Evaluation of the immunomodulatory effect of the 14 kDa protein isolated from aged garlic extract on dendritic cells

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1. Introduction

Garlic (Allium sativum, Liliaceae) is used all over the world for treatment of different diseases. In the last decade, garlic was reported to display antibiotic properties including antifungal [1] and antibacterial activities [2]. It is also reported to have hypolipidemic, antiatherosclerosis [3], and anticarcinogenesis activities [4]. Furthermore, numerous studies have indicated that garlic modulates immune responses.

According to our previous studies, it has been demonstrated that garlic enhances natural killer (NK) cell activity [5] and T-lymphocyte proliferation [6]. Also, garlic extract and a garlic protein fraction were shown to augment the oxidative burst in peritoneal macrophages of BALB/c mice [7]. Ghazanfari et al. [8] showed that garlic extract induces a shift in cytokine pattern in Leishmania major-infected BALB/c mice and the outcome of the immune response with regard to Th1 (IFN-γ, IL-2). In different study Zamani et al. [9] demonstrated that oral treatment with garlic enhanced Th2 immune response in rat. A unique garlic preparation called “aged garlic extract” (AGE) has been reported to have an array of pharmacologic effects including immunomodulation, tumor cell growth inhibition, and chemopreventative effects [10]. In rodents, AGE and its constituents have been reported to inhibit the development of chemically induced tumors in the bladder, mammary glands [11], colon, esophagus, lung, skin, and stomach [12].

One major protein has been isolated and purified from garlic extract; it is the 14 kDa protein. This protein has been shown to have immunomodulatory effects. Previous study showed that 14 kDa protein isolated from garlic extract augmented delayed type hypersensitivity (DTH) response compared to garlic extract and other fractions purified from it [13]. Also, the 14 kDa protein could induce augmented NK cell activity against K562 tumor cell line and induced a resistance to the growth of spontaneous mammary carcinoma in BALB/c mice [14]. Nikoo et al. [15] showed that 14 kDa protein isolated from AGE suppresses indoleamine 2,3-dioxygenase metabolites in mononuclear cells in vitro. Also Daneshmandi et al. [16] showed that 14 kDa protein is able to suppress nitric oxide (NO) production from macrophages in vitro and this protein cannot induce augmented macrophage activity against WEHI-164 fibrosarcoma cells.

Dendritic cells (DC) are the most powerful antigen presenting cells (APC), with a potent capacity to initiate naïve T cells, and play a critical role in the induction of primary immune responses [17].
DC are present in almost all tissues in an immature state and are characterized by a high ability for antigen uptake and processing. The exposure to inflammatory stimuli (e.g., TNF-α, interleukin-1β) or microbial agents (e.g., lipopolysaccharide (LPS)) causes the induction of a complex maturation process in DC which enhances their antigen presentation ability to T cells [18]. Mature DC up-regulate surface molecules like major histocompatibility complex class I (MHC-I) and II (MHC-II), CD80, CD86, CD40, and CD54, and thus become potent inducers of T cell activation [19]. Moreover, several cytokines such as IL-12, TNF-α, and IL-10 are released by DC during maturation.

In the present study, we investigated the effect of the 14 kDa protein isolated from AGE on mouse DC in vitro. Based on the extraordinary role of DC in induction of primary T cells, we analyzed the modulation of immature DC by the 14 kDa protein isolated from AGE. Surface expression of co-stimulatory molecules and the activation of naive T cells were examined.

2. Materials and methods

2.1. Animals

Eight- to 10-week-old inbred female BALB/c mice and C57BL/6 mice were purchased from the Pasteur Institute of Iran (Tehran, Iran). Mice were kept under optimal conditions of hygiene, temperature, and humidity with 12 h light:12 h darkness cycle and were allowed food and water ad libitum. All of the procedures of this study complied with the ethical guideline set down by the Institute for Animal experimentation.

2.2. Isolation of 14 kDa protein from aged garlic extract

Fresh garlic bulbs were obtained from Hamadan, Iran. Dry garlic bulbs were peeled and kept in the freezer for 6 months in −20 °C. Aqueous AGE was prepared by the method used by Mantis et al. [20]. Briefly, garlic samples were homogenized with two parts of distilled water in a varying blender. The homogenized blend was then filtered under vacuum through Whatman filter paper (number 1) and the filtrate was centrifuged at 3400 g for 30 min. The clear supernatant was collected. Twenty-seven grams of NH₄SO₄ (Merck, Germany) was added to 1 L of the supernatant and centrifuged at 3400 g for 30 min. The residue was subsequently suspended in saline and dialyzed against buffer saline. The isolated fractions were then run on a Sephadex G-50 (Sigma, USA) gel filtration chromatography column for further isolation of the 14 kDa protein as measured by Bradford assay and evaluated by SDS-PAGE.

2.3. SDS–PAGE electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to judge an analysis of the molecular weight and purity of the protein isolated from AGE. The protein molecules were run on 12% (w/v) sodium SDS–PAGE electrophoresis. After electrophoresis, the gel was fixed with methanol and acetic acid formaldehyde for 60 min and stained with coomassie blue (Sigma, USA).

2.4. Enrichment of splenic dendritic cells

A DC-enriched population was prepared from mice spleen according to the method reported by Vremec et al. [21], with minor modifications. Briefly, after cervical dislocation, spleens were taken from BALB/c mice under aseptic conditions. Tissues were cut into small fragments with scissors, suspended in 5–10 ml RPMI-1640 (Gibco, UK) containing collagenase D (1 mg/ml; Roche, Germany) and DNase (0.02 mg/ml; Roche, Germany), and then digested for 30 min at 37 °C in a 5% CO₂ incubator. To disrupt cell aggregations or DC–T cell complexes, EDTA (5 mM, pH 7.2) was added at the end of incubation period and the cell suspension was pipetted several times. Undigested stromal fragments were afterwards removed by passing the suspension through a stainless steel sieve. The cell suspension was washed twice with phosphate-buffered saline (PBS) containing 5 mM EDTA at 4 °C, 300g for 10 min. The pellet was immediately resuspended in 2–3 ml RPMI, added slowly on 2 ml Nycodenz 13% (w/v), d = 1.068 (Axis-Shield, Norway) and centrifuged at 4 °C, 600g for 15 min. Low-density cells were recovered from the interface, washed twice with RPMI, and cultured in complete RPMI medium containing 5% fetal calf serum, non-essential amino acids, L-glutamine, penicillin, and streptomycin (all from Gibco, UK) for 120 min. Non-adherent cells were then removed by gently washing of the plates with warm RPMI and adherent cells were cultured for another 16–20 h in complete RPMI medium. Afterwards, the non-adherent cells were collected and used as enriched DC in allogeneic mixed lymphocyte reaction (MLR).

2.5. Treatment of dendritic cells with 14 kDa protein isolated from aged garlic extract

To evaluate the effect of the 14 kDa protein isolated from AGE on the maturation and function of DC, different concentrations of this protein were added to the overnight culture of DC obtained from BALB/c mice. For this purpose, cells were divided into three groups. The first group was pulsed with the 14 kDa protein isolated from AGE, while the second and third groups were used as positive and negative controls, respectively. After removing the non-adherent cells, in order to optimize the concentration of Ag needed for DC maturation, the enriched DC were treated with different Ag concentrations from 14 kDa protein including: 5, 10, and 20 μg/ml during the overnight culture.

DC were treated with RPMI 1640 containing 0.5% normal mouse serum in the absence of 14 kDa protein as the negative control group. The positive control group contained TNF-α treated DC cultured in complete RPMI in the absence of 14 kDa protein. After incubation time of 14–16 h, the non-adherent cells (enriched DC) were collected and analyzed using flow cytometry (BD, Partec).

2.6. Preparation of T cells from lymph nodes

Scrubbed nylon wools (Zeptometrix Corporation) were put into a 10 ml syringe and then autoclaved for sterility. Before use, the columns were equilibrated by washing with 20 ml RPMI tissue culture media, sealed, and incubated for 30 min at 37 °C and 5% CO₂. Brachial lymph nodes were removed from C57BL/6 mice and single cell suspensions were prepared by grinding the nodes through a wire mesh.

Cell suspensions were cultured in complete RPMI medium for 2 h, and the non-adherent fraction was collected and loaded onto a nylon wool column, and washed with 2 ml warm RPMI. The column was sealed and incubated at 37 °C and 5% CO₂ for 45 min. Cells were then eluted with 10 ml warm RPMI. T cells were obtained by depletion of nylon wool adherent cells. Purity of T cells isolated with nylon wools determined by flow cytometry.

2.7. Flowcytometric analysis

In order to evaluate the purity of isolated DC and T cells, and the effect of 14 kDa protein isolated from AGE on expression of DC surface markers, DC were stained for their phenotypic markers by a standard direct procedure using monoclonal antibodies. In brief, DC were treated on ice with 5% normal mouse serum for 15 min
and then with hamster anti-mouse CD11c (Pharmingen, Australia) diluted to 1 µg/10^6 cell in PBS containing 2% FCS (PBS–FCS) for 30 min at 4°C. After washing twice, the cells were stained with FITC-conjugated monoclonal antibodies against MHC-II, CD86, or CD40 (Pharmingen, Australia) for the next 30 min. Cells were washed twice and resuspended in 0.5 ml cold PBS–FCS and retained on ice until analysis with a flow cytometer (Partec, Germany). T cells were stained directly by PE-conjugated rat anti-mouse CD3. Appropriate isotype controls were used for all staining.

### 2.8. Primary MLR

MLR was set up in triplicate in 96-well round-bottom microplates (Nunc, Denmark) with graded ratios of DC:T cells (1:5, 1:10, 1:20, 1:40, and 1:80) using decreasing numbers of irradiated (3000 rad) DC as stimulator and 1 × 10^5 T cells as responder in a volume of 200 µl complete RPMI medium. Cultures were incubated for 72 h in a 5% CO2 incubator. After 72 h of stimulation, MTT (5 mg/ml in PBS) in 0.1 of total volume was added to wells and incubated for 4 h at 37°C and 5% CO2. Later, supernatants were gently removed and 100 µl of acidic isopropanol (0.04 M HCl in isopropanol) was added in order to dissolve the formazan crystals generated by MTT reduction in living cells. Absorbance was read at wavelength of 540 nm. The test results were expressed as Stimulation Index (STI), which is optical density at 540 nm (OD540) of the test samples/OD540 of negative control. Our preliminary study showed that the stimulatory effects of 14 kDa protein isolated from AGE are more prominent when the DC:T ratio is 1:10. For this reason this ratio was used in all MLR experiments.

### 2.9. Statistical analysis

All data were presented as mean ± SD and Mann–Whitney test was used in our experiments. P value ≤0.05 was regarded as statistically significant.

### 3. Results

#### 3.1. Evaluation of the 14 kDa protein isolated from aged garlic extract

Different protein fractions were purified from the AGE by ammonium sulfate precipitation and G-50 gel chromatography. In order to evaluate the purity of 14 kDa protein, this protein was run on the SDS/PAGE electrophoresis and the results indicated the presence of 95% purified band of 14 kDa protein (Figs. 1 and 2).

#### 3.2. Evaluation of the enriched DC from mice spleen and T cells isolated from brachial lymph nodes

In this study, 10–15 × 10^7 mononuclear cells were obtained from each BALB/c mouse spleen and their viability was higher than 95%. After separation of low-density cells by Nycodenz gradient (12.2% v/w), the amount of recovered mononuclear cells was evaluated to be 4–6%. After 2 h as culture period and removing non-adherent cells, only about 0.4% of all spleen cells remained attached to the plate. Adherent cells contained immature DC, as well as very low populations of B lymphocytes and macrophages. The purity of DC was estimated using a phase-contrast microscope (Trade mark & country). Overnight culture, resulting in maturation of DC, caused these cells to be floated. Floating cells were stained for DC specific marker by a standard direct procedure using CD11c monoclonal antibody. Results showed that yield of purified DC from each mouse spleen was 3–6 × 10^6 cells with more than 90% purity (94.5 ± 3.2%).

T cells from C57BL/6 mice were purified from brachial lymph nodes by nylon column. After the first round of enrichment, purity was approximately 70%. In order to increase the purity of T cells, nylon wool non-adherent cells were again loaded on the nylon wool column. Purified T cells were stained for T cell specific marker using a standard direct procedure by PE-conjugated rat anti-mouse CD3. The obtained results indicated that the purity of T cells isolated from mouse brachial lymph nodes was more than 90% (94.5 ± 3.2%).

#### 3.3. Effect of 14 kDa protein on maturation markers of DC

To evaluate the maturation of DC, 5 µg/ml of 14 kDa purified protein of AGE was added to 1 × 10^5 DC and cultured overnight. The results of flowcytometric analysis using double staining of CD11c and MHC-II, CD86 or CD40 on treated AGE showed no significant effect on expression of MHC-II and CD86 molecules, while AGE significantly (P < 0.05) increased the expression of CD40, as is observed in Figs. 3 and 4.

#### 3.4. Functional activity of 14 kDa protein-treated DC assessed by MLR

In order to examine the effect of 14 kDa protein on allostimulatory activity of DC, the 14 kDa protein isolated from AGE was added to the overnight culture of DC at the 5 µg/ml concentration. After 72 h, pulsed DC with 14 kDa protein co-cultured with allogenic T cells from C57BL/6 mice at the DC:T cells ratio of 1:10 and T cells proliferation was detected by MTT assay. Our results showed that capacity of pulsed DC with 14 kDa protein was not affected significantly to induce proliferation responses of allogenic T cells compared to non-pulsed DC (Fig. 5). (The viability percentage of DC was similar in treated and untreated cells (90–95% viable, assessed by trypan blue exclusion.)

### 4. Discussion

In this study, we examined the effect of 14 kDa protein isolated from AGE on splenic DC maturation and function. Our results showed that the 14 kDa protein isolated from AGE is capable of increasing the expression of CD40 molecule on DC; but it did not influence CD86 and MHCII molecules. Furthermore, no significant
differences were noticed in the pulsed-DC with 14 kDa protein and non-pulsed DC on the MLR.

Various studies have indicated that garlic modulates immune responses [22]. Previous studies showed that garlic enhances the activity of NK cells [5] and T-lymphocyte proliferation [6]. Garlic has been reported to inhibit tumor growth in vitro and in vivo. Also, garlic extract and a garlic protein fraction have been shown to augment the oxidative burst in peritoneal macrophages of BALB/c mice [7]. Lau et al. [23] showed that AGE is an efficient candidate as an immune modifier compared to fresh garlic extract, which maintains the homeostasis of immune functions. Our previous studies demonstrated that aged garlic (kept in dry garlic bulbs in freezer for 6 months at −20 °C) possesses enriched immunostimulator fractions and reduced immunosuppressor fractions [15]. In this study, we therefore used AGE instead of fresh garlic extract.

Ghazanfari and Hassan [6] demonstrated that 14 kDa protein isolated from AGE was the major immunomodulatory fraction in AGE that enhanced the DTH response, T-cell proliferation and NK cell activity [14], shifted the cytokine pattern to Th1 (IFN-γ, IL-2) [13] and exhibited enhancement of peritoneal macrophage phagocytic activity against Leishmania major. According to the reports, we suppose that this fraction can be a good candidate for DC maturation and activation.

DC have the potential for cancer immunotherapy due to their ability to process and present antigens to T-cells and also to stimulate immune responses. Nevertheless, DC-based vaccines have only exhibited minimal effectiveness against established diseases in animal model and humans. The use of appropriate adjuvant enhances the efficacy of DC vaccines in treating tumors and infectious diseases [24]. In this study, we supposed that the 14 kDa protein isolated from AGE could be an efficient adjuvant for DC vaccines.

We found in the present study that pulsed DC with 14 kDa protein isolated from AGE increased significantly (P < 0.05) the expression of CD40 molecule on pulsed DC in comparison with non-pulsed DC; but we observed no statistical difference between expression of MHC-II and CD86 on pulsed DC with 14 kDa protein.
isolated from AGE and non-pulsed DC (Figs. 3 and 4). Result of all-ogenic MLR showed that pulsed DC with 14 kDa protein isolated from AGE and non-pulsed DC had similar potency of T-cell proliferation (Fig. 5). In spite of these results, it is questionable whether 14 kDa protein isolated from AGE induced DC maturation and increased immunostimulatory activity.

DC have an unequaled capacity to initiate primary and secondary immune responses, including tolerogenic responses [25]. DC function is dependent on the stage of DC maturation. Winzler et al. [26] demonstrated that DC have three sequential stages of DC maturation including immature, mature, and apoptotic stage. Immature DC (stage 1) have low expression of co-stimulatory molecules and MHCII, and are specialized for antigen capture with low APC ability [27]. Upon maturation of DC (stage 2), antigen uptake and presentation of native protein antigen is reduced. In contrast, presentation of immunogenic peptides and allostimulatory activity become very efficient. This functional DC maturation ended with apoptotic cell death (stage III), and no reversion to the immature phenotype was observed. McLellan et al. showed that immature DC are CD80 and only weakly express CD86, but both molecules are rapidly induced during culture on DC [28]. Furthermore, Helleman and Eriksson [29] showed that the earliest change in protein expression detected after stimulating peripheral DC with LPS was increased production of the chemokine interleukin 8 (IL-8) and then respectively increased expression of MHC-II, CD80, CD86 and CD40. They also demonstrated that, compared to CD80 and CD86, the CD40 molecule has slower kinetic expression on DC.

In the current research, overnight culture of pulsed DC with 14 kDa protein isolated from AGE and non-pulsed DC provided adequate time for over-expression of CD86 and MHC-II on DC and resulted in the exhibition of the same pattern of expression pertaining to these molecules. In our study, slow kinetics of CD40 gene expression on DC led to a significant difference between pulsed DC and non-pulsed DC for this molecule. These findings demonstrated that 14 kDa protein isolated form AGE enables the induction of DC maturation and its activation.

The interaction of CD28 on T cells with the B7 family (B7–1/CD80 and B7-2/CD86) molecules on DC provides a crucial and adequate co-stimulatory signal for T cell activation. Ligation of the CD28 by CD80 or CD86 co-stimulates T-cell proliferation, cytokine production, and generation of cytotoxic activity [30]. Although interaction between CD80 or CD86 and CD28 is adequate for T cell activation and proliferation, the interaction between CD40 on DC and CD40L on T cells provides an additional effect of priming for T cells, a mechanism which is essential for achieving long-term immunity [31]. Previous studies showed that an inducible CD40 receptor enhanced DC migration in vitro and extended the pro-stimulatory state of DC within lymphoid tissues [32].

**Fig. 4.** Effect 14 kDa protein isolated from AGE on DCs. The expression of the CD40, CD86, and MHC-II surface molecules on dendritic cells was assessed by flow cytometry after different treatments. AGE, aged garlic extract; DC, dendritic cells.

**Fig. 5.** Allogenic MLR utilizing Pulsed DCs with 14 kDa protein. Splenic DCs were purified from BALB/c mice co-cultured with allogenic T cells from C57BL/6 mice at the DCT ratio 1:10. After 72 h incubation, the T cells proliferation was determined by the MTT assay. DCs (DC) or T cells alone (T) do not show considerable OD. Results are expressed as the mean OD ± SD. MLR, mixed leukocyte reactions; DC, dendritic cells; OD, optical density.
The 14 kDa protein isolated from AGE is probably a lectin compound. Lectins are multifunctional molecules that mediate a number of diverse physiologic processes. Reports have demonstrated that, the biological function of lectin is the result of binding of these molecules to cell surface sugars [35]. It is therefore plausible to propose the hypothesis that, the 14 kDa protein isolated from AGE which was added to overnight culture of DC medium binds to cell surface sugars and exerts upregulation in CD40 molecule on these cells. Although several questions such as those about precise chemical nature of 14 kDa protein isolated from AGE, its binding sites on DC, and its signal transduction mechanism still remain unresolved at present, its effect on functional and phenotypic maturation of DC may provide us with a rational basis for its efficacy in its clinical application. In future study, we plan to evaluate the mechanism of action of the 14 kDa protein against the DC.

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References


