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• *BRIEF REPORTS* •

Co-expression of heat shock protein 70 and glucose-regulated protein 94 in human gastric carcinoma cell line BGC-823

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Abstract

AIM: To investigate the co-expression and significance of heat shock protein 70 (HSP70) and glucose-regulated protein 94 (grp94) in human gastric carcinoma cell line BGC-823.

METHODS: The expression and localization of HSP70 and grp94 in human gastric carcinoma cell line BGC-823 were determined by immunocytochemistry and indirect immunofluorescence cytochemical staining. Flow cytometry was used to analyze the correlation between expression of HSP70, grp94 and cell cycle in BGC-823 cell line.

RESULTS: Gastric cancer cell line BGC-823 expressed high level of HSP70 and grp94. The positive rate of HSP70 and grp94 was 84.9±4.94% and 79.6±5.16%, respectively. Both of them were stained in cell plasma. There was a significant difference compared with control group (1.9±0.94%, $P<0.01$). During the cell cycle, HSP70 and grp94 were continuously expressed in BGC-823.

CONCLUSION: HSP70 and grp94 are highly expressed in human gastric carcinoma BGC-823 cells through the whole cell cycle. There is no relationship between expression of HSP70, grp94 and cell cycle.

Key words: Heat shock protein 70; Glucose-regulated protein 94; Gastric carcinoma

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INTRODUCTION

Eukaryotic and prokaryotic cells exposed to adverse environmental conditions, such as heat stress, hypoxia, glucose deprivation, virus infection and the presence of metal ions, exhibit an accurately regulated and highly conserved cellular response by inducing synthesis of a specific set of proteins known as heat shock proteins (HSPs). HSPs are molecular chaperones, which are biochemical regulators of cell growth, apoptosis, protein homeostasis and cellular targets of peptides[1-3]. In tumor cells, several HSPs are overexpressed and display an important role in cytoprotection *in vitro* and *in vivo*[4,5]. Previous studies have demonstrated that heat shock protein 70 (HSP70) or glucose-regulated protein 94 (grp94) peptide complexes derived from a tumor can elicit cancer-specific immunity against the same tumor by binding to tumor-specific peptides[6]. Further researches indicate that tumor-specific immunity induced by HSP70 or grp94 peptide complex can be mediated by CD8+ T lymphocytes, γδT+ cells or NK cells, and its mechanism involves MHC-1 molecule-restricted and non-MHC-1 molecule-restricted responses[7-9]. Gastric cancer is one of the most malignant cancers. However, limited information is available on the HSP70 and grp94 molecules in gastric cancer. In this study, by immunocytochemistry and flow cytometry analysis, we investigated the expression and significance of HSP70 and grp94 on cell cycle of gastric cancer cell line BGC-823.

MATERIALS AND METHODS

Reagents

Rabbit anti-human HSP70 antibody, mouse anti-human grp94 monoclonal antibody, TRITC-labeled goat anti-rabbit antibody and FITC-labeled goat anti-mouse antibody were purchased from Santa Cruz Company. EnVision™ kits were purchased from Dako Biological Technology Company.

Cell line and cell culture

Human gastric cancer cell line BGC-823 was provided by the Institute of Oncology, Chinese Academy of Medical Sciences, Beijing, China. The derived cell lines were grown in RPMI 1640 medium supplemented with 100 mL/L heat-inactivated fetal calf serum, 50 000 U/L penicillin, and 0.05 g/L streptomycin. The cells were maintained at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2. Viability of the cells used in these experiments was consistently more than 95% when evaluated by the trypan blue exclusion method. Cells with a density of 2.5×10^5/mL were seeded onto six-well plates for 24 h, washed with PBS, fixed by

adding 2 mL of cold 500 mL/L methanol-acetone and stored at 4 ℃ for 20 min, then washed with PBS and dried at room temperature.

Immunocytochemistry

All cells in six-well plates were hydrated with graded alcohol. Endogenous peroxidase was then blocked with 3 mL/L H_2O_2 diluted in methanol for 30 min at room temperature. Antigen retrieval was performed by treating the slides in citrate buffer, in a microwave for 10 min. The cells were incubated in a moist chamber with HSP70 rabbit antibody (1∶100) or grp94 mouse monoclonal antibody (1∶100) at 4 ℃ overnight respectively. After a complete wash in PBS, the cells were treated with HRP-labeled goat anti-rabbit and goat anti-mouse antibody (1∶100) for 45 min at 37 ℃. After a complete wash in PBS, all cells were developed in 0.5 g/L freshly prepared diaminobenzedine solution (Sigma Co.) for 8 min, and then counterstained with hematoxylin, dehydrated, air dried, and mounted. AFP was used as a substitute for the primary antibody as negative control.

Indirect immunofluorescence cytochemistry

All cells in six-well plates were hydrated with graded alcohol. The cells were blocked in 10 mL/L bovine serum albumin for 30 min at room temperature, and then incubated with HSP70 rabbit antibody (1∶100) or grp94 mouse monoclonal antibody (1∶100) at 4 ℃ overnight respectively. After a complete wash in PBS, the cells were treated with TRITC-labeled goat anti-rabbit antibody or FITC-labeled goat anti-mouse antibody (1∶20) for 40 min at room temperature. After extensive washing, the stained cells were observed under an immunofluorescence microscope. AFP was used as a substitute for the primary antibody as negative control.

Flow cytometric analysis

Flow cytometry was employed to determine the DNA content and the expression of HSP70 and grp94 in gastric cancer cell line BGC-823. Cells with a density of 2.5×10^5/mL were seeded onto six-well plates for 24 h, harvested, trypsinized, washed with PBS, fixed by adding 2 mL of cold 850 mL/L acetone and stored at 4 ℃ for 20 min. After fixation, the cells were washed, centrifuged, and incubated with HSP70 rabbit antibody (1∶100) or grp94 mouse monoclonal antibody (1∶100) at 4 ℃ overnight respectively. After a complete wash in PBS, the cells were treated with TRITC-labeled goat anti-rabbit antibody or FITC-labeled goat anti-mouse antibody for 40 min at room temperature. After a complete wash in PBS, the cells were centrifuged and resuspended in 0.05 g/L propidium iodide in PBS. The sample was incubated at room temperature for 30 min, and analyzed on a FACSCalibur (BD PharMingen, Franklin Lakes, USA). AFP was used as a substitute for the primary antibody as negative control.

Statistical analysis

Results were expressed as mean±SD. HSP70 and grp94 expression differences between gastric cancer cell line BGC-823 and control group were analyzed statistically using χ^2 test. $P<0.05$ was considered statistically significant.

RESULTS

Expression of HSP70 and grp94 in gastric cancer cell line BGC-823

Positive expression of HSP70 and grp94 showed brown staining in the nuclei or cytoplasm, more than 500 cells were calculated in different microscopic fields of each well, and percentage of positive cells were evaluated.

Immunocytochemical staining showed that the positive rate of HSP70 was 92.0%, and grp94 was 84.0%, while that in control group was 6.0%. There was a significant difference between experimental group and control group ($P<0.01$). HSP70 and grp94 were mainly stained in cell cytoplasm (Table 1 and Figure 1).

Table 1 Expression of HSP70 and grp94 in gastric cancer cell line BGC-823 detected by immunocytochemistry

Groups	Cells counter	Positive cells counter	Positive rate (%)
HSP70[b]	500	460	92.0
grp94[d]	500	420	84.0
AFP	500	30	6.0

[b]$P<0.01$ *vs* AFP group; [d]$P<0.01$ *vs* AFP group.

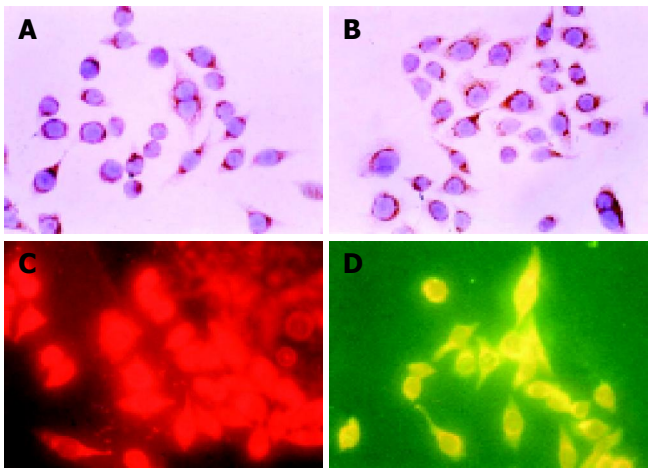

Figure 1 Expressions of HSP70 and grp94 in gastric cancer cell line BGC-823 by immunocytochemistry and immunofluorescence cytochemistry, ×400. **A**: HSP70 positive expression in cytoplasm; **B**: grp94 immunostaining in cytoplasm; **C**: HSP70 immunostaining in cytoplasm; **D**: grp94 green immunofluorescence in cytoplasm.

Relationship between expression of HSP70, grp94 and cell cycle in gastric cancer cell line BGC-823

The results showed that the expression rate of HSP70 in BGC-823 was 84.9±4.94%, while that of grp94 was 79.6±5.16%, there was a significant difference when compared with control group (1.9±0.94%, $P<0.01$). DNA content analysis showed that the expression rate of G_1 phase and G_2 cells was 59.2% and 14.9% respectively, $G_1/G_2 = 1.879$. During the cell cycle, there existed a continuous expression of HSP70 and grp94 in BGC-823. The results suggested that there was no significant relationship between expression of HSP70, grp94 and cell cycle in BGC-823 (Figure 2).

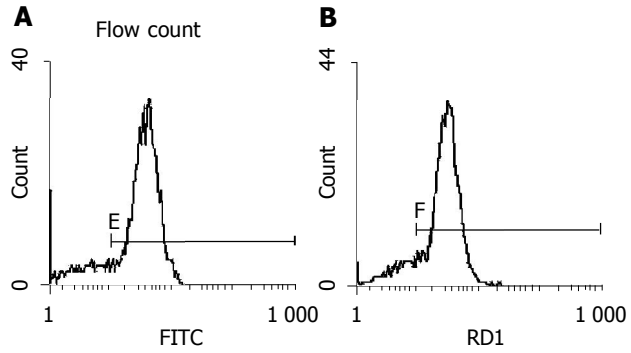

Figure 2 Expression of grp94 (**A**) and HSP70 (**B**) in cell cycle of gastric cancer cell line BGC-823.

DISCUSSION

In this study we examined the expression of HSP70 and grp94 in gastric cancer cell line BGC-823 by immunocytochemistry, immunofluorescence cytochemistry and flow cytometry analysis. The results showed that almost all gastric cancer cells expressed high level of HSP70 and grp94, which had a significant difference compared with control group. By immunofluorescence cytochemistry and flow cytometry analysis, we found that there was no correlation between expression of HSP70, grp94 and cell cycle of gastric cancer cell line BGC-823. During the entire cell cycle, BGC-823 expressed HSP70 and grp94 continuously. In the study, HSP70 and grp94 were mainly localized in cytoplasm. These results are consistent with the tumor results of several other groups[10-12].

HSP is a group of highly conserved proteins synthesized after heat induction[1-3]. In mammalian cells, this system is divided into HSPs and glucose-regulated proteins (grps) that appear to be structurally and functionally related[2]. In normal cells, HSP70 is constitutively expressed at low levels but the expression is dramatically enhanced under stressful conditions[3]. Grps sharing highly in amino acid identity to HSPs are thought to act as molecular chaperones, helping in transporting, folding and processing of their target proteins. However, grps do differ from HSPs, which are localized in cytoplasm and mitochondria, whereas grps are mostly located in endoplasmic reticulum[2]. Although the implication of the increased production of these proteins is unknown, it may be expected that every stressful response would enhance capacity of the pathway (s) in which these proteins function and perhaps protect the associated cellular compartments from damage via abnormal protein interactions. Several studies have suggested that HSPs and grps play some roles during cell survival and cell proliferation of tumors[10-12]. Continuous expression of HSPs and grps in tumor cells may be required to serve as molecular chaperones in regulating and stabilizing tumor growth process. Some studies have suggested that high-level expression of HSP70 and grp94 contributes to tumorigenicity of certain tumors[11,12], but its role in tumorigenicity is not clear. During the growth of murine tumors, the levels of grp94 are increased, correlating with the size of the tumor[13]. Furthermore, HSP70 and grp94 have been implicated to protect neoplastic cells and tumors against cytotoxic T-lymphocyte-mediated cytotoxicity and confer drug resistance[14]. Suppression of grp94 by antisense oligonucleotides results in increased sensitivity to cytotoxic T-lymphocytes and inhibition of tumor progression *in vivo*[15]. It is reasonable to propose that HSP70 and grp94 upexpression in these tumor cells is closely related with the tumor cell survival and proliferation[16]. Our data showed that there was an overexpression of grp94 in gastric cancer cells. The expression level of HSP70 and grp94 may be used as a diagnostic or prognostic marker and the result may be useful in the study of immunity between the expression of HSPs and tumor growth.

Cancer cells differ from normal cells in many important characteristics, including loss of differentiation and decrease of apoptosis[17,18]. Cell cycle plays an important role in modulating tumor cell growth and apoptosis[19]. Completion of a cell cycle requires co-ordination of a variety of molecular synthesis, assemblies, and movements[19,20]. Earlier studies suggested that, during the growth and development of normal cells, HSPs and grps are controlled by cell cycle[4,5], but they are continuously expressed at high level independently of cell cycle in tumor cells without any stimulation[6]. Recent studies have proposed that HSPs, in addition to facilitating protein folding and assembly, may take part in cell growth and proliferation by several ways such as signal transduction and cell cycle regulation through combining with certain proto-oncogene products[21,22], indicating that the proliferating cells need much more HSPs to maintain the protein activities. It is believed that tumor cells are a group of high proliferation heterogeneous cells, which progress gradually through mutant oncogene products[23]. Continuous expression of HSP proteins in tumor cells may be required to serve as molecular chaperones in regulating and stabilizing these mutant oncogene products during tumor growth. The existence of mutant or oncogene products may stimulate HSP synthesis[24,25]. It has been verified that HSP70 interacts with mutant p53 to stabilize its function, conversely wild-type p53 may downregulate HSP70 expression[26]. ErbB2 is the receptor tyrosine kinase, which takes part in signal transduction and cell cycle. The finding that grp94 forms a complex with mutant erbB2 in human carcinoma, stabilizing erbB2 and maintaining its proper intracellular distribution demonstrates the importance of grp94[27]. It has been found that erbB2 is overexpressed in carcinoma of breast, ovary and prostate, and is associated with poor prognosis[26-28]. Our results showed that HSP70 and grp94 were highly expressed in gastric carcinoma BGC-823 cells through the whole cell cycle, indicating that upexpression of HSP70 and grp94 is likely to have some relationship with proliferation, development and poor prognosis of gastric cancer.

Previous investigations have shown that HSP itself has no antigenicity and its immunogenicity is attributed to the chaperoned peptide[8]. It has been verified that HSP70 and grp94 are better molecular chaperones which process and present weak viral, bacterial or tumor antigen to host APC, gendering specific T-cell response and CTL reaction[7-9]. Several studies have shown that HSP70-associated peptides directly activate γδT+ lymphocytes or natural killer cells as superantigens independent of the stimulation of MHC-I

class molecules[29,30]. Exogenously introduced HSP70 or grp94-peptide complexes assist MHC-I peptide loading through the up-take of this complex by HSP70 or grp94 receptors on the antigen presented cell surfaces[31]. Our data suggest that HSP70 and grp94 are highly expressed in human gastric carcinoma BGC-823 cells through the entire cell cycle. There is no significant relationship between expression of HSP70, grp94 and cell cycle. The fact that gastric carcinoma cells could synthesize more HSP70 and grp94 may be useful in the study of immunity between HSP70, grp94 expression and tumor growth or design of tumor vaccine against gastric cancers.

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