Des-γ-carboxy Prothrombin Is a Potential Autologous Growth Factor for Hepatocellular Carcinoma*

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Des-γ-carboxy prothrombin (DCP) is a well recognized tumor marker for hepatocellular carcinoma (HCC). In the present study, we demonstrate that DCP has a mitogenic effect on HCC cell lines. Purified DCP stimulated DNA synthesis of Hep3B and SK-Hep-1 cells in a dose-dependent manner. DCP was found to bind with cell surface receptor Met causing Met autophosphorylation and also to activate STAT3 signaling pathway through Janus kinase 1. Luciferase gene reporter analysis showed that DCP induced STAT3-related transcription. Small interfering RNAs against both STAT3 and Met abrogated DCP-induced cell proliferation. DCP did not affect the mitogen-activated protein kinase pathway, Myc signaling pathway, or phosphoinositide 3-kinase/Akt pathway. Based on these results, we believe that DCP acts as an autologous mitogen for HCC cell lines. The Met-Janus kinase 1-STAT3 signaling pathway may be a major signaling pathway for DCP-induced cell proliferation.

Des-γ-carboxy prothrombin (DCP) is a well recognized tumor marker for its high sensitivity and specificity in the screening and diagnosis of hepatocellular carcinoma (HCC). Forty-four to eighty-one percent of HCC patients have elevated serum DCP levels (1–5). DCP is a prothrombin precursor with no coagulation activity. Prothrombin is synthesized in the liver depending on the presence of vitamin K-dependent γ-glutamyl carboxylase. The prothrombin precursor has 10 Glu residues in the N terminus that are converted into carboxylase. The prothrombin precursor has 10 Glu residues in a dose-dependent manner. DCP was found to bind with cell surface receptor Met causing Met autophosphorylation and also to activate STAT3 signaling pathway through Janus kinase 1. Luciferase gene reporter analysis showed that DCP induced STAT3-related transcription. Small interfering RNAs against both STAT3 and Met abrogated DCP-induced cell proliferation. DCP did not affect the mitogen-activated protein kinase pathway, Myc signaling pathway, or phosphoinositide 3-kinase/Akt pathway. Based on these results, we believe that DCP acts as an autologous mitogen for HCC cell lines. The Met-Janus kinase 1-STAT3 signaling pathway may be a major signaling pathway for DCP-induced cell proliferation.

For example, serum and tissue DCP expressions are thought to reflect the biological malignant potential of HCC (10–14), serum DCP level is used as a clinical parameter for the development of portal venous invasion of HCC (15), and cell proliferation markers have been seen to correlate with tissue DCP expression in clinical pathological studies of HCC (10, 16).

In exploring the structure of DCP, there are two kringle domains similar to those of hepatocyte growth factor (HGF), which was originally identified as a potent mitogen for mature hepatocytes (17, 18). Kringle domains are mandatory for HGF to bind with Met, and their presence implies that DCP interacts with Met. We hypothesize that DCP stimulates HCC cell proliferation through Met. Met is a membrane-spanning receptor tyrosine kinase that mediates biological responses to various tissues including cell scattering, growth stimulation, and the branching morphogenesis of cells in various tissues (19–22). Recent studies revealed that the coupling between HGF and Met integrates biological processes, such as the invasive and metastasis progression to cancer cells (23, 24).

In this study, we found that DCP stimulated the proliferation of HCC cell lines. During our investigation of the receptor for DCP, DCP was found to bind with Met. The transdutional apparatus of DCP was identified to activate Janus kinase 1 (JAK1)/signal transducers and activators of the transcription 3 (STAT3) signaling pathway during cancer proliferation. These findings provide a description of a novel autocrine/paracrine growth stimulatory mechanism behind the development of HCC.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Two human HCC cell lines, Hep3B (ATCC, Manassas, VA) and PLC/PRF/5 (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen). Another human HCC cell line, SK-Hep-1 (ATCC), was maintained in Eagle’s minimum essential medium (Sigma). The human colon cancer cell line HT-29 (ATCC) was cultured in McCoy’s 5A medium (Sigma). All of the media were supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 1% nonessential amino acid (Sigma), 1% sodium pyruvate (Sigma), and 1% penicillin/streptomycin solution (Sigma). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

Determination of DCP Levels Produced by HCC Cell Lines—DCP levels produced by HCC cell lines were determined by an electrochemiluminescence immunoassay (Picolumi PIVKA-II™, Eisai Co., Ltd., Tokyo, Japan). 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 rhenylated. An electrochemically triggered light reaction was quantified by an electrochemiluminescence detection system.

Purification of DCP—The DCP-producing cell line PLC/PRF/5 was cultured in the presence of warfarin sodium (10 μg/ml) to enhance DCP production. DCP was purified from the conditioned media by affinity chromatography with an anti-prothrombin antibody (Eisai). The purified DCP was then separated by SDS-PAGE and transferred to an
Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was stained with Blot-FastStain™ protein-staining reagent (Chemicon International, Inc., Temecula, CA) according to the manufacturer’s protocol. Purified DCP sample was developed by SDS-PAGE and blotted with anti-DCP antibody (Eisai). DCP could be distinguished from normal prothrombin by high performance liquid chromatography (HPLC) analysis (6). HPLC analysis was performed using a column (TSKgel; DDS-80TS, Tosoh Biosciences, Tokyo, Japan).

Cell Proliferation Assay—The cells were grown to confluence in 12-well plastic tissue culture plates. The cells were kept inactive for 24 h and then treated with DCP, prothrombin (Sigma), and apolipoprotein A (Calbiochem, San Diego, CA) at the indicated concentrations. After a 12-h incubation, 5 µCi of [3H]thymidine (Amersham Biosciences) was added to each well and maintained for 8 h. The cells were treated with 5% trichloroacetic acid for 30 min at 4 °C. The cells were then harvested, and we used a liquid scintillation counter (Beckman Coulter, Fullerton, CA) to analyze the samples.

Western Blot Analysis—The cells were plated into 6-well plates and grown to confluence. After 24 h of quiescence, the cells were treated with DCP, prothrombin, HGF (PeproTech, Rocky Hill, NJ), or epidermal growth factor (Sigma) at the indicated concentrations for 15 min at 37 °C. Cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore). The membranes were blocked using Tris-buffered saline with Tween 20 (Sigma) (TBS-T) buffer containing 5% bovine serum albumin for 1 h. Cell lysates containing the same amount of protein were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were then blocked with 5% bovine serum albumin in 37 °C. Cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore). The membranes were blocked using Tris-buffered saline with Tween 20 (Sigma) (TBS-T) buffer containing 5% bovine serum albumin for 1 h. The membranes were then incubated with anti-phospho-Met (Tyr1022/1023) antibody (Cell Signaling Technology, Beverly, MA), anti-phospho-p85 phosphoinositide 3-kinase (PI3K)-binding motif antibody (Cell Signaling Technology), and anti-phospho-Akt antibody (Cell Signaling Technology) overnight at 4 °C. The membranes were washed three times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibody before being developed with an ECL Western blotting detection system (Amersham Biosciences) using enhanced chemiluminescence.

Luciferase Gene Reporter Assay—The reporter plasmids, 30 µg of pFA2-Etk plasmid (PathDetect™ Trans-Reporting System, Stratagene, La Jolla, CA), and 7 µg of pRL-TK control Renilla luciferase plasmid (Promega, Madison, WI) per 105 cells, were co-transfected by electroporation (voltage, 300 V; capacitor, 950 microfarad; cuvette gap, 4 mm) and plated into 6-well plates. For the STAT3 activity reporter assay, 30 µg of pSTAT3-Luc (Clontech laboratories, Palo Alto, CA) and 7 µg of pRL-TK control Renilla luciferase plasmid (Promega) were co-transfected per 105 cells. After 26 h, the media were changed just before the following treatment to avoid the effect of self-produced DCP. The cells were stimulated with DCP (2-200 ng/ml), prothrombin (2-200 ng/ml), and/or epidermal growth factor (10 ng/ml) and incubated for 12 h. The cells were then lysed, and the activities of firefly and Renilla luciferases were measured sequentially with a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Far Western Blot Analysis—DCP protein was biotinylated using biotinamido hexanoic acid 3-ethylmaleimide ester with an ECL biotinylation module (Sigma). The cell lysates were developed on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were then blocked with 5% bovine serum albumin in TBS-T and left to react overnight with biotin-labeled DCP in TBS-T containing 1% bovine serum albumin at 4 °C. Excess amounts of the probe were removed by four washes in TBS-T. The membranes were incubated with ExtrAvidin™ Peroxidase (Sigma) for detection. After three times washing with TBS-T, the membrane was developed using an ECL detection system (Amersham Biosciences).

Immunoprecipitation—Cell lysates containing the same amount of proteins were immunoprecipitated with anti-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The precipitates were analyzed using 7.5% SDS-PAGE and immunoblotted with an anti-DCP antibody (Eisai), anti-phosphotyrosine 20 (PY-20) antibody (BD Transduction Laboratories, Lexington, KY), and anti-Met antibody.

Inhibitors for Met-JAK-STAT Signaling Pathway—A function-blocking anti-Met antibody (R & B Systems, Minneapolis, MN) was utilized for function blocking assay against Met signaling. The cells were pre-treated with function-blocking anti-Met antibody (1 µg/ml) for 1 h before DCP stimulation. JAK inhibitor AG490 and STAT3 inhibitor peptide were purchased from Calbiochem.

Gene Silencing with Small Interfering RNAs—siRNA duplexes targeting STAT3 and Met sequences were obtained from Dharmaco SMARTpool™ pool technology (Dharmacon Research, Lafayette, CO). Control (nonsilencing, 5'-AATTTCGCGAGTTCAGCT-3') and Met-JAK-STAT5 siRNA duplexes were purchased from Sigma. siRNAs were transfected into cells using the RNAiFect™ transfection reagent (Qiagen). The cells were incubated for 12 h and used for the following analysis.

Total RNA was purified from siRNA transfected cells with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Lamin A/C mRNA expression level was determined by real time PCR using Quantitect™ gene expression assay system (Qiagen) and LightCycler™ (Roche Applied Science) to verify that nonspecific suppression did not occur by siRNA transfection.

The interferences of STAT3 and Met protein expression were confirmed by Western blot analysis using an anti-STAT3 antibody (Cell Signaling Technology) and anti-Met antibody (Santa Cruz Biotechnology). Luciferase gene reporter assays for STAT3 activation and a cell proliferation assay were then performed as described above.
RESULTS

Purification of DCP from PLC/PRF/5—The purified DCP was analyzed by SDS-PAGE and HPLC analyses. More than 99% was DCP in SDS-PAGE and densitometry analysis (Fig. 1A). The purified DCP was analyzed by Western blot with anti-DCP antibody to demonstrate that the DCP was uncontaminated (Fig. 1B). Purified DCP from warfarin sodium-treated PLC/PRF/5 cells contained no detectable level of normal prothrombin in HPLC analysis (Fig. 1C, lower panel). HPLC analysis showed that DCP produced by PLC/PRF/5 cell has 6–8 Gla residues. This result is compatible with a former report (6).

Self-production of DCP by HCC Cell Lines—The DCP produced by each cell line was determined using an electrochemiluminescence immunoassay. Hep3B and PLC/PRF/5 produced DCP at rates of 0.49 ± 0.04 and 2.33 ± 0.04 ng/ml/day/10^6 cells, respectively. SK-Hep-1 and HT-29 did not produce a detectable DCP level.

DCP Stimulates the Proliferation of HCC Cell Lines—To investigate the mitogenic effect of DCP, we incorporated [3H]thymidine into DNA as an index of proliferation. As shown in Fig. 2, increasing concentrations of DCP led to increased incorporation of [3H]thymidine in each cell line. DCP stimulated cell proliferation 1.50 ± 0.16-fold at a concentration of 20 ng/ml in the DCP self-producing Hep3B cell, whereas normal prothrombin stimulated cell proliferation 1.15 ± 0.07-fold at the same concentration. This effect reached a plateau with a DCP stimulus of 200 ng/ml (data not shown). In SK-Hep-1 non-DCP-producing cells, DCP showed a significant potential mitogenic effect of 2.10 ± 0.05-fold at a concentration of 20 ng/ml. Normal prothrombin hardly affected proliferation of the SK-Hep-1 cell line. In fact, cell proliferation in SK-Hep-1 cells was not even induced with 100 μg/ml of prothrombin, the physiological plasma concentration (data not shown). Moreover, we assessed the effect of DCP in colon cancer cell line HT-29, which does not produce DCP but expresses Met. DCP stimulated cell proliferation 1.91 ± 0.40-fold at a concentration of 20 ng/ml in HT-29 cells, but prothrombin had little effect. Moreover, apolipoprotein A was employed as a control serum protein that contains kringle domains (25). A polipoprotein A did not affect cell proliferative activity at its physiological serum concentration (280 ng/ml) in HCC cells (1.13 ± 0.17, 0.90 ± 0.04, and 0.96 ± 0.03-fold for Hep3B, SK-Hep1, and HT-29, respectively; not significant).

DCP Binds to HGF Receptor Met and Phosphorylates Tyrosine Residues of Met—Met expression in Hep3B, SK-Hep-1, and HT-29 cells was confirmed by Western blot analysis. Far Western blot analysis using a biotin-labeled DCP probe showed that DCP interacted with Met. In all of the cell lysates, a 145-kDa protein was detected by this probe and identified as Met (Fig. 3A). The extracts of Hep3B, SK-Hep-1, and HT-29 cells were immunoprecipitated with anti-Met antibodies and blotted with an anti-DCP antibody. When anti-Met antibody was used for immunoprecipitation, DCP could be visualized with an anti-DCP antibody (Fig. 3B). When Met is activated by its ligand HGF, specific tyrosine residues are reported to be autophosphorylated (26, 27). HGF treatment induced the autophosphorylation of Met as detected by the anti-PY-20 antibody in

FIG. 2. Effect of DCP on DNA synthesis in Hep3B, SK-Hep-1, and HT-29 cells. The cells were grown to confluence in 12-well dishes and kept quiescent for 24 h. The cells were then treated with DCP or prothrombin for 20 h. A cell proliferation assay was performed as described under “Experimental Procedures” using [3H]thymidine. The data are shown as ratios to untreated control cells and are the means ± S.E. of more than three independent studies. *, p < 0.05; **, p < 0.01 (versus nontreatment of DCP). Student’s t test.

FIG. 3. Far Western blot analysis (A) and co-immunoprecipitation analysis (B) for Met and analysis for phosphorylation of Met (C and D). A, cells were grown to confluence in a 10-cm tissue culture dish and lysed with buffer containing sodium vanadate and sodium pyrophosphate. Cell lysates were developed on SDS-PAGE with nonreduced condition and transferred to a polyvinylidene difluoride membrane. The membrane was probed with biotin-labeled DCP, and the Far Western blot was developed with streptavidin-conjugated alkaline phosphatase. Shown is the representative blot of two independent studies. B, cells were grown to confluence in a 10-cm dish and kept quiescent for 24 h. The cells were then stimulated with DCP (20 ng/ml) for 15 min. Met protein was immunoprecipitated with anti-Met antibody (Santa Cruz Biotechnology) from total cell lysates. The immunoprecipitates were analyzed by Western blot using anti-DCP antibody. Shown is the representative blot of two independent studies. IP, immunoprecipitation; IB, immunoblot. C, Hep3B cells were grown to confluence in a 10-cm dish and kept quiescent for 24 h. The cells were stimulated with HGF (50 ng/ml), DCP (20 ng/ml), and prothrombin (20 ng/ml) for 15 min. Met protein was immunoprecipitated from total cell lysates. The precipitates were then analyzed by SDS-PAGE and immunoblotted with anti-PY-20 antibody and anti-Met antibody. Shown are the representative blot of two independent studies. D, Western blots demonstrating the effects of DCP on tyrosine phosphorylation of Met in the Hep3B cell line. The cells were kept quiescent for 24 h and then stimulated with HGF (50 ng/ml), DCP (20 ng/ml), and prothrombin (20 ng/ml) for 15 min. The cell extracts were analyzed using Western blot and anti-phospho-specific Met Tyr^{1234/1235} and Tyr^{1349} antibodies. Shown are the representative blots of three independent studies. n.t., nontreated control.
FIG. 4. **JAK1-STAT3 signals induced by DCP stimulation.** A, Hep3B cells were kept quiescent for 24 h and then stimulated with HGF (50 ng/ml), prothrombin (PT, 20 ng/ml), and DCP (20 ng/ml) for 15 min. The cell extracts were analyzed using Western blot with an anti-phospho-specific JAK1 antibody (**upper panel**) and an anti-phospho-specific STAT3 antibody (**lower panel**). Shown are the representative blots of more than three independent studies. B, STAT3 activation was determined using the luciferase reporter assay. Hep3B cells were transiently transfected with pSTAT3-Luc reporter plasmid and pRL-TK control Renilla luciferase plasmid. After 36-h of quiescence, the cells were treated with HGF (50 ng/ml),
prothrombin (20 ng/ml), DCP (20 ng/ml), AG490 (80 μM), function-blocking anti-Met antibody (R&D Systems), and/or STAT3 inhibitor peptide (100 μM) for 12 h. Cellular extracts were collected, and luciferase activities were measured. C, a cell proliferation assay was performed as described under “Experimental Procedures” with DCP (20 ng/ml), function-blocking anti-Met antibody (1 μg/ml), AG490 (80 μM), and/or STAT3 inhibitor peptide (100 μM). The data are shown as the ratios to untreated control cells and are the means ± S.E. of more than three independent studies. **, p < 0.05; ***, p < 0.01 (versus control nonsilencing siRNA); Student’s t test.

FIG. 5. The effect of Met and STAT3 gene silencing on DCP-induced STAT3 activation (A) and cell proliferation (B). A, STAT3 activation was determined using a luciferase reporter assay. Met siRNA duplexes (100 nM), STAT3 siRNA duplexes (100 nM), and control (nonsilencing) siRNA duplexes (100 nM) were transfected into cells and incubated for 12 h. The cells were transiently transfected with pSTAT3-Luc reporter plasmid and pRL-TK control Renilla luciferase plasmid. After 36-h of quiescence, the cells were treated with DCP (20 ng/ml) for 12 h. Cellular extracts were collected, and luciferase activities were measured. The STAT3 activity, depending on luciferase activity, was normalized to the constitutive active Renilla luciferase activity. B, after treatment with siRNA duplexes, a cell proliferation assay was performed as described under “Experimental Procedures” using [3H]thymidine. The data are shown as the ratios to DCP-un-treated control cells and are the means ± S.E. of more than three independent studies. NS, p > 0.05; *, p < 0.05; **, p < 0.01 (versus control nonsilencing siRNA); Student’s t test.

Hep3B cell line and 66.6% in the SK-Hep-1 cell line (Fig. 5B). Moreover, STAT3 siRNA transfection decreased DCP-induced incorporation of [3H]thymidine by 56.8% in the Hep3B cell line and by 100% in the SK-Hep-1 cell line (Fig. 5B).

DCP Did Not Affect the Raf-MEK-ERK-MAPK Pathway—HCC cell lines were treated with DCP for the indicated period. Raf and ERK-MAPK phosphorylation was analyzed by Western blot analysis. DCP did not phosphorylate either Raf (data not shown) or ERK-MAPK (Fig. 6). In addition, we performed luciferase reporter assays for Elk activation and found that DCP did not raise Elk-dependent luciferase activity in Hep3B cells (Fig. 6). We further investigated whether DCP stimulates
FIG. 6. The effect of DCP on Raf-MAPK signaling. Hep3B cells were grown to confluence in a 6-well tissue culture dish and kept quiescent for 24 h. The cells were treated with epidermal growth factor (10 nm), DCP (2–200 ng/ml), and prothrombin (2–200 ng/ml) for 15 min. The cell extracts were analyzed by Western blot using a phospho-specific p44/42 MAPK (Thr202/Tyr204) antibody. MAPK-related transcriptional factor Elk activities were analyzed using a luciferase reporter assay. The cells were transiently transfected with Elk reporter plasmid and then treated with epidermal growth factor, DCP, and prothrombin for 12 h. The cellular extracts were collected, and luciferase activities were measured. The data are shown as the ratios to untreated control cells and are the means ± S.E. of more than three independent studies.

DISCUSSION
In the current study, we present DCP as a novel autocrine/paracrine mitogen for HCC cell lines. Clinical studies show a significant correlation between serum DCP level and the clinical malignancy of HCC (11–15, 33); however, the molecular basis for this correlation is still poorly understood. Herein, DCP was demonstrated to stimulate the Met-JAK-STAT signaling pathway in HCC cell lines. To the best of our knowledge, this is the first study that clearly demonstrates a mitogenic effect of DCP in HCC cell lines.

Proto-oncogene c-Met, which encodes the cell surface tyrosine kinase receptor for HGF, is overexpressed in a significant proportion of HCC cases as well as in other human cancers (19, 24, 34–39). Met ligand HGF is known to be produced in various cancer cells (40, 41), and extensive studies suggest that autologous Met activation causes cancer cell growth and invasion (42–44). ERK-MAPK, STAT3, and the PI3K-Akt signaling pathway were elicited by Met activation (45). Through ERK-MAPK, HGF stimulates proliferative activity of the HCC cells (46). In contrast, some studies have shown that HGF has an inhibitory effect or no effect on HCC cell growth (47, 48). In keeping with these studies, we found that HGF stimulated neither cell proliferation nor the ERK-MAPK signaling pathway (data not shown) in Hep3B and SK-Hep1 cells. Both DCP and HGF bound to Met and caused its autophosphorylation. HGF induced full phosphorylation of the Met autophosphorylation site, including Tyr1234/1235 and Tyr1349, whereas DCP only stimulated Tyr1234/1235. This partial phosphorylation may cause a different biological effect on cell proliferation. Although the biological response through a single receptor that binds to different ligands is usually indistinguishable, some receptors reportedly can discriminate between ligands and induce different biological responses (49, 50). We found, however, that DCP did not activate the ERK-MAPK signaling pathway. Thus, further study is needed to elucidate ligand discrimination in the autophosphorylation and biological response of the Met receptor.

In searching for the downstream signaling pathway of DCP, the JAK-STAT signaling pathway was found to be a promising candidate. A wide variety of extracellular signals activate the STAT class of transcription factors. Many cytokines, lymphokines, and growth factors signal through a related superfAMILY of cell surface receptor tyrosine kinases. Other signaling pathways were not affected significantly by DCP (data not shown). Similar results were obtained with SK-Hep-1 cells (data not shown).

other Met signaling pathways were screened using an antibody array (MaxArray™ RTK Antibody Array) (Zymed Laboratories Inc., Laboratories, South San Francisco, CA), which contains antibodies for proteins related to receptor tyrosine kinases. Other signaling pathways were not affected significantly by DCP (data not shown). In the first study that clearly demonstrates a mitogenic effect of DCP in HCC cell lines.

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