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Original Article

The Pro-apoptotic Effect of Allicin on Human Colon Adenocarcinoma Cell Line HT29

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ABSTRACT

Background and Objective: **The management of apoptotic cell death has been considered as a putative therapeutic strategy for cancer treatment. In the present study we investigated the putative pro-apoptotic effect of allicin, the main garlic organosulfur component with repeatedly claimed chemopreventive potency, on the human adenocarcinoma cell line HT29 as an apoptosis resistant cell line, in vitro.**

Materials and Methods: **The HT29 cells were incubated with different concentrations of allicin (0-40µg/ml) and for different time periods (6-48h) to investigate its effect on cell proliferation and apoptotic cell death.**

Results: **Five and 10µg/ml allicin could induce a significant cell death only after 12h, whereas concentrations of 20 and 40µg/ml resulted in a significant cell loss as soon as 6h. The results of the TUNEL assay, presented as percentage of apoptotic cells to total cell loss, indicated that concentrations ≥5µg/ml significantly increased the apoptotic features in time periods 6-24h, but after 48h no significant changes could be detected. The ratio of the sum of the apoptotic features of the four studied time points to the total cell loss calculated after 48h was about 0.5.**

Conclusion: **Allicin can induce apoptosis in a concentration- and time-dependent manner with most considerable effects achieved at 24h and by concentrations higher than 10µg/ml.**

Keywords: **Allicin, Cell line, Apoptosis, Colon, Adenocarcinoma**

Introduction

Adenocarcinomas account for most cases of colorectal cancer and HT29 cell line, a well-known apoptosis resistant human adenocarcinoma cell line, is routinely used in a wide bulk of studies on cancer and its putative treatments. Concerning the multistep nature of prolonged tumorigenesis process, most malignancies can be fought on multiple fronts. Cancer chemopreven-

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tion can be attributed to therapeutic strategies which slow down the progression or inhibit carcinogenesis in healthy objects, and may involve the elimination of transformed cells through the induction of apoptosis (1). Insufficient apoptosis as a well-controlled process of cell death plays an essential role in tumor progression and therapy resistance (2).

A bulk of evidence claiming lower cancer rates among populations who consume large quantities of fruit and vegetables has led to the identification of natural chemopreventives (3), such as Liliaceae family members' onion and garlic (4). Garlic contains organosulfurs which can selectively inhibit tumor proliferation through controlling DNA repair mechanisms, chromosomal stability and cell cycle regulation (5). Experimentally, garlic and its associated organosulfurs are reported to suppress the incidence of tumors of rodent models in breast, colon, skin, uterus, oesophagus and lung (6, 7).

Allicin (diallylthiosulphate), one of the most biologically active garlic organosulfur compounds (8), is produced by the interaction between the non-protein amino acid alliin and the enzyme alliinase. Allicin is unstable and readily decomposes under uncontrollable chemical reactions to produce diallyl sulfide, diallyl disulfide, diallyl trisulfate, allyl methyl sulfide, dithiins and ajoene (6, 9). Since lipid bilayers do not constitute a barrier for allicin diffusion (10), the significance of allicin as a biological effecter molecule can also be attributed to its accessibility resulting from high membrane permeability (11). Despite the controversies regarding stability of allicin in biological fluids, since most of the biologically active compounds of garlic are derived from allicin, its estimation may be a necessity for varietal evaluations (12).

Because some of the garlic organosulfur compounds can induce apoptosis in tumor cells of different tissue origins (13), apoptosis could be a potential mechanism of anticarcinogenic activities of individual garlic components (6). Although in vitro studies have provided evidence for pro-apoptotic effects of allicin against various cancer cells, the mechanism by which allicin exerts its apoptotic effect is not fully understood (14, 15).

Following our last study indicating the apoptosis-inducing effect of the garlic extract on the HT29 cells (1), in the present study we investigated the putative pro-apoptotic effect of allicin, the major biologically active component of garlic and a precursor of a number of secondary products formed in garlic preparations, on HT29 cell line as an apoptosis resistant cell line.

Materials & Methods

Allicin

Allicin (LKT Laborataries, Inc. St Paul. MN. USA) was dissolved as 10 mg/ml in methanol/water/formic acid (60/40/0.1) and stored as the stock solution at -20℃. Five calibration standards of allicin with concentrations of 1,5,10, 20, and 40µg/ml were prepared after appropriate dilutions of the standard stock solution.

Cell line & cell culture

The human colon adenocarcinoma cell line HT29**,** obtained from Pasteur Institute Cell Bank Tehran/Iran, were cultured in DMEM (Gibco-Uk) supplemented with 10% heat-inactivated FCS (Chemicon-Uk), 50U/ml penicillin, and 50µg/ml streptomycin (Gibco- Uk). Cells were grown in monolayers, incubated in a water-saturated atmosphere of 5% CO_2/95% relative humidity at 37℃. The medium was refreshed every 48h and the culture was passaged routinely by tripsin-EDTA 0.025% (Gibco-Uk). Cells were seeded ($5x10^5$cells/well) onto 6-well plates (Falcon-USA) containing sterile glass coverslips at their bottom and allowed to adhere for 24h. HT29 cells were treated with increasing concentrations of Allicin (1,5,10,20 and 40µg/ml) for 6,12,24 and 48h. For each time point, a group of cells was incubated with the equal volume of the vehicle (methanol/water/formic acid: 60/40/0.1) as the

control group. All treatment and control groups were treated at least in triplets.

Cell viability analysis:

Cell viability was settled by the trypan blue dye exclusion assay, briefly 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). Dead cells picked the dye and the viable cells could be counted with a hemocytometer. The experiment was repeated independently three times.

Assessment of apoptosis by TUNEL assay

Cells grown on coverslips were washed twice with PBS, air dried and fixed for 60min 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 2min at 4℃. 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 50µl of TUNEL reaction mixture (5µl enzyme solution containing terminal deoxynucleotidyltransferase (TdT) from calf thymus in storage buffer, and 45µl label solution containing FITC-labeled dUTP nucleotides in reaction buffer) for 2h at 37℃ 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 10µg/ml DNase I in 50mM Tris-HCl, pH 7.4, 1mM MgCl2 and 1mg/ml BSA for 10min at room temperature, to induce DNA strand breaks artificially, served as positive control. Cells were washed with PBS and incubated for 30min in a humidified chamber, at 37℃ with 50µl converter-POD (Anti-fluorescein antibody, Fab fragment from sheep, conjugated with horse-radish peroxidase). After rinsing in PBS, the samples were incubated for 10min with 100µl Diaminobenzidine (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 shrunken dark brown cells. At least 100 cells were counted and the percentage of apoptotic cells was calculated.

Statistical analysis

Each experiment was performed at least three times, and the results were expressed as mean values ± S.D. To compare the effect of different concentrations of Allicin 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

The cell viability analysis in different experimental groups:

The cell viability was analyzed in the experimental groups treated with different concentrations of allicin (1, 5, 10, 20, 40µg/ml) for 6, 12, 24 and 48h. In all time points the number of viable cells in the vehicle treated control groups, was considered as 100% and the values of other groups were calculated relative to it. One µg/ml allicin did not exert any significant effect on cell growth, so that even after 48 hours still about 90% of the cells were viable (Fig. 1). The data of viability tests of 5 and 10µg/ml concentrations of allicin indicated that these two doses needed a least 12h treatment period to induce a statistically significant cell death ($P<0.05$). Nevertheless higher concentrations of 20 and 40µg/ml allicin induce a significant cell death as soon as a 6h treatment ($P<0.05$), with obviously more considerable effects after 12h ($P<0.01$) and 24 or 48h ($P<0.001$). The above data can be summa-

rized as that the 5µg/ml allicin could induce a statistically significant cell death (P<0.05) after 12h, but yet concentrations equal and higher than 10µg/ml were needed to obtain obviously more significant effects (P<0.01).

On the other hand, regarding the duration of the allicin treatment, it could be shown that a 6 hour period is not an adequate time for the drug to make apparent its cell death-inducing effect, unless concentrations higher than 20µg/ml were used, but after 12h a concentration as low as 5µg/ml was enough to induce a significant cell death (P<0.05). Although all of the studied concentrations of allicin, except 1µg/ml, induced an obviously high incidence of cell death after 48h, in each of the three experimental groups treated with 10, 20 and 40µg/ml, the difference between the induced cell death at 12h and 24h is more prominent (P<0.05) than the difference between 24h and 48h (P>0.05), indicating that allicin exerts its effect mostly between 12 and 24 hours following treatment (Fig. 1). The above mentioned findings of cell viability assay indicated that allicin could inhibit cell growth in a concentration- and time-dependent manner with most considerable effects achieved after 24h and by concentrations higher than 10µg/ml.

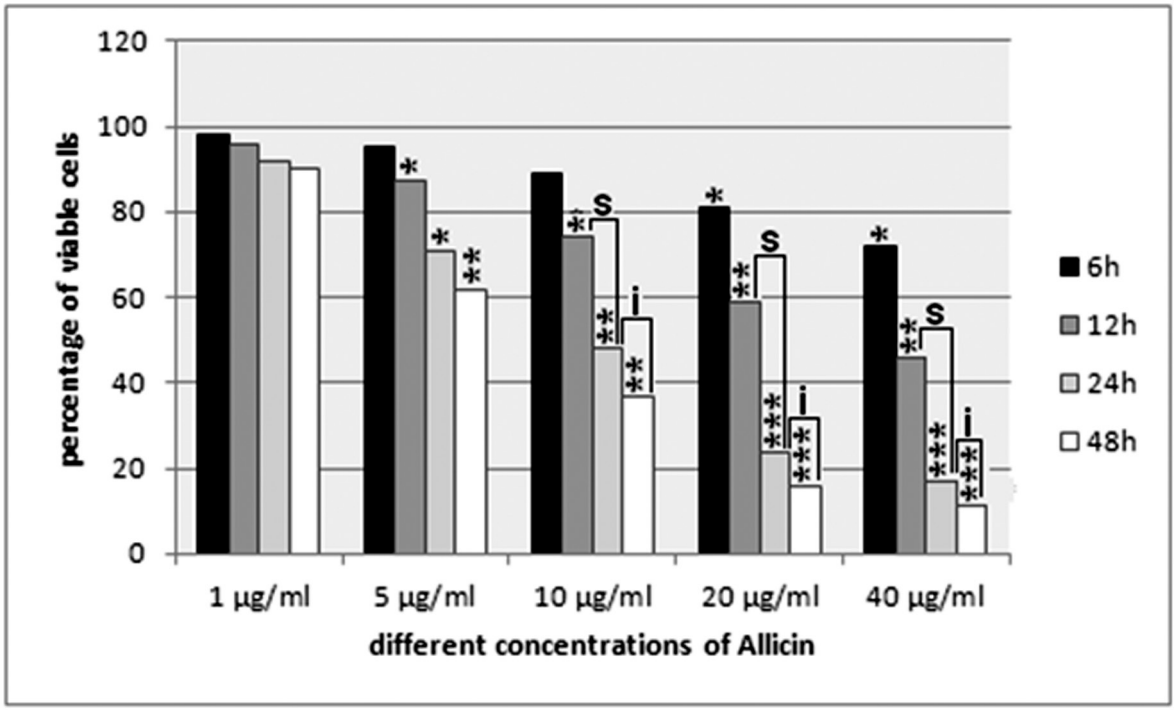

Fig.1: The percentage of viable HT29 cells after treatment with various concentrations of allicin in different time points. The stars indicate the statistical significance between each of the treated groups and the control group in every time point (*= P<0.05, **= P<0.01, ***= P<0.001). s and i indicate the significant differences between 12h and 24h, and the insignificant differences between 24h and 48h treatments respectively, in each of the allicin treated groups

The apoptosis rate induced by different concentrations of allicin:

In the TUNEL assay prepared micrographs, the apoptotic cells could be seen as condensed shrunken dark brown features (Fig. 2). The data of TUNEL assay, as percentage of apoptotic cells induced by different concentrations of allicin, have been summarized in Table 1. The values of apoptotic features at different time points in the group treated with 1µg/ml of allicin represented more or less similar results with the control group receiving only vehicle (first column of Table 1),

indicating the inadequacy of 1µg/ml of allicin to significantly affect the apoptosis rate. Treatment of the cells with allicin for 6 hours indicated that higher concentrations of allicin could induce progressively more increased apoptotic percentage, with the highest values obtained by 40µg/ml. Although the 24h treatment of allicin induced a statistically significant increase in apoptotic percentage with the concentrations 5 and 10µg/ml, but in the presence of higher doses of 20 and 40µg/ml no more increase occurred. And finally incubation of the cells for 48 hours with each of the different concentrations of allicin could not increase the apoptotic percentage significantly (Table 2 and Fig. 3).

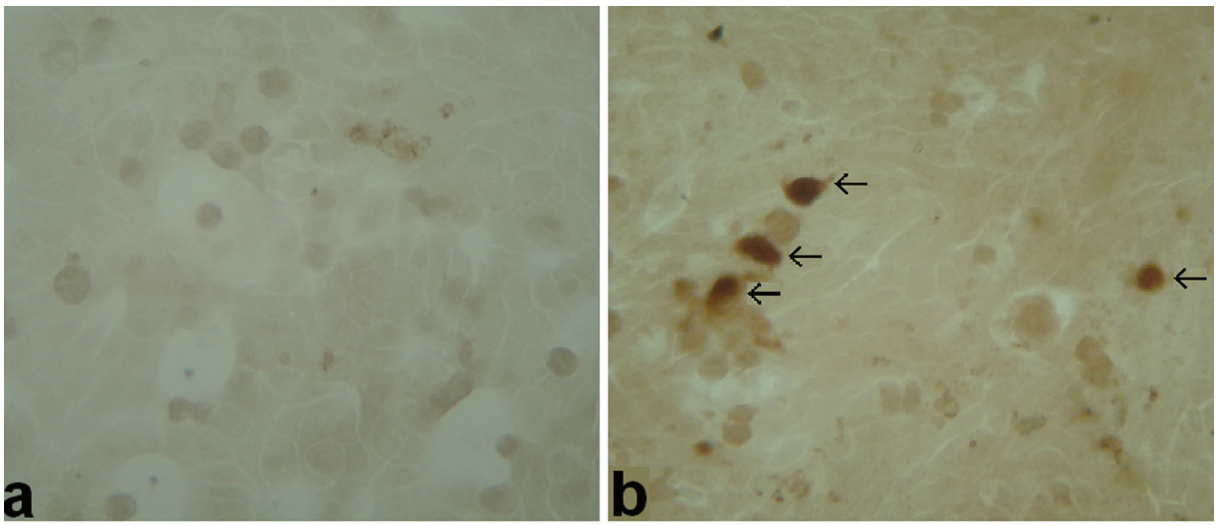

Fig. 2: The photomicrograph of TUNEL assay stained with DAB as chromogen, demonstrating the control (a) and the experimental group treated with 10µg/ml allicin for 48h (b). The arrows indicate the apoptotic features of HT29 cells (×400)

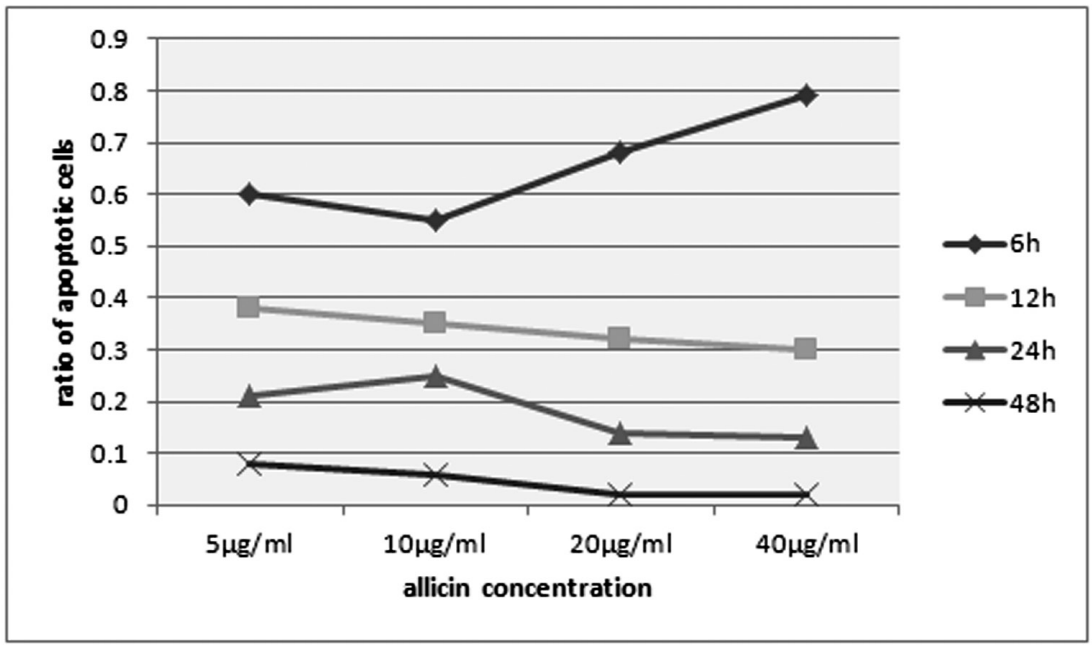

Fig. 3: The ratio of apoptotic features detected by TUNEL assay, to the calculated cell loss in different time points, indicating the most and least prominent ratios at 6 and 48 hours following the treatment, respectively. The data of 1µg/ml, which were almost similar to the control group, have not been presented in this figure

Table 1: The mean percentage of apoptotic HT29 cells after the in vitro treatment with different concentrations of allicin as experimental groups and the vehicle treated control groups.
The last row represents the sum of apoptotic features of all of the four time points in each of the control and experimental groups. The stars indicate the statistical significance between each of the experimental and the control group (vehicle) in every time point (*= $P<0.05$, **= $P<0.01$, ***= $P<0.001$)

	vehicle (control)	Experimental allicin-treated groups				
		1µg/ml	5µg/ml	10µg/ml	20µg/ml	40µg/ml
6h	1	2	3*	6**	13***	22***
12h	0	1	5*	9**	13***	16***
24h	2	2	6*	13**	11**	11**
48h	1	0	3	4	2	2
Sum	4	5	17*	32**	39**	51***

Table 2: The ratio of the sum of apoptotic features of all studied time points to the cell loss occurred after 48h treatment with different concentrations of allicin.

	1µg/ml	5µg/ml	10µg/ml	20µg/ml	40µg/ml
The sum of apoptotic values	5	17	32	39	51
Cell loss at 48h	10	38	63	84	89
The ratio	0.5	0.45	0.51	0.46	0.57

The sum of the apoptotic features induced by each of the examined concentrations, as well as the cell loss occurred following 48 hours increased progressively by higher doses of allicin, and the ratio of these two values in every experimental group, is more or less equal to 0.5 (Table 2). In other words, only half of the total dead cells could be detected as apoptotic features in the four selected time points.

Discussion

The findings of this study indicated a concentration- and time-dependent pro-apoptotic effect of allicin on HT29 adenocarcinoma cells, so that concentrations equal or higher than 5µg/ml and treatment durations longer than 12h were needed to induce a statistically significant cell death ($P<0.05$), and to achieve the most prominent cell loss, ($P<0.001$) yet higher concentration of 20 and 40 µg/ml, and least treatment durations as long as 24h were required. Also we have indicated that though the highest cell death has been achieved at 48h, but most of this cell loss occurred between 12 and 24h following the treatment.

A 22h treatment of HL60 cells with 5 and 10µM of allicin could inhibit the cell proliferation about 50 and 80% respectively, and that a 22h treatment of U937 cells with 15 and 20µM concentrations resulted in 50 and 60% inhibition of cell proliferation respectively (14). Moreover Park *et al.* following exposing the human gastric carcinoma AGS cells to different doses of allicin (1-100µg/ml) and for different time periods (6-72h), reported a dose- and time-dependent anti-proliferative effect where for example 10µg/ml of allicin could induce an almost 78.5% cell death following 24h treatment (15). The data of

both above studies showed some diversities with our findings, which may be due to the different cell types, and indicates that various cells can respond differently to a common signal.

Our data indicated that in each of the studied time points except the 6h, the ratio of apoptotic features to the cell loss percentage is clearly low, so that the ratio of the sum value of apoptotic features seen in all four studied time points is only about half of the cell loss occurred following 48h (Table 2). However at 6h the percentage of apoptotic features is clearly much higher than other time points, because apoptosis is usually a fast phenomenon starting very quickly in the first hours after injury, and so most of them can be detected easily following a short period. On the other hand since the apoptotic cells will be omitted from the tissue very quickly through different mechanisms, only a little proportion of the apoptotic cells can be detected after longer time periods (Fig. 3).

Apoptotic cell death can be considered as a target for therapeutic strategies in the research of cancer chemoprevention (7). A bulk of in-vivo and in-vitro studies have indicated the potent chemopreventive effect of garlic and its contained organosufur compounds on several types of cancer cells, which can inhibit both initiation and promotion of tumorigenesis through inhibiting cell proliferation, arresting the cell cycle, inducing cell apoptosis, blocking invasion and metastasis, inhibiting the metabolism, inhibition of angiogenesis and inhibition of oncogene activity or DNA synthesis (9, 16). 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 (17).

It has been demonstrated that the organosulfur allicin is the most powerful medicinal compound of garlic which provides its greatest reputed benefits. Allicin does not occur in ordinary garlic, but after chopping or crushing of the cloves will be produced by the interaction of alliin and alliinase, which are enclosed in different compartments within garlic clove cells with proportions of 1.7% and 2.8% of dry weight of garlic bulbs respectively (14). Allicin starts to degrade immediately after it is produced, so its medical effectiveness decreases over time and due to its unstable nature it has been doubted whether the observed effects of allicin is due to the allicin itself or its other metabolites (18).

Allicin can easily diffuse through phospholipid cell membranes to the cytoplasm which may strengthen its biological activity in the target cells (6). Allicin can exert its biological effect by reacting with free intracellular thiols, such as reduced glutathione which is the most abundant thiol molecule in the cytosol and mitochondria (18).

Apoptosis induction by diallyl disulfide, which is produced as a result of decomposition of allicin, involves different apoptotic genes and enzymes such as up-regulation of bax, down regulation of bcl-2 and bcl-XL, release of cytochrome c, activation of caspase-3 and caspase-9, increase of intracellular free calcium, cleavage of caspase-3 and PARP (19), all of which suggest the classical mitochondrial pathway of diallyl disulfide-induced apoptosis (7). The increased intracellular H_2O_2 and other ROS within 30 minutes following treatment with diallyl disulfide indicated that ROS formation is the earliest event in the cascade of induced apoptosis (20), and oxidative stress may be the triggering factor in diallyl disulfide-induced apoptosis (7).

The rapid consumption and disappearance of allicin from the medium, due to its penetration into the cells and its reaction with SH groups, enables full activity of SH-dependent apoptotic enzymes such as the cysteine proteases and caspases 9 and 3 (21). Allicin and its prominent derivative diallyl sulfide, through increasing the level of p53 and bax, and decreasing the antiapoptotic protein bcl-2 can release cytochrome-c from

the mitochondria to the cytosol, which is a prerequisite for apoptosis, and that bcl-xL does not protect the treated cells from allicin-induced apoptosis (15, 22).

Many studies have demonstrated the apoptosis-inducing capacity of allicin in several other tumor cell lines: SiHa (human cervical caner cell line), L929 (murine fibrosarcoma), SW480 (human colon cancer), HeLa (human cervical cancer cell line), AGS (human gastric cancer), SGC-7901 cells (gastric cancer cells), HL60 and U937 cell lines (lukemia) (6, 15, 23, 24)**.** Allicin induces apoptosis in human epithelial carcinoma through a caspase-independent pathway, mediated by the release of apoptotic-inducing factor (AIF) from mitochondria and protein kinase A (PKA) activation (15). On the contrary, some certain tumor cells could evade the allicin-induced apoptosis which has been suggested to be because of their genetic background and the changes of the contained apoptosis-associated proteins, such as overexpression of antiapoptotic protein bcl-2, downregulation and mutation of proapoptotic gene bax, decreased release of mitochondrial cytochrome-c, alterations of p53, phosphatidylinositol 3-kinase/Akt or nuclear factor kappa B (NF-nB), and decrease of caspase-3 (23, 25). Cho and his colleagues after studying the effects of allicin on depleted nutritional macrophages, and indicating its apoptosis-inhibiting effect on malnourished immune cells, reported that the treatment of allicin may be a benefical therapeutic treatment for immune-suppressive diseases caused by malnutrition (23).

Conclusion

The findings of this study have proved the hypothesized pro-apoptotic and anti-proliferative effects of allicin on the apoptosis-resistant colorectal cancer cell line HT29, which act in a dose- and duration-dependent manner, with the most prominent effects achieved by concentrations higher than 10µg/ml and treatment durations longer than 24 hours. These first results can be considered as the basis for the analysis of the molecular signal transduction mechanisms behind them.

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