Heat shock protein 20 (HSPB6) regulates apoptosis in human hepatocellular carcinoma cells: Direct association with Bax

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Abstract. A small heat shock protein (HSP), HSP20 (HSPB6) is ubiquitously expressed in various tissues and has several functions. We previously reported that the expression of HSP20 protein in human hepatocellular carcinoma (HCC) cells is inversely proportional to the progression of HCC. In addition, we showed that HSP20 is associated with phosphoinositide 3-kinase (PI3K) and inhibits the proliferation of HCC cells via suppression of the AKT signaling pathway. However, the relationship between HSP20 and apoptosis in HCC has not yet been elucidated. To clarify whether HSP20 is implicated in the apoptosis of HCC cells, in the present study, we examined the effect of HSP20 on caspases, the central regulators of apoptosis, using human HCC-derived HuH7 cells that are transfected with wild-type human HSP20 (HSP20-overexpressing cells). The cleavage of caspase-3 and caspase-7 in HSP20-overexpressing cells was enhanced compared with the empty vector-transfected cells (control cells). In addition, the cleavage of nuclear poly (ADP-ribose) polymerase (PARP) in HSP20-overexpressing cells was also strengthened. We further investigated the direct targets of HSP20 focusing on Bcl-2 family proteins in the HSP20-overexpressing cells. HSP20 proteins in the cells were coimmunoprecipitated with Bax. On the contrary, Bad, Bcl-2 and Bcl-xL were not coimmunoprecipitated with HSP20. These findings strongly suggest that HSP20 directly associates with Bax and stimulates caspase cascade in human HCC cells.

Introduction

Heat shock protein 20 (HSPB6) is a member of the small HSP family (HSPB) and is ubiquitously expressed in many tissues

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including liver (1,2). HSP20 has a variety of functions in addition to a molecular chaperoning function. We previously showed that HSP20 acts as an extracellular inhibitor of human platelet aggregation induced by thrombin or botrocetin (3,4). Additionally, it has been reported that HSP20 acts in processes ranging from insulin resistance to prevention of vasospasms, to airway smooth muscle relaxation, and it has also been demonstrated to have a protective function in the heart (5-8). However, the exact roles of HSP20 (HSPB6) remain to be elucidated.

Human hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and is the third leading cause of cancer-related mortality (9). Even after resection of the primary HCC, recurrence frequently develops. The survival rate of HCC is 30-40% at five years post-surgery. A significant number of the molecular events altered in HCC progression, compromise the balance between survival and apoptotic signals in the tumor cells. We previously reported that HSP20 protein levels in HCC inversely correlate with the TNM stage (10). In our previous studies on HCC (11,12), we demonstrated that the HSP20 protein directly interacts with phosphoinositide 3-kinase (PI3K) which activates AKT, a major mediator of cell survival, and suppresses its activity resulting in reducing the cell proliferation (11,12).

Accumulating evidence suggests that apoptosis is important in hepatocarcinogenesis, from the initial genotoxic insult (initiation), through the clonal expansion from a premalignant to a tumorous lesion (promotion) and finally to the progression of tumor cell growth by further clonal expansion (13). Caspases, a family of cysteine proteases, are central regulators of apoptosis (14). Caspases hydrolyze peptide bonds after certain aspartic acid residues in the substrate. Caspases are initially produced as inactive form, procaspases, and require cleavage for activation. Caspase-3 has a critical role for apoptosis, and subsequently the activated caspase-3 cleaves many key proteins, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (15). Since PARP is involved in DNA repair and helps cells to maintain their viability, the cleavage of PARP leads to apoptosis (15,16). Upstream of the caspase pathways, mitochondria play a pivotal role in apoptosis, inducing cytochrome *c* release, which subsequently activates caspases (13). In the mitochondrial-mediated regulation of apoptosis, particularly the Bcl-2 family of proteins, which

include the members of both pro- and anti-apoptotic effects, act as important regulators (17). The balance between pro- and anti-apoptogenic Bcl-2 family member activities and their interactions plays central roles in the mitochondrial-mediated apoptosis pathway. In response to mitochondrial pathway stimulation, processing of caspases is induced. An imbalance in the pro- and anti-apoptotic members of the Bcl-2 family has been observed in HCC (17). Bcl-xL is overexpressed, whereas pro-apoptotic members of the family, such as Bax, are down-regulated in HCC (17). However, the relationships between HSP20 and apoptosis in HCC remain to be elucidated. The aim of the present study was to clarify the effect of HSP20 protein expression on apoptosis in human HCC. We herein demonstrated that HSP20 directly interacts with Bax and activates caspase cascade in human HCC cells.

Materials and methods

Materials. HSP20 antibodies were purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Antibodies against caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, cleaved PARP, Bad, Bcl-2, Bcl-xL, Bax and peroxidase-conjugated anti-rabbit-IgG (conformation specific) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies and normal rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Wild-type human HSP20 cDNA (clone ID 6074542), which was obtained from Open Biosystems, Inc. (Huntsville, AL, USA), was subcloned into the eukaryotic expression vector, pcDNA 3.1(+), as previously described (11). The eukaryotic expression vector, pcDNA 3.1(+) and Dynabeads protein A were purchased from Life Technologies Corp. (Carlsbad, CA, USA). The BCA protein assay kit was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Cell culture. Human HCC-derived HuH7 cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). The HuH7 cells were maintained in RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 1% fetal calf serum (FCS; Hyclone Corp., Logan, UT, USA).

Stable transfections. To analyze caspase activity, the stably HSP20-overexpressing HuH7 cells and the control empty vector-transfected HuH7 cells were used. These cells were established as described previously, by means of Tet-Off™ gene expression systems (Clontech Laboratories Inc., Palo Alto, CA, USA) according to the manufacturer's instructions (11). Induction of HSP20 protein expression in the HSP20-overexpressing cells can be controlled by the presence of doxycycline (Sigma-Aldrich). The HSP20-overexpressing cells and the control cells were maintained in RPMI-1640 supplemented with 1% FCS, 200 μg/ml G418 (Invitrogen), 100 μg/ml hygromycin B (Merck KGaA, Darmstadt, Germany) and 1 μg/ml doxycycline. For western blotting, both cells were cultured under serum-starvation for the indicated days.

Transient transfections. The transiently HSP20-overexpressing HuH7 cells and the control empty vector-transfected HuH7

cells were used for immunoprecipitation as previously described (12). For transient transfections, the HuH7 cells were cultured in 90 mm diameter dishes (1x10^6 cells/dish) and were transfected with 4 μg of the wild-type HSP20 plasmid or the control empty pcDNA 3.1(+) vector using the UniFector transfection reagent (B-Bridge International, Mountain View, CA, USA) in 4 ml of RPMI-1640 medium without FCS. One day after transfection, the medium was changed to 6 ml of RPMI-1640 medium with 1% FCS. The cells were then cultured for another 24 h.

Protein preparation. For coimmunoprecipitation, the transfected cells were lysed in ice-cold TNE lysis buffer [10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium fluoride, 1 mM sodium vanadate and protease inhibitor cocktail (Roche Diagnostics K.K.)]. The lysates were then centrifuged at 10,000 x g at 4°C for 30 min, and the supernatant was collected as TNE-soluble proteins, as previously described (12). For the western blot analysis, the serum-starved cells were lysed, homogenized and sonicated in lysis buffer, as previously described (11).

Coimmunoprecipitation. Coimmunoprecipitation was performed as previously described (12). The indicated antibodies were added to the TNE-soluble proteins, and the mixture was shaken gently overnight at 4°C, followed by the addition of 50 μl of Dynabeads protein A and incubation for a further 1 h with continuous mixing. Protein immuno-complexes were isolated with the use of a magnetic particle concentrator (6-tube magnetic separation rack; New England BioLabs Inc., Ipswich, MA, USA). The immunoprecipitated proteins and TNE-soluble proteins (for analysis total protein) were resuspended in the loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), heated at 95°C for 5 min, and analyzed by western blot analysis.

Western blot analysis. Western blot analysis was performed as previously described (10). Briefly, SDS-PAGE was performed by the method described by Laemmli (18). The proteins in the gel were transferred onto polyvinylidene fluoride (PVDF) membranes, which were then blocked with 5% fat-free dry milk in phosphate-buffered saline (PBS) with 0.1% Tween-20 for 1 h before incubation with the indicated primary antibodies. Peroxidase-labeled anti-rabbit IgG antibodies were used as secondary antibodies. The peroxidase activity on the PVDF membranes was visualized on X-ray film by means of an ECL western blotting detection system (GE Healthcare, Waukesha, WI, USA) as described in the manufacturer's protocol.

Results

Increased cleavage of caspase-3 and caspase-7 by HSP20 overexpression in HCC cells. In our previous studies (10-12), we showed that the HSP20 protein is expressed in the tumor tissue of human HCC, although the expression level is lower than in non-tumor tissues. However, the HSP20 protein is not expressed in human HCC cell lines. Therefore, we transfected wild-type HSP20 cDNA into HuH7 cells, a HCC-derived cell line, to make them express the HSP20 protein, and then
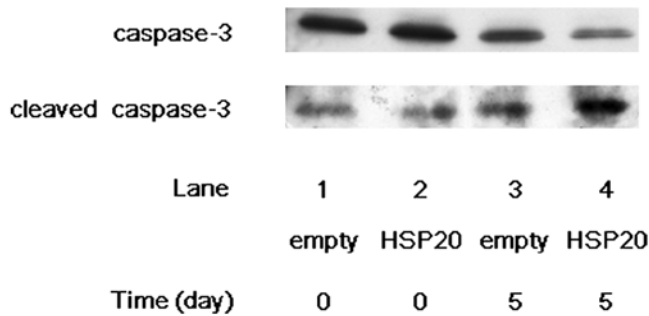
Figure 1. HSP20 enhances cleavage of caspase-3 in the HSP20-overexpressing HCC cells. The empty vector-transfected (empty) and HSP20-overexpressing (HSP20) HuH7 cells were cultured without FCS for the indicated days. The protein levels of caspase-3 and cleaved caspase-3 in the empty and HSP20 cell extracts were analyzed by western blotting using caspase-3 and cleaved caspase-3 antibodies.
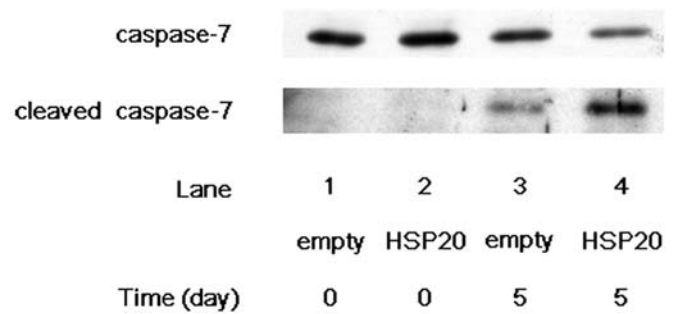

Figure 2. HSP20 enhances cleavage of caspase-7 in the HSP20-overexpressing HCC cells. The empty vector-transfected (empty) and HSP20-overexpressing (HSP20) HuH7 cells were cultured without FCS for the indicated days. The protein levels of caspase-7 and cleaved caspase-7 in the empty and HSP20 cell extracts were analyzed by western blotting using caspase-7 and cleaved caspase-7 antibodies.

analyzed its function. We first examined the effect of HSP20 expression on the cleavage of caspase-3 in the HSP20-overexpressing HCC cells. After 5 days of incubation without FCS, the level of cleaved caspase-3 markedly increased in the HSP20-overexpressing HuH7 cells compared with that in the empty vector-transfected cells (Fig. 1, lane 4 compared with lane 3). On the other hand, the level of caspase-3 was decreased by HSP20 overexpression on day 5 (Fig. 1, lane 4 compared with lane 3). We next examined the effect of HSP20 expression on the cleavage of caspase-7 in the HSP20-overexpressing HCC cells. After 5 days of incubation without FCS, the expression level of cleaved caspase-7 showed marked increase in the HSP20-overexpressing cells compared with that in the empty vector-transfected cells (Fig. 2, lane 4 compared with lane 3), while the level of caspase-7 was decreased by HSP20 overexpression at day 5 (Fig. 2, lane 4 compared with lane 3). These findings suggest that the HSP20 protein plays a role activating the cascade of caspases in the HCC cells.

Increased cleavage of PARP by HSP20 overexpression in HCC cells. PARP, which helps cells to maintain their viability, is a main cleavage target of caspase-3, and cleaved PARP induces apoptosis, indicating that cleaved PARP is observed in the cells undergoing apoptosis (15,16). After 5 days of incubation without FCS, the cleavage of PARP markedly increased in the HSP20-overexpressing HuH7 cells compared with that in the empty vector-transfected cells (Fig. 3, lane 4 compared with lane 3), suggesting that HSP20 induces the caspase cascade which leads to apoptosis.

HSP20 directly interacts with Bax among the Bcl-2 family proteins. Among several apoptotic pathways, mitochondria are key participants (14). The mitochondrial pathway is coupled to the activation of caspase-3 and caspase-7. It is well known that the Bcl-2 family proteins are critical death regulators for mitochondria-mediated apoptosis (17). Therefore, we next examined whether HSP20 interacts with the Bcl-2 family proteins, Bad, Bcl-2, Bcl-xL and Bax in the HCC cells. Bad, Bcl-2, Bcl-xL and Bax proteins were expressed in both the empty vector-transfected and HSP20-overexpressing HuH7 cells (Fig. 4). However, HSP20 protein in the HSP20-overexpressing cells was not coimmunoprecipitated with Bad, Bcl-2 or Bcl-xL
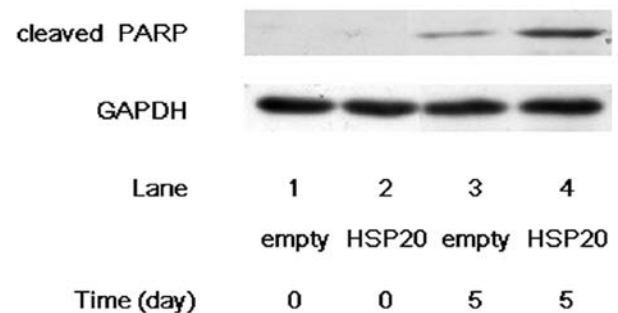

Figure 3. HSP20 enhances cleavage of PARP in the HSP20-overexpressing HCC cells. The empty vector-transfected (empty) and HSP20-overexpressing (HSP20) HuH7 cells were cultured without FCS for the indicated days. The cleaved PARP and GAPDH in the empty and HSP20 cell extracts were analyzed by western blotting using cleaved PARP and GAPDH antibodies.

proteins (Fig. 4A-C). On the other hand, as shown in Fig. 4D, the HSP20 protein in the HSP20-overexpressing cells was markedly coimmunoprecipitated with Bax (Fig. 4D, lane 2 in comparison with lane 1). We confirmed that the HSP20 protein was not coimmunoprecipitated with normal rabbit IgG (Fig. 4D). These results suggest that the HSP20 protein directly interacts with the Bax protein but not with the Bad, Bcl-2 and Bcl-xL proteins in the HCC cells.

Discussion

We have previously shown that HSP20 suppresses HCC cell growth by downregulation of proliferation signals via the AKT and mitogen-activated protein kinase pathways (11,12). Cell growth is affected by both the survival and apoptosis signals. Therefore, it led us to consider the relationship between HSP20 and apoptosis in HCC. In the present study, we demonstrated that caspase cascade, such as caspase-3 and caspase-7, the central regulatory system of apoptosis signals is activated in HSP20 protein-overexpressing human HCC cells compared with that in the control HCC cells. In addition, we showed that the level of cleaved PARP is increased in the HSP20-overexpressing HuH7 cells. It is firmly established that PARP is involved in DNA repair and maintains cell viability (15,16). The cleavage of PARP facilitates cellular disassembly,
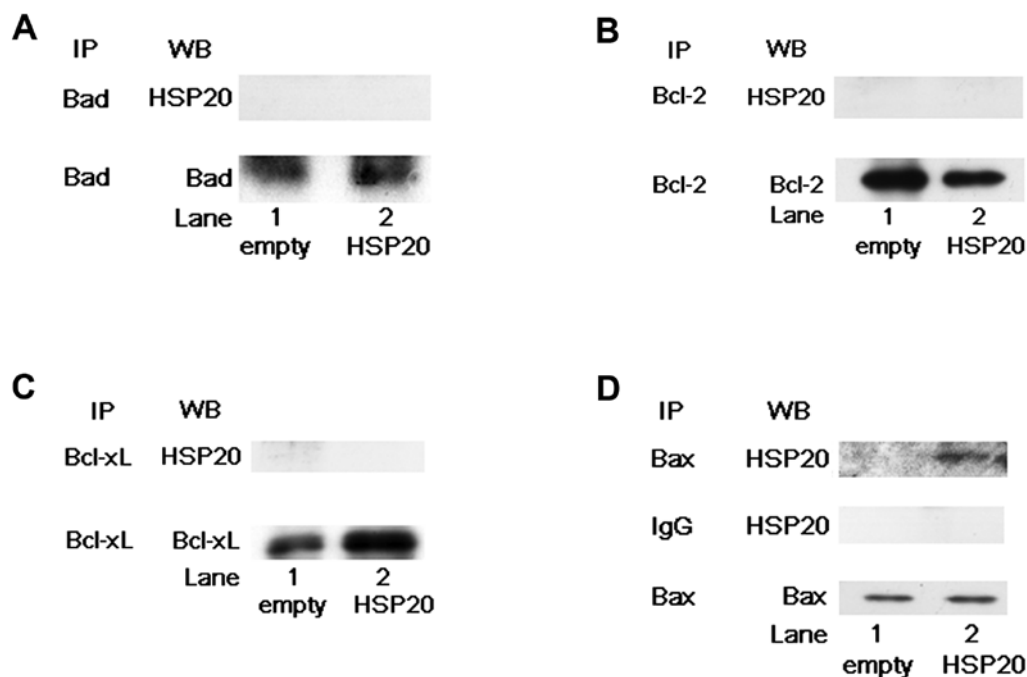
Figure 4. HSP20 does not directly interact with Bad, Bcl-2 or Bcl-xL, but it does with Bax. (A) The empty vector-transfected (empty, lane 1) and HSP20-overexpressing (HSP20, lane 2) HuH7 cell lysates were immunoprecipitated with Bad antibodies followed by western blotting (WB) using HSP20 antibodies. Immunoprecipitation (IP) of Bad proteins in the cells was confirmed by WB using Bad antibodies. (B) The empty vector-transfected (empty, lane 1) and HSP20-overexpressing (HSP20, lane 2) HuH7 cell lysates were immunoprecipitated with Bcl-2 antibodies, followed by WB using HSP20 antibodies. IP of Bcl-2 proteins in the cells was confirmed by WB using Bcl-2 antibodies. (C) The empty vector-transfected (empty, lane 1) and HSP20-overexpressing (HSP20, lane 2) HuH7 cell lysates were immunoprecipitated with Bcl-xL antibodies, followed by WB using HSP20 antibodies. IP of Bcl-xL proteins in the cells was confirmed by WB using Bcl-xL antibodies. (D) The empty vector-transfected (empty, lane 1) and HSP20-overexpressing (HSP20, lane 2) HuH7 cell lysates were immunoprecipitated with Bax antibodies and normal rabbit IgG, followed by WB using HSP20 antibodies. IP of Bax proteins in the cells with Bax antibodies was confirmed by WB using Bax antibodies.

serving as a marker of cells undergoing apoptosis. Based on our findings, it is most likely that expression of HSP20 protein might suppress HCC cell growth via both the downregulation of cell proliferation signals and the activation of apoptosis pathway.

We next demonstrated that the HSP20 protein directly interacts with Bax but not with Bad, Bcl-2 or Bcl-xL among the Bcl-2 family proteins in the HCC cells. The Bcl-2 family consists of pro-apoptotic members, such as Bad and Bax, and anti-apoptotic members, such as Bcl-2 and Bcl-xL (17). Regarding the Bcl-2 family proteins in HCC, it has been reported that Bcl-xL, an anti-apoptotic member, is overexpressed whereas Bax, a pro-apoptotic member, is down-regulated (17). The activities of the Bcl-2 family members are affected by the dimerization of these proteins, and mutant forms of Bcl-2 that fail to heterodimerize with Bax reportedly lose their ability to protect cells from apoptosis (13). Bax alone has been shown to be sufficient for induction of apoptosis. It is generally recognized that the Bcl-2 family proteins act as regulators for the mitochondria-mediated apoptosis, coupling to the activation of caspase-3 and caspase-7 (13). Thus, it is probable that HSP20 interfere Bcl-2 binding to Bax protein, and exert the effects to the mitochondria-caspase signals to induce apoptosis in the HCC cells. Activated AKT reportedly phosphorylates and inhibits Bax, and, as a result, prevents apoptosis (13). We have previously shown that HSP20 directly interacts with PI3K and inhibits AKT pathway activation in the HCC cells (12). Therefore, suppression of AKT activities

by HSP20 protein in the HCC cells might affect not only cell proliferation but also apoptosis in HCC.

In normal mouse heart, overexpressed HSP20 reportedly interacts with the Bax protein and protects the heart against ischemia/reperfusion injury (19). It has also been reported that acute expression of HSP20 in rat cardiomyocytes is protective against apoptosis (20). However, the exact mechanism of HSP20 underlying apoptosis of HCC remains to be clarified. Further investigations are necessary to elucidate the detailed role of HSP20.

In conclusion, our findings strongly suggest that HSP20 directly interacts with Bax and activates caspase cascade, resulting in the induction of apoptosis in HCC.

Acknowledgements

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