Article

Mitoxantrone-Mediated Apoptotic B16-F1 Cells Induce Specific Anti-tumor Immune Response

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In the process of cell apoptosis induced by specific reagents, calreticulin (CRT) in endoplasmic reticulum is transferred and coated onto the cell membrane. As a sort of specific ligand, the CRT on the surface of apoptotic cells could mediate recognition and clearance of apoptotic cells by phagocytes. In this research we discovered that mitoxantrone could induce apoptosis of mouse melonoma B16-F1 tumor cells, accompanied by the membrane translocation and coating of CRT. When mitoxantrone-treated B16-F1 cells were used as antigen to inoculate mice, the mice acquired an ability to suppress proliferation of homologous tumor cells. Splenocytes from these mice showed an increased cytolytic effect on homologous B16-F1 cells but no such effect on non-homologous H22 tumor cells. All these results suggested that mitoxantrone-treated apoptotic B16-F1 cells could be used as a sort of cell vaccine to initiate effective anti-tumor immunoresponse in mice. *Cellular & Molecular Immunology*. 2009;6(6):469-475.

Key Words: calreticulin, tumor immune, mitoxantrone, apoptosis

Introduction

One important factor of tumor generation is that mutated tumor cells escape from immunological surveillance by lowering the expression of membrane marker molecules which play essential roles in the process of cell recognition and phagocytosis (1-4). It has been expected to achieve an effective tumor prevention and treatment by restoring or stimulating body's immune response against the cancers.

Calreticulin (CRT) is a highly conservative Ca^{2+}-binding protein which is ubiquitous in mammalian and mostly presents at the endoplasmic reticulum (ER) lumen. CRT has multiple biological functions which are relevant to its subcellular localization, including chaperone functions, lectin binding, activation of store-operated Ca^{2+} influx, regulation of cell adhesion and removal of apoptotic cells, etc (6-11). Recently, it has been proved that CRT in the membrane of apoptotic cells was the key molecule involved in the recognition and clearance of apoptotic cells (12-16). In 2006, Obeid M and his colleagues discovered that treating CT-26 cells (mouse colon carcinoma cells) with anthracycline, one class of antitumor drugs used in the clinic, resulted in translocation of CRT from ER to cell surface along with the cell apoptosis. When these CRT-coated apoptotic CT-26 cells were used as antigen to immune animals, the specific anti-tumor immune response was elicited in tumor bearing mice. The possible mechanism was that, as a specific marker, the CRT on the surface of apoptotic cells was recognized by dendritic cells or other antigen presenting cells (APCs) that lead to the collective phagocytosis of apoptotic cells. Within the APCs, tumor associated antigens (TAA) or tumor special antigens (TSA) were subsequently processed and presented to $CD4^+$ and $CD8^+$ T lymphocytes, and finally specific anti-tumor immune response was elicited in the experimental animals (18, 19). These researches suggest a potential importance of CRT in tumor immunotherapy.

In order to further prove the universal value of CRT in anti-tumor immunity, in this study, the effect of specific anti-tumor immune response mediated by mitoxantrone-treated B16-F1 cells was evaluated. Results demonstrated that mitoxantrone, a member of the anthracycline family, could also induce apoptosis and CRT membrane translocation in mouse melanoma B16-F1 cells. And when the mitoxantrone-treated cells were used as cell-antigen to immune mice, the specific immune response against homologous tumor cells was observed.

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Received Nov 25, 2009. Accepted Dec 7, 2009.

Abbreviations: CRT, calreticulin; NVI, mitoxantrone; LDH, lactate dehydrogenase; NBT, nitrotetrazoliumbluechloride; PMS, phenazine methosulfate; DAPI, 4',6'-diamidino-2-phenylindole

Materials and Methods

Experimental animals and cell lines

BALB/c mice (7-8 weeks old) were purchased from Hubei Experimental Animal Center (No. 0001839). All the animals were housed under specific pathogen free conditions. The mouse melanoma cell line B16-F1 and hepatocellular carcinoma cell line H22 were maintained in our laboratory.

Drugs and chemicals

Trizol total RNA purification reagent, Lipofectamine2000^{TM}, RPMI-1640, penicillin, streptomycin, 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phenazine methosulfate and nitrotetrazoliumbluechlorid were purchased from Sigma Co. (St. Louis, MO, USA). Mitoxantrone was product of Hisun Pharmaceutical Co. (Zhejiang, China). SYBR PrimeScript Kit was obtained from TaKaRa Co. (Otsu, Japan). Interferon-γ assay kit was purchased from R&D Systems Inc. (Minneapolis, MN, USA). PVDF membrane was obtained from Bio-Rad (Hercules, CA, USA). ECL detection system was product of Amersham Pharmacia Biotech (Uppsala, Sweden). Lymphocyte separation medium was from DaKewe Biotech Co. (Shengzhen, China). Rabbit anti-human recombinant CRT pAb and rabbit anti-mouse β-actin mAb were obtained from Stressgen Co. (Ann Arbor, MI, USA). Goat anti-rabbit IgG-HRP and goat anti-rabbit IgG-Rhodamine123 were from Zhongshan Goldenbrige Biotechnology Co. (Beijing, China). All primers synthesis was performed by Sangon Biological Engineering Technology & Services Co. (Shanghai, China).

Cell culture

Cells were cultured in flasks with RPMI-1640 medium supplemented with 10% (v/v) calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin in a humidified 5% CO_2 and 95% air incubator at 37°C.

Cellular proliferation assay

B16-F1 cells were seeded into 96-well culture plates (4,000 cells/well in 50 μL standard RPMI-1640 medium) in triplicate. Twenty four hours later, the cells were treated with mitoxantrone at different concentrations in 50 μL medium. The cells treated with equal amount of normal medium instead of drugs were served as control. After drug exposure for 24 h or 48 h, the medium in each well was removed and 200 μl MTT solution (0.25 g/L in RPMI-1640 medium) was added. After 4 h incubation at 37°C, the medium was removed and 200 μl dimethyl sulfoxide was added into each well. After 10 min incubation at room temperature, absorbance (A) at 570 nm was recorded. The cell survival rate was calculated as follows: Cell survival rate = A_{drug} / $A_{control}$ × 100%

Detection of apoptosis by DNA fragmentation analysis

B16-F1 cells were cultured in RPMI-1640 medium containing mitoxantrone (1 μg/ml) for 0 h, 4 h, 12 h and 24 h. Harvested cells were then lysed in 0.3 ml cell lysis buffer (50 mM Tris-HCL, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) for 20 min, on ice. Nuclei was removed from cell lysate by centrifuge at 12000 r/min for 10 min and then the supernatant was extracted with phenol/chloroform. The DNA fragments in extracted supernatant were precipitated with 2 volumes of 100% ethanol and 1/10 volume of 3 mol/L sodium acetate (pH 5.2), and the pellets were resuspended in 0.1 × SSC buffer. After treatment with DNase-free RNase A (50 mg/ml) for 30 min at 37°C to remove RNA, NaCl was added into the solution for a final concentration of 1 mol/L and the solution was extracted again with phenol/chloroform and DNA was precipitated by ethanol. The pellets were suspended in 30 μL ddH_2O and loaded to a 2% (w/v) agarose gel for electrophoresis.

Western blot analysis

B16-F1 cells were cultured in RPMI-1640 medium containing mitoxantrone (1 μg/ml) for 12 h and harvested. Cells were then treated with cell lysis buffer (as described above) for 20 min on ice. Nuclei and cytoplasm were separated from each other by centrifuge at 1000 r/min for 5 min. A total of 5 μg of nuclei or cytoplasm proteins from each sample was denatured by boiling, loaded onto a 10% (w/v) SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% (w/v) non-fat milk, the blots were probed with specific primary Abs and visualized with the HRP-conjugated secondary Abs and ECL detection system. β-actin was used as a loading control in this sutdy.

Quantitative real-time PCR

The CRT mRNA levels in B16-F1 cells before and after mitoxantrone (1 μg/ml) treatment were detected by quantitative realtime PCR and expressed relatively to the expression of β-actin. The first-strand cDNA was synthesized from total RNA of B16-F1 cells by reverse transcription. Real-time PCR was performed with SYBR PrimeScript^{TM} Kit using Opticn2 Thermal Cycler (MJ Research Inc., Walthan, MA, USA). Primers used for amplifying CRT were 5'-GAT GGA TGG AGA GTG GGA ACC-3'/5'-GAG ATC TAG GCC CAG TAC AGC-3' and primers for β-actin were 5'-TGG CAC CCA GCA CAA TGA A-3'/5'-CTA AGT CAT AGT CCG CCT AGA-3'. The PCR cycling condition was as follows: 95°C 5 min; 94°C 30 s, 60°C 30 s, 72°C 30 s, 78°C 1 s for plate reading, 30 cycles; 72°C 10 min. The melting curves and quantitative analysis of the data were performed using Opticon Monitor 2.02.24 software. The relative difference of gene expression was calculated as follows: $\Delta\Delta CT = (CT_{CRT} - CTactin)_{treatment} - (CT_{CRT} - CTactin)_{control}$. The relative difference of gene expression = $2^{-\Delta\Delta CT}$

Immunofluorescence detection of calreticulin in B16-F1

Immunofluorescence was used to determine the subcellular localization of CRT. B16-F1 cells were seeded into 12-well culture plates (2×10^4/well in 1 ml standard RPMI-1640 medium). Twenty four hours later, the cells were treated with mitoxantrone (1 μg/ml) in 1 ml medium for 12 h. B16-F1 cells treated with equal amount of normal medium instead of

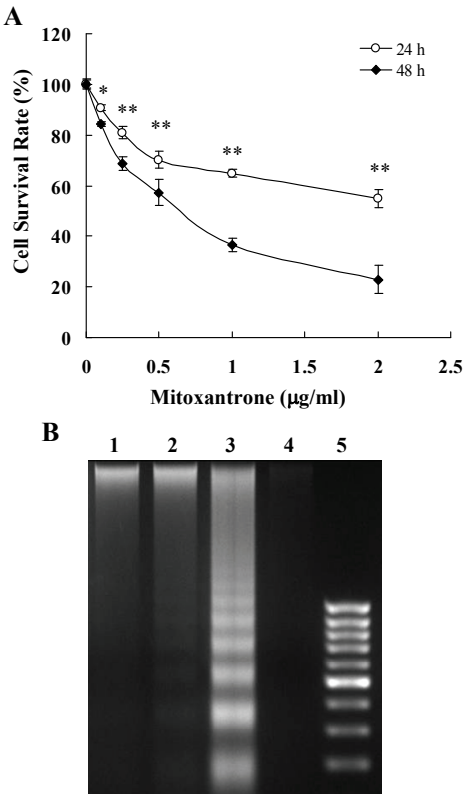

Figure 1. Mitoxantrone inhibited growth of B16-F1 cells by inducing apoptosis. (A) Survival rates of B16-F1 cells after treatment with 0~2 μg/L mitoxantrone for 24 h and 48 h. Data expressed as the mean ± SD (n = 3), $^{**}p < 0.01$ and $^{*}p < 0.05$ vs B16-F1 control group. (B) DNA fragmentation induced by mitoxantrone (1 μg/ml) for 4~24 h. Line 1, B16-F1 cells treated by mitoxantrone for 4 h; Line 2, B16-F1 cells treated by mitoxantrone for 12 h; Line 3, B16-F1 cells treated by mitoxantrone for 24 h; Line 4, B16-F1 control cells; Line 5, DNA Marker.

drugs were served as the control group. After 12 h, the medium was discarded and the cells were washed with PBS and treated with 4% (w/v) paraformaldehyde for fixation. The cells were then washed with PBS, incubated with 10% (v/v) goat serum for blocking background and reacted with anti-CRT pAbs over night at 4°C. The cells were then washed with PBS, incubated with Rhodamine-123 labeled anti-rabbit IgG for 45 min at 37°C. Finally, the cells were stained with 300 nmol/L DAPI for nuclei, washed with PBS, enclosed in 50% glycerol (diluted in 0.01 mol/L PBS, pH 8.0) and examined by fluorescence/phase contrast microscopy (TE2000S, Nikon, Japan).

Animal experiments

BALB/c mice (26 male, 27 female, n = 53) were randomly divided into 3 groups: 1) PBS group (blank control, n = 14), 2) B16 group (tumor control, n = 20) and 3）B16-NVI group (immune-inoculated group, n = 19). Apoptotic B16-F1 cells induced by mitoxantrone (1 μg/ml) for 12 h were subcutaneouly inoculated into the back of each mouse in B16-NVI group, PBS instead of mitoxantrone-treated B16-F1 cells was used to inoculate other two control groups of mice at the same time. The second-time animal immunization was performed 10 days after the first-time immunization. Ten days after the second immunization, living B16-F1 cells (5.0×10^5 cells in 100 μL PBS for each) were subcutaneouly injected into the back of each mouse in B16 group and B16-NVI group. The mice in PBS group were subcutaneouly injected PBS as the tumor-free group. Then mice were monitored once every two days after injection. Appearance of black-blue spots in inoculated area was regarded as tumor begins to generate.

LDH release assay

The splenocytes were obtained from the experimental mice and isolated with lymphocyte separation medium and then used as effector cells in the LDH release assay. The splenocytes were seeded into 96-well culture plates (1×10^6 cells/well) in 100 μl RPMI-1640 medium without phenol-sulphonphthalein in triplicate. B16-F1 cells, used as target cells, were then seeded into the wells that contained splenocytes in 100 μl in RPMI-1640 medium in three different effector/target cells ratio (1 : 25, 1 : 50, 1 : 100). In the natural release group, target cells were replaced by equal volume of medium. In the maximum release group, effector cells were replaced by equal volume of 0.1% NP-40. After incubation for 3 h, the cell culture plates were centrifugated at 2000 r/min for 5 min. Cell medium was then transfered to fresh culture plates (100 μl/well) and equal volume of LDH substrate reaction buffer (80 μg/ml phenazine methosulfate, 320 μg/ml nitrotetrazoliumbluechlorid, 800 μg/ml codehydrogenase I, sodium lactate 40 mmol/L) was added. After 10-min incubation at 37°C in dark, enzyme reaction was stopped by citric acid (30 μL/well) and then absorbance (A) at 450 nm was recorded at room temperature. The cell killing rate was calculated as follow: Rate of cytolysis = $(A_{effector} - A_{natural\ release}) / A_{maximum\ release} \times 100\%$

Detection of IFN-γ in mouse serum by ELISA

Blood was collected from experimental mice and incubated at 37°C for 30 min to obtain serum IFN-γ concentration in mouse serum was determined by ELISA assay according the manufacturer's protocol.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Statistical analysis between groups was assessed by Student's two-tailed *t*-test. Data for animal tumor model experiment were evaluated by Fisher's exact probabilities in 2 × 2 table, *p*-values less than 0.05 were regarded as statistically significant.

Results

Mitoxantrone inhibited growth of B16-F1 cells by inducing apoptosis

It has been proved that anti-tumor chemical mitoxantrone

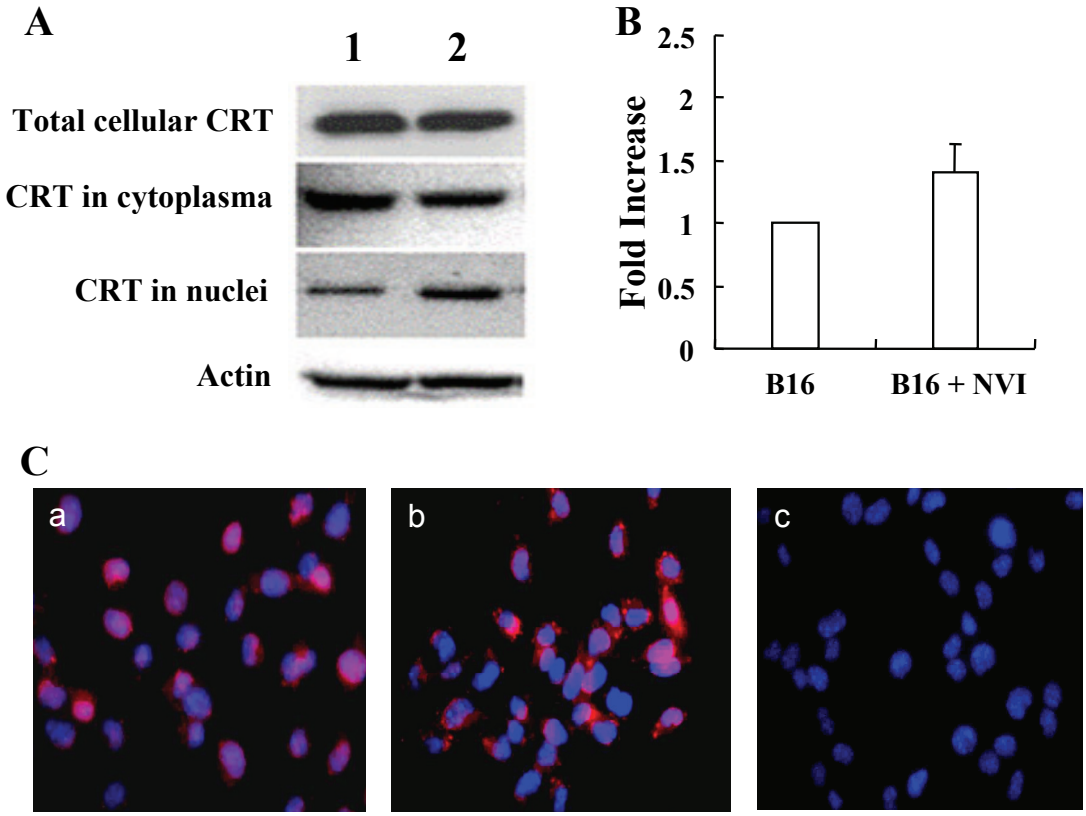

Figure 2. Effect of mitoxantrone on CRT expression and its subcellular localization in B16-F1 cells. (A) Western blot assay was used to determine effect of mitoxantrone on CRT expression in protein level. Lane 1, B16-F1 cells treated with mitoxantrone (1 μg/ml) for 12 h; Lane 2, B16-F1 control cells. (B) QT-RT-PCR was used to determine the CRT expression at mRNA levels in mitoxantrone-treated and control B16-F1 cells. The B16-F1 cells were treated by mitoxantrone (1 μg/ml) for 12 h; the mRNA level was represented relatively to the expression of β-actin. Each bar presents the mean ± SD (n = 3). (C) Immunofluorescence assay was performed to elucidate redistribution of CRT in B16-F1 cells. The red color represented CRT recognized by rabbit anti-CRT antibody and Rhodamine-123 labeled anti-rabbit IgG. Blue color indicates the nuclei stained with DAPI. (a) B16-F1 control cells. (b) B16-F1 cells treated by mitoxantrone (1 μg/ml) for 12 h. (c) B16-F1 control cells in which PBS was used in place of the rabbit anti-CRT antibody in the process of immunofluorescence detection to eliminate the non-specific binding of Rhodamine-123 labled anti-rabbit IgG.

could induce apoptosis of mouse colon carcinoma cells along with the translocation of CRT from ER to cell surface. In order to determine whether mitoxantrone was also toxic to B16-F1 cells, MTT cellular survival assay and DNA fragmentation assay were performed in this study. As shown in the Figure 1A, mitoxantrone exhibited significant inhibition on the growth of B16-F1 cells in a dose- and time-dependent manner. DNA fragmenttation assay showed that after treatment with mitoxantrone (1 μg/ml) for different times (4 h, 12 h and 24 h), typical DNA ladders for apoptosis were observed in the cell cytosol and the intensity of DNA ladder was increased with the extension of time (Figure 1B), suggesting that mitoxantrone could effectively induce apoptosis of B16-F1 cells.

Effects of mitoxantrone on CRT expression and its subcellular localization in B16-F1 cells

To validate whether mitoxantrone treatment could affect CRT expression and its subcellular localization in B16-F1 cells along with the apoptosis, the Western blot assay was used to assay CRT protein content in whole cell lysate, cytosol and nuclei. QT-RT-PCR was performed to determine CRT mRNA level, and immunofluorescence assay was used to observe the subcellular localization of CRT in B16-F1 cells. Western blot assay showed that mitoxantrone treatment for 12 h did not change total cellular CRT level, but resulted in a decreased CRT content in nuclei and an increased CRT content in the cytoplasm (Figure 2A). This observation was also supported by QT-RT-PCR assay for CRT mRNA. As shown in Figure 2B, no significant differences on the mRNA levels of CRT were determined between mitoxantrone-treated and control B16-F1 cells. The subcellular localization of CRT in B16-F1 cells was also evaluated by immunofluorescence detection. Results showed that mitoxantrone treatment induced an obvious membrane translocation of CRT and, very importantly, the CRT coated on the membrane was found to crow together on the cell surface (Figure 2C). In B16-F1 control cells, CRT mainly distributed in cytoplasm as described by Ostwald and his colleagues (5).

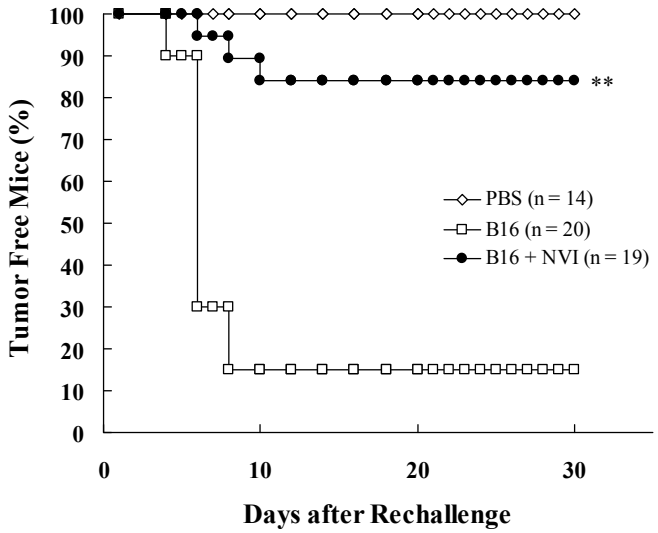

Figure 3. Immunizing Balb/C mice with mitoxantrone-treated B16-F1 cells resulted in a specific antitumor effect against the homogeneous tumor. BALB/c mice were divided into 3 groups: PBS group (blank control); B16 group (tumor control) and B16-NVI group (immune-inoculated group). Apoptotic B16-F1 cells induced by mitoxantrone (1 μg/ml) for 12 h were subcutaneouly inoculated into the back of each mouse in B16-NVI group and PBS was used to inoculate other two control groups of mice at the same time. The second immunization was performed 10 days after the first immunization. Ten days after the second immunization, living B16-F1 cells were subcutaneouly injected into the back of each mouse in B16 group and B16-NVI group. The mice in PBS group were injected PBS as the tumor-free group. Then mice were monitored once every two days after injection. Appearance of black-blue spots in inoculated area was regarded as tumor begins to generation. Data was assessed by *Fisher*'s exact probabilities in a 2 × 2 table. $^{**}p < 0.01$ *vs* B16 group.

Mitoxantrone-treated B16-F1 cells induced a specific antitumor immunological effect against homogeneous tumor cells in mice

Since mitoxantrone treatment resulted in obvious CRT membrane translocation in B16-F1 cells, it is possible that apoptotic B16-F1 cells induced by mitoxantrone could also stimulate antitumor immunological effect in the experimental animals as mitoxantrone-treated CT-26 cells (mouse colon carcinoma cells). To address this issue, in this experiment, apoptotic B16-F1 cells induced by mitoxantrone were used as the cell-antigen to immunize mice. The results showed that the specific antitumor effect against homogeneous tumor cells was clearly induced in immunized animals (Figure 3). Ten days after rechallenge by live B16-F1 cells, a much lower percentage of tumor generation (15%) in the mice of B16-NVI group (immunized by apoptotic B16-F1) was observed, compared with a much higher percentage (85%) in the mice of B16 group (without immunization).

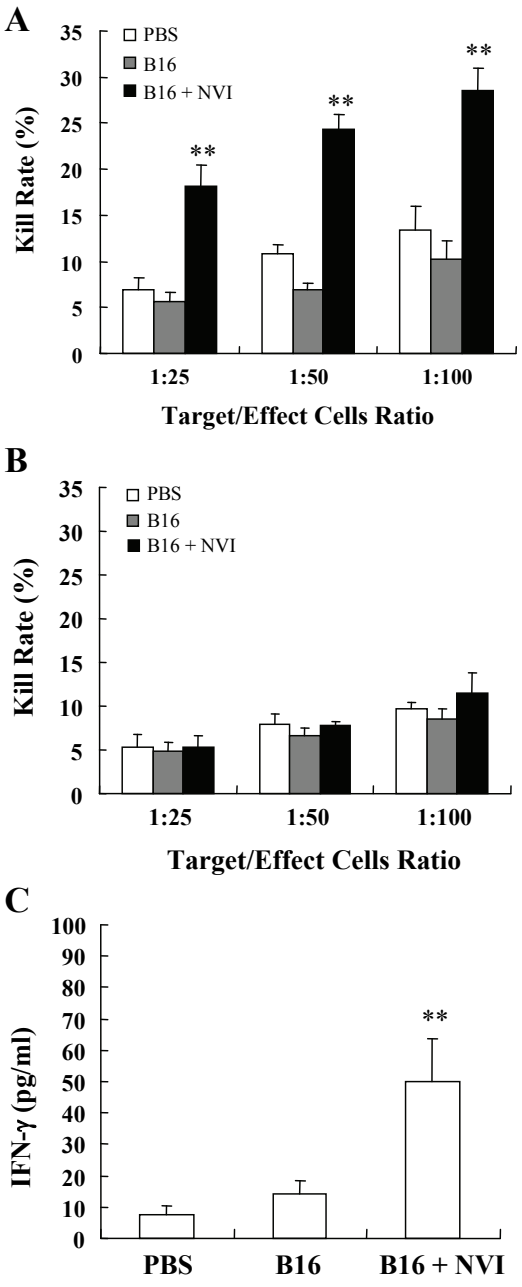

Figure 4. Immune activation stimulated by apoptotic B16-F1 cells (A) Immunizing mice with mitoxantrone-treated B16-F1 cells increased cytolytic effect of the splenocytes. B16-F1 cells were used as Target cells. (B) No differences in cytolytic effect of the splenocytes to H22 cells were observed between immunized and control mice. H22 cells were used as Target cells. (C) Immunizing mice with mitoxantrone-treated B16-F1 cells increased the serum level of interferon-γ. Blood was collected from experimental mice and incubated at 37°C for 30 min to obtain serum. IFN-γ concentration in mouse serum was determined by ELISA assay. Each bar presents the mean ± SD (n = 3), $^{**}p < 0.01$ *vs* PBS group.

Immunizing mice with mitoxantrone-treated B16-F1 cells increased cytolytic effect of splenocytes

In order to prove the effect of immune activation stimulated by apoptotic B16-F1 cells, the specific cytolytic effect of the splenocytes on B16-F1 cells was determined by LDH release assay in which the splenocytes from three groups of mice

were used as effectors and B16-F1 cells were used as targets. In a parallel assay, H22 cells, as a sort of nonhomologous tumor cells, were used as target cells to prove the specificity of cytolysis. The results showed that, compared with the splenocytes from the mice in PBS group and B16 group, the splenocytes from immune-inoculated mice (B16-NVI group) exhibited a significant higher cytolytic efficiency against B16-F1 cells (Figure 4A). In the parallel experiment, the splenocytes from all three different groups of mice exhibited a similar cytolytic efficiency against H22 cells (Figure 4B). These results suggested that the immune response induced by mitoxantrone-treated B16-F1 cells was specifically against homogeneous tumor cells.

IFN-γ is a cytokine that is critical for innate and adaptive immunity for tumor control. It is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops. To validate whether immunizing mice with mitoxantrone-treated B16-F1 cells can increase release of IFN-γ by immune cells, the interferon-γ level in mouse serum was determined by ELISA method. Data showed that the serum interferon-γ levers in the mice of B16-NVI group were obviously higher than in the mice of other two groups (Figure 4C).

Discussion

Mitoxantrone, as a membrane of anthracyclines family, is an anti-tumor drug widely used in the clinic. The studies have pointed out that mitoxantrone could induce apoptosis of CT-26 colon cancer cells along with the CRT membrane translocation and aggregation on the cell surface. When these CRT-coated apoptotic cells were used as the antigen to inoculate mice, a specific anti-tumor effect against homogeneous tumor cells were observed in the experimental animals (20-22), suggesting a potential importance of membrane CRT in mediating anti-tumor immune response.

In this research, we discovered that mitoxantrone was also toxic to mouse B16-F1 melanoma cell line. Treatment with mitoxantrone (1 μg/ml) could induce apoptosis of B16-F1 and, in the same time, CRT was transferred to and aggregated on the cell surface. Subsequently, we immunized BALB/c mice with mitoxantrone-treated B16-F1 cells as antigen and rechallenged the animals with live B16-F1 cells 20 days later. The results showed that the immunization by mitoxantrone-treated B16-F1 cells induced a protective effect against attack of live B16-F1 cancer cells in the mice. Ten days after rechallenge by live B16-F1 cells, the percent of tumor-bearing mice in the immune-inoculating group (15%) was much lower than that in non-inoculating group (85%). The data from LDH release assay showed that the splenocytes from immune-inoculated mice exhibited a more potent cytotoxic effect on B16-F1 cells than those from the control mice, but no such difference was observed when the non-homologous H22 cancer cells were used as the target cells, suggesting a specific immune activation against homologous cell-antigen. ELISA assay also demonstrated that serum concentrations of interferon-γ were obviously increased only in immune-inoculated mice. Interferon-γ was an essential immune effector released by activated immunocytes, so that the serum concentration of interferon-γ could indirectly represent the active extent of immune system *in vivo*.

The possible mechanism underlying the CRT-mediated anti-tumor immunity is that CRT coating induced by mitoxantrone-treatment gives B16-F1 cells a stronger immunogenicity. When these CRT-coated cells were inoculated into the animals, the cells can be recognized by and phagocytosed into the immunocytes in which tumor-specific antigens are processed and presented, which finally resulted in a specific anti-tumor immunity against the homologous tumor cells.

On the whole, all our data indicate a potential role of membrane CRT in mediating anti-tumor immunity. This study provides a new idea and approach for tumor immune prevention and treatment. But the tumor-antigens and antigen-presenting immune cells that involve in the immune process still need to be identified in the further experiments.

Aknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No.30973445).

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