shRNA plasmids were transfected into the cells by 5.0 µl Lipofectamine 2000, according to the protocol from SuperArray. The transfected medium was changed by 10% FBS-DMEM with Geneticin (G418), 1200 mg/l, after 24 h. The G418 media were changed every 3 days. The knock-down expression of HDGF protein was confirmed by Western blot in SK-Hep-1 cells selected by G418 media.

A quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of HDGF mRNA levels

HDGF mRNA expression was measured by a quantitative real-time PCR according to the method previously reported.[18] In brief, the total RNA was extracted with the AGPC method using Isogen (Nippongene, Tokyo, Japan); 5 µg deoxyribonuclease I-treated total RNA was used for the reverse transcriptase reaction. An aliquot representing 100 ng input RNA was amplified by using a TaqMan PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM 7700 sequence Detection System (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s, and 60°C for 1 min. The forward primer 5′-AAGTTTGGCAAGCCCAACA-3′, reverse primer 5′-GGCTCTTCCACACAGCTCTTT-3′, and probe

5′-FAM-AACCCTACTGTCAAGGCTTCCGGCT-TAMRA-3′ were used for HDGF. As an internal control, beta-actin mRNA was used. The RNA extracted from HuH-7 cells was used as a standard. After reverse transcription (RT), standard complementary DNA (cDNA) was serially diluted to obtain five standard solutions for use in the PCR reaction to generate the reference curve. The relative amount of cDNA in each sample was measured by the interpolation of the standard curve, and then the relative ratio of the HDGF/beta-actin expression was calculated for each sample.

Luciferase assay of HDGF promoter activity

Constructs of luciferase-reporter plasmids of the HDGF promoter region. The DNA from the HuH-7 cells was extracted by the Isogen method. Thereafter, the DNA was digested by Tth111 I, purified by phenol/chloroform, and precipitated by ethanol. The HDGF promoter DNA was acquired by a nested PCR. First, 0.5 μg digested and purified DNA was amplified by the forward primer HDGF P5F5 (5′-TACGACATCAGGAGTTCG AAACCA-3′) and the reverse primer HDGF P3R (5′-TGCGCGCTCGTCGAGTTGTTTGT-3′) using a LA TAKARA Taq Kit (RR002A) (Takara, Kyoto, Japan). This PCR product was used as the template of the second amplification. The second PCR was done by series primer pairs designed by DNASIS software using a TAKARA Taq Kit. The DNA amplification was performed in the condition of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, for 35 cycles.

Plasmid constructs

The PCR products were purified, polished, and inserted into the pGL3 Basic luciferase-reporter vector predigested by *Sma*I, and transfected into *Escherichia coli* by a PCR Cloning Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. The isolated plasmids containing the desired HDGF promoters were verified by *Kpn*I plus *Xho*I digestions and sequenced using the RV Primer 3 from the 5′-end and the GL Primer 2 from the 3′-end.

Luciferase assay of luciferase-reporter plasmids

The HepG2 cells (2×10^5 cells/well) were seeded in a 6-well culture dish (Iwaki, Funabashi, Chiba, Japan) in phenol red-free DMEM containing 5% charcoal-dextran-stripped fetal bovine serum (FBS-CCS). The cells were transfected with 2 μg luciferase-reporter vectors by using a Fugene 6 transfection reagent kit (Roche), according to the manufacturer's recommendations. Twenty-four hours later, the culture media were changed to the fresh media with several concentrations (0, 10, 30, and 100 μM) of vitamin K_2. After incubation for 24 h, the cells were harvested and lysed with luciferase lysis buffer (Promega, Madison, WI, USA). The proteins were measured by a BCA protein assay kit. The luciferase activity of each sample was measured by a luciferase assay kit (Promega). The level of induction was calculated by dividing the mean luciferase activity of the samples treated with vitamin K_2 by the mean activity of the untreated control samples. All experiments were carried out in triplicate and repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA) of HDGF protein

The cells were lysed with the RIPA buffer, as described above. After centrifugation at 10000 rpm for 30 min, the supernatants of the cell lysate were used for the measurement of the HDGF protein by an ELISA. An ELISA for the HDGF was developed by the sandwich method using a monoclonal antibody and a polyclonal antibody against HDGF.

Statistical analysis

The results are expressed as the means ± SE. At least three separate experiments were performed for each data point. The statistical analyses were done using Student's unpaired *t* test (two tailed).

Results

Effect of vitamin K_2 on HCC cells

In patients whom vitamin K_2 are administered at clinically used doses, the serum concentration is calculated to reach to about 30 μM. Thus, we used three doses—10, 30, and 100 μM—of vitamin K_2 in the subsequent experiments. Vitamin K_2 suppressed the proliferation of three HCC cell lines in a dose-dependent manner. The inhibitory effects by vitamin K_2 after 96 h treatment at 30 μM and 100 μM are shown for the three HCC cell lines in Fig. 1.

HDGF was expressed in three cell lines (Fig. 2a). The intracellular HDGF amounts in these cell lines were measured by an ELISA (Fig. 2b). HCC cells with higher production of the HDGF protein seem to show higher inhibition of HCC cell proliferation by vitamin K_2, although not significantly. Some growth factors are involved in the proliferation of HCC cells. Next, we knocked down the HDGF expression and assessed its participating level on the proliferation of HCC cells. We obtained two stable HDGF-knock-down clones after transfection of HDGF-shRNA into SK-Hep-1 cells. In

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).

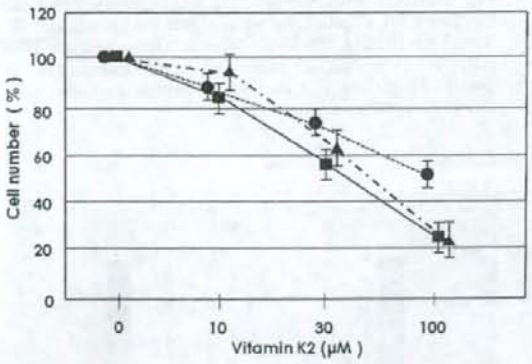

Fig. 1. Vitamin K_2 suppressed the proliferation of human hepatocellular cells dose-dependently. HepG2 (●), HuH-7 (▲), and SK-Hep-1 (■) were treated with various concentrations of vitamin K_2. After vitamin K_2 treatment for 96 h, the cell numbers were determined by the MTT method. Data are mean ± SE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

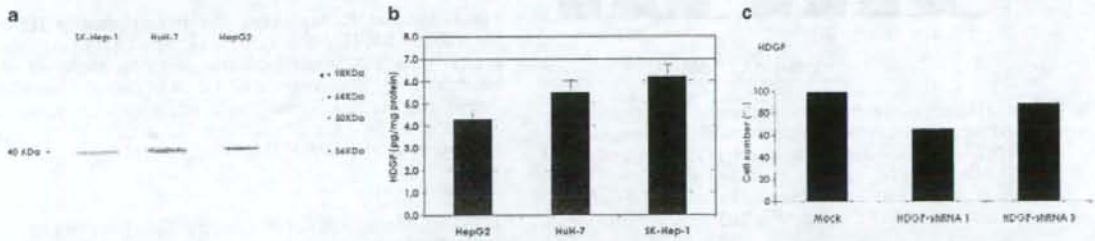

Fig. 2. Expression of hepatoma-derived growth factor (HDGF) protein in human HCC cell lines. Three HCC cell lines were lysed and vortexed with lysis buffer after 48 h culture. After centrifugation, the supernatants of each cell line were used for analysis. **a** Western blot analysis. The cell lysate 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

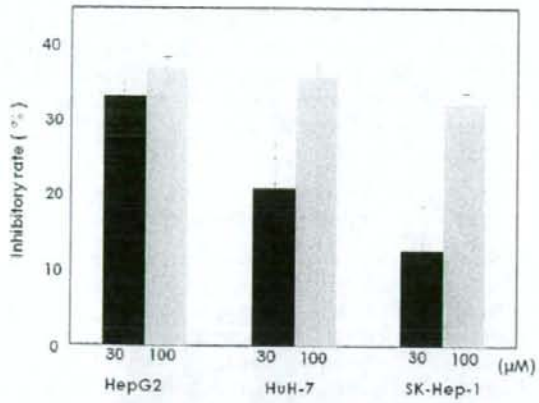

Fig. 3. HDGF protein expression in HCC cells was suppressed by vitamin K_2. Inhibitory rate of HDGF protein expression by vitamin K_2 is shown. Three HCC cell lines were treated with vitamin K_2 at the dose of 30 μM or 100 μM for 96 h. The HCC cells were lysed and vortexed with lysis buffer, and the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

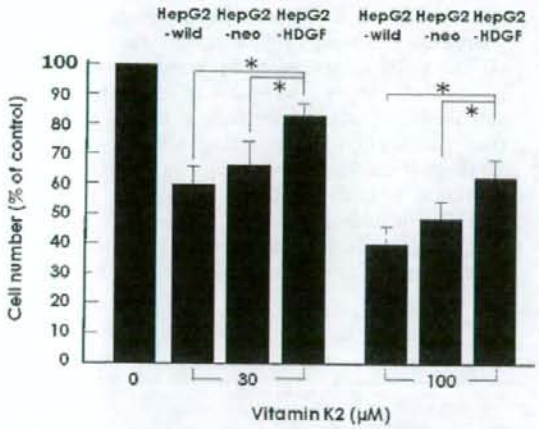

Fig. 4. HDGF overexpression recovered the vitamin K_2-induced suppression of cell proliferation. HDGF-overexpressing HepG2 (HepG2-HDGF), mock (HepG2-neo), and parent HepG2 (HepG2-wild) cells were treated with 30 or 100 μM vitamin K_2. Ninety-six hours later, cell numbers of each well were measured by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

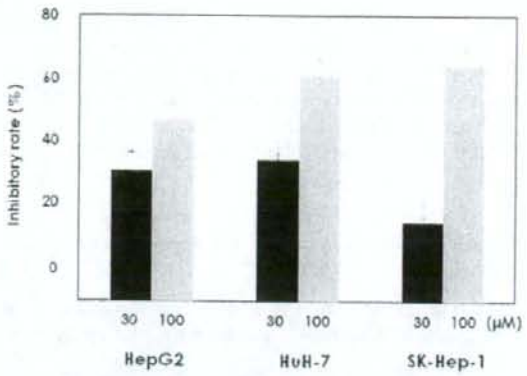

Fig. 5. HDGF mRNA expression was suppressed by vitamin K_2. Three HCC cell lines were treated with vitamin K_2 at 30 μM or 100 μM for 96 h. After RNA extraction, HDGF mRNA expression was measured by the quantitative real-time PCR method. The data are shown as the mean ± SE of three independent experiments

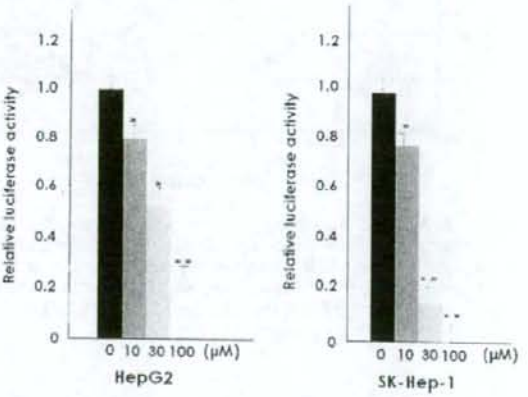

Fig. 6. Vitamin K_2 suppressed the transcription of HDGF. HepG2 and SK-Hep-1 were transfected with 1 μg promoter vector (pLuc-H2). After incubation with the indicated concentrations (μM) of vitamin K_2 for 24 h, the cells were harvested and the relative luciferase activities were measured. The data are 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-

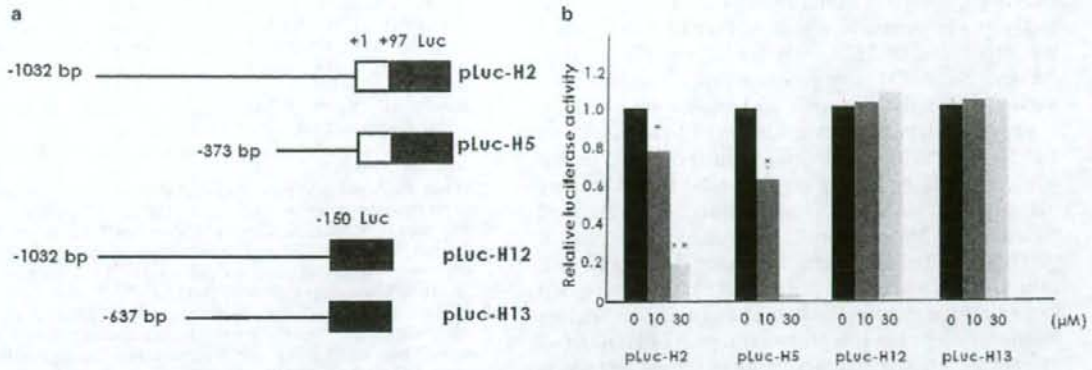

Fig. 7. Possible action site of vitamin K_2 in the promoter of the HDGF gene. **a** The construct of the luciferase-reporter assay plasmids for the HDGF promoter region. **b** SK-Hep-1 cell was transfected with 1 μg each promoter vector (pLuc-H2, -H5, -H12, -H13). After incubation with the indicated 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.

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Journal of Gastroenterology

Involvement of hepatoma-derived growth factor in the growth inhibition of hepatocellular carcinoma cells by vitamin K_2

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Background. Vitamin K_2 has been reported to suppress the growth of human hepatocellular carcinoma (HCC) in vitro and hepatocarcinogenesis in hepatitis C virus (HCV)-related cirrhosis in vivo. Hepatoma-derived growth factor (HDGF) is a unique nuclear targeting growth factor that is highly expressed in HCC cells and is a possible prognostic factor for patients with HCC. We investigated the regulation of HDGF expression by vitamin K_2. ***Methods.*** Three HCC-derived cell lines, HepG2, HuH-7, and SK-Hep-1, were used. Cell number was determined with the MTT assay. The expression levels of HDGF mRNA and protein were measured by the real-time reverse transcriptase-polymerase chain reaction (PCR) method and ELISA and Western blot analysis, respectively. The HDGF promoter activity was measured by a dual luciferase-reporter assay. ***Results.*** Vitamin K_2 suppressed the growth of the three HCC cell lines in a dose-dependent manner. Vitamin K_2 significantly suppressed the expression of the HDGF protein and mRNA in three cell lines. By a luciferase assay, vitamin K_2 significantly suppressed the promoter activity of the HDGF protein. Based on some luciferase-reporter plasmids containing truncated promoter regions, the possible responsive site of vitamin K_2 seems to reside in the region −1 to −150 bp of the HDGF gene. ***Conclusions.*** These findings suggested that regulation of the HDGF gene expression is one of the crucial mechanisms of vitamin K_2-induced cell growth suppression for HCC.

Key words: HDGF, HCC, vitamin K_2, luciferase assay

Introduction

Hepatoma-derived growth factor (HDGF) is a unique nuclear targeting growth factor with heparin affinity that was purified and cloned from a human hepatocellular carcinoma (HCC) cell line.[1–4] HDGF has both oncogenic and angiogenic activity.[5,6] HDGF stimulates the proliferation of HCC cells, in addition to fibroblasts, endothelial cells, vascular smooth muscle cells, and fetal hepatocytes, after translocation to the nucleus by use of the bipartite nuclear localization signals.[1–9] HDGF is highly expressed in several cancers including HCC and is closely related to the aggressive biological potential of cancer cells.[10–16] A downregulation of HDGF by antisense oligonucleotides or siRNA treatment suppresses the proliferation of cancer cells that express HDGF endogenously.[14,17] Recently, a significant correlation has been shown between HDGF expression and the prognosis for the recurrence-free and overall survival in patients with HCC.[18,19] HDGF is considered to play an important role in both hepatocarcinogenesis and cancer progression. If HDGF expression is suppressed by drugs or chemical agents, then the growth of HCC cells should be regulated efficiently. However, the regulation mechanism of HDGF expression has not yet been clarified.

Vitamin K, an essential hydrophobic vitamin, and its derivatives have been shown to inhibit the proliferation of cancer cells including HCC.[20–23] However, the precise mechanism of their growth inhibitory action has not yet been clarified. Vitamin K consists of different forms, vitamin K_1–K_5. Vitamin K_2 (menaquinone) is produced by the intestinal flora and is used as an oral medication for patients with osteoporosis. The in vivo preventive effect of vitamin K_2 on the development of HCC, or the recurrence after treatment of HCC, in patients with HCV-related cirrhosis has been reported.[24,25] Recent in vitro studies have advocated some molecular mechanisms for the growth inhibition by vitamin K_2: a pathway

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via protein kinase A activation, induction of the cell cycle-regulating proteins including p21, and reduced expression of the cyclin-dependent kinases.[26-29] However, these mechanisms cannot explain the entire suppressive effects of vitamin K_2 on the proliferation of HCC cells. Other unknown mechanisms have been suggested for the cell proliferation inhibitory effects of vitamin K. The transcriptional regulation of the growth factor genes or growth factor receptor genes by vitamin K_2 has not yet been reported.

In the present study, we investigated the regulation of HDGF expression by vitamin K_2 in HCC cells.

Materials and methods

Materials

Vitamin K_2 (menatetrenone, MK-4) was supplied from Eisai Co. (Tokyo, Japan). The human HCC cell lines HepG2, HuH-7, and SK-Hep-1 were purchased from American Type Culture Collection (ATCC). These cell lines 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 proliferation assays

The cells were seeded onto 96-well plates at a density of 2.5×10^3 cells. After a 24-h culture, 100 µl fresh medium containing different concentrations of vitamin K_2 (10, 30, and 100 µM) was added in each well. Vitamin K_2 was dissolved in 99% ethanol at the concentration of 10 mM and then diluted with DMEM to the appropriate concentrations for the experiments. Forty-eight hours later, the culture medium was replaced with fresh medium containing different concentrations of vitamin K_2. The control cells were cultured in DMEM containing the corresponding concentration of ethanol to each dose of the vitamin K_2. After a 4-day culture with vitamin K_2 treatment, the number of viable cells in each well was determined with the 3-(4,5-dimethyl-2-thiazolyl-2,5-diophenyl-2*H*-tetrazolium bromide) (MTT) assay (Roche, Nutley, NJ, USA) according to the manufacturer's instructions.

All experiments were carried out in four wells concurrently, and then were repeated three times.

Western blotting

After a 96-h culture with vitamin K_2, the 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 dodecyl sulfate (SDS), 100 µg/ml phenylmethylsulfonyl fluoride, 45 µg/ml aprotinin, 100 mM sodium orthovanadate]. The supernatant of the homogenate was used for protein determination with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and electrophoresis. The samples with 5 µg total protein were electrophoresed on a 12.5% SDS-polyacrylamide gel under reducing conditions and blotted to a polyvinylidene difluoride (PVDF) membrane by electroblotting. The membranes were blotted with the anti-C terminus of the HDGF polyclonal antibody at a dilution of 1:10000, which was generated by the New Zealand White rabbit.[3] The signals were developed with an ABC kit (Vector, Burlingame, CA, USA) and diaminobenzidine.

HDGF-overexpressing HepG2 cells

We constructed *myc*-tagged human HDGF in pEF-BOS plasmids and selected and cloned stable transfectants after transfection to HepG2 as described previously.[3]

HDGF-knock-down SK-Hep-1 cells by shRNA

SuperSilencing shRNA plasmid for human HDGF was purchased from SuperArray Bioscience Corporation (catalog number: KH10419N). SK-Hep-1 was seeded at 1×10^5 cells in 6-well plates with 2 ml 10% FBS-DMEM medium. Next day, 2 µg HDGF-shRNA and the negative control shRNA plasmids were transfected into the cells by 5.0 µl Lipofectamine 2000, according to the protocol from SuperArray. The transfected medium was changed by 10% FBS-DMEM with Geneticin (G418), 1200 mg/l, after 24 h. The G418 media were changed every 3 days. The knock-down expression of HDGF protein was confirmed by Western blot in SK-Hep-1 cells selected by G418 media.

A quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of HDGF mRNA levels

HDGF mRNA expression was measured by a quantitative real-time PCR according to the method previously reported.[18] In brief, the total RNA was extracted with the AGPC method using Isogen (Nippongene, Tokyo, Japan); 5 µg deoxyribonuclease I-treated total RNA was used for the reverse transcriptase reaction. An aliquot representing 100 ng input RNA was amplified by using a TaqMan PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM 7700 sequence Detection System (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s, and 60°C for 1 min. The forward primer 5′-AAGTTTGGCAAGCCCAACA-3′, reverse primer 5′-GGCTCTTCCACACAGCTCTTT-3′, and probe

5′-FAM-AACCCTACTGTCAAGGCTTCCGGCT-TAMRA-3′ were used for HDGF. As an internal control, beta-actin mRNA was used. The RNA extracted from HuH-7 cells was used as a standard. After reverse transcription (RT), standard complementary DNA (cDNA) was serially diluted to obtain five standard solutions for use in the PCR reaction to generate the reference curve. The relative amount of cDNA in each sample was measured by the interpolation of the standard curve, and then the relative ratio of the HDGF/beta-actin expression was calculated for each sample.

Luciferase assay of HDGF promoter activity

Constructs of luciferase-reporter plasmids of the HDGF promoter region. The DNA from the HuH-7 cells was extracted by the Isogen method. Thereafter, the DNA was digested by Tth111 I, purified by phenol/chloroform, and precipitated by ethanol. The HDGF promoter DNA was acquired by a nested PCR. First, 0.5 μg digested and purified DNA was amplified by the forward primer HDGF P5F5 (5′-TACGACATCAGGAGTTCG AAACCA-3′) and the reverse primer HDGF P3R (5′-TGCGCGCTCGTCGAGTTGTTTGT-3′) using a LA TAKARA Taq Kit (RR002A) (Takara, Kyoto, Japan). This PCR product was used as the template of the second amplification. The second PCR was done by series primer pairs designed by DNASIS software using a TAKARA Taq Kit. The DNA amplification was performed in the condition of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, for 35 cycles.

Plasmid constructs

The PCR products were purified, polished, and inserted into the pGL3 Basic luciferase-reporter vector predigested by *Sma*I, and transfected into *Escherichia coli* by a PCR Cloning Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. The isolated plasmids containing the desired HDGF promoters were verified by *Kpn*I plus *Xho*I digestions and sequenced using the RV Primer 3 from the 5′-end and the GL Primer 2 from the 3′-end.

Luciferase assay of luciferase-reporter plasmids

The HepG2 cells (2×10^5 cells/well) were seeded in a 6-well culture dish (Iwaki, Funabashi, Chiba, Japan) in phenol red-free DMEM containing 5% charcoal-dextran-stripped fetal bovine serum (FBS-CCS). The cells were transfected with 2 μg luciferase-reporter vectors by using a Fugene 6 transfection reagent kit (Roche), according to the manufacturer's recommendations. Twenty-four hours later, the culture media were changed to the fresh media with several concentrations (0, 10, 30, and 100 μM) of vitamin K_2. After incubation for 24 h, the cells were harvested and lysed with luciferase lysis buffer (Promega, Madison, WI, USA). The proteins were measured by a BCA protein assay kit. The luciferase activity of each sample was measured by a luciferase assay kit (Promega). The level of induction was calculated by dividing the mean luciferase activity of the samples treated with vitamin K_2 by the mean activity of the untreated control samples. All experiments were carried out in triplicate and repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA) of HDGF protein

The cells were lysed with the RIPA buffer, as described above. After centrifugation at 10000 rpm for 30 min, the supernatants of the cell lysate were used for the measurement of the HDGF protein by an ELISA. An ELISA for the HDGF was developed by the sandwich method using a monoclonal antibody and a polyclonal antibody against HDGF.

Statistical analysis

The results are expressed as the means ± SE. At least three separate experiments were performed for each data point. The statistical analyses were done using Student's unpaired *t* test (two tailed).

Results

Effect of vitamin K_2 on HCC cells

In patients whom vitamin K_2 are administered at clinically used doses, the serum concentration is calculated to reach to about 30 μM. Thus, we used three doses—10, 30, and 100 μM—of vitamin K_2 in the subsequent experiments. Vitamin K_2 suppressed the proliferation of three HCC cell lines in a dose-dependent manner. The inhibitory effects by vitamin K_2 after 96 h treatment at 30 μM and 100 μM are shown for the three HCC cell lines in Fig. 1.

HDGF was expressed in three cell lines (Fig. 2a). The intracellular HDGF amounts in these cell lines were measured by an ELISA (Fig. 2b). HCC cells with higher production of the HDGF protein seem to show higher inhibition of HCC cell proliferation by vitamin K_2, although not significantly. Some growth factors are involved in the proliferation of HCC cells. Next, we knocked down the HDGF expression and assessed its participating level on the proliferation of HCC cells. We obtained two stable HDGF-knock-down clones after transfection of HDGF-shRNA into SK-Hep-1 cells. In

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).

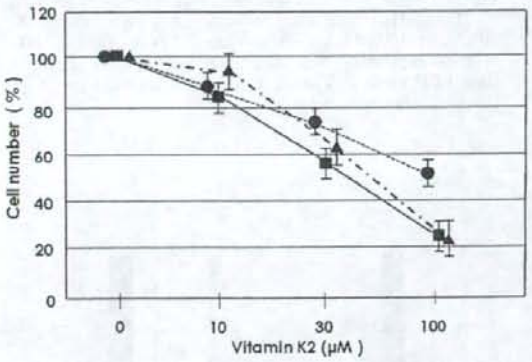

Fig. 1. Vitamin K_2 suppressed the proliferation of human hepatocellular cells dose-dependently. HepG2 (●), HuH-7 (▲), and SK-Hep-1 (■) were treated with various concentrations of vitamin K_2. After vitamin K_2 treatment for 96 h, the cell numbers were determined by the MTT method. Data are mean ± SE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

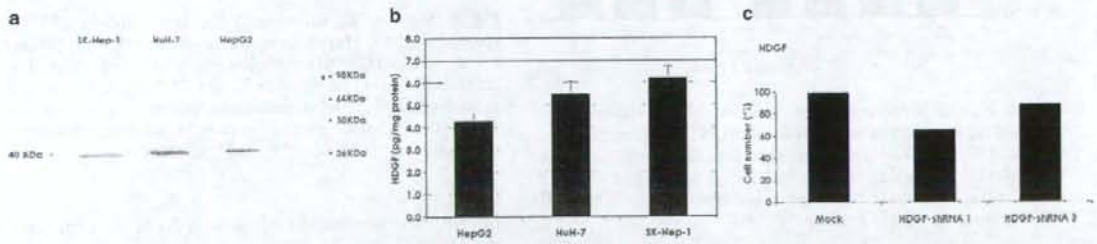

Fig. 2. Expression of hepatoma-derived growth factor (HDGF) protein in human HCC cell lines. Three HCC cell lines were lysed and vortexed with lysis buffer after 48 h culture. After centrifugation, the supernatants of each cell line were used for analysis. **a** Western blot analysis. The cell lysate 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

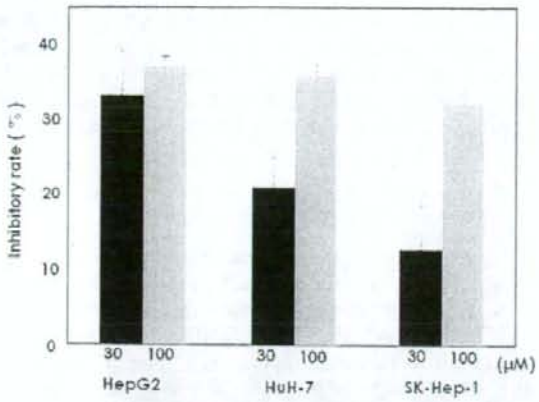

Fig. 3. HDGF protein expression in HCC cells was suppressed by vitamin K_2. Inhibitory rate of HDGF protein expression by vitamin K_2 is shown. Three HCC cell lines were treated with vitamin K_2 at the dose of 30 μM or 100 μM for 96 h. The HCC cells were lysed and vortexed with lysis buffer, and the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

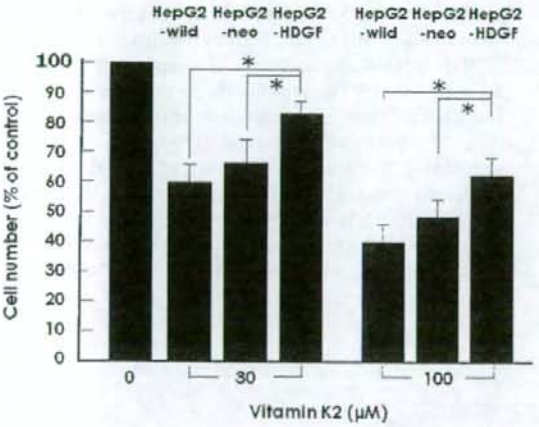

Fig. 4. HDGF overexpression recovered the vitamin K_2-induced suppression of cell proliferation. HDGF-overexpressing HepG2 (HepG2-HDGF), mock (HepG2-neo), and parent HepG2 (HepG2-wild) cells were treated with 30 or 100 μM vitamin K_2. Ninety-six hours later, cell numbers of each well were measured by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

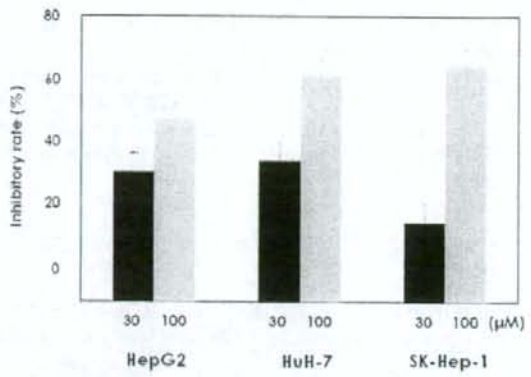

Fig. 5. HDGF mRNA expression was suppressed by vitamin K_2. Three HCC cell lines were treated with vitamin K_2 at 30 μM or 100 μM for 96 h. After RNA extraction, HDGF mRNA expression was measured by the quantitative real-time PCR method. The data are shown as the mean ± SE of three independent experiments

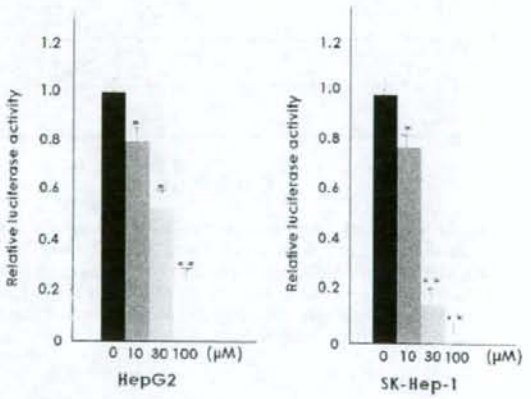

Fig. 6. Vitamin K_2 suppressed the transcription of HDGF. HepG2 and SK-Hep-1 were transfected with 1 μg promoter vector (pLuc-H2). After incubation with the indicated concentrations (μM) of vitamin K_2 for 24 h, the cells were harvested and the relative luciferase activities were measured. The data are 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-

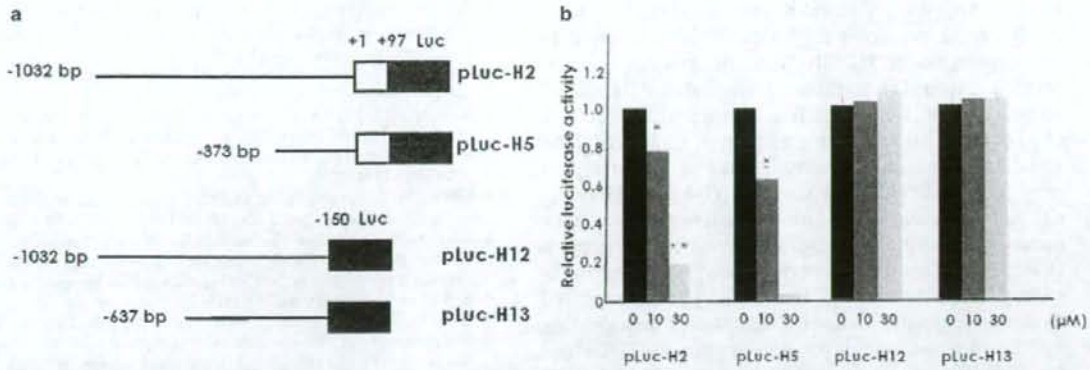

Fig. 7. Possible action site of vitamin K_2 in the promoter of the HDGF gene. **a** The construct of the luciferase-reporter assay plasmids for the HDGF promoter region. **b** SK-Hep-1 cell was transfected with 1 μg each promoter vector (pLuc-H2, -H5, -H12, -H13). After incubation with the indicated 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.

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