Allicin stimulates lymphocytes and elicits an antitumor effect: a possible role of p21 ras

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Abstract

Allicin, the main organic allyl sulfur component in garlic, exhibits immune-stimulatory and antitumor properties. Allicin stimulated [3H]thymidine incorporation in mouse splenocytes and enhanced cell-mediated cytotoxicity in human peripheral mononuclear cells. Multiple administration (i.p.) of allicin elicited a marked antitumor effect in mice inoculated with B-16 melanoma and MCA-105 fibrosarcoma. The immune-stimulatory and antitumor effects of allicin are characterized by a bell-shaped curve, i.e. allicin at high, supra-optimal concentrations is less effective or inhibitory. Allicin induced activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in human peripheral mononuclear cells, and also in wild-type Jurkat T-cells. Allicin failed to activate ERK1/2 in Jurkat T cells that express p21 ras, in which Cys118 was replaced by Ser. These cells are not susceptible to redox-stress modification and activation. We postulate that the immune stimulatory effect of allicin is mediated by redox-sensitive signaling such as activation of p21 ras. It is suggested that the antitumor effect of allicin is related to its immune-stimulatory properties.

Introduction

It is claimed that garlic is effective against a broad spectrum of diseases. During the past 20 years, studies on garlic have been conducted primarily in the field of cardiovascular and cancer research. It has been suggested that garlic decreases cholesterol and triglyceride levels, and may reduce the atherosclerosis process (1–3). Anti-thrombotic and vasodilatory effects of garlic have also been noted (4,5). The in vitro stimulatory effect of garlic extracts on rat and human lymphocytes has been reported (6,7). Diallyltetrathiol has been shown to augment concanavalin A activation of T lymphocytes (8). Garlic extracts have been found to elevate IL-10 and IL-12 in monocytes, while suppressing other pro-inflammatory cytokines, including IL-2 (9). A protein fraction (F4), isolated from aged garlic extract, enhanced the cytotoxicity of human peripheral blood lymphocytes against NK-sensitive and -resistant cell lines (7). Aged garlic extracts have an immunomodulatory effect (10) and, upon administration intravesically, were effective against murine transitional cell carcinoma, similar to that of BCG (11). Both preparations induced lymphocyte and macrophage infiltration and cytokine release. It is apparent that garlic components induce immune stimulation by mitogenic activation (e.g. allicin) and by providing an adjuvant effect (e.g. protein F4).

Reports have indicated that garlic plays a role in reduction of death caused by malignant diseases (12–14). Garlic constituents were shown to exhibit antitumor and cytotoxic action, both in vitro and in laboratory animals (15–17), and also to possess antibacterial, antifungal and antiparasitic activities (18,19). Pre-clinical and clinical studies indicated that agents stimulating the immune system in a non-specific manner, exhibit antitumor properties (20–23). Hemin, an iron-containing compound generating free radicals, exhibits immune-stimulatory and antitumor properties (24). We have recently reported (25) that ferrocene, a synthetic iron-containing compound, induces lymphocyte activation and antitumor activity by redox-sensitive signaling.

The organic allyl sulfur components in garlic (mainly allicin) were implicated to mediate its biological activity. It has been
suggested that the biological activities of these compounds may be related to their SH modification and antioxidant properties (26–28). The proto-oncogene p21ras has been identified as a key molecular switch involved in regulating T cell activation, triggered by different mitogens (29–32). We have previously described the immune stimulatory properties of agents that generate free radicals (33–37). We found that p21ras was a primary target for these agents and have identified a single cysteine residue, Cys118, modified by free radicals (38–40). This modification triggers the activation of p21ras, mimics the effect of guanosine nucleotide exchange factor, and leads to downstream signaling events such as the activation of mitogen-activated protein kinase, activation of phosphatidylinositol 3′-kinase and transcription factor activation (41,42).

We explored the hypothesis that p21ras plays a role in mediating the effect of allicin (as a SH modifier) to induce lymphocyte activation and elicit an antitumor effect.

Methods

Materials

Alliin was synthesized from L-cysteine and allyl bromide after oxidation by H₂O₂ using the procedure described by Stoll and Seebeck (43). Pure alliin was produced by applying synthetic alliin into immobilized alliinase (44), followed by its purification according to Jansen et al. (45). Phytohemagglutinin (PHA) was obtained from Sigma (Rehovot, Israel) and human recombinant IL-2 (23 SD 107 U/mg) was obtained from PeproTech (Rocky Hill, NJ).

Jurkat T cells stably expressing p21ras C118S(1–189)

Jurkat T cells stably expressing p21ras C118S(1–189), in which Cys118 was replaced by Ser, were prepared as previously described (39,40). These transfected cells expressed 7- to 10-fold more p21ras than the wild-type cells determined by western blotting with anti-p21ras antibody, Y13-259, and did not respond to thiol modifiers by stimulating ERK1/2. This antibody cannot distinguish between wild-type and p21ras C118S, suggesting that although endogenous p21ras was not specifically inhibited, ectopic expression of high levels of mutant p21ras apparently prevented its signaling (39). This dominant-negative activity of p21ras C118S toward thiol modification may be due to its high level of expression.

Mice

C57BL female mice, 6–8 weeks of age, were obtained from the Animal Breeding Center at Tel Aviv University, Tel Aviv, Israel.

Tumor models

Two mouse tumor models in C57BL mice were used: B-16 melanoma and MCA-105 (a methylcholanthrene-induced fibrosarcoma). B-16 and MCA-105 were grown in culture and obtained from a large stock of frozen cells. The stock of B-16 was prepared from in vivo s.c. growth and that of MCA-105 was obtained from i.p. growth, both in C57BL mice. B-16 and MCA 105 cells were injected i.v. at 5 × 10⁴ and 2 × 10⁵/mouse,

Isolation of cells and culture conditions

Human peripheral blood mononuclear cells (PBMC) were obtained from normal, healthy subjects by Ficoll-Hypaque density gradient centrifugation, and contained 70–90% lymphocytes and 10–30% monocytes. They were suspended (1 × 10⁵/ml) in RPMI 1640 medium containing 5% FCS and distributed (0.2 ml aliquots) in flat-bottom microwells, and various additions were made. Cells were then incubated at 37°C in a 95% air, 5% CO₂ atmosphere for the time indicated and [³H]thymidine incorporation (2 µCi/well) into DNA during the final 20 h of incubation was determined. Triplicate cultures were examined and the means ± SD were determined.

Mouse splenocytes

Spleens were removed aseptically and crushed in RPMI 1640 supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 5 × 10⁻⁵ 2-mercaptoethanol, 100 µg/ml streptomycin, 100 U/ml penicillin, 0.03% fresh glutamine and 5% heat-inactivated FCS (complete medium (CM)). Cells were then washed twice with PBS, resuspended in CM (2 × 10⁶/ml), distributed (0.2 ml aliquots) in flat-bottomed microwells and incubated at 37°C for the time indicated. [³H]Thymidine incorporation (2 µCi/well) into DNA during the final 20 h of incubation was determined. Triplicate cultures were examined and the means ± SD were determined.

In vitro cytotoxicity assay

Cytotoxicity assays were performed as follows: target cells were labeled with 400 µCi of sodium ⁵¹CrO₄ for 1 h in CM. They were then washed 3 times and resuspended in CM at 5 × 10⁶ cells/ml. Effector cells were prepared as above, washed and mixed with target cells at various E:T ratios, and incubated in 96-well, round-bottomed microplates for 3 h at 37°C in a 5% CO₂ incubator. Cell cultures were harvested and supernatants counted in a liquid scintillation counter. Maximum isotope release (MR) was produced by incubation of the targets with 0.1% Triton X-100. Spontaneous release (SR) was measured by incubation of the targets with medium alone. The percentage of cell lysis was calculated by: ER = SR/MR − SR × 100, where ER was the experimental effector release.

Western blot analysis

Cells were solubilized with lysis buffer containing 50 mM Tris–HCl (pH 7.5), 0.5% Triton X-100, 3 mM EGTA, 12 mM β-glycerophosphate, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium vanadate, 2 mM DTT, 1 mM PMSF, 1 mM aprotinin and 0.1% 2-mercaptoethanol. Cell lysates were then applied to a 10% polyacrylamide gel. The electrophoresed proteins were transferred onto a nitrocellulose membrane. After blocking, the membranes were incubated with a mouse monoclonal anti-MAP kinase, activated (diphosphorylated ERK1 and ERK2), clone MAPK-YT (Sigma, St Louis, MO; M-8159). The membrane was then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody and developed with enhanced chemiluminescence mixture.
**Fig. 1.** Allicin stimulates [³H]thymidine incorporation in mouse splenocytes (A) and in human PBMC (B). Cells were incubated for 3–5 (A) and 3 (B) days under conditions that were described in Methods. *P < 0.05, **P < 0.01, ***P < 0.001 versus cell cultures without allicin.

**Fig. 2.** Allicin enhances cell-mediated cytotoxicity in human PBMC. Human PBMC (10⁶/ml complete medium) were incubated for 6 days in the absence (A) and presence of IL-2 (10 U/ml) (B). Cytotoxicity assays were performed as described in Methods. K562, a human erythroleukemia cell line (NK sensitive), was used as target. E:T ratios of 50:1 and 25:1 (A) and 25:1 (B). Cells were cultured in duplicate and the results are expressed as the means. Deviation from the mean did not exceed 10%.
Statistical analysis
Results are expressed as means ± SD. Differences between means were determined using Student’s t-test.

Results

Lymphocyte activation induced by allicin

Stimulation of [3H]thymidine incorporation. Allicin stimulated [3H]thymidine incorporation in mouse splenocytes in a bell-shaped pattern. Maximal stimulation was attained by allicin at concentrations of 0.5–1 μg/ml. At higher concentrations, allicin was less stimulatory (Fig. 1A). Allicin also stimulates [3H]thymidine incorporation in human PBMC (Fig. 2B).

Enhancement of cell-mediated cytotoxicity. Human PBMC incubated for 6 days with allicin elicited cell-mediated cytotoxicity against NK-susceptible human erythroleukemic cells (K562) (Fig. 2A). Allicin induction of cell-mediated cytotoxicity revealed a ‘bell-shaped’ pattern, i.e. allicin, at high concentrations, was less effective. Allicin further enhanced IL-2-induced cell-mediated cytotoxicity in human PBMC (Fig. 2B).

Fig. 3. Anti-tumor properties of allicin. (A) Allicin was administered (i.p.) to B-16 melanoma inoculated mice (six or seven mice were included in each group), at different doses, 4 times each, on days T2, T6, T9 and T13 (T0 = day of tumor inoculation). Tumor load was assessed by weighing the lungs 25 days post-tumor inoculation. (B) Allicin was administered (i.p.) to MCA-105 fibrosarcoma-inoculated mice (seven or eight mice were included in each group), at different doses, 3 times each, on days T2, T9 and T14. Tumor load was assessed by weighing the lungs 25 days post-tumor inoculation. **P < 0.01 versus mice that were treated with PBS.
Antitumor properties of allicin

The antitumor effect of allicin was evaluated in mice bearing established lung metastases, following injection of the tumor cells into the tail vein. Mice were sacrificed 25 days post-tumor inoculation. The weight of lungs in tumor-inoculated mice, above that of controls, represents tumor load.

The antitumor effect of allicin in B-16 melanoma bearing mice is shown in Fig. 3(A). Allicin was administered i.p. at different doses, 4 times each, on days T1, T2, T9, and T13 (T0 = day of tumor inoculation). Tumor load was assessed by weighing the lungs at T25. Maximal antitumor effect was attained by administration of allicin at multiple doses of 5 mg/kg, as outlined above. Allicin at higher doses was less or not effective.

The antitumor effect of allicin in MCA-105 fibrosarcoma-bearing mice is shown in Fig. 3(B). Allicin, at different doses, was administered (i.p.) on days T2, T9, and T13. Tumor load was assessed by weighing the lungs 25 days post-tumor inoculation. Maximal antitumor effect was attained by administration of allicin at multiple doses of 12.5 mg/kg as outlined above. Allicin at a higher dose (25 mg/kg) was significantly less effective.

Mechanism of action of allicin

We postulate that p21ras is a primary molecular target of allicin in stimulating lymphocytes. p21ras plays a key role in T lymphocyte activation and has previously been shown to be activated by cellular redox stress. ERK1/2, which belong to the mitogen-activated protein kinase family, are downstream of ras and are activated (phosphorylated) by a variety of growth factors. Figure 4(A) demonstrates the activation of ERK1/2 in human PBMC treated with allicin (10 μg/ml). The levels of activated ERK1/2 were determined by western blot analysis at different time intervals following allicin addition, maximal stimulation being attained after 10 min, diminishing gradually with time. Thirty minutes after allicin was added, the level of activated ERK1/2 was similar to that of control, untreated cells. As a positive control, cells were treated with PHA (2 μg/ml) for 10 min. To establish that p21ras Cys118 is a primary target site for oxidation by allicin, we investigated the stimulatory effect of allicin on ERK1/2 (which is mediated by ras) in wild-type Jurkat T cells and in Jurkat T cells that express p21ras in which Cys118 was replaced by a Ser residue (referred to as p21ras C118S, see Methods), which is not susceptible to redox-stress modification. As depicted in Fig. 4(B), allicin activates ERK1/2
Allicin-induced redox-triggered signaling

Fig. 5. A scheme of a proposed mechanism for allicin-induced p21\textsuperscript{ras} activation.

Discussion

The many beneficial effects of garlic have been reported. They include anticancer activity, immunomodulatory effects, antibacterial and antiparasitic effects, and lowering serum cholesterol, triglycerides and blood pressure (3). It is plausible that the different compounds comprising garlic ingredients are involved in eliciting their multiple effects.

The most characterized non-protein fraction of garlic includes the organosulfur compounds. Allicin is the main biologically active component of this group and its action was attributed to either antioxidant activity or interaction with thiol-containing proteins. In this paper we reported the immunostimulatory properties of a pure preparation of allicin. In vitro, allicin induced proliferation of both murine splenocytes and human PBMC, enhanced cytotoxicity of human PBMC, and ferrocene (25).

Further studies are required to demonstrate the direct modification of p21\textsuperscript{ras} by allicin, and elucidating the mechanism which couples allicin-induced immune stimulation and its antitumor effect.

It is unlikely that the redox-triggered mechanism of allicin can be extended to all the biological effects attributed to garlic. The different components of garlic may exert their biological effect by various mechanisms.

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Abbreviations

CM complete medium
ERK extracellular signal-regulated kinase
PBMC peripheral blood mononuclear cell
PHA phytohemagglutinin

References

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