EUROPEAN JOURNAL OF CANCER 47 (2011) 1115–1124

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Des-γ-carboxyl prothrombin induces matrix metalloproteinase activity in hepatocellular carcinoma cells by involving the ERK1/2 MAPK signalling pathway

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ARTICLE INFO

Article history:
Received 24 November 2010
Received in revised form 18 January 2011
Accepted 26 January 2011
Available online 23 February 2011

Keywords:
Des-γ-carboxy prothrombin
Hepatocellular carcinoma
Matrix metalloproteinase
ERK1/2 MAPK

ABSTRACT

Des-γ-carboxy prothrombin (DCP), an aberrant prothrombin produced by hepatocellular carcinoma (HCC) cells, has been shown to be associated with the biological malignant potential of HCC. The aim of this study was to evaluate the effect of DCP on HCC cell growth and metastasis, and to explore the underlying molecular mechanisms. DCP significantly stimulated HCC cell growth, as measured by cell counting kit-8 assay. Transwell chamber assay showed that DCP increased HCC cell migration through reconstituted extracellular matrix (Matrigel). Gelatin zymography assay and Western blot analysis demonstrated that DCP increased the secretion and expression of matrix metalloproteinase (MMP)-2 and MMP-9 in the supernatant of cultured HCC cells and on tumour cell membranes. DCP was found to bind to the cell surface receptor Met, resulting in Met phosphorylation and subsequent activation of the epidermal growth factor receptor (EGFR). Western blot analysis demonstrated that DCP stimulated a sequential kinase phosphorylation cascade including ERK1/2, MEK1/2 and c-Raf, indicating activation of the extracellular signal-regulated kinase/mitogen activated protein kinase (ERK1/2 MAPK) signalling pathway. Furthermore, blocking ERK1/2 MAPK activation with ERK1/2 inhibitor PD98059 essentially abolished the DCP-induced MMP-2 and MMP-9 activity, confirming the signalling pathway of DCP stimulation. Taken together, these results suggested that DCP stimulates HCC growth and promotes HCC metastasis by increasing the activity of MMP-2 and MMP-9 through activation of the ERK1/2 MAPK signalling pathway.

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1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide in terms of number of cases (626,000/year) but because of its very poor prognosis, it is the third most common cause of death from cancer (598,000/year).[1] Although advancement in surgical techniques has improved the immediate post-surgical survival, the long-term prognosis remains

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doi:10.1016/j.ejca.2011.01.017

disappointing because of the high incidence of intrahepatic metastasis and of recurrence of the neoplasm after surgical removal.[2,3] Malignant progression and metastasis of HCC has been shown (by us and others) to be associated with the activity of matrix metalloproteinases (MMP)-2 and MMP-9 in tumour cells.[3–5] Understanding the molecular processes that regulate tumour cell expression of these MMPs subtypes could potentially lead to the development of more effective therapeutic strategies that might eventually improve the long-term survival of patients with HCC.[6]

Des-γ-carboxy prothrombin (DCP, also known as protein induced by vitamin K absence or antagonist-II, PIVKA-II) is an aberrant prothrombin that lacks the ability to interact with other coagulation factors. The difference between normal prothrombin and DCP is the composition of amino acid residues.[7,8] The exact cause of DCP production in HCC tissues has not been understood as yet. The prothrombin molecule has some functional domain structures, and there are 10 γ-carboxylated glutamic acid (Gla) residues in the N-terminal domain, called the Gla domain. These Gla residues are originally glutamic acid (Glu) residues in prothrombin precursor and these residues are completely synthesised by the vitamin K-dependent enzymatic reaction of γ-glutamyl carboxylase as post-translational modification. When this reaction is insufficient in conditions like a vitamin K deficiency, DCP with Glu residues remaining in the Gla domain without γ-carboxylation is expressed and secreted extracellularly.[9–11] Since Liebman reported an increased serum DCP level in patients with HCC,[12] DCP has been found to be a useful diagnostic marker of HCC. Elevated serum DCP levels have been found in 44–81% of HCC patients.[13] Recently, several reports revealed that DCP may play important roles in determining the growth and invasion ability of HCC. High level of serum DCP has been associated with large tumour size.[13,14] Tumour recurrence and metastasis are more frequent in HCC patients with positive DCP than in HCC patients with negative DCP.[14] High level of serum DCP is related to portal vein invasion and/or intrahepatic metastasis, which significantly affects prognosis for patients with HCC.[15] Several basic studies showed that the levels of DNA synthesis in HCC cell lines were significantly increased by adding DCP and this increase was more marked in non-DCP-producing cell lines than in DCP-producing cell lines.[11] Further studies revealed that DCP may have the ability to bind with Met and this binding causes Met autophosphorylation and subsequent activation of the STAT3 signalling pathway through Janus kinase 1.[16,17] Therefore, DCP has been considered as a potential growth factor in HCC proliferation and may play significant roles in cancer progression. In a previous study, we observed the proliferation of HCC cells HepG2 and SMMC-7721 upon exposure to DCP both *in vitro* and *in vivo*.[18] HCC proliferation was accompanied by the elevated levels of angiogenic factors including vascular endothelial growth factor (VEGF), transforming growth factor-alpha (TGF-α) and basic fibroblast growth factor (bFGF).[18] DCP was also found to increase the migration of human umbilical vein endothelial cells and to activate MMP-2.[19] However, the role of DCP in the malignant progression of HCC has not been completely defined and the mechanism underlying the processes of tumour cell proliferation and metastasis has remained largely unknown.

In this study, we first examined the stimulatory effect of DCP on HCC cell growth and migration within reconstituted extracellular matrix (Matrigel). We then investigated the effect of DCP on tumour cell production of MMP-2 and MMP-9. Furthermore, we evaluated the involvement of the ERK1/2 MAPK signalling pathway in the process of DCP-induced MMP-2 and MMP-9 activity. Findings from this study provide insights into the molecular mechanisms of the mitogenic and pro-metastatic effects of DCP on HCC cells.

2. Materials and methods

2.1. Des-γ-carboxy prothrombin (DCP)

DCP was a kind gift from Eisai Co., Ltd., Tokyo, Japan. DCP was purified from the conditioned media of the DCP-producing cell line PLC/PRF/5 by affinity chromatography with an anti-prothrombin antibody.[16–19] DCP was distinguished from normal prothrombin using high performance liquid chromatography (HPLC) analysis.[16–19]

2.2. Cell Lines and cell culture

The human HCC cell lines HLE and SK-Hep which are the DCP-negative cells were purchased from the American Type Culture Collection (Manassas, USA).[20] HCC cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated foetal 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% ethylenediamine tetraacetic acid–phosphate buffered saline (EDTA–PBS) (ICN, Aurora, USA).

2.3. Cell proliferation assay

The proliferation of HCC cells was evaluated based on the viable cell number as estimated by cell counting kit-8 (CCK-8) (Dojindo Laboratories, Japan).[21] HCC cells (1.5×10^3 per well) seeded in 96-well plates were incubated with increasing concentrations of DCP or human normal prothrombin (negative control, Sigma, USA) for the indicated time. About 10 μl of CCK-8 solution was added to each well and the 96-well plate was continuously incubated at 37 °C for 120 min. Absorbance at 450 nm was measured using a multilabel plate reader (Perkin–Elmer, USA). The results were plotted as mean ± SD of three separate experiments having six determinations per experiment.

2.4. Transwell chamber assay

The motility and invasive ability of HCC cells induced by DCP were evaluated in 24-well transwell plates (Corning, USA).[19] The upper surface of polycarbonate filters with 8 μm pores was coated with 100 μg of reconstituted extracellular matrix Matrigel (Sigma, USA). HLE or SK-Hep cells (2×10^5 cells/100 μl) were seeded in the upper chambers of transwell plates and then incubated with increasing concentrations of DCP. The lower chambers were filled with 600 μl of medium. Cells were allowed to migrate for 8 h at 37 °C. Migration was

terminated by removing the cells from the upper compartment of the filter with a cotton swab. Cells that had invaded through the Matrigel and reached the lower surface of the filter were quantified by counting the number of cells that migrated in five random microscopic fields per filter at a magnification of ×400.

2.5. Gelatin zymography analysis

The activities of MMP-2 and MMP-9 were estimated by gelatin zymography analysis as described previously.[22–24] HLE or SK-Hep cells (1×10^5 cells, 80% confluent in 24-well plates) were washed twice with PBS and then cultured in 24-well plates. The cells were incubated with increasing concentrations of DCP in 2 ml of serum-free medium for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Ten microlitres of the supernatant containing 10 μg of protein, determined using the Bradford method,[25] were subjected to electrophoresis on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis copolymerised with 1 mg/ml gelatin as a substrate. The gels were electrophoresed at a constant voltage of 125 V for approximately 2 h. Following electrophoresis, the gels were rinsed in distilled water and washed with 2% Triton X-100 solution (Sigma, USA) to remove SDS and then incubated on a rotary shaker in an activation buffer (50 mmol/l Tris, 5 mmol/l $CaCl_2$, 0.5 mmol/l $ZnCl_2$, pH 7.4) for 16 h at 37 °C. The gels were rinsed and stained by incubation with 0.05% Coomassie brilliant blue R-250 (40% methanol, 10% acetic acid, and 0.5% brilliant blue R-250, Sigma, USA) for 1 h and then destained with a solution of acetic acid, methanol and water (10:50:40) to maximise contrast between proteolytic areas and non-digested areas. The identity of the proteases was determined by analysis of the distance that the bands migrated on the gels, compared with the distance for migration of molecular weight standards. Non-staining regions of the gel corresponding to MMP-2 and MMP-9 activity were quantified by densitometry using an Electrophoresis Image Analysis System (Bio-profile Bio, ID image analyzer, USA).

2.6. Western blot analysis

HCC cells (3×10^5) seeded in 6-well plates were incubated with various concentrations of DCP for 24 h or the indicated time periods. The cells were harvested and then lysed in lysis buffer [50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.1% SDS, 1 mM dithiothreitol, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin] and centrifuged at 10,000*g*. Supernatant was collected and total protein was determined using the Bradford method.[25] Cell lysates (30 μg of protein per lane) were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and then electro-transferred onto nitrocellulose membranes. After blocking with TBST buffer (20 mM Tris–buffered saline and 0.1% Tween-20) containing 5% (w/v) non-fat dry milk for 1 h at room temperature, the membranes were incubated with primary antibodies for 2 h, followed by three washes and exposure to HRP-conjugated secondary antibodies (Santa Cruz, USA) for 1 h at room temperature. The primary antibodies included rabbit monoclonal antibodies against MMP-2 (Epitomics, USA) and MMP-9 (Santa Cruz, USA), rabbit polyclonal antibody against epidermal growth factor receptor (EGFR) (Boster Biological Technology Ltd., China), rabbit polyclonal antibody against phospho-EGFR (Thr669, Cell Signaling, USA), rabbit monoclonal antibody against phospho-Met (Tyr1234/1235, Cell Signaling, USA), rabbit polyclonal antibody against phospho-p44/42 MAPK (ERK1/2) (Thr-202/204, Cell Signaling, USA), rabbit polyclonal antibody against phospho-MEK1/2 (Ser217/221, Cell Signaling, USA), rabbit polyclonal antibody against phospho-c-Raf (Ser338, Cell Signaling, USA) and anti-β-actin (ab6276, Abcam). The bound antibodies were visualised using an ECL system (Amersham Pharmacia Biotech, USA) and quantified by densitometry using a Bio-profile Bio, ID image analyzer.

2.7. Inhibitor for ERK1/2 MAPK signalling pathway

A function-blocking ERK1/2 MAPK inhibitor PD98059 was used to explore the signalling pathway of DCP activation.[26] HLE or SK-Hep cells (3×10^5) seeded in 6-well plates were pretreated with PD98059 (50 or 80 μM, Cell Signaling, USA) for 1 h before DCP exposure. The cells were harvested and the levels of phospho-p44/42 ERK1/2 and the production of MMP-2 and MMP-9 were estimated by Western blot analysis as described above.

2.8. Statistical analysis

Statistical significance was determined by the Student's two-tailed *t*-test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. DCP stimulates HCC cell proliferation

HCC cell lines HLE or SK-Hep were incubated with DCP for up to 48 h and cell proliferation was evaluated by the CCK-8 assay. As shown in Fig. 1, DCP in the range of 10–160 ng/ml had a dose-dependent stimulatory effect on HLE cell growth (Fig. 1A, $p < 0.05$ versus untreated control, $r = 0.99$). This mitogenic effect plateaued at a 51.2% enhancement over untreated cells when the cells were exposed to DCP at a concentration of 160 ng/ml for 48 h.

A similar proliferation profile was observed for SK-Hep cells incubated with DCP (Fig. 1B, $p < 0.05$ versus untreated control, $r = 0.99$).

HLE and SK-Hep cells were also exposed to normal prothrombin (10–160 ng/ml) for up to 48 h and proliferation was evaluated as described above. As shown in Fig. 1C and D, normal prothrombin at each of these concentrations did not affect cell proliferation of HLE (Fig. 1C) and SK-Hep (Fig. 1D) ($p > 0.05$ versus untreated cells).

3.2. DCP increases HCC cell invasion and migration

We evaluated the motility and invasive ability of HCC cells migrating through reconstituted extracellular matrix (Matrigel). The percentage of HCC cells migrating through Matrigel was significantly increased by a 24 h incubation with DCP (Fig. 2). In the range of 10–160 ng/ml of DCP, the number of HLE cells migrating through the Matrigel coated membrane

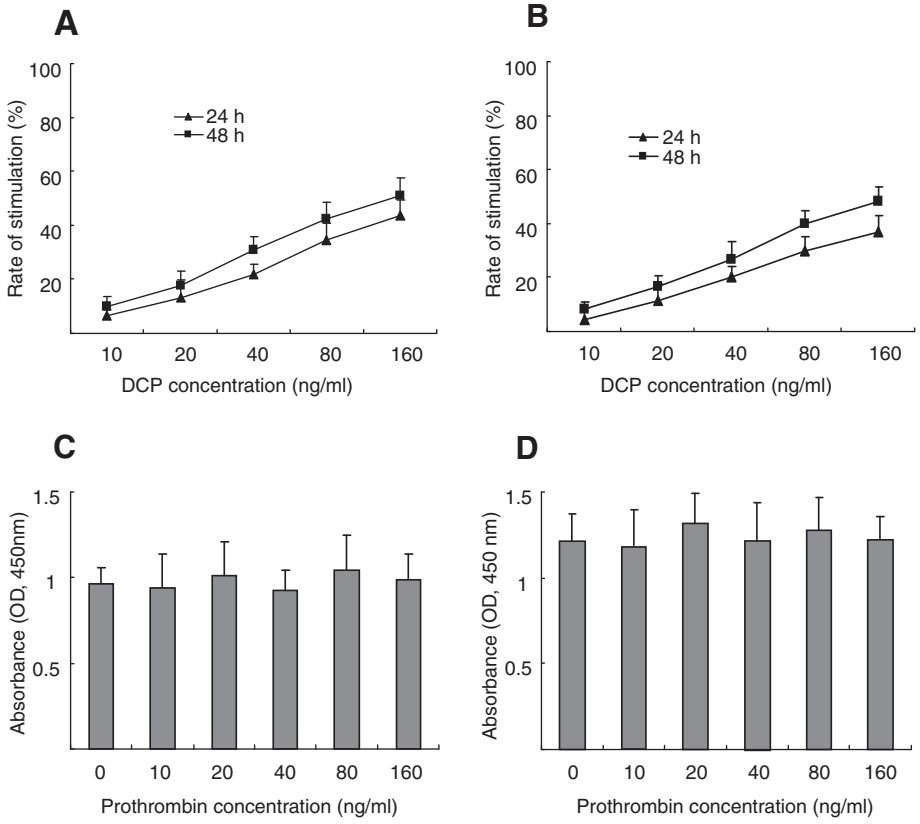

Fig. 1 – The effects of des-γ-carboxy prothrombin (DCP) or normal prothrombin on hepatocellular carcinoma (HCC) cell proliferation. HLE or SK-Hep cells were incubated with increasing concentrations of DCP (A and B) or normal prothrombin (C and D) for up to 48 h. Viable cells were evaluated by cell counting kit-8 (CCK-8) assay and denoted as a percentage of untreated controls at the concurrent time point. The bars indicate mean ± SD ($n = 6$). $^{*}p < 0.05$ (versus control).

was increased by 36.3%, 52.2%, 65.7%, 86.9% and 127.3%, respectively (Fig. 2A, $p < 0.05$ versus untreated control). Fig. 2B showed the increasing number of SK-Hep cells migrating through the Matrigel coated membrane when exposed to DCP. The rates of migration were increased from 30.0% to a maximum increase of 169.5% after 24 h of incubation (Fig. 2B, $p < 0.05$ versus untreated control).

3.3. *DCP stimulates secretion and activity of MMP-2 and MMP-9*

The gelatin zymography assay was employed to evaluate the activity of MMPs produced by HCC cells. As shown in Fig. 3A, the levels of MMP-2 and MMP-9 in supernatants of the cultured HLE cells were significantly increased following DCP stimulation. The percentages of increase by 10, 40, and 160 ng/ml of DCP were 59.2%, 66.3%, and 79.7%, respectively, for MMP-2 and 57.2%, 84.7%, and 109.8%, respectively, for MMP-9.

Fig. 3B showed the increase of MMP-2 and MMP-9 in supernatants of cultured SK-Hep cells exposed to DCP. DCP treatment increased the secretion rate of MMP-2 up to 86.5% and of MMP-9 up to 140.5% (Fig. 3B).

Western blot analysis was then used to estimate the expressions of MMP-2 and MMP-9 in HCC cells. Increasing levels of MMP-2 and MMP-9 were detected in a DCP concentration-dependent manner when compared with untreated controls. In the concentration range of 10–160 ng/ml of DCP, the percentages of increase varied from 21.4% to a maximum increase of 220.0% for MMP-2 and from 30.0% to a maximum increase of 169.5% for MMP-9 (Fig. 3C).

Similar results were observed in SK-Hep cells exposed to DCP (Fig. 3D).

3.4. *DCP activates Met phosphorylation and increases EGFR*

We measured the levels of c-Met and EGFR in HCC cells using Western blot assay. DCP significantly increased phospho-Met expression. As shown in Fig. 4, in the range of 10–160 ng/ml of DCP, the levels of phospho-Met were elevated by up to 106.2% in HLE cells (Fig. 4A) and 86.5% in SK-Hep cells (Fig. 4B), compared with control untreated cells, after a 24 h incubation with DCP ($p < 0.05$ versus untreated control).

Fig. 4C and D showed the levels of EGFR in HCC cells after a 24 h incubation with DCP. The percentages of EGFR expression were increased up to 50.6% in HLE cells (Fig. 4C, $p < 0.05$ versus untreated control) and increased up to 114.3% in SK-Hep cells by 160 ng/ml of DCP stimulation (Fig. 4D, $p < 0.05$ versus untreated control).

Further, the autophosphorylation of EGFR was examined in HCC cells exposure to DCP. HCC cells were cultured in serum free medium for 24 h and then exposed to DCP for various times (5, 15, 30, 60 and 120 min). As shown in Fig. 4E (HLE) and

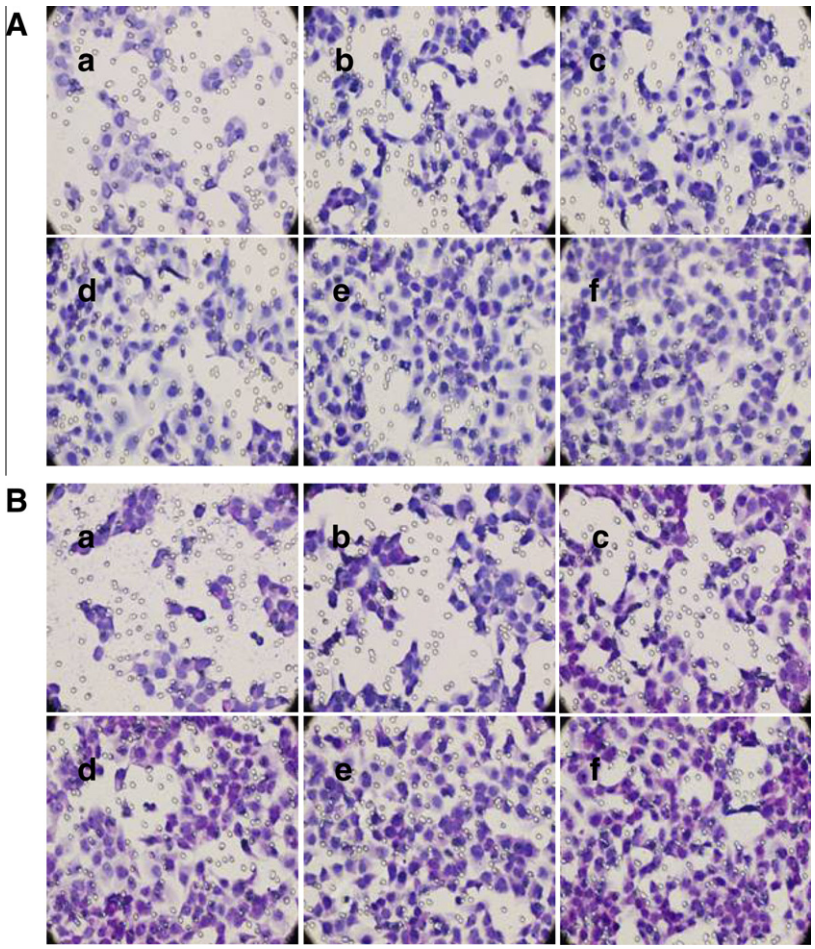

Fig. 2 – DCP stimulated HCC cell invasion and migration. HLE (A) or SK-Hep (B) cells with increasing concentrations of DCP (10–160 ng/ml) were placed in the upper chambers of the reconstituted extracellular matrix (Matrigel)-coated filters in 24-well transwell plates for 8 h. The number of cells passing through the filter was counted after staining with haematoxylin (original magnification, ×400). (a) without DCP; (b–f) cells exposed to 10, 20, 40, 80, and 160 ng/ml of DCP, respectively.

F (SK-Hep), DCP at 160 ng/ml activated EGFR phosphorylation after 30 min exposure. However, the amount of phospho-EGFR was slightly decreased in 5 and 15 min. A drastic activation of phospho-EGFR was detected in 60 and 120 min. The increasing expression rates were up to 76.3% increase in HLE cells and 110.7% increase in SK-Hep cells in 120 min (Fig. 4E and F).

3.5. *DCP activates ERK1/2 MAPK signalling pathway*

To explore the signalling pathway of DCP activation, we examined the expression levels of phospho-p44/42 ERK1/2, phospho-MEK1/2 and phospho-c-Raf following DCP stimulation. HLE cells were cultured for 2 days, serum starved for 24 h and then exposed to DCP. As shown in Fig. 5A, DCP at 160 ng/ml activated ERK1/2 phosphorylation as early as 5 min and was at its maximum by 120 min. The levels of phospho-p44/42 ERK1/2 were increased by up to 272.0% compared with control untreated cells after a 120 min exposure (Fig. 5A). Further experiments were carried out to examine the status of MEK1/2 and c-Raf in HCC cells after 120 min of DCP stimulation. The levels of phospho-MEK1/2 (Ser217/221) were increased after 120 min of DCP stimulation. As shown in Fig. 5B, in the range of 10–160 ng/ml of DCP, the percentages of increase varied from 37.5% to a maximum increase of 135.8% (Fig. 5B). Fig. 5C showed the increasing expression of phospho-MEK1/2 (Ser217/221) in SK-Hep cells. c-Raf is the effector recruited by GTP-bound Ras to activate the ERK-MAP kinase pathway. Activation of c-Raf has been shown to phosphorylate the ERK-MAP kinase pathway. Herein, we observed that the levels of phospho-c-Raf were increased following DCP stimulation. As shown in Fig. 5D, the increasing levels of phospho-c-Raf in HLE cells were significant and concentration dependent when compared with control untreated cells, after a 120 min exposure to DCP ($p < 0.05$ versus untreated control).

Increasing expression of phospho-c-Raf was also observed in SK-Hep cells following 120 min of DCP stimulation (Fig. 5E, $p < 0.05$ versus untreated control).

3.6. *Activation of ERK1/2 MAPK signalling pathway is involved in the production of MMP-2 and MMP-9*

To identify the role of ERK1/2 MAPK signalling pathway in DCP-induced MMP-2 and MMP-9 activity, HCC cells were pretreated with PD98059, a specific ERK inhibitor, before DCP

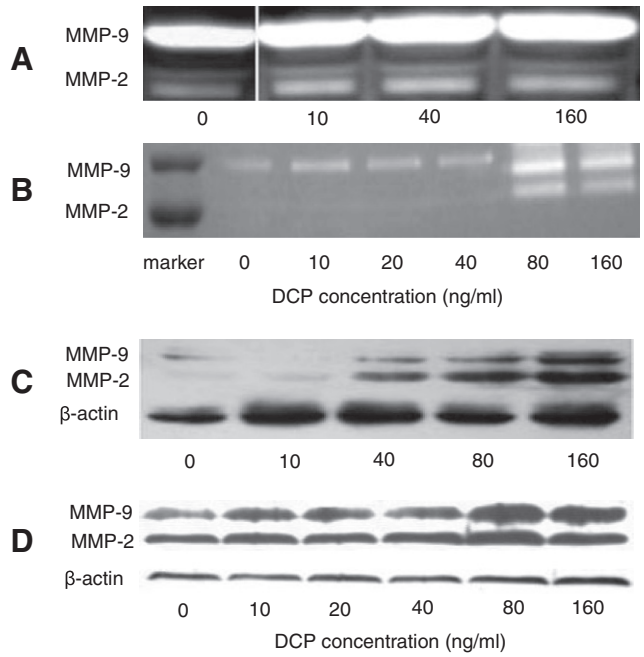

Fig. 3 – DCP stimulated activities of matrix metalloproteinase (MMP)-2 and MMP-9 in HCC cells. (A and B) Proteolytic activity of MMP-2 and MMP-9 estimated by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) gelatin zymography. HCC cells seeded in 24-well plates were exposed to increasing concentrations of DCP in serum-free medium for 24 h. The activities of MMP-2 and MMP-9 in the medium were evaluated as two destained bands by SDS–polyacrylamide gel electrophoresis gelatin zymography. The percentage of increase was estimated by comparing the densitometry in the presence or absence of DCP. (A) HLE cells; (B) SK-Hep cells. (C and D) Expressions of MMP-2 and MMP-9 evaluated by Western blot assay. HLE (C) and SK-Hep (D) cells were exposed to increasing concentrations of DCP for 24 h. Cells were lysed and subjected to Western blot analysis as described in Section 2.

(80 ng/ml) treatment. Blockade of ERK1/2 activation abolished DCP-stimulated MMP-2 and MMP-9 activity. As shown in Fig. 6A, PD98059 (50 μM) pretreatment for 1 h resulted in a 65.8% decrease in phospho-p44/42 ERK1/2 expression in HLE cells. Consequently, the levels of MMP-2 and MMP-9 were decreased by 35.5% and 47.0%, respectively (Fig. 6A, $p < 0.05$ versus untreated control). Again, 80 μM of PD98059 pretreatment completely blocked DCP-stimulated phospho-ERK1/2 activation. The levels of MMP-2 and MMP-9 were decreased by 95.4% and 80.1%, respectively (Fig. 6B, $p < 0.05$ versus untreated control).

A similar abolishment of DCP-stimulated MMP-2 and MMP-9 activity was observed in SK-Hep cells pretreated with PD98059 (Fig. 6C). PD98059 (50 μM) pretreatment resulted in a 43.6% decrease of phospho-p44/42 ERK1/2 expression and the levels of MMP-2 and MMP-9 were decreased by 31.3% and 67.1%, respectively (Fig. 6C, $p < 0.05$ versus untreated control). These results imply that DCP can induce the production of MMP-2 and MMP-9 in HCC cells through activation of ERK1/2 MAPK signal pathway.

4. Discussion

In our previous studies, we identified DCP as a novel autocrine/paracrine mitogen for HCC cell lines.[9,18,19] Incubation of HCC cells with DCP increases the proliferation of tumour cell growth as well as the expressions of VEGF, TGF-α and bFGF, which are angiogenic growth factors for endothelial cells. Further examination in nude mice bearing HCC xenografts showed that injection of DCP could stimulate growth of HCC cancer tissues. Immunohistochemical analysis revealed that the microvessel density in HCC xenograft tissues was increased following DCP stimulation. Furthermore, a recent *in vitro* study showed that DCP could stimulate growth and invasion of human vascular endothelial cells. DCP induced overexpression of EGFR, VEGF and MMP-2 (latent and active) in vascular endothelial cells. These findings indicate that DCP secreted from HCC cells can stimulate the proliferation of HCC cells and induce angiogenesis in HCC tissues. Activation of MMPs activity in HCC and endothelial cells may involve in the progression of HCC.[19] In the current study, the DCP-negative HCC cell lines of HLE and SK-Hep were used to further investigate the signalling pathway of HCC invasion and metastasis. DCP was found to stimulate HCC proliferation and to increase the activities of MMP-2 and MMP-9 through the ERK1/2 MAPK signalling pathway. These observations provide further understanding of the molecular mechanisms of the mitogenic and pro-metastatic effects of DCP on HCC cells.

DCP is an abnormal prothrombin lacking carboxylation of 10 glutamic acid residues at its N-terminus and is devoid of coagulation activity.[27,28] Due to the absence of carboxylation of the carbon atom at the γ-position, it is called des-γ-carboxy prothrombin.[14,29] In the structure of DCP there are two kringle domains which resemble those of hepatocyte growth factor (HGF).[17,19] HGF has been identified as a mitogenic factor involved in proliferation, migration, survival, morphogenesis, angiogenesis, and tissue regeneration of HCC.[30] Accordingly, kringle domains may bind with c-Met (the HGF transmembrane tyrosine kinase receptor) to stimulate HCC cell proliferation.[17,19] c-Met is hypothesised to be a membrane-spanning receptor tyrosine kinase that mediates biological responses to DCP. Binding of DCP to c-Met, therefore, activates various downstream effectors including autophosphorylation of EGFR and many other mitogenic factors to increase HCC proliferation and invasion.[18,19] This hypothesis is also supported by our previous observation that DCP increases EGFR expression in vascular endothelial cells and stimulates proliferation and angiogenesis in HCC cells.[19] Activation of EGFR phosphorylation may provide the basic drive for the binding of ligands such as epidermal growth factor, TGF-α, amphiregulin β-cellulin, heparin-binding EGF-like growth factor, and epiregulin.[31,32] Such binding may lead to activation of a cascade of biochemical and physiological responses involved in mitogenic signal transduction of HCC cells.[17] In this study, DCP was found to induce Met phosphorylation, increase autophosphorylation of EGFR and consequently the proliferation and migration of HCC cells were increased.

Invasion and metastasis are the main factors that are responsible for the poor prognosis of HCC in patients. In

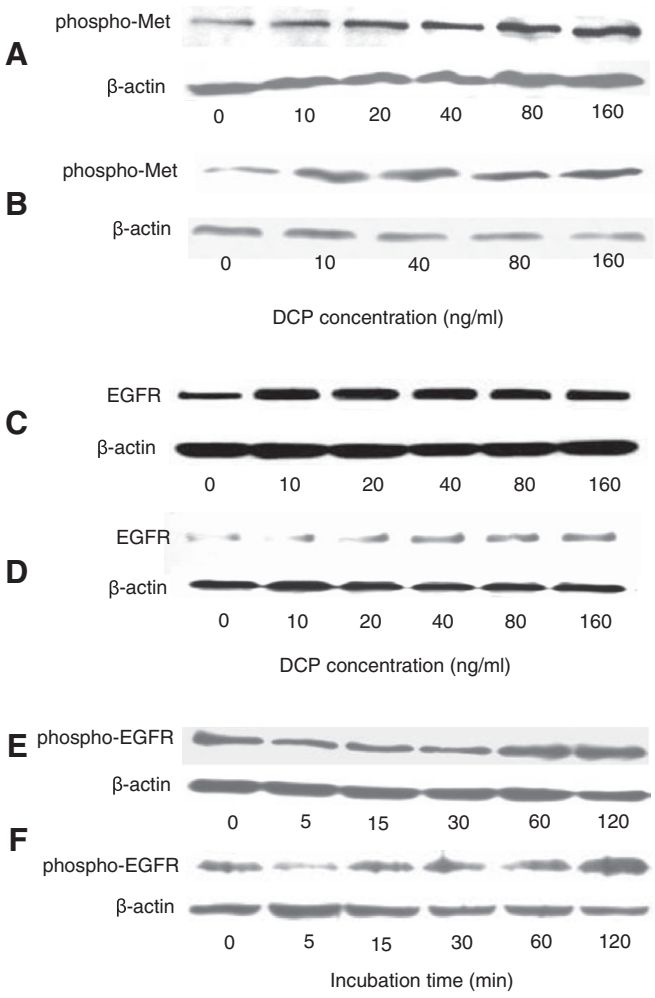

Fig. 4 – Western blot analysis of phosphor-Met and epidermal growth factor receptor (EGFR) in HCC cells. (A–D) HLE and SK-Hep cells were exposed to increasing concentrations of DCP for 24 h. Cells were lysed and subjected to Western blot assay as described in Section 2. (A) Activation of phospho-Met in HLE cells; (B) activation of phosphor-Met in SK-Hep cells; (C) EGFR expression in HLE cells; (D) EGFR expression in SK-Hep cells. (E and F) HLE and SK-Hep cells were serum starved for 24 h and then exposed to DCP (160 ng/ml) for the indicated time periods. Cell extracts were subjected to Western blot analysis using phospho-EGFR antibody. (E) HLE cells; (F) SK-Hep cells.

the early stages of cell migration, invasion, and metastasis, extracellular matrix breakdown is primarily mediated by the actions of MMP-2 and MMP-9. Thus, MMP-2 and MMP-9 have been considered to be critical for HCC invasion and metastasis.[33,34] In our earlier study, we observed activation of MMP-2 in vascular endothelial cells upon exposure to DCP. The activated MMP-2 has a great affinity for fibronectin, laminins, elastin, and collagens, degrading them and promoting endothelial cell invasion into the surrounding interstitial matrix.[35,36] MMP-2 may also activate mitogenic factors including EGFR, VEGF, FGF-2 and TGF-β which have a role in the proliferation of HCC in addition to the degradation of extracellular matrix (ECM) in HCC.[37,38] In this study, DCP was found to increase MMP-2 and MMP-9 secretion and activity in HCC cells, which correlated well with the proliferation and migration of HCC cells through Matrigel. To the best of our knowledge, this is the first study that shows activation of MMP-2 and MMP-9 following DCP stimulation in HCC.

In searching for the downstream signalling pathway of DCP, the ERK1/2 MAPK kinase cascade has been found to be involved in regulating MMP-2 and MMP-9 activity. There is mounting evidence that multiple signalling cascades are involved in the activation of MMP-2 and MMP-9, especially for the ERK1/2 MAPK signalling pathway.[39–41] Mitogen-activated protein kinases (MAPKs) are a widely conserved family of serine/threonine protein kinases involved in many cellular programs such as cell proliferation, motility, differentiation and invasion in response to diverse extracellular stimuli.[42] The p44/42 ERK1/2 MAPK kinase cascade can be activated in response to a diverse range of extracellular stimuli including mitogens, growth factors, and cytokines.[43] Our results show that DCP activated p44/42 ERK1/2 phosphorylation as early as 5 min and reached its maximum expression at 120 min. Accordingly, ERK1/2 is a downstream component of an evolutionarily conserved signalling module that is activated by the Raf serine/threonine kinases. Raf activates the MAPK/ERK kinase (MEK)1/2 dual-specificity protein kinases, which then

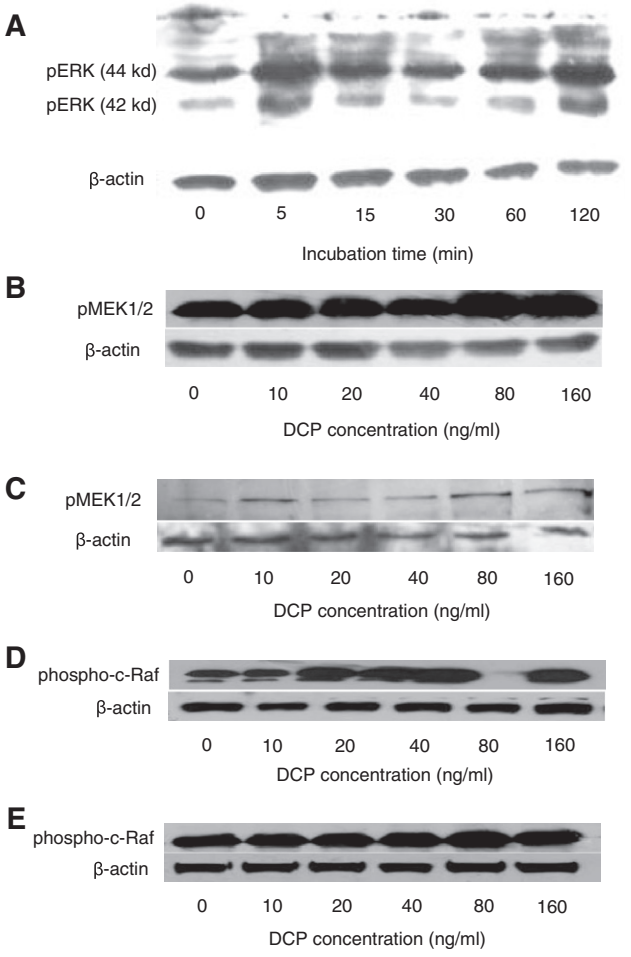

Fig. 5 – Western blot analysis of extracellular signal-regulated kinase/mitogen activated protein kinase (ERK1/2 MAPK) kinase cascade after DCP stimulation. (A) HLE cells were serum starved for 24 h and then exposed to DCP (160 ng/ml) for the indicated time periods. Cell extracts were subjected to Western blot analysis using phospho-ERK1/2 antibodies. (B–E) HLE or SK-Hep cells were serum starved for 24 h and exposed to increasing concentrations of DCP for 120 min. Cell extracts were subjected to Western blot analysis using phospho-specific antibodies. The percentage of stimulation was evaluated by comparing the densitometry in the presence and absence of DCP.

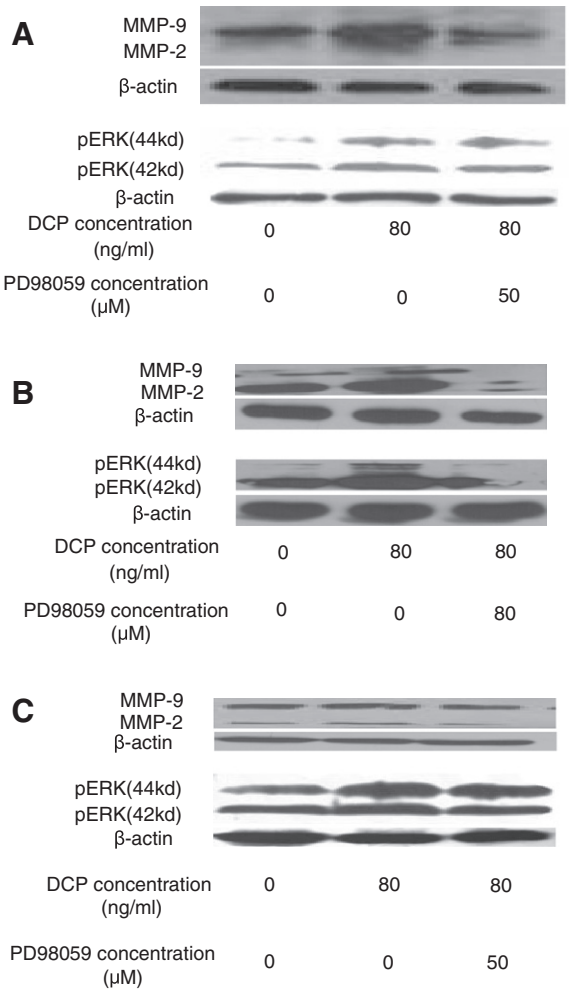

Fig. 6 – Effects of blocking ERK1/2 signal pathway on DCP-stimulated MMP-2 and MMP-9 activity. HLE or SK-Hep cells were serum starved for 24 h. The cells were treated with PD98059 (50 or 80 μM) for 1 h to block ERK1/2 signal pathway and then exposed to DCP (80 ng/ml) for 120 min. Cell extracts were subjected to Western blot analysis using phospho-ERK1/2 antibodies and monoclonal antibodies against MMP-2 and MMP-9. The percentage of stimulation was estimated by comparing the densitometry in the presence and absence of DCP. (A) PD98059 (50 μM) blockade of DCP-stimulated ERK1/2 signalling in HLE cells. (B) PD98059 (80 μM) completely blocked DCP-stimulated phospho-ERK1/2 activation in HLE cells. (C) PD98059 (50 μM) blockade of DCP-stimulated ERK1/2 signalling in SK-Hep cells.

activate ERK1/2.[44–46] Therefore, we measured the levels of phosphorylation-MEK1/2 (Ser217/221) and phosphorylation-c-Raf after DCP stimulation. Additionally, the Raf–MEK–ERK pathway is a key downstream effector of the Ras small GTPase.[43] Ras is a key downstream effector of EGFR. The activation of Raf–MEK–MAPK pathway may depend on the phosphorylation of EGFR.[39–41] In this study, the amount of phosphorylation-EGFR was decreased within 15 min of DCP exposure. Accordingly, the phosphorylation-EGFR internalisation might occur during the short time of DCP stimulation.[47] The increase of phosphorylation-EGFR was detected after 30 min and the maximum expression was observed at 120 min. Thus, we hypothesise that the EGFR–Ras–Raf–MEK–ERK kinase cascade might be involved in the signalling pathway of DCP stimulation responsible for HCC proliferation and MMPs activation.

Furthermore, we blocked the ERK1/2 MAPK signalling pathway with the ERK1/2 inhibitor PD98059 and then examined production of MMP-2 and MMP-9. Inhibition of ERK1/2 activation essentially abolished the DCP-induced MMP-2 and MMP-9 activity. Taken together the observation that DCP rapidly activates the ERK/MAPK signalling pathway and sustains its maximum expression for 120 min, and that PD98059 inhibits DCP induced MMP-2 and MMP-9 activity, our results

suggest that the stimulatory effects of DCP on production of MMP-2 and MMP-9 are mediated via activation of the ERK1/2 MAPK signalling pathway.

In conclusion, we found that DCP, the aberrant prothrombin produced in HCC cells, has a stimulatory effect on proliferation and migration of HCC cells. The mechanism behind these actions of DCP may be related to the up-regulation of MMP-2 and MMP-9 and many other mitogenic factors. Activation of the ERK1/2 MAPK signalling pathway is involved in the process of DCP-induced MMPs activation. Identification of these HCC mitogenic factors and an understanding of the molecular mechanisms responsible for the activation of MMPs will be crucial in the development of pharmacologic inhibitors for HCC treatment.

Conflict of interest statement

None declared.

Acknowledgements

This project was supported by Shandong Provincial Foundation for Natural Science (2009ZRB01798), the Doctoral Science Foundation of the Ministry of Education of China (20090131110063) and the Natural Science Foundation of China (30973550, 30901833). This work was also supported by Department of Pathology and Laboratory Medicine, University of Calgary and Calgary Laboratory Services, Canada.

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