Effects of disodium cantharidinate on dendritic cells of patients with bladder carcinoma

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Abstract. The present study explored the effects of disodium cantharidinate (DC) on the peripheral blood-derived dendritic cells of patients with bladder carcinoma. The peripheral blood mononuclear cells from the 15 cases of urinary bladder carcinoma of middle and advanced stage were separated, and dendritic cells were prepared. The morphological changes of dendritic cells were observed. Flow cytometry was used to detect the expression levels of CD1a and CD83 on dendritic cell surface. MTT assay was utilized to measure the proliferation ability of allogeneic lymphocyte stimulated by DC. Annexin V-FITC/propidium iodide (PI) double staining flow cytometry method was carried out to detect cell apoptosis after treatment with DC. The changes in caspase-3 and PARP expression levels were investigated by western blot method. The high-dose DC resulted in a significant increase in the expressions of dendritic cell phenotypic molecules CDla and CD83 as compared to control group. In addition, the proliferation index of allogenic lymphocyte stimulated by DC was significantly higher than that of control group. Moreover, MTT assay showed significant inhibition of the growth of BIU-87 cells. After 24 h of DC treatment, double staining flow cytometry confirmed the ability of DC to induce cell apoptosis. Further, western blot method showed a significant elevation of caspase-3 and PARP protein expression after DC treatment. In conclusion, DC treatment could induce dendritic cell maturation of patient with carcinoma of urinary bladder and promote its functional changes. Furthermore, DC was able to inhibit the proliferation of cell BIU-87 and also has the ability to induce cell apoptosis.

Introduction

Bladder tumor is one of the most common tumors, featuring high incidence rate, high recurrence rate and low-grade malignancy (1). The main symptom of patients with carcinoma of urinary bladder is hematuresis. Clinically, radiography and cystoscopy are often taken as the diagnostic methods of carcinoma of urinary bladder (2). The patients with carcinoma of urinary bladder could be divided into superficial bladder cancer and invasive bladder cancer. Superficial bladder cancer has the features of high recurrence rate and low metastasis rate, while the features of invasive bladder cancer are high invasion and metastasis rate (3).

Dendritic cell is a kind of antigen presenting cell that plays a key role in the process of tumor immunity (4). Further, studies in the recent past revealed that a decrease in the dendritic cells could affect immune response of lymphocytes, leading to immune tolerance of organism (5-7). As a consequence, cancer cells would escape from the organism immune system. Thus, dendritic cells play an important role in the process of tumor occurrence, development and metastasis (5). The study confirmed that dendritic cells have a close relation with carcinoma of urinary bladder, which could involve in the treatment of carcinoma of urinary bladder. Moreover, dendritic cells are also reported as makers of urinary bladder carcinoma (6,7).

Disodium cantharidinate (DC) is a ramification of cantharidin, which is being used clinically for the cure of liver cancer and stomach cancer. DC has the characteristics of low virulence and irritability as compared to lead compound cantharidin (8-11). The antitumor mechanisms of DC are complicated and multiple. The different approaches used by DC against carcinogenesis include interdicting cell cycle, inducing cell apoptosis, inhibiting the invasion/metastasis of tumor cells and improving the immunity of the organism (12-14). In the present study, the effect of DC on the maturation and function of peripheral blood-derived dendritic cells in urinary bladder cancer patients was studied, as these cells are crucial for immune response against tumors. We provide research basis for the clinical application of DC for the patients with carcinoma of urinary bladder.

Materials and methods

Materials. rh-GM-CSF and rh-IL-4 were procured from R&D Systems (Minneapolis, MN, USA). Mouse anti-human CDla and CD83 mococlonal antibodies were labeled by FITC from Invitrogen (Carlsbad, CA, USA) (dilution, 1:500;

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cat. nos. 11-0015-42 and 11-0839-42). Mouse anti-human caspase-3, PARP, GAPDH primary polyclonal antibody (dilution, 1:1,000; cat. nos. ABP57612, ABP57383 and ABP57259) and goat anti-mouse HRP-labeled second polyclonal antibody (dilution, 1:2,000; cat. no. A21010) were procured from AmyJet Scientific Co., Ltd. (Wuhan, China). Ficoll paque plus and lymphocyte separation medium were obtained from Amersham Biosciences Corp. (Piscataway, NJ, USA). RPMI-1640 culture medium and fetal calf serum were provided by HyClone Laboratories (Logan, UT, USA). BCA protein assay kit and Annexin V-FITC apoptosis detection kit were procured from Beyotime Institute of Biotechnology (Nantong, China).

Dendritic cell culture in vitro and medication. Fifteen patients with carcinoma of urinary bladder and 10 healthy persons were selected as study subjects. Twenty milliliters venous blood was extracted from each person, and peripheral blood mononuclear cells were separated by Ficoll paque plus. Further, lymphocyte separation medium was cultured for 2 h under the condition of 37˚C and 5% CO_2. Suspension cells were removed by washing to obtain adherent cells. Then medium RPMI-1640 that contained GM-CSF (1,000 IU/ml), IL-4 (500 IU/ml) and 10% autoserum was applied to culture, which was changed to half after every 24 h. The fifth day, the cultured cells were divided into groups as follows: Control group; low-dose DC group (0.01 μg/ml); middle-dose DC group (0.05 μg/ml); high-dose DC group (0.25 μg/ml); healthy people group. Related detections were conducted for the eighth day after cells were collected.

Expression of CDla and CD83 of dendritic cells by flow cytometry. Cells from the eighth day were collected. The fluorescent antibodies CD1a and CD83 were then added, which was followed by incubation for 30 min under room temperature and darkroom. Precooling phosphate buffer (2 ml) was then added. At last, flow cytometry was applied to detect the expression levels of CD1a and CD83 on the surface of dendritic cells from each group.

Influence of DC on lymphocyte proliferation of dendritic cells detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Venous blood of healthy people was extracted and separated to monocyte that was cultivated under the condition of 37˚C and 5% CO_2. After 2 h, the cells that were not adherent to the walls, were absorbed to obtain lymphocytes. Dendritic cells in each group of the eighth day were collected and incubated for 30 min by mitomycin C (50 g/ml). The cell density was adjusted to $1x10^7$/liter dendritic cells and lymphocyte were mixed by the proportion of l:10. The mixed cells were gathered on 96-well plates and were cultured for 48 h. MTT method was used to test the cell activity. Absorbance values on the position of 570 nm were measured to calculate the stimulation index of lymphocytes according to the formula, stimulation index = experimental group OD value/control OD value.

Inhibition effects of DC on urinary bladder carcinoma BIU-87 cells. BIU-87 was cultivated by RPMI-1640 that contained 10% fetal calf serum under the condition of 37˚C and 5% CO_2. There were 3 groups viz. control group (without drugs), 10 μg/ml DC group and 20 μg/ml DC group. Six pores were set in each group and MTT treatment fluid was added 12, 24, 36 and 48 h after cultivation. The absorbance values were recorded at 570 nm and the enzyme-linked immunosorbent assay (ELISA) was used to calculate cell viability.

Influence of DC on apoptosis of urinary bladder carcinoma BIU-87 cells detected by Annexin V-propidium iodide (PI) double staining method. On the basis of the groups in 2.5, BIU-87 of logarithmic phase was inoculated to 6 pore plates respectively, after 24 h of drug function. The cells were collected from digestion and centrifugation. Binding buffer solution (0.3 ml) was added and blended. Annexin V of 5 μl and PI of 5 μl were added to each test sample and incubated for 15 min under room temperature. Binding buffer solution (0.2 ml) was refilled. Flow cytometry was applied to detect cell apoptosis.

Statistical analysis. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) analyzed the experimental data. One-way ANOVA was applied to analyze the data. $P<0.05$ is considered to indicate a statistically significant difference.

Results

Expression of CDla and CD83 of dendritic cells by flow cytometery. The image result of inverted microscope is shown in Fig. 1. The cells of DC group were larger as compared to control group. The cells of high-dose DC group (Fig. 1D) showed protuberance and was shaped as a branch, which was similar to the cellular morphology of healthy people group (Fig. 1E). The results of flow cytometry detection are shown in Fig. 2. Compared with control group, CD83 and CDla of DC treatment group increased significantly. In addition, the expression levels of CD83 and CDla of high-dose DC group were high but the difference had no statistical significance.

Detection of proliferation ability of allogeneic lymphocyte stimulated by DC. The proliferation index of allogenic lymphocyte stimulated by DC was significantly higher than that of control group (Fig. 3). Besides, different dose of DC in each group, the proliferation index of lymphocyte stimulated by DC in the high-dose group was the highest.

Inhibition effects of DC detected by MTT. The cell viability of each medication administration group reduced to different extent in comparison with control group (Fig. 4). The inhibition effect of drug increased significantly with increase in the concentration of DC.

Influence of DC on apoptosis of urinary bladder carcinoma BIU-87 cells (detected by Annexin V-PI double staining method). Compared with control group, the apoptosis rate of DC groups dramatically increased, and the difference was statistically significant ($P<0.01$). So, this result demonstrated that DC could induce BIU-87 apoptosis (Fig. 5).

Changes of caspase-3 and PARP protein expression detected by western blot method. Compared with control group, caspase-3 and PARP protein expression of BIU-87 from DC

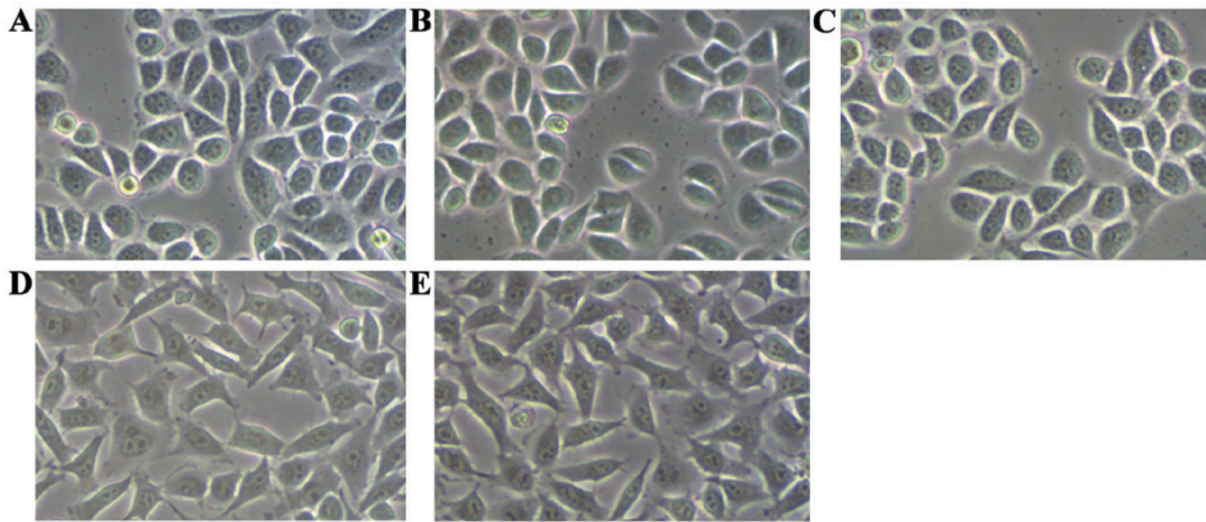

Figure 1. Morphology of dendritic cells (x400): (A) Control; (B) low-dose; (C) middle-dose; (D) high-dose; (E) healthy.

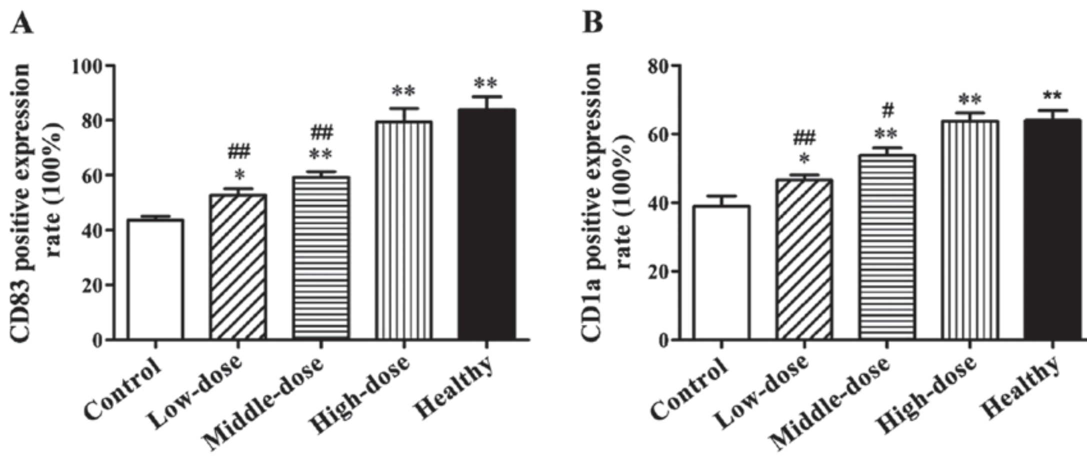

Figure 2. Expression of CD83 and CD1a in dendritic cells. *p<0.05 and **p<0.01 compared with control group; #p<0.05 and ##p<0.01 compared with healthy group.

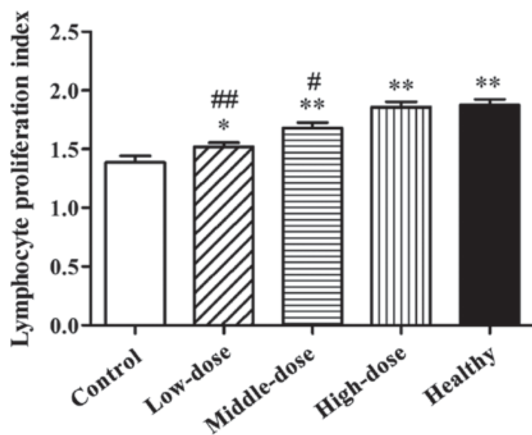

Figure 3. Cell proliferation of allogenic lymphocyte stimulated by disodium cantharidinate. *p<0.05 and **p<0.01 compared with control group; #p<0.05 and ##p<0.01 compared with healthy group.

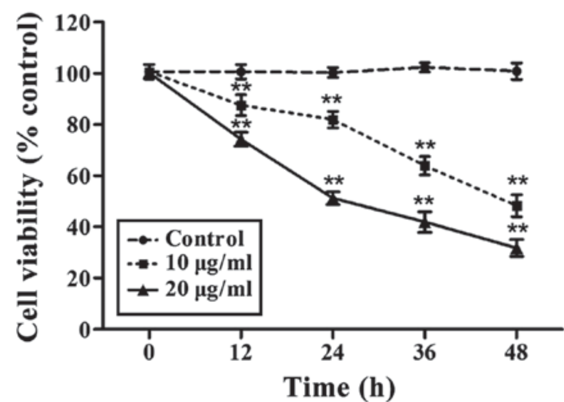

Figure 4. Cell viability treated by different concentrations of disodium cantharidinate, detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **p<0.01 compared with control group.

groups were significantly enhanced (Fig. 6) and the difference was statistically significant (P<0.01).

Discussion

Carcinoma of urinary bladder is one of the most common tumors, which is mainly treated by the operation that reserves

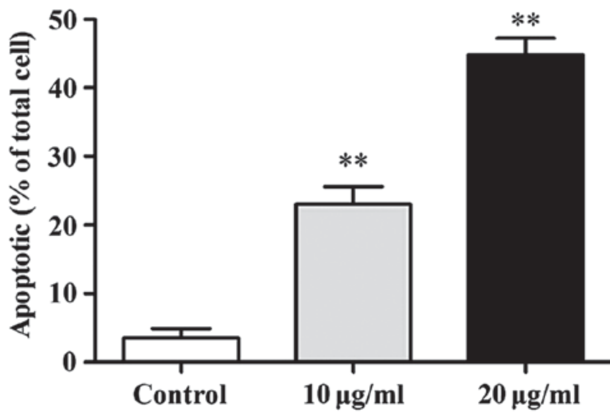

Figure 5. Apoptosis of different treatments of groups detected by flow cytometry. **p<0.01 compared with control group.

bladder in clinic. However, this therapeutic method may result in incomplete excision, as the recurrence rate of a half of patients is extremely high. At present, to solve this malpractice, drug therapy of intravesical instillation was applied to the patients after operation in clinic, but drug resistance is often associated with bladder tumor.

DC has not only antineoplastic activity but also has ability to affect the immune function of organism. The molecular weight of DC is small enough to enter cells. It could also improve the immunity of patients and make the antitumor effect stronger. Currently, the combination of DC and other antitumor drugs in clinic is mainly applied for the treatment of lung cancer, stomach cancer and old-aged bladder cancer (15-17). DC has the ability to improve the immune function of organism, by increasing the number of leucocytes so as to stimulate the secretion of cytokines (18,19). Furthermore, the specific molecule on the surface of dendritic cell membrane could combine with the corresponding ligand of lymphocyte surface; as a result, antigen-specificity T cells are activated and proliferated, which in turn activates immune response (20-22). Multiple approaches to produce a large number of dendritic cells have been developed, and the sources mainly include marrow, cord blood and peripheral blood, among which peripheral blood is the most common approach (23).

In the present study, CDla revealed the number of dendritic cells, in addition, CD83 acted as a marker for the maturity of dendritic cells (24,25). The results of this experiment proved that the surface of cultured cells produced protuberance and shape as branch after DC and dendritic cells were incubated together. At the same time, the expressions of CDla and CD80 on the surface of dendritic cells dramatically increased. The dendritic cells in high-dose DC group were compared with that of healthy group, and the difference had no statistical significance. Furthermore, MTT method was applied to detect the proliferation ability of lymphocyte stimulated by DC. The results showed that DC could improve the proliferation ability of lymphocytes, which suggested that DC could improve the immunity of patients.

The change of intercellular environment brings about emergency reaction of cells leading to self-destructing death of cells, called apoptosis. Multiple genes are involved in the whole process of cell apoptosis, among which caspase-3 plays a key role. When caspase-3 is activated, cells enter into an irreversible apoptosis process. Moreover, most of apoptosis induced by multiple factors are through caspase-3 (26).

Further, MTT method demonstrated that DC could significantly inhibit the growth of BIU-87, showing dose- and time-dependence. Annexin V-PI double staining method testified that DC could induce BIU-87 apoptosis. Further the influence of DC on apoptin caspase-3 and PARP expressions was also noted. As a result, in comparison with control group, caspase-3 and PARP protein expression of BIU-87 in DC groups increased, to promote BIU-87 cell apoptosis.

To sum up, on the one hand, DC could promote the maturation of dendritic cells for the patients with carcinoma of urinary bladder and enhance the proliferation of lymphocytes stimulated by dendritic cells. On the other hand, it could inhibit the proliferation of bladder cell BIU-87 by induction of apoptosis. This study provides a new strategy for the clinical application of DC during urinary bladder carcinoma.

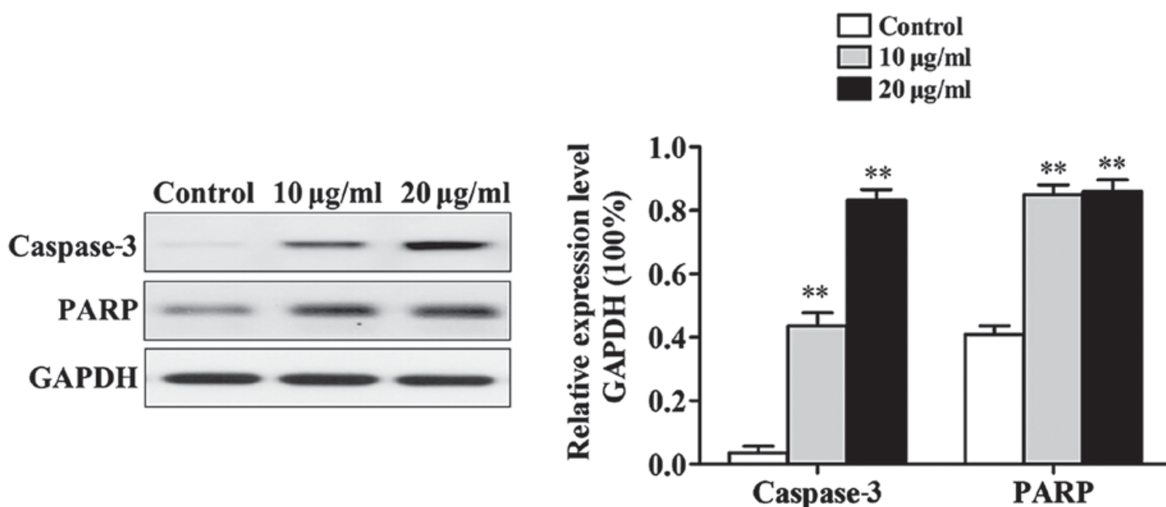

Figure 6. Caspase-3 and PARP protein expression detected by western blot analysis. **p<0.01 compared with the control group.

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