RESEARCH COMMUNICATION

Antitumoral Activity of Allicin in Murine Lymphoma L5178Y

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Abstract

Epidemiological studies link increased garlic (*Allium sativum*) consumption with a reduced incidence of cancer in various human populations. Experimental carcinogenesis studies in animal models and in cell culture systems indicate that several allium-derived compounds exhibit inhibitory effects and that the underlying mechanisms may involve apoptosis. To provide a better understanding of the effects of allium derivatives regarding prevention of cancer, we examined antitumoral activity of allicin, a major component of garlic, in L5178Y lymphoma bearing mice. For *in vitro* studies, we utilized cell proliferation and apoptosis in the same tumor cell line. We found that allicin inhibited the growth of tumor cells at doses two fold superior to that in normal splenocytes. Allicin also induced apoptosis, and this was associated with an increase in caspase3 activity.

Keywords: Allicin - cell proliferation - apoptosis - antitumoral - lymphoma L5178Y

Asian Pacific J Cancer Prev, **11**, 1241-1244

Introduction

Garlic (*Allium sativum*) has been used among centuries for treating various diseases, its consumption has been related to reduce cancer risk (Galeone et al., 2006). Some garlic constituents have been shown to alter activation of carcinogens and to cause growth inhibition of tumor cells (Ali and Thomson, 2003). Allicin, the major component present in freshly crushed garlic, is one of the most biologically active compounds of garlic (Rivlin, 2001).

It has been reported that allicin inhibit various cancer cells. However, the exact pathway by which allicin affects the growth of tumor cell lines is still not clear (Hirsch et al., 2000; Jakubikova and Sedlak, 2006). Lower cytotoxicity in normal cells compared to tumor cells is a prerequisite for any chemopreventive agent. Therefore, the present study was carried out to determine the antitumor activity of allicin in murine test system L5178Y lymphoma and and to compare the cellular proliferation and apoptosis induction in both tumor and normal cells.

Materials and Methods

Materials

Allicin was obtained from Chromadex Company (USA). Other materials were of analytical grade and obtained from Sigma Aldrich (USA).

Mice

Male BALB/c mice (20-25 g), were obtained from the *Zooterio de la Universidad de Guadalajara* (Mexico) and maintained under conventional laboratory conditions, according to the guidelines for the use and care of laboratory animals and World Medical Association Declaration of Helsinki. Animal protocols were approved by the Biomedicine Sciences Committee.

Tumor models

In vivo antitumor activity was evaluated by survival. Animals (10 per group) were inoculated i.p. with 0.1 ml of suspension ascitic fluid, containing L5178Y lymphoma $2X10^4$ cells/mouse, on day 0. This tumour line was derived from murine thymic lymphoma (H-2d) haplotype (Puebla et al., 1998). The treatement with allicin, started 24 h after inoculation, and it was extended over 7 days. Mice groups were daily observed to evaluate their general conditions. Survival was represented as Kaplan–Meier curves.

Cell viability assay

Cell viability assays were carried out as described with slight modifications (Anto et al., 2000). Briefly, cells obtained from lymphoma L5178Y were cultured in RPMI medium, at 37°C in a 5% CO_2 incubator, with increasing amounts of allicin. Spleen cells obtained from healthy mice were utilized as control. Viability of cells was determinded by assaying for the reduction of MTT to formazan, and the absorbance was measured spectrophotometrically at 570 nm. The blank control contained cell culture medium only and the absorbance of untreated cultures was set at 100%. At least three independent experiments were performed.

Detection of morfological apoptosis

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using acridine orange

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and ethidium bromide staining method (Oommen et al., 2004). Briefly, tumor and normal cells were cultured and then treated with allicin for 24 h. After washing once with phosphate-buffered saline, the cells were stained with a mixture (1:1) of acridine orange–ethidium bromide solutions. The cells were immediately washed with phosphate-buffered saline, and viewed under a fluorescent microscope.

Caspase-3 determination

Caspase-3 activity in cells treated with allicin was determined with the CaspACE™ Assay System, Colorimetric kit (Promega Corporation, Madison, WI, USA) (Babich et al., 2005). The procedure was as follows. Tumor and normal cells, were incubated with allicin for 24 h, washed with cold PBS, treated with cell lysis buffer, freeze-thawed to prepare the cell lysate, and then harvested by centrifugation. Supernatants of cell extracts were inoculated into microtiter wells containing caspase assay buffer, dimethyl sulfoxide, dithiothreitol, and colorimetric caspase-3 substrate labeled with the chromophore, p-nitroaniline (Ac-DEVD-pNA). The plates were incubated at 37°C for 3 h and absorbance was read at 405 nm with a microtiter plate spectrophotometer.

Statistical analysis

Results of the vivo experiments were analysed using Kaplan-Meier estimation of survival and Log Rank test. Cell proliferation studies were analyzed with probit. Apoptosis and caspace induction are expressed as means + SD. Differences between means were determined using Student´s t-test.. The *P*-value of the effect had to be ≤ 0.05% to be considered significant.

Results

The effect of allicin on tumor growth has been studied in L5178Y murine lymphoma model (Figure 1). Intraperitoneal injection of allicin was performed every day, following tumor cell inoculation. It resulted in an inhibition of tumor growth that has been quantified by survival time at the end of the experiment. We evaluated several doses (data not shown). Maximal antitumor effect was attained by administration of allicin at doses of 20.0 mg/kg.

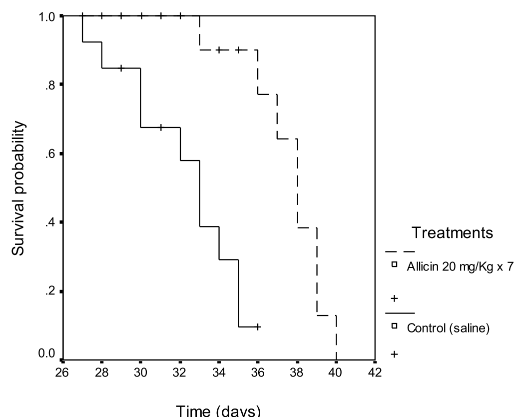

Figure 1. Antitumour Effect of Allicin Evaluated by the Survival Rate of Mice with Lymphoma L5178Y, Comparing the Control Group (Placebo Treatment).

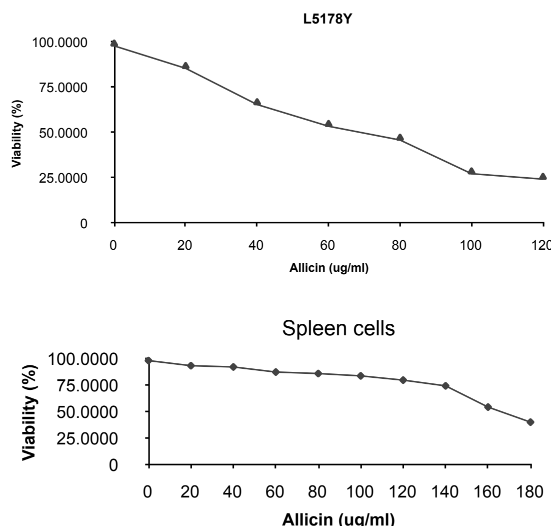

Figure 2. Inhibitory Effects of Allicin on the Growth of L5178Y Tumor Cells and Normal Spleen Cells. Viability was determined by MTT assay. Data are mean+ SD of results for three independent experiments

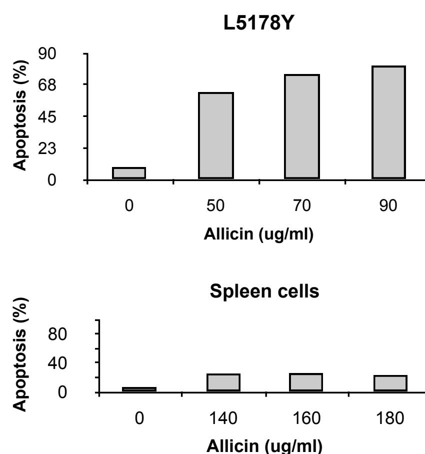

Figure 3. Effects of Allicin on the Induction of Apoptosis in L5178Y and Spleen Cells

The L5178Y tumor cells and normal spleen cells were choosen to determine the antiproliferative activity of allicin. A typical dose-dependent inhibition of cell growth is shown in Figure 2 documenting the survival cell porcentage plotted versus allicin concentration. After a 24 h incubation, increasing concentrations of allicin (0-120 ug/ml) led to a gradual decrease of the viable cells. IC_{50} value was 72 ug/ml. This effect was lowest in spleen cells, with an IC50 value of 177 ug/ml.

Spontaneous apoptosis occurred in 6-8% of the L5178Y and spleen cells, as assessed by staining with ethidium bromide and acridine orange. The addition of allicin in cytotoxic concentrations to cultured cells induced an increment in apoptosis porcentage. This effect was greater in the L5178Y cells than in the normal spleen cells, which is consistent with the fact that spleen cells were more resistant with respect to growth inhibition (Figure 3).

To assess the participation of caspases in the allicin-induced apoptosis, the activity of caspase-3 was

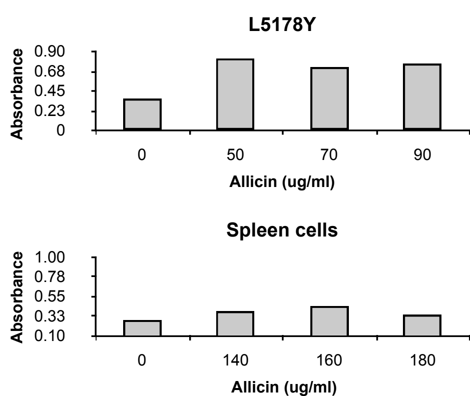

Figure 4. Induction of Caspase-3 Activity in Tumor and Normal Cells

measured. Upon treatment of L5178Y and spleen cells with doses near their respecives IC_{50} values, caspase-3 activity significantly increased only in tumor cells (Figure 4). This signal transduction pathway resembled the Fas and TNF pathways.

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 (Tattelman, 2005).

Allicin, one of garlic constituents, was implicated to mediate its biological activity. Siegers and coworkers (1999) also suggested a major role for breakdown products of allicin, such as dialyl sulfide, dialyl dislfudie and ajoene, in the growth inhibitory effect of garlic preparations.

In the present study, we reported the antitumoral and apoptotic properties of allicin. In vivo, the compound shown an antitumor effect in murine L5178Y, whereas In vitro, allicin inhibited growth of L5178Y cells and induced apoptosis through the activation of caspase 3. Comparatively normal spleen cells were lowest affected.

Information about antitumoral effect of allicin in vivo is limited. Our results are in agreement with those reported in mice bearing B-16 melanoma and MCA-105 fibrosarcoma with maximal antitumor effect attained at doses of 5 and 12.5 mg/Kg. Interestingly, allicin at higher doses was less or not effective (Patya et al., 2004).

The antiproliferative activity of allicin has been demostrated in several tumor cell types at low concentrations (Hirsch et al., 2000; Pinto and Rivlin, 2001). Here we observed the antiproliferative effect in high concentrations of allicin like others authors (Jakubikova and Sedlak, 2006). Only a few articles evaluated the antiproliferative activity of allicin in normal cells, Hirsch has demostrated the growth supression cancer cells as well as in human fibroblast lines, albeit at higher concentrations. (Hirsch et al., 2000). We demostrated the antiproliferative effect in normal spleen cells about two folds higher concentration than required for lymphoma cells. This is important in the context of cancer chemoprevention.

Recently, it has demonstrated that cytotoxic concentrations of allicin induced apoptosis in tumor cells (Sun and Wang, 2003; Oomen et al., 2004; Jakubikova and Sedlak, 2006; Zhang et al., 2006). In this work, apoptotic cell morphology were noted in L5178Y cells treated with allicin, but not in normal splenocytes.

The studies presented herein also demonstrated the greater sensitivity of tumor L5178Y cells, over normal spleen cells, towards allicin-induced inhibition of proliferation and the induction of apoptosis.

The mechanism of apoptosis allicin-induced is not well stablished. The caspases are cystein proteases that play key roles in the execution phase of apoptosis. Among the family of caspases, caspase-3 has been reported to be the most frequently activated caspase protease in apoptotic cells, indicating its crucial role in the cell death process. In the present study, our data show the allicin induced apoptosis through a caspase 3 pathway.

This has been demonstrated previously in tumor cells of human and murine origin (Oomen et al., 2004). We postulated that the apoptotic effect in L5178Y tumor cells reported here is mediated by caspase 3 activation.. However, This contrasts with apoptosis in allicin-treated gastric carcinoma cell line, where allicin induces caspase-independent apoptosis (Park et al., 2005). Such different findings to our results could be due to the difference in cell type used.. Indeed, recent studies demonstrated that several parallel apoptosis pathways could be initiated by same inducer (Cummings and Schnellmann, 2002).

The unstable nature of allicin raises the question whether all the effects of allicin observed by us and others are due to allicin itself or its metabolites. There is evidence that allicin is formed from its metabolite, diallyl disulfide, in human liver microsomes indicating that allicin may also be acting intracellularly (Teyssier et al., 1999).

Additional studies are needed to clarify the mechanisms and intracellular mediators of the antitumoral activity of allicin.

Acknowledgments

This work was supported in part by CONACyT-SAGARPA, Grant 185236.

References

Ali M, Thomson M (2003). Garlic (Allium sativum): A review of its potential use as an anti-cancer agent. *Curr Cancer Drug Targ*, **3**, 67-81.

Anto RJ, Maliekal TT, Karunagaran D, et al (2000). L-929 cells harboring ectopically expressed RelA resist curcumin-induced apoptosis. *J Biol Chem*, **275**, 15601-4.

Babich H, Krupka ME, Nissim HA, et al (2005). Differential in vitro cytotoxicity of (–)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity. *Toxicol in vitro*, **19**, 231-42.

Cummings B, Schnellmann R (2002). Cisplatin-induced renal cell apoptosis: Caspase 3-dependent and -independent pathways. *J Pharmacol Exp Ther*, **302**, 8-17.

Galeone C, Pelucchi C, Levi F, et al (2006). Onion and garlic use and human cancer. *Am J Clin Nutr*, **84**, 1027-32.

Hirsch K, Danilenko M, Giat J, et al (2000). Effect of purified allicin, the major ingredient of freshly crushed garlic, on

cancer cell proliferation. *Nutr Cancer*, **38**, 245-54.

Jakubikova J, Sedlak J, (2006). Garlic-derived organosulfides induce cytotoxicity, apoptosis, cell cycle arrest and oxidative stress in human colon carcinoma cell lines. *Neoplasma*, **53**, 191-199.

Oommen S, Anto RJ, Srinivas G, et al (2004). Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells. *Eur J Pharmacology*, **485**, 97-103.

Park S, Cho S, Kwon H, et al (2005). Caspase-independent cell death by allicin in human epithelial carcinoma cells: involvement of PKA. *Cancer Letters*, **224**, 123-32.

Patya M, Zahalka M, Vanichkin A, et al (2004). Allicin stimulates lymphocytes and elicits an antitumor effect: a possible role of p21ras. *Internat Immunol*, **16**, 275-81.

Pinto J., Rivlin RS (2001). Antiproliferative effects of allium derivatives from garlic. *J Nutr*, **131**, 1058S-60S.

Puebla AM, Huacuja L, Rodríguez G, et al (1998). Cytotoxic and antitumour activity from *Bursera fagaroides* ethanol extract in mice with L5178Y lymphoma. *Phytother Res*, **12**, 545-8.

Rivlin RS (2001) Historical perspective on the use of garlic. *J Nutr*, **131**, 951S-4S.

Siegers CP, Robke S, Pentz R (1999). Effects of garlic preparations against human tumor cell proliferation. *Phytomedicine*, **6**, 13-16.

Sun L, Wang X (2003). Effects of allicin on both telomerase activity and apoptosis in gastric cancer SGC-7901 cells. *World J Gastroenterol*, **9**, 1930-4.

Tattelman E (2005). Health effects of garlic. *Am Fam Phys*. **72**, 103-6.

Teyssier T, Guenot L, Suschetet M, et al (1999). Metabolism of diallyl disulfide by human liver microsomal cytochromes P-450 and flavin-containing monooxygenases. *Drug Metab Dispos*, **27**, 835–41.

Zhang Z, Zhong N, Gao H, et al (2006). Inducing apoptosis and upregulation of Bax and Fas ligand expression by allicin in hepatocellular carcinoma in Balb/c nude mice. *Chin Med J*, **119**, 422-5.