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Suppression by Heat Shock Protein 20 of Hepatocellular Carcinoma Cell Proliferation via Inhibition of the Mitogen-Activated Protein Kinases and AKT Pathways

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ABSTRACT

Heat shock protein (HSP) 20, one of the low-molecular weight HSPs, is known to have versatile functions, such as vasorelaxation. However, its precise role in cancer proliferation remains to be elucidated. While HSP20 is constitutively expressed in various tissues including the liver, we have previously reported that HSP20 protein levels in human hepatocellular carcinoma (HCC) cells inversely correlate with the progression of HCC. In this study, we investigated the role of HSP20 in HCC proliferation. The activities of extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and AKT were negatively correlated with the HSP20 protein levels in human HCC tissues. Since HSP20 proteins were hardly detected in HCC-derived cell lines, the effects of HSP20 expression were evaluated using human HCC-derived HuH7 cells that were stably transfected with wild-type human HSP20 (HSP20 overexpressing cells). In HSP20 overexpressing cells, cell proliferation was retarded, and the activation of the mitogen-activated protein kinases (MAPKs) signaling pathways, including the ERK and JNK, and AKT pathways, as well as cyclin D1 accumulation induced by either transforming growth factor-α (TGFα) or hepatocyte growth factor, were significantly suppressed compared with the empty vector-transfected cells (control cells). Taken together, our findings strongly suggest that HSP20 suppresses the growth of HCC cells via the MAPKs and AKT signaling pathways, thus suggesting that the HSP20 could be a new therapeutic target for HCC. J. Cell. Biochem. 112: 3430–3439, 2011.

KEY WORDS: HSP20; HEPATOCCELLULAR CARCINOMA; GROWTH FACTOR; MAPK; AKT

Small heat shock proteins (HSPs) have molecular masses from 15 to 30 kDa based on their apparent molecular sizes. These proteins have significant similarities in terms of their amino acid sequences, known as the α-crystallin domain [Kappé et al., 2003; Taylor and Benjamin, 2005]. HSPs, which are well recognized to function as molecular chaperones, have been reported to be involved in a wide range of human cancers such as skin, breast, lung, prostate, colon and liver, and implicated in tumor cell proliferation, differentiation, invasion, metastasis, death, and recognition by the immune system [Ciocca and Calderwood, 2005]. HSP20 (HSPB6) was first identified from skeletal muscle [Kato et al., 1994; Taylor and Benjamin, 2005], and it is highly expressed in normal skeletal and smooth muscle, heart and liver tissues, where it may be essential [Kato et al., 1994]. HSP20 shows versatile functions, such as suppression of platelet aggregation [Kozawa et al., 2002], association with insulin resistance [Wang et al., 2001], prevention of vasospasms [Flynn et al., 2005], and airway smooth muscle relaxation [Komalavilas et al., 2008]. In addition, recent studies

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showed that HSP20 has protective functions in the heart [Fan et al., 2006, 2008; Fan and Kranias, 2010].

Hepatocellular carcinoma (HCC) is the third of most lethal cancer worldwide [Greten et al., 2009]. Even after resection, the overall survival of patients with HCC is still unsatisfactory due to frequent recurrence [Ercolani et al., 2003]. There is increasing evidence that protumorigenic growth factor signaling pathways, such as the transforming growth factor-α (TGFα)/epidermal growth factor (EGF)-signaling and/or hepatocyte growth factor (HGF) signaling pathways, are dysregulated in human HCC [Daveau et al., 2003; Breuhahn et al., 2006]. TGFα has been reported to correlate with differentiation and proliferation, which is likely to affect the early stages of hepatocarcinogenesis [Breuhahn et al., 2006]. HGF is also a potent growth factor for hepatocytes, and has been shown to be increased in clinical HCC tissues. Moreover, the HGF levels in the serum negatively correlate with the survival of patients and positively correlate with tumor size [Breuhahn et al., 2006]. MET, a HGF receptor, is an oncogene, and has been reported to be dysregulated in a subset of human HCCs, and its expression is related to more aggressive phenotype and poor prognosis of HCC [Daveau et al., 2003].

Mitogen-activated protein kinases (MAPKs) and AKT are essential components of intracellular signal transduction, and are activated by phosphorylation in response to various extracellular stimuli, including growth factors, such as TGFα and HGF [Breuhahn et al., 2006]. Among the MAPK superfamily, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) are key molecules that transfer signals into the nuclei to induce proliferation and differentiation [Ito et al., 1998; Kojima et al., 2004]. ERK has been reported to act as a potent proliferative factor in HCC and to be constitutively activated in the human HCC cells and tissues [Ito et al., 1998; Matsushima-Nishiwaki et al., 2001; Kojima et al., 2004]. JNK is also known as a potent inducer of proliferation in HCC [Hui et al., 2008]. As for AKT, while it generally regulates multiple processes, such as apoptosis, cell proliferation, glucose utilization, and anchorage-dependency [Datta et al., 1999], increasing evidence suggests that it also plays a major role in carcinogenesis and drug resistance in numerous types of cancer, including HCC [Datta et al., 1999; Nakanishi et al., 2005]. In particular, AKT phosphorylation has been reported as a significant risk factor for early HCC recurrence and poor prognosis [Nakanishi et al., 2005]. By inhibiting glycogen synthase kinase (GSK)-3β, both AKT and ERK-mediated growth factor pathways promote dephosphorylation and stabilization of cyclin D1 [Cohen and Frame, 2001]. Therefore, cyclin D1 plays a crucial role in neoplastic transformation and growth [Kim and Diehl, 2009], and its expression has been reported to be increased in HCC [Zhang et al., 1993].

Our previous studies showed that the level of HSP20 in human HCC tissues inversely correlates with the tumor size and the TNM stage of HCC [Noda et al., 2007]. However, the exact role of HSP20 in cancer progression remains to be clarified. The present study aimed to unveil the role of HSP20 in HCC in vivo and in vitro. Using a HCC-derived cell line that was stably transfected with wild-type HSP20, we demonstrated that HSP20 expression affects the proliferation of HCC cells and both the MAPK and AKT signaling pathways induced by TGFα and HGF.

MATERIALS AND METHODS

ANTIBODIES, CHEMICALS, AND PLASMIDS

Anti-HSP20 antibodies were purchased from Stressgen Biotechnologies Corporation (Atlanta, GA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against ERK (p44/p42 MAPK), phospho-ERK (threonine (Thr) 202/tyrosine (Tyr) 204), MEK, phospho-MEK (serine (Ser) 217/221), JNK, phospho-JNK (Thr183/Tyr185), c-Jun, phospho-c-Jun (Ser63), Elk-1, phospho-Elk-1 (Ser383), AKT, phospho-AKT (Thr308), GSK-3β, phospho-GSK-3β (Ser9), phosphoinositide-dependent protein kinase (PDK)1, phospho-PDK1 (Ser 241), and cyclin D1 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Recombinant human TGFα and HGF were obtained from R&D systems Inc. (Minneapolis, MN). Wild-type human HSP20 cDNA (clone ID 6074542), which was obtained from Open Biosystems, Inc. (Huntsville, AL), was cut out using restriction enzymes (*EcoRI* and *Xho* I) and subcloned into the eukaryotic expression vector pcDNA 3.1(+) (Invitrogen Corp., Carlsbad, CA).

TISSUE SPECIMENS

HCC tissues were obtained by surgical resection from 23 patients in the Department of Surgery, Ogaki Municipal Hospital (Gifu, Japan) according to a protocol approved by the committee for the conduct of human research at Ogaki Municipal Hospital and at Gifu University Graduate School of Medicine. Informed consent was obtained from all of the patients.

CELL CULTURES

Human HCC-derived HuH7, HLE, HLF, and PLC/PRF/5 cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA). HuH7 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma–Aldrich, MO) supplemented with 1% fetal calf serum (FCS) (Hyclone Co, Logan, UT) and other cell lines were cultured in DMEM (Sigma–Aldrich) with 10% FCS.

ESTABLISHMENT OF A STABLY TRANSFORMED CELL LINE

We newly established two new HuH7 cell lines, which were transfected with either a wild-type HSP20 vector or an empty control vector (called HSP20 overexpressing cells and control cells, respectively), by means of Tet-Off^{TM} gene expression systems (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions. Induction of HSP20 protein expression in HSP20 overexpressing cells can be controlled by the presence of doxycycline (Sigma–Aldrich). HSP20 overexpressing cells and control cells were maintained in RPMI1640 supplemented with 1% FCS, 200 μg/ml G418 (Invitrogen), 100 μg/ml hygromycin B (Merck KGaA Co. Darmstadt, Germany) and 1 μg/ml doxycycline.

PREPARATION OF PROTEIN SAMPLES AND THE WESTERN BLOT ANALYSIS

In order to induce HSP20 expression, HSP20 overexpressing cells and control cells were incubated in RPMI1640 without doxycycline

for 24 h. The cells were then cultured under serum-starvation for another 24 h and subsequently stimulated with 20 ng/ml TGFα or 20 ng/ml HGF for the indicated periods. A Western blot analysis of the lysates from HCC tissues and the cultured cells was performed as described previously [Noda et al., 2007; Matsushima-Nishiwaki et al., 2008]. To quantify the protein from the HCC tissue extracts, 0.3 μl of MagicMark XP Western protein standard (Invitrogen), a marker protein, was run in every gel. The samples from the cell cultures to be quantitatively compared by the Western blot analyses were run in the same gel. The data of the normalized values of the protein bands were statistically analyzed as described in the Statistical analysis section.

CELL COUNTING ASSAY AND BROMODEOXYURIDINE (BRDU) INCORPORATION ASSAYS

HSP20 overexpressing cells and control cells were plated on 96-well dishes (3×10^3 cells/well) in RPMI1640 medium with 10% FCS without doxycycline. For the cell counting assay, the medium was exchanged for RPMI1640 with 1% FCS 24 h after seeding, and cells were incubated for the indicated periods. The cell numbers were then counted using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions and the trypan blue dye exclusion method. For the BrdU incorporation assay, the medium was exchanged for RPMI1640 with 1% FCS 24 h after seeding, and cells were incubated for an additional 24 h. BrdU incorporation was then quantified using Cell proliferation enzyme-linked immunosorbent assay (ELISA), BrdU (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions.

DENSITOMETRIC ANALYSIS

The densitometric analysis was performed using a scanner and an image analysis software program (image J version 1,32). The phosphorylated protein levels were calculated as follows: the background-subtracted signal intensity of each phosphorylation signal was normalized to the respective total protein signal and plotted as the fold increase in comparison to control cells without stimulation.

STATISTICAL ANALYSIS

Data are expressed as the means ± SD. The statistical significance of the data from the cell culture experiments was analyzed using the Mann–Whitney *U*-test, and the patient clinical data were analyzed using the Spearman's correlation coefficient (r). All *P* values were derived from two-tailed tests and $P < 0.05$ was accepted as statistically significant. A Spearman's correlation coefficient of $|r| > 0.400$ was accepted as a positive correlation.

RESULTS

THE LEVELS OF HSP20 INVERSELY CORRELATED WITH THE LEVELS OF ERK, JNK, AND AKT ACTIVATION IN HUMAN HCC TISSUES

In this study, we first examined the correlation between HSP20 protein levels and the expression levels of phosphorylated ERK, JNK, and AKT in the clinical HCC tissues. The clinical and pathological characteristics of the patients with HCC are shown in Figure 1A. We found that the HSP20 protein levels were negatively correlated with the phosphorylation level of ERK ($r = -0.730$, $P = 0.0006$; Fig. 1B), JNK ($r = -0.555$, $P = 0.0092$; Fig. 1C), and AKT ($r = -0.646$, $P = 0.0024$; Fig. 1D). These results led us to further investigate the role of HSP20 in vitro.

THE EXPRESSION OF THE HSP20 PROTEIN IN HCC CELL LINES

In our previous study [Noda et al., 2007], we performed an immunohistochemical analysis of HSP20 in HCC specimens from several patients with HCC. We showed that HSP20 expression was detected in both HCC cells and the hepatocytes of non-tumor tissue. We have also provided some Western blot analysis data about HSP20 expression in HCC tissues [Noda et al., 2007, Supplementary Fig. 1A). Therefore, we decided to use an HCC cell line in order to analyze the role of HSP20 in the present study. We examined the endogenous expression levels of the HSP20 protein in five HCC cell lines, HuH7, HepG2, HLE, HLF, and PLC/PRF/5. Unexpectedly, HSP20 protein was hardly detected in any of the HCC cell lines (Supplementary Fig. 1A).

OVEREXPRESSION OF HSP20 DECREASES THE PROLIFERATION OF HCC CELLS

Based on our finding that HSP20 was absent or minimally expressed in HCC cell lines and tumor tissue specimens, we next established HuH7 cell lines which were transfected with wild-type HSP20 (HSP20 overexpressing cells) or an empty vector (control cells), as described in the Materials and Methods section and investigated the effect of HSP20 on cell proliferation in these HCC cells. A Western blot analysis demonstrated that HSP20 was overexpressed in the HSP20 overexpressing cells, but not control cells (Supplementary Fig. 1B). In our experiments, the results from the naive cells were almost the same as those from the control cells (data not shown).

To clarify the relationship between the expression of the HSP20 protein and cell growth, we first performed a cell counting assay using these two cell lines. The cell growth of the HSP20 overexpressing cells was remarkably suppressed in comparison to that of the control cells (Fig. 2). In addition to retarding cell proliferation, the cell viability of the HSP20 overexpressing cells also decreased more than that of the control cells. The percent cell viability of the HSP20 overexpressing cells and control cells were 50.5% and 85.9% after 7 days, and 50.3% and 71.0% after 8 days, respectively. To further investigate the inhibitory effect of HSP20 on HCC cell proliferation, BrdU incorporation, an indicator of cell proliferation, was analyzed. As expected, the BrdU incorporation in HSP20 overexpressing cells was significantly decreased in comparison to that of control cells (Supplementary Fig. 1C).

OVEREXPRESSION OF HSP20 REPRESSES THE ACTIVATION OF MEK–ERK AND JNK SIGNALING IN HCC CELLS

We hypothesized that the suppressive effect of HSP20 on the HCC cell proliferation was caused by a decrease in their response to growth factors. TGFα and MET, a unique HGF receptor, are frequently overexpressed in human HCCs, and enhanced signaling via these growth factor pathways correlates with cell proliferation [Breuhahn et al., 2006]. In addition, the expression of TGFα mRNA has been reported in HuH7 cells [Nakamura et al., 1996]. We

A

Gender	Male; n=16	Female; n=7		
Underlying disease	Liver cirrhosis; n=10	Chronic hepatitis; n=13		
Etiology of liver disease	HBV; n=4	HCV; n=13	Other; n=6	
Number of tumors	Solitary; n=19	Multiple; n=4		
Tumor size (mm)	<20; n=5	20-50; n=16	>50; n=2	
Vascular invasion	Negative; n=19	Positive; n=4		
Tumor stage	Stage I; n=6	Stage II; n=10	Stage III; n=7	Stage IV; n=0
Histological classification (differentiation)	Well; n=7	Moderately; n=15	Poorly; n=1	

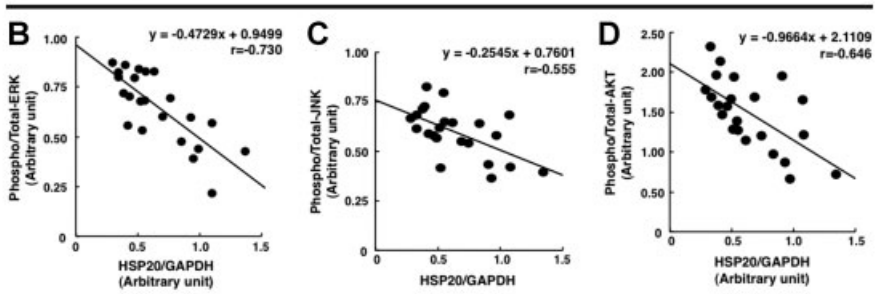

Fig. 1. There is a significant inverse correlation between the levels of HSP20 and the activation of ERK, JNK, and AKT in human HCC tissues. A: The clinical and pathological characteristics of patients with HCC. B–D: The correlations between the expression levels of HSP20 and the levels of phopsho-ERK (B), phospho-JNK (C) or phospho-AKT (D) in human HCC tissues. The expression levels of HSP20, phospho- and total ERK, phospho- and total JNK, phospho- and total AKT, and GAPDH were determined by the band intensities obtained from the Western blot analyses. The band intensities of HSP20, phospho-ERK, phospho-JNK, and phospho-AKT were normalized to those of GAPDH, total ERK, total JNK and total AKT, respectively. The data were analyzed with Spearman's correlation coefficient (r), and $|r| > 0.400$ was accepted as a positive correlation. $P < 0.05$ was considered to be statistically significant.

observed that HuH7 cells indeed released TGFα, and that when the cells were treated with neutralizing antibodies against TGFα, the cell number and BrdU incorporation of the HuH7 cells were significantly inhibited by 11% and 19%, respectively [data not shown). Since HGF is also a potent growth factor for hepatocytes and we detected MET protein expression in HuH7 cells (data not shown), we examined the effects of TGFα and HGF on several molecules related to cell proliferation in both of the HSP20 overexpressing and control HuH7 cells. Twenty nanogram per millilitre of growth factors was found to be the minimum concentration that could be used to obtain a significant effect.

We examined whether the retardation of cell growth in HSP20 overexpressing cells correlates with the ERK activity in these cells. The basal levels of phosphorylated ERK were similar between HSP20 overexpressing cells and control cells (Fig. 3A,B, lane 1 compared with lane 2, respectively). Although the phosphorylation levels of ERK (phospho-ERK level) in HSP20 overexpressing cells and control cells were similarly increased after stimulation with 20 ng/ml of TGFα for 10 min, it in HSP20 overexpressing cells was significantly decreased in comparison to that in control cells for 30 or 60 min after the stimulation (Fig. 3A, lanes 3–8). With regard to HGF, while 20 ng/ml of HGF also induced the phosphorylation of ERK in HSP20 overexpressing cells and control cells, the level in HSP20 overexpressing cells was significantly lower than that in control cells when the cells were stimulated with HGF for 10, 30, or 60 min (Fig. 3B, lanes 3–8). Neither TGFα nor HGF affected the total ERK protein levels in HSP20 overexpressing cells or control cells.

We next focused on the upstream kinase, MEK, because it regulates ERK activity. MEK was not affected in a manner similar to ERK, and in particular, the inhibition of MEK by TGFα and HGF had different kinetics (Fig. 3C,D). Most probably, this was due to the different behavior of the controls. Additionally, we did not detect any differences in the phosphorylation levels of c-Raf, an upstream kinase of MEK, between HSP20 overexpressing cells and the control cells stimulated with TGFα or HGF (data not shown). We also examined whether the retardation of cell growth in HSP20 overexpressing cells correlated with the JNK activity. The basal levels of phosphorylated JNK were similar between HSP20

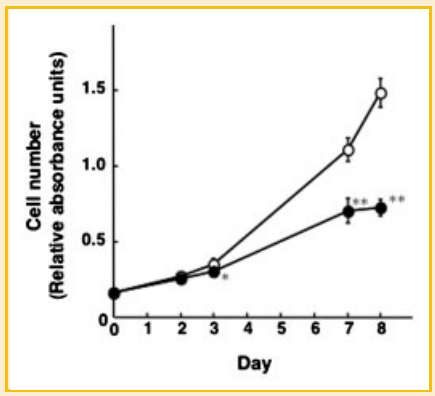

Fig. 2. The cell proliferation of HSP20-overexpressing HuH7 (HSP20 overexpressing) cells. The cell growth curves of HSP20 overexpressing cells (closed circle) compared with control cells (open circle). The data are the means ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ versus curves form the control cells (open circle) at the indicated days.

overexpressing cells and control cells (Fig. 3E,F, lanes 1 and 2). TGFα or HGF caused a marked phosphorylation of JNK in the control cells at 10 min after stimulation (Fig. 3E,F, lane 3 compared to lane 1). The phospho-JNK levels in the control cells were greatly decreased starting from 30 min after stimulation. On the other hand, TGFα or HGF had little effect on the phosphorylation of JNK in the HSP20 overexpressing cells (Fig. 3E,F, lanes 4, 6, and 8). At 30 and 60 min, the levels between the control cells and HSP20 overexpressing cells were very similar. Neither TGFα nor HGF affected the total expression of JNK protein in either cell line.

HSP20 INHIBITS THE C-JUN AND ELK-1 ACTIVITIES IN THE HCC CELLS

ERK and JNK are known to activate the transcription factors Elk-1 and c-Jun by phosphorylation [Su and Karin, 1996]. Elk-1 contributes to an increased expression of the c-Fos oncoprotein [Su and Karin, 1996]. The heterodimerized complex of c-Jun and c-Fos is called activation protein-1 (AP-1), which is known as a potent activator of HCC proliferation [Koike and Moriya, 2005]. Therefore, we next examined whether the retardation of cell growth in HSP20 overexpressing cells correlated with the activities of c-Jun and Elk-1. We observed that the basal levels of phosphorylated c-Jun and Elk-1 were similar between HSP20 overexpressing and control cells (Fig. 4, lanes 1 and 2). However, the phospho-c-Jun levels in HSP20 overexpressing cells were markedly decreased in comparison to control cells when they were stimulated with TGFα (Fig. 4A, lanes 4, 6, and 8 compared to lanes 3, 5, and 7, respectively). As for HGF, while it also induced the phosphorylation of c-Jun in HSP20 overexpressing cells and control cells, the phosphorylation levels in HSP20 overexpressing cells were significantly lower than those in the control cells (Fig. 4B, lanes 4 and 6 compared to lanes 3 and 5, respectively). In addition, similar effects were observed for Elk-1 in cells that were stimulated with TGFα or HGF (Fig. 4C,D). Although

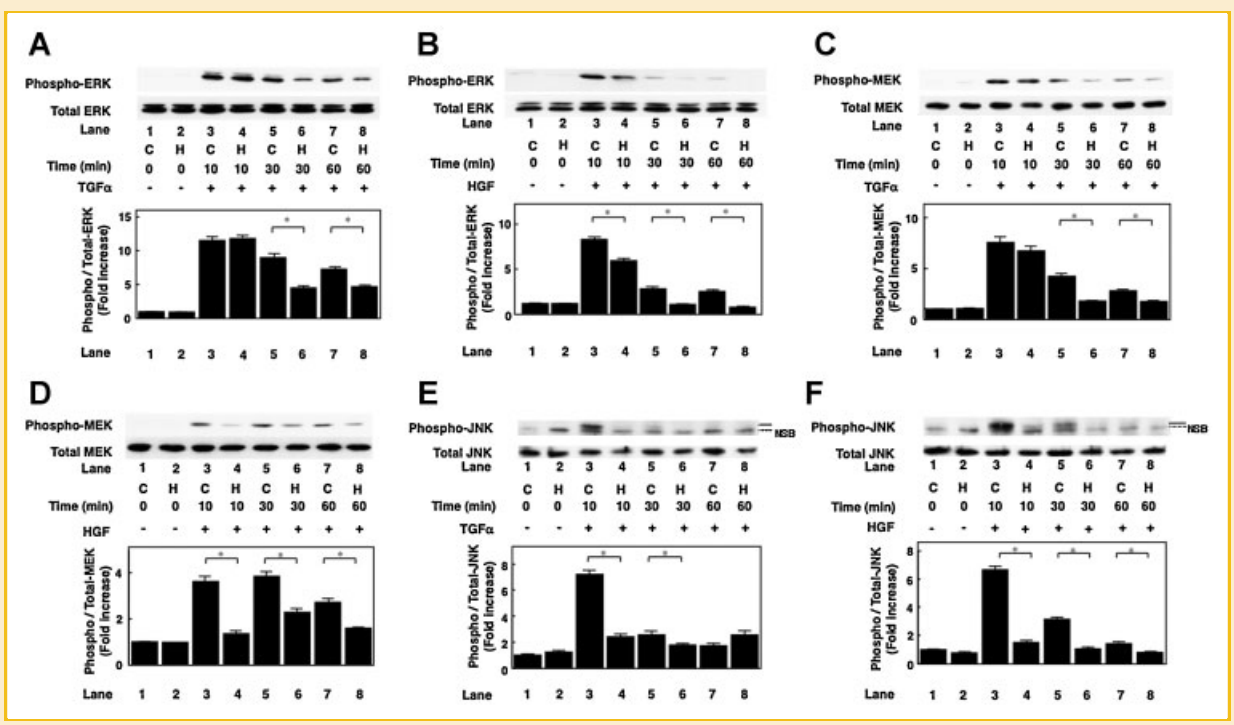

Fig. 3. The phosphorylation of MEK-ERK and JNK signaling induced by TGFα or HGF in HSP20 overexpressing cells (H) and control cells (C). HSP20 overexpressing cells and control cells were stimulated with 20 ng/ml TGFα (A, C, and E) or HGF (B, D, and F) for the indicated periods and the levels of phospho-ERK (A,B), phospho-MEK (C,D) and phospho-JNK (E,F) were determined by a Western blot analysis. The lower bar graph shows the quantification data for the relative levels of phospho-ERK, phospho-MEK, or phospho-JNK after normalization with respect to the total proteins, as determined by a densitometry analysis. The values are the means ± SD (n = 3). $^{*}P < 0.05$. NSB, non-specific band.

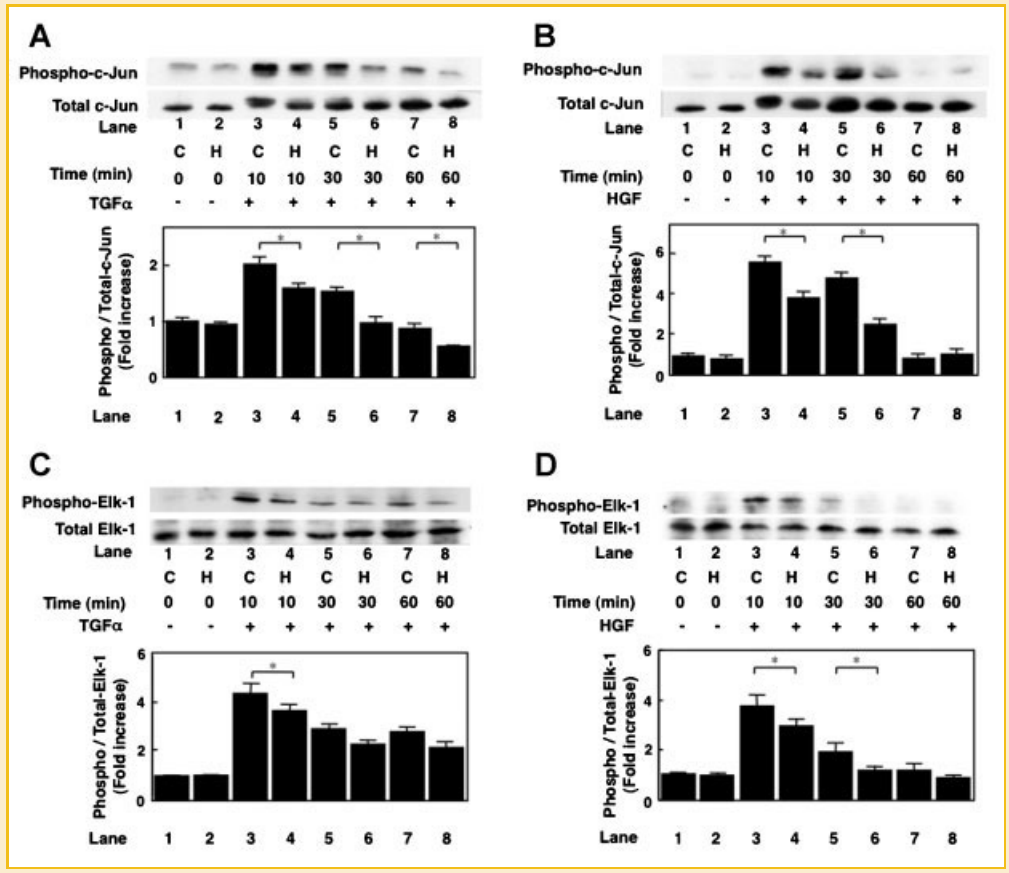

Fig. 4. The phosphorylation of c-Jun and Elk-1 after treatment with TGFα or HGF in HSP20 overexpressing cells (H) and control cells (C). HSP20 overexpressing cells and control cells were stimulated with 20 ng/ml TGFα (A,C) or HGF (B,D) for the indicated periods and the levels of phospho-c-Jun (A,B) and phospho-Elk-1 (C,D) were determined by a Western blot analysis. The lower bar graph shows the quantification data for the relative levels of phospho-c-Jun and phospho-Elk-1, after normalization with respect to the total protein, as determined by a densitometry analysis. The values are the means ± SD (n = 3). $^{*}P < 0.05$.

the bands of phosphorylated c-Jun were shifted and shown as broad bands by a Western blot analysis, the total c-Jun and Elk-1 proteins were expressed at similar level in both the HSP20 overexpressing cells and control cells, regardless of whether or not they were stimulated with TGFα or HGF.

HSP20 INHIBITS THE ACTIVATION OF THE AKT SIGNALING PATHWAY IN HCC CELLS

PDK1 plays a critical role in growth factor pathways [Datta et al., 1999]. The phosphorylation of the activation loop threonine 308 by PDK1 is necessary for AKT activity [Datta et al., 1999]. Therefore, we examined whether the retardation of cell growth in HSP20 overexpressing cells correlated with the phosphorylation status of PDK1. The basal level of phospho-PDK1 in HSP20 overexpressing cells was significantly lower than that in the control cells (Fig. 5A). Neither TGFα nor HGF affected the phosphorylation level of PDK1 (data not shown), which is consistent with a previous study showing that PDK1 is a constitutively active enzyme, and that its activity is not enhanced by AKT activators [Datta et al., 1999].

While TGFα caused a marked increase in the phosphorylation of AKT in control cells (Fig. 5B, lane 3 compared to lane 1), TGFα had little effect on the phospho-AKT levels in HSP20 overexpressing cells (Fig. 5B, lanes 4, 6, and 8). Similarly, HGF also induced the phosphorylation of AKT in control cells at a peak of 30 min after stimulation, and the phosphorylation of AKT in HSP20 overexpressing cells was induced to a much lower extent, compared to the control cells (Fig. 5C).

Unlike most protein kinases, GSK3β is constitutively active in unstimulated cells [Force and Woodgett, 2009]. AKT and ERK signaling cascades are known to phosphorylate and inactivate the growth suppressive activity of GSK3β [Cohen and Frame, 2001]. Surprisingly, the basal phosphorylation level of GSK3β was dramatically lower in HSP20 overexpressing cells than that in control cells (Fig. 5D,E, lane 1 compared with lane 2, respectively). The phosphorylation levels of GSK3β in HSP20 overexpressing cells seem to be lower than those in the control cells, regardless of the addition of TGFα or HGF.

CYCLIN D1 EXPRESSION IS SUPPRESSED IN THE HCC CELLS OVEREXPRESSING HSP20

Increased ERK and JNK activities and the downregulation of GSK3β contribute to the induction of AP-1 transcriptional activity, and AP-1 activates the cyclin D1 promoter to induce cell proliferation [Cohen and Frame, 2001; Lavoie et al., 1996]. Moreover, cyclin D1 is

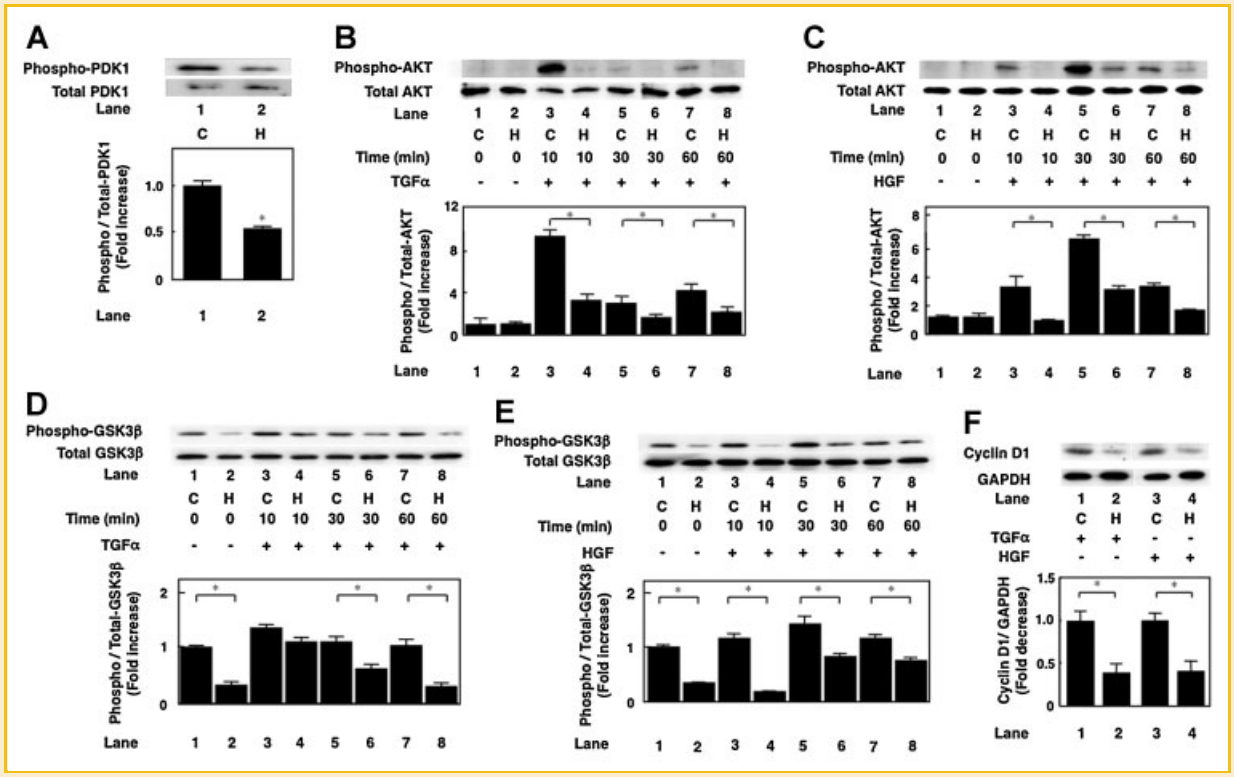

Fig. 5. The PDK1–AKT–GSK3β signaling pathway and cyclin D1 protein expression in HSP20 overexpressing cells (H) and control cells (C). A: The phosphorylation status of PDK1 in HSP20 overexpressing cells and control cells. HSP20 overexpressing cells and control cells were collected without stimulation, and protein levels were determined by a Western blot analysis. B–E: The phosphorylation of AKT and GSK3β by TGFα or HGF in HSP20 overexpressing cells and control cells. HSP20 overexpressing cells and control cells were stimulated with 20 ng/ml TGFα (B,D) or HGF (C,E) for the indicated periods, and the levels of phospho-AKT (B,C) and phospho-GSK3β (D,E) were determined by a Western blot analysis. F: The expression level of cyclin D1 in HSP20 overexpressing cells and control cells. HSP20 overexpressing cells and control cells were stimulated with TGFα or HGF for 24 h and protein levels were determined by a Western blot analysis. Each lower bar graph shows the quantification data for the relative phosphorylation level, after normalization with respect to the total protein. The values are the means ± SD (n = 3). $^{*}P < 0.05$ versus control cells (A), $^{*}P < 0.05$ (B–F).

phosphorylated by GSK3β at Thr286, which is a target for ubiquitination and subsequent proteolytic destruction [Cohen and Frame, 2001]. Therefore, we examined the involvement of HSP20 in the expression levels of cyclin D1 protein in HSP20 overexpressing cells and control cells. When cultured in the presence of either TGFα or HGF for 24 h, the expression level of cyclin D1 protein in HSP20 overexpressing cells was significantly lower than that in control cells (Fig. 5F), thus suggesting a suppressive role for HSP20 in cell proliferation.

DISCUSSION

Although a variety of functions of HSP20 have been previously reported, the precise role of HSP20 in tumor progression still remains unknown. We previously reported that the HSP20 levels in human HCC tissues are reduced compared with the non-tumor tissues, and that the expression levels of HSP20 in tumors are inversely correlated with the tumor stage based on the TNM classification, presence of microvascular invasion, and tumor size [Noda et al., 2007]. It is generally accepted that the activation of the ERK, JNK, and AKT signaling pathways lead to mitogenic effects in HCC [Ito et al., 1998; Datta et al., 1999; Llovet and Bruix, 2008; Min et al., 2011]. In this study, we demonstrated that the expression of HSP20 was inversely correlated with the activity of ERK, JNK, and AKT in the clinical specimens from patients with HCC, and when HSP20 was overexpressed in an HCC cell line (HSP20 overexpressing cells). Our present findings strongly suggest that the decrease in HSP20 protein expression in tumors accelerates HCC progression. The decreased expression of the HSP20 protein may result in an increased or infinite proliferative capacity of HCC cell lines.

The dysregulation of cell signaling caused by growth factors, such as TGFα and HGF, is commonly found in human HCC [Daveau et al., 2003; Llovet and Bruix, 2008; Greten et al., 2009]. Various substances that promote HCC proliferation, such as the hepatitis C virus core protein and ethanol, induce TGFα expression [Sato et al., 2006; Hennig et al., 2009], and HGF promotes hepatocarcinogenesis through MET activation in HCC [Horiguchi et al., 2002]. We therefore examined the function of HSP20 in HCC cells in the presence of these growth factors. The downregulation of the MAPKs and AKT pathways in HSP20 overexpressing cells were followed by attenuated cyclin D1 expression. A decrease in cyclin D1 due to inhibition of the MAPKs and AKT pathways might be a mechanism by which HSP20 controls of HCC proliferation. In contrast, whereas TGFα and HGF induced EGF receptor (EGFR) and

MET phosphorylation, respectively, there was little difference in the phosphorylation levels of these proteins between HSP20 and control cells (data not shown), which led us to speculate that HSP20 targets a protein downstream of the EGFR and MET. The potential mechanism by which HSP20 regulates the proliferation of HCC cells is summarized in Figure 6.

HSP20 was recently reported to form a complex with 14-3-3 protein, and this association affects the activities of other 14-3-3 binding proteins [Chernik et al., 2007; Dreiza et al., 2010]. MAPK/ERK kinase kinase 3 (MEKK3), a common upstream kinase of ERK and JNK, was also reported to bind 14-3-3, and its association with 14-3-3 is required for sustained MEKK3 kinase ativity [Fritz et al., 2006]. In our present study, HSP20 did not interfere with Raf-1 kinase activity, but did inhibit MEK activity in HCC cells, thus suggesting that HSP20 might compete for 14-3-3 binding with MEKK3 in HCC. Furthermore, 14-3-3 directly interacts with and regulates the activities of PDK1 and GSK3β [Sato et al., 2002; Agarwal-Mawal et al., 2003]. We showed that the phosphorylation levels of GSK3β in HSP20 overexpressing cells were lower than those in the control cells, regardless of TGFα or HGF. In addition, the basal level of phospho-PDK1 in HSP20 overexpressing cells was lower than that in the control cells. Therefore, it is likely that 14-3-3 might mediate the signaling between HSP20 and its targets proteins, such as GSK3β and PDK1, and HSP20 might regulate the activity of GSK3β or PDK1 via 14-3-3.

Besides 14-3-3, phosphorylated HSP20 has also been reported to interact with AKT, actin, apoptosis signal-regulating kinase 1 (ASK1), Bax, Bag3, Beclin-1 HSP22, and HSP27 [Fan and Kranias, 2010]. In addition, wild-type HSP27 has been reported to associate with and inhibit the rate of phosphorylation of HSP20 [Bukach et al., 2009]. Taken together with our previous study showing that phosphorylated HSP27 represses the growth of HCC cells via inhibition of the ERK signaling pathway [Matsushima-Nishiwaki et al., 2008], the interaction between HSP20 and HSP27 might have an effect on the ERK signaling in HCC. The attenuation of β-adrenergic agonist-mediated cardiac remodeling by HSP20 is associated with an inhibitory effect on the apoptosis signal-regulating kinase 1 (ASK1)-JNK/p38 MAPK signaling cascade [Fan et al., 2006]. In addition, phosphorylated HSP20 has been reported to interact with phospho-AKT and preserve AKT activities in cardiomyocytes, leading to cardioprotective effects against doxorubicin-triggered cardiac toxicity [Fan et al., 2008]. The overexpression of HSP20 also reportedly protects mesenchymal stem cells against oxidative stress-induced cell death, and this effect is associated with enhanced AKT activation and increased secretion of growth factors [Wang et al., 2009]. The HSP20 in cardiomyocytes and mesemchymal cells showed opposite activities on AKT activity from our results in HCC. These discrete effects of HSP20 might be caused by the differences in the phosphorylation status of HSP20 and/or the conditions of the other cellular proteins that form a complex with HSP20.

The activation of signaling pathways, such as the ERK or AKT pathways, predicts poor prognosis and early recurrence of HCC [Matsushima-Nishiwaki et al., 2001; Schmitz et al., 2008], and the development of targeted therapies against the ERK and AKT pathways are ongoing [Porta and Paglino, 2010]. AZX100, a cell permeable phosphopeptide analog of HSP20, is currently under development as a candidate therapeutic agent for its cardioprotective and smooth muscle relaxation effects [Lopes et al., 2009; Furnish et al., 2010]. The HSP20 expression has been recently reported to decrease not only in HCC, but also in melanoma, cervical squamous carcinoma, lung adenoma and in glioma [Edwards et al., 2011]. HSP20 could therefore represent a new therapeutic target for cancer. Although further detailed analyses, especially on the phosphorylation status and activities of HSP20 and its associated proteins are necessary, our findings suggest that the inhibition of MAPK and AKT signaling pathways by HSP20 in HCC could represent a novel therapeutic strategy.

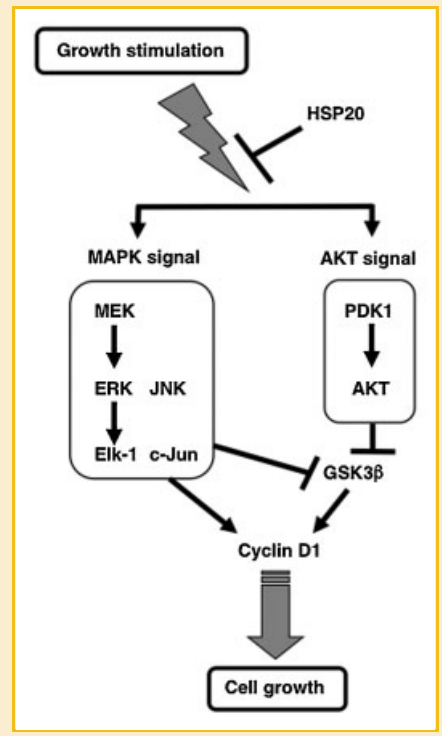

Fig. 6. Role of HSP20 in HCC cell growth. In a liver with HCC, growth factors, such as TGFα or HGF, cause the activation of the ERK/JNK (MAPK) signaling pathway and/or AKT signaling pathway, leading to the subsequent increase in the expression of cyclin D1. We speculate that HSP20 functions as a negative regulator upstream of MEK or PDK1.

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