

Ajoene, a Compound of Garlic, Induces Apoptosis in Human Promyeloleukemic Cells, Accompanied by Generation of Reactive Oxygen Species and Activation of Nuclear Factor κ B

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ABSTRACT

The pharmacological role of garlic in prevention and treatment of cancer has received increasing attention, but thorough investigations into the molecular mechanisms of action of garlic compounds are rare. The present study demonstrates that ajoene, a major compound of garlic induces apoptosis in human leukemic cells, but not in peripheral mononuclear blood cells of healthy donors. The effect was dose and time dependent. Apoptosis was judged by three criteria, morphology of cells, quantification of subdiploid DNA content by flow cytometry, and detection of DNA fragmentation by gel electrophoresis. Ajoene increased the production of intracellular peroxide in a dose- and time-dependent fashion, which could be partially

blocked by preincubation of the human leukemic cells with the antioxidant *N*-acetylcysteine. Interestingly, *N*-acetylcysteine-treated cells showed a 50% loss of ajoene-induced apoptosis. Moreover, ajoene was demonstrated to activate nuclear translocation of the transcription factor nuclear factor κ B, an effect that was abrogated in *N*-acetylcysteine-loaded cells. These results suggested that ajoene might induce apoptosis in human leukemic cells via stimulation of peroxide production and activation of nuclear factor κ B. This is a novel aspect in the biological profile of this garlic compound and an important step in elucidating the underlying molecular mechanisms of its antitumor action.

The role of dietary compounds as drugs in cancer prevention and treatment is widely discussed (Pezzuto, 1993; Agarwal, 1996; Koch and Lawson, 1996; Milner, 1996). In this regard the potential chemopreventive effect of garlic (*Allium sativum*) was subject of various clinical trials (Dorant *et al.*, 1993; Steinmetz *et al.*, 1994; Agarwal, 1996; Dorant *et al.*, 1996; Koch and Lawson, 1996; Lea, 1996; Milner, 1996). The results, however, were quite contradictory depending on the type of tumor examined and the garlic preparation used (Dorant *et al.*, 1993). Because crude garlic extracts contain numerous pharmacologically active substances, including organosulfur compounds with varying stability and biological activity (Milner, 1996), more detailed studies of the effects of chemically defined garlic compounds on tumor genesis are needed. Some garlic constituents have been shown to alter the activation of several carcinogens and to cause growth inhibition and/or death of tumor cells (Takeyama *et al.*, 1993; Hatono *et al.*, 1996; Koch and Lawson, 1996; Singh *et al.*, 1996). However, the molecular mechanisms underlying the

tumor cytotoxicity of garlic substances are poorly defined. The cytotoxicity of most classical antitumor drugs is thought to be mediated by their ability to induce apoptosis (Sen and D'Incalci, 1992). Apoptosis is a form of physiological cell death, characterized by chromatin condensation, cytoplasmic blebbing, and DNA fragmentation (Wyllie *et al.*, 1980). Apoptosis can be initiated by alterations in signaling pathways (Jones *et al.*, 1989; Hsu *et al.*, 1996) or by oxidative stress mediated by the generation of ROS (Buttke and Sandstrom, 1994). It has been shown that oxidative stress activates the transcription factor NF- κ B and that activation of NF- κ B is involved in inducing the apoptotic cell death in some cells (Grimm *et al.*, 1996).

The aim of the present study was first to examine whether ajoene [(*E,Z*)-4,5,9-trithiadodeca-1,6,11-triene-9-oxide] (Fig. 1), a major compound of crushed garlic (Agarwal, 1996), was able to induce apoptosis in the human promyelocytic leukemia cell line HL-60. This cell line provides a valid model system for testing antileukemic or general antitumoral compounds (Suh *et al.*, 1995). Second, we investigated the mechanisms underlying apoptosis induction. We examined the generation of ROS by ajoene and the effect of ajoene on the

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ABBREVIATIONS: ROS, reactive oxygen species; DHR, dihydrorhodamine; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EMSA, electrophoretic mobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HL, human leukemic cells; NF, nuclear factor; NAC, *N*-acetylcysteine; PI, propidium iodide; PBS, phosphate-buffered saline; PMBC, peripheral mononuclear blood cells.

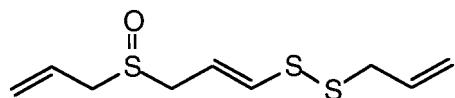


Fig. 1. Chemical structure of *trans*-ajoene [(*E*)-4,5,9-trithiadodeca-1,6,11-triene-9-oxide]

activation of the transcription factor NF- κ B. Apoptosis was assessed by morphological analysis of cells as well as characterization and quantification of DNA degradation by flow cytometry and gel electrophoresis. The ROS formation of HL-60 cells exposed to ajoene was monitored by oxidation of the dye DHR-123. Activation of NF- κ B was examined by its DNA-binding activity using EMSA.

Materials and Methods

Cell cultures. The human promyelocytic leukemia HL-60 cell line and in addition the human colon adenocarcinoma DLD-1 cell line, human squamous carcinoma cells (A431), and human neuroblastoma cells (SH-SY5Y) were cultured (37° and 5% CO₂) in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin/100 μ g/ml streptomycin, and L-glutamine (2 mM) (all from GIBCO/BRL, Eggenstein, Germany). For experiments 1–5 $\times 10^5$ cells/well were seeded (1-ml, 24-well plates; Peske, Aindling-Pichl, Germany) and grown overnight.

Human PMBC were recovered from heparin-anticoagulated blood of healthy volunteers by centrifugation with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's instructions and cultivated as described for the HL-60 cells. In some experiments, PMBC were stimulated with phytohemagglutinin (1 μ g/ml) for either 24 or 48 hr. In addition, PMBC of a patient (male, 54 years old) with a chronic myelogenous leukemia undergoing a myeloid blast crisis were purified and cultured as described above. Blood cell differentiation analysis of the patient revealed 70% myeloblastocytes.

General viability of cultured cells was determined by either trypan-blue exclusion or by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (Mosmann, 1983).

Ajoene (GBF, Braunschweig, Germany), which was a mixture of *cis*- and *trans*-ajoene, was dissolved in 10% dimethylsulfoxide and further diluted in PBS. Ajoene was added to cells in various concentrations (1–80 μ M) for various periods of time as indicated in Results. Final dimethylsulfoxide concentration was less than 0.1% and has been tested not to interfere with the test systems employed.

Apoptosis. Apoptosis was examined by cell morphology, DNA gel electrophoresis, and flow cytometry. Cells after treatment with ajoene were cytospun, fixed in methanol/acetone (1:1) (4°, 5 min), and stained with Wright solution (Merck, Darmstadt, Germany) for light microscopic determination of morphological changes.

Apoptotic cells were detected by flow cytometry using PI (Nicoletti *et al.*, 1991). Briefly, after incubation with test substances, cells were harvested and fixed in 70% ethanol for 2 hr at –20°. Carcinoma cells had to be detached by trypsinization. Cells were washed and resuspended in PBS (0.2 ml) containing 0.5 mg/ml RNase and 0.1 mg/ml PI (both Sigma, Deisenhofen, Germany). Samples were kept in the dark for 30 min. Cytometry was performed using the FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with a 488-nm argon-ion laser. DNA content per cell was estimated by collecting PI-fluorescence through 585-nm filter (FL-2). Only those events with detectable and linear FL-2 area versus width were included in analysis. In addition forward and side scatter of cells were recorded to observe morphological changes of cells. Ten thousand events were collected per sample. Experiments were performed in triplicate and repeated at least three times. All analyses were performed with the Cell Quest software (Becton Dickinson).

DNA isolation and electrophoresis was performed (Tuosto *et al.*,

1994). After centrifugation (200 $\times g$, 5 min), cell pellets (containing about 10⁶ cells) were resuspended in 1 ml of lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0) containing 0.5% sodium dodecyl sulfate and 0.5 mg/ml RNase A (Sigma) and incubated at 37° for 1 hr followed by treatment with 0.5 mg/ml proteinase K (Sigma) for another 1 h at 50°. The lysate was extracted twice with phenol/chloroform (1:1) and the DNA precipitated with ethanol. After centrifugation, pellets were dissolved in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA was analyzed after separation by gel electrophoresis (1.8% agarose) and staining by ethidium bromide. Experiments with HL-60 and PBMC were repeated at least three times, experiments with carcinoma cells at least twice.

Measurement of ROS generation. Production of ROS (peroxide) by HL-60 was measured by flow cytometry as previously described (Vollmar *et al.*, 1997). Briefly, HL-60 cells suspended in 5 mM saline buffered with HEPES (2 $\times 10^6$ /ml) were placed in polypropylene tubes (200 μ l; Becton-Dickinson) and loaded for 15 min with 0.4 mM DHR-123 (Molecular Probes, Eugene, OR) (dissolved in *N,N*-dimethylformamide at a stock concentration of 43.3 mM) and thereafter washed with HEPES-buffered saline. DHR-containing cells were stimulated with ajoene (1–20 μ M) for various times (5–40 min). To estimate intracellular peroxide production, fluorescence intensity (FL1, 530 nm) of 10,000 cells was recorded. Cells incubated with DHR only were employed to monitor basal peroxide synthesis. Fluorescence intensity was obtained as histogram statistics. Triplicates of three independent experiments were performed.

Detection of activation of NF- κ B by EMSA. Nuclear extracts were prepared as described previously (Schreiber *et al.*, 1989). In brief, cells (10⁶ per tube) were washed with PBS and resuspended in 400 μ l of ice-cold buffer A [10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride], kept on ice for 15 min, followed by addition of 25 μ l of 10% Nonidet NP-40. Tubes were vigorously vortexed for 10 sec and the homogenate centrifuged (30 sec, 10,000 $\times g$). The pellet was resuspended in 50 μ l of buffer B (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM phenylmethylsulfonyl fluoride) and vigorously rocked for 15 min (4°). The nuclear extract was centrifuged (5 min, 10,000 $\times g$), and after determination of protein concentration (method of Lowry), aliquots were either frozen at –70° or immediately used for EMSA as previously described (Boese *et al.*, 1996). Briefly, an oligonucleotide containing the most common NF- κ B consensus sequence (22mer; Promega, Heidelberg, Germany) was end-labeled with [γ -³²P]ATP (300 Ci/mmol; Hartman, Braunschweig, Germany) using the T4 polynucleotide kinase (Promega). Binding reactions were performed incubating 50,000–200,000 cpm of ³²P-labeled DNA with nuclear protein extract (10 μ g of protein) in a final volume of 15 μ l of buffer [5 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM DTT, 5% glycerol, 1 mM EDTA, 1 μ g poly dI-dC (Promega)] for 30 min (22°). The mixture was electrophoresed on a 4.5% nondenaturing polyacrylamide gel (100 V) and the gel was exposed to X-ray film overnight. Three independent experiments were performed. Films were evaluated by densitometry (EASY plus system; Herolab, Wiesloch, Germany).

Results

Induction of apoptosis by ajoene. Ajoene, whose chemical structure is shown in Fig. 1, induced morphological changes that are characteristic of apoptosis in HL-60 cells. Whereas untreated cells exhibit typical nonadherent, fairly round morphology, cells exposed to ajoene (10 μ M, 20 hr) frequently display condensation of chromatin and appearance of apoptotic bodies (data not shown).

Flow cytometric analysis of HL-60 cells exposed to ajoene (10 μ M, 20 hr) confirmed the morphological observations. Ajoene-treated cells (Fig. 2B) contained a population of cells with higher side scattering of cells than untreated HL-60

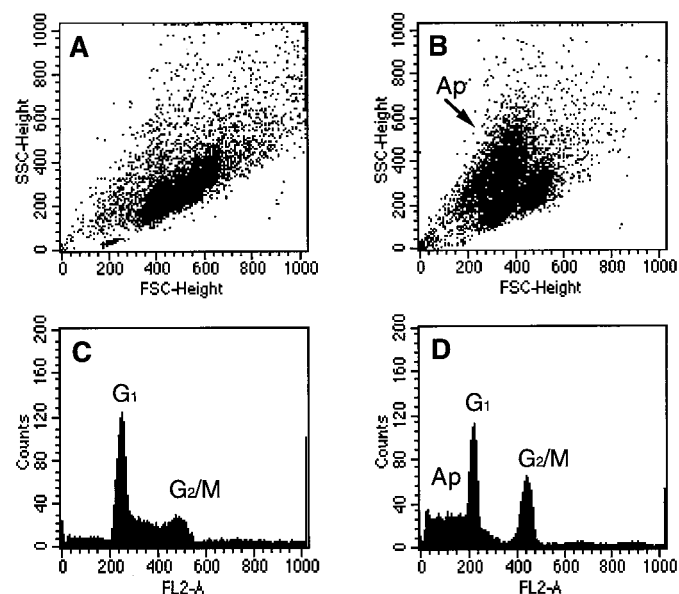


Fig. 2. Flow cytometric analysis of apoptosis. A, Cell size (forward scatter) and granularity (side scatter) analysis of untreated cells. B, Forward and side scatter analyses of ajoene-treated ($10 \mu\text{M}$, 20 hr) cells. Histograms of PI-fluorescence: C, Untreated HL-60 cells. D, Appearance of cells with subdiploid DNA content (*Ap*) after treatment with ajoene. *Ap*, apoptotic cells.

cells, in accord with the different nucleus/cytoplasm consistency of apoptotic cells (Tuosto *et al.*, 1994) (Fig. 2A). The DNA fluorescence histograms of PI-stained cells showed the low DNA stainability of the ajoene-treated, apoptotic cells (Fig. 2D), which resulted in a distinct, quantifiable region below the G_1 peak. In contrast, the G_1 peak predominates in control cells (Fig. 2C).

Finally, agarose gel electrophoresis showed the typical DNA fragmentation pattern and thus confirmed the apoptosis-inducing effects of ajoene (Fig. 3A). DNA fragmentation

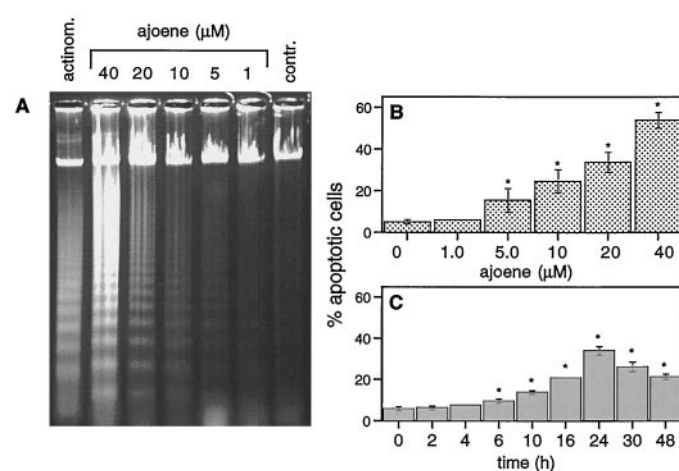


Fig. 3. A, Demonstration of apoptosis by gel electrophoresis. DNA extracted from HL-60 cells untreated (*contr.*) or exposed to increasing concentrations of ajoene ($1\text{--}40 \mu\text{M}$) for 20 hr was separated by agarose gel (1.8%) and stained with ethidium bromide. As a positive control, cells were exposed to actinomycin D ($1 \mu\text{g}/\text{ml}$, 20 hr). B, Dose-dependent induction of apoptosis by ajoene. Cells were incubated with increasing concentrations of ajoene for 20 hr. C, Time dependency. Cells were incubated with $10 \mu\text{M}$ ajoene for increasing amounts of time. % apoptotic cells, percentage of cells with subdiploid DNA content as described under Methods. Bars, mean \pm standard deviation of three experiments performed in triplicate. *, $p < 0.01$ (t test).

by ajoene was dose dependent; the intensity of DNA fragments increased as increasing amounts of ajoene ($1\text{--}40 \mu\text{M}$) were added to the cells. As a positive control, cells were treated with actinomycin D ($1 \mu\text{g}/\text{ml}$, 20 hr) (Naora *et al.*, 1996). Quantification of dose dependency was done by monitoring the amount of nuclei with subdiploid DNA content by flow cytometry (Fig. 3B). Ajoene in concentrations higher than $5 \mu\text{M}$ incubated for 20 hr increased the percentage of apoptotic cells significantly, up to 60% ($40 \mu\text{M}$). Time dependency of this effect was assessed by exposing cells to $10 \mu\text{M}$ ajoene for the periods of time indicated (Fig. 3C). As early as 6 hr after ajoene addition, apoptotic cells could be detected by flow cytometry. Maximal apoptotic cells detection was reached after 24 hr and declined after 30-hr and 48-hr incubation times.

Interestingly, ajoene dose dependently induced apoptosis in PBMC of a chronic leukemic patient (Fig. 4). On the basis of blood cell differentiation, the patient was diagnosed as suffering from a myeloid blast crisis with the percentage of myeloblasts at 70%. Fig. 4A shows the appearance of the characteristic DNA ladder in the patient's ajoene-exposed PBMC. Quantification of apoptotic cells by flow cytometry (Fig. 4B) revealed similar potency in ajoene with respect to inducing apoptosis in patient leukemia cells as observed in HL-60 cells.

Importantly, ajoene did not induce apoptosis in quiescent or proliferating PBMC of healthy humans. Proliferation was induced by incubating cells with the mitogen phytohemagglutinin ($1 \mu\text{g}/\text{ml}$; 24 and 48 hr, respectively). Cells were exposed to ajoene ($10\text{--}40 \mu\text{M}$, 20 hr), and neither morphological and flow cytometric analysis nor DNA electrophoresis revealed any sign of apoptosis (data not shown). At a concentration of $40 \mu\text{M}$ ajoene, phytohemagglutinin-stimulated cells started to round up, without, however, significant loss of viability ($<10\%$) even after 48 hr incubation [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium test; data not shown].

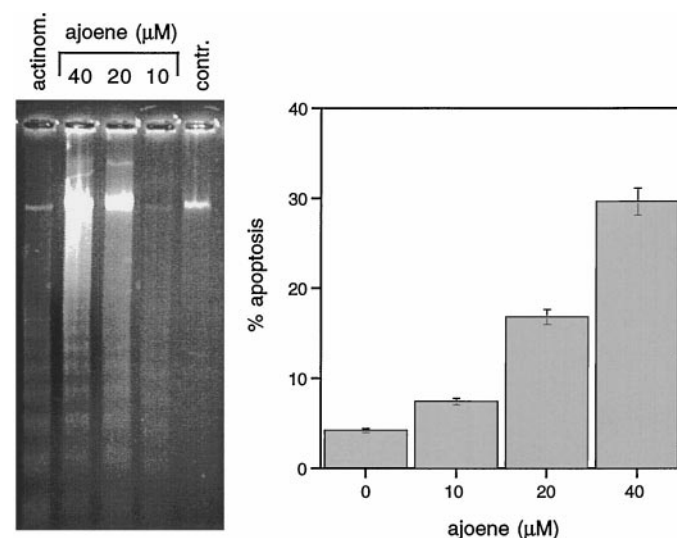


Fig. 4. Effect of ajoene on PBMC of a leukemic patient with a myeloid blast crisis. A, Gel electrophoretic analysis of DNA from patient PBMC 95 either untreated (*contr.*) or exposed to ajoene ($10\text{--}40 \mu\text{M}$, 20 hr). For control, PBMC were incubated with actinomycin D (*actinom.*, $1 \mu\text{g}/\text{ml}$). B, Quantification of leukemic PBMC with subdiploid DNA content (% apoptotic cells) after incubation with ajoene ($10\text{--}40 \mu\text{M}$, 20 hr). Experiment was performed in quadruplets and the mean \pm standard deviation is presented.

Apoptotic activity of ajoene was also examined in two human carcinoma cell lines, colon carcinoma cells (DLD-1) and the squamous carcinoma cell line A431, as well as in human neuroblastoma cells (SH-SY5Y). Ajoene added to cells in a concentration range of 10 to 80 μM for 20 to 48 hr did not induce apoptotic DNA fragmentation (data not shown). When each of the three cell lines was exposed to very high concentrations of ajoene (40 and 80 μM , each concentration for 24hr), however, viability was decreased (DLD-1, $15 \pm 2\%$ and $30 \pm 7\%$, respectively; SH-SY5Y, $42 \pm 8\%$ and $62 \pm 10\%$, respectively; A431, $< 10\%$ and $25 \pm 4\%$, respectively).

Induction of peroxide production by ajoene. An important mechanism by which compounds induce apoptosis is through generation of ROS, predominantly peroxides. To assess intracellular peroxide production, we measured oxidation of DHR-123 in HL-60 cells by flow cytometry.

Treatment of HL-60 cells with ajoene caused a dose- and time-dependent increase in peroxide production. Ajoene (5 μM) increased ROS production by 35% after 5 min (Fig. 5A), with production reaching a plateau after 30 min, followed by a slight decrease after 60 min (data not shown). Preincubation of cells with the antioxidant NAC inhibited ajoene-induced ROS production by 50% (Fig. 5B). As a control for the reaction, we added catalase, which was unable to permeate

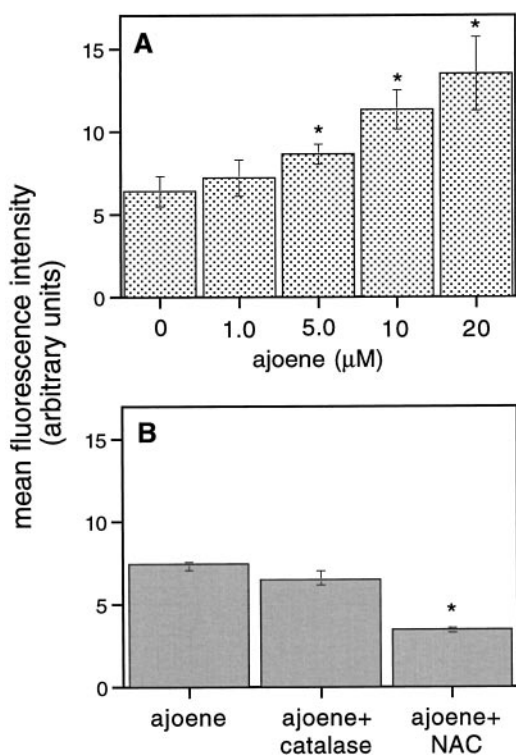


Fig. 5. Increase of peroxide production by ajoene. A, DHR-123-loaded HL-60 cells were incubated with increasing concentrations of ajoene for 15 min. Intracellular peroxide levels were detected by measuring the FL-1 intensity values due to oxidation of DHR-123. Data are given as mean fluorescence (arbitrary units) and represent the mean \pm standard deviation of four experiments run in triplicate. * $p < 0.01$ (t test). B, Peroxide levels are shown in cells treated with ajoene (10 μM , 20 min) only, in cells exposed to ajoene and catalase (200 units/ml), and cells preloaded with *N*-acetylcysteine (15 mM, 3 hr) and exposed to ajoene. Neither catalase nor NAC alone significantly altered basal cellular peroxide level (data not shown). Bars represent relative FL-1 intensities (i.e., FL-1 values of ajoene-treated cells minus FL-1 values of cells not exposed to ajoene) and are the mean \pm standard deviation of three experiments in triplicate. * $p < 0.01$.

into the cell in our experimental condition. Catalase did not prevent the ajoene-induced increase in peroxide production.

A link between peroxide generation and apoptosis in HL-60 cells exposed to ajoene is suggested by the fact that loading cells with NAC leads to a significant decrease in apoptotic activity of the garlic compound (Fig. 6).

Activation of NF- κB by ajoene. Induction of apoptosis and peroxide production by ajoene may be linked through activation of NF- κB , which is known to be induced by oxidative stress and to be involved in signaling of apoptotic processes (Grimm *et al.*, 1996). As demonstrated by EMSA (Fig. 7), ajoene (10 μM , 3 hr) was able to activate the nuclear translocation of NF- κB , and importantly, cells pretreated with 15 mM NAC displayed significantly (i.e., 50–65%) less NF- κB activation when exposed to ajoene than cells treated with ajoene only.

Discussion

The present study elucidates the biological effect of ajoene, a major component of garlic, on human leukemia cells and supports the notion of garlic as a chemopreventive or chemotherapeutic drug. We showed that ajoene induces apoptosis in a human promyeloleukemic cell line (HL-60) as well as in peripheral blood cells of a chronic leukemic patient suffering from a myeloid blast crisis. In contrast, ajoene does not induce apoptosis in proliferating as well as nonproliferating PMBC of healthy human donors. We demonstrated further that ajoene stimulates reactive oxygen production in HL-60 cells and activates the nuclear translocation of the transcription factor NF- κB in these cells.

Apoptosis is a highly controlled form of cell death and plays an important role in maintaining normal tissue homeostasis as well as in the development of various diseases including cancer (Fisher, 1994; McConkey *et al.*, 1996). Recently, interest has focused on the manipulation of apoptotic processes in the treatment and prevention of cancer. Thus, much effort has been directed toward the search for compounds that influence apoptosis and their mechanism of action. The signaling cascade leading to programmed cell death seems to involve ROS as second messengers (Khan and Wilson, 1995).

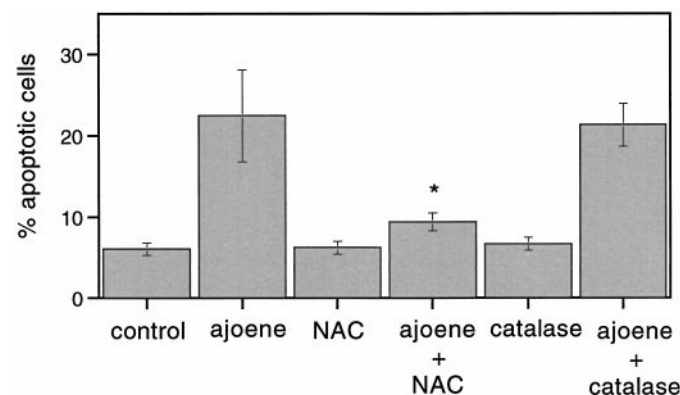


Fig. 6. Reduction of ajoene-induced apoptosis in cells loaded with NAC. The percentage of apoptotic cells measured as described in Fig. 3 is shown for untreated cells (control); cells exposed to ajoene (10 μM , 20 hr); cells preloaded with 15 mM NAC for 3 hr, either exposed or not exposed to ajoene (10 μM , 20 hr); and cells incubated with catalase (200 units/ml, 20 hr) in the absence or presence of ajoene (10 μM , 20 hr). The graph represents the mean \pm standard deviation of three independent experiments in triplicate. * $p < 0.01$ (t test).

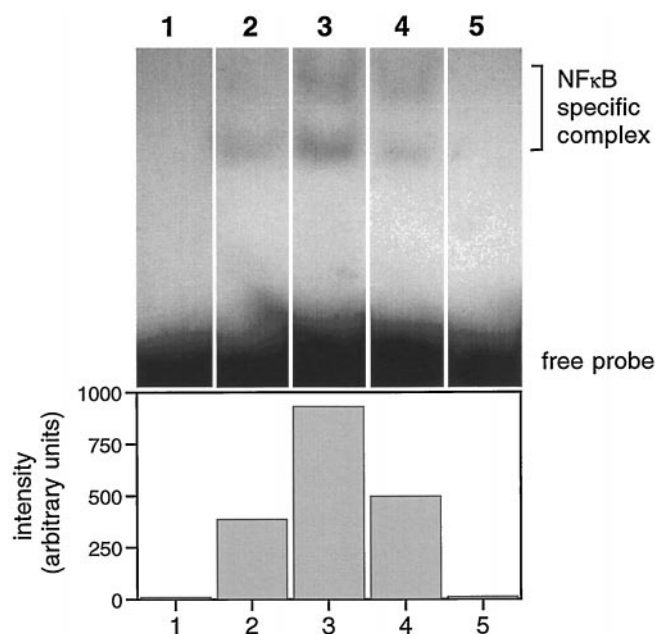


Fig. 7. Induction of NF- κ B DNA-binding activity upon ajoene exposure of cells. *Top*, Equal amounts of nuclear cell extracts (10 μ g) were subjected to EMSA as described in Methods. *Lane 1*, untreated cells; *lane 2*, cells preloaded with NAC (15 mM, 3 hr); *lane 3*, cells treated with ajoene (10 μ M, 3 hr); *lane 4*, NAC-loaded cells treated with ajoene; *lane 5*, nucleic extract of ajoene-treated cells incubated with a 100-fold excess of unlabeled NF- κ B-specific oligonucleotide. *Bottom*, Densitometric analysis was performed on the autoradiograph shown above. Signal intensities are expressed as arbitrary units.

This is in agreement with the fact that Bcl-2, a protooncogene product that has been shown to protect cells from apoptosis, is suggested to interfere with signal transduction events caused by oxidative stress. In fact, the antiapoptotic effect of Bcl-2 has been at least partially explained by its antioxidant properties (Khan and Wilson, 1995; Grimm *et al.*, 1996). Today, a large body of evidence suggests that oxidative stress induces apoptosis and that a concomitant increase of hydrogen peroxide might function as a cellular messenger (Khan and Wilson, 1995; Grimm *et al.*, 1996).

Substances such as ajoene that alter the redox status of the cell may be potent tools for corresponding mechanistic investigations.

Observations that NF- κ B, a transcription factor known to respond to oxidative stress, is activated by certain apoptotic stimuli and is down-regulated by Bcl-2 (Grimm *et al.*, 1996) led to the speculation that this transcription factor may mediate aspects of programmed cell death.

Substances causing oxidative stress may induce cell apoptosis via activation of NF- κ B. Our findings that ajoene not only induces apoptosis but also stimulates production of ROS and NF- κ B activation in HL-60 cells would fit into the above proposed model. At this time we cannot prove a functional association of the three observations. However, the fact that incubation of cells loaded with the antioxidant *N*-acetylcysteine resulted in decreased apoptosis and ROS formation as well as NF- κ B activation suggests a correlation of these events. Ajoene may serve as a valuable tool for investigating the mechanisms underlying apoptotic processes.

The ability of ajoene to induce programmed cell death in leukemic cells but not in peripheral blood cells of healthy

subjects represents a novel and important aspect of the discussion of the antitumoral effects of garlic compounds. The observation that a variety of carcinoma cell lines exposed to ajoene were not affected by apoptosis may suggest that the apoptotic activity of ajoene is specific to leukemic cells. Clearly, the significance of these findings in a broader context has to be proven by further studies employing various other tumor cells as well as established xenograft tumor models.

The data presented here, however, provide evidence for the antileukemic activity of the garlic compound ajoene and thus support the contention of a benefit of garlic intake in cancer prevention and treatment.

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