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Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells

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Abstract

Garlic (*Allium sativum*) has been used for centuries for treating various ailments, and its consumption is said to reduce cancer risk and its extracts and components effectively block experimentally induced tumors. Allicin, the major component present in freshly crushed garlic, is one of the most biologically active compounds of garlic. We found that allicin inhibited the growth of cancer cells of murine and human origin. Allicin induced the formation of apoptotic bodies, nuclear condensation and a typical DNA ladder in cancer cells. Furthermore, activation of caspases-3, -8 and -9 and cleavage of poly(ADP-ribose) polymerase were induced by allicin. The present results demonstrating allicin-induced apoptosis of cancer cells are novel since allicin has not been shown to induce apoptosis previously. Our results also provide a mechanistic basis for the antiproliferative effects of allicin and partly account for the chemopreventive action of garlic extracts reported by earlier workers.

Keywords: Allicin; Apoptosis; SiHa; HeLa; SW480; L-929; Garlic

1. Introduction

Egyptians, Greeks, Chinese and Indians used garlic (*Allium sativum*) for centuries for treating various ailments such as heart disease, arthritis, pulmonary complaints, abdominal growths (particularly uterine), diarrhea and worm infestation (Rivlin, 2001). Garlic consumption is correlated to reduced cancer risk, and its extracts and components effectively blocked experimentally induced tumors in a variety of sites including skin, breast, uterine cervix and colon, suggesting a general mechanism of action (Hussain et al., 1990; Milner, 1996, 2001). A recent report suggested that aqueous garlic extract might exert its chemo-preventive effect by inducing apoptosis (Balasenthil et al., 2002). Many organosulfur compounds, the major active principles in garlic, inhibited the proliferation of cancer cells, and some of them induced apoptosis in tumor cells of different tissue origin (Dirsch et al., 1998; Kwon et al., 2002; Pinto et al., 2001; Shirin et al., 2001; Sigounas et al., 1997). Hence, apoptosis could be a potential general mechanism providing a mechanistic basis for the anticarcinogenic activities of individual garlic components, although the actual mechanism is not known. Apoptosis or programmed cell death is characterized by cell shrinkage, chromatin condensation, DNA fragmentation and the activation of specific cysteine proteases known as caspases. Two pathways that converge on caspase-3, one involving caspase-8, and the other involving mitochondrial release of cytochrome *c* and activation of caspase-9 have been described (Steller, 1995; Sun et al., 1999).

Allicin, the major component present in freshly crushed garlic, is one of the most biologically active compounds of garlic (Ali et al., 2000), and its chemical structure is shown in Fig. 1. Allicin has antibacterial, antiviral and antiparasitic effects and is said to decrease platelet aggregation, cholesterol biosynthesis, serum lipid profile of hyperlipidemic rabbits and intraocular pressure in normal rabbits, and it also acts as a vasodilator (Agarwal, 1996; Eilat et al., 1995; Hirsch et al., 2000; Mayeux et al., 1988). Allicin is formed from alliin by the action of allinase and gets metabolized rapidly into diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, *S*-allylmercaptocysteine, *S*-allyl cysteine and vinyl dithiines (Dirsch et al., 1998; Hirsch et al., 2000; Sigounas et al., 1997; Sundaram and Milner, 1993; Welch et al., 1992). Interestingly, the garlic extract with high concentration of alliin was unable to inhibit cellular proliferation, whereas the supplementation of garlic powder (which contains enzyme allinase) exhibited a concentration-depen-

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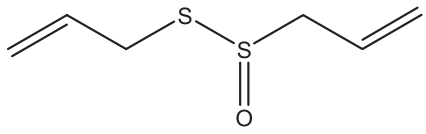

Fig. 1. Chemical structure of allicin [*S*-(2-propenyl) 2-propene-1-sulfinothioate or diallyl thiosulfinate].

dent inhibition of tumor cell growth indicating that the antiproliferative effects of garlic may be due to breakdown products of alliin, such as allicin or polysulfides, rather than alliin itself (Siegers et al., 1999). Garlic was used to treat uterine ailments (Moyers, 1996), and garlic extract was cytotoxic to HeLa, a human cervical cancer cell line (Weber et al., 1992). Chemo-preventive action of garlic on methylcholanthrene-induced carcinogenesis in the uterine cervix of mice was demonstrated (Hussain et al., 1990). Hence, the aim of the present study was first to examine whether allicin was able to inhibit growth in cancer cell lines in general and those of human cervical origin in particular and the involvement of apoptosis in this process. Here we show that allicin inhibits the proliferation of cancer cells and induces apoptosis with typical features such as apoptotic bodies, DNA fragmentation, activation of caspases and poly(ADP-ribose) polymerase cleavage, and thus, these effects of allicin may account partly for the anticarcinogenic properties of garlic.

2. Materials and methods

2.1. Cell culture and maintenance

SiHa cells (human cervical cancer cell line) were obtained from Dr. Sudhir Krishna, National Centre for Biological Sciences, Bangalore, India. L-929 (murine fibrosarcoma), SW480 (human colon cancer) and HeLa (human cervical cancer) cell lines were obtained from the National Centre for Cell Science, Pune, India. The cells were grown in monolayer culture in Dulbecco's Modified Eagle's Medium (Life Technologies, USA) containing 10% fetal bovine serum (Sigma, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Reagents and antibodies

Allicin (Flavourcin™ 1500) was a gift from Brittania Natural Products, UK, (presently known as Overseal Foods) and involved alliin synthesis and converting this to allicin with allinase (purified from garlic) immobilized on an inert support (Eilat et al., 1995; Rabinkov et al., 1995). The purified allicin (1 mg/ml in 50 mM phosphate buffer, pH 4.5) we used was stable (due to the low pH and conditions used in the manufacturing process that involved immediate removal of the reactants after product formation preventing its contact with other components) with 20% loss after 3 weeks at ambient temperature and no loss of activity at 4 °C up to 10 weeks. Antibodies to caspases-8 and -9 and poly(ADP-ribose) polymerase were purchased from Cell Signaling, USA. Caspase-3 substrate (AcDEVD-AFC) was bought from Calbiochem, USA, and the enhanced chemiluminescence detection kit was procured from USB, Amersham, UK.

2.3. Cell viability assay

Cell viability assays were carried out as described with slight modifications (Anto et al., 2000). Briefly, cells were seeded at a density of 3×10^4cells/well into 24-well plates. After 24 h, allicin was added to the medium at various concentrations and incubated for 24 or 48 h as indicated. At the end of the incubation, 50 μl of 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) (2 mg/ml) per well was added, and the formazan crystals formed were solubilized in acidified isopropanol after aspirating the medium. The extent of MTT reduction was measured spectrophotometrically at 570 nm, and the cell survival was expressed as percentage over the untreated control.

2.4. Thymidine incorporation assay

Cells were cultured and treated with allicin as above, and [^{3}H]thymidine was added (0.2 μCi/well) and incubated for 6 h. The culture medium was removed, and wells were washed twice with phosphate-buffered saline, and the proteins were precipitated with 5% trichloroacetic acid. The supernatant was removed, and after washing with ethanol, the cells were solubilized with 0.2N NaOH, and the radioactivity was measured using a β-scintillation counter.

2.5. Acridine orange–ethidium bromide staining

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using acridine orange and ethidium bromide staining method. Briefly, cells were seeded in 12-well plates at seeding densities of 5×10^5cells and then treated with allicin (50 μM) for 24 h. After washing once with phosphate-buffered saline, the cells were stained with 100 μl of a mixture (1:1) of acridine orange–ethidium bromide (4 μg/ml) solutions. The cells were immediately washed with phosphate-buffered saline, viewed under a Nikon inverted fluorescent microscope (TE-Eclipse 300) attached with a camera, and photographs were taken under phase contrast and fluorescent conditions.

2.6. Terminal deoxynucleotidyl transferase-mediated biotin dUTP Nick End Labeling assay

To detect apoptotic cells, in situ end labelling of the 3′ OH end of the DNA fragments generated by apoptosis-associated endonucleases was performed using the Dead End apoptosis detection kit (dUTP Nick End Labeling,

TUNEL assay) from Promega (Madison, USA). Briefly, the cells were grown in cover slips and treated with allicin for 24 h. The cells were washed in phosphate-buffered saline and fixed by immersing the slides in 4% paraformaldehyde for 25 min. All the steps were performed at room temperature, unless otherwise specified. They were then washed twice by immersing in fresh phosphate-buffered saline for 5 min. Cells were permeabilised with 0.2% Triton X-100 solution in phosphate-buffered saline for 5 min, washed twice in phosphate-buffered saline, and then covered with 100 μl of equilibration buffer and kept for 5–10 min. The equilibrated areas were blotted around with tissue paper, and 100 μl of terminal deoxynucleotidyl transferase (TdT) reaction mix was added to the sections on the slide and were then incubated at 37 °C for 60 min inside a humidified chamber for the end-labelling reaction to occur. Immersing the slides in 2 × sodium chloride–sodium citrate buffer for 15 min terminated the reactions. The slides were washed thrice by immersing in fresh phosphate-buffered saline for 5 min to remove unincorporated biotinylated nucleotides. The endogenous peroxidase activity was blocked by immersing the slides in 0.3% H_2O_2. After washing, horseradish-peroxidase-labeled streptavidin solution was applied, and the slides were incubated for 30 min. After incubation, the color was developed with the peroxidase substrate (hydrogen peroxide) and the stable chromogen (diaminobenzidine). The slides were then mounted and examined with a light microscope.

2.7. DNA fragmentation assay

SiHa cells were seeded in 60-mm petri dishes at a seeding density of 4×10^5 cells/plate and treated with allicin or tetradecanoyl phorbol acetate or without any treatment for 48 h. Tetradecanoyl phorbol acetate-treated cells were taken as positive control. The cells were harvested and washed with phosphate-buffered saline. The oligonucleosomal DNA fragments were isolated and analysed as described previously (Anto et al., 2000). DNA in the gels was visualized under ultraviolet light after staining with ethidium bromide.

2.8. Western blot analysis

The cells treated with or without allicin were washed with phosphate-buffered saline and lysed in ice-cold radioimmunoprecipitation buffer (10 mM phenyl methyl sulfonyl fluoride, 1 μg/ml of aprotinin, 100 mM EGTA, 100 mM sodium orthovanadate and 100 mM dithiothreitol). Whole cell extracts (60 μg protein) were resolved on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, probed with corresponding antibodies to caspase-8 or -9, or poly(ADP-ribose) polymerase and detected by enhanced chemiluminescense as per the manufacturer's protocol (USB).

2.9. Caspase-3 assay

The activity of caspase-3 was assayed as described (Sun et al., 1999). Briefly, 10^6 cells treated with or without allicin were washed with phosphate-buffered saline and lysed in radioimmunoprecipitation buffer. The supernatant after centrifugation at 15000 rpm for 10 min was removed, and caspase activity was detected by measuring the proteolytic cleavage of the fluorogenic substrate, AcDEVD-AFC. For this, 50 μg of each sample was incubated with caspase assay

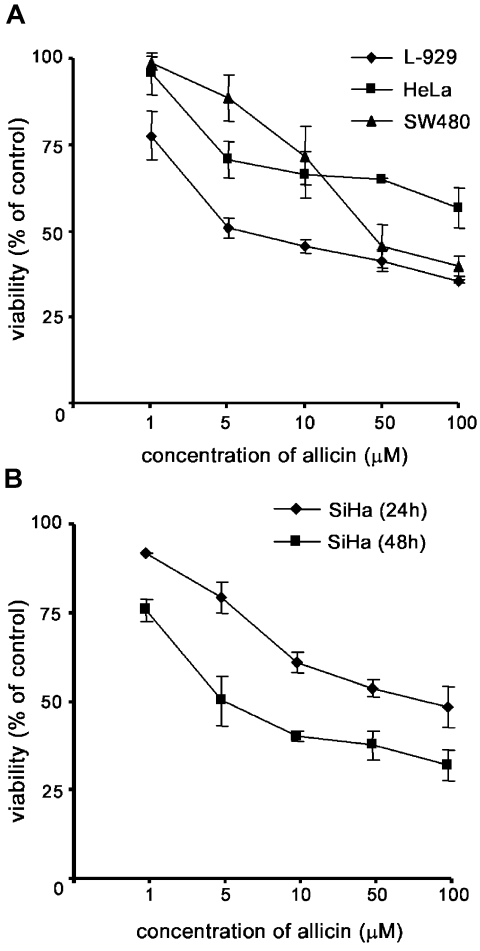

Fig. 2. Inhibition of cell viability of allicin-treated cancer cells. (A) HeLa, SW480 and L-929 grown in 96-well plates were treated without or with the indicated concentrations of allicin and incubated for 24 h. At the end of the incubation, MTT was added, and the cell viability was assessed by MTT assay with quadruplicate samples as described in Materials and methods. The results are expressed as the mean percentage over control, and experiments were repeated three times with similar results and the error bars indicate standard deviations. The differences among the mean values were analyzed using one-way ANOVA, and the average mean values of cell survival differed significantly as a function of concentration of allicin ($P < 0.001$). (B) Viability of SiHa cells treated with or without allicin and incubated for 24 or 48 h was assessed by MTT assay with quadruplicate samples as described above. The experiments were repeated three times with similar results, and the error bars indicate standard deviations. The one-way ANOVA revealed that the average mean values of cell survival differed significantly as a function of concentration of allicin ($P < 0.001$).

buffer and substrate at 37 °C for 45 min. The caspase activity was measured using a spectrofluorimeter (LS 50B model, Perkin Elmer, USA) set with an excitation at 400 nm and emission at 505 nm.

3. Results

3.1. Allicin inhibits the proliferation of cancer cells

L-929, SW480 and HeLa cells were treated with or without allicin for 24 h as indicated and the cell viability assayed by MTT (expressed as percentage over control) decreased with increase in the concentration of allicin (Fig. 2A). In order to show the time-dependent action of allicin in tumor cells, SiHa cells were treated with indicated concentrations of allicin and viability assessed after 24 or 48 h (Fig. 2B), and at 72 h, most of the cancer cells taken up in the present study were killed with 100 μM concentration of allicin (data not shown). We carried out all the experiments using aliquots of a single batch of allicin showing similar pattern and extent of growth inhibition over a range of allicin concentrations by MTT assay. DNA synthesis as measured by the incorporation of labeled thymidine was also decreased by allicin in a concentration-dependent manner in cancer cells (data not shown). These results indicate that allicin inhibits the proliferation of cancer cells in a concentration- and time-dependent manner.

3.2. Morphological alterations and nuclear condensation induced by allicin

While the untreated L-929 or SiHa cells (control) were well spread with flattened morphology, many apoptotic bodies could be noticed in those treated with 50 μM allicin for 24 h (Fig. 3). Similarly, the nuclei were of the normal size (examined by staining the cells with ethidium bromide

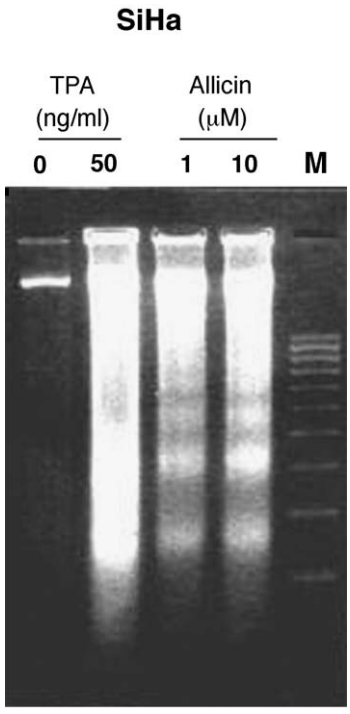

Fig. 4. Allicin-induced DNA fragmentation. SiHa cells grown in 60-mm petri dishes were treated for 24 h with allicin (1 or 10 μM) or tetradecanoyl phorbol acetate (50 ng/ml) as positive control or left untreated (0). M denotes molecular weight marker. The cells were harvested, and the oligonucleosomal DNA fragments were isolated, separated by gel electrophoresis and analysed as described under Materials and methods. The results were similar when another experiment was carried out under the same conditions.

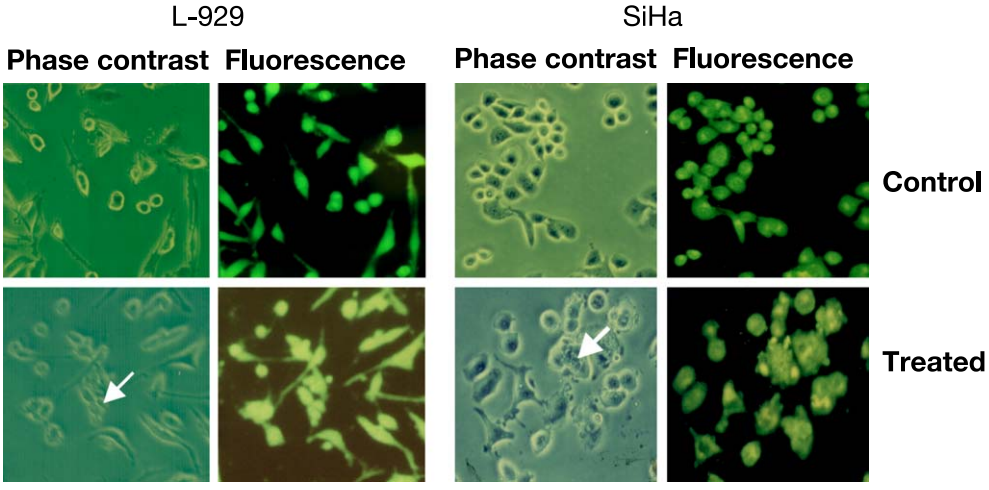

Fig. 3. Changes in nuclear morphology induced by allicin. Cells were seeded in 12-well plates and then treated with or without allicin for 24 h. After washing with phosphate-buffered saline, the cells were stained with a mixture of acridine orange–ethidium bromide solutions. The cells were viewed under an inverted fluorescent microscope and photographed as described under Materials and methods. L-929 or SiHa cells without (control) or with allicin (50 μM) treatment stained by acridine orange–ethidium bromide mixture viewed under phase contrast or fluorescent illumination are shown. These results were confirmed in another independent experiment and the arrows indicate apoptotic bodies. Bar = 50 μm.

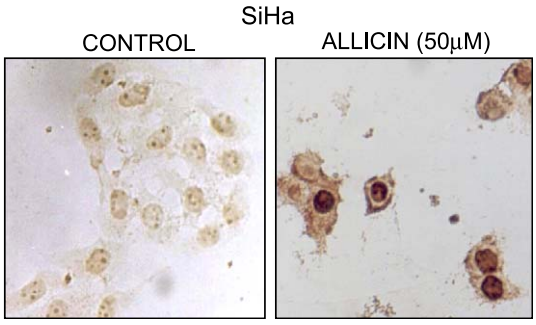

Fig. 5. Changes in TUNEL reactivity induced by allicin. SiHa cells were grown in cover slips and treated with or without allicin for 24 h. The cells were fixed, permeabilised with 0.2% Triton X-100, end-labelled with terminal deoxynucleotidyl transferase reaction mix, and the TUNEL reactivity was visualized as described in Materials and methods. TUNEL reactivity of SiHa cells without (control) or with 50 μM allicin is shown. Bar = 25 μm.

and acridine orange) in untreated cells, whereas treatment with 50 μM of allicin for 24 h resulted in condensation of nuclei (Fig. 3). Thus, the cancer cells treated with allicin form apoptotic bodies and exhibit nuclear condensation, features characteristic of apoptosis.

3.3. Allicin induces DNA fragmentation

The degradation of DNA into multiple internucleosomal fragments of 180–200 base pairs is a distinct biochemical hallmark for apoptosis. To confirm whether the effects induced by allicin in cancer cells involve DNA fragmentation, it was assessed by agarose gel electrophoresis and TUNEL assays. For DNA fragmentation assay, the nuclear DNA isolated from cells was separated by agarose gel electrophoresis and stained with ethidium bromide, and a typical ladder formation was observed upon 24 h treatment with 1 μM allicin or 50 ng/ml tetradecanoyl phorbol acetate (positive control) in SiHa cells, whereas the untreated cells did not show a typical ladder (Fig. 4). Allicin also induced DNA fragmentation in a similar way in L-929 cells (data not shown). SiHa cells without allicin did not show a positive TUNEL reaction, whereas the cells treated with allicin (50 μM) incorporated the labeled nucleotide into DNA (Fig. 5).

3.4. Activation of caspases and poly(ADP-ribose) polymerase cleavage are induced by allicin

We also analyzed the cell extracts for the activation of caspases-8, -9 and -3 upon treatment with allicin. Caspase-3 activity was studied using a fluorogenic substrate, and Fig. 6 shows activation of caspase-3 in both L-929 and SiHa cells by allicin (at two different concentrations) and etoposide (positive control). The cleaved forms of caspase-9 (35 and 37 kDa) were detected by Western blotting, which significantly increased with allicin treatment compared to the control in SiHa cells (Fig. 7A). We also detected the cleaved caspase-8 (18 kDa) upon treatment with allicin in SiHa cells but not in control (Fig. 7B). Furthermore, we examined the cleavage of a well-characterized caspase-3 substrate, poly (ADP-ribose) polymerase, from its 116-kDa intact form into 89-kDa fragment by Western blotting. Poly(ADP-ribose) polymerase was processed to its predicted cleavage product of 89 kDa after allicin treatment, but the processing was absent in the untreated cells (Fig. 7C). These results suggest the involvement of poly(ADP-ribose) polymerase cleavage and activation of caspase-3, -8 and -9 during allicin-induced apoptosis in SiHa cells.

Taken together, the above results suggest that allicin inhibits the growth of cancer cells, and the antiproliferative effects of allicin are mediated through the induction of apoptosis characterized by DNA fragmentation and nuclear

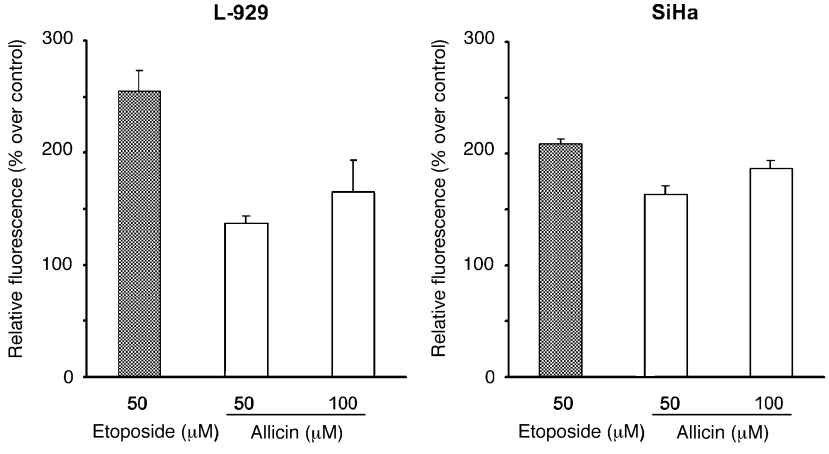

Fig. 6. Activation of caspase-3 by allicin. L-929 or SiHa cells treated for 24 h with the indicated concentrations of allicin or etoposide (positive control) or left untreated were lysed and centrifuged, and the supernatant was used for assaying caspase-3 activity with a fluorogenic substrate. The experiment was repeated another time with similar results and the caspase-3 activity was expressed as relative fluorescence units in percentage over the control. The mean fold activation was significantly higher than the control sample ($P < 0.01$).

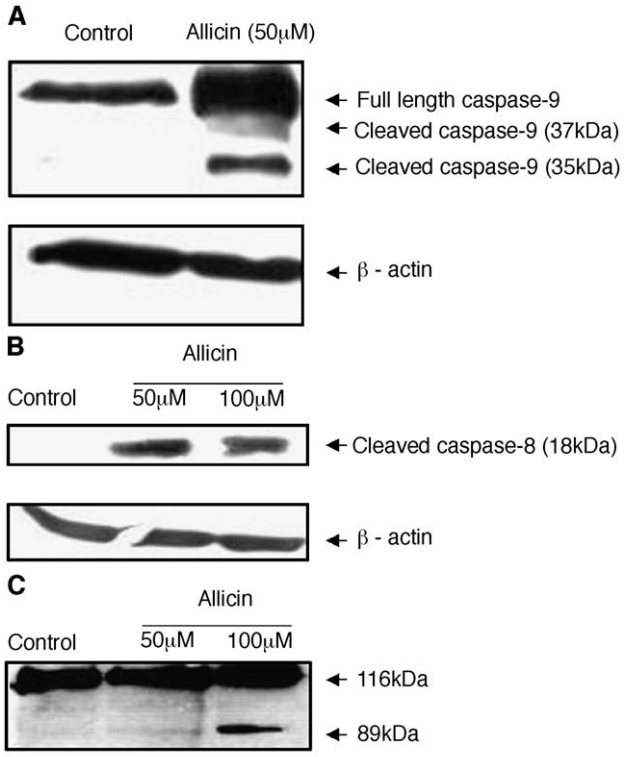

Fig. 7. Cleavage of caspases and poly(ADP-ribose) polymerase by allicin. SiHa cells treated with or without allicin for 24 h were lysed, resolved on sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blotting was carried out to detect the cleaved products of activation of caspase-9 or -8, or poly(ADP-ribose) polymerase shown respectively, in (A), (B) and (C). The experiments were repeated at least two times with similar results, and β-actin was used as loading control.

condensation. Moreover, allicin-induced apoptosis is caspase-dependent, as shown by the activation of caspases-3, -8 and -9 and cleavage of poly(ADP-ribose) polymerase.

4. Discussion

Allicin is readily permeable through phospholipid membranes aiding its biological activity, but its unstable nature raises the question whether all the effects of allicin observed by us and others are due to allicin itself or its metabolites (Hirsch et al., 2000; Miron et al., 2000). There is evidence that allicin is formed from its metabolite, diallyl disulfide, in human liver microsomes indicating that allicin may also be acting intracellularly (Teyssier et al., 1999). In agreement with the present results, allicin inhibited the proliferation of a tumorigenic lymphoid cell line (Scharfenberg et al., 1990) and purified allicin, but not its precursor, alliin, inhibited the growth of human breast, endometrial and colon cancer cell lines (Hirsch et al., 2000). Antiproliferative effects of allicin were attributed to cell cycle arrest and its ability to decrease intracellular glutathione levels rather than its antioxidant activity (Hirsch et al., 2000). The present results demonstrating allicin-induced apoptosis of cancer cells are novel since allicin has not been shown to induce apoptosis previously. Diallyl disulfide induces apoptosis of human leukemia HL-60 cells and triggers the generation of hydrogen peroxide, activation of caspase-3, degradation of poly (ADP-ribose) polymerase and fragmentation of DNA (Kwon et al., 2002). Diallyl trisulfide also induces apoptosis of HL-60 cells, although this was initially mistaken to be an effect of allicin (Zheng et al., 1997; 2000). Other products of allicin transformation, ajoene and *S*-allylmercaptocysteine, also induce apoptosis in human promyeloleukemic and erythroleukemia cell lines, respectively (Dirsch et al., 1998; Sigounas et al., 1997).

Our results support the notion that apoptosis is a potential mechanism by which allicin exerts its antiproliferative effects; however, allicin did not induce DNA degradation and poly(ADP-ribose) polymerase cleavage in MCF-7 (breast cancer) and HT-29 (colon cancer) cells (Hirsch et al., 2000). MCF-7 cells are known to be deficient in caspase-3 (Saunders et al., 2000), but whether that accounts for the failure of allicin to induce apoptosis in these cells is not known. Allicin-induced apoptosis is presumably regulated differently in cells with different genetic background, and changes in the expression of proteins that regulate apoptosis in tumor cells could also account for the failure of allicin to induce apoptosis in certain cells. In fact, tumor cells often evade apoptosis by expressing several anti-apoptotic proteins such as Bcl-2, downregulation and mutation of pro-apoptotic genes and alterations of p53, phosphatidylinositol 3-kinase/Akt or nuclear factor kappa B (NF-κB) pathways that give them survival advantage (Igney and Krammer, 2002). It is relevant to point out that ajoene activates NF-κB (Dirsch et al., 1998) and decreases Bcl-2 expression (Ahmed et al., 2001). Diallyl sulfide increases the level of p53 and Bax, but decreases Bcl-2 in H460 non-small cell lung cancer cells (Hong et al., 2000). It will be of interest to study whether such pro- and anti-apoptotic proteins play any role in allicin-induced apoptosis.

Our results provide a mechanistic basis for the antiproliferative effects of allicin and partly account for the chemopreventive action of garlic extracts reported by earlier workers (Hirsch et al., 2000; Milner, 1996; Scharfenberg et al., 1990; Siegers et al., 1999). Our results also provide a mechanistic basis to the previously observed effects of garlic on cervical cancer cells. Further studies are still needed to understand the various mechanisms regulating the antiproliferative effects and apoptosis induced by allicin.

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