Mechanistic Study on Growth Suppression and Apoptosis Induction by Targeting Hepatoma-derived Growth Factor in Human Hepatocellular Carcinoma HepG2 Cells

Tsun Yee Tsang^a, Wan Yee Tang^{a,b}, Wing Pui Tsang^a, Ngai Na Co^a,
Siu Kai Kong^a and Tim Tak Kwok^a

^aDepartment of Biochemistry, The Chinese University of Hong Kong, Hong Kong SAR, ^bPresent address: Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, Ohio

Key Words

HDGF • Apoptosis • Anchorage-independent growth
• Fas • Bad • NF- κ B/I κ B

Abstract

Hepatoma-derived growth factor (HDGF) is frequently overexpressed in human cancer. The growth factor was previously demonstrated to be a survival factor as knock-down of HDGF suppresses the growth and induces apoptosis in human cancer cells through the Bad-mediated intrinsic apoptotic pathway. However, inactivation of Bad cannot completely repress the apoptosis induced upon HDGF knock-down, indicating the presence of other unidentified pathways. In the present study, HDGF knock-down was shown to trigger the Fas-mediated extrinsic apoptotic pathway in human hepatocellular carcinoma HepG2 cells through NF- κ B signaling pathway. Increases in Fas expression and *fas* promoter activity were detected upon HDGF knock-down by Western blot analysis and luciferase reporter assay. Knock-down of *fas* inhibited HDGF knock-down effect on apoptosis induction and growth suppression as revealed by annexin V binding

assay and soft agar assay. Down-regulation of I κ B α was also observed upon HDGF knock-down. Overexpression of I κ B α by transient transfection or inhibition of NF- κ B by BAY11-7082 suppressed HDGF knock-down effect on *fas* promoter activation, Fas up-regulation, apoptosis induction and growth suppression. Furthermore, the interaction of Fas-mediated extrinsic and Bad-mediated intrinsic apoptotic pathways was demonstrated as a stronger inhibition on apoptosis induction and growth suppression upon HDGF knock-down was observed when both pathways were inactivated. The results therefore suggested that, through both intrinsic and extrinsic apoptotic pathways, HDGF may function as a survival factor and be a potential target for cancer therapy.

Copyright © 2009 S. Karger AG, Basel

Introduction

Hepatoma-derived growth factor (HDGF) is a heparin-binding growth factor originally isolated from the conditioned media of human hepatoma HuH-7 cells [1].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2009 S. Karger AG, Basel
1015-8987/09/0244-0253\$26.00/0

Accessible online at:
www.karger.com/cpb

Tim Tak Kwok
Department of Biochemistry, The Chinese University of Hong Kong
Shatin, Hong Kong SAR, (China)
Tel. +852-26096311, Fax +852-26037246
E-Mail kwok2020@cuhk.edu.hk

It functions as a mitogen and was reported to stimulate the proliferation of hepatoma cells, fibroblasts, vascular smooth muscle cells and endothelial cells [2-4].

Overexpression of HDGF can be found in a number of human cancer, including hepatocellular carcinoma (HCC) [5-8], gastric cancer [9, 10], colorectal cancer [11], non-small cell lung cancer [12], pancreatic cancer [13] and melanoma [14]. Elevated tumor level of HDGF is correlated with poor prognosis in HCC and non-small cell lung cancer patients as they have lower disease-free and overall survival rate [6, 12], presumably due to the HDGF growth stimulating effect.

As reported previously, knock-down of HDGF induced apoptosis in human cancer cells through the Bad-mediated intrinsic apoptotic pathway [15]. However, blocking of this apoptotic pathway alone cannot fully abrogate the apoptosis induction. It therefore indicates the possible involvement of other yet to be identified pathways. Since more and more evidence indicates that the intrinsic and extrinsic apoptotic pathways may work together in regulating apoptosis [16], it is therefore speculated that the extrinsic apoptotic pathway may also be involved in the apoptotic effect of HDGF.

The extrinsic apoptotic pathway is activated upon the binding of cytokine ligands such as Fas ligand (FasL) to members of the TNF α receptor superfamily which are usually called the death receptors, such as Fas (CD95 or APO-1) [17]. The extracellular region of Fas contains FasL binding sites while the intracellular region contains a death domain (DD). Binding of Fas with FasL triggers the trimerization of Fas which stimulates the binding of Fas with Fas-associated protein with death domain (FADD). FADD is a cytosolic protein. After binding with Fas, FADD interacts with pro-caspase-8 through the death effector domain (DED). The binding of Fas, FADD and pro-caspase-8 forms an intracellular multi-protein complex known as the death inducing signaling complex (DISC) [18]. Once formed, the DISC promotes the proximity-induced activation of caspase-8, which is then further processed via an auto-proteolysis mechanism [19]. Activated caspase-8 activates effector caspases, such as caspase-3, and thus leads to apoptosis.

As revealed in the present study, knock-down of HDGF activates not only the intrinsic apoptotic pathway but also the Fas-mediated extrinsic apoptotic pathway which eventually suppresses the anchorage-independent growth of cancer cells. Since the expression level of HDGF is related to the development and progression of human cancer, a better understanding on the mechanism for HDGF regulation of cell proliferation and apoptosis

may lead to the design of new strategies for cancer therapy.

Materials and Methods

Cell culture

Human hepatocellular carcinoma HepG2 cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal bovine serum (Invitrogen, USA) and L-glutamine (Invitrogen, USA) at 37°C in a humidified atmosphere with 10% CO₂.

Antisense oligonucleotides and siRNA transfection

The sequence for the 21-base HDGF antisense oligonucleotides is complementary to the coding region of *hdgf* (nt 1027-1038, 5'-GGT TGG ATC GCG ACA TGG CGG-3') while the sequence of the sense oligonucleotides is the same as the coding sequence of *hdgf* (5'-CCG CCA TGT CGC GAT CCA ACC-3'). All bases were phosphorothioated to increase the stability of the nucleotides. The sequence for the 19-base *fas* siRNA is complementary to the *fas* mRNA (5'-CAC UUG GGC AUU AAC ACU UdT dT-3') and the 19-base *bad* siRNA is complementary to the *bad* mRNA (5'-GAA GGG ACU UCC UCG CCC GdT dT-3'). The cells were transfected with 0.2 μ M HDGF antisense or sense oligos and/or 0.1 μ M *fas* siRNA, *bad* siRNA or scramble siRNA with the aid of Oligofectamine (Invitrogen, USA) according to manufacturer's protocol.

I κ B α full length cDNA transfection

The full-length I κ B α cDNA in pCMV6-XL5 vector was purchased from OriGene Technologies, USA. The cells were transfected with 1.2 μ g/ml I κ B α -pCMV6 or empty vector with the aid of Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's protocol.

Soft agar assay

HepG2 cells were transfected with HDGF antisense oligos. After transfection, 1000 cells in 0.5ml of 0.3% noble agar with DMEM supplemented with 20% FBS were plated onto the top of the existing 0.5% bottom noble agar in each well of 24-well tissue culture plates. The plates were incubated at 37°C in a humidified atmosphere with 10% CO₂ for three weeks. At the end of two weeks incubation, the colonies were stained with 0.05% crystal violet solution. Total number of colonies in each well was counted.

DNA fragmentation assay

The cells were collected by trypsinization and lysed by lysis solution with 5mM Tris-HCl buffer (pH 8.0), 100mM EDTA (pH 8.0), 1% SDS and 0.2mg/ml proteinase K. After incubation at 45°C for 2h, the DNA in the crude lysate was extracted by phenol-chloroform and then precipitated by ethanol. The extracted DNA was dissolved in Tris-EDTA buffer with 0.2mg/ml RNaseA and incubated at 37°C overnight. Thereafter, 30 μ g DNA was loaded into 1.5% EtBr staining agarose gel for electrophoresis. The DNA fragmentation pattern was

viewed under UV light box.

Annexin V binding assay

After trypsinization, the cells were resuspended in Annexin V binding buffer containing 10mM HEPES, 140mM NaCl and 2.5mM CaCl₂. After that the cells were stained with 5μl Annexin V-GFP and 1 μg/ml propidium iodide (PI) at room temperature for 15min. The cells were subjected to flow cytometry using the BD FACSCanto flow cytometer (Becton Dickinson, USA).

RT-PCR

The cells were lysed by Tri Reagent (Molecular Research Centre, USA) for RNA extraction. After that, total RNA was subjected to reverse transcription with oligo dT primer (Invitrogen, USA) by MMLV Reverse Transcriptase (Promega, USA) at 42°C for 1h and heated to 70°C for 15 min. PCR amplification of cDNAs using the pair of primers specific to *fas* open reading frame was carried out for 30 cycles of 94°C for 30s, 55°C for 30s and 70°C for 30s; forward primer (5'-AAG GAG TAC ACA GAC AAA GCC C-3') and reverse primer (5'-AAG AAG AAG ACA AAG CCA CCC-3'). RT-PCR assay with primers specific for β-actin was also performed.

Western blot analysis

The cells were lysed in lysis solution containing 1% Triton X-100, 4.9mM MgCl₂, 1mM sodium ortho-vanadate, 2.1μg/ml aprotinin, 0.5μg/ml leupeptin, and 1mM PMSF. The cell lysate was centrifuged and the protein content in the supernatant was determined by the BCA Assay Kit (Thermo, USA). Protein from each sample was separated by SDS-PAGE according to size and was transferred onto PVDF membrane (Millipore, USA) by electro-blotting. The membrane was incubated with 5% non-fat dry milk in PBS at 4°C overnight and then probed with primary antibody for 2h and with respective horse-radish peroxidase conjugated secondary antibody for 1h. The primary antibodies are anti-caspase-3, Bad and Fas (Santa Cruz, USA), caspase-8 (Stressgene, USA), NF-κB p60, NF-κB p50, IκBα, IκBβ, IKKα and IKKβ (Cell signaling, USA), HDGF (R&D Systems, USA) and β-actin (Sigma, USA). The protein bands were detected by the ECL Western blot detection reagent (Amersham Pharmacia, USA). The protein band intensity was scanned and analyzed by ImageJ (version 1.38x) software.

Construction of *fas* promoter reporter vector

The 442bp *fas* promoter was amplified by PCR reaction from the genomic DNA of HepG2 cells using Platinum Taq DNA polymerase (Invitrogen, USA). The PCR was carried out for 40 cycles of 94°C for 30s, 54°C for 30s and 68°C for 1min. The primers were designed to amplify the *fas* promoter sequence of nt -460 to -19 upstream of the Fas mRNA transcription start site. Forward primer (5' - GAG AGC TAG CCT CGA GGT CCT CAC CTG A-3') and reverse primer (5' - CTC TAA GCT TAG AGC TTC CCC AAC TCC G-3'). The forward and reverse primers contain the HindIII and NheI restriction site (underlined) respectively for the cloning of the *fas* promoter sequence into the pGL3-Basic luciferase reporter

Fig. 1. The effects of HDGF knock-down on apoptotic cell death and anchorage-independent growth in HepG2 cells. HepG2 cells were transfected with HDGF antisense oligos (AS) or HDGF sense oligos (S). Cells with no transfection (N) were also included as control. (A) Protein was extracted from cells at 24h after transfection and the expression level of HDGF was determined. The number beneath the band was the relative expression level of HDGF. It was first normalized with β-actin and then calculated in relation to that with no transfection. (B) The cells were allowed to grow for 24, 48 and 72h after transfection followed by DNA fragmentation assay. M: 100bp DNA ladder. (C) The cells were allowed to grow for 48h after transfection followed by Annexin V binding assay. The number indicated the percentage of cells in the specific quadrant. The bar chart indicated the percentage of early apoptotic cells (grey bar) and late apoptotic cells (white bar). (D) 1000 cells from each treatment were plated in soft agar assay. The number of colonies in each well was counted and was shown in the bar chart. The experiments have been repeated at least three times with similar results and the one shown is the representative one. Error bar: the standard deviation from at least 3 separate experiments. **p*<0.05.

vector (pGL3) forming the *fas* promoter reporter vector (Fas-pGL3).

Firefly dual luciferase reporter assay

1μg of reporter vectors and 40ng of pRL-CMV Renilla normalizing luciferase vector were transfected into cells by Lipofectamine 2000 reagent (Invitrogen, USA) according to manufacturer's protocol. After transfection, cell extracts and

luciferase reagents were prepared with the Promega Dual-Luciferase Reporter Assay System (Promega, USA). Both firefly and Renilla luciferase activities were monitored with luminometer. The firefly luciferase activity was normalized by the Renilla luciferase activity. Relative luciferase activity is then calculated as the normalized activity of the Fas-pGL3 transfected samples relative to the pGL3 transfected sample.

Chromatin immunoprecipitation (ChIP) assay

HepG2 cells were incubated with PBS containing 1% formaldehyde for 10min at 37°C for DNA/protein cross-linking. Cells were lysed and DNA in the supernatant was sheared by sonication. An aliquot of the sonicated sample (Input) was separated for PCR analysis and the remaining sample was used for immunoprecipitation. The nonspecific background was pre-cleared with salmon sperm DNA/Protein A agarose-50% slurry for 30 min at 4°C with rotation. It was then immunoprecipitated with nonspecific rabbit IgG or anti-NF-κB p65 antibody (Santa Cruz, USA) with constant mixing at 4°C overnight. The antibody/protein/DNA complex was collected after the incubation of salmon sperm DNA/Protein A agarose-50% slurry for 1h at 4°C with rotation. Protein A beads were collected by centrifugation and protein/DNA complexes were eluted from the beads followed by a cross-link reversal step, addition of 5M NaCl to the eluted sample and incubation for 4h at 65 °C. DNA from Input and each IP reaction were PCR amplified by 35 cycles of 94°C 30s, 54°C for 30s, and 70°C for 1 min using the primer pair that constructs the *fas* promoter reporter vector.

Results

Induction of apoptosis and suppression of cell growth after knock-down of HDGF

HDGF may act as a survival factor in human cancer cells [15]. As reported previously, knock-down of HDGF by HDGF specific antisense oligonucleotides as well as HDGF specific siRNA suppressed cell proliferation and induced apoptosis in human cancer cells including human hepatocellular carcinoma HepG2 and Hep3B cells, human squamous cell carcinoma A431 cells, human lung carcinoma Calu-6 cells, human cervical carcinoma SiHa and CaSki cells [15]. In this report, we further confirmed that knock-down of HDGF can induce apoptosis as well as suppress the anchorage-independent growth of HepG2 cells (Fig. 1). A fewer number of colonies was observed in HepG2 cells transfected with HDGF antisense oligos than did cells with HDGF sense oligos or with no transfection in soft agar assay (Fig. 1D). Similar results were found in human colorectal carcinoma HT-29 cells (data not shown). Since apoptosis is one of the possible causes for reduction in cell growth, further investigation was therefore carried out to reveal the mechanisms for the HDGF knock-down effect on apoptosis.

Fig. 2. Cleavage of caspase-3 and -8 in relation to apoptosis induction after knock-down of HDGF in HepG2 cells. HepG2 cells were transfected with HDGF antisense oligos (AS) or HDGF sense oligos (S). The cells with no transfection (N) were also included as control. (A) Protein was extracted from cells at 0, 4, 24h after transfection. Western blot analysis was performed and the expression levels of the pro and the active forms of caspases-3 and -8 were determined. The number beneath the band was the relative expression level of that protein. It was calculated first by normalizing with the β -actin and then in relation to the expression at 0h of that treatment. * $p < 0.05$ as compared with 0h of the same treatment. (B) After transfection, the cells were incubated with 30 μ M caspase-3 and -8 inhibitors for 72h followed by DNA fragmentation assay. M: 100bp DNA ladder. The experiments have been repeated at least three times with similar results and the one shown is the representative one. (C) The cells were incubated with caspase-3 and -8 inhibitors for 48h after transfection followed by Annexin V binding assay. The % increase in apoptotic cells was calculated by subtracting the % apoptotic cell in the group transfected with HDGF antisense oligos from that of the corresponding group transfected with HDGF sense oligos. Error bar: the standard deviation from at least 3 separate experiments. * $p < 0.05$ as compared with the no inhibitor control.

Activation of the extrinsic apoptotic pathway by knock-down of HDGF

In our previous report, HDGF knock-down was found to trigger apoptosis through the Bad-mediated intrinsic apoptotic pathway that involved the up-regulation of Bad,

Fig. 3. Induction of Fas expression upon HDGF knock-down in relation to apoptotic cell death in HepG2 cells. (A-B) HepG2 cells were transfected with HDGF antisense oligos (AS) or HDGF sense oligos (S). The cells with no transfection (N) were also included as control. (A) Protein was extracted from cells at 0, 4, 24h after transfection. Western blot analysis was performed and the expression level of Fas was determined. The relative expression level of Fas was calculated first by normalizing with β -actin and then in relation to the expression at 0h of that treatment. * $p < 0.05$ as compared with 0h of the same treatment. (B) RNA was extracted from cells at 16h after transfection. RT-PCR was performed and the expression level of *fas* was determined. The number beneath the band was the relative expression level of *fas*. The result shown above is the representative one of three independent experiments. $\blacktriangle p < 0.05$ as compared with the sample transfected with HDGF sense oligos. (C-E) HepG2 cells were transfected with HDGF antisense oligos (AS) or HDGF sense oligos (S) in combination with *fas* siRNA (Fi) or scramble siRNA (Si). (C) Protein was extracted from cells at 48h after transfection. Western blot analysis was performed and the expression level of HDGF and Fas were determined. The number beneath the band was the relative expression levels of HDGF or Fas in cells under different conditions in relation to that of the control. $\blacktriangle p < 0.05$ as compared with the sample transfected with HDGF sense oligos; $\dagger p < 0.05$ as compared with the sample transfected with scramble siRNA; $\clubsuit p < 0.05$ as compared with sample transfected with HDGF sense oligos and scramble siRNA. (D) Annexin V binding assay was performed at 48h after transfection. The percentages of early apoptotic and late apoptotic cells were shown in the bar chart on the left panel. The bar chart on the right panel showed the % increase in apoptotic cells which was calculated by subtracting the % apoptotic cell in the group transfected with HDGF antisense oligos from that of the corresponding group transfected with HDGF sense oligos. * $p < 0.05$. (E) 1000 cells from each treatment were plated for soft agar assay at 24h after transfection. The number of colonies in each well was counted and was shown in the bar chart on the left panel. The bar chart on the right panel indicated the decrease in colony number which was calculated by subtracting the number of colony in the group transfected with HDGF antisense oligos from that of the corresponding group transfected with HDGF sense oligos. All the data are average from three independent experiments. Error bar: the standard deviation from at least three separated experiments. * $p < 0.05$.

the activation of caspase-3 and -9 [15]. However, inactivation of the intrinsic pathway alone cannot fully overcome the HDGF knock-down effect on apoptosis. Since the intrinsic and extrinsic pathways may work together in triggering apoptosis, the effect of HDGF knock-down on the extrinsic apoptotic pathway was therefore also examined. Western blot analysis showed that there were increases in the expressions of the active forms and decreases in the pro-forms of caspase-3 and -8 after knock-down of HDGF (Fig. 2A), indicating the increases in cleavage of caspase-3 and -8. Moreover, caspases-3 and -8 inhibitors abrogated the apoptosis induced by the knock-down of HDGF, as determined by DNA fragmentation assay (Fig. 2B) and Annexin V binding assay (Fig. 2C). On the other hand, a significant increase in the protein and also mRNA levels of the death

receptor Fas was observed in HepG2 cells after knock-down of HDGF (Fig. 3A and B). Taken together it suggests that knock-down of HDGF may also activate the extrinsic apoptotic pathway which involved the up-regulation of Fas, the cleavage of caspase-8 and -3.

Involvement of Fas in apoptosis induction and cell growth suppression upon HDGF knock-down

Death receptor Fas (CD95) is one of the key activators of the extrinsic apoptotic pathway. Since Fas was up-regulated after knock-down of HDGF, the role of Fas in apoptosis induction was investigated. Western blot analysis showed that the Fas siRNA counteracted the Fas up-regulation upon HDGF knock-down (Fig. 3C). At the same time, the Fas siRNA also reduced the level of apoptosis induction upon HDGF knock-down; a

Fig. 4. The increase in *fas* promoter activity and the NF- κ B signaling pathway upon HDGF knock-down in HepG2 cells. (A) The cells were transfected with empty reporter vector (pGL3) or the *fas* promoter reporter vector (Fas-pGL3) in combination with HDGF antisense oligos (AS) or HDGF sense oligos (S). Transfection with the reporter vectors alone was also performed as control (N); 40ng of pRL-CMV vector was co-transfected for normalization. After transfection, firefly dual luciferase assay was performed for the determination of the luciferase activity. Relative fold activity was calculated as the ratio between the normalized Fas-pGL3 activity and the normalized pGL3 value. * $p < 0.05$. (B) Binding of NF- κ B p65 on *fas* promoter in HepG2 cells was determined by ChIP assay. The genomic DNA of HepG2 cells was immunoprecipitated by NF- κ B p65 specific antibody. The immunoprecipitates were analyzed by PCR for the presence of the *fas* promoter sequence. Chromatin starting materials not subjected to immunoprecipitation (Input) and immunoprecipitation with non-specific rabbit IgG (-ve) were also performed as the positive and negative control respectively. M: 100bp DNA ladder. (C) HepG2 cells were transfected with HDGF antisense oligos and protein was extracted at 0, 4 and 24 hours after transfection. Western blot analysis was performed and the expression levels of IkB α , IkB β , NF- κ B p65, NF- κ B p50, IKK α and IKK β were determined. The number beneath the band of the specific protein was the relative expression level of that protein. It was calculated first by normalizing with β -actin and then in relation to the expression at 0h of that treatment. The results shown above are the representative one of three independent experiments. Error bar: the standard deviation from at least three separated experiments. * $p < 0.05$ as compared with the sample at 0h of the corresponding treatment.

smaller number of apoptotic cells was detected by knocking down of both Fas and HDGF when compared with HDGF alone (Fig. 3D). It supports that the death receptor Fas may mediate the effect of HDGF knock-down on apoptosis induction in HepG2 cells.

To further determine whether Fas may also be involved in the suppression of anchorage-independent growth upon knock-down of HDGF, soft agar assay was performed. A smaller reduction in the number of colonies was observed in HepG2 cells after knock-down of both Fas and HDGF when compared with HDGF alone (Fig. 3E). It therefore suggests that Fas may also be responsible for the suppression of anchorage-independent growth after knock-down of HDGF in HepG2 cells.

Activation of *fas* promoter upon HDGF knock-down

To further investigate the mechanism that leads to Fas up-regulation, the promoter activity of *fas* was determined. A 442bp *fas* promoter sequence upstream of the *fas* transcriptional start site (-460 to -19) was amplified from the genomic DNA of HepG2 cells and cloned into the pGL3-Basic luciferase vector. As shown

in Fig 4A, a higher luciferase activity was demonstrated in cells when the *fas* promoter construct was co-transfected with HDGF antisense oligos than with HDGF sense oligos. The results indicate the activation of *fas* promoter upon HDGF knock-down in HepG2 cells.

NF- κ B pathway mediates the effect of HDGF knock-down on Fas up-regulation and apoptosis induction

Since *fas* promoter activity was elevated upon HDGF knock-down, further study was carried out to identify the transcription factors that may involve. According to the literature, *fas* promoter can be activated by NF- κ B (nuclear factor-kappaB) [20]. Therefore, ChIP assay was performed to determine whether there was interaction between NF- κ B and *fas* promoter sequence in HepG2 cells. DNA/protein cross-linked sample was extracted from HepG2 cells. Immunoprecipitation with anti-NF- κ B p65 antibody revealed the presence of the 442bp *fas* promoter sequence (-460 to -19) in the sample while it was absent in the case with non-specific rabbit IgG (Fig. 4B). The results indicate the interaction of NF- κ B p65 with the *fas* promoter sequence in HepG2 cells.

Fig. 5. The inhibitory effect of I κ B α overexpression on the *fas* promoter activity and the apoptotic cell death upon knock-down of HDGF. HepG2 cells were transfected with HDGF antisense oligos (AS) or HDGF sense oligos (S) in combination with I κ B α vector (I) or empty vector (P). The cells with no transfection (N) were also included as control. (A) Protein was extracted from cells at 48h after transfection. Western blot analysis was performed and the expression levels of HDGF and Fas were determined. The number beneath the band was the relative expression level of the specific protein in cells with different treatments in relation to that of the control. $\blacktriangle$ $p < 0.05$ as compared with the sample transfected with HDGF sense oligos; $\dagger p < 0.05$ as compared with the sample transfected with empty vector; $\clubsuit p < 0.05$ as compared with sample transfected with HDGF sense oligos and empty vector. (B) HepG2 cells were transfected with HDGF antisense oligos (AS) or HDGF sense oligos (S), I κ B α vector (I) or empty vector (P) together with *fas* promoter reporter vector (Fas-pGL3) or empty reporter vector (pGL3). Transfection with the reporter vectors alone was also performed as control (N); 40ng of pRL-CMV vector was co-transfected for normalization. After transfection, firefly dual luciferase assay was performed for the determination of the luciferase activity. The bar chart on the left panel indicates the relative luciferase activity calculated as the ratio between the normalized Fas-pGL3 activity of each treatment and the normalized pGL3 value. The bar chart on the right panel indicates the HDGF effect on luciferase activity calculated as the ratio between the normalized Fas-pGL3 activity of the group transfected with HDGF antisense oligos and that of the corresponding group transfected with HDGF sense oligos. $*p < 0.05$. (C) Annexin V binding assay was performed 48h after transfection with HDGF antisense oligos and I κ B α carrying vector. The percentage of early apoptotic and late apoptotic cell were shown in the bar chart on the left panel. The bar chart on the right panel shows the % increase in apoptotic cells which was calculated by subtracting the % apoptotic cell in the group transfected with HDGF antisense oligos from that of the corresponding group transfected with HDGF sense oligos. Error bar: the standard deviation from at least three separated experiments. $*p < 0.05$.

Fig. 6. The effect of caspase-3, -8 and -9 inhibitors on the level of HDGF knock-down induced apoptosis in HepG2 cells. HepG2 cells were transfected with HDGF antisense oligos (AS) or HDGF sense oligos (S). After transfection, the cells were incubated with 30 μ M caspase-3, -8 and -9 inhibitors for 48h followed by Annexin V binding assay. The percentage of early apoptotic and late apoptotic cell were determined. The % increase in apoptotic cells was calculated by subtracting the % apoptotic cell in the group transfected with HDGF antisense oligos from that of the corresponding group transfected with HDGF sense oligos. The data are average from three independent experiments. Mean $\pm$ SEM. $*p < 0.05$.

Moreover, NF- κ B inhibitor BAY11-7082 was found to reduce *fas* promoter activity and Fas protein expression in HepG2 cells (data not shown). It therefore supports that NF- κ B is involved in the regulation of Fas expression in HepG2 cells.

NF- κ B is a transcription factor. Its activity can be activated by I κ B (Inhibitor of κ B) and IKK (I κ B kinase). Upon stimulation, IKK is phosphorylated and triggers the phosphorylation and degradation of I κ B and that leads to the activation of NF- κ B. After NF- κ B was confirmed to

Fig. 7. Induction of Fas and Bad expression upon HDGF knock-down in relation to apoptotic cell death and anchorage-independent growth in HepG2 cells. HepG2 cells were transfected with HDGF antisense oligos (AS) or HDGF sense oligos (S) in combination with *fas* siRNA (Fi), Bad siRNA (Bi) or scramble siRNA (Si). (A) Annexin V binding assay was performed at 48h after transfection. The bar chart shows the % increase in apoptotic cells which was calculated by subtracting the % apoptotic cell in the group transfected with HDGF antisense oligos from that of the corresponding group transfected with HDGF sense oligos. (B) 1000 cells from each treatment were subjected to soft agar assay at 24h after transfection. The number of colonies in each well was counted. The bar chart indicates the decrease in colony count which was calculated by subtracting the number of colony in the group transfected with HDGF antisense oligos from that of the corresponding group transfected with HDGF sense oligos. The data are average from three independent experiments. Error bar: the standard deviation from at least three separated experiments.

regulate the *fas* promoter activity, the effect of HDGF knock-down on the expressions of the NF- κ B family proteins were therefore investigated. By Western blot analysis, significant down-regulations of I κ B α (~80%) and I κ B β (~50%) were observed while there were no obvious changes in the levels of NF- κ B p65, NF- κ B p50, IKK α and IKK β in HepG2 cells after knock-down of HDGF (Fig. 4C).

Even though there is no obvious change in the NF- κ B expression after HDGF knock-down, a significant decrease in I κ B may still indicate the activation of the NF- κ B pathway for *fas* promoter activation and Fas up-regulation in HepG2 cells. The idea is supported as overexpression of I κ B α by transfection with I κ B α expression vector reversed the HDGF knock-down effect on Fas up-regulation (Fig. 5A) as well as *fas* promoter activation (Fig. 5B). The NF- κ B inhibitor BAY11-7082 also inhibited HDGF knock-down effect on Fas up-regulation and *fas* promoter activation (data not shown). On the other hand, less apoptotic cells were detected when HepG2 cells were co-transfected with I κ B α expression vector and HDGF antisense oligos than those with HDGF antisense oligos only (Fig. 5C). Taken together, the results from Fig. 5 suggest that down-regulation of I κ B α is important in mediating the HDGF knock-down effect on Fas up-regulation and apoptosis induction.

Interaction between the extrinsic and intrinsic apoptotic pathways in apoptosis induced by HDGF knock-down

Since both the Bad-mediated intrinsic apoptotic pathway and the Fas-mediated extrinsic apoptotic pathway

are conferred to be triggered upon HDGF knock-down, we would therefore like to find out how the two pathways may interact in apoptosis regulation. As shown in Fig. 6, both caspase-9 and caspase-8 inhibitors greatly reduced the apoptosis induced by HDGF knock-down while the combination of both inhibitors demonstrated an even greater suppression effect (Fig. 6).

Knock-down of Fas and Bad respectively decreased the HDGF knock-down effect on the induction of apoptosis and suppression of anchorage-independent cell growth. Similarly, greater decreases were demonstrated by knock-down of both Fas and Bad together (Fig. 7). The results therefore indicate that both the extrinsic and intrinsic apoptotic pathways are important and both pathways work together in mediating the effect of HDGF knock-down on apoptosis and cancer growth.

Discussion

Up-regulation of Fas upon HDGF knock-down was triggered by the NF- κ B transactivation pathway. NF- κ B is a transcription factor and the most common form is the p50/p65 heterodimer. According to literature, the NF- κ B p50/p65 heterodimer activated the *fas* promoter by binding onto the 10bp Sp1- κ B binding sequence at -295 to -286 of the 442bp *fas* promoter sequence, -460 to -19bp upstream of the *fas* transcription start site [20]. In HepG2 cells, the Fas expression is also regulated by NF- κ B as NF- κ B p65 was found to bind onto the 442bp *fas* promoter (Fig. 4B) and NF- κ B inhibitor BAY11-7082 suppressed both *fas* promoter activity and Fas expression (data not shown). At the same time, HDGF knock-down was found

to activate the *fas* promoter and up-regulate Fas expression. The activity of NF- κ B is regulated by the upstream signaling pathway which involves I κ B (Inhibitor of κ B). There are three major I κ B proteins, I κ B α , I κ B β and I κ B ϵ . Among them, I κ B α and I κ B β which contain the PEST C-terminal domain for binding with NF- κ B are more important in regulating the activity of NF- κ B; especially I κ B α , which is the most abundant I κ B proteins [21]. Upon activation, I κ B becomes phosphorylated and undergoes ubiquitination and proteasomal degradation. It then leads to the release of NF- κ B. The free NF- κ B dimerizes and enters the nucleus to regulate gene expressions [22]. Knock-down of HDGF decreased the expressions of I κ B α and I κ B β but not the other NF κ B family proteins (Fig. 4C). I κ B α and I κ B β are important in suppressing the NF- κ B activity in cells. It was reported that loss of function mutation of I κ B α can lead to constitutive activation of NF- κ B in Hodgkin's disease-derived cell lines indicating the importance of I κ B α in regulating the NF- κ B activity [23]. Therefore, even though there is no obvious change in NF- κ B expression level after knock-down of HDGF, down-regulation of I κ B will probably still allow NF- κ B to stimulate the gene expressions, e.g. Fas. This is confirmed as overexpression of I κ B α reversed the HDGF knock-down effect on Fas up-regulation, Fas promoter activation, apoptosis induction, and consequently, cell growth retardation (Fig. 5). Therefore, it indicates that I κ B α is up-stream of Fas in mediating the HDGF knock-down effect on cells.

HDGF may act as a survival factor for human cancer cells at least through the regulation of the apoptotic pathways. HDGF knock-down was found to suppress the anchorage-dependent growth [15] as well as the anchorage-independent growth and triggers apoptosis in a number of human cancer cells (Fig. 1B-C). In addition to the intrinsic pathway, in the present study, the extrinsic apoptotic pathway was also confirmed to be involved. Up-regulation of death receptor Fas and increases in the cleavage of both caspase-8 and -3 were observed upon HDGF knock-down. Furthermore, inactivation by Fas siRNA or inhibitors of caspase-8 and -3 revert the apoptosis induction by HDGF knock-down. Both the extrinsic and intrinsic apoptotic pathways are important in mediating the HDGF knock-down effect on apoptosis and cell growth (Fig. 8). Inactivation of either pathway alone demonstrated less suppressive effect than inactivation of both pathways together, as in the case of using caspase-8 and -9 inhibitors (Fig. 6) and knock-down of Bad and Fas (Fig. 7).

The extrinsic and intrinsic apoptotic pathways may

Fig. 8. An overview of the extrinsic and intrinsic apoptotic pathways activated upon HDGF knock-down.

interact with each other through different mediator molecules, e.g. Bid. The activation of caspase-8 in the extrinsic pathway may trigger the cleavage of Bid into truncated Bid (tBid). The truncated Bid then exerts its pro-apoptotic effect by translocation to the mitochondria where it promotes oligomerization of Bax and Bak on the mitochondria leading to the activation of the intrinsic pathway [24]. To support that, increases in Bid and tBid were detected in HepG2 cells upon knock-down of HDGF (data not shown). In addition to Bid, whether the two pathways may interact at any part upstream is still not clear. Besides up-regulation of Bad, knock-down of HDGF was previously reported to suppress the MEK/ERK pathway [15]. Whether the MEK/ERK pathway may cross talk with the NF- κ B pathway [25] in apoptosis regulation upon HDGF knock-down will require further investigation.

The up-stream mechanisms for HDGF to regulate the intracellular signaling pathways are also not clear. The growth factor may exert its effect by binding onto the yet to be identified cell surface receptor. Or, the growth factor, which contains a NLS and DNA binding PWWP domain, may translocate into the nucleus and interact with DNA to regulate gene expression directly [26]. Nevertheless, the present findings support that HDGF may control cell survival through the regulation of the Fas-mediated extrinsic apoptotic pathway and the Bad-

mediated intrinsic apoptotic pathway via the NF- κ B and the MEK/ERK pathways. A better understanding of the action mechanism for HDGF to regulate cancer cell growth may help to design new strategy for targeting HDGF in cancer therapy.

Acknowledgements

The work is supported by a grant from Hong Kong Research Grants Council Earmarked Grant CUHK4428/05M and a grant from the Shanghai-Hong Kong Anson Research Foundation, Hong Kong.

References

- Nakamura H, Kambe H, Egawa T, Kimura Y, Ito H, Hayashi E, Yamamoto H, Sato J, Kishimoto S: Partial purification and characterization of human hepatoma-derived growth factor. *Clin Chim Acta* 1989;183:273-284.
- Nakamura H, Izumoto Y, Kambe H, Kuroda T, Mori T, Kawamura K, Yamamoto H, Kishimoto T: Molecular cloning of complementary DNA for a novel human hepatoma-derived growth factor. Its homology with high mobility group-I protein. *J Biol Chem* 1994;269:25143-25149.
- Everett AD, Lobe DR, Matsumura ME, Nakamura H, McNamara CA: Hepatoma-derived growth factor stimulates smooth muscle cell growth and is expressed in vascular development. *J Clin Invest* 2000;105:567-575.
- Oliver JA, Al-Awqati Q: An endothelial growth factor involved in rat renal development. *J Clin Invest* 1998;102:1208-1219.
- Hu TH, Huang CC, Liu LF, Lin PR, Liu SY, Chang HW, Changchien CS, Lee CM, Chuang JH, Tai MH: Expression of hepatoma-derived growth factor in hepatocellular carcinoma. *Cancer* 2003;98:1444-1456.
- Yoshida K, Nakamura H, Okuda Y, Enomoto H, Kishima Y, Uyama H, Ito H, Hirasawa T, Inagaki S, Kawase I: Expression of hepatoma-derived growth factor in hepatocarcinogenesis. *J Gastroenterol Hepatol* 2003;18:1293-1301.
- Huang JS, Chao CC, Su TL, Yeh SH, Chen DS, Chen CT, Chen PJ, Jou YS: Diverse cellular transformation capability of overexpressed genes in human hepatocellular carcinoma. *Biochem Biophys Res Commun* 2004;315:950-958.
- Yoshida K, Tomita Y, Okuda Y, Yamamoto S, Enomoto H, Uyama H, Ito H, Hoshida Y, Aozasa K, Nagano H, Sakon M, Kawase I, Monden M, Nakamura H: Hepatoma-derived growth factor is a novel prognostic factor for hepatocellular carcinoma. *Ann Surg Oncol* 2006;13:159-167.
- Yamamoto S, Tomita Y, Hoshida Y, Takiguchi S, Fujiwara Y, Yasuda T, Doki Y, Yoshida K, Aozasa K, Nakamura H, Monden M: Expression of hepatoma-derived growth factor is correlated with lymph node metastasis and prognosis of gastric carcinoma. *Clin Cancer Res* 2006;12:117-122.
- Chang KC, Tai MH, Lin JW, Wang CC, Huang CC, Hung CH, Chen CH, Lu SN, Lee CM, Changchien CS, Hu TH: Hepatoma-derived growth factor is a novel prognostic factor for gastrointestinal stromal tumors. *Int J Cancer* 2007;121:1059-1065.
- Lepourcelet M, Tou L, Cai L, Sawada J, Lazar AJ, Glickman JN, Williamson JA, Everett AD, Redston M, Fox EA, Nakatani Y, Shivdasani RA: Insights into developmental mechanisms and cancers in the mammalian intestine derived from serial analysis of gene expression and study of the hepatoma-derived growth factor (HDGF). *Development* 2005;132:415-427.
- Ren H, Tang X, Lee JJ, Feng L, Everett AD, Hong WK, Khuri FR, Mao L: Expression of hepatoma-derived growth factor is a strong prognostic predictor for patients with early-stage non-small-cell lung cancer. *J Clin Oncol* 2004;22:3230-3237.
- Uyama H, Tomita Y, Nakamura H, Nakamori S, Zhang B, Hoshida Y, Enomoto H, Okuda Y, Sakon M, Aozasa K, Kawase I, Hayashi N, Monden M: Hepatoma-derived growth factor is a novel prognostic factor for patients with pancreatic cancer. *Clin Cancer Res* 2006;12:6043-6048.
- Bernard K, Litman E, Fitzpatrick JL, Shellman YG, Argast G, Polvinen K, Everett AD, Fukasawa K, Norris DA, Ahn NG, Resing KA: Functional proteomic analysis of melanoma progression. *Cancer Res* 2003;63:6716-6725.
- Tsang TY, Tang WY, Tsang WP, Co NN, Kong SK, Kwok TT: Downregulation of hepatoma-derived growth factor activates the Bad-mediated apoptotic pathway in human cancer cells. *Apoptosis* 2008;13:1135-1147.
- Khosravi-Far R, Esposti MD: Death receptor signals to mitochondria. *Cancer Biol Ther* 2004;3:1051-1057.
- Debatin KM, Krammer PH: Death receptors in chemotherapy and cancer. *Oncogene* 2004;23:2950-2966.
- Walczak H, Sprick MR: Biochemistry and function of the DISC. *Trends Biochem Sci* 2001;26:452-453.
- Donepudi M, Mac Sweeney A, Briand C, Grutter MG: Insights into the regulatory mechanism for caspase-8 activation. *Mol Cell* 2003;11:543-549.
- Chan H, Bartos DP, Owen-Schaub LB: Activation-dependent transcriptional regulation of the human Fas promoter requires NF- κ B p50-p65 recruitment. *Mol Cell Biol* 1999;19:2098-2108.
- Whiteside ST, Israel A: I kappa B proteins: structure, function and regulation. *Semin Cancer Biol* 1997;8:75-82.
- Karin M, Ben-Neriah Y: Phosphorylation meets ubiquitination: the control of NF- κ B activity. *Annu Rev Immunol* 2000;18:621-663.
- Cabannes E, Khan G, Aillet F, Jarrett RF, Hay RT: Mutations in the I κ B α gene in Hodgkin's disease suggest a tumour suppressor role for I κ B α . *Oncogene* 1999;18:3063-3070.
- Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ: tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* 2000;14:2060-2071.
- Maeng YS, Min JK, Kim JH, Yamagishi A, Mochizuki N, Kwon JY, Park YW, Kim YM, Kwon YG: ERK is an anti-inflammatory signal that suppresses expression of NF- κ B-dependent inflammatory genes by inhibiting IKK activity in endothelial cells. *Cell Signal* 2006;18:994-1005.
- Nameki N, Tochio N, Koshiba S, Inoue M, Yabuki T, Aoki M, Seki E, Matsuda T, Fujikura Y, Saito M, Ikari M, Watanabe M, Terada T, Shirouzu M, Yoshida M, Hirota H, Tanaka A, Hayashizaki Y, Guntert P, Kigawa T, Yokoyama S: Solution structure of the PWWP domain of the hepatoma-derived growth factor family. *Protein Sci* 2005;14:756-764.