Effect of hyperthermic CO$_2$-treated dendritic cell-derived exosomes on the human gastric cancer AGS cell line

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Abstract. The aim of the present study was to determine the antitumor effects of hyperthermic CO$_2$ (HT-CO$_2$)-treated dendritic cell (DC)-derived exosomes (Dex) on human gastric cancer AGS cells. Mouse-derived DCs were incubated in HT-CO$_2$ at 43˚C for 4 h. The exosomes in the cell culture supernatant were then isolated. Cell proliferation was analyzed using the cell counting kit-8 (CCK-8) assay. Cell apoptosis was observed using flow cytometry, Hoechst 33258 staining and the analysis of caspase-3 activity. In addition, the proliferation of tumor cells was evaluated in xenotransplant nude mice. HT-CO$_2$ markedly inhibited cell proliferation, as assessed by the CCK-8 assay, and also induced apoptosis in a time-dependent manner, as demonstrated by Annexin V/propidium iodide flow cytometry, caspase-3 activity and morphological analysis using Hoechst fluorescent dye. It was also revealed that HT-CO$_2$-treated Dex decreased the expression of heat shock protein 70 and inhibited tumor growth in nude mice. In conclusion, HT-CO$_2$ exerted an efficacious immune-enhancing effect on DCs. These findings may provide a novel strategy for the elimination of free cancer cells during laparoscopic resection. However, the potential cellular mechanisms underlying this process require further investigation.

Introduction

Gastric cancer is the fourth most common cancer worldwide, and the second leading cause of cancer-associated mortalities (1). Important factors underlying the poor prognosis of gastric cancer are recurrence and distant metastases, which may be a result of free cancer cells being shed into the peritoneal cavity during surgical manipulation (2). Therefore, reducing the incidence of recurrence and metastasis is crucial for improving the survival of patients. At present, laparoscopic resection is the first choice of treatment for gastric cancer. This involves the percutaneous injection of CO$_2$ into the region surrounding the target tumor in order to physically separate the lesion from adjacent structures; however, this approach enhances the spread of free cancer cells into the peritoneal cavity (3).

Emerging treatments for gastric cancer with peritoneal carcinomatosis include the use of regional chemotherapy, specifically hyperthermic intraperitoneal chemotherapy, which may improve the prognosis of patients (4). The exposure of tumors to hyperthermic conditions has been revealed to be an effective adjuvant therapy to radiotherapy and chemotherapy for a range of cancers, including locally advanced head and neck cancer (1), melanoma (2), esophageal cancer (3,4), locally advanced cervical cancer (5) and gliomas (6). These results suggest that hyperthermic conditions confer a beneficial effect during laparoscopic resection of gastric cancer (5). CO$_2$ pneumoperitoneum is used in laparoscopic surgery for the creation of an operative field. Previous data has revealed that compared with ambient temperature CO$_2$, hyperthermic CO$_2$ (HT-CO$_2$) pneumoperitoneum directly inhibits cell proliferation and induces cell apoptosis in gastric and colorectal cancers (5,6). However, the oncological effect and underlying mechanism of HT-CO$_2$ on tumor immune responses remains unclear.

Dendritic cells (DCs) act as specialized accessories to induce immunity and tolerance (7,8). A number of previous studies have established that DC-derived exosomes (Dex) are able to modulate tumor-associated immune responses (9). Exosomes are small, membrane-bound vesicles measuring ~100 nm in diameter that are involved in the endocytic pathway and externalized by a variety of cell types. Exosomes are formed by a fusion between multivesicular bodies and the plasma membrane, which is followed by exocytosis (10,11). It has been demonstrated that Dex pulsed with tumor antigens induce potent T cell-dependent antitumor effects in tumor-bearing hosts (12).

HT-CO$_2$ pneumoperitoneum has been identified to exert an efficacious cytotoxic effect on cancer cells, and may also enhance immune function by improving the antitumor...
activity of DCs, which inhibit free gastric cancer cell proliferation and disrupt gastric cancer recurrence. This hypothesis encourages the investigation of the mechanisms for HT-CO₂ pneumoperitoneum on DC immune responses.

The present study examined the effects of HT-CO₂ on the antitumor activity of Dex in gastric cancer cells. It was identified that Dex treated with HT-CO₂ induced a significant decrease in AGS cell proliferation in vitro and in vivo. In addition, Dex treated with HT-CO₂ increased the apoptosis of AGS cells.

Materials and methods

In vitro HT-CO₂ model. An in vitro HT-CO₂ model was constructed in order to simulate the HT-CO₂ pneumoperitoneum in the human body. The model enabled the exposure of cells to HT-CO₂ at a constant temperature, pressure, flow rate and humidity, with an accurate stability of ±0.3°C, ±1 mmHg, ±0.5/min and ±5% CO₂, respectively. The model was designed by the present research group and has approved patent protection from the State Intellectual Property Office of China (patent no., ZL201220519625.5).

Preparation and treatment of DCs. DCs were prepared as primary spleen cultures from inbred mice, as previously described (13). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Guangzhou, Guangdong, China) containing 10% fetal calf serum (Invitrogen) in 5% CO₂ in air at 37°C. The medium was changed as required. After several weeks, the splenic cells consisted of a stromal cell monolayer of fibroblastic and endothelial cells that continuously supported the proliferation and differentiation of hemopoietic cells into non-adherent dendritic-like cells. The supernatant was then transferred to a Falcon® 100 mm Cell Culture Dish (BD Biosciences, Franklin Lakes, NJ, USA) for the next step of purification. The cells in the supernatant were cultured in DMEM containing 10% fetal calf serum in 5% CO₂ in air at 37°C. On day 8 of culture, DCs were harvested at medium as non-adherent cells released into the culture supernatant. Cell viability was assessed using the Trypan Blue exclusion method (Sigma-Aldrich, St. Louis, MO, USA). In total, 10⁴ cells were cultured under normal conditions or at 43°C, in a 95% CO₂ atmosphere or at 43°C with a 95% CO₂ atmosphere for 4 h prior to exosome preparation.

Exosome preparation and characterization. Exosomes in the cell culture supernatant were prepared using a total exosome isolation kit (Invitrogen) according to the manufacturer's instructions. The isolated products were then examined by electron microscopy (JEM-2010HR; JEOL, Tokyo, Japan). The purified exosomes underwent sterile filtration through a 0.22-µm membrane and stored at -80°C until use.

Cell culture and cell proliferation assays. The gastric cancer AGS cell line was used in the present study. The cells were cultured in RPMI-1640 (Invitrogen) containing 10% fetal bovine serum (FBS) at 37°C under a humidified atmosphere containing 5% CO₂. For the cell proliferation assay, AGS cells were plated into 96-well plates (Corning Incorporated, Corning, New York, NY, USA) containing a medium supplemented with 10% FBS at a density of ~1,000 cells per well 24 h after exosome treatment. For the quantification of cell viability, cultures were stained using the cell counting kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) at various time points. In brief, 20 µl CCK-8 solution was added to each well and incubated for 4 h at 37°C. Each solution was then measured spectrophotometrically at 450 nm using a Multiskan Ascent microplate reader (ELx800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Analysis of cell apoptosis. Subsequent to a 24-h incubation with Dex, an Annexin V-fluorescein isothiocyanate apoptosis detection kit was used to assess cellular apoptosis (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Apoptosis was analyzed using a FACSCanto flow cytometer (BD Biosciences). For each sample, at least 10⁴ cells were analyzed using FCAP Array™ v3.0.1 software (BD Biosciences). For the Hoechst 33258 (Sigma-Aldrich) fluorescence staining, AGS cells were seeded into 24-well plates (Corning Incorporated) and treated with 5 µl of 20 µg/ml Hoechst 33258 for 30 min in the dark. Morphological changes in the nuclei of AGS cells were analyzed and counted using a fluorescence microscope (Zeiss, Oberkochen, Germany) in five different fields in order to discriminate between normal and apoptotic cells. Images of the cells were captured and then processed using Adobe Photoshop software version 7.0 (Adobe Systems, Inc., San Jose, CA, USA). Caspase-3 activity was determined using a caspase assay kit, according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). A spectrophotometer with an excitation wavelength of 400 nm and an emission wavelength of 505 nm was used to measure the level of free 7-amino-4-trifluoromethyl coumarin.

Western blot analysis. The exosome protein concentration was assessed using Bradford reagent (Bio-Rad, Guangzhou, Guangdong, China). Overall, 100 µM total protein was separated using SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (Millipore China Ltd., Guangzhou, Guangdong, China). The membrane was then incubated with the primary antibodies at 4°C overnight. The primary polyclonal rabbit anti-mouse heat shock protein (HSP)70 (1:1,000; cat. no. 4876) and CD63 (1:1,000; cat. no. 14023) antibodies, and the horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (1:3,000; cat. no. 7074P2) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The β-actin primary antibody was purchased from Bioworld Technology.
An enhanced chemiluminescence substrate kit (Thermo Fisher Scientific, Guangzhou, Guangdong, China) was used to visualize the protein bands. The percentage reduction in band intensity was calculated based on the untreated samples and then normalized to β-actin.

**In vivo carcinogenesis assay.** In total, 20 specific pathogen‑free grade BALB/c nude mice aged 6-8 weeks old were purchased from the Animal Experimental Center of the Guangdong Medical College (Foshan, Guangdong, China). The AGS cells, at a density of 2x10^6 cells per 100 µl serum-free medium, were injected into the right flank of each mouse. After 4 weeks, the animals were sacrificed by sodium barbital injection (50 mg/kg) and the tumors were weighed. The animal experiments were approved by the Experimental Animal Care and Use Committee of Guangdong Medical College.

**Statistical analysis.** The data from at least three separate experiments are expressed as the mean ± standard deviation. Unless stated otherwise, the Student’s t-test was used for comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Generation of DCs.** The DCs were prepared as primary spleen cultures from inbred mice. The cells were harvested at medium as non-adherent cells released into culture supernatant. The cell viability was determined using the Trypan Blue exclusion method and the cell viability was >90% in all experiments. In total, 10^6 cells were collected prior to exosome preparation.

**Exosome preparation and characterization.** The exosomes were successfully prepared from DCs. Electron microscopy was performed in order to confirm the morphology of the isolated products. It was revealed that the isolated exosomes were universal membrane vesicles with a diameter of 60-100 nm (Fig. 1A). Western blot analysis of Dex revealed the presence of the exosome marker CD63 (Fig. 1B).

**Dex inhibit gastric cancer cell proliferation.** The present study analyzed the effect of Dex repression on the growth of AGS cells. The proliferation rate was determined by the CCK-8 assay. It was established that treatment with Dex resulted in a significant inhibition of the proliferation of
AGS cells (45.3±9.1%; Fig. 2B). This finding demonstrates that HT-CO$_2$-treated Dex downregulated the growth of gastric cancer cells.

**Induction of apoptosis following Dex treatments.** AGS cells that had been treated with Dex for 48 h were evaluated for apoptosis. The apoptotic rate of the CO$_2$, heat- and HT-CO$_2$-treated Dex groups were 5.10±1.40, 13.30±1.32 and 27.70±1.87%, respectively (Fig. 3B). These values were significantly higher compared with the control group (3.1±0.44%). Compared with the group treated with heat alone, the HT-CO$_2$ group exhibited an increased apoptotic rate. These results indicate that the heat and CO$_2$-treated Dex are more effective than a single treatment for inducing AGS cell apoptosis. Consistent results were obtained from Hoechst 33258 fluorescence staining and the analysis of caspase-3 activity (Fig. 3A and C).

**Western blot analysis.** The protein composition of the DC-derived exosomes was analyzed by SDS-PAGE and then compared with that of the DC lysate. As shown in Fig. 4, the expression of CD63 was enriched in the exosome preparations compared with the DC lysate, which indicated a successful exosome extraction. The analysis of HSP70 expression levels following treatment revealed that HT-CO$_2$-treated Dex
induced an increase in the level of HSP70. This suggests that HSP70 may have a key role in the antitumor effect of Dex.

Tumor growth. For the in vivo experiments performed in mice, the antitumor effects of Dex were investigated in the gastric cancer AGS cell line. Tumor growth was significantly reduced in the heat and CO$_2$-treated Dex group. By contrast, no marked effects were observed in the control group, and heat- and CO$_2$-treated Dex groups (Fig. 5A). The tumor volumes are shown in Fig. 5B, with a mean tumor volume of 823.9±330.2 mm$^3$ in the control group, and 809.4±241.5 and 793.5±231.1 mm$^3$ in the heat- and CO$_2$-treated Dex groups, respectively. Treatment with HT-CO$_2$-treated Dex reduced the tumor volume to 533.4±286.6 mm$^3$ (P<0.05).

Discussion

The tumor-free principle is a primary rule for the radical resection of tumors. The no-touch isolation technique has been developed in order to prevent cancer cells from being shed into the peritoneal cavity during the surgical resection of gastric cancers. However, free cancer cells have been detected in the blood and peritoneal cavity following radical resection (14). Prognosis is particularly unfavorable for patients with peritoneal carcinomatosis. Establishing how to control free cancer cells is important in order to prevent gastric cancer recurrence and metastasis (4).

DCs are antigen-presenting cells that have an important role in the initiation of antitumor immune responses. DCs acquire antigens from apoptotic tumor cells to induce the activation of major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes, and also initiate antitumor immunity (15). Exosomes are nanovesicles that originate from late endosomal compartments and are secreted by the majority of living cells in ex vivo cell cultures. Dex are able to modulate immune responses, either directly, by exposing MHC and costimulatory molecules, or indirectly, by conveying internal components to surrounding cells (9). A previous study revealed that Dex are able to stimulate the proliferation of T lymphocytes in vitro and generate antitumor immune responses in vivo (16). A further study revealed that, in patients with malignant glioma, tumor-derived exosome-leaded DCs elicited a specific CD8(+) cytotoxic T-lymphocyte response against autologous tumor cells (17). The functional role of exosomes has been characterized according to their protein composition (18). HSP70 is a major host component in exosomes. Previous evidence has indicated that hematopoietic and tumor cells may secrete HSPs into the circulation through exosome-mediated, granule-mediated or lipid raft-mediated exocytosis (19). Such extracellular HSPs have been identified to activate innate immune responses through the use of Toll-like receptors (20). The analysis of the specific actions of HSPs should lead to the identification of effective HSP-based immunotherapies. It is known that HSP70 is involved in the
regulation of immune cells (21). A previous study revealed that when peripheral blood mononuclear cells were treated at 40°C for 1 h and then recovered at 37°C for 4 h, the level of HSP70 in the exosomal vesicles increased (22). The exposure of tumors to hyperthermic conditions has been established to be an effective adjuvant therapy to radiotherapy and chemotherapy. It has been hypothesized that elevated temperatures may have the effect of enhancing antitumor immunity (23).

In the present study, DCs were treated with HT-CO$_2$, or with heat or CO$_2$ alone. Dex from the HT-CO$_2$-treated group significantly decreased AGS cell proliferation. In addition, flow cytometry, Hoechst 33258 fluorescence staining and the analysis of caspase-3 activity revealed that it enhanced the apoptosis of AGS cells. Furthermore, an effective suppression of tumor growth was observed in mice treated with HT-CO$_2$ Dex.

To the best of our knowledge, the present study was the first to demonstrate that HT-CO$_2$-treated Dex reduced the proliferation and induced the apoptosis of the gastric cancer AGS cell line in vitro and in vivo. Therefore, Dex may confer an effective free tumor cell ablation benefit in induction of antitumor immunity.

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