Effect of hyperthermic CO_2-treated dendritic cell-derived exosomes on the human gastric cancer AGS cell line

JINLIN WANG[1], ZHIYONG WANG[2], YANXIA MO[1], ZHAOHUI ZENG[1], PEI WEI[2] and TAO LI[2]

[1]Department of General Surgery, Dongguan People's Hospital, Dongguan, Guangdong 523059;
[2]Guangdong Provincial Key Laboratory of Medical Molecular Diagnostics,
Guangdong Medical College, Dongguan, Guangdong 523808, P.R. China

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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

Correspondence to: Mr. Tao Li, Guangdong Provincial Key Laboratory of Medical Molecular Diagnostics, Guangdong Medical College, 1 Xincheng Broadway, Dongguan, Guangdong 523808, P.R. China
E-mail: taoliby@hotmail.com

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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-CO_2 pneumoperitoneum on DC immune responses.

The present study examined the effects of HT-CO_2 on the antitumor activity of Dex in gastric cancer cells. It was identified that Dex treated with HT-CO_2 induced a significant decrease in AGS cell proliferation *in vitro* and *in vivo*. In addition, Dex treated with HT-CO_2 increased the apoptosis of AGS cells.

Materials and methods

In vitro HT-CO_2 model. An *in vitro* HT-CO_2 model was constructed in order to simulate the HT-CO_2 pneumoperitoneum in the human body. The model enabled the exposure of cells to HT-CO_2 at a constant temperature, pressure, flow rate and humidity, with an accurate stability of ±0.3˚C, ±1 mmHg, ±0.5 l/min and ±5% CO_2, 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% CO_2 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% CO_2 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, 10^6 cells were cultured under normal conditions or at 43˚C, in a 95% CO_2 atmosphere or at 43˚C with a 95% CO_2 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-2010; JEOL Ltd., Tokyo, Japan) in order to confirm the morphology, and then cluster of differentiation (CD)63 detection was performed through western blotting. Next, the purified exosomes were fixed for 1 h in 4% paraformaldehyde and washed once with phosphate-buffered saline. The pellets were then fixed in 2.5% glutaraldehyde, loaded on Formvar/carbon-coated electron microscopy grids, post-fixed in 1% glutaraldehyde, and contrasted successively in 2% methycellulose/0.4% uranyl acetate (pH 4.0). Observations were made using a JEM-2010HR electron microscope (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% CO_2. 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 10^4 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 spectrofluorometer 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

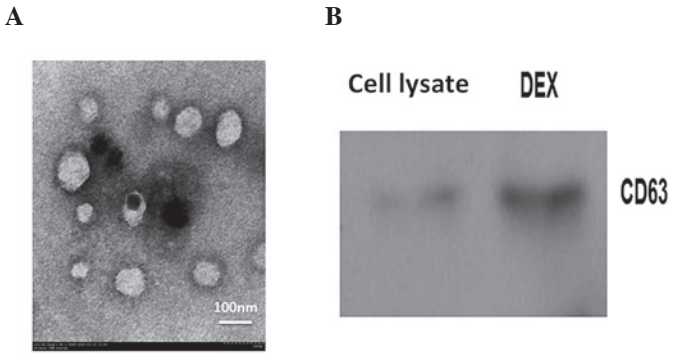

Figure 1. Phenotypical analysis of Dex. (A) Electron microscope image revealing exosomal membrane vesicles purified from DCs. The purified pellets were observed by transmission electron microscopy. (B) Western blot analysis of Dex. The Dex protein was detected by the presence of the exosome marker CD63. Dex, dendritic cell-derived exosomes; DC, dendritic cell; CD63, cluster of differentiation 63.

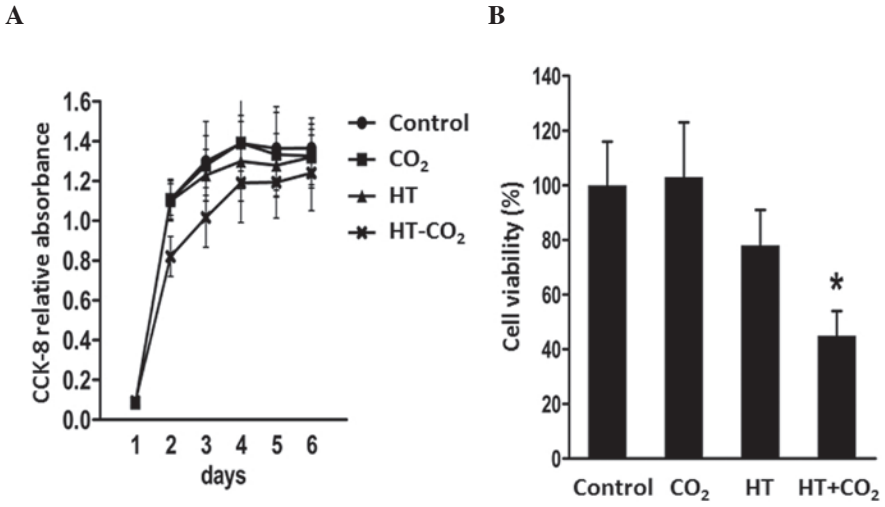

Figure 2. Cell proliferation was determined by the CCK-8 assay. (A) The effect of treated dendritic cell-derived exosomes on the viability of the gastric adenocarcinoma AGS cell line was measured in a time-dependent manner. (B) Relative cell viability. The data were normalized to a normal saline control and are presented as the mean ± standard deviation of triplicates. CCK-8, cell counting kit-8; HT, hyperthermic.

(catalog no. BSAP0060; Shanghai, China). 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

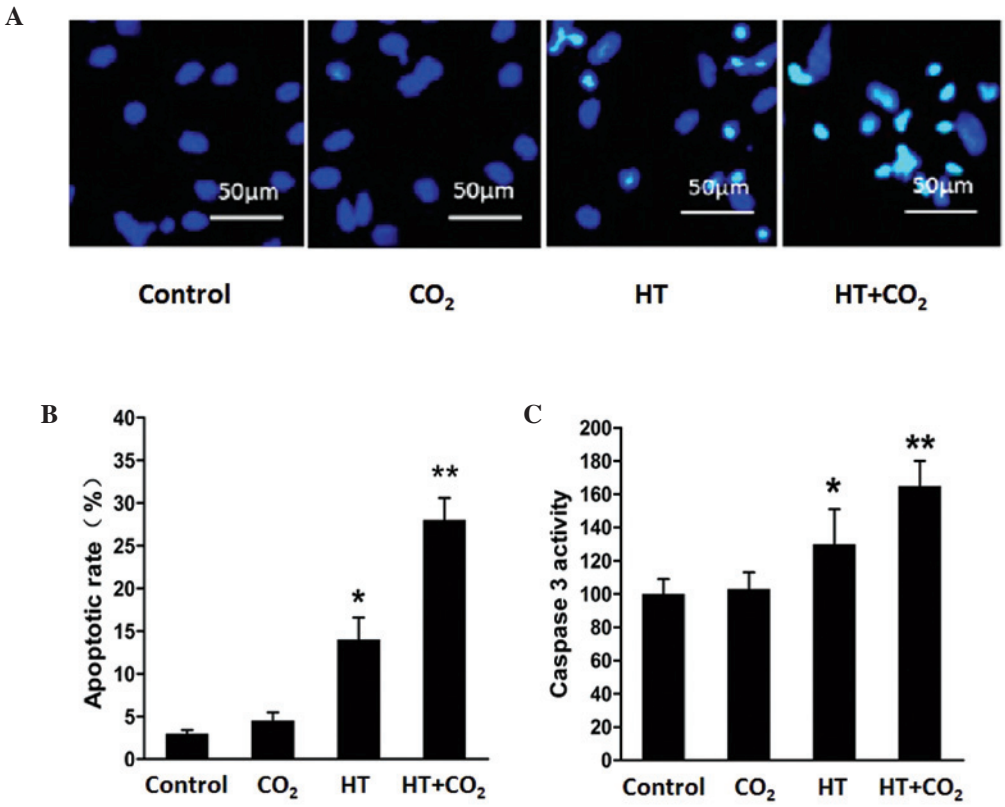

Figure 3. Induction of apoptosis in the gastric adenocarcinoma AGS cell line following treatment with dendritic cell-derived exosomes. (A) Cellular apoptosis was observed by Hoechst 33258 staining, which detects chromosomal condensation and nuclear fragmentation. (B) Early cellular apoptosis was analyzed by flow cytometry. (C) Caspase-3 activity was assessed and presented as a percentage of the change in the mean values derived from three separate experiments and then compared with the control group. The data were normalized to a normal saline control and are presented as the mean ± standard deviation of triplicates. *P<0.05 compared with the control. HT, hyperthermic.

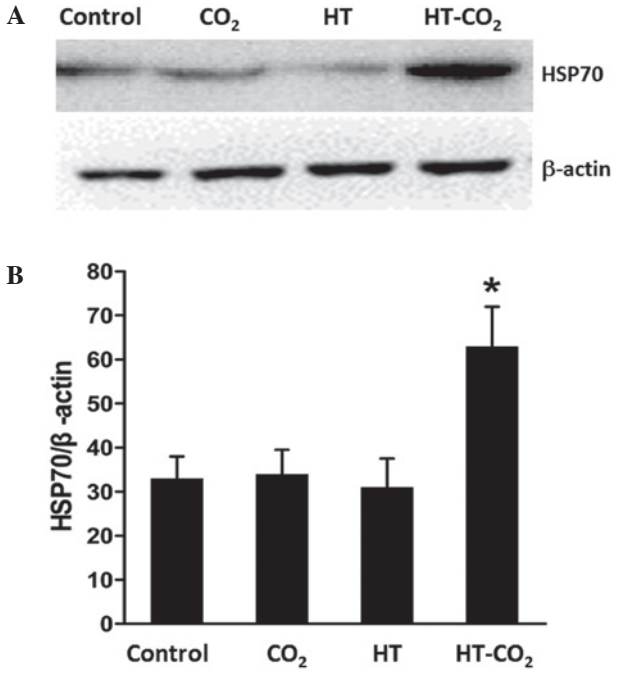

Figure 4. Western blot analysis of HSP70 expression in treated dendritic cell-derived exosomes. (A) The corresponding bands were detected using an enhanced chemiluminescence detection kit. The western blot analysis was stripped and reprobed with a β-actin antibody in order to ensure equal loading of total protein. (B) Histogram showing the relative expression levels of HSP70 normalized to β-actin. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the parental group. HSP70, heat shock protein 70; HT, hyperthermic.

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). The caspase-3 activity (OD_{405}) of the heat- and HT-CO_2-treated Dex groups was markedly higher than that of the control group. However, no significant difference was observed between the CO_2 alone-treated group and the control group.

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

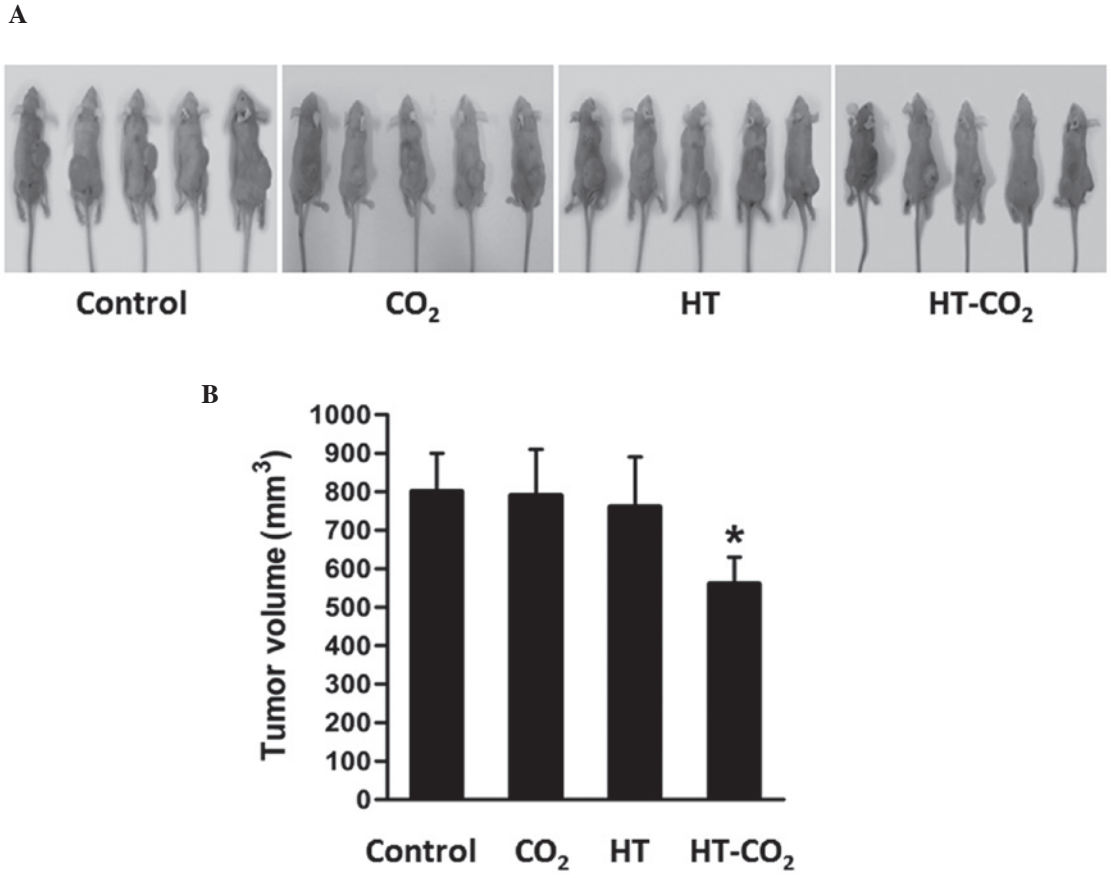

Figure 5. Tumorigenicity of gastric adenocarcinoma cell line incubated with treated DC-derived exosomes. (A) The appearance of the xenograft subcutaneous gastric adenocarcinoma cell line incubated with treated DC-derived exosomes. (B) Tumor volumes obtained from the nude mice. Data are presented as the mean ± standard deviation. Values are presented from five mice. *P<0.05 vs. control group. DC, dendritic cell.

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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