A hyposensitive anticancer drug induces higher surface expression and release of heat shock proteins in a human hepatocellular carcinoma cell line

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Abstract. Heat shock proteins (HSPs) respond to multiple stresses and have been implicated as essential immune chaperones that regulate innate and adaptive immunity. The exposure of HSPs containing tumour peptide complex to immune surveillance elements may elicit a specific anti-tumour response. The present study examined the potential of anticancer drugs to induce apoptosis of HepG2 cells and elicit the expression of HSP proteins, including HSP70 and gp96, on the membrane or their release to the extracellular environment, leading to HSP exposure. In the present study, etoposide and carboplatin were classified by an adenosine triphosphate assay as representatives of hypersensitive and hyposensitive anticancer drugs, respectively. Flow cytometry, immunofluorescence, ELIZA and reverse transcription quantitative polymerase chain reaction were all used to detect changes in the HSPs. The results demonstrated that etoposide and carboplatin induced apoptosis of HepG2 cells. In addition, following treatment with etoposide or carboplatin, HSP70/gp96 expression increased, demonstrating a ‘transfer expression’ pattern: The cytosol expression decreased while the surface expression increased. These alterations progressed steadily with notable alterations following treatment with etoposide for 24 h or carboplatin for 72 h. Additionally, at the end of treatment, release of HSP70/gp96 to the extracellular environment increased. Notably, following treatment with the hyposensitive anticancer drug carboplatin for 72 h, the surface expression of gp96 in HepG2 cells was significantly increased. These results suggest that when combined with cancer cell apoptosis, anticancer drugs induce the membrane expression and release of HSP70/gp96 in hepatocellular carcinoma (HCC) cells, which may represent a crucial event in the immune anti-tumour response. Notably, treatment with the hyposensitive anticancer drug for a longer time period resulted in greater surface expression and release of gp96, which suggests a potential use for hyposensitive anticancer drugs in HSP-based dendritic cell vaccine preparation and chemoimmunotherapy for HCC patients.

Introduction

Hepatocellular carcinoma (HCC) is a type of tumour with increasing incidence and a high mortality rate worldwide, ranking as the second most prevalent cancer in China (1-4). However, since it remains clinically asymptomatic until the late stages, therapeutic options are limited. Even at the early stages, treatments, including partial liver resection, chemotherapy, radiotherapy and molecular targeted therapy have demonstrated only a modest clinical benefit (5). Since HCC has been demonstrated to be immunogenic (6), immunotherapy, which aims at inducing the immune system to eradicate malignant, transformed cells, is considered to be a potential approach for the treatment HCC (7). However, in clinical practice, chemotherapy (8,9) or immunotherapy (10) alone cannot achieve satisfactory therapeutic efficacy. Thus, the use of chemioimmunotherapy has been proposed. The putative theory is that traditional chemotherapy can lower the tumour burden and promote antigen presentation, which elicits an immune response. A therapeutic chemodrug strategy using GOLF (gemcitabine, oxaliplatin, leucovorin and 5-fluorouracil), followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 to treat metastatic colorectal carcinoma patients resulted in a good therapeutic response and disease control rate and
effectively delayed disease progression (11). In vitro, it was found that GOLF therapy elicited an antigen-specific cytotoxic T lymphocyte (CTL) immune response mediated by dendritic cells (DCs) (12). This immune reaction may be due to the presentation of a ‘risk signal’ induced by chemodrugs (12). It is well established that chemotherapy causes tumour cell apoptosis (13); however, apoptotic cells stimulate variable outcomes (i.e., immunotolerance or immunoreactions) under different conditions. A previous study demonstrated that apoptotic cells induced by radioactive rays or retrogradation suppress immunoreactions as the immune system interprets this apoptotic phenomenon as normal (14). By contrast, apoptotic cells induced by infection, heat shock or certain chemodrugs could activate antigen-presenting cells and then elicit an immune reaction; this apoptosis was considered to be a ‘danger’ and thus abnormal (15). Therefore, tumour cells were induced to undergo apoptosis and to express heat shock proteins (HSPs), well-known protein chaperone molecules, by gene transfection to elicit a ‘danger signal’ and enhance tumour immunogenicity. Notably, when combined with ‘danger signal’ HSPs, apoptotic tumour cells elicited an immune response and subsequently destroyed residual tumour cells (16).

HSPS, including HSP70, gp96, HSP27 and HSP60, are intracellular molecular chaperones of nascent proteins and function during protein synthesis, folding, assembly, transport and stabilization (8). HSPS are synthesised under different types of stress conditions, including cell growth and differentiation, infection, inflammation, malignancy, heat shock and oxygen radicals (17). An immune response is elicited by HSPS isolated from cancer cells, not from normal cells, due to the tumour antigen peptide associating with HSPS. In this response, HSPS act as a ‘danger signal’ to attract and activate DCs (18). Those HSPS eliciting tumour immunogenicity are marked not by the constitutive but by the inducible HSPS (19), which may be exposed under the immune surveillance elements. Thus, the exposure of HSPS represents an important event in the anti-tumour response (17).

A comparison of HCC and normal samples demonstrated HSP deregulation in tumour cells (20). A comparison of HCC and normal samples demonstrated HSP deregulation in tumour cells (20). HSP70 and gp96 are stress sensitive members of the HSP family and have been the focus of numerous studies (21,22). Using flow cytometry, confocal microscopy, ELISA and reverse transcription quantitative polymerase chain reaction, the present study examined the potential of anticancer drugs to induce apoptosis of HepG2 cells and determine the protein expression levels of HSPs, including HSP70 and gp96, on the membrane or their release into the extracellular environment, leading to HSP exposure.

Materials and methods

Cell culture and reagents. The human HCC cell line HepG2 used in the present study was purchased from the American Type Culture Collection (Manassas, VA, USA) and routinely maintained in complete Dulbecco’s modified Eagle’s culture medium (25 mM D-glucose, 4 mM L-glutamine and 1 mM sodium pyruvate; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Gibco-BRL) at 37°C in 5% CO₂.

Growth inhibition assay. An adenosine triphosphate-tumour chemosensitivity assay (ATP-TCA; Biothera AB, Handen, Sweden) was used to determine the growth inhibitory effects of anticancer drugs on HepG2 cells. All anticancer drugs, including paclitaxel [100% test drug concentration (TDC) 13.6 µg/ml; Bristol-Myers Squibb Co., New York, NY, USA], etoposide (100% TDC 48 µg/ml; Jiangsu Hengrui Medicine Co., Nanjing, China), fluorouracil (100% TDC 0.5 µg/ml; Pfizer, Inc., New York, NY, USA), carboptatin (100% TDC 15.8 µg/ml; Bristol-Myers Squibb Co.), irinotecan hydrochloride (100% TDC 14 µg/ml; Pfizer, Inc.) and mitomycin (100% TDC 0.23 µg/ml; Zhejiang Hisun Pharmaceutical Co., Taizhou, China) were prepared according to the manufacturer’s instructions of the ATP-TCA kit. In total, 3x10⁴ cancer cells growing in a 96-well plate were incubated with different anticancer drugs at different concentrations [6,25, 12.5, 25, 50, 100 and 200% test drug concentration (TDC)] in complete growth medium at 37°C and 5% CO₂. The cells were collected for the ATP-TCA assay 72 h later according to the manufacturer’s instructions. Specifically, 100 µl ATP extraction solution was added to the cells, mixed and incubated for 20-30 min at room temperature. Following that, 500 µl of the mixture was added to the detection plate and mixed with 50 µl fluorescence-luciferase. It was then analysed using a Luminescence analyser (Synergy™ MX; BioTek, Winooski, VT, USA). Data were analysed by Microsoft Excel 2010 software and hyper- and hyposensitive drugs of HepG2 cells were profiled.

Anticancer drug treatment on HepG2 cells. The hypersensitive drug etoposide and the hyposensitive drug carboptatin were selected for the experiments. HepG2 cells were cultured in a 6-well plate at 37°C and 5% CO₂. Etoposide or carboptatin with 100% TDC was added and co-cultured for the indicated time intervals: 1, 2, 4, 6, 12, 18, 24, 48 or 72 h. The etoposide-treated group was not assessed for 48 or 72 h as it caused marked apoptosis after 24 h of treatment. At each time interval, the cells were harvested for flow cytometry or immunostaining analysis.

Flow cytometry. Following treatment with anticancer drugs, cancer cells were collected for intracellular and surface immunolabelling of HSP70/gp96. Briefly, for intracellular labelling, cells were fixed and permeabilised using 70% ethanol at 4°C overnight and then incubated with each of the following antibodies: Phyceroerythrin-conjugated mouse anti-HSP70 polyclonal antibody (1:100; cat. no. sc-1060; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit anti-human GRP94 (gp96) (1:100; cat. no. AHP848; AbD Serotec, Raleigh, NC, USA) followed by the secondary antibody [goat anti rabbit-fluorescein isothiocyanate (FITC); 1:200; cat. no. 172-1506; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA] for 20 min at room temperature and protected from light. For surface HSP70 or gp96 staining, tumour cells were dissociated into a single cell suspension and incubated under the same conditions as described above without fixation and permeabilisation. All labelled cells were washed and measured immediately using a FACS Calibur flow cytometer (Beckman-Coulter, Inc., Miami FL, USA). More than 1x10⁴ cells were analysed in each analysis and the mean fluorescence intensity (MFI) was used for the assessment.
of HSP70 and gp96 expression. Controls were routinely performed in all cases.

**Immunofluorescence.** Tumour cells were incubated with hypersensitive or hyposensitive drugs in a 6-well plate with a coverslip for different time intervals: Etoposide for 24 h and carboplatin for 72 h. Coverslips with cells were collected for indirect immunofluorescence staining. Cells were fixed and permeabilised using 70% ethanol at 4°C overnight and blocked by normal goat serum confining liquid for 30 min at room temperature. Subsequently, the cells were incubated with the following specific antibodies for 1 h at room temperature: Mouse anti-HSP70 (1:100; cat. no. BM0368; Wuhan Boster Biological Technology, Ltd., Wuhan, China) and rabbit anti-GRP94 (gp96) monoclonal antibody (1:100; cat.no. AHP848; ABD Serotec, Inc.). Secondary antibodies used for the visualization of HSP70 or gp96 were FITC-conjugated goat anti-mouse IgG (Wuhan Boster Biological Technology, Ltd.) and Cy3-conjugated sheep anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA). Hoechst was used for nuclear staining. During incubation with secondary antibodies and Hoechst, the cells were protected from light. Control staining was performed in all cases. Following staining, the cells were observed and images were captured under a fluorescent confocal microscope (LS< 510 META; Carl Zeiss, Jena, Germany).

**Apoptosis.** An annexin V detection kit used to determine apoptosis was provided by BioVision, Inc. (Milpitas, CA, USA; cat. no. K102-25). Tumour cells treated with drugs were dissociated into single cell suspensions in 200 µl binding buffer. Subsequently, 10 µl FITC-conjugated annexin V and 5 µl propidium iodide were added, and then the cells were incubated for 30 min at room temperature while protected from light. Next, 300 µl binding buffer was added and the cells were analysed using a FACS Calibur flow cytometer (Beckman-Coulter, Inc.).

**ELISA.** HSP70 (Surveyor™ IC; R&D Systems, Minneapolis, MN, USA; cat. no. SUV1663) and gp96 (MyBioSource, San Diego, CA, USA; cat no. MBS705033) ELISA kits were used and the quantity of released HSP70/gp96 from HepG2 cells treated with chemodrugs was analysed according to the manufacturer's instructions. Briefly, culture supernatants in which HepG2 cells were cultured with etoposide for 24 h or carboplatin for 24 or 72 h were collected and 100 µl of them were added to the microtitre that was pre-coated with anti-HSP70 or gp96 antibody. The cells were then incubated for 2 h at room temperature and washed three times with PBS. Subsequently, 100 µl HSP70 or gp96 detection antibody was added and incubated for 2 h at room temperature. The HSP70 or gp96 antibody was washed off and streptavidin horseradish peroxidase (HRP; HSP70) or HRP avidin (gp96) was added and incubated at room temperature for an additional 20 min. Following that, 100 µl substrates were added and incubated for 20 min. Finally, 50 µl stop solution was added and the optical density was determined at 450 nm using a Luminescence analyzer. The HSP70 or gp96 standard was used to make a standard curve by proportional dilution and the formula was produced for the concentration and optical density. Subsequently, the HSP70 or gp96 concentration in each sample was calculated.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** HepG2 cells treated with etoposide for 24 h and carboplatin for 24 or 72 h were harvested. Total RNAs were extracted and reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). RT-qPCR was performed using the Lightcycler-Faststart DNA master SYBR green I PCR kit (Roche Diagnostics, Madison, WI, USA) in a Roche Lightcycler 1.2 Real Time PCR System (Roche Diagnostics) according to the manufacturer's instructions: Initiation with a 10-min denaturation at 95°C, followed by 40 cycles of amplification with 15 sec of denaturation at 95°C, 5 sec of annealing at 50-60°C, 15 sec of extension at 72°C, and then the plate was read for fluorescence data collection at 76°C. The primer sequences were as follows: HSP70, forward 5'TGGTGTTCTACTCGTATCCC-3' and reverse 5'TGACATCCAAGACGCAAAAT-3'; gp96, forward 5' GCCCTGTCAGGGTTATTTT-3' and reverse 5'CACCTTTGCATCAGGGTCAAT-3'; GAPDH, forward 5'TGGTGGCATCAATGACCCCTT-3' and reverse 5'CTCCAAGACGTACTCAAGCG-3'. The comparative threshold cycle (CT) method was used for the calculation of amplification fold. The expression level of each gene was normalised by dividing by the expression level of the GAPDH gene transcript.

**Statistical analysis.** All data are expressed as the mean ± standard deviation. Statistical analyses were performed using paired Student's t-test with Microsoft Excel 2010 (Microsoft, Albuquerque, NM, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results.**

**Chemosensitivity of HepG2 cells to anticancer drugs and apoptosis caused by hyper- or hyposensitive anticancer drugs.** To determine the chemosensitivity of HepG2 cells to anticancer drugs, the present study selected six commonly used drugs to treat HepG2 cells and then used those cells to conduct ATP-TCA assays. For each single anticancer drug, the chemosensitivity was categorised as sensitive (100% TDC>90% and 50% TDC<70%) or resistant (100% TDC<70% and 50% TDC>50%). Dose-response curves for HepG2 cells following a continuous 24 h exposure to anticancer drugs at various concentrations using the ATP-TCA assay are depicted. The results demonstrated that etoposide (VP-16), taxol (TAX) and phomorubicin had high inhibitory rates and were thus classified as sensitive drugs. By contrast, mitomycin, irinotecan and carboplatin had low inhibitory rates (Fig. 1A and B) and were classified as resistant drugs. According to the results, the hypersensitive drug etoposide (VP-16), a cell cycle-specific anti-tumour drug that targets the S phase or G2 phase and the hyposensitive drug carboplatin, a cell cycle-non-specific drug, which inhibits DNA duplication and transcription, were selected for subsequent experiments. Subsequently, HepG2 cells were treated with hypersensitive (etoposide) or hyposensitive (carboplatin) drugs for the indicated time intervals and cell apoptosis levels were determined using an annexin V apoptosis detection kit. Apoptosis of drug-treated HepG2 cells slowly increased with time. Etoposide caused marked apoptosis in a relatively short time period, with
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the maximal annexin V positive rate achieved after 24 h. Carboplatin-treated cells took longer (72 h) to arrive at the maximal annexin V positive rate (Fig. 1C).

Alterations in the mRNA level and secretion of HSP70/gp96 in etoposide- or carboplatin-treated HepG2 cells. HepG2 cells were treated with etoposide for 24 h or carboplatin for 72 h. The cells were lysed and the mRNA levels of HSP70/gp96 were determined by RT-qPCR (Fig. 2A and B). In addition, cell culture supernatants were collected and the quantity of secretory HSP70 or gp96 was quantified by ELISA (Fig. 2C and D). The data indicated that although etoposide and carboplatin increased the mRNA level of HSP70 and gp96 in HepG2 cells, only carboplatin-treated HepG2 cells produced increased secretory HSP70 and gp96.

Gradual alterations in cytoplasmic and surface HSP70/gp96 expression in etoposide- or carboplatin-treated HepG2 cells. HepG2 cells were treated with etoposide or carboplatin for the indicated time intervals: 0, 1, 2, 4, 6, 12, 18, 24, 48 and 72 h. The cytoplasmic and surface HSP70 (Fig. 3A) and gp96 (Fig. 3B) expression levels were determined by flow cytometry and the parameter MFI was used to quantify the alterations. As shown in Fig. 3, prior to treatment with chemodrugs, HepG2 cells exhibited strong intracellular (cytoplasmic) HSP70/gp96 expression but weak surface expression. However, following treatment with chemodrugs, cytoplasmic HSP70/gp96 expression decreased and cancer cells exhibited upregulated surface HSP70/gp96 expression. Marked alterations were observed following 24 h treatment with etoposide and 72 h treatment with carboplatin. Following treatment with carboplatin for 72 h, the surface expression of gp96 in HepG2 cells was significantly increased.

Cytoplasmic and surface expression alterations in HSP70/gp96 in drug-treated HepG2 cells. HepG2 cells were treated with etoposide for 24 h or carboplatin for 72 h and immunofluorescence staining was used to demonstrate HSP70/gp96 expression. Consistent with flow cytometric analysis, the confocal images demonstrated a decrease in cytoplasmic HSP70/gp96 expression (Fig. 4A) and a clear increase in the surface expression of HSP70/gp96 (Fig. 4B). However, the increase in the surface expression of HSP70 was not significant.

Discussion

Almost all cells contain HSPs, which are present in a variety of intracellular locations, including the cytosol, endoplasmic reticulum, nuclei and mitochondria. HSPs function in normal cells as molecular chaperones to assist protein folding, unfolding, degradation and assembly (8). When cells become
malignant and proteins mutate or change, HSPs accumulate to adapt to the condition and protect cells from damage. For example, if tumour cells suffer from stress, such as following photodynamic therapy (17), HSPs not only markedly increase but also alter their distribution. They translocate from the cytosol to the cell surface and even release into the extracellular environment (23). In this circumstance, antigens chaperoned by HSPs are exposed to immune surveillance elements. Hence, HSP surface expression levels have been reported to correlate with tumour immunogenicity (19) and HSPs are considered to be ‘risk signals’, alerting to the existence of a threat and priming a self-protection system (24,25). Thus, upregulating cell surface HSPs in cancer cells or stimulating HSP release and exposure may be a crucial method to enhance anti-tumour activity.

Chemotherapy is considered to be an important treatment for HCC, but its acute and cumulative toxicity limit its application. However, immunotherapy, with its different functional mechanisms, can be combined with chemotherapy. Chemotherapy is
an immunological priming factor. It reduces tumour burden, augments tumour immunogenicity and provides the basis for an effective immunoresponse. A rational combination of chemo- and immunotherapy would lead to solid tumour regression and generate immunological memory (26). Furthermore, immunotherapy based on HSPs has demonstrated such effectiveness that it is not necessary to identify each tumour-specific antigen (27). In contrast to previous studies in which heat shock, ultraviolet radiation or pathogenic microorganisms were applied as a stressor, the present study employed chemodrugs as a stressor to examine their effects on HSPs in the hepatoma cell line HepG2. The present study primarily detected alterations in HSP70 and gp96 that are well investigated and can aid in eliciting an anticancer immunity response (28,29). As the data show, these chemodrugs were able to cause HepG2 cell apoptosis. The hypersensitive anticancer drug etoposide induced rapid apoptosis of cancer cells and after a 24 h treatment, the majority of cells died, thus cancer cell apoptosis and HSP alterations could only be recorded for 24 h. Conversely, the hyposensitive anticancer drug carboplatin induced apoptosis relatively slowly and 72 h were required to reach the peak of apoptosis. Along with demonstrating apoptosis, the cancer cells presented the ‘danger signals’ HSP70/gp96. Following treatment with etoposide for 24 h or carboplatin for 72 h, cancer cells were collected to determine the expression of HSP70/gp96. RT-qPCR data demonstrated that the mRNA levels of HSP70/gp96 increased. In addition, when treated with carboplatin for 72 h, HSP70 expression and, even more markedly, gp96 expression exhibited a transfer pattern from the cytoplasm to the cell surface and released into the extracellular environment. This external exposure of HSP70/gp96 may result in an enhancement of tumour immunogenicity. These results have greater significance than the simple upregulation of HSP genes established in previous studies (20). Therefore, it would be beneficial to re-evaluate the effects of hyposensitive drugs and adjust therapeutic strategies.

Previous studies have verified that HSP-peptide complex could present tumour antigens to activate a specific CTL response (30-32). These studies claimed that HSPs could be
employed for cancer treatment. However, in cancer patients, increased HSPs did not elicit an effective anti-tumour response and the patients often appeared to be immunotolerant (33). In addition to a large tumour burden, the possibility that the tumour antigens are not fully exposed to the immune surveil-

lance system is an important factor to consider. Therefore, in addition to degrading the tumour burden, challenging tumour cells with a stressor and eliciting antigen expression and presen-

tation are of significance. The present study successfully employed chemodrugs as a stressor. They induced cancer cell apoptosis and augmented surface HSP70/gp96 expression, resulting in HSP70/gp96 release to the microenvironment. Thus, combined with the HSP70/gp96 ‘danger signal’, apoptotic tumour cells treated with chemodrugs would elicit an immune response and subsequently eradicate residual tumour cells. Notably, the present study found that although the hypersensitive drug etoposide could promote rapid cancer cell apoptosis, it induced less HSP70/gp96 exposure. The continuous treat-

ment of cancer cells by the hypersensitive drug carboplatin was more effective, and it induced stronger surface expression and release of gp96, which suggests a potential use of hypossensi-

tive drugs for HSP-based DC vaccine preparation in vitro and chemoimmunotherapy for HCC patients. The present study provided an experimental basis for patient-specific chemoimmunotherapy for HCC. The results demonstrated that chemodrugs boosted surface HSP expression and release, which may facilitate tumour immunogenicity. Further studies are required to investigate the potential of this therapy.

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