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RAPID COMMUNICATION

Enhancement of CD4$^+$ T cell activities and modulation of Th1/Th2 lineage development in radiated tumor-bearing rats treated with male zooid of Antheraea pernyi extracts

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Abstract

AIM: To investigate whether supplementation of male zooid of Antheraea pernyi extracts (MZAPE) could enhance immune function of radiated tumor-bearing rats.

METHODS: Eighty male Wistar rats were randomly divided into a control group, a simple radiation group, a MZAPE group, and a radiation plus MZAPE group. With the tumor model established by implanting Walker-256 ascites tumor cells, tumor weight and tumor control rate were calculated. The rats in the simple radiation and radiation plus MZAPE groups were underwent to radiation at 10 Gy within 2 d. In the MZAPE and radiation plus MZAPE groups, the MZAPE was gavaged at a dose of 16.53 mg/kg once a day for 7 d. T cell subsets in peripheral blood were determined by flow cytometry and the expression of IL-2, IFN-γ, IL-4 and IL-10 in sera were determined by ELISA on the 8th d.

RESULTS: The tumor weight of simple radiation group, MZAPE group and radiation plus MZAPE group was lower than that of control group ($P < 0.01$) and tumor control rates were 63.08% ± 6.43%, 69.86% ± 7.12% and 35.30% ± 7.67%, respectively. CD4$^+$ T and CD8$^+$ T cells in the peripheral blood of the simple radiation group were fewer than in control group. In the MZAPE and radiation plus MZAPE groups, the number of CD4$^+$ T cells was higher while CD8$^+$ T cells was lower than in the control and simple radiation groups. Expression of IL-2 and INF-γ in the radiation group was lower than in control group, and significantly enhanced during MZAPE therapy ($P < 0.05$). Expression of IL-4 and IL-10 in the radiation group had no significant changes compared with the control group, and decreased significantly after MZAPE treatment ($P < 0.01$).

CONCLUSION: MZAPE administration may help improve the immune function of the radiated tumor-bearing rats and reverse the radiation-induced immune inhibition by promoting the proliferation of T helper cells and inducing the transdifferentiation from Th2 to Th1.

Key words: Antheraea pernyi; Male zooid; Rats; Radiotherapy; Immune suppression

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INTRODUCTION

Patients and experimental animals with advanced cancer often exhibit a poorly functioning immune system[1], which was manifested by allergy to skin-test antigens[2], decreased T-cell proliferation[3], alterations in signal transducing molecules[4], reduced CD4:CD8 ratios[5] and deficient production of Th1 cytokines[6]. These alterations correlate with the severity of the disease and poor survival[7]. Large doses of radiotherapy in tumor treatment

is associated with normalization of cytokine producing capacity in cancer patients[6]. Moreover it will inevitably hurt normal tissues and organs, resulting in a weak body and poor immune functions. Radiotherapy-induced immune suppression could contribute to the spread of the disease and constitute a barrier to immunotherapeutic interventions. Many experimental models have confirmed the correlation between the diversity of clinical or pathological features in immune related diseases in cancer patients. Multiple factors may contribute to radiotherapy-induced immune suppression. These include the number of immunocompetent cells, which are mainly the T lymphocytes that are most sensitive to radiation, and the balance of Th1/Th2 cytokine production[8]. The T cell subgroups are general indicators for evaluating the immune state and immune competence of the body. The Th1/Th2 classification scheme is useful in terms of correlation between overall cytokine production patterns and clinical outcomes in a variety of pathological states[9]. INF-γ secreted from Th1 cells is known to stimulate the differentiation of naive $CD4^+$ T cells into Th1 cells and to inhibit the proliferation of Th2 cells[10]. In addition, IL-4 and IL-10 secreted from Th2 cells are known to induce the differentiation of naive $CD4^+$ T cells to Th2 cells and to inhibit the function of Th1 cells[11,12]. In addition, the regulation of the immune balance of Th1/Th2 cell responses has been shown to be critically important for anti-tumor immune responses, such as inhibition of tumor growth and metastasis, and improvement of survival rate[13-15]. As described above, the tumor tissue and radiotherapy-induced immune suppression mainly express Th2 cytokines, resulting in a drift from Th1 to Th2, which further induces immune suppression. Therefore, it is important to improve immune function and to drift Th2 to Th1. Therapeutic interventions aimed at protecting the immune system from damage caused by radiotherapy in cancer patients may, therefore, enhance their immune competence. Seo[16] demonstrated that Chinese herbal medicine could change the Th1/Th2 balance by directly weakening the activities of the T cells, and the Th1 cytokines could take the predominant position.

The male zooid of Antheraea pernyi (MZAPE) has long been used as a pure preparation of traditional Chinese medicine to treat many illnesses and promote longevity. Our previous study demonstrated that the concentrated liquor of the male zooid could improve immune function, promote recognition and lethal effects toward tumor cells, and it possessed a unique priority for adjunctive therapy against tumors[17]. We investigated the mechanism regarding the reversion of radiation-induced immune suppression by the active ingredients of MZAPE extracts in tumor-bearing rats.

MATERIALS AND METHODS

Materials

The unmated MZAPE was obtained from the Silkworm Research Institute of Shandong Academy of Agricultural Science. In total, 250 g of zooid was ground into powder and then passed through a mesh screen (#80) and suspended in aqueous ethanol (95%; 20 L) for 24 h. After filtration, the residue was resuspended in aqueous ethanol (95%; 20 L) for an additional 24 h and refiltered. We then concentrated MZAPE by combined liquid-phase filtration.

Experimental animals and grouping

Eighty male Wistar rats with an average weight of 120 ± 10 g were purchased from the Animal Center of Shangdong University. All animals were housed in groups of 4-6 in 29 cm × 18 cm × 13 cm polyethylene cages. The animal room was maintained at 22℃-24℃ on a fixed light: dark cycle (12 h: 12 h). Rats were looked after according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C.). Our hospital Ethical Committee for Animal Welfare approved all the experiments. Hen egg lysozyme (HEL) was purchased from Sigma-Aldrich (St. Louis, MO) and recrystallized three times. Rats were immunized via the hind footpads with 1 nmol/rat of native HEL emulsified in colonization factor antigen (CFA) (Difco, Detroit, MI). The rats were weighed and randomly divided into a control group, a simple radiation group, a MZAPE group, and a radiation plus MZAPE group, and there were 20 rats in each group. The ascites cells of rats bearing with Walker 256 carcinoma were collected, counted and diluted at 2×10^7/mL, and 0.25 mL was applied to the right axilla of each rat *via* hypodermic inoculation. The experiments were carried out when the tumors grew to a diameter of 0.8-1.0 cm.

A Siemens PRIUS medical electronic linear accelerator (Shandong Tumor Hospital) was used. The radiation dose rate was 100 cGy/min, and the source-skin distance was set at 205 cm. The rats in the simple radiotherapy and radiation plus MZAPE groups underwent radiation with 5 Gy/d of routine radiotherapy for two days (10Gy in total). After 24 h, the MZAPE was gavaged at 16.53 mg/kg once a day for seven days in the MZAPE and radiation plus MZAPE groups. The rats in the simple radiation group and control group were gavaged with 2 mL normal saline for seven days. The rats were sacrificed on the first day after MZAPE administration. Tumor tissues were harvested and weighed, and tumor-inhibiting rate was calculated.

T cell proliferation assay

Spleens were removed on the first day after MZAPE gavage and spleen cell suspensions were prepared. The erythrocytes in the cell suspensions were lysed with Tris-NH_4Cl. A total of 5×10^6 cells/mL in 100 μL RPMI 1640 containing 1 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5×10^{-5} mol/L 2-mercaptoethanol and 1% heat-inactivated autologous rat serum were added to each well, followed by the addition of 100 mg/L HEL. The cells were cultured for 72 h. Each well was pulsed with 0.5 μCi tritiated thymidine, and the cells were cultured for another 16 h. The cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques.

Measurement of HEL-specific antibodies

Blood was collected on the first day after MZAPE gavage, and sera were heat-inactivated at 56℃ for 30 min.

Table 1 Effect of MZAPE on tumor weight, tumor-inhibiting rate, suppression of anti-HEL IgG antibody production, and proliferative responses of PBMC to HEL (n = 20, mean ± SE)

Groups	Tumor weight (g)	Tumor-inhibiting rate (%)	Anti-HEL IgG antibody (A_{405})	Proliferation (× 1000 cpm)
Control	4.27 ± 0.60		0.17 ± 0.02	6.0 ± 0.7
Simple irradiation	1.58 ± 0.40[b]	63.08 ± 6.43	0.09 ± 0.01[b]	5.31 ± 0.8[d]
Radiation plus MZAPE	1.29 ± 0.33[b]	69.86 ± 7.12	0.34 ± 0.01[b]	27.4 ± 2.8[b]
MZAPE	2.76 ± 0.37[b]	35.30 ± 7.67	0.44 ± 0.04[b]	49.6 ± 3.1[b]

[b]P < 0.001 and [d]P = 0.006 *vs* control group.

IgG, IgG1 and IgG2a antibodies specific for HEL were measured with ELISA (BD Pharmingen, San Diego, CA, USA). In brief, 96-well flat-bottomed microtiter plates were coated with 100 μL/well HEL (100 mg/L) at 37℃ for 1 h and washed three times with PBS. The wells were then blocked by incubation with 100 μL PBS containing 1% ovalbumin at 37℃ for 1 h. After washing, the plates were incubated with 100 μL of a 1:10000 dilution of each serum sample at 37℃ for 30 min. The plates were washed, and 100 μL/well of a 1:1000 dilution of rabbit anti-rat IgG, IgG1 or IgG2a labeled with alkaline phosphatase was added and incubated at 37℃ for 1 h. After washing, 100 μL of 3 mmol/L p-nitrophenylphosphate was added to each well, and the plates were incubated in the dark at room temperature for 15 min. Absorbance was then measured at 405 nm in a Titertec Multiscan spectrophotometer (EFLAB, Helsinki, Finland). The results were expressed as absorbance units at A_{405} ± SEM.

Cytokine assays

Single-cell suspensions from spleens were prepared as described above and 5 × 10^6 cells/mL were cultured in 1 mL aliquots in 24-well tissue culture plates with 100 mg/L HEL. Forty-eight hours later, the supernatants were harvested and stored at -70℃ until assayed. The changes in expression of Th1 cytokines (IL-2, INF-γ) and Th2 cytokines (IL-4, IL-10) were detected with ELISA. IL-2, INF-γ, IL-10, IL-4 ELISA sets were purchased from BD Pharmingen (San Diego, CA, USA).

Preparation of lymphocytes

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood of the rats by Ficoll-Conray method, and the number was counted under microscope. 1 × 10^6 cells diluted in 1 mL PBS were added to each tube and the phenotypic alternations of the peripheral blood lymphocytes were analyzed by flow cytometry.

FACS analysis

The cells were first stained for surface antigens (30 min at 4℃) with anti-CD3-FITC, anti-CD4-FITC, anti-CD8-FITC, and anti-CD57-FITC (BDIS Biosciences, Stockholm, Sweden). Thereafter, the lymphocytes were permeabilized with FACS-lysing solution and FACS permeabilizing solution (BDIS Biosciences). The staining protocol included isotype controls for both surface and cytoplasmic staining. After staining, the cells were fixed with CellFix (BDIS Biosciences) and acquisition was performed within 2 h. Flow cytometric measurements were performed using a FACS Calibur (Becton Dickinson, Stockholm, Sweden) and at least 10000 cells/sample were collected. Data analysis was done using Cell Quest software (BDIS Biosciences) according to a standardized pattern-protocol. The background fluorescence was determined with markers applied on the isotype control cytograms and was < 1% in all cases.

Table 2 Effect of MZAPE on anti-HEL IgG2a and IgG1 antibody production (n = 20, mean ± SE)

Groups	Anti-HEL IgG2a antibody (A_{405})	P	Anti-HEL IgG1 antibody (A_{405})	P
Control	0.08 ± 0.01		0.25 ± 0.02	
Simple irradiation	0.06 ± 0.01	< 0.001	0.51 ± 0.03	< 0.001
Radiation plus MZAPE group	0.15 ± 0.02	< 0.001	0.396 ± 0.02	< 0.001
MZAPE group	0.32 ± 0.04	< 0.001	0.24 ± 0.01	0.053

Statistical analysis

Statistical analysis was performed using a two-tailed, paired Student's t test. Significance was accepted at P < 0.05.

RESULTS

Tumor weight and tumor-inhibiting rate

The weight and volume of tumors in the simple radiation, radiation plus MZAPE and MZAPE groups were significantly lower than those in the control group (P < 0.001); moreover the tumor-inhibiting rates in the simple radiation, radiation plus MZAPE and MZAPE groups were 63.08% ± 6.43%, 69.86% ± 7.12% and 35.30% ± 7.67%, respectively, (Table 1).

Effect of MZAPE on anti-HEL IgG antibody production, proliferative responses to HEL and anti-HEL IgG2a and IgG1 antibody production in rats

The antigen-specific IgG antibodies in sera, the proliferative responses, anti-HEL IgG2a and IgG1 antibody production of rats immunized with HEL and administered MZAPE were measured on the 8th day. The anti-HEL IgG antibody production markedly decreased in simple irradiation group while increased in radiation plus MZAPE group and MZAPE group when compared to control group (P < 0.001). The proliferative responses of PBMC to HEL were significantly enhanced in the radiation plus MZAPE group and MZAPE group compared with the control group (P < 0.001). The anti-HEL IgG2a antibody production

Table 3 Effect of MZAPE on peripheral blood CD4 and CD8 cells (n = 20, mean ± SE)

	Lymphocyte subgroups (%)					
	$CD4^+$ (%)	*P*	$CD8^+$ (%)	*P*	CD4/CD8	*P*
Control group	38.05 ± 5.05		19.30 ± 2.17		1.97 ± 0.35	
Simple irradiation group	30.36 ± 2.12	< 0.001[b]	17.70 ± 2.34	0.031	1.72 ± 0.23	0.011
Radiation plus MZAPE group	36.99 ± 5.24	0.519	17.30 ± 3.44	0.034	2.13 ± 0.32	0.140
MZAPE group	32.13 ± 3.85	< 0.001[b]	16.70 ± 1.82	< 0.001[b]	1.92 ± 0.26	0.611

[b]$P < 0.001$ *vs* control group.

Table 4 Effect of MZAPE on Th1 and Th2 cytokines (n = 20, mean ± SE) (pg/mL)

	IL-2	*P*	INF-γ	*P*	IL-4	*P*	IL-10	*P*
Control group	181.99 ± 19.33		89.06 ± 21.44		97.69 ± 7.11		180.14 ± 19.56	
Simple irradiation group	129.14 ± 16.96	< 0.001[b]	74.09 ± 39.29	0.143	100.57 ± 12.01	0.362	179.26 ± 18.13	0.883
Radiation plus MZAPE group	138.16 ± 23.15	< 0.001[b]	91.25 ± 27.00	0.778	74.78 ± 8.28[d]	< 0.001[b,d]	180.25 ± 17.32	0.985
MZAPE group	166.73 ± 13.34[d]	0.006[b] < 0.001[d]	103.85 ± 6.55[d]	0.005[b] 0.002[d]	66.02 ± 7.14[d]	< 0.001[b,d]	136.29 ± 18.21[d]	< 0.001[b,d]

[b]$P < 0.001$ *vs* control group; [d]$P < 0.001$ *vs* simple irradiation group.

markedly increased in radiation plus MZAPE group and MZAPE group as against the control group ($P < 0.001$). The anti-HEL IgG1 antibody production significantly increased in simple irradiation group and radiation plus MZAPE group ($P < 0.001$). There was no significant change of anti-HEL IgG1 antibody production in MZAPE group (Tables 1 and 2).

Effect of MZAPE on peripheral blood lymphocyte changes of rats

The number of $CD4^+$, $CD8^+$ lymphocytes in the peripheral blood of the simple irradiation group and the ratio of $CD4^+/CD8^+$ were both decreased. The $CD4^+$ lymphocytes in the peripheral blood of the radiation plus MZAPE and MZAPE groups were higher than that in the simple radiation and control groups, and the ratio of $CD4^+/CD8^+$ increased. The $CD8^+$ lymphocytes of the radiation plus MZAPE and MZAPE groups showed no significant changes compared to the simple irradiation and control groups (Table 3).

Effect of MZAPE on expression of Th1 (IL-2, INF-γ) cytokines and Th2 (IL-4, IL-10) cytokines in rats

The expression of IL-4 and IL-10 in the spleens of the simple radiation group showed no significant changes, while the expression of IL-2 decreased ($P < 0.001$). In radiation plus MZAPE group, the expression of IL-2 and IL-4 significantly increased ($P < 0.001$). In the MZAPE group, the expression of IL-2, INF-γ, IL-4 and IL-10 significantly increased when compared with the control group and simple radiation group ($P < 0.001$) (Table 4).

DISCUSSION

The male zooid, a kind of bombycidae, is an animal derived medicine in China, and disintegrates from the male tussor chrysalis. According to the Dictionary of Traditional Chinese Medicine, the major components of the male zooid are proteins, with more than 20 kinds of free amino acids and cytochrome c. Actually, the male zooid also contains various active substances, such as brain hormone, prothymosin, hormone of Antheraea pernyi, and diuretics, which can regulate metabolism and restore immune functions[18]. In addition, such substances may have certain pharmacological activities such as strengthening Yang Qi and astringing essence, promoting host defense mechanisms, and have potentials of anti-aging, anti-tumor and immunity enhancement[19]. It can be clinically used to treat impotence, seminal emission and stranguria with hematuria. A number of studies have shown the multiple effects of the male zooid in immune responses. The immune system in the Chinese oak silk moth, Antheraea pernyi, originated from a single ancestral gene with that of the Cecropia moth, whose antibacterial activity has been tested against nine different bacterial species[20]. Zhang *et al*[21] reported that the cecropins from the Chinese oak silkworm Antheraea pernyi possess effective anti-tumor activity with no cytotoxicity against normal eukaryotic cells, and impede the neoplastic process in murine large intestines.

It is documented that cytokines such as IL-4 and IL-10 can induce the differentiation of Th1 to Th2 and inhibits the production of Th1 cytokines[22], affecting immune system and the anti-tumor defense functions. In addition, the immune system of malignant tumor patients could be in immune suppression status and manifest Th2 cell superiority[23], and radiotherapy could aggravate immunologic injury of the body while it killed tumor cells, resulting in the progressive deterioration of the body's immune function. When the immune function becomes weak, the tumor would easily relapse or transfer to other sites.

In our study, the number of $CD4^+$ T cells in the radiated tumor-bearing rats increased after administration of the MZAPE. Such results allow us to hypothesize that

the extracts could strengthen cell immunity by promoting $CD4^+$ T cells. In contrast, its effect on $CD8^+$ T cells was not obvious and the exact reason for that needs to be investigated in the future. The MZAPE could inhibit the production of Th2 cytokines and reduce the induction of Th2 cytokines in Th1 cells, thereby increasing Th1 cells. The extracts could stimulate the expression of Th1 cytokines, which might induce the transformation of Th0 cells to Th1 cells. We presume that Th1 cells or large number of cytokines secreted by them would activate or enforce immune cell function. Further experiments are needed to elucidate the above-mentioned process. In conclusion, our results demonstrate that MZAPE selectively alters Th1/Th2 cytokine secretion pattern, strengthens immune function of the body and reverses immune suppression induced by radiotherapy. The extracts have a significant effect on enhancing cell immunity by inducing the transformation of Th2 to Th1. This study provides the pharmacological basis for the clinical application of MZAPE.

COMMENTS

Background

Hypoimmunity of tumor patients caused by radiotherapy is one of the major reasons for the failure of treatment and death of patients. Our previous study demonstrated that the concentrated liquor of the male zooid could improve immune function, promote recognition and lethal effects toward tumor cells, and it possessed a unique priority for adjunctive therapy against tumors. In this study the authors evaluated whether supplementation of the male zooid of Antheraea pernyi extracts (MZAPE) could enhance the immune function of the radiated tumor-bearing rats.

Research frontiers

The male zooid contains various active substances, which may have certain pharmacological activities such as strengthening Yang Qi and astringing essence, promoting host defense mechanisms, and has potentials of anti-aging, anti-tumor and immunity enhancement. Some studies have shown the male zooid's immunoregulatory function and anti-tumor activity.

Related publications

Our previous study entitled "Immunization of mice with concentrated liquor from male zooid of Antheraea pernyi", which was published at World Journal of Gastroenterology, demonstrated that the concentrated liquor of the male zooid could improve immune function, promote recognition and lethal effects toward tumor cells, and it possessed a unique priority for adjunctive therapy against tumors. Other studies also reported that the cecropins from the Chinese oak silkworm Antheraea pernyi possess effective anti-tumor activity with no cytotoxicity against normal eukaryotic cells, and impede the neoplastic process in murine large intestines.

Innovations and breakthroughs

The MZAPE administration may improve immune function in radiated tumor-bearing rats and reverse the radiation-induced immune inhibition by promoting the proliferation of T helper cells and inducing the differentiation from Th2 to Th1, and the MZAPE could strengthen the immune function of the body and reverse the immune suppression induced by radiotherapy.

Applications

The MZAPE selectively alters the Th1/Th2 cytokine secretion pattern, strengthens the immune function of the body and reverses the immune suppression induced by radiotherapy. The study provides the pharmacological basis for the clinical application of the male zooid of Antheraea pernyi extracts.

Terminology

The male zooid of Antheraea pernyi extracts (MZAPE): it has long been used as a pure preparation of traditional Chinese medicine, which possesses many health-care functions. According to The Great Dictionary of Traditional Chinese Medicine, Antheraea pernyi is the matured insect of silkworm.

Peer review

This is an interesting study showing the impact of MZAPE on immune system using a tumor-bearing rat model. The study may benefit from powerful calculation to decide the number of animals needed in each group.

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