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Original Article

Garlic Extract Can Induce Apoptotic Cell Death in The Human Colon Adenocarcinoma HT29 Cell Line

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ABSTRACT

Background and Objective: **Garlic has been known worldwide as a dietary constituent with many pharmacological effects. The present in vitro study was designed to investigate the putative anticancer effect of garlic extract on cancer cells, and if this effect was through apoptosis induction.**

Material and Methods: **Human colon adenocarcinoma cells HT29 were treated with different doses (1, 5 and 10 mg/ml) of fresh garlic extract in cell culture at 24, 48 and 72 h. Cell death was assessed by viability test, and its apoptotic nature was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. All experiments were statistically analyzed using SPSS 11.5 software**

Results: **Garlic extract induced a dose-dependent cell death with the highest values at 72 hours. The apoptosis analyses by TUNEL assay demonstrated that in each dose/time group the apoptotic values are much less prominent than the related cell death percentage found in viability test. In every dose, the sum of apoptotic cells in all three-time points was nearly equal to the percentage of cell death at 72 hours.**

Conclusion: **Fresh garlic extract can induce a dose-dependent apoptotic cell death in cultured HT29 cell line.**

Keywords: Garlic , HT29 cell , Apoptosis , TUNEL

Introduction

Cancer is still one of the most serious causes of death worldwide with no major progress in reducing its morbidity and mortality. Understanding the multistep nature of prolonged tumorigenesis process has led to the realization that most malignancies can be fought on multiple fronts. Thus in addition to cancer therapy, cancer prevention has become an important approach to control cancer (1). One of the common prevention strategies include cancer chemoprevention, which uses agents that slow the progression of or inhibits carcinogenesis in healthy subjects (2). A chemopreventive approach can involve the elimination of transformed cells through the induction of apoptosis. A list of putative cancer chemoprevention agents derived from dietary

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constituents and their synthetic derivatives are supposed to trigger apoptosis in tumor cells (3).

A large number of epidemiological studies have reported lower cancer rates among populations who consume large quantities of fruit and vegetables. This has led to considerable interest in the identification and characterization of natural chemopreventives found in diet (4). Among these, vegetables of the Liliaceae family, such as garlic and onion have received widespread support as beneficial nutrients which can prevent malignant disease. There has been growing interest on biological and pharmacological properties of garlic and its components particularly on cardiovascular system and prevention of cancer (5).

Garlic is a perennial plant made up of 65% water, 30% carbohydrates and 5% other components, mainly sulfur-containing compounds with pharmacological antiviral, antibacterial, antifungal, antioxidative, anticancer, and immunoreactive effects (6).

The present in vitro study was designed to asses the putative anticarcinogenic effect and the apoptosis induction capability of garlic extract on the human colon adenocarcinoma cell line HT29.

Materials and Methods

Preparation of garlic extract:

Native raw garlic (Hamadan/Iran) was commercially available. Following peeling, the chopped garlic cloves were spread on sterile filter papers and dried at 40±3°C temperature in a hot air oven. After cooling, cloves were powdered in a grinder. Ten gr of dried garlic powder was dissolved in 50 ml absolute ethanol and distilled water (2:1) for 24 hours at room temperature. The mixture was centrifuged for 15 min at 2000 rpm and the supernatants were filtered through sterile filter papers 0.2 µm (Whatman-Uk). Filtrates were concentrated 5 times at 37°C in an incubator so that a final volume of 10 ml was obtained as a 1 g/ml stock solution. The pH of stock solution was adjusted to 7.4 with NaOH (Merck-Germany) (7). The required final concentrations were obtained by diluting aliquots of the stock solution in culture medium supplemented with 2% FCS (Chemicon-Uk).

Cell line & cell culture:

Human colon adenocarcinoma cell line HT29**,** obtained from Pasteur Institute Cell Bank Tehran/Iran, was cultured in DMEM (Gibco-Uk) supplemented with 10% heat-inactivated FCS (Chemicon-Uk), 50 U/ml penicillin, and 50 µg/ml streptomycin (Gibco-Uk). Cells were grown in monolayers, incubated in a water-saturated atmosphere of 5% CO295%/ relative humidity at 37 °C. The medium was refreshed every 48h and the culture was passaged routinely by tripsin-EDTA 0.025% (Gibco-Uk). Cells were seeded onto 6-well plates (Falcon-USA) containing sterile glass coverslip at their bottom and allowed to adhere for 24 h (8). HT29 cells were treated with increasing concentrations of garlic extract (1, 5 and 10 mg/ml) for 24, 48 and 72 h. For each time point, a group of cells was incubated without garlic extract as control group. All treatment and control groups were treated at least in triplates.

Cell viability analysis:

HT29 cells cultured to about 80% confluence were harvested and viability was assessed by mixing aliquots of all suspensions with an equal volume of 0.4% trypan blue (Gibco-Uk). Cells that picked the dye were considered as dead and the viable cells were counted with a hemocytometer. The experiment was repeated independently three times (9).

Assessment of apoptosis:

Cells grown on coverslips were washed twice with PBS, air dried and fixed for 60 min in freshly prepared 4% paraformaldehyde / PBS (Sigma-Germany), pH 7.4, at room temperature. Then the cells were washed again twice with PBS and incubated with 3% H_2O_2/ methanol (Merck-Germany) for 10 min. Following washing with PBS, cells were permeabilized in 0.1% Triton X-100/PBS (Sigma-Germany) for 2 min at 4°C. Permeabilized cells were washed in PBS and the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay was performed as described in the In Situ Cell Death Detection Kit, POD (Roche-Germany). Briefly samples were incubated in 50 µl of TUNEL reaction mixture (5 µl enzyme solution containing terminal deoxynucleotidyltransferase (TdT) from calf thymus

in storage buffer, and 45 µl label solution containing FITC-labeled dUTP nucleotides in reaction buffer) for 2 h at 37°C in a humidified chamber and in the dark, covered with parafilm. Omission of TdT provided the negative control for the assay, and preincubation of cells with 10 µg/ml DNase I in 50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$ and 1 mg/ml BSA for 10 min at room temperature to induce DNA strand breaks artificially, served as positive control. Cells were washed with PBS and incubated for 30 min in a humidified chamber, at 37°C with 50 µl converter-POD (Anti-fluorescein antibody, Fab fragment from sheep, conjugated with horse-radish peroxidase). After rinsing in PBS, the samples were incubated for 10 min with 100 µl DAB (Sigma-Germany) substrate in the dark. At the end, the samples were mounted and analyzed under light microscope, where the apoptotic cells could be seen as condensed shrinked dark brown cells. At least 100 cells were counted and the percentage of apoptotic cells was calculated.

Statistical analysis:

All experiments were statistically analyzed using SPSS 11.5 software. To compare the effect of different concentrations of garlic extract on viability and apoptosis induction the two-tailed unpaired t-test and one-way ANOVA was used and the *P* value < 0.05 was assumed as significant.

Results

Viability analysis:

Viability test showed that the garlic extract induced cell death of HT29 cells in a dose dependent manner. As indicated in Table 1, 1 mg/ml of garlic extract induced 16, 24 and 31% cell death compared to controls after 24, 48 and 72 h respectively. Five mg/ml of garlic extract induced 23, 32 and 37%, and 10 mg/ml resulted in 29, 38 and 41% cell death at the same time points respectively. In all groups, the number of cells in the control samples at each time point was considered as 100% and the percentage of cell death was calculated according to it. Statistical analysis indicated a significant induction of cell death by all three doses of garlic extract compared to controls. The comparison of values related to each dose (rows of Table 1), indicated that in each dose the difference between percentage of cell death at different time points was statistically significant, except between 48 and 72 h in the dose of 10 mg/ml (38 and 41%). In addition, the comparison of values resulted from different doses at each time point (columns of table 1), indicated significant differences between variant doses, except between doses 5 and 10 mg/ml at 72 h (37% and 41%).

Table 1: The findings of viability test.

Doses of garlic extract	**Time points of treatment**		
	24 h	48 h	72 h
1 mg/ml	16	24	31
5 mg/ml	23	32	37[b]
10 mg/ml	29	38[a]	41[a,b]

The values are indicated as percentage of cell death induced by different doses of garlic extract and in various time points, compared to controls. The cell death of control group for each time point has been considered as zero. ***a*** indicates insignificant difference between values related to 48 and 72 hours in 10 mg/ml, and ***b*** indicates insignificant difference of values related to 5 and 10 mg/ml at 72 hours .

Apoptosis analysis:

To determine further if garlic extract-induced cell death was caused by apoptosis, we used TUNEL assay and DAB as chromogen, which demonstrates apoptotic cells as highly condensed brown, cells (Fig. 1). Table 2 represents the results of TUNEL assay as the percentage of apoptotic cells to total cells. One mg/ml garlic extract revealed 14, 10 and 8%; 5 mg/ml resulted in 18, 12 and 9%, and 10 mg/ml caused 20, 13 and 11% apoptosis at 24, 48 and 72 h respectively. In all three doses of garlic extract (rows of Table 2), the difference between values of 48 and 72 h were statistically insignificant. In all three doses, the sum of apoptotic cells of the three time points was calculated (The last column of Table 2), and the comparison of the two tables indicates that although at each time point the apoptosis rate is much less than the cell death values, the sum of apoptotic cells is nearly the same as the cell death percentage at 72 h.

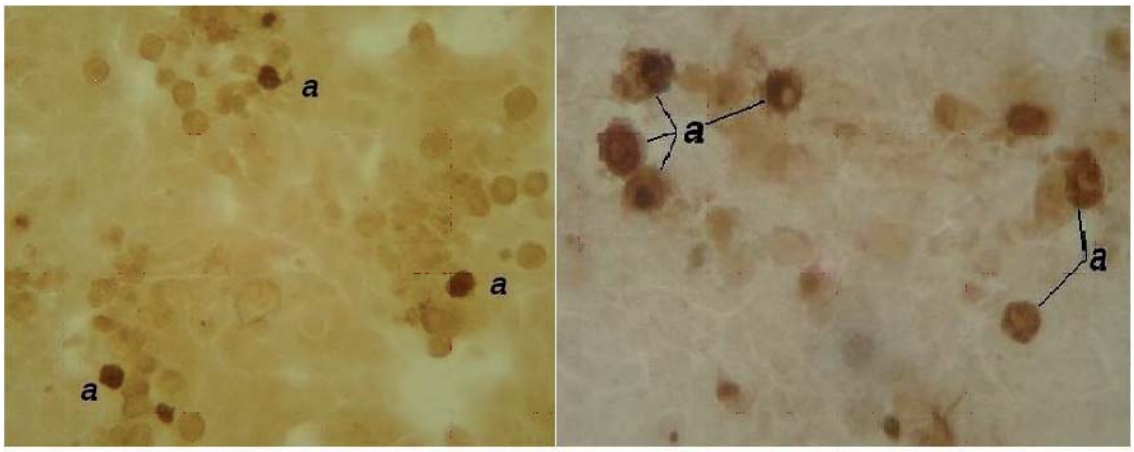

Fig. 1: Two photomicrographs of TUNEL assay stained with DAB as chromogen, demonstrating experimental groups treated with 1 (**A**) and 10 mg/ml (**B**) garlic extract for 72 h. Among the nuclei of many healthy HT29 cells, apoptotic cells (**a**) can be seen as dark brown condensed figures

Table 2: The findings of apoptosis analysis.

Doses of garlic extract	Time points of treatment			The sum of apoptotic cells
	24 h	48 h	72 h	
1 mg/ml	14	10 [a]	8 [a]	32
5 mg/ml	18	12 [b]	9 [b]	39
10 mg/ml	20	13 [c]	11 [c]	44

The values are indicated as percentage mean of apoptotic cells to total cells in each dose/time point. The last column represents the sum of apoptotic cells in all three-time points. ***a***, ***b*** and ***c*** represent insignificant difference between values related to 48 and 72 hours in 1, 5 and 10 mg/ml groups respectively

Discussion

Our findings indicate a dose-dependent induction of HT29 cell death by garlic extract, which for doses of 1 and 5 mg/ml is most obvious at 72 h, but for 10 mg/ml, where the difference between cell death values at 48 and 72 h is insignificant, the most efficiency can be obtained at 48 h. The TUNEL assay proved that the nature of induced cell death was mainly apoptosis. Table 2 demonstrates that at each time point the percentage of apoptosis is much less than the related percentage of cell death at the same time, which can be explained by the fact that apoptosis is a fast process and the apoptotic cells will be rapidly removed from the field and only some of the apoptotic cells can be detected at each moment. So at 72 hours, when the highest cell death can be calculated, most of the apoptotic cells already have been removed, but the sum of apoptotic cells of all three-time points is nearly equal to the cell death percentage at 72 h.

The modern era of the use of garlic as anticancer agent begins in the 1950s when it was demonstrated *in vitro* and *in vivo* that thiosulfinate extracts from garlic inhibited the tumor cells growth. Since these investigations, many epidemiological and laboratory studies have been developed to evidence the garlic chemopreventive or anticarcinogen effects (10). Experimental carcinogenesis studies indicate that components of garlic inhibit both the initiation and promotion of tumorigenesis in various types of cancer, including colorectal, lung and skin cancers (11). It has been reported that garlic powder in the diet could inhibit mammary tumors (12), and a garlic extract decreased the incidence of cervical carcinoma (13).

Several mechanisms have been proposed to explain the cancer-preventive effects of *Allium* vegetables

and related organosulfur compounds. These include inhibition of mutagenesis by inhibiting the metabolism, inhibition of DNA adduct formation, free radical scavenging, and effects on cell proliferation and tumor growth. Although there are evidences supporting these mechanisms for organosulfur compounds, they are still speculative and further research is needed to support causality between such properties and the cancer-preventive activity in experimental animals (5).

Recent findings support a growing body of evidence that garlic works as an anticarcinogen in both prevention and treatment in sarcoma, mammary carcinoma, hepatoma, colon cancer, and squamous cell carcinoma of the skin and esophagus (14, 15). A number of studies have demonstrated the chemopreventive activity of garlic by using different garlic preparations including fresh garlic extract, aged garlic, garlic oil, and a number of organosulfur compounds derived from garlic. Biological effects of garlic are attributed to its characteristic organosulfur compounds such as allicin (diallylthiosulphate) which is the principal active substance of fresh garlic extract. Allicin, which is produced by the interaction between the non-protein amino acid alliin and the enzyme alliinase, is unstable and readily decomposes under uncontrollable chemical reactions to produce diallyl sulfide, diallyl disulfide, diallyl trisulfate, allyl methyl sulfide, dithiins and ajoene (15). Alicin can easily diffuse into the internal volume of vesicles or into the cytoplasm of red blood cells, and lipid bilayers do not constitute a barrier for allicin penetration and its diffusion (16). These findings raise the possibility that in biological systems allicin can penetrate very rapidly into different compartments of the cells and exert its biological effects. Thus, the significance of allicin as a biological effecter molecule is not only due to its high reactivity with low and high molecular weight thiols and its prominent antioxidant activity, but also to its accessibility resulting from high membrane permeability (17). Allicin inhibited proliferation of human mammary, endometrial and colon (HT29) cancer cells (18).

Another water-soluble garlic derivative S-Allylmercaptocysteine has been reported that can inhibit growth, arrest cell cycle progression at M-phase, and induce apoptosis in HT29 cells (19). The activation of JNK1 and caspase-3, and depolymerization of cellular microtubules play important roles in S-Allylmercaptocysteine-induced apoptosis (11). Prevention of malignant transformation may result from inhibition of procarcinogens by garlic's effect on cytochrome P450 enzymes, antioxidant activity, or detoxification by binding sulfur compounds in garlic (15).

Several investigations have shown that both water- and lipid-soluble sulfur compounds from garlic provide their anticarcinogen benefits (10). Karasaki *et al*. reported that after treatment of human histiocytic lymphoma cells (U937) with different doses of garlic lectin for 72 h, nearly 30% of cells underwent apoptosis, which is almost similar to our results; they proposed that garlic lectin binds to cellular membrane of tumor cells to exert its cytotoxic effect (20). Direct inhibition of cancer cell growth in tissue culture has been documented in sarcoma as well as gastric, colon, bladder and prostate carcinoma cell lines (15).

Conclusion

Fresh garlic extract can induce a dose-dependent apoptotic cell death in cultured HT29 cell line. We have found the highest percentage of cell death after 72 hours, which is nearly equal to the sum of apoptotic values of all three-time points.

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