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Des-gamma-carboxy prothrombin increases the expression of angiogenic factors in human hepatocellular carcinoma cells

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ABSTRACT

Aims: Des-γ-carboxyl prothrombin (DCP) is a serum protein produced by hepatocellular carcinoma (HCC) cells. The aim of this study was to evaluate the angiogenic activity of DCP in HCC cells.
Main methods: The proliferation of HCC cells was measured by 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. The growth of HCC cells was also evaluated in vivo by using the xenografts in nude mice. The enzyme-linked immunosorbent assay (ELISA) was employed to measure the levels of angiogenic factors in supernatant of cell culture. The expression of angiogenic factors was examined by Western blot analysis and immunohistochemical staining.
Key findings: DCP displayed the stimulation of HCC cell growth in a dose (5–80 ng/ml) and time (24–96 h) dependent manner. The increase of cell growth was also observed in nude mice bearing well-established, palpable HepG2 and SMMC-7721 xenografts after 2 weeks administration of DCP. HCC cell growth was accompanied by the elevated levels of angiogenic factors. The levels of vascular endothelial growth factor (VEGF), transforming growth factor-alpha (TGF-α) and basic fibroblast growth factor (bFGF) in supernatant of SMMC-7721 cells were increased from 47, 126, and 60 pg/10^6 cells/24 h to 400, 208, and 298 pg/10^6 cells/24 h, respectively, after 72 h incubation with 80 ng/ml of DCP. The results of Western blot analysis and immunohistochemical staining of HCC xenografts also showed the significant increase of VEGF, TGF-α, and bFGF in HCC cells.
Significance: These results provide the information that DCP is a type of growth factor in progression of HCC.

Introduction

Emerging evidence has shown that tumor growth beyond a few millimeters is dependent on the induction of angiogenesis mediated by the release of angiogenic factors secreted by the tumor cells (Yoshiji et al., 2002; Inagaki et al., 2008). It is believed that angiogenic factors are secreted by cancer cells to increase the endothelial cell growth through paracrine mechanisms and contribute to the tumor progression via autocrine signaling stimulation (de Jong et al., 1998; Chakraborty et al., 2008). To date, many angiogenic factors that contribute to tumor development have been identified. Among these, vascular endothelial growth factor (VEGF), transforming growth factor-alpha (TGF-α), and basic fibroblast growth factor (bFGF), appear to be the most potent and best-characterized factors in the development of hepatocellular carcinoma (HCC) (Pang et al., 2008). VEGF activates the angiogenic switch in vivo and enhances vascular permeability (Lien and Lowman 2008). bFGF secreted by HCC cells may promote the growth and metastasis of HCC by an autocrine stimulatory effect on tumor cell proliferation and motility (Poon et al., 2001). TGF-α is a major factor in the activation of HCC and endothelial cell growth, migration and capillary tube formation during the invasion of HCC (Pang et al., 2008). Therefore, these angiogenic factors have been well documented for tumor angiogenic phenotype in HCC.

Des-γ-carboxyl prothrombin (DCP) is a well recognized tumor marker for its high sensitivity and specificity in the screening and diagnosis of hepatocellular carcinoma (HCC) (Kim do et al., 2007; Shirabe et al., 2007). Reportedly, 44–81% of hepatocellular carcinoma patients have elevated serum DCP levels (Yuen and Lai 2005). High level of DCP correlates with the presence of vascular invasion or intrahepatic metastases (Hakamada et al., 2008). However, the role of DCP in HCC development is poorly understood. It is hypothesized that as DCP is secreted from HCC cells, DCP might work as an autologous growth factor for hepatocellular carcinoma development (Suzuki

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et al., 2005). Moreover, DCP might work as a paracrine interaction factor between HCC cells and vascular endothelial cells (Fujikawa et al., 2007). To our knowledge no studies describe the role of DCP in promoting the expression of angiogenic factors in human hepatocellular carcinoma cells. Herein, we assessed the effect of DCP on stimulation of HCC cell growth and expression of angiogenic factors.

Materials and methods

DCP

Des-gamma-carboxy prothrombin (DCP) is a gift from Eisai Co., Ltd., Tokyo, Japan. The DCP was purified from the DCP-producing cell line PLC/PRF/5 in the conditioned media by affinity chromatography with an anti-prothrombin antibody (Suzuki et al., 2005). DCP could be distinguished from normal prothrombin by high performance liquid chromatography (HPLC) analysis (Suzuki et al., 2005).

Cell lines and cell culture

The human hepatocellular carcinoma cell lines HepG2 and SMMC-7721 were purchased from Shanghai Cell Bank, the Institute of Cell Biology, China National Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin–streptomycin (100 IU/ml–100 μg/ml), 2 mM glutamine, and 10 mM Hepes buffer at 37 °C in a humid atmosphere (5% CO_2–95% air) and were harvested by brief incubation in 0.02% EDTA-PBS.

Determination of DCP levels produced by HCC cells

Cells (3×10^5 per well) seeded in 6-well plates were incubated at 37 °C in a humid atmosphere (5% CO_2–95% air) for the indicated time. DCP levels produced by HCC cells were determined by an electrochemiluminescence immunoassay (Picolumi PIVKA-II™; Eisai Co., Ltd., Tokyo, Japan) every 24 h for five consecutive days (Suzuki et al., 2005). The electrochemiluminescence immunoassay method uses a mouse monoclonal anti-DCP antibody coated on solid phase beads and a rabbit polyclonal anti-prothrombin that has been ruthenylated. An electrochemically triggered light reaction was quantified by an electrochemiluminescence detection system (Suzuki et al., 2005).

Cell proliferation assay

Cells ($2–5\times10^4$ per well) seeded in 96-well plates for 12 h were treated with different concentrations of DCP or human normal prothrombin (Sigma, St Louis, MO, USA) for the indicated time. The medium was then removed and the wells were washed with phosphate buffered saline (PBS). MTT assay was performed by adding 20 μl MTT (5 mg/ml, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma, St Louis, MO, USA) for 4 h (Choi et al., 2007). Light absorbance of the solution was measured at 540 nm on a plate reader (TECAN, Harrogate, UK). Triplicate experiments with triplicate samples were performed.

HCC cell xenografts in nude mice

HCC cell growth was assessed in nude mice after administration with DCP. Balb/c athymic (nu+/nu+) female mice, 4–6 weeks of age, were purchased from Experimental Animal Laboratory of China Academy of Medical Sciences (Beijing, China). Animals were housed under pathogen-free conditions. HCC cells (1×10^7) were suspended in 100 μl of Matrigel (Collaborative Biomedical, Bedford, MA, USA) and were injected subcutaneously into the right anterior flank of mice. After 2 weeks, when established tumors of approximately 0.1 to 0.2 cm^3 in diameter were detected, 8 mice/group were administered via tail vein on days 1–6 of each week for two weeks DCP at the indicated doses. Tumor growth stimulation rates were defined as a ratio to the control tumor weight (Liu et al., 2007).

ELISA assay

The secretion of VEGF, TGF-α, and bFGF was evaluated using commercially available sandwich ELISA kits according to the manufacturer's instructions. The ELISA kits for VEGF and for bFGF were purchased from R&D Systems (Minneapolis, MN, USA). The ELISA kit for TGF-α was purchased from Oncogene Research Products (Cambridge, MA, USA). HCC cells were plated in 6-well plates and treated for 72 h with different concentrations of DCP (Lai et al., 2007). Assays were performed using 24-h-collected, serum-free medium. Results were normalized for the number of producing cells and reported as pg of growth factors/10^6 cells/24 h.

Western blot analysis

The expression of angiogenic factors was evaluated using Western blot analysis. Cells (3×10^5) seeded in 6-well plates were treated with different concentrations of DCP for 72 h. The medium was removed and the cells were washed with PBS. Cells were lysed with 200 ml of 5 g/L SDS, and centrifuged at 10 000 ×*g*. Total protein was determined using Bradford method (Okutucu et al., 2007). Equal amounts of protein in the cell extracts were fractionated by 10% SDS-PAGE and then electrotransferred onto nitrocellulose membranes. After blocking with TBST buffer (20 mM Tris-buffered saline and 0.1% Tween) containing 5% nonfat dry milk for 1 h at room temperature, the membranes were incubated with primary antibodies for 2 h, which was followed by washing for 3 times and reaction with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) for 1 h at room temperature. The primary antibodies included mouse monoclonal antibody against human VEGF (sc-7269), mouse monoclonal antibody against human TGF-α (sc-36), and goat polyclonal antibody against human bFGF (sc-1359) (Santa Cruz Biotechnology, USA). The bound antibodies were visualized using an ECL system (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified by densitometry using an electrophoresis image analysis system (FR980, Shanghai Furi Science & Technology, Shanghai, China) (Prasad et al., 2007).

Immunohistochemical analysis

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections (5 μm) of HepG2 and SMMC-7721 xenografts as reported previously (Tang et al., 2003; Mirza et al., 2007). After overnight incubation with the appropriate primary antibody at 4 °C, sections were washed and treated with an appropriate secondary biotinylated antibody (1:200 dilution; Bo Shide, Wu Han, China), washed, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide, as described previously (Ciardiello et al., 2000). The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. The following antibodies were used: an anti-VEGF mouse monoclonal antibody, an anti-TGF-α mouse monoclonal antibody, and an anti-bFGF goat polyclonal antibody (Santa Cruz Biotechnology, USA). To determine the percentage of positive cells, at least 1000 cancer cells/slide were counted and scored. Both the percentage of specifically stained cells and the intensity of immunostaining were recorded, as reported previously (Ciardiello et al., 2000, 2001). New blood vessels were detected as previously described by Weidner et al. (1991), using a monoclonal antibody raised against human CD34 (ZB-0046, Santa Cruz Biotechnology, USA). Each slide was scanned at low power (×10–100), and the area with the higher number of new vessels was identified (hot spot). This region

was then scanned at ×200. Five fields were analyzed, and for each of them, the number of stained blood vessels was counted. For individual tumors, MVD was scored by averaging the five field counts (Ciardiello et al., 2001; Khorana et al., 2007).

Statistical analysis

Statistical significance was determined by the Student's two-tailed *t*-test. The limit of statistical significance was $P<0.05$.

Results

Expression of DCP by HCC cell lines

The endogenous level of DCP in each cell line was determined using an electrochemiluminescence immunoassay. Low levels of DCP were measured in the supernatant of HepG2 and SMMC-7721 cells culture. Fig. 1 showed the baselines of DCP production by individual cell line after five days of culture. Significant differences were seen between the two cell lines ($P<0.05$). Cells cultured at 120 h, HepG2 and SMMC-7721 cells produced DCP at rates of 0.76±0.1 and 0.53±0.11 (ng/ml/10^6 cells), respectively. Normal human hepatocyte line HL-7702 did not produce a detectable DCP level (data not shown).

DCP increases the proliferation of HCC cell lines

To investigate the mitogenic effect of DCP, HepG2 and SMMC-7721 were examined using MTT assay. As shown in Fig. 2, HCC cell growth was increased in a dose (5–80 ng/ml) and time (24–96 h) dependent (Fig. 2). This effect reached a plateau with a DCP stimulus of 160 ng/ml at 120 h incubation. In SMMC-7721 lower level DCP producing cells, proliferation rates at 72 h incubation were increased by 20.1, 26.8, 32.9, 42.8, 54.3 and 60.1% respectively (Fig. 2A), whereas DCP increased HepG2 cell proliferation by 13.8, 20.7, 25.7, 27.1, 35.7 and 41.1% respectively at the same concentrations (Fig. 2B).

The mitogenic effect of DCP on human hepatoma cell xenografts in nude mice was then examined. As shown in Table 1, the growth of HepG2 and SMMC-7721 cells transplanted into mice was significantly increased by administration of DCP in a dose-dependent manner. The mitogenic effect on human hepatoma cell xenografts was also more pronounced in SMMC-7721 than in HepG2 cells. Normal prothrombin did not affect cell proliferative activity in the experiment (data not shown).

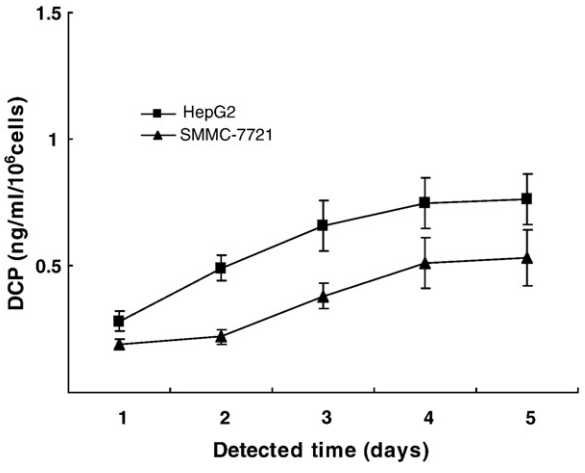

Fig. 1. The production of DCP by HepG2 and SMMC-7721 cells. HCC cells were cultured for the indicated time and DCP levels were determined using an electrochemiluminescence immunoassay as described in Materials and methods. The bars indicate means±S.D. ($n=3$).

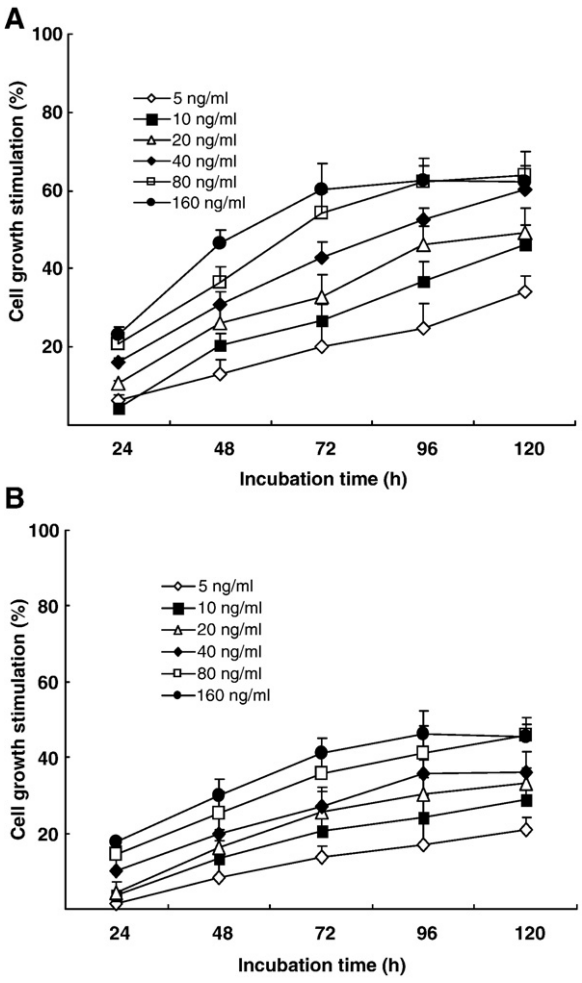

Fig. 2. DCP increases the growth of SMMC-7721 (A) and HepG2 (B) cells. HCC cells were cultured in the presence of DCP (5, 10, 20, 40, 80 and 160 ng/ml) for up to 120 h. Cell growth was evaluated by MTT assay and the percentage of stimulation was calculated by comparing to the untreated control at the concurrent time point. The bars indicate means±S.D. ($n=3$).

DCP increases the expression of angiogenic factors

We first measured the levels of angiogenic factors in HepG2 and SMMC-7721 cells by ELISA assay. Both VEGF, TGF-α and bFGF were

Table 1
The growth of HCC cell xenografts in nude mice and immunohistochemical analysis of angiogenic proteins after treatment with DCP ($n=8$)

Cell line	Dosage (μg/kg)	Tumor weight (g; mean±SD)	Tumor growth stimulation (%)	% positive cells (±SD)			
				VEGF	TGF-α	bFGF	CD34*
HepG2							
	0	0.92±0.37	–	38±6	30±3	44±3	14±3
	20	1.16±0.27[a]	26.1	56±5[b]	46±5[a]	64±3[b]	19±2[a]
	40	1.31±0.43[b]	42.4	68±8[b]	64±3[b]	66±5[b]	26±1[b]
SMMC-7721							
	0	0.83±0.52	–	26±3	33±4	28±5	12±1
	20	1.11±0.40[a]	45.8	51±6[b]	58±6[b]	44±6[b]	20±1[a]
	40	1.39±0.39[b]	67.5	68±5[b]	70±8[b]	64±5[b]	30±2[b]

Analysis was performed on day 28 after tumor cell injection. The percentage (±SD) of specifically stained HCC cells for VEGF, TGF-α, and bFGF was recorded. To determine the percentage of positive cells, at least 1000 HCC cells/slide were counted and scored. The number of microvessels for field (±SD) was measured using a monoclonal antibody raised against human CD34 and was scored by averaging five field counts of three individual tumors for each group.
[a]$P<0.05$, [b]$P<0.01$ *vs.* untreated group.
* Figures for CD34 are number of positively staining microvessels.

Table 2
The expression of VEGF, TGF-α, and bFGF in HCC cell lines after treatment with DCP

Dose of DCP (ng/ml)	HepG2 (pg/10^6 cells/24 h)			SMMC-7721 (pg/10^6 cells/24 h)		
	VEGF	TGF-α	bFGF	VEGF	TGF-α	bFGF
0	67±8	168±21	87±16	47±10	126±17	60±12
5	71±12	189±41[a]	98±23[a]	52±9	102±36	68±11[a]
10	102±16[b]	231±55[b]	102±36[b]	64±12[a]	150±41[a]	105±25[b]
20	123±23[b]	301±61[b]	200±45[b]	203±26[b]	169±31[b]	136±40[b]
40	206±14[b]	332±42[b]	261±51[b]	273±39[b]	197±40[b]	251±22[b]
80	261±32[b]	421±69[b]	316±28[b]	400±61[b]	208±21[b]	298±36[b]
160	321±45[b]	409±71[b]	351±27[b]	415±62[b]	241±36[b]	321±41[b]

The secretion of VEGF, TGF-α, and bFGF was determined on 24-h-collected serum-free medium by using ELISA kits, as described in "Materials and methods." Results were normalized for the number of producing cells and reported as pg of growth factors/10^6 cells/24 h.
[a]$P<0.05$, [b]$P<0.01$ *vs.* untreated group.

secreted by the two cell lines at low levels. DCP treatment resulted in a markedly increased secretion of the three angiogenic factors in a dose-dependent manner. As shown in Table 2, the levels of VEGF, TGF-α, and bFGF were 47, 126, and 60 pg/10^6cells/24 h, respectively, in SMMC-7721 cells under control conditions, while the levels were increased to 400, 208, and 298 pg/10^6cells/24 h after 72 h of exposure to 80 ng/ml, respectively. A similar dose-dependent increase in the secretion of angiogenic factors was observed in HepG2 cells exposed to DCP (Table 2).

We then evaluated the expressions of angiogenic proteins in HCC cells using Western blotting analysis. Figs. 3 and 4 showed the expressions of VEGF, TGF-α and bFGF in HepG2 and SMMC-7721 cells exposed to DCP. A dose-dependent increase was observed within the concentrations (10 to 160 ng/ml) after 72 h incubation with DCP. In HepG2 cells, the rates of stimulation were 7.6, 12.8, 65.3, 67.1, and 86.7%, respectively, for VEGF (Fig. 3A); 5.3, 10.1, 33.6, 70.1, and 76.1%, respectively, for TGF-α (Fig. 3B), and 8.7, 10.2, 21.3, 31.9, and 76.5%, respectively, for bFGF (Fig. 3C). The similar results in the expressions of VEGF, TGF-α and bFGF were also observed in SMMC-7721 cells (Fig. 4A, B, C).

Finally, we examined whether DCP induced high expressions of angiogenesis in hepatoma cells bearing in nude mice. DCP increased HCC cells proliferation with a parallel increase of angiogenic proteins, as assessed by immunohistochemical staining. As shown in Table 1,

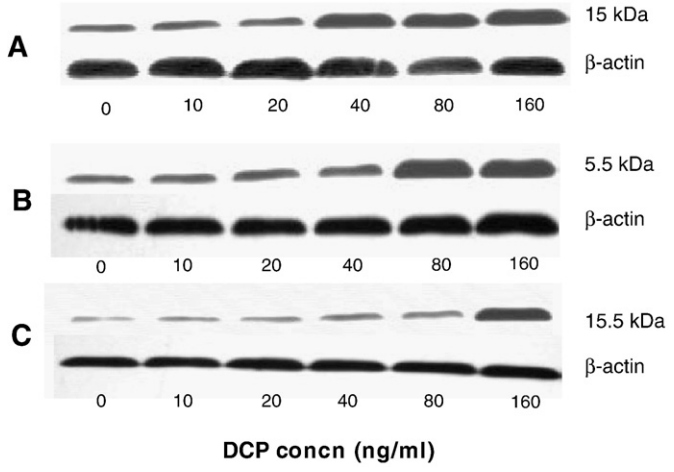

Fig. 3. Western blot analysis of VEGF (A), TGF-α (B), and bFGF (C) in HepG2 cells. Total cell proteins (50 μg) were fractionated through 10% SDS-PAGE, transferred to nitrocellulose filters, and incubated with specific anti-VEGF antibody, anti-TGF-α antibody, and anti-bFGF antibody, respectively. Immunoreactive proteins were visualized using an ECL system. The rates of increase of angiogenic proteins were estimated by comparing to the untreated control for each individual protein.

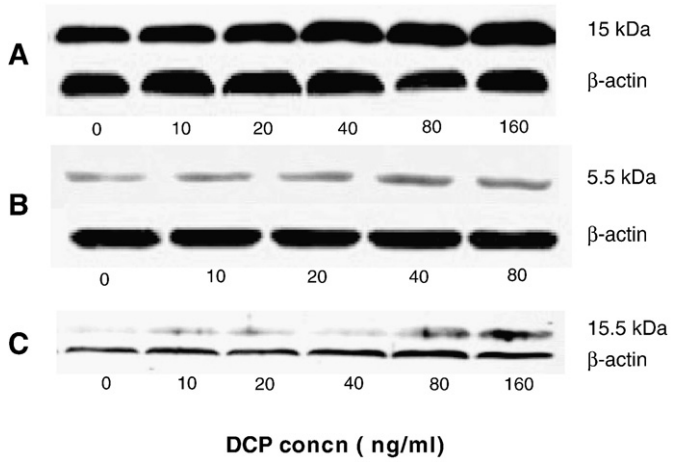

Fig. 4. Western blot analysis of VEGF (A), TGF-α (B), and bFGF (C) in SMMC-7721 cells. Total cell proteins (50 μg) were fractionated through 10% SDS-PAGE, transferred to nitrocellulose filters, and incubated with specific anti-VEGF antibody, anti-TGF-α antibody, and anti-bFGF antibody, respectively. Immunoreactive proteins were visualized using an ECL system. The rates of increase of angiogenic proteins were estimated by comparing to the untreated control for each individual protein.

the levels of VEGF, TGF-α, and bFGF, which were performed on xenografts after two weeks DCP stimulation, revealed a marked and dose-dependent increase in the percentage of positive HCC cells for all three angiogenic factors. The rates of positive cells were well correlated with the increase of tumor growth induced by DCP (Table 1). DCP-induced vascularization was quantified by immunohistochemistry as microvessel count (MVD) in the areas of most intense neovascularization, using an anti-CD34 monoclonal antibody. As shown in Table 1, DCP stimulation substantially increased MVD in a dose-dependent manner.

Discussion

This study involves the assessment of DCP, the prothrombin precursor, as autocrine/paracrine mitogen for hepatocellular carcinoma. Incubation of HCC cells with DCP increases the proliferation of tumor cell growth as well as the expressions of VEGF, TGF-α and bFGF, which are angiogenic growth factors for endothelial cells. In this respect, the increase of HCC cell growth and a high score of tumor microvessel density (MVD) by CD34 immunostaining were observed in hepatoma cells bearing in nude mice.

DCP is a prothrombin precursor with no coagulation activity (Naraki et al., 2002). The precise mechanism underlying DCP production and the role of DCP in HCC development are still poorly understood. Prothrombin is synthesized in the liver depending on the presence of vitamin K-dependent γ-glutamyl carboxylase (Huisse et al., 1994). The prothrombin precursor has 10 Glu residues in the N terminus that are converted into γ-carboxy-glutamic acid (Gla) residues by γ-glutamyl carboxylase (Liebman 1989). All of these Glu residues must be converted into Gla residues before prothrombin can obtain coagulation activity (Uehara et al., 1999). In DCP, not all of the 10 Gla residues are transformed. Instead, some remain as Glu residues (Uehara et al., 1999). Therefore, low levels of DCP were measured in the supernatant of HepG2 and SMMC-7721 cells culture. Previously, DCP was found to stimulate DNA synthesis of Hep3B and SK-Hep-1 cells (Sugimoto et al., 2003). In the present study, we demonstrated the increase of HCC cell growth after DCP stimulation in vitro and xenografts in nude mice. Normal prothrombin did not increase the proliferative activity of HepG2 and SMMC-7721 cells and DCP weakly affected normal human hepatocytes HL-7702 growth (data not shown). These results suggest that DCP is a potential growth factor for hepatocellular carcinoma.

Growth factors regulate cancer development through several mechanisms. These include uncontrolled cell growth attributable to the autocrine production of growth factors by cancer cells and stimulation of tumor neovascularization as a result of paracrine stimulation of endothelial cells by angiogenic growth factors secreted by cancer cells (Bonfil et al., 2007; Weigand et al., 2005). From that point of view, we assessed the DCP-induced expressions of VEGF, TGF-α and bFGF in HCC carcinoma. The elevated angiogenic factors may contribute to the progression and metastasis of HCC through many pathways. It is reported that bFGF accelerated the proliferation of KYN-2, KYN-3, HAK-1A, and HAK-1B HCC cells and the secretion of VEGF (Yamaguchi et al., 1998). TGF-α may take an important role in the proliferation of HCC carcinoma via binding its receptor EGFR in HCC tissue (Yu et al., 2007; Okano et al., 2006). VEGF is a potent and specific mitogen for endothelial cells that activates the angiogenic switch in vivo and enhances vascular permeability (Lien and Lowman 2008). Therefore, administration of DCP in mice bearing established xenografts of the human HCC carcinoma determines the growth stimulation that is accompanied by a promotion in bFGF, TGF-α and VEGF production by cancer cells. The high score of MVD by CD34 immunostaining was observed.

HCC is one of the most hypervascular tumors, and angiogenesis contributes to its malignant biological characteristics like vascular invasion, which results in metastasis and a high rate of recurrence even with surgical resection (Pang et al., 2008). Pathological correlation studies have demonstrated that high VEGF expression in HCC is associated with a high proliferative index, poor encapsulation of tumors, and venous tumor emboli portal vein thrombosis (Pang et al., 2008). Recent studies have shown that a high tumor microvessel density was associated with worse disease-free survival and early recurrence after resection of HCC (Poon et al., 2002). In the present study, DCP increases tumor microvessel density and the expression of angiogenic factors in HCC cells, suggesting that these angiogenic proteins may provide additional prognostic information in the detection of HCC.

The mechanism of stimulation of DCP on tumor angiogenesis has remained unknown. In searching for the structure of DCP, there are two kringle domains similar to those of hepatocyte growth factor (HGF), which has been identified as a member of angiogenic growth factors in HCC cells (Suzuki et al., 2005). Kringle domains are mandatory for HGF to bind with c-Met (HGF receptor), suggesting that c-Met may involve in the downstream signaling pathway of DCP (Suzuki et al., 2005). It was previously reported that DCP binds to c-Met and cause its autophosphorylation in Tyr1234/1235 site (Suzuki et al., 2005). JAK-STAT (Janus kinase-signal transducers and activators of transcription) signaling pathway was also found to be the downstream pathway of DCP because both STAT3 inhibitor peptide and siRNA against STAT3 abrogated DCP-induced cell proliferation as well as STAT3-induced transcription (Suzuki et al., 2005). Therefore, the DCP-Met-JAK1-STAT3 pathway is considered to be a signaling pathway for expression of angiogenic factors in HCC cells (Suzuki et al., 2005). Further study is needed to elucidate the biological effects induced by DCP in different cancer cells.

In conclusions, the results of the present study demonstrate that the mitogenic activity of DCP is accompanied by significant angiogenic activity in HCC cells. These results provide the information about the role of DCP in progression of HCC. Further work on mechanism of action of DCP is warranted.

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