Synergistic effect of aged garlic extract and naltrexone on improving immune responses to experimentally induced fibrosarcoma tumor in BALB/c mice

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ABSTRACT

Background: Garlic, a medicinal plant, and Naltrexone (NTX), an opioid receptor antagonist, both have immunomodulatory and antitumor effects. Current study was designed to evaluate synergistic antitumor effects of aged garlic extract (AGE) and NTX. **Materials and Methods:** WEHI-164 fibrosarcoma cells were implanted subcutaneously on day 0 into right flank of 80 BALB/c mice at age of 8 weeks. Mice were randomly categorized in four separate groups: The first group received AGE (100 mg/kg, i.p.), the second group received NTX (0.5 mg/kg, i.p.), the third group received both of them, and the fourth group received phosphate buffered saline as control group. Treatments were administered three times per week. Tumor growth was measured and morbidity was recorded. Subpopulations of CD4+/CD8+ T cells were determined using flowcytometery. WEHI-164 cell specific cytotoxicity of splenocytes and *in vitro* production of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) cytokines were measured. All statistical analyses were conducted with SPSS 16 software and $P < 0.05$ was considered to be statistically significant. **Results:** The mice who received AGE+NTX had significantly longer survival time compared with the mice treated with AGE or NTX alone. An enhanced inhibitory effect on tumor growth was seen in combination therapy group. The CD4+/CD8+ ratio and *in vitro* IFN-γ production of splenocytes were significantly increased in AGE+NTX and NTX groups. WEHI-164 specific cytotoxicity of splenocytes was also significantly increased at 25:1 E:T ratio in AGE+NTX treated mice. Coadministration of AGE with NTX resulted in improvement of immune responses against experimentally implanted fibrosarcoma tumors in BALB/c mice. **Conclusions:** AGE showed synergistic effects with NTX on inhibition of tumor growth and increment of survival times.

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INTRODUCTION

Cancer has accounted for 7.6 million deaths globally in 2008 and it is estimated to continue to rise to over 11 million in 2030. Different therapies are available for different cancers based on the types and stages of the cancer and the tissues involved. While common therapies, like chemotherapy, are associated with numerous side effects,[1] supplemental therapy of cancers using dietetic ingredients, like alpha lipoic acid or medicinal herbs, in combination with medicinal drugs may result in enhanced efficacy of the generic therapies and improvement of quality of life of cancer patients.[2]

Naltrexone (NTX) a long-acting opioid receptor antagonist has extensively been used for managing opioid overdoses and addiction. NTX has also been used to inhibit tumor growth and different mechanisms were proposed to involve in its antitumor activity.[3] Indeed, binding of cell surface opioid receptors to low dose NTX leads to blockade of the receptors for 3–6 hours, followed by over expression of the receptors and related endogenous opioids, named opioid growth factors (OGFs, chemical term [Met(5)]-enkephalin).

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Over-expression of opioid receptors on cancerous cells and further binding of OGFs leads to regulation of cell metabolism and inhibition of uncontrolled proliferation of tumor cells. In other words, amplification of OGF–OGFr interface by NTX results in depression of neoplasia and angiogenesis. The OGF interacts with the OGF receptor (OGF–OGFr axis) to form an important growth-regulating pathway contributing to homeostasis and neoplasia. OGF bound to OGFr has inhibitory effects on cell proliferation.[4]

Exogenous opioids have immuno-modifying properties.[5] The cells involved in immunity have opioid receptors and binding of opioid to the receptors at regular doses leads to apoptosis of the cells.[6] Opioids have adverse effects on function of cytotoxic and natural killer cells, which may explain the high prevalence of malignancies in drug abusers. Administration of low doses of NTX inhibits proliferation and angiogenesis of tumor cells,[7] improves cytotoxic activity of natural killer cells,[8] enhances the proliferation potential of lymphocytes, shifts T-helper polarization toward Th1 cells, and also leads to an increase in CD4+ T cell counts.[9]

Garlic, Allium sativum, has immunomodulatory and antitumor effects *in vitro* and *in vivo*. Garlic shifts polarization of CD4+T cells toward Th1,[10] increases number and function of natural killer cells,[11,12] enhances frequency and proliferation potential of lymphocytes,[9] improves CD4+/CD8+ T cell ratio, and has antiproliferative and antiangiogenesis effects on tumor cells.[13]

Several purified chemicals from garlic or its crude extracts have been demonstrated to have obvious antitumor activities.[14] Direct preexposure of tumor cells with garlic extracts resulted in complete growth inhibition of implanted tumor cells. It is believed that malignancies are associated with increased production and release of potentially harmful free reactive oxygen species (ROS) and aged garlic extract (AGE) has antioxidant potential for scavenging the ROS. Furthermore, AGE acts as an enhancer for cellular antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), and also increases glutathione levels in the cells.[15] Antioxidant constituents of garlic result in enhanced antioxidant capacity of the body, improved immunity, and effective scavenging of free radicals.[16]

Garlic and NTX each have been individually investigated for their antitumor activities and their numerous positive effects have been confirmed. The present study investigated the effect of concomitant administration of AGE and NTX on experimentally induced fibrosarcoma cell tumors in BALB/c mice. Survival times, tumor volumes, CD4+/CD8+ T cell ratios in spleen, tumor specific cytotoxicity of splenocytes, and *in vitro* production of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) cytokines were analyzed and compared.

MATERIALS AND METHODS

Female inbred BALB/c mice and WEHI-164 fibrosarcoma cell line were purchased from Pasteur Institute, Tehran, Iran. Other reagents used for *in vitro* measurements were as follows: Naltrexone hydrochloride (Sigma, Germany), FITC-conjugated anti-CD4 (Serotec, UK), PE-conjugated anti-CD8 (Serotec, UK), Lactate dehydrogenase (LDH) release kit (Roche-Applied, Germany), IL-4 and IFN-γ cytokines kits (Quantikine, UK).

Garlic bulbs were peeled and minced in an aqueous-alcoholic solution and kept under anaerobic conditions for 8 months. Aged garlic was crushed using mortar and pestle and homogenized in distilled water. The homogenized preparation was filtered through Whatman paper No.1 and the filtrate was centrifuged at 4500*g* for 30 min. The clear supernatant was collected and used. The AGE (containing 0.4 g of garlic materials per ml) was diluted in distilled water and 100 mg/kg of the preparation was administered intraperitoneally to each mouse.

WEHI-164 cell line was used for generation of tumors in mice and as a target cell for Cytotoxic T-Lymphocyte assay. The cells were cultured using Roswell Park Memorial Institute (RPMI) 1640 (Gibco, USA) media, supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco, USA), and were incubated in 37°C in a humidified, 5% CO_2 atmosphere. Cells in logarithmic growth phase were used to establish tumor model by subcutaneously implantation of 1×10^6 cells/200 μl into the right flanks of the mice. Tumor dimensions were measured on 7 days intervals using Vernier caliper. Tumor volume (mm^3) was calculated by the formula length × width $2 \times \pi/6$.

A total number of 80 BALB/c mice at 6–8 weeks of age went under experimental induction of tumor by injection of WEHI-164 cells. Tumorized mice were randomly divided into 4 groups, the first group received AGE (100 mg/kg, i.p.), the second group received NTX (0.5 mg/kg, i.p.), the third group received both of them, and the fourth group (the control or PBS group) received only phosphate buffered saline (PBS). All injections were made every other day (three times per week) until the time of sacrifice of the mice (for *in vitro* study) reached or death occurred (for survival analysis). Half of the mice in each treated group were devoted to survival study and tumor volume measurements and the other half were euthanized on day 28 (contemporary with the first mortality in PBS group) for *in vitro* studies of

immunologic parameters. All procedures done on animals were in consistence with laboratory animal care and use law of Pasteur Institute of Iran.

For *in vitro* studies mice were euthanized on day 28 and the splenocytes were isolated as a single-cell suspension. Erythrocytes were then lysed at room temperature using ACK lysis buffer (NH_4Cl, $KHCO_3$, Na_2EDTA).

The isolated splenocytes were used for *in vitro* measurements after three times of being washed with PBS. The CD4+ and CD8+ T cells percentages, specific cytotoxicity of splenocytes against WEHI-164 cells and *in vitro* production of both IL-4 and IFNγ cytokines were determined.

Viability of isolated cells was determined by trypan blue exclusion method. The cells were resuspended in RPMI 1640 (GIBCO) supplemented with 10% FCS (GIBCO). The freshly prepared cells were analyzed by flow cytometry after immunostaining with two fluorochrome labeled antibodies (FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies). Each sample was immunostained with the antibodies for 45 min at 4°C. The cells were washed in washing buffer and fixed with 2% paraformaldehyde. The percentage of CD4+ and CD8+ T cells was determined by flow cytometric analysis of immunostained cells using an EPICS flow cytometer.

The cytolytic activity of splenocytes was measured by LDH release assay. Single cell suspension of splenocytes was prepared and used as effector cells. WEHI-164 cells (1×10^4 cells per 100 μl), as target cells, were incubated with 100 μl of effector cells suspension at effector/target ratios of 1:10, 1:25 or 1:50. LDH release assaying kit was used to measure amounts of LDH enzyme released from lysed cells. For low and high control wells (spontaneous release and maximum release, respectively) 100 μl of assay medium or 2% Triton × 100 in assay medium was added. All experiments were done in triplicate and percentage of specific cytotoxicity was determined using the following formula:

$$\text{Cytotoxicity}(\%) = 100 \times \frac{OD_{\text{experiment}} - OD_{\text{effector spontaneous}} - OD_{\text{target spontaneous}}}{OD_{\text{target maximum}} - OD_{\text{target spontaneous}}}$$

Cytokine release after *in vitro* stimulation of splenocytes with tumor cell lysate was measured using standard Enzyme link immune sorbent assay (ELISA) kits. Splenocytes at the concentration of 1×10^6 cells/well were cultured in the 24 well plates in presence of 50 μg/ml WEHI-164 cell lysate (prepared by sonication of the cells), 8 μg/ml PHA or complete media, in the total volume of 1 ml. RPMI-1640 supplemented with 10% heat inactivated FBS, 100 μg/ml streptomycin, 100 U/ml penicillin was used. The plates were incubated for 48 h at 37 °C in a humidified 5% CO2 atmosphere. Supernatants were collected after centrifugation, IFN-γ and IL-4 concentrations were determined using commercial Quantikine® sandwich ELISA assay kits.

Statistical analysis

Results from triplicate measurements were averaged. Statistical analysis was performed using the Student's *t*-test. Kaplan–Meier analysis was applied to compare survival time between groups and log-rank test was used for pair wise comparisons. A $P < 0.05$ was considered to be statistically significant. All statistical analyses were conducted with Statistical Product and Service Solutions (SPSS) 16 software.

RESULTS

Survival Analysis

Survival analysis was conducted parallel with *in vitro* study. Survival recordings were terminated when the last living mouse was dead. The *in vitro* study begun at day 28 when the first mouse died of tumor.

Survival analysis was done for 40 tumor bearing mice that had been treated using four different treatment protocols. Death of each mouse as a terminal outcome was recorded in respect with time. Kaplan-Meier analysis and simultaneous log-rank test were applied for total and pair wise comparisons. The increase in life span (ILS) of the treated groups was expressed as a percentage (ILS %). Median survival times (MST) was determined, and the percentage of ILS was calculated as:

ILS% = {[MST (days) of treated mice/MST (days) of control mice] − 1} × 100.

There were no noticeable differences between garlic and NTX treated groups considering mean survival times ($P = 0.706$). Garlic and NTX treated groups had increased ILS (60.6% and 54.5%, respectively), but the value was highest in the group receiving both of them (154.5%). Mean ± SE of survival times (day) in AGE+ NTX treated mice were 83.9 ± 4.2, in AGE treated group was 52.4 ± 2.1, in NTX treated group was 51.9 ± 2.2, and in control group was 35 ± 1.8.

Tumor volume following treatments

Mean ± SE of tumor volume was calculated for the four groups and expressed in mm^3. Time for tumor appearance was retarded significantly in combination therapy group. All mice in control group had palpable tumors on day 14 postimplantation, while the percentage of tumor bearing mice in AGE, NTX, and AGE+NTX groups were 2%, 4%, and 0% respectively. Tumors implanted in mice treated concomitantly with garlic and NTX showed significantly

slower growth rate ($P < 0.001$) compared with the other three groups. Garlic or NTX in this study's conditions (garlic: 100 mg/kg and NTX: 0.5 mg/kg three times per week) exerted approximately the same inhibitory effects on tumor growth and the effect of combined treatment was additive [Figure. 1].

Flow cytometric analysis of T CD4 + and T CD8 + cells in spleen

The effect of administration of garlic and NTX on spleen lymphocyte subpopulations was analyzed by flow cytometry. The results showed significant increase in CD4+/CD8+ ratios in AGE + NTX and NTX ($P < 0.001$) groups compared with the controls. AGE treated mice had modest increase in the ratio and the differences were not statistically significant ($P = 0.62$) in comparison to the control group. The mice receiving NTX had also significantly higher CD4+/CD8+ ratio in comparison to the group treated with AGE [Table. 1].

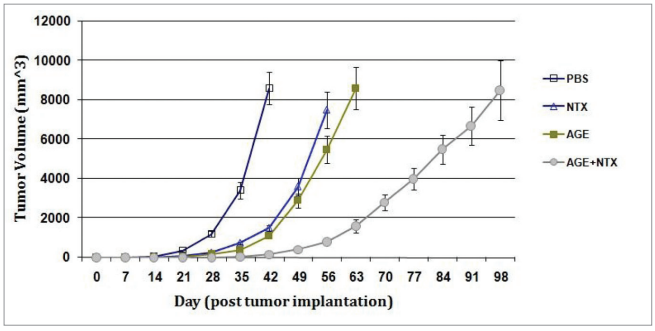

Figure 1: Tumor volume (mm^3) with respect of time (day) in different treatment groups

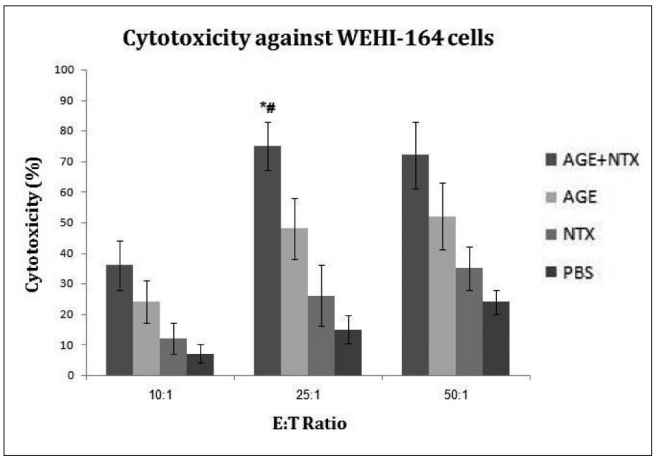

Figure 2: Percentage of cytotoxicity of splenocytes against WEHI-164 cells in three different E:T ratios. *Significant difference in comparison to PBS receiving group ($P < 0.01$). #Significant difference in comparison to NTX receiving group ($P < 0.01$) The observed cytotoxicity was also WEHI-164 specific because the splenocytes did not release considerable amount of LDH enzyme during the incubation period with colon carcinoma cell line CT-26 (data not shown)

In vitro cytotoxic activity of splenocytes

The cytotoxic activity of RBC-free splenocytes against WEHI-164 cells was assessed using LDH release assay. The splenocytes from AGE + NTX treated mice showed a significant increase in cytotoxic activity against the target WEHI-164 cells in comparison to the control and NTX groups ($P < 0.01$) (at E:T ratio 25:1), while, there was no significant difference between E:T ratios of AGE and NTX groups at all [Figure 2].

In vitro cytokine production of stimulated splenocytes

The splenocytes from the mice were analyzed for *in vitro* production of IFN-γ and IL-4 cytokines in response to stimulation by exposure to WEHI-164 cell lysate. The splenocytes of combined treated mice showed significant ($P < 0.01$) increase in the level of IFN-γ comparing the NTX or control groups. *In vitro* production of IFN-γ was also increased in AGE treated and NTX treated groups, but the differences were only significant between AGE and control groups ($P < 0.05$) [Figure 3].

There were no noticeable differences between groups in *in vitro* IL-4 production level [Figure 4].

Table 1: CD4+/CD8+ Ratio in splenocytes of different groups

CD4+/CD8+ Ratio in splenocyte		
Treatment	**Mean ± SE**	***P* value**
AGE + NTX *	5.02 ± 0.51	= 0.033
AGE	4.85 ± 0.51	= 0.062
NTX *	6.91 ± 0.70	< 0.001
PBS	3.6 ± 0.36	–

* Significance of differences compared with PBS group

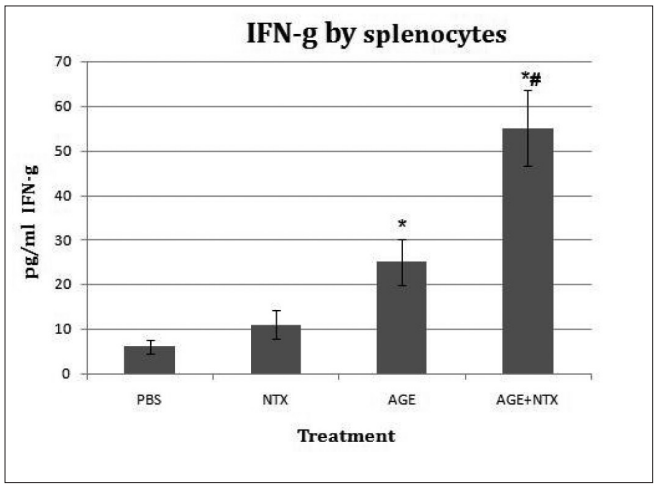

Figure 3: *In vitro* IFN-γ production of splenocytes after exposure to WEHI-164 cell lysate. *Significant difference in comparison to PBS treated group ($P < 0.05$) #Significant difference in comparison to NTX treated group ($P < 0.05$)

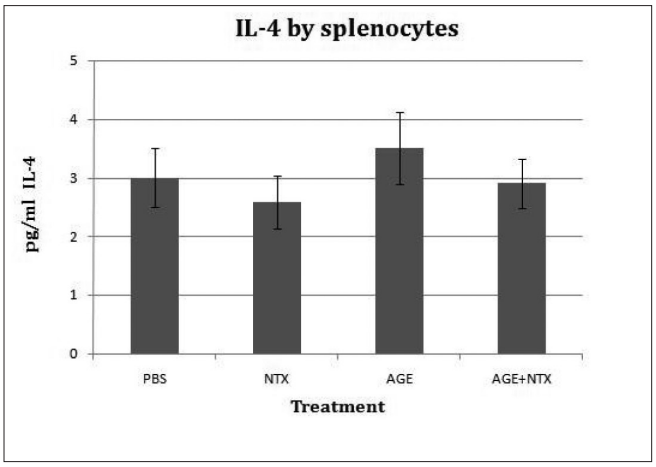

Figure 4: *In vitro* IL-4 production of splenocytes after exposure to WEHI-164 cell lysate

DISCUSSION

Accumulating evidence suggests that low dose Naltrexone (LDN) can promote health by supporting immune-modulation, which may reduce various oncogenic and inflammatory autoimmune processes.[17] Garlic can also modify the immune responses by different ways. Garlic increases antioxidant capacity of the cells and prepare improved circumstances for proper deciding and acting against threats. In this way, garlic exerts its modulatory effects on innate and adaptive immunity.[18] Intraperitoneal administration of AGE has led to an increase in macrophage counts and enhanced killing activities of the cells.[19]

In the present study it was decided to use the benefits of both AGE and NTX to inhibit fibrosarcoma tumor growth in BALB/c mice. NTX or AGE groups showed significant retardation in tumor appearance and combined administration of AGE and NTX resulted in further additive effects. All of PBS treated control mice showed visible tumors (100%) 14 days postimplantation, while combined treated mice had no palpable tumors on implantation sites (0%) and the tumors appeared about 35 days postimplantation. Administration of LDN and 100 mg/kg of AGE efficiently inhibited tumor growth and significantly increased life spans of these mice in comparison to controls.

LDN increased the latency from visible to measurable tumors up to 1.6-folds and markedly reduced tumor volume and weight, and decreased DNA synthesis in squamous cell carcinoma of the head and neck.[20] LDN treatment upregulated the expression of the OGF and its receptor, OGFr. Previous tissue culture studies have reported that OGF is the only opioid peptide with antiproliferative activity on ovarian cancer cells. The alteration of growth by NTX was also detected in cells representative of pancreatic, colorectal, and squamous cell carcinomas.[21]

It has been shown by using an *in vitro* assessment that direct exposure of tumor cells with AGE results in suppression of tumor cell growth and inhibition of the cell migration. It was concluded that garlic, as a natural plant may play a role in fighting cancer without significant side effects.[15] Present study with AGE (IP) administered 1 day after tumor implantation also showed significant retardation in tumor appearance and ILS (60.6% compared with control group). Combination of AGE with NTX resulted in further improvement in mice survival and tumor growth inhibition.

AGE inhibited the development of putative preneoplastic lesions in rat hepatocarcinogenesis, inducing a slowing in the proliferation rate of liver cells after partial hepatectomy.[22] It also has a chemopreventive effect on colon carcinogenesis through suppression of cell proliferation.[16]

For preliminary elucidation of the mechanisms involved in mouse resistance against the tumor, we assayed several immune parameters using splenocytes. For that splenocytes were used and CD4+/CD8+ ratio was calculated. The ratio was the highest in NTX treated mice ($P < 0.001$) and was high in AGE+NTX group ($P < 0.033$) but there were no significant differences between AGE and control groups. Explanation for lower ratio seen in combined treated mice (compared with NTX alone) is probably that administration of AGE results in increase in both CD4+ and CD8+ T cell counts, thus the ratio undergoes minimum changes.

The present study showed increased cytotoxic activity of splenocytes in AGE or NTX treated mice but the differences were statistically significant only in combined AGE+ NTX treated group. In addition, concomitant administration of AGE and NTX had an enhanced effect on fighting against tumor cells.

IFN-γ secretion by CD4+ Th1 cells, CD8+ T cells, gamma/delta T cells and activated NK cells plays an important role in activating lymphocytes to enhance antimicrobial and antitumor responses. Boyadjieva has reported that subcutaneous implantation of a NTX pellet for 2 weeks in ethanol consumed mice has led to significant increase in production of IL-2, IL-4, and IL-6 and IFN-γ .[9] It has been reported that Met-enkephalin (an endogenous opioid that is upregulated by LDN administration) effects the *in vitro* production of IFN-γ and IL-4 cytokines.[5] We found significant increase in IFN-γ production of splenocytes of AGE or combined treated mice. It seems garlic administration has a profound effect on IFN-γ production of splenocytes compared with low doses of NTX. Our findings showed that concomitant administration of

AGE and LDN induced effective immune responses against fibrosarcoma tumor in BALB/c mice and led to a significant inhibition of tumor growth and an increase in survival times of mice. Synergistic effects of garlic with NTX seen in the current study have potential application in prevention or treatment of various cancers.

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