Caspase-independent cell death by allicin in human epithelial carcinoma cells: involvement of PKA

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Abstracts

Allicin, the major component of Garlic (Allium sativum) was examined for the ability to induce apoptosis and the mechanism of the induction of apoptosis in human epithelial carcinoma cells. Allicin inhibited cell growth and induced apoptosis in gastric epithelial cells. Treatment with allicin resulted in morphological changes, DNA fragmentation, hypodiploid DNA contents and the translocation of Bax to mitochondria. The release of cytochrome c from mitochondria into the cytosol, which is an initiator of the activation of caspase cascades, was observed in allicin-treated cells. However, pretreatment with Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), a broad spectrum of caspase inhibitor, could not rescue apoptotic cells from allicin toxicity. Coincidently, caspase-3 activation and cleavage of PARP were not detected. In addition, caspase independent apoptosis-inducing factor (AIF) was released from mitochondria after treatment with allicin. After pre-incubation of cells with the protein kinase A (PKA) inhibitor H-89, allicin was not capable of inducing an increase of the rate of apoptosis with affecting the expression levels of Bax and AIF. These data demonstrate that allicin induces a caspase-independent apoptotic pathway mediated by mitochondrial release of AIF and PKA appears to be involved in allicin-induced apoptosis in gastric epithelial cells.

1. Introduction

Garlic (Allium sativum) has been used as a general food and a remedy in Oriental for a long time. Previous investigations have shown that garlic plays an important pharmacological role as an anti-microbial [1], anti-thrombotic [2], anti-hypertensive [3,4], anti-hyperglycemic [5] and anti-hyperlipemic [6,7] agent. It has also been known that garlic reduced the risk of hypercholesterolemia which was derived from the induction of iNOS [8]. In addition, it has been recently demonstrated that garlic may reduce some cancer risk. Pre-incubation of tumor cells with garlic renders these cells as non-tumorigenic, possibly by interfering with tumor cell metabolism through inactivating essential sulphydryl enzymes or...
by damaging certain ultrastructures of the tumor cells [9]. Some reports supported that garlic and related constituents were effective on the modification of the incidence of cancer caused by several chemical carcinogens [10]. However, the cytotoxic mechanisms of garlic components that are responsible for this anti-tumor activity have not yet been clearly defined.

Allicin is a major component of garlic and a precursor of a number of secondary products formed in aged garlic and crushed garlic preparations. Allicin has been shown to have various biological activities including anti-microbial and anti-tumor activities [11].

Apoptosis, or programmed cell death, is a genetically controlled process whereby the cell actively participates in its own destruction in response to environmental or developmental cues. Apoptosis is morphologically characterized by membrane blebbing, cytoplasmic, nuclear and chromatin condensation, and DNA fragmentation [12]. Recently, evidence for pro-apoptotic effects of allicin against various cancer cells was provided by in vitro studies [13,14]. However, the mechanism by which allicin exerts its apoptotic effects are not fully understood. In the present study, our results provided further evidence that allicin induces apoptosis of the cells through caspase-independent apoptosis pathway, which was accompanied by the mitochondrial release of AIF and protein kinase A (PKA) appears to play an important role in the caspase-independent apoptosis.

2. Materials and methods

2.1. Cell culture and purification of allicin

The human gastric carcinoma cell line AGS was purchased from the ATCC (Rockville, MD) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (GIBCO BRL, Life Technologies, Grand Island, NY) in a humidified atmosphere with 5% CO₂ incubator at 37 °C.

The HPLC system used for the isolation and the analysis of allicin was a Gilson medel with 306 pump and UV/VIS 112 detector (254 nm). The preparative HPLC column used was Econosil Silica 10 μm (10×250 mm) and the analytical column was Econosil C 18 5 μm (4.6×250 mm). Flash column chromatography was carried out over Lichroprep® RP-18 gel (40–63 μm, Merck 113900). The powder of nine authentic allicin tablets (Madaus Murdock, Inc., 10 Mountain Spring Parkway, Springville, UT, USA) was extracted with H₂O (14 ml) four times at room temperature. The resultant aqueous portion was subjected to C-18 reverse phase flash column chromatography eluting with H₂O (1 L) and methanol (500 mL). The methanol fraction was evaporated in vacuo at about 10 °C and purified by preparative HPLC (Hexane–tetrahydrofuran, 90:10) to afford allicin (5 mg, flow rate 3.0 mL/min, Rₜ 15.0 min), whose purity was analyzed by HPLC with Econosil C 18 5 μm column (4.6×250 mm). Mobile phase was methanol–water containing 0.1% formic acid (65:35) with flow rate of 0.8 mL/min and the retention time of allicin was 5.17 min. The purified allicin was diluted to 0.01% (w/v) solution with diethylether and stored at −40 °C. Allicin was obtained as colorless oil. ¹H-NMR (500 MHz, CDCl₃) δ 3.6–3.9 (4H, m), 5.0–6.30 (6H, m).

2.2. Cell proliferation assay

Gastric carcinoma cells were seeded at a concentration of 5×10⁴ cells/well in 96-well tissue culture plates and incubated with various concentrations of allicin for different time period. After treatment, cell viability was assessed by incubating the cells with 25 μg/ml of MTT (Sigma, St Louis, MO) for another 4 h. Then, the MTT-formazan produced by viable cells was dissolved in dimethyl sulfoxide and a molecular device microplate reader (Menlo Park, CA) was used to measure absorbance at 560 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.

2.3. Detection of morphological apoptosis

Hoechst staining of nuclei was performed to detect morphological changes. Briefly, cells were seeded at a concentration of 1×10⁶ cells/well in 6-well tissue culture plates (Nunc, Denmark) and treated with various doses of allicin for 24 h. After treatment, cells were fixed with 2% glutaraldehyde solution (TAAB Lab., Aldermastone, England) for 1 h and stained with Hoechst 33342 (100 μg/ml) for 30 min to visualize
the location of DNA. Cells were examined with a fluorescence microscope (Olympus BH2, Tokyo, Japan) for determination of fragmentation of nuclei and/or condensation of chromatin. Condensed or fragmented nuclei were scored as apoptotic. Gastric carcinoma cells grown simultaneously in the absence of allicin served as controls.

2.4. Agarose-gel analysis for DNA fragmentation

Cells were treated with various doses of allicin for 24 h. DNA was extracted by the use of apoptotic DNA ladder kits (Roche MB, Germany) according to the manufacturer’s instructions. Samples (10 μg/lane) were loaded on a 1.5% agarose gel.

2.5. Cell cycle analysis and sub-G1 DNA measurement

Cells were seeded at a density of 2×10^6 cells in 100 mm petri-dishes and cultured with only RPMI-1640 for 48 h. After 48 h serum-starvation, cells treated with indicated concentration of allicin for 24 h. For growth inhibition analysis and sub-G1 DNA content measurement, cells were collected and fixed in ice-cold 70% ethanol in PBS and stored at −20 °C before use. After resuspension, cells were washed and incubated with 100 μl of RNase I (1 mg/ml) (Sigma) and 100 μl of propidium iodide (400 μg/ml) (Sigma) at 37 °C for 30 min. After staining, flow cytometry with Modfit software (Bio-Rad Laboratories, Hercules, CA) was used for analysis of cell cycle phase and sub-G1 DNA content.

2.6. Caspase-3 determination

Cells were cultured in the presence or absence of allicin, then harvested at 24 h. Allicin-treated and non-treated control cells were lysed and their caspase-3 activities were determined using the BIOMOL Quantzyme Assay kit (Plymouth Meeting, PA) according to the manufacture’s instructions.

2.7. Western blotting

Western blot analysis was performed by modification of the technique described previously [15]. After treatment, cells were washed twice in PBS and suspended in homogenizing buffer (20 mM Hepes–KOH (pH 7.5), 10 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepstatin and 12.5 mg/ml of N-acetyl-Leu-Leu-Norleu-Ala) and homogenized by a Dounce homogenizer (Bellco Biotechnology, Vineland, NJ). After centrifugation at 23,100×g for 30 min at 4 °C, the supernatant was collected and the pellet containing mitochondria was suspended in lysis buffer {150 mM NaCl, 0.5% Triton×100, 50 mM Tris–HCl (pH 7.4), 20 mM EGTA, 1 mM DT, 1 mM sodium orthovanadate, protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany)} and frozen at −70 °C until use. The supernatant was further centrifuged at 23,100×g for 1 h at 4 °C and stored at −70 °C until used for electrophoresis.

Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Lab, Hercules, CA) with BSA (Sigma) as standard. Protein (20 μg) was resolved by 10–15% SDS-polyacrylamide gel, transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington Heights, IL) and probed with anti-AIF antibody (BD Pharmingen, San Diego, CA), anti-cytochrome c antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax antibody (Santa Cruz Biotechnology), anti-Bcl-xL antibody (Santa Cruz Biotechnology) and anti-PARP antibody (BD Pharmingen), respectively. The blots were developed using the enhanced chemoluminescence (ECL) kit (Amersham). In all immunoblotting experiments, blots were reprobed with an anti-β-actin (Sigma) antibody to control for protein loading.

2.8. Statistical analysis

Data are means of at least 2 different experiments ± SE. Results were compared using a two-tailed Student’s t-test and considered significant if the P-values were < 0.05.

3. Results

3.1. Allicin-induced cell death

The anti-proliferative effect of allicin on human gastric carcinoma cell line, AGS cells, was examined
by exposing them to different concentrations of allicin for 6–72 h. Cell viability was determined by MTT assay. When AGS cells were exposed to allicin (1–100 μg/ml), the growth of cell was inhibited in a dose- and time-dependent manner (Fig. 1A).

The induction of apoptosis by allicin in AGS cells was confirmed by electrophoresis of fragmented DNA (Fig. 1B). Low-molecular weight DNA fragments extracted from cells cultured with various doses of allicin for 24 h showed typical oligonucleosomal ladders at 5–20 μg/ml. Negligible cleavage of DNA into nucleosomal fragments was seen with untreated cells. AGS cells were also stained with the Hoechst dye to visualize nuclear morphology. We observed that cells began to change in appearance at 12–24 h of allicin treatment. Allicin caused morphological changes characteristic of apoptosis, including degeneration of neuritis, shrinkage of cell bodies and condensation of nuclei, whereas very few condensed nuclei were found in untreated cells (Fig. 2). The same situations were seen when camptothecin, a chemical inducer of apoptosis, was administrated to cells.

Since in some paradigms de novo protein synthesis is required for cell death, we examined whether allicin-induced apoptosis was an active type of cell death, that is, requires new mRNA or protein synthesis. It was found that allicin-induced cell death was attenuated by a protein synthesis inhibitor, cycloheximide (CHX), but unaffected by the RNA synthesis inhibitor, actinomycin (ActD) (Fig. 3). These results suggest that translation of pre-existing mRNA mediates allicin-induced apoptosis.

3.2. Flow cytometric analysis of allicin-induced apoptotic cell death

Flow cytometric measurement was used to quantify the extent of apoptosis in the total cell population, combining both adherent and floating cells. Apoptotic nuclei were distinguishable by the hypodiploid DNA contents as compared with the diploid DNA contents of normal cells. Exponentially growing AGS cells contained a low level (4.5%) of an apoptotic population with hypodiploid DNA content (Fig. 4). Treatment with allicin (20 μg/ml) for 24 h greatly increased this apoptotic cell population to 45.2%. There is an increase in the sub-G1 DNA content and decrease in the proportion in cells of S phase. These results further support the notion that allicin induces apoptosis in AGS cells.
3.3. Defective activation of caspases in allicin-induced cell death

To investigate the involvement of caspases in allicin-induced apoptosis, cells were treated with allicin for 24 h. Caspase-3 protease activity was assessed in cell lysates by measuring hydrolysis of colorimetric caspase-3 substrate DEVD-pNA. As shown in Fig. 5A, the lysate from cells treated with allicin featured levels of caspase-3 like activity similar to those detected in cell lysates from control cells. Additionally, the activity of caspase-8 was not increased in allicin-treated cells (data not shown).

Cleavage of the p116/PARP to its characteristic p85/PARP fragments is considered as a marker of apoptosis and a substrate for caspase-3. To further corroborate the colorimetric assay results, AGS cells were treated with allicin, and the cell lysates were analyzed by Western blot assay using a specific PARP antibody. As shown in Fig. 5B, allicin treatment did not lead to PARP cleavage. In addition, at 24 h after treatment, cells exposed to allicin had a similar percentage of apoptotic cells as cell pretreated with a broad spectrum of caspase inhibitor, Z-VAD-fmk (Fig. 5C). It was also found that pretreatment with Z-VAD-fmk was not able to reduce the extent of characteristic changes of apoptosis in morphology (data not shown).

3.4. Effects of allicin on cytochrome c, Bax, Bcl-xL and AIF protein levels

To further evaluate the mechanism of allicin-induced apoptosis in gastric cancer cell, we examined the changes in protein expression of apoptosis-related genes, including cytochrome c, Bax, Bcl-xL and apoptosis-inducing factor (AIF) by Western blot analysis using antibodies directed against the respective proteins.

Release of Cyt c from the intermembrane spaces of the mitochondria into the cytosol is a key event in apoptosis via a mitochondria-dependent pathway. The cells were treated with various doses of allicin and the release of Cyt c was examined. As shown in Fig. 6, Cyt c in the cytosol of cells was detected after allicin treatment with a loss of mitochondria Cyt c. Since treatment of cells with allicin results in the release of Cyt c, the signaling pathway upstream of
the mitochondria was examined. As shown by the data (Fig. 6), mitochondrial Bax protein level was increased in a dose-dependent manner and simultaneously decreased in the cytosolic fraction. In addition, we tested whether caspase-independent pro-apoptotic effect of allicin might be mediated by AIF. Treatment of allicin resulted in the release of mitochondrial AIF into the cytosol in a concentration-dependent manner (Fig. 6). However, anti-apoptotic Bcl-xL expression was found to be unaffected by allcin treatment (data not shown).

3.5. Involvement of PKA in allicin-induced apoptosis

Several protein kinase pathways are known to regulate cell proliferation and survival. To determine the potential involvement of these protein kinases in allicin-induced apoptosis in AGS cells, various signaling inhibitors were used to specifically block distinct signal transduction pathways. After pre-incubation of cells with inhibitors for 1 h, only PKA inhibitor (H-89) was capable of reducing apoptosis of allicin-treated cells (Table 1). However, other signal inhibitors did not affect the allicin-induced apoptosis in AGS cells, suggesting that the activation of PKA pathway is involved in allicin-induced apoptosis. H-89 did not influence the rate of apoptosis when the cells were incubated with this substance.

Since H-89 treatment prevented the induction of apoptosis by allicin, we analyzed the expression levels of Bax, Bcl-xL and AIF to verify whether inhibition of PKA pathway influenced this apoptotic checkpoint. Interestingly, inhibition of PKA pathway by H-89 resulted in significant reductions in the expression of Bax and AIF (Fig. 7). Indeed, the expression of Bax was partially inhibited by 52% after 24 h. Treatment with H-89 inhibitor also decreased AIF expression by 73%. In contrast, the expression of Bcl-xL was not
Fig. 5. Allicin-induced cell death occurs independent of caspase activation. (A) AGS cells were cultured for 24 h with various doses of allicin or without allicin. The activity of caspase-3 was estimated. (B) AGS cells were treated with allicin for 24 h. Whole-cell protein lysates were prepared for Western blotting analysis using anti-PARP and -β-actin antibodies. Equal loading was confirmed with anti-β-actin antibodies. (C) AGS cells exposed to allicin were compared with cell pretreated with 40 μM of Z-VAD-fmk. Apoptosis was assessed by PI staining. Camptothecin (CPT) was used as positive control. Data are means of at least two different experiments ± SE. *P < 0.01 vs untreated control.
significantly affected by the H-89 treatment (data not shown). These results indicated that the expression of Bax and AIF are regulated through a PKA-mediated signal transduction pathway.

4. Discussion

Garlic has been known to be effective against a broad spectrum of diseases. Allicin, one of garlic constituents, was implicated to mediate its biological activity. In the present study, our results demonstrate that allicin inhibited growth and induced apoptosis in AGS cells, gastric carcinoma cells bearing wild-type p53 gene through a caspase-independent pathway and allicin-induced apoptosis appears to be mediated via activation of PKA. Additionally, this form of cell death requires de novo protein synthesis.

Concerning the potential chemical mediators of apoptosis signaling induced by allicin, experiments with superoxide dismutase (SOD) and catalase indicated that allicin induction of caspase-independent apoptosis was not inhibited by the addition of SOD or catalase (data not shown), indicating that allicin action was not likely mediated by either superoxide anion or hydrogen peroxide.

Mitochondria play a central role in commitment of cells to apoptosis via increased permeability of the outer mitochondrial membrane, decreased transmembrane potential, release of Cyt c and apoptosis-inducing factor (AIF), and production of reactive oxygen species (ROS) [16,17]. Anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-XL) can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members (Bax, Bak, Bad) can trigger these changes. In our experiments, mitochondrial changes were observed in AGS cells. Allicin treatment resulted in elevated level of mitochondrial Bax and Cyt c release from the mitochondria to the cytosol. However, Bcl-xL expression remained the same (data not shown), implicating that Bcl-xL does not protect cells from allicin-induced apoptosis. Cyt c release from mitochondria that occurs after allicin treatment is possibly controlled by Bax. Previous results have shown that the translocation of Bax to mitochondria can alter the outer mitochondrial membrane permeability, that leads to the release of Cyt c to cytosol [18] and then activates caspase cascade from apical caspases to effector caspases leading to apoptotic cell death. In AGS cells, Bax protein level was increased in the mitochondria after treatment, which was subsequent to Cyt c release. Based on these finding, it is conceivable that sufficient Bax appears to reside at the mitochondrial membrane to induce Cyt c release after allicin treatment.

One of the main consequences of mitochondrial Cyt c release is the activation of caspase-3. 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 [19]. In the present study, our data show...
that allicin-induced apoptosis was not inhibited by the broad-spectrum caspase inhibitor Z-VAD-fmk, with no activation of caspase-3 suggesting a caspase-independent signal transduction pathway. This contrasts with apoptosis in allicin-treated fibrosarcoma and cervical cancer cells, however, where allicin induces caspase-mediated apoptosis [14]. Such different findings to our results could be due to the difference in cell type used. These opposing results also raise the possibility that there are fundamental differences of the individual gene expression pattern between cell types or even between various cell lines of the same cell type. Indeed, recent studies demonstrated that several parallel apoptosis pathways could be initiated by same inducer [20,21].

AIF, which translocates via the cytosol to nuclei, is a hallmark of caspase-independent apoptosis [22]. In this report, our data demonstrate that allicin treatment induces AIF release from mitochondria, which provides obvious support for the contention that allicin is able to induce caspase-independent apoptosis. Thus, the present results suggest that allicin appears to induce at least two different signaling pathways of cell death, depending on cell type.

Several protein kinase pathways are known to regulate cell proliferation and apoptosis signaling in various model systems [23–26]. The data from experiments with various signaling inhibitors showed that blocking PKA significantly reduced the ability of allicin to produce apoptosis. In addition, inhibition of PKA pathway caused a down-regulation of the expression levels of Bax and AIF. These results suggest that PKA is involved in the allicin-driven apoptotic process and the expression of Bax and AIF are regulated through a PKA-mediated pathway in AGS cells. However, our data do not totally rule out the possibility that allicin modulates the activities of protein kinases and other enzymes through phosphorylation-independent mechanisms.

In conclusion, we have demonstrated in this study that allicin conducts a caspase-independent pathway through the collapse of the membrane potential of mitochondria and the mitochondrial release of AIF and PKA signal transduction pathway may play an important role in the apoptosis. Moreover, anti-proliferative activity through cell cycle arrest and induction of apoptosis are expected to contribute to the direct actions of allicin on target epithelial cells to inhibit the growth of cancerous lesions in the context of cancer chemoprevention.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Mean ± SE</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>15.3 ± 2.1</td>
</tr>
<tr>
<td>Allicin</td>
<td>78.5 ± 1.2</td>
</tr>
<tr>
<td>Allicin + JNK inhibitor</td>
<td>83.1 ± 3.2</td>
</tr>
<tr>
<td>Allicin + U0126</td>
<td>77.5 ± 4.5*</td>
</tr>
<tr>
<td>Allicin + H-89</td>
<td>44.6 ± 2.2</td>
</tr>
<tr>
<td>Allicin + Akt inhibitor</td>
<td>70.3 ± 3.5</td>
</tr>
<tr>
<td>Allicin + bisindolymalide</td>
<td>76.2 ± 4.9</td>
</tr>
<tr>
<td>Allicin + tryphostin</td>
<td>81.1 ± 2.7</td>
</tr>
<tr>
<td>Allicin + SB 203580</td>
<td>84.9 ± 4.3</td>
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</table>

AGS cells were pretreated with or without various signal inhibitors for 1 h and then treated with 20 μg/ml allicin for 24 h. The percentage of apoptotic cells was analyzed by PI. JNK inhibitor (JNK inhibitor I, 1 μM); MEK inhibitor (U0126, 100 nM); PKA inhibitor (H-89, 4 μM); Akt inhibitor (5 μM); PKC inhibitor (Bisindolymalide I, 100 nM); PTK inhibitor (Tryphostin AG490, 200 nM); p38MAPK inhibitor (SB 203580, 600 nM). Cell viability was not influenced by pretreatment of inhibitors. Data shown are the mean ± SE of triplicate cultures. The results are represented as the mean of three independent experiments. *, significant at P<0.01.

Fig. 7. Expression of Bax (mitochondria fraction) and AIF (cytosolic fraction) in H-89-treated AGS cells. AGS cells were pretreated with or without H-89 (4 μM) for 1 h and then treated with 20 μg/ml allicin for 24 h. Mitochondrial and Cytosolic fractions were subjected to 10-15% SDS-PAGE and analyzed by Western blotting with Bax and AIF antibodies. Results are shown from representative studies that were repeated at least two times. β-actin was used as an internal control.
Acknowledgements

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References