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Curcumin-Induced Apoptosis of A-431 Cells Involves Caspase-3 Activation

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Curcumin, a yellow pigment from *Curcuma longa*, has been known to possess antioxidative and anticarcinogenic properties, as well as to induce apoptosis in some cancer cells. There have been, however, several contradictory reports that hypothesized curcumin (a hydrophobic molecule) can bind a membrane lipid bilayer and induce nonspecific cytotoxicity in some cell lines. Why curcumin shows these contradictory effects is unknown. In A-431 cells, growth inhibition by curcumin is due mostly to the specific inhibition of the intrinsic tyrosine kinase activity of the epidermal growth factor receptor, as reported earlier by Korutla *et al.* Thus, we assumed that the cell death of A-431 by curcumin might be due to the specific induction of apoptosis. In this paper we clearly show that curcumin induces apoptosis in A-431 cells. The curcumin-induced cell death of A-431 exhibited various apoptotic features, including DNA fragmentation and nuclear condensation. Furthermore, the curcumin-induced apoptosis of A-431 cells involved activation of caspase-3-like cysteine protease. Involvement of caspase-3 was further confirmed by using a caspase-3 specific inhibitor, DEVD-CHO. In another study, decreased nitric oxide (NO) production was also shown in A-431 cells treated with curcumin, which seems to be the result of the inhibition of the iNOS expression by curcumin, as in other cell lines. However, 24 h after treatment of curcumin there was increased NO production in A-431 cells. This observation has not yet been clearly explained. We assumed that the increased NO production may be related to denitrosylation of the enzyme catalytic site in caspase-3 when activated. Taken together, this study shows that the cell death of A-431 by curcumin is due to the induction of apoptosis, which involves caspase-3 activation.

Keywords: Apoptosis, Caspase-3, Curcumin, DEVD-CHO, Nitric oxides.

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Introduction

Curcumin is a yellow phenolic compound found in *Curcuma longa*. It has been widely used as a traditional medicine in Asia. It is known to possess antioxidative and anticancer properties. Recent strategies to tackle various cancers have turned toward apoptosis, a key mode in organisms that involves embryonic development, metamorphosis, and hormone-dependent atrophy (regulating the cell number and eliminating damaged cells). Unlike necrosis, apoptotic cells shrink and are rapidly digested by neighboring cells before any content of the damaged cells spill over. Thus, the specific induction of apoptosis against tumors can be seen as an exceptional strategy that yields little or no side effects. Curcumin has been reported to induce apoptosis in HL-60, the human leukemic cell line (Kuo *et al.*, 1996). Khar *et al.* (1999) showed that curcumin induces apoptosis in AK-5 cells. They also confirmed that the curcumin-induced apoptosis of AK-5 cells is mediated by caspase-3 activation. On the other hand, Mehta *et al.* (1997) reported that although sensitive to curcumin, breast cancer cell lines showed no apoptotic cell death despite cell growth inhibition. A recent study by Jaruga *et al.* (1998) showed that curcumin (a hydrophobic molecule) passes easily through the plasma membrane, and spreads throughout the lipid phase of membranous structures, i.e. ER, mitochondria, and the nuclear envelope. Thus, curcumin can enter into the lipid bilayer, and evoke structural and functional changes of cellular membranes, such as membrane blebbing and phosphatidyl serine exposure. These effects of curcumin are a non-specific way and seem to be a mimic of apoptosis. These contradictory reports led us to investigate whether curcumin can specifically induce apoptosis in other human cell lines. According to Korutla *et al.* (1994), curcumin inhibited the intrinsic tyrosine kinase activity of the epidermal growth factor receptor (EGF-R) in A-431 cells. This effect occurred as early as 15 min after being treated with curcumin. So, we presumed that the growth inhibitory effect of curcumin on A-431 cells may be mainly due to the inhibition of EGF-R tyrosine kinase activity, and the cell death of A-431 by

curcumin might involve the specific induction of apoptosis. In this study, we conducted several apoptosis assays, including DNA fragmentation, TUNEL, and caspase-3 assay. The apoptotic cell death of A-431 by curcumin, and modulation of NO production at the time when caspase-3 is activated, also will be discussed.

Materials and Methods

Materials Curcumin was purchased from the Sigma Chemical Co. (St. Louis, USA). DMEM, fetal bovine serum, and antibiotic-antimycotic were purchased from Gibco BRL (Grand Island, USA). Proteinase K was purchased from Roche Molecular Biochemical (Mannheim, Germany). The TUNEL assay kit was bought from the Intergen Company. Griess Reagent was purchased from Fluka. Ac-DEVD-pNA, Ac-YVAD-pNA, and Ac-DEVD-CHO were purchased from the Sigma Chemical Co. All other chemicals and reagents were the highest grade commercially available.

Cell culture The A-431 (human epithelial carcinoma) cell line was kindly provided by KRIBB (Korean Research Institute of Bioscience and Biotechnology; Taejon, Korea). The cells were maintained in a DMEM medium supplemented with 10% fetal bovine serum with 1% antibiotic-antimycotic at 37°C in a humidified atmosphere of 10% CO_2.

MTT assay Cytotoxicity was measured using the MTT assay. The MTT assay was performed using a modified method by Plumb *et al.* (1989). Briefly, exponential growing cells were trypsinized and the cell numbers were counted using either a hemocytometer or Coulter Counter (Coulter, UK). These cells were then inoculated at 3×10^3 cells/well using 96-well plates supplemented with 150 μl of DMEM. After 24 h, various concentrations of curcumin were added. After 3 days, 50 μl of MTT (2 mg/ml stock solution, Sigma) was added and the plates were incubated for an additional 4 h. 150 μl of DMSO was added to dissolve the formazan and 20 μl of Sorenson's glycine buffer was added to obtain the highest absorbance at 570 nm. After 10 min of gentle shaking, each plate was read at 570 nm using a microplate reader (Molecular Devices Co.). The IC_{50} values were determined by plotting the drug concentration versus the survival ratio of the treated cells.

Analysis of DNA fragmentation The 2×10^3 cells were seeded in 6-well plates and after 2 days were treated with curcumin at various concentrations. Cells were harvested with a cell scraper and washed twice with PBS, then centrifuged at 2000 rpm for 5 min at 4°C. Cells were lysed in a 500 μl lysis buffer at 37°C for 2 h and centrifuged at 1000 g for 10 min to remove cell debris. Supernatants were incubated for at least 2 h or overnight in 50 μg/ml RNase A and 120 μg/ml proteinase K. Then, DNA was extracted with phenol in 0.5 M Tris (pH 8.0), phenol/chloroform/isoamylalcohol (25 : 24 : 1). The supernatant containing DNA was then precipitated with 100% ethanol and 0.3 M sodium acetate at -70°C, then washed with 70% alcohol. DNA samples were electrophoresed through a 1.5% agarose gel containing ethidium bromide, and DNA bands were visualized under UV light.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) assay Cells were prepared as described previously in 24-well plates with coverslips. After curcumin administration, a TUNEL assay was performed according to the protocol provided by the manufacturer. Briefly, cells were fixed in 1% paraformaldehyde and precooled ethanol : acetic acid (2 : 1), and equilibrated with a equilibration buffer. Samples were treated with TdT enzyme and incubated in a humidified chamber at 37°C for 1 h. After washing in PBS, samples were applied with an anti-digoxigenin peroxidase conjugate, and incubated in a humidified chamber for 30 min at room temperature. Samples were washed with PBS and stained with peroxidase substrate (diaminobenzidine) for 5 min at room temperature. Counterstain was performed with 0.5% (w/v) methyl green. The stained cells were observed under a microscope.

Caspase Assay The 2×10^5 cells were seeded in 6-well plates, and after 2 days were treated with curcumin at various time intervals. The 5×10^6 cells were harvested and washed with cold PBS, then resuspended in 200 μl of a lysis buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% sucrose). After incubation on ice for 30 min, lysates were centrifuged for 5 min at 13,000 rpm and the clear supernatants were removed for determination of caspase activity. An aliquot of each sample was saved for protein quantification. The protease reactions were carried out with 100 μg of protein extract and 200 μM substrate in a lysis buffer. Reaction mixtures were placed into a flat-bottomed mitrotiter plate and read with a 405 nm filter using a microtiter plate reader. Caspase activities were detected by measuring the proteolytic cleavage of the colored substrates Ac-DEVD-pNA and Ac-YVAD-pNA. The cleaved pNA concentration was determined by absorbance at 405 nm in comparison with standard solutions of p-nitroaniline prepared in the same buffer.

Measurement of Nitric Oxides The nitric oxide (NO) production was measured as the amount of nitrite, the stable end product of NO, released into the culture supernatant. The nitrite concentration was determined in culture supernatants using the spectrophotometric method based on the Griess reaction. Samples were mixed with equal volumes of Griess reagent and incubated at room temperature for 10 min. The nitrite concentration was determined by absorbance at 540 nm in comparison with standard solutions of sodium nitrite prepared in the same medium.

Results

The Effect of Curcumin on A-431 Cell Growth The A-431 cells were incubated with various concentrations of curcumin, and the viability of the treated cells was compared with that of solvent-treated controls. Curcumin showed the potent growth inhibition at micromolar concentrations, as determined by a MTT assay (Fig. 1). The IC_{50} value of curcumin for A-431 was 13 μM. After 50 μM of curcumin treatment, the cell viability was decreased by less than 10%.

Analysis of Internucleosomal DNA Fragmentation In order to further investigate the mode of cell death induced by

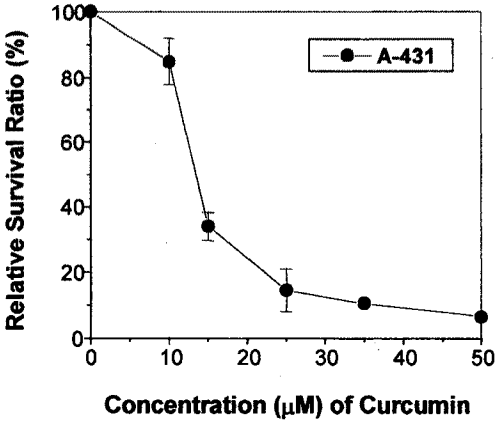

Fig. 1. Cell survival ratio of A-431 cells treated with curcumin for 48 h. Data points represent means ± S.D. from three separate experiments.

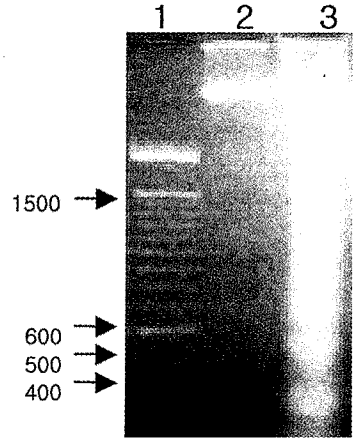

Fig. 2. DNA extracted from A-431 cells at 24 h after being treated with curcumin. Lane 1, 100 bp DNA ladder marker; Lane 2, control; Lane 3, 40 μM curcumin.

curcumin, we examined the biochemical changes of the genomic DNA of A-431 cells following treatment with curcumin. DNA fragmentation is one of many traits that apoptotic cells undergo. Therefore, to determine the effect of curcumin we judged the appearance of a DNA ladder by gel electrophoresis on A-431 cells. The DNA fragmentation was induced at 24 h after treatment with curcumin at a concentration of 40 μM (Fig. 2). As compared to the 100 bp DNA ladder marker, curcumin induced internucleosomal DNA fragmentation in A-431 cells.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) Assay To determine the effect of curcumin on high and low molecular weight DNA fragmentation microscopically, we applied TUNEL assay. DNA fragmentation was observed when A-431 cells were treated with curcumin for 24 h at the concentration of 40 μM (Fig. 3). Because A-431 cells were detached during apoptotic processes, the number of observed cells was much fewer than

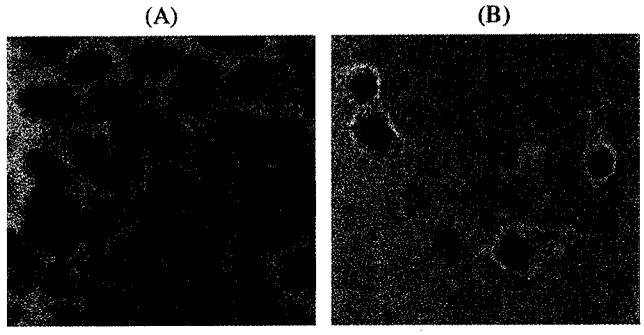

Fig. 3. TUNEL-labeled A-431 cells after being treated with 40 mM curcumin. Blue colors represent normal nuclei and dark-brown colors exhibit fragmented DNA. A, control; B, A-431 cells treated with 40 μM of curcumin for 24 h. (×400 magnification).

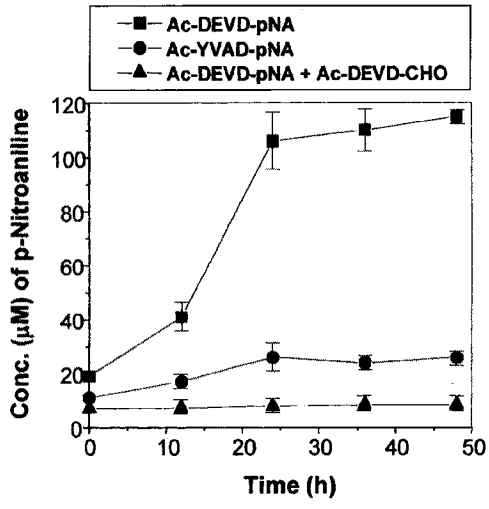

Fig. 4. Caspase-1 and caspase-3 activities in 40 μM curcumin treated A-431 cells at different time points. Caspase activities are expressed as the concentration of cleaved colored substrate, p-nitroaniline, using a standard curve for p-nitroaniline. Data are means ± S.D. from three separate experiments.

that of the control. In Fig. 3B, black dots represent condensed chromatins.

Activation of Caspase-3 There is considerable evidence that during apoptosis, members of a family of cysteine protease with aspartate specificity (the caspases) undergo proteolytic processing and activation, and then orchestrate the central apoptotic events. We examined whether or not caspase-3 and caspase-1 are involved in curcumin-induced apoptosis. Two of the tetra-peptides, Ac-DEVD-pNA and Ac-YVAD-pNA, were used as substrates for caspase-3 and caspase-1 respectively. In our results, caspase-1 activity was not noticeable, whereas caspase-3 activity was significantly higher after a 24 h curcumin treatment (Fig. 4). The involvement of caspase-3 during curcumin-induced apoptosis in A-431 cells was further confirmed by using the caspase-3 specific inhibitor, Ac-DEVD-

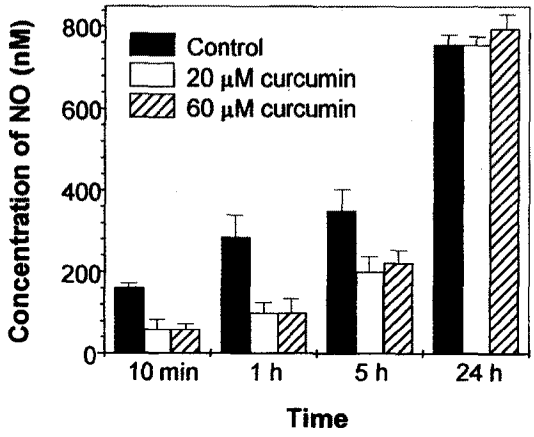

Fig. 5. Production of nitric oxides (NO) in A-431 cells treated with or without curcumin. Data are means ± S.D. from three separate experiments.

CHO. Caspase-3 activation during curcumin-induced apoptosis in A-431 cells was completely inhibited by Ac-DEVD-CHO (Fig. 4). These results suggest that caspase-3 is a major cysteine protease and is, in part, involved in curcumin-induced apoptosis in A-431 cells.

Measurement of Nitric Oxides (NO) The reactive oxygen species (ROS) have been focused on their involvement in the cellular signaling events, which exists at a low concentration. Particularly, they play a major role in the cell proliferative signaling pathways such as NF-kB activation, EGF signaling, and Ras activation. Among the ROS, nitric oxides (NO) have been known to be signaling molecules that respond to inflammation. So, we examined the inhibitory effect of curcumin on nitric oxide (NO) production in A-431 cells.

Curcumin down-regulated the nitric oxide production in A-431 cells, even 10 min after treatment (Fig. 5). However, 24 hours later the NO were increased at nearly the same concentration as the ones in the control (Fig. 5). Chan *et al.* (1998) and other reports showed that curcumin down-regulates the iNOS expression, which results in decreased NO production. So, the early inhibitory effect of curcumin on NO production in A-431 cells seems to be the result of the down-regulation of the iNOS expression by curcumin. However, at the time when cells are dying, curcumin increases in the NO release in A-431 cells. This observation has not been fully explained. A recent study by Mannick *et al.* (1999), as well as other reports, showed that caspase-3 activation requires denitrosylation of their catalytic site. Considering these reports, we assumed that the increase in the NO release in A-431 cells at the late stage of curcumin treatment is related to the activation of caspase-3. This assumption is consistent with the result that shows caspase-3 activation at 24 h after treatment with curcumin (Fig. 4).

Discussion

Curcumin is a well known anticancer agent. Apoptosis induction by curcumin has been reported in human leukemia cells (Kuo *et al.*, 1996). According to Jaruga *et al.* (1998), curcumin (a hydrophobic molecule) passes easily through the plasma membrane and spreads throughout the lipid phase of membranous structures, i.e. ER, mitochondria, and nuclear envelope. Thus, curcumin can enter into the lipid bilayer, and evoke structural and functional changes of cellular membranes, such as membrane blebbing and phosphatidyl serine exposure. These effects of curcumin are a non-specific way and seem to be a mimic of apoptosis. However, curcumin specifically induced apoptosis in AK-5 cells with caspase-3 activation (Khar *et al.*, 1999). In our study, curcumin induced apoptosis in A-431 cells, as confirmed by DNA fragmentation and nuclear condensation. Moreover, caspase-3 was activated in the apoptotic cell death of A-431. Although we did not perform the experiment that shows that caspase-3 mediates curcumin-induced apoptosis, (at least in part) caspase-3 is involved in the curcumin-induced apoptosis of A-431 cells. These results suggest that curcumin could induce apoptosis in a more specific way. Inducible nitric oxide synthase (iNOS) appears to regulate the cyclooxygenase-2 (COX-2) expression and production of proinflammatory prostaglandins, which are known to play a key role in various tumor developments. Curcumin has been reported as an inhibitor of the iNOS expression (Chan *et al.*, 1998; Rao *et al.*, 1999). Down-regulation of the NO production by curcumin in our results is likely due to the suppression of the iNOS expression. At the late stage of curcumin treatment, NO were released into the culture supernatant at the same control levels. A recent study by Mannick *et al.* (1999) showed that the caspase-3 activation in Fas-induced apoptosis requires denitrosylation of its catalytic site. Although the exact molecular mechanism of curcumin remains to be investigated further, increased NO production in A-431 cells by curcumin seems to be related to caspase-3 activation.

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