Phosphorylated Heat Shock Protein 27 Represses Growth of Hepatocellular Carcinoma via Inhibition of Extracellular Signal-regulated Kinase*

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Heat shock protein 27, one of the low molecular weight stress proteins, is recognized as a molecular chaperone; however, other functions have not yet been well established. Phosphorylated heat shock protein 27 levels inversely correlate with the progression of human hepatocellular carcinoma. This study shows that phosphorylated heat shock protein 27 interferes with cell growth of the hepatocellular carcinoma-derived HuH7 cells in the presence of the proinflammatory cytokine, tumor necrosis factor-α, via inhibition of the sustained activation of the extracellular signal-regulated kinase signal pathway. The activities of Raf/extracellular signal-regulated kinase and subsequent activator protein-1 transactivation and the induction levels of p38 mitogen-activated protein kinase and mitogen-activated protein kinase kinase of the WDPF motif, an inhibitory protein of extracellular signal-regulated kinase. These results indicate that phosphorylated heat shock protein 27 might suppress the extracellular signal-regulated kinase activity in the hepatocellular carcinoma cells via two separate pathways in an inflammatory state. The extracellular signal-regulated kinase activity is inversely correlated with phosphorylated heat shock protein 27 at serine 15 and also in human hepatocellular carcinoma tissues in vivo. Because the extracellular signal-regulated kinase signal pathway is a major proliferation signal of hepatocellular carcinoma, activator protein-1 activation is an early event in hepatocarcinogenesis. These findings strongly suggest that the control of the phosphorylated heat shock protein 27 levels could be a new therapeutic strategy especially to counter the recurrence of hepatocellular carcinoma.

The mammalian small stress protein, heat shock protein (HSP) is a widely expressed 27-kDa protein, and it is one of 10 members of the human low molecular weight HSP family. HSPs are classified into high molecular weight HSPs such as HSP70 and HSP90, and low molecular weight HSPs with molecular masses from 10 to 30 kDa based on their apparent molecular sizes. Low molecular weight HSPs have significant similarities in terms of amino acid sequences, known as the α-crystallin domain and WDPF motif (1, 2). The high molecular weight HSPs act as molecular chaperones in protein folding, oligomerization, and translocation (1). Although the functions of low molecular weight HSPs are not well characterized as those of the high molecular weight HSPs, it is recognized that they may have chaperone activities (1). The functions of HSP27 are regulated by post-translational modifications such as phosphorylation (3, 4). Mouse HSP27 is phosphorylated at two sites (Ser-15 and Ser-82), whereas human HSP27 is phosphorylated at three sites (Ser-15, Ser-78, and Ser-82) (3). Ser-78 and Ser-82 of HSP27 are adjacent to the amino-terminal sequence of the α-crystallin domain, whereas Ser-15 is on the amino terminus of the WDPF motif. HSP27 can form oligomers up to 1000 kDa and interfere with cell death induced by several stimuli (1, 5). The oligomerization is regulated by phosphorylation of Ser-78 and/or Ser-82 and the WDPF motif, although phosphorylation of Ser-15 is unrelated to oligomerization (2). HSP27 is reportedly phosphorylated through the following activation of the p38 mitogen-activated protein kinase (MAPK) pathway by the MAPK-activated protein kinase (MAPKAP) 2 and 3 (1). Phosphorylated HSP27 forms a dimer, and the chaperone function is diminished (1). However, the role of phosphorylated HSP27 has not yet been precisely elucidated.

Proinflammatory stimuli, such as tumor necrosis factor-α (TNFα), are involved in the pathophysiology of viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease (6). TNFα plays a dichotomous role in the liver, where it not only acts as a mediator of cell death but also induces hepatocyte proliferation and liver regeneration. HSP27 was reported to be able to suppress TNFα-induced apoptosis and enhance NF-κB activation via promotion of the proteasome-dependent degradation of IkB in a human leukemic cell line (7). Otherwise, TNFα activated protein kinase; MAPKAP, mitogen-activated protein kinase-activated protein kinase; MEK, MAPK/ERK kinase; MKK, mitogen-activated protein kinase kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; TNFα, tumor necrosis factor-α; WT, wild type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Phosphorylated HSP27 Represses ERK-dependent HCC Cell Growth

Plasmids—Wild-type (WT) and mutant human HSP27s subcloned into pcDNA3.1 mammalian expression vector were kindly provided by Dr. C. Schäfer (Klinikum Grosshadern, Ludwig-Maximilians University Munich, Munich, Germany). For mutant HSP27 vectors, the cDNA of HSP27 had been mutated at serine residues 15, 78, and 82 to aspartate (3D) to imitate the mutant HSP27 vectors, the cDNA of HSP27 had been mutated at the same residues to aspartate (3D) to imitate the phosphorylated HSP27 form or mutated at the same residues to alanine (3A) to prevent phosphorylation of HSP27 (17). A constitutively active MEK1 cDNA was the generous gift from Dr. N. G. Ahn (Howard Hughes Medical Institute, University of Colorado, Boulder) (18).

Antibodies and Chemicals—HSP27 antibodies, phosphorylated HSP27 (Ser-15) antibodies, and phosphorylated HSP27 (Ser-78) antibodies were purchased from StressGen Biotechnologies Corp. (Atlanta, GA). Phosphorylated HSP27 (Ser-82) antibodies were obtained from Biomol (Plymouth Meeting, PA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma, respectively. Caspase 9 antibodies, ERK (p44/p42 MAPK) antibodies, phospho-ERK antibodies, MEK antibodies, phospho-MEK antibodies, phospho-c-Raf antibodies, cyclin D1 antibodies, p38 MAPK antibodies, phospho-p38 MAPK antibodies, and phospho-MKP-1 (Ser-359) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Recombinant human TNFα was a kind gift from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Caspase-9 inhibitor 1 (benzoxoycarbonyl-LEHD-fluoromethyl ketone), a cell-permeable and irreversible inhibitor of caspase 9, was purchased from Merck.

Cell Culture and Stable Transfections—Human HCC-derived HuH7 cells, which originated from well differentiated HCC tissues, were obtained from the Japanese Cancer Research Resources Bank. HuH7 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 1% fetal calf serum. For stable transfections, 4×10^5 HuH7 cells were cultured in 6-well dishes and then transfected with 2 μg of the WT or mutant HSP27 plasmids that expresses geneticin (G418) resistance using 12 μl of UniFECTOR transfection reagent (B-Bridge International, Mountain View, CA) in 1 ml of RPMI 1640 medium without fetal calf serum per well. One ml/well medium with 10% fetal calf serum was added 5 h after transfection. The cells were subcultured and grown in the presence of 1 mg/ml of G418 (EMD Chemicals, Inc., San Diego) 2 days later. After about 2 weeks, single G418-resistant colonies were obtained by serial dilution in 96-well dishes. The colonies then were maintained and analyzed individually for the expression of HSP27s.

Cell Growth Assay—Empty vector-transfected, WT, or mutant HSP27s stably expressing HuH7 cells were plated on 96-well dishes (1×10^3 cells/well). Twenty four h after seeding, the cells were treated with or without 1 nM TNFα for the indicated time, and cell numbers were counted using the trypan blue dye exclusion method or using WST-1 reagent (Roche Diagnostics) according to the manufacturer’s instructions. To investigate the influence of caspase 9 on the cell growth, WT, or the 3D HSP27, stably expressed HuH7 cells were treated with caspase-9 inhibitor I simultaneously with or without 1 nM TNFα for 6 days.

Western Blotting—The cultured cells, which overexpressed WT or mutant HSP27s, were stimulated with or without TNFα for the indicated time. The cells or the snap-frozen human HCC samples were lysed, homogenized, and sonicated in lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 50 mM diethiothreitol, and 10% glycerol. A Western blot analysis was performed as described previously (16, 19). Band intensities were visualized on x-ray film with the ECL Western blotting detection system (GE Healthcare). The protein band intensities were determined by integrating the optical density over the band area (band volume) using NIH image software. The samples from the cell cultures to be quantitatively compared by
Western blots were run in the same gel. Values represent the amount of phospho-ERK or phospho-MEK divided by those of total ERK or total MEK, respectively. The values represent the amount of full-length and cleaved caspase 9, phospho-c-Raf, cyclin D1, phospho-p38 MAPK, and phospho-MKP-1 divided by those of GAPDH. To quantify the protein from the HCC tissue extracts, 0.25 μl of MagicMark XP Western protein standard (Invitrogen), the marker protein, was run in every gel. Based on the intensity of the marker protein band on x-ray film, the proteins of the tissue samples were quantitatively compared. After being normalized by the intensity of the marker protein, values represent the amount of phospho- and total HSP27s or phospho-ERK divided by those of β-actin or total-ERK, respectively. The data of the normalized values of the protein bands were statistically analyzed as described under “Statistics.”

**Luciferase Reporter Assay**—A reporter plasmid, activator protein-1 (AP-1)-Luc was kindly provided by Dr. S. Kojima (RIKEN, Wako, Japan). The cells were stimulated with or without 1 nM TNFα for 48 h before transfection. At 5 h after transfection, another 24 h of stimulation of TNFα was performed. Transient transfection with the AP-1-Luc reporter (1 μg/35-mm dish) and measurement of luciferase activity of cell lysates were performed using UniFECTOR transfection reagent and a dual luciferase reporter assay system (Promega Corp., Madison, WI) as described previously (20). The cells were cotransfected with pRL-CMV (Renilla luciferase; 100 ng/35-mm dish) as an internal standard to normalize transfection efficiency. To examine the involvement of MEK-ERK system in AP-1-mediated transactivation activity within the 3D HSP27 mutant overexpressed cells, active MEK1 was cotransfected with the reporter plasmid.

**Tissue Specimens**—HCC tissues were obtained by surgical resection from 44 patients infected with hepatitis viruses B (10 cases) or C (31 cases) and 3 patients with alcoholic cirrhosis at the Department of Surgery, Ogaki Municipal Hospital. No patient had previously undergone chemotherapy. The resected tissues were snap-frozen in liquid nitrogen and then stored at −80 °C until used for the Western blot analysis. The resected HCC specimens were obtained according to protocol approved by the Committee for the Conduct of Human Research at Ogaki Municipal Hospital. Informed consent was obtained from all patients.

**Statistics**—Data are expressed as the means ± S.D. Statistical significance of the data from the cell cultures was analyzed using one-way analysis of variance, followed by Dunnett’s test, and the patient clinical data were analyzed using the Pearson correlation coefficient (r). All p values were derived from two-tailed tests, and p < 0.05 was accepted as statistically significant. A Pearson correlation coefficient of |r| > 0.400 was accepted as a positive correlation.

**RESULTS**

**Expression of HSP27 in Wild-type, Unphosphorylated Type, or Phospho-mimic Type HSP27-transfected HuH7 Cells**—To investigate the effect of phosphorylated HSP27 on HCC cell growth, human HCC-derived HuH7 cells were stably transfected with cDNAs of mutant HSP27s with alanine 15, alanine 78, and alanine 82 (3A) that mimicked the unphosphorylated type or with aspartate 15, aspartate 78, and aspartate 82 (3D) that mimicked the phosphorylated type. For comparison purposes, HuH7 cells were also transfected with wild-type (WT) HSP27-overexpressed HuH7 cells was remarkably delayed in the presence of TNFα. To clarify the relationship between phosphorylation of HSP27 and HCC cell growth, we first studied whether the cell growth of phosphorylated HSP27-overexpressed HuH7 cells was suppressed compared with that of unphosphorylated HSP27-overexpressed cells. HCC commonly arises in the liver with chronic inflammation (10, 21). In the liver, the levels of TNFα, a proinflammatory stimuli, in patients with cirrhosis and HCC have been reported to be significantly higher than those in normal individuals (22). Therefore, the cell growth of phosphorylated HSP27-overexpressed HuH7 cells was examined both in the presence and in the absence of TNFα. In the absence of TNFα, all WT, 3A, or 3D HSP27-overexpressed cell lines and the empty vector-transfected cell line showed almost the same growth curve (Fig. 2A, curves 1–4). Even in the presence of 1 nM TNFα, the empty vector or 3A HSP27 vector-transfected HuH7 cells also exhibited almost similar growth rate as in the absence of TNFα (Fig. 2A, curves 5 and 7). However, the cell growth of WT and 3D HSP27-overexpressed HuH7 cells was remarkably delayed in the presence of TNFα (Fig. 2A, curves 2 and 6).
**Phosphorylated HSP27 Represses ERK-dependent HCC Cell Growth**

FIGURE 2. Cell growth of the phosphorylated HSP27-overexpressed HCC cells was suppressed in the presence of TNFα and was not associated with caspase 9 activation. A, cell growth curve. HuH7 cells were stably transfected either with empty (curves 1 and 5), WT (curves 2 and 6), 3A (curves 3 and 7), or 3D (curves 4 and 8) HSP27 vectors. These cells were cultured either in the absence (curves 1–4) or in the presence (curves 5–8) of 1 nM TNFα. Data are the mean ± S.D. (n = 6). The levels of full-length (B) and cleaved (C) caspase 9 of the HuH7 cells that were stably transfected either with empty (lanes and columns 1 and 2), WT (lanes and columns 3 and 4), 3A (lanes and columns 5 and 6), or 3D (lanes and columns 7 and 8) HSP27 vectors were determined by a Western blot analysis. The cells were stimulated with vehicle (lanes and columns 1, 3, 5, and 7) or 1 nM TNFα (lanes and columns 2, 4, 6, and 8) for 2 h. Values represent the amount of full-length (B) or cleaved (C) caspase 9 divided by those of GAPDH and were plotted as fold induction in comparison with those in the empty vector-transfected cells without TNFα stimulation (mean ± S.D., n = 3). D, effects of caspase-9 inhibitor I on cell growth. WT or 3D HSP27 vectors-transfected HuH7 cells were cultured in the absence (columns 1, 2, 5, and 6) or in the presence (columns 3, 4, 7, and 8) of 1 nM TNFα with (columns 2, 4, 6, and 8) or without (columns 1, 3, 5, and 7) 20 μM caspase-9 inhibitor I for 6 days. Data are the mean ± S.D. (n = 6). **, p < 0.01 versus curves 1–5 and 7 at the indicated day (A). **, p < 0.01 versus column 1; + + , p < 0.01 (B and C). **, p < 0.01 versus column 1; + + , p < 0.01 versus column 5 (D).

Therefore, it seems unlikely that caspase-dependent apoptosis caused the growth retardation of the phosphorylated HSP27-overexpressed cells in the presence of TNFα.
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Phosphorylated HSP27 Inhibited Prolonged Activation of ERK Signal Transduction in the HCC Cells—ERK has been reported to act as a potent proliferative factor of HCC and be constitutively activated in the human HCC cells and tissues (12, 23). Does the cell growth retardation of the phosphorylated HSP27-overexpressed cells correlate with the ERK activity? The basal levels of phosphorylated ERK were similar among all HSP27 cDNA-transfected cells (Fig. 3A). Although the ERK phosphorylation levels in all HSP27 cDNA-transfected cells were similarly increased after 2 h of stimulation with 1 nM TNFα, phospho-ERK levels in both WT and the 3D HSP27-overexpressed HuH7 cells significantly decreased in comparison with those in 3A HSP27 cDNA or empty vector-transfected HuH7 cells after 72 h of stimulation with TNFα (Fig. 3A). Total ERK proteins were expressed at almost the same levels among all HSP27 cDNA-transfected cells regardless of whether or not they were stimulated with TNFα. The ERK activity is regulated by upstream kinases MEK and c-Raf. As shown in Fig. 3B, a significant decline of MEK activity in WT and the 3D HSP27-overexpressed HCC cells was observed in comparison with that in 3A HSP27 cDNA or empty vector-transfected cells after 72 h of stimulation of TNFα. Furthermore, significant attenuation of c-Raf activity was also shown in WT and the 3D HSP27-overexpressed HCC cells following 48 h of TNFα stimulation (Fig. 3C). Therefore, phosphorylated HSP27 might act as a repressor for prolonged activation of ERK signaling pathway at a point upstream of c-Raf in the HCC cells.

Transactivation Activities of AP-1 and Cyclin D1 Expression Were Suppressed in the 3D HSP27-overexpressed HCC Cells—ERK contributes to the induction of AP-1 transcriptional activity, and AP-1 activates the cyclin D1 promoter to induce cell proliferation (13, 24). Therefore, the effect of the phosphorylated HSP27 on AP-1 transactivation activity was assessed (Fig. 4A). After 72 h of stimulation with TNFα, WT and the 3D HSP27-overexpressed HuH7 cells expressed significantly less transactivation activity of AP-1 than 3A HSP27-introduced cells (Fig. 4A, columns 6 and 8, in comparison with column 7). A remarkable decrease of AP-1 transactivation activity occurred because of the ERK signaling pathway, constitutive active MEK1 cDNA was transfected into the 3D HSP27-overexpressed HuH7 cells. The active MEK1 restored AP-1 transactivation activity of the 3D HSP27-overexpressed HuH7 cells to the similar level as the 3A HSP27-overexpressed or empty vector-transfected HuH7 cells (Fig. 4A, column 9 in comparison with columns 7 or 5). In the absence of TNFα, no significant difference of the AP-1 transactivation activity among empty vector and all HSP27 cDNAs-transfected cells was shown (Fig. 4A, columns 1–4). Therefore, phosphorylated HSP27 presumably reduced AP-1-mediated cell proliferation via ERK signaling pathway in the HCC tissues under inflammatory conditions. Next, cyclin D1 protein expression levels in empty vector and all HSP27 cDNA-transfected cells in the presence and absence of TNFα were examined. In WT and the 3D HSP27-transfected cells treated with TNFα for 72 h, cyclin D1 levels significantly decreased in comparison with those in the 3A HSP27-transfected cells (Fig. 4B, columns and lanes 6 and 8 in comparison with column and lane 7). In the absence of TNFα, no significant difference of cyclin D1 protein level among empty vector and all HSP27 cDNA-transfected cells was shown (Fig. 4B, columns 1–4). Therefore, phosphorylated HSP27 presumably reduced AP-1-mediated cell proliferation via ERK signaling pathway in the HCC tissues.

Increased Expression Level and Activation of p38 MAPK That Were Followed by the Induction of Active MKP1 Were Observed in the Phosphorylated HSP27-overexpressed HCC Cells—In eukaryotic cells, there are another two MAPKs, p38 MAPK and...
versus column 1

The maximum activity of ERK was observed after 2 h stimulation with TNFα, and the activated form of MKP-1, phosphorylated MKP-1 (27). Phosphorylated HSP27 was significantly induced in WT and the 3D HSP27-introduced HuH7 cells in the presence of TNFα (26). The effect of phosphorylated HSP27 was analyzed on an active form of MKP-1, phosphorylated MKP-1 (27). Phosphorylated MKP-1 was significantly induced in WT and the 3D HSP27-overexpressed cells after 72 h stimulation of TNFα in comparison with that in the 3A HSP27 cDNA-transfected cells (Fig. 5B, lanes 2 and 4 in comparison with lane 3). Phosphorylated HSP27 represses ERK-dependent HCC cell growth.

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JNK, in addition to ERK (11). Although p38 MAPK and JNK are less sensitive to growth signals than ERK, their activation is preferentially triggered by pro-inflammatory cytokines and environmental stresses (25). Therefore, p38 MAPK activities were next examined in the unphosphorylated and in the phosphorylated HSP27-overexpressed HCC cells. Because the maximum activity of ERK was observed after 2 h stimulation with TNFα, the amount of p38 MAPK and phosphorylated p38 MAPK in the cells was also examined after 2 h of stimulation with TNFα. Fig. 5A shows that the p38 MAPK level was increased in WT and especially in the 3D HSP27-overexpressed HuH7 cells in the presence of TNFα in comparison with that in the 3A HSP27 cDNA or empty vector-transfected cells. The increased levels of p38 MAPK were also observed even in the absence of TNFα in both WT and the 3D HSP27-overexpressed HuH7 cells (data not shown). Furthermore, TNFα stimulation significantly induced the p38 MAPK phosphorylation in both WT and the 3D HSP27 cDNA-transfected HuH7 cells in comparison with the empty vector and 3A HSP27 cDNA-transfected cells (Fig. 5A, lanes 2 and 4 in comparison with lanes 1 or 3). The similar tendency of enhanced activation of p38 MAPK in WT and 3D HSP27-introduced HuH7 cells was observed in 72-h TNFα-stimulated cells, although the activity was less than after 2 h of stimulation (data not shown). HSP27 is phosphorylated by the p38 MAPK pathway (1). On the other hand, phosphorylated HSP27 enhanced p38 MAPK expression and activation in this experiment. This is probably the first report showing such a positive feedback from phosphorylated HSP27 to p38 MAPK. The activation of p38 MAPK is reported to induce MKP-1, a phosphatase that inactivates ERK (26). The effect of phosphorylated HSP27 was analyzed on an active form of MKP-1, phosphorylated MKP-1 (27). Phosphorylated MKP-1 was significantly induced in WT and the 3D HSP27-overexpressed cells after 72 h stimulation of TNFα in comparison with that in the 3A HSP27 cDNA-transfected cells (Fig. 5B, lanes 2 and 4 in comparison with lane 3). Phosphorylated HSP27 represses ERK-dependent HCC cell growth.
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FIGURE 6. Significant inverse correlation between the levels of phosphorylated HSP27 (Ser-15) and activation of ERK in human HCC Tissues. Correlation between the expression levels of phospho-ERK and the levels of phosphorylated HSP27 (Ser-15) (A), phosphorylated HSP27 (Ser-78) (B), phosphorylated HSP27 (Ser-82) (C), and total HSP27 in human HCC tissues (D). The expression levels of phospho-ERK and total ERK, phosphorylated and total HSP27, and β-actin were determined by the band intensities obtained from a Western blot analysis. Based on the intensity of the same concentration of the marker protein that runs in every gel, the values of the tissue samples protein on separate gels were normalized. The values represent the amount of phosphorylated HSP27 (Ser-15) (r = -0.468), phosphorylated HSP27 (Ser-78) (r = -0.158), phosphorylated HSP27 (Ser-82) (r = 0.114), and total HSP27 (r = 0.123). Not only the presence of the phosphorylated HSP27 but also the total amount of HSP27 might play some part in the induction of active MKP-1. The activity of JNK, the other MAPK, was not significantly changed in these experiments (data not shown).

The Levels of Phosphorylated HSP27 (Ser-15) Inversely Correlated with the Levels of ERK Activation in Human HCC Tissues—The ERK activities were down-regulated in the WT and the 3D HSP27-transfected cells also in the absence of TNFα (data not shown). Not only the phosphorylated HSP27 but also the total amount of HSP27 might play some part in the induction of active MKP-1. The activity of JNK, the other MAPK, was not significantly changed in these experiments (data not shown).

DISCUSSION

The influence of post-translational modification, such as phosphorylation on the function of HSP27, is precisely unknown. In this study, phosphorylated HSP27 reduced the cell growth rate of the HuH7 cells in the presence of TNFα (Fig. 2A). HSP27 reportedly inhibits the caspases to protect the cells from apoptosis, and the phosphorylation status of HSP27 influences that function (1, 5). A transcription factor, NF-κB, has been implicated in suppression of apoptosis, cell survival, proliferation, viral replication, inflammation, tumorigenesis, and metastasis, and all members of the TNF superfamily are known to activate it (28). The ability of HSP27 to interact with IKKβ has been reported to be enhanced via the TNFα-induced activation of MAPK-dependent phosphorylation of HSP27, thus leading to the enhanced inhibition of IKK activity, reduced iκB degradation, and consequent suppression of NF-κB activation in HeLa cells (9). The overexpression of phosphorylated HSP27 increased caspase 9 protein levels and activity (Fig. 2, B and C). Phosphorylated HSP27 may therefore increase apoptosis in the HCC cells. However, the caspase activities in the phosphorylated HSP27-overexpressed cells were not enhanced by TNFα (Fig. 2C), and the caspase inhibitor did not restore the cell growth retardation of WT and phosphorylated HSP27-overexpressed cells in the presence of TNFα (Fig. 2D). These results suggest that the influence of some mechanisms other than apoptosis might play important roles in the control of the HCC cell proliferation by phosphorylated HSP27 in the presence of an inflammatory cytokine like TNFα. However, the HCC cell growth retardation by phosphorylated HSP27 may be caused, in part, by the suppression of NF-κB.

In human HCC, it is generally accepted that the activation of the ERK signal pathway leads to a mitogenic effect (12, 20). This study presented novel evidence that phosphorylated HSP27 inhibits the sustained activation of the c-Raf-MEK-ERK pathway in an inflammatory environment (Fig. 3). The phosphorylation of HSP27 significantly correlated with the activity of ERK in not only HCC cells in vitro but also the specimens in patients with HCC in vivo (Fig. 6). There have so far been few reports addressing the influence of HSP27 on the ERK activation, except for the study by Lee and co-workers (29, 30) where the overexpression of HSP25, the same species as human HSP27 in the mouse, was shown to down-regulate ERK expression, while also inhibiting their activation in mouse fibroblast L929 cells by a reduction in reactive oxygen species. Contrary to our results, HSP25 overexpression attenuated the H$_2$O$_2$-ERK pathway-mediated apoptosis in their experiments. The role of the HSP27-ERK pathway might be different in the mouse fibroblasts as compared with the human HCC cells. The attenuation of phosphorylated HSP27, especially phosphorylated at Ser-15, is correlated with HCC progression (15). In addition, the expression levels of HSP20, one of the low molecular weight HSP family

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Although HSP27 phosphorylation is generally known to be a reversible process catalyzed by the MAPKAP2, a downstream substrate of p38 MAPK (1), phosphorylated HSP27 surprisingly induced p38 MAPK (Fig. 5A). The activation of the p38 MAPK cascade is preferentially triggered by pro-inflammatory cytokines, such as TNFα and environmental stress. The α-isooform of protein kinase C, an essential molecule of malignant cancer cells, has been reported to activate p38 MAPK while also stimulating cell migration and invasion in poorly differentiated human HCC cell lines (34). Transforming growth factor β mediated the activation of p38 MAPK, and its downstream HSP27 may increase the invasive potential and matrix metalloprotease (MMP)-2 expression in human prostate cancer cells (35). On the contrary, p38 MAPK and p38 MAPK kinase (MKK3) have been shown to significantly inhibit mitogen-induced cyclin D1 expression in the constitutively active Raf-1 and estrogen receptor fusion protein stably expressed CCL39 cells (13). The association of human HCC with nearby normal tissues has been shown to reduce p38 MAPK and MKK6 activities especially in larger tumors (36). Moreover, it has been reported recently that p38 MAPKα suppresses liver cancer development by antagonizing the JNK-c-Jun pathway (14). There are at least four isoforms of p38 MAPK that have been identified and characterized, and the activation of p38 MAPK is mediated by MKK3, -4, and -6 (37). The role of p38 MAPK and its isoforms in HCC cell growth have not yet been established. It has been shown that p38 MAPK induces MKP-1, a major negative regulator for ERK (26). This study also showed that phosphorylated HSP27 activated p38 MAPK and subsequently induced phosphorylated MKP-1 (Fig. 5B). These findings suggest that the cross-talk among phosphorylated HSP27, p38 MAPK, and MKP-1 might also regulate ERK activity in addition to the down-regulation of c-Raf-MEK-ERK signal transduction by phosphorylated HSP27, thus resulting in the suppression of HCC cell proliferation. The potential mechanism of phosphorylated HSP27 in HCC shown here is summarized in Fig. 7.

A number of studies have shown that the redox state plays a role in the regulation of TNFα intracellular signaling and ERK activation (38, 39). HSP27 has been reported to regulate the intracellular reactive oxygen species and/or glutathione level (30, 40). However, reactive oxygen species inhibitors do not suppress TNF-induced AP-1 activation (40). No change in the total glutathione levels is observed with the decrease in ERK phosphorylation in response to oxidative stress in primary cultured rat hepatocytes (41). It was recently reported that HSP27 is required for interleukin-1-activated transforming growth factor-β-activated kinase-1 (TAK1) and downstream signaling by p38 MAPK, JNK, and their activator kinases and IKKβ (42). TAK1 is the most upstream common signaling protein of both interleukin-1 and TNFα. However, the activation of ERK...
Phosphorylated HSP27 Represses ERK-dependent HCC Cell Growth

induced by interleukin-1 appears to be independent of HSP27 (42). Further studies should therefore investigate where and how phosphorylated HSP27 acts at a point upstream of c-Raf.

The activation of AP-1 is known as an early event in HCC carcinogenesis. The prevention of the recurrence of HCC at early stage is therefore urgently needed to enhance long term survival. Recently, an oral multikinase inhibitor, sorafenib, was developed, and the clinical trials against human HCC are in progress (44). Sorafenib blocks tumor cell proliferation by targeting Raf/MEK/ERK signaling at the level of Raf kinase (45). The HCC cell growth retardation via inhibition of ERK pathway by phosphorylated HSP27 shown here could therefore be a novel promising therapeutic strategy to prevent the recurrence of HCC.

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