Antitumor activity of curcumin is mediated through the induction of apoptosis in AK-5 tumor cells

Ashok Khar*, A. Mubarak Ali, B.V.V. Pardhasaradhi, Zareena Begum, Rana Anjum

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Received 29 December 1998

Abstract Curcumin, the yellow pigment of turmeric (*Curcuma longa*), used commonly as a spice, has been shown to possess anticarcinogenic activity. Curcumin inhibited AK-5 tumor growth and induced apoptosis in AK-5 cells. Curcumin induced apoptosis is mediated through the activation of caspase-3, which is specifically inhibited by the tetrapeptide Ac-DEVD-CHO. In addition, curcumin induced tumor cell death is caused through the generation of reactive oxygen intermediates which is inhibited by *N*-acetyl-L-cysteine. Our studies suggest that the apoptotic process induced by curcumin is the mechanism mediating AK-5 tumor cell death.

Key words: AK-5 apoptosis; Curcumin; Tumor growth; Caspase-3

1. Introduction

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], the active component of turmeric, a common spice used in the Indian cuisine and routinely used in Indian natural medicine [1], has been shown to exhibit antimutagenic and anticarcinogenic activities in addition to the anti-inflammatory activity [2,3]. Curcumin has also been shown to possess bactericidal [4], anthelmintic activity and reduce cholesterol levels when given orally [5]. Recently, curcumin has been shown to induce apoptosis-like changes in rat thymocytes [6]. Curcumin has also been shown to inhibit cell proliferation thus affecting the cell cycle [7]. Several mechanisms for curcumin action, like inhibition of protein kinase C [8] and redox regulation [9], have been proposed.

AK-5, a rat histiocytic tumor [10], is regressed when injected s.c. but kills 100% of hosts when transplanted i.p. [11]. Tumor cells are killed by necrosis and apoptosis [12]. In this paper, we have studied the antitumor activity of curcumin in i.p. tumor transplanted animals. Eighty percent of the animals transplanted with AK-5 cells did not develop ascites after treatment with curcumin. Curcumin kills the tumor cells by apoptosis, and induces typical apoptotic features such as the formation of apoptotic bodies, DNA fragmentation in AK-5 tumor cells involving caspase-3 which is specifically inhibited by the tetrapeptide DEVD-CHO. *N*-Acetylcysteine, an antioxidant, also inhibited curcumin mediated apoptosis of AK-5 cells.

2. Materials and methods

2.1. Materials

AK-5 tumor was maintained as ascites in an inbred colony of Wistar rats by injecting 5×10^6 tumor cells i.p. We also used a single cell clone of AK-5 adapted to grow in vitro, called BC-8, in these studies to avoid ambiguity in results due to tumor heterogeneity [13]. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), curcumin, and *N*-acetyl-L-cysteine were procured from Sigma Chemical Co., USA. Propidium iodide was from Calbiochem, YVAD-AMC and DEVD-AMC were purchased from Bachem, Switzerland and the ECL detection kit was procured from Amersham, UK.

2.2. Apoptosis assay and flow cytometry

BC-8 cells (1×10^6) were plated in DMEM-FCS. The cells were incubated with 45% ammonium sulfate fraction of anti-AK-5 serum called serum factor (10%) which served as the positive inducer of apoptosis. Cells were treated with curcumin for different time periods. After the incubation, the cells were washed with phosphate buffered saline, fixed in ethanol, and stained with propidium iodide (propidium iodide 50 μg/ml in 0.1% sodium citrate containing 0.1% Triton X-100) and analyzed by flow cytometry.

2.3. DNA extraction and electrophoresis

Fixed tumor cells were washed, suspended in citrate-phosphate buffer and the fragmented DNA was extracted as described earlier [14]. Fragmented DNA with loading buffer was electrophoresed on 0.8% agarose gel at 2 V/cm for 16 h. DNA in the gels was visualized under UV light after staining with 5 μg/ml ethidium bromide.

2.4. Inhibition of apoptosis

AK-5 or BC-8 cells (2×10^6) after treatment with curcumin (25 μM) were extracted with lysis buffer. The cell extract was preincubated with the tetrapeptide Ac-DEVD-CHO for 15 min and the extracts were used for caspase assays.

2.5. Caspase assays

The activities of caspase-1 and caspase-3-like proteases were assayed as described earlier [15]. Briefly, 10^6 cells were lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin) for 15 min at 4°C followed by centrifugation ($20\,000\times g$) for 5 min. Caspase activities were detected in the supernatants by measuring the proteolytic cleavage of the fluorogenic substrates YVAD-AMC and DEVD-AMC [16] respectively in assay buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, 2 mM DTT, 1 mM PMSF, 1 μg/ml each of aprotinin and leupeptin) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

2.6. Tumor growth

Ten animals in each group were transplanted with 2×10^6 AK-5 cells i.p. The curcumin group was administered 40 mg of curcumin per rat/injection i.p. on days 0, 2, 4, 6, 8 and, 10 after tumor transplantation. Animals were regularly monitored for the appearance of peritoneal bulge and survival up to 40 days.

2.7. Inhibition of apoptosis by N-acetyl-L-cysteine

BC-8 cells (1×10^6) were cultured in DMEM-FCS in the presence of curcumin (25 μM). Cells were also treated with different concentrations of *N*-acetyl-L-cysteine (0.25–1 mM). After incubation the cells were fixed, stained with propidium iodide and analyzed by flow cytometry.

*Corresponding author. Fax: (91) (40) 7171195.
E-mail: khar@ccmb.ap.nic.in

PII: S0014-5793(99)00114-3

2.8. Western blotting

After treatment of AK-5 cells with curcumin, the cells were washed, boiled in Laemmli's sample buffer and the proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membrane and probed for 1 h with an appropriate dilution of antibody to PARP or caspase-3. The blots were washed and treated with either alkaline phosphatase or horseradish peroxidase-conjugated second antibody for 30 min. PARP blot was developed for alkaline phosphatase and the caspase-3 blot was developed with the ECL detection kit.

3. Results

3.1. Effect of curcumin on tumor growth

We have studied the effect of curcumin on the growth of AK-5 tumor as ascites. There was a significant inhibition of tumor growth by curcumin (Table 1). All the control animals developed peritoneal bulge and 90% of animals died by day 10. However, in curcumin treated animals, no peritoneal bulge was observed in 80% of the animals and the animals survived even after 40 days. These observations confirm the antitumor activity of curcumin.

3.2. Curcumin induced apoptosis in AK-5 cells

In order to understand the exact mechanism of killing of AK-5 tumor cells by curcumin, we incubated AK-5 cells with different concentrations of curcumin in culture. Curcumin at 10 μM concentration induced apoptosis in about 40% of tumor cells during 4 h incubation. The same concentration of curcumin induced apoptosis in 100% of cells after 18 h incubation. Induction of apoptosis was confirmed by the formation of apoptotic bodies (Fig. 1B) and fragmentation of cellular DNA (Fig. 1C). Curcumin induced apoptosis was quantitated by flow cytometry after staining the cells with propidium iodide. We also used a factor present in the serum of tumor rejecting animals and dexamethasone as positive inducers of apoptosis in AK-5 cells (data not shown).

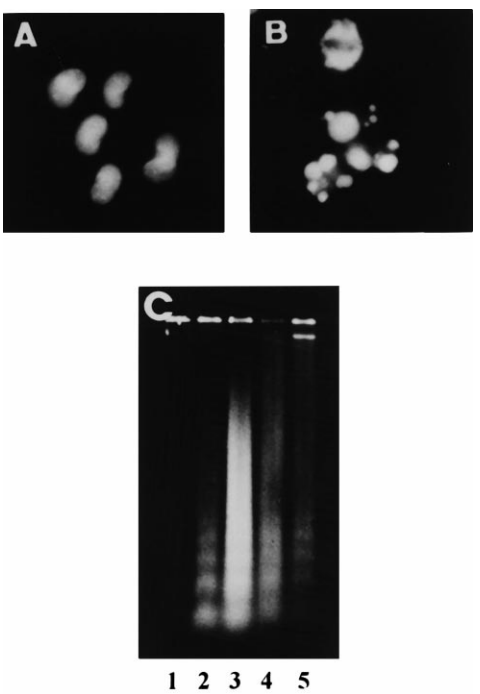

Fig. 1. Curcumin induced formation of apoptotic bodies in AK-5 cells. A: Control cells. B: Curcumin treated propidium iodide stained cells. C: Curcumin induced DNA fragmentation. Lanes 1, control; 2, serum factor treated positive control; 3, 25 μM curcumin; 4, curcumin+DEVD 50 μM; 5, curcumin+DEVD 100 μM.

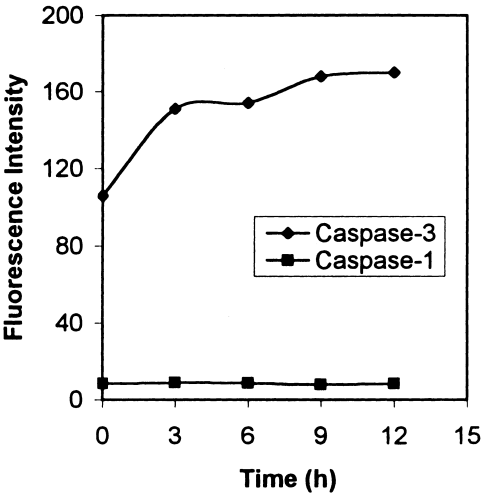

Fig. 2. Caspase-1 and caspase-3 activities in curcumin treated AK-5 cells at different time points. Caspase activities are expressed as relative fluorescence units.

3.3. Role of caspases in curcumin induced apoptosis

Caspases have been shown to participate in the execution phase of the apoptotic process. After curcumin treatment of AK-5 cells, we analyzed the cell extracts for caspase-1 and caspase-3 activities using fluorogenic substrates. Caspase-1 activity was not detected up to 12 h after curcumin treatment of AK-5 cells, whereas caspase-3 activity was significantly higher after 3 h curcumin treatment (Fig. 2). These results suggest the participation of caspase-3 during curcumin induced apoptosis in AK-5 cells.

3.4. Specific inhibition of caspase-3 activity

In order to confirm the participation of caspase-3 during curcumin induced apoptosis in AK-5 cells, we used a caspase-3 specific tetrapeptide inhibitor Ac-DEVD-CHO (DEVD). Caspase-3 activity was completely inhibited by 1 μM DEVD (Fig. 3A). DEVD also inhibited the curcumin induced DNA fragmentation in AK-5 cells (Fig. 1C, lanes 4 and 5). These observations suggest caspase-3 to be a major cysteine protease involved in curcumin induced apoptosis. Fig. 3B shows the effect of *N*-acetyl-L-cysteine on curcumin induced apoptosis in AK-5 cells. A significant inhibition of apoptosis in tumor cells was observed with *N*-acetyl-L-cysteine concentrations ranging

Table 1
Effect of curcumin on the growth of AK-5 tumors[a]

Group	Number of animals dead/number of animals injected
Control	9/10 (10%)
Curcumin treated	2/10 (80%)

[a]AK-5 tumor cells were transplanted i.p. Curcumin (40 mg/rat) was administered i.p. on days 0, 2, 4, 6, 8, and 10 after the tumor transplantation and the animals were monitored for tumor growth and survival up to day 40. Control animals died by day 10 of AK-5 transplantation. The values in parentheses denote percent survival.

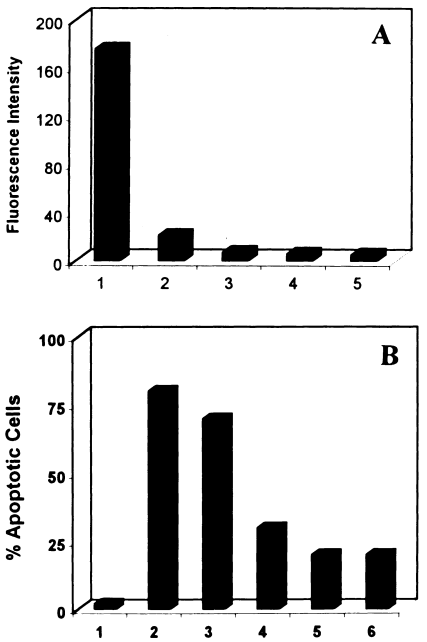

Fig. 3. A: Inhibition of caspase-3 activity with different concentrations of DEVD. 1, positive control; 2, DEVD 50 nM; 3, DEVD 100 nM; 4, DEVD 500 nM; 5, DEVD 1 μM. B: Inhibition of curcumin induced apoptosis in AK-5 cells with different concentrations of *N*-acetyl-L-cysteine (NAC). 1, control; 2, curcumin 25 μM; 3, curcumin+NAC 0.25 mM; 4, curcumin+NAC 0.5 mM; 5, curcumin+NAC 0.75 mM; 6, curcumin+NAC 1 mM.

from 0.5 to 1.0 mM. These observations suggest the involvement of reactive oxygen intermediates during curcumin mediated apoptosis in AK-5 cells.

3.5. Activation of caspase-3

We also confirmed the cleavage of procaspase-3 to its active form of 17 kDa by Western blotting (Fig. 4B, lane 3). Lane 2 is the serum factor treated tumor cells which acted as the positive control. Similarly PARP was also degraded to its cleavage product of 85 kDa (Fig. 4A, lane 3). Thus these observations confirm participation of caspase-3 in curcumin induced apoptosis in AK-5 cells.

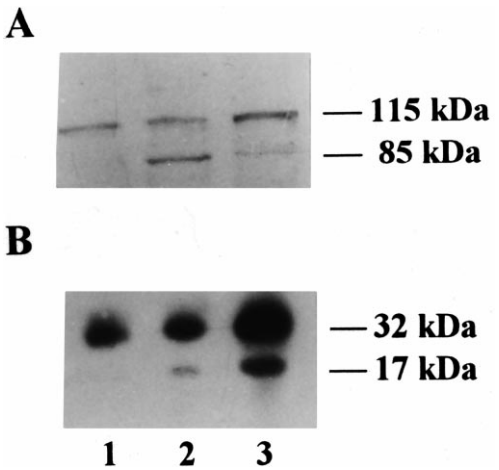

Fig. 4. Western blotting of PARP degradation (A) and caspase-3 activation (B). Lanes 1, control AK-5 cells; 2, AK-5 cells+serum factor; 3, AK-5 cells+curcumin 25 μM.

4. Discussion

Immunological rejection of AK-5 tumor in syngeneic hosts is mediated through necrosis and apoptosis [12] induced by NK cells through perforin and granzyme B respectively [17]. AK-5 tumor cells transplanted i.p. are not rejected and kill 100% of hosts. This differential response to the same tumor in syngeneic animals has been ascribed to the overexpression of interleukin-12 (p40 subunit) [18] and regulated expression of Fas-L by the AK-5 cells transplanted i.p. which help the tumor cells in immune escape [19]. In our studies curcumin was highly effective in inhibiting intraperitoneal tumor growth. Therefore, it was interesting to study the mechanism of action of curcumin on AK-5 tumor cells.

Curcumin, being a hydrophobic molecule [20], passes easily through the plasma membrane into the cytosol. Recently curcumin was shown to cause phosphatidylserine exposure, increased plasma membrane permeability and decreased mitochondrial membrane potential and cell shrinkage [6], which are typical features of apoptotic cells. In AK-5 cells too, curcumin was able to induce the formation of apoptotic bodies and fragmentation of nuclear DNA (Fig. 1). We excluded the possibility of necrotic death in AK-5 cells induced after curcumin treatment as there was no cell swelling and the membrane remained intact at least during the initial phase of curcumin treatment, which was further confirmed through the involvement of caspase-3. However, caspase-1 does not seem to be activated during curcumin induced apoptosis in AK-5 cells. Participation of caspase-3 was further confirmed using DEVD as the specific inhibitor which inhibited curcumin induced apoptosis and fragmentation of AK-5 cell DNA.

Curcumin is a known antioxidant and acts as a scavenger of free radicals [21]. Antioxidants such as superoxide dismutase or catalase prevented curcumin induced apoptosis in human leukemia cells [22]. We have recently shown the participation of reactive nitrogen intermediates in AK-5 cell apoptosis [23] and our preliminary observations indicate a role for reactive oxygen intermediates during apoptosis (unpublished observations). This was further confirmed using *N*-acetyl-L-cysteine, which is a well known scavenger of reactive oxygen intermediates, and inhibited curcumin induced apoptosis in AK-5 cells. Similar observations were made during curcumin induced apoptosis in human leukemic cells [22]. Curcumin also increased the level of glutathione which would prevent thiol depletion occurring during apoptosis [6]. Thus the role of curcumin in redox balance needs further investigation. Curcumin is an interesting molecule because of the variety of biological effects it possesses in addition to its potent anticancer activity. However, its exact mechanism of action is not very clear. Our present studies suggest that its anticancer activity could be mainly due to its ability to induce apoptosis in tumor cells.

Acknowledgements: The authors are grateful to Dr. Anu Srinivasan for anti-caspase-3 antibody and Dr. Guy Poirier for anti-PARP antibody. Excellent technical help provided by Mr. P. Jayaraman in handling animals is acknowledged. Mr. K. Kennady helped us in flow cytometry and Ms. T. Hemalatha in typing the manuscript. Financial support was provided by the Department of Biotechnology, Government of India.

References

[1] Nadkarni, K.M. (1976) in: Indian Materia Medica (Nadkarni, K.M., Ed.), pp. 414–417, Popular Prakashan, Bombay.
[2] Stoner, G.D. and Mukhtar, H. (1995) J. Cell Biochem. Suppl. 22, 169–180.
[3] Kuttan, R., Bhanumathy, P., Nirmala, K. and George, M.C. (1985) Cancer Lett. 29, 197–202.
[4] Ramprasad, C. and Sirsi, M. (1956) J. Sci. Ind. Res. (India) 156, 239–241.
[5] Pachuari, S.P. and Mukherjee, S.K. (1970) J. Res. Indian Med. 6, 27–31.
[6] Jaruga, E., Salvioli, S., Dobrucki, J., Chrul, S., Bandorowicz-Pikula, J., Sickora, E., Franceschi, C., Cossarizza, A. and Bartosz, G. (1998) FEBS Lett. 433, 287–293.
[7] Hanif, R., Qiao, L., Shiff, S.J. and Rigas, B. (1997) J. Lab. Clin. Med. 130, 576–584.
[8] Liu, J.-Y., Lin, S.-J. and Lin, J.-K. (1993) Carcinogenesis 14, 857–861.
[9] Pulla Reddy, A.Ch. and Lokesh, B.R. (1994) Food Chem. Toxicol. 32, 279–283.
[10] Khar, A. (1986) J. Natl. Cancer Inst. 76, 871–878.
[11] Khar, A. (1993) Int. J. Oncol. 2, 393–398.
[12] Kausalya, S., Hegde, S.P., Bright, J.J. and Khar, A. (1994) Exp. Cell. Res. 212, 285–290.
[13] Khar, A. and Ali, A.M. (1990) In Vitro Cell. Dev. Biol. 26, 1024–1025.
[14] Bright, J.J., Kausalya, S. and Khar, A. (1995) Immunology 85, 638–644.
[15] Dimmeler, B.S., Haendeler, J., Nehls, M. and Zeiher, A.M. (1997) J. Exp. Med. 185, 601–607.
[16] Gurtu, V., Kain, S.R. and Zhang, G. (1997) Anal. Biochem. 251, 98–102.
[17] Khar, A., Pardhasaradhi, B.V.V., Varalakshmi, Ch., Ali, A.M. and Kumari, A.L. (1997) Cell. Immunol. 177, 86–92.
[18] Nandakumar, K.S., Leelakumari, A. and Khar, A. (1997) Cytokines Cell. Mol. Ther. 3, 225–232.
[19] Khar, A., Varalakshmi, Ch., Pardhasaradhi, B.V.V., Ali, A.M. and Kumari, A.L. (1998) Cell. Immunol. 189, 85–91.
[20] Oetari, S., Sudibyo, M., Cammandeur, J.N.M., Samhoedi, R. and Vermeulen, N.P.E. (1996) Biochem. Pharmacol. 51, 39–45.
[21] Tonnensen, H.H. and Greenhill, J.V. (1992) Int. J. Pharm. 87, 79–87.
[22] Kuo, M.-L., Huang, T.-S. and Lin, J.-K. (1996) Biochim. Biophys. Acta 1317, 95–100.
[23] Bhaumik, S. and Khar, A. (1998) Apoptosis (in press).