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The 14kDa Protein Molecule Isolated from Garlic Suppresses Indoleamine 2, 3-Dioxygenase Metabolites in Mononuclear Cells *In vitro*

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ABSTRACT

A wide range of biological activities of garlic *in vitro* and *in vivo* have been verified including its antioxidant, antitumor and anti-inflammatory effects. Indoleamine 2,3-dioxygenase (IDO) is an enzyme widely distributed in mammals and is inducible preferentially by IFN-γ. IDO degrades the essential amino acid tryptophan to form N-formyl kynurenine.

In the present *in vitro* study, the modulatory effect of 14kDa molecule isolated from garlic on IDO induction was tested. Cultures of mononuclear cells were exposed to 14kDa garlic fraction. Then, their proliferation responses and IDO metabolites were measured.

A significant down-regulatory effect of garlic on IDO activity was found and also the proliferation responses of mononuclear cells increased.

If these results are verified *in vivo*, an explanation will be provided on how garlic may interfere in IDO induction, which paves the way for elucidating its specific therapeutic effect in preventing tumor progress.

Key words: Garlic; Indoleamine 2, 3-dioxygenase; Proliferation; Tryptophan

INTRODUCTION

For five decades, garlic has had a worldwide reputation as a formidable prophylactic and therapeutic medicinal agent.[1] More than 3000 publications in previous and this century have provided evidence for the efficacy of garlic in the prevention and treatment of a variety of diseases, and for validating its traditional uses. Many favorable biological and pharmacologic effects of garlic preparations have been reported experimentally and clinically. Garlic has been shown to reduce risk factors of cardiovascular diseases, i.e., lowering serum cholesterol and triglycerides, inhibiting blood coagulation, improving blood circulation and lowering blood pressure.[2] Many *in vitro* and *in vivo* studies have suggested possible cancer-preventive effects of garlic preparations and their respective constituents.[3] In 1990, the U.S. National Cancer Institute initiated the Designer Food Program to determine which foods played an important role in cancer prevention. They concluded that garlic may be the most potent food

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having cancer-preventive properties.[4] In addition to the above mentioned pharmacologic activities, garlic has been shown to be a possible biological response modifier. Weisberger and Pensky (1957) first reported the augmentation of tumor immunity by garlic; subsequently a variety of immunostimulatory effects of garlic were reported.[5] Since certain diseases can be caused by immune dysfunction, modification of immune functions by garlic may contribute to the treatment and prevention of such diseases. Thus, some pharmacologic effects of garlic might be mediated through immunomodification. A unique garlic preparation called "aged garlic extract" (AGE) has been reported to have an array of pharmacologic effects including immunomodulation,[6] tumor cell growth inhibition and chemopreventative effects. In rodents, AGE and its constituents have been reported to inhibit the development of chemically induced tumors in the bladder,[6] mammary glands,[8] colon,[9] esophagus,[10] lung,[11] skin[12] and stomach.[13] Possible anticarcinogenic mechanisms of AGE and its constituents may include the inhibition of carcinogen activation,[14] enhancement of detoxification[15] and excretion[16] and protection of DNA from the activated carcinogens.[17] AGE significantly increases the release of four cytokines from mouse splenic cells (IL-2, IL-12, IFN-γ, TNF-α). Albeit garlic function is dose dependent and IFN-γ decreases in the presence of low dose garlic.[18] Indoleamine 2,3-dioxygenase (IDO) enzyme, which is mainly produced by antigen presenting cells (APCs), is widely distributed in mammals and is inducible preferentially by IFN-γ and has the ability to suppress T lymphocytes. IDO degrades the essential amino acid tryptophan to form N-formyl kynurenine, which, depending on the cell type and enzymatic repertoires, is subsequently converted into the finally form, niacin.[19] The first evidence for a tumoral immune resistance mechanism based on tryptophan degradation was provided by Uyttenhove *et al*. in a murine model, in which they showed that the immunomodulatory enzyme of IDO reduces antitumoral activity of T cell.[20] Cell proliferation of alloreactive T cells is thereby arrested in the G1 phase of the cell cycle via local tryptophan deprivation and the accumulation of toxic proapoptotic catabolites.[21]

As we described above 14kDa molecule isolated from garlic increases the proliferative response of Mononuclear cells (MNCs) but the exact mechanism of this effect is not clarified yet, therefore in this study, we decided to investigate whether 14kDa molecule isolated from garlic induces this proliferation through IDO suppression or inhibition of the toixic effects of the tryptophan metabolites such as kynurenine.

MATERIALS AND METHODS

Animals

Eight- to ten-week-old inbred Balb/c mice were purchased from the Pasteur Institute of Iran, (Tehran, Iran). They were given standard mouse chow ad libitum throughout the study.

Isolation of 14 kDa Fraction from Garlic

Fresh garlic bulbs were obtained from Hamadan, Iran. Dry garlic bulbs were peeled and kept in the freezer for six months in -20C^{o}. The obtained garlics were homogenized with two parts of distilled water in a varying blender. The homogenized blend was then filtered under vacuum through Whatman paper (number 1) and the filtrate was centrifuged at 3400g for 30 minutes. The clear supernatant was collected. Twenty seven grams of NH_4SO_4 was added to one liter of the supernatant and centrifuged at 3400g for 30 minutes. The residue was resuspended in saline and dialyzed against buffer saline. The isolated fractions were then run on G50 gel chromatography for further isolation of the 14 kDa as measured by Bradford assay and evaluated by SDS-PAGE.

SDS-PAGE Electrophoresis

A 12% (Weight/volume) polyacrylamide gel was used to judge the purity of molecules and to estimate the molecular mass with standard protein. After electrophoresis, the gel was fixed with methanol and acetic acid formaldehyde for 60 minutes and stained with coomassie blue.

Isolation of Mononuclear Cells

Mononuclear cells (MNC) were obtained from normal mouse spleen. The spleen was injected with cell culture media and the cell suspension was collected and kept at 4C^{o}. The injected spleen was then cut into pieces and passed through a metal mesh. The cell suspension was collected and added to the cells of previous step (spleen injection). The whole splenic cells were centrifuged twice with PBS-EDTA (2mM) (360g, 10mins). The MNCs were isolated by density centrifugation (600g, 20mins, 20^{o}C) (Ficoll, Baharafshan, Iran) and maintained in RPMI 1640

(Sigma-aldrich, USA), supplemented with 10% heat-inactivated fetal calf serum (Gibco, England), 2mM L-glutamine (Serva, Heidelberg, Germany). The MNCs were seeded at a density of 3×10^6/ml in complete RPMI-1640 medium and exposed to 5-40 µg/ml of 14 kDa garlic fractions. After 48 h incubation at 37°C, 5% CO2, the culture supernatants were harvested by centrifugation and frozen at -20^0C and the tryptophan and kynurenine were measured by HPLC.

Cell Viability and Proliferation Test

After 48 hrs incubation, the mononuclear proliferation was determined by the MTT [3, (4, 5-dimethylthiazal-2-yl)-2, 5-diphenyl tetrazolium bromide] assay. The cells, were cultured in a 96-well plate and incubated for another 4 hrs in presence of MTT (5 mg/ml) (Sigma, USA), followed by the addition of 0.1 ml dimethyl sulfoxide (DMSO MW=78.13) in order to dissolve the formazan crystals. Subsequently the absorbance was read at 490nm by ELISA reader.

Measurement of Tryptophan and Kynurenine Concentrations by High Performance Liquid Chromatography (HPLC)

The culture supernatants (150 µl) were extracted with 1.3 ml of methanol. Then the precipitate was removed by centrifugation, and the supernatant was dried under vacuum. The Samples were resuspended in an initial mobile phase (deionized water), injected onto a C18 column (Luna C-18; 250 ×4.6 mm, 5µm; Phenomenex, Torrance, CA), and eluted with a linear gradient of acetonitrile in water (0–80% over 20 min). Absorbance was measured at 225 nm and compared against the standard curves for tryptophan and kynurenine. By calculating the ratio of tryptophan versus kynurenine (trp/kyn), IDO enzyme activity was estimated as described by Munn *et al*.[22]

Statistical Analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. The p-values less than 0.05 were considered to indicate significant differences.

RESULTS

Isolation of 14 kDa Fraction of Garlic

Immunomodulatory molecules were purified from the garlic extract by means of Ammonium sulphate and the fractions were collected by centrifugation. The isolated fractions were then run on G 50 gel chromatography for further isolation of the 14 kDa protein. In order to evaluate the purity of 14 kDa protein, this protein molecule was run on the SDS/PAGE electrophoresis and the results indicated the presence of 95% purified band of 14 kDa molecule (Figure 1).

Mononuclear cell Viability

In order to assess the proliferative effect of 14 kDa protein on the mononuclear cells, spleens of mice were examined by the MTT assay**.** As the test groups, MNCs were treated with different concentrations of 14kDa molecule isolated from garlic and in some wells these cells were cultured in the absence of 14kDa molecule isolated from garlic as the control group. The results showed that proliferative responses were significantly increased ($p<0.05$) when mononuclear cells were treated in different concentrations of 14 kDa protein of the garlic compared with the control group (MNCs which were cultured in the absence of 14kDa protein) (Figure 2).

The Tryptophan/ Kynurenine Ratio

The supernatants of MNC were collected and examined for trp/kyn ratio. In addition, different concentrations of 14 kDa fraction of the garlic were used (0 µg concentration as the control group). The results showed a significant increase ($p<0.05$) in the trp/kyn ratio in the test groups compared with the control group (Figure 3).

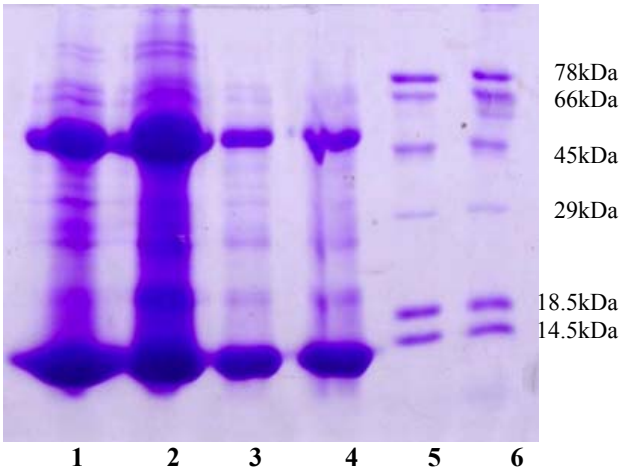

Figure 1. garlic 14 kDa fraction band evaluated by SDS-PAGE electrophoresis. In order to evaluate the purity of 14 kDa protein, the protein molecule was run on the SDS/PAGE electrophoresis and the results indicated the presence of 95% purified band of 14 kDa molecule.
1- 113µg/1λ garlic extract, 2- 88µg/1λ garlic extract, 3- 55µg/1λ garlic extract, 4- 27µg/1λ garlic extract, 5&6- marker

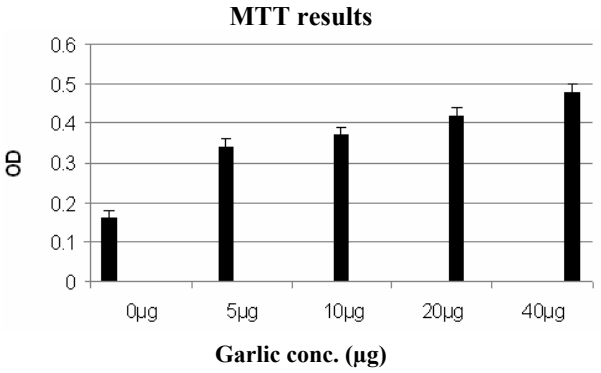

Figure 2. Proliferation responses of 14kDa molecule isolated from garlic treated mononuclear cells. Proliferations were measured after 48 h. Columns show means ± SD

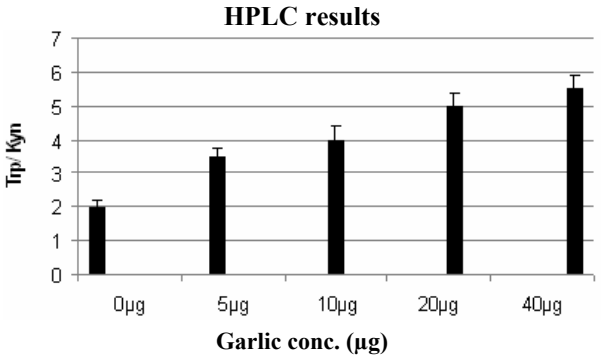

Figure 3. Activity of the indoleamine 2,3-dioxygenase (IDO) in mononuclear cells influenced by 14kDa molecule isolated from garlic. Mononuclear cells were treated with 14kDa molecule isolated from garlic 5-40 µg. Tryptophan and kynurenine concentrations were measured in culture supernatants after 48 h. IDO activity was calculated by the ratio of tryptophan per kynurenine . Columns show means ± SD

DISCUSSION

In this study we showed that 14kDa molecule isolated from garlic increased the proliferative responses in splenic MNCs and also the kynurenine concentration was decreased after the splenic MNCs were treated with 14kDa molecule isolated from garlic.

Various researches have indicated that garlic modulates immune responses.[23] Our previous studies also demonstrated that garlic enhances natural killer cell (NK) cell activity[24] and T-lymphocyte proliferation.[25] Garlic also modulates thioredoxin reductase, protein disulfide isomerase, quinone reductase, glutathione reductase, and intracellular redox status, which, lead to regulation cell signal transduction, transcription factor activation, and DNA repair.[26]

Our previous studies demonstrated that 14kDa molecule isolated from garlic enhanced the delayed-type hypersensitivity (DTH) response[5], T-cell proliferation[25] and natural killer (NK) cell activity,[24] shifted the cytokine pattern to Th1 (IFNγ, IL-2)[27] and exhibited enhancement of peritoneal Macrophage Phagocytic activity against Leishmania major.[28]

The exact mechanism through which 14kDa molecule isolated from garlic induces its proliferative responses was not known. Therefore the major aim of this study was to determine if there is any relationship between 14kDa molecule isolated from garlic effects and the activity of the indoleamin 2,3-dioxygenase (IDO).

As we described in introduction IDO enzyme degrades the essential amino acid tryptophan to form N-formyl kynurenine, which, depending on the cell type and enzymatic repertoires, is subsequently converted into the final form: niacin[20] and the cell proliferation of alloreactive T cells is thereby arrested in the G1 phase of the cell cycle via local tryptophan deprivation and the accumulation of toxic proapoptotic catabolites such as kynurenine.[21] Recently, Banerjee *et al*.[29] showed that the *in vivo* antitumor mechanism of action of natural product-based brassinins is the inhibition of indoleamine 2,3-dioxygenase.

Therefore we hypothesized that 14kDa molecule isolated from garlic had increased the MNCs' proliferative responses through a decrease in IDO production or inhibition of the toxic effects of the tryptophan metabolites.

Tryptophan and kynurenine concentrations measurement in the supernatant of the cultures of MNCs which were treated with 14kDa molecule isolated from garlic showed that tryptophan concentration did not show any significant differences in supernatant of the test group (14kDa molecule isolated from garlic treated cells) in comparison to the control (MNCs cultured in the absence of the 14 kDa molecule isolated from garlic). But the kynurenine concentration was decreased in the supernatant of the test group. The equal concentrations of tryptophan in the supernatants of the test and control groups showed that 14kDa molecule isolated from garlic did not have any effect of IDO activity but the lower concentration

of kynurenine in the supernatant of the culture of test group compared with the control suggests that 14kDa molecule isolated from garlic has inhibited the toxic effects of the tryptophan metabolites such as kynurenine.

It is well known that the function of tryptophan metabolites is influenced by the redox potential of the microenvironment.[30] It has been shown that among the tryptophan metabolites generated by IDO, 3-OH-kynurenine and 3-OH-anthranilic acid are the most important mediators of immunosuppression.[31] Both compounds are good electron donors that reduce cytochrome C and are readily oxidized under aerobic conditions.[32] Their oxidations lead to the generation of quinone-imines, which oxidatively modify various amino acid side chains of proteins.[33] Thus, 3-OH-kynurenine and 3-OH-anthranilic acid, two reducing molecules, are the immediate precursors of potentially oxidizing agents '*in vivo*', contributing to oxidation stress. It has also been shown that 14 kDa molecule isolated from garlic modulates several reductase enzymes such as quinone reductase. The decreased concentrations of kynurenine after 14 kDa molecule isolated from garlic treatment showed that 14 kDa molecule isolated from garlic affected the redox potential of the microenvironment through modulating the reductase enzymes and subsequently decreased the concentration of tryptophan toxic metabolites such as kynurenine.

The antitumor effects of the garlic have been shown through several studies[5] and also in cancer patients, significantly accelerated degradation of tryptophan with lowered serum concentrations of tryptophan and increased kynurenine as well as increased IDO activity have been previously recognized and this phenomenon could be best explained by IDO expression within the tumors[34] therefore understanding the exact relationship between the mechanisms through which 14 kDa molecule isolated from garlic induces its effects are very important and IDO function would help us to find more effective ways of using garlic in clinics.

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