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Curcumin inhibits telomerase activity in human cancer cell lines

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Abstract. Curcumin, one of the major components of tumeric, the dried rhizome of *Curcuma longa* L, has been shown to have anti-proliferating and anti-carcinogenic properties. In this study, we examined the effects of curcumin on cell growth and telomerase activity in human cancer cell lines Bel7402, HL60 and SGC7901. Curcumin (1-32 μM) showed anti-proliferating effects on these cell lines in a dose-dependent manner *in vitro*, and anti-tumor effects when curcumin (50-200 mg/kg) was orally administered to nude mice transplanted with the cancer cells. When the cells were treated with 1 μM of curcumin for 120 h, apoptotic cells were observed by means of the adridine orange/ethidium bromide staining method, single cell microgel electrophoresis and flow cytometric analysis. On the other hand, suppression of telomerase activity in extracts of the cells treated with 1 μM of curcumin was observed by means of a telomeric repeat amplification protocol - silver staining assay. These results suggest that curcumin could suppress telomerase activity in the cancer cell lines and that the decrease of telomerase expression followed by induction of apoptosis might be involved in the anti-proliferating effect of curcumin.

Introduction

Despite significant advances in medical technology for diagnosis and treatment, cancer is still widely posing a threat of mortality. Considerable attention has been focused on identifying naturally occurring chemopreventive substances capable of inhibiting, retarding or reversing the process of multistage carcinogenesis. A wide spectrum of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess substantial anti-carcinogenic and anti-mutagenic effects (1,2). The majority of these substances retain anti-oxidative and anti-inflammatory properties, which appear to contribute to their chemopreventive activity (3). Curcumin (diferulolylmethane) is one of the major components of turmeric, the dried rhizome of *Curcuma longa* L, which has been used for centuries as a naturally occurring herbal remedy for treatment of inflammatory disorders as well as many other diseases (4). Curcumin has been shown to be an anti-oxidant that has anti-proliferating and anti-carcinogenic properties (5). It has been reported that the induction of apoptosis by curcumin is responsible for such an anti-proliferating effect (6,7), although the mechanism remains to be elucidated. On the other hand, it has been suggested that maintenance of telomere integrity protect cells from apoptosis and that inhibition of telomerase, an enzyme participating in telomeric DNA elongation, elicits an apoptotic response in cancer cells (8,9).

In this study, in order to clarify the mechanism of the anti-proliferating effect of curcumin, we confirmed cell growth inhibition and induction of apoptosis by curcumin and then examined the effect of curcumin on telomerase activity in various human cancer cell lines.

Materials and methods

Chemicals. Curcumin was purchased from Sigma-Aldrich (St. Louis, MO), and dissolved in dimethylsulfoxide (DMSO) for *in vitro* study or in 5% amylum for *in vivo* study.

Cells. HL60 cells were provided by P. Yang of Experimental Hematology, Institute of Hematology, Tianjin, China. Bel7402 and SGC7901 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China. Cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin and streptomycin in a humidified atmosphere (5% CO_2) at 37˚C. Cells were fed every 3-4 days, and Bel7402 and

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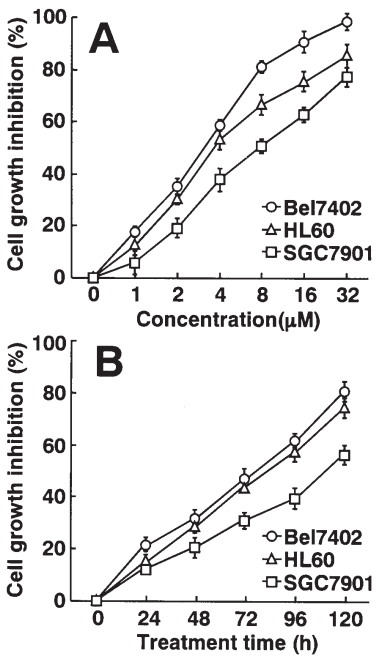


Figure 1. Cell growth inhibition by curcumin *in vitro*. Bel7402, HL60 and SGC7901 cells were treated with various concentrations of curcumin for 96 h (A) or with 4 μM of curcumin for various incubation times (B). Viable cell numbers were evaluated by the MTT assay and are denoted as a percent of that of untreated controls at the concurrent time point. The bars indicate means ± SD (n=3).

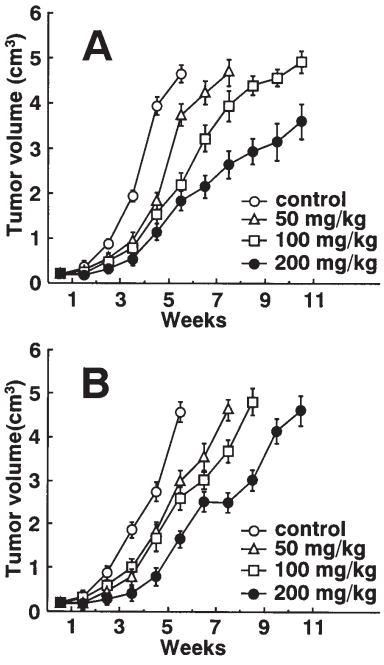


Figure 2. Anti-tumour activity of curcumin *in vivo*. Nude mice were injected s.c. in the dorsal flank with $1x10^7$ cells of Bel7402 (A) or SGC7901 (B), and then the indicated doses of curcumin were orally administered, as described in Materials and methods. Tumor volume was measured every week during the life span. The bars indicate means ± SD (n=10).

SGC7901 cells were harvested by brief incubation with 0.02% EDTA. Cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay (10).

Xenografts in nude mice. Female BALB/c athymic (nu+/nu+) mice, 4-6 weeks of age, were obtained from Experimental Animal Laboratory at the Chinese Academy of Medical Sciences (Beijing, China). The research protocol was approved in accordance with the institutional guidelines of the Shandong Academy of Medical Sciences Animal Care and Use Committee. Mice were injected s.c. with $1x10^7$ cancer cells suspended in 200 μl of matrigel (Collaborative Biomedical Products, Bedford, MA) into the dorsal flank. After 7 days, when tumor volume reached ~0.2-0.3 cm^3, the mice were orally administered 0-200 mg/kg of curcumin everyday for 5 consecutive weeks. Tumor size was measured every week as reported elsewhere (11).

Assessment of apoptosis induction. Induction of apoptosis by curcumin was assessed by means of an adridine orange/ethidium bromide staining method, single cell microgel electrophoresis, and flow cytometric analysis. For adridine orange/ethidium bromide staining (12), 25 μl of cell suspension (10^6 cells/ml) was incubated with 1 μl of the adridine orange/ethidium bromide solution (50 μg/ml each in PBS) followed by observation with a fluorescence microscope (magnification, x400). To analyze the level of DNA damage in individual cells for single cell microgel electrophoresis (13), the cells were embedded in 1% agarose and then put in a weak electric field followed by staining the DNA with acridine orange. For flow cytometric analysis (14), cells were subjected to PI staining and then analyzed by flow cytometry (FACS-Calibur; BD Biosciences, San Jose, CA) equipped with a 5 W argon ion laser tuned to 488 nm at 200 mW and CellQuest software.

Telomerase activity. Telomerase activity in cells was measured using a telomeric repeat amplification protocol (TRAP)-silver staining assay (15). In brief, curcumin-treated or untreated cells (10^6 cells each) were lysed with a lysis buffer on ice for 30 min and then centrifuged at 12,000 rpm for 20 min at 4˚C. The supernatant (1 μg protein) was added to 48 μl of the reaction mixture containing TRAP buffer, dNTP mix, TS primer, RP primer, Ki primer and 2 units of Taq polymerase. PCR conditions were cycles at 94˚C for 30 sec, and 59˚C for 30 sec. The PCR products were separated on 12.5% non-denaturing polyacrylamide gels and stained with silver. Quantification of the PCR products was performed as described elsewhere (16).

Results

Inhibitory effects of curcumin on cancer cell growth in vitro and in vivo. Effect of curcumin on cell growth was examined with Bel7402, HL60, and SGC7901 cells. As shown in Fig. 1, growth of these three cell lines was inhibited by curcumin in a dose- and time-dependent manner. When the cells were exposed to curcumin for 96 h, IC_{50}s for HL60, Bel7402, and SGC7901 cell lines were 3.11, 3.83, and 8.00 μM, respectively.

Effects of curcumin on Bel7402 and SGC7901 xenografts in nude mice were then examined. As shown in Fig. 2,

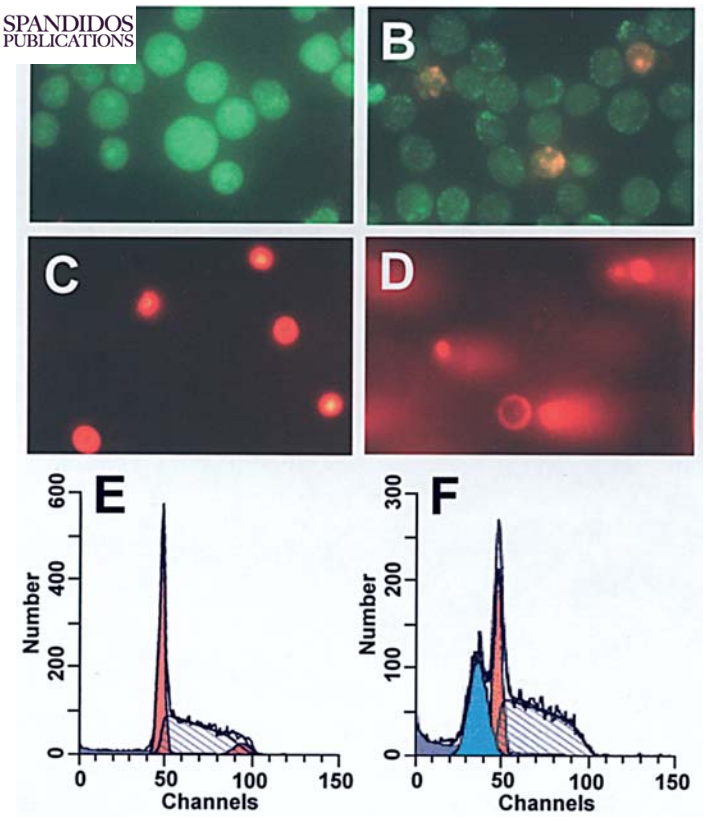


Figure 3. Induction of apoptosis by curcumin. HL60 cells were treated without (A, C and E) or with (B, D and F) 1.0 μM of curcumin for 120 h. The cells were then subjected to adridine orange/ethidium bromide staining of apoptotic cells (A and B; original magnification, x400), single cell electrophoresis analysis to examine nuclear DNA strand breakage (C and D), and flow cytometry after PI staining for detection of DNA fragmentation (E and F).

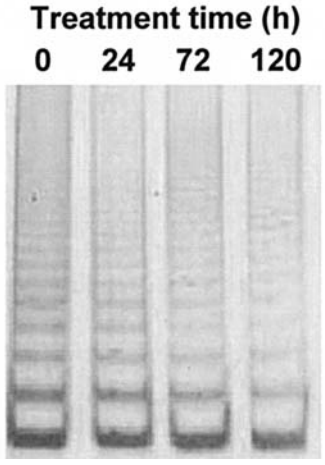


Figure 4. Typical separation profiles of PCR products on 12.5% non-denaturing polyacrylamide gel. Bel7407 cells were treated with 1 μM of curcumin for the indicated incubation time. The cell extracts were subjected to a TRAP-silver staining assay.

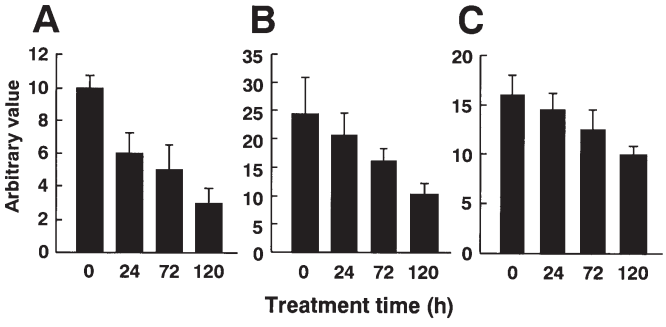


Figure 5. Suppression of telomerase activity in cancer cell lines by curcumin. Bel7402 (A), HL60 (B) and SGC7901 (C) cells were treated with 1 μM of curcumin for the indicated incubation time. Telomerase activity in the cell extracts was measured by a TRAP-silver staining assay as described in Materials and methods. The bars indicate means ± SD (n=3).

curcumin treatment produced a dose-dependent inhibition of tumor growth. This inhibitory effect on xenografts was more pronounced in Bel7402 cells than in SGC7901 cells. Delayed tumor growth was accompanied by a prolonged life span of mice which was significantly longer than that of untreated controls (data not shown). Curcumin treatment was generally well tolerated by mice with no signs of acute or delayed toxicity or reduction in body weight (data not shown). In the following *in vitro* experiments, we preferably chose a low concentration of curcumin (1 μM), giving consideration to the future clinical application.

Induction of apoptosis by curcumin. We next examined whether curcumin can induce apoptosis using HL60 cells *in vitro*. Curcumin-untreated and -treated cells were subjected to an adridine orange/ethidium bromide staining method (Fig. 3A and B, respectively). Early apoptotic cells, which contained bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation, and late apoptotic cells, which showed orange-colored condensation of nuclei, were observed after treatment with 1.0 μM of curcumin for 120 h (Fig. 3B). Single cell electrophoresis analysis was also performed to examine nuclear DNA strand breakage (Fig. 3C and D). As shown in Fig. 3D, curcumin-treated cells migrated like a 'comet' with a brightly fluorescent head and a long tail region, suggesting an increment of broken DNA in the cells. Furthermore, apoptotic body formation was confirmed by means of flow cytometric analysis after PI staining of the cells treated without or with 1.0 μM curcumin (Fig. 3E and F, respectively). As shown in Fig. 3F, a sub-diploid peak (blue-colored) was observed in curcumin-treated HL60 cells, suggesting the appearance of apoptotic nuclei as a result of the partial loss of DNA due to leakage of low-molecular-weight fragments generated during apoptosis. The area of the sub-diploid peak was enlarged in a treatment time-dependent manner (data not shown). These results suggest that curcumin can induce apoptosis.

Inhibitory effect of curcumin on telomerase activity in cancer cell lines. Telomerase activity in cancer cell lines after exposure to curcumin for different incubation times was assessed by a TRAP-silver staining assay. Fig. 4 shows an example of typical analysis of the PCR products by telomerase in cell extracts. Quantification of the PCR products showed that telomerase activity in all three cell lines was decreased by the incubation with 1.0 μM curcumin in a time-dependent manner (Fig. 5). At 120 h-incubation time, the percent of inhibition of

telomerase activity in Bel7402, HL60 and SGC7901 cell lines was 70.0, 58.0, and 37.4%, respectively. Inhibitory efficiency was closely correlated with the sensitivity of anti-proliferating effects of curcumin on each of the three cell lines. These results suggest that curcumin might inhibit cell growth through reducing telomerase activity followed by induction of apoptosis.

Discussion

A significant amount of research has showed that curcumin might affect several cellular processes involved in tumorigenesis and progression (4,17). In the present study, we focused on the effect of curcumin on telomerase activity to clarify the anti-proliferating effect of curcumin. Curcumin exerted an anti-proliferating effect on human cancer cell lines *in vitro* and *in vivo* (Figs. 1 and 2). Furthermore, curcumin-treated cells proved that curcumin induces apoptosis and suppresses telomerase activity (Figs. 3 and 4). It has been suggested that tumor cell growth inhibition is associated with the promotion of apoptosis and decrease in telomerase expression (8,9,18,19). Therefore, curcumin may inhibit cancer cell growth and induce apoptosis via reducing telomerase expression.

Telomerase is a ribonucleoprotein reverse transcriptase that is suppressed in normal somatic cells and is activated in cancer cells and immortalized cell lines (20). The catalytic core of human telomerase consists of an RNA template (hTR), a catalytic protein subunit with reverse transcriptase activity (hTERT) and additional telomerase-associated proteins such as hTEP1, p23, Hsp90, and dyskerin (21). hTERT acts as a limiting factor for telomerase activity. Expression of hTERT mRNA closely coincides with the presence of telomerase activity in cancer (22-25). Inhibition of hTERT expression results in loss of telomere and limits the growth of cancer cells. It has been proven that curcumin can down-regulate expression of several genes such as mutation-type of *p53, bcl-2, c-myc* pro-oncogenes and protein kinase C in cancer cells (26-28), which induce suppression of cell proliferation. Although the mechanism of modulation of telomerase expression by curcumin in cancer cells is still unknown, down-regulation of hTERT expression might be one of the physiological actions of curcumin, which promotes an apoptotic response in cancer cells.

Curcumin has been reported to inhibit reactive oxygen-generating enzymes such as lipooxygenase/cyclooxygenase, xanthine dehydroxygenase/oxidase, and nitric oxide synthase (29-33), as well as epidermal growth factor receptor intrinsic kinase activity (34), NF-κB activity (35), and protein kinase C activity (26). In contrast, curcumin has been found to be fairly safe without toxic side effects (17), which is also suggested in the present study involving cancer xenografts in immunodeficient mice (data not shown). This study suggests the inhibitory effect of curcumin on telomerase activity. Further understanding of the physiological mechanisms of curcumin would establish the value of curcumin as a chemopreventive or therapeutic agent.

In summary, curcumin exerts an anti-proliferation effect on human cancer cell lines *in vitro* and *in vivo*. Biological effect of curcumin is multi-functional and the inhibition of telomerase expression followed by induction of apoptosis may be one of the major components that curcumin provides pertaining to anti-proliferation of cancer cells.

Acknowledgements

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