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SHORT COMMUNICATION

Nonselective Inhibition of Proliferation of Transformed and Nontransformed Cells by the Anticancer Agent Curcumin (Diferuloylmethane)

Subhash C. Gautam, Yong X. Xu, Kirit R. Pindolia, Nalini Janakiraman and Robert A. Chapman*

Division of Hematology/Oncology, Department of Medicine, Henry Ford Hospital, Detroit, MI 48202, U.S.A.

ABSTRACT. We have investigated the antiproliferative effect of curcumin, an antitumor agent with antioxidant and anti-inflammatory properties, against a variety of transformed and nontransformed cell types. At equimolar concentrations ranging from 6.25 to 50 μM, curcumin inhibited DNA synthesis, as revealed by ^{3}H-incorporation, in five leukemia lines, three nontransformed hematopoietic progenitor cell populations, and four nontransformed fibroblastic cell lines in a concentration-dependent manner. Curcumin also inhibited the cellular growth of both transformed and nontransformed cells in clonogenic assays. Without discriminating between transformed or nontransformed cells, the inhibition of cell proliferation by curcumin was not always associated with programmed cell death. These findings have implications for developing curcumin-based anticancer and anti-inflammation therapies. BIOCHEM PHARMACOL **55**;8:1333–1337, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. curcumin; leukemia; hematopoietic progenitors; apoptosis

Curcumin (diferuloylmethane), the yellow pigment and active component of turmeric (*Curcuma longa*), a widely used food coloring agent and spice, possesses both antioxidant and anti-inflammatory activity [1–3]. This compound has been shown to inhibit chemically induced initiation and promotion of skin tumors in mice [4, 5]. Curcumin has also shown growth inhibitory activity against tumor cell lines *in vitro* and *in vivo* and chemoprevented gastrointestinal carcinogenesis by benzo[*a*]pyrene, azoxymethane, and 7,12-dimethyl benz[*a*]anthracene [6–10]. In addition, curcumin has been shown to inhibit the replication of type 1 human immunodeficiency virus [11] and the production of cytokines [12, 13]. Similarly, the activation of nuclear transcription factors such as NF-κB† and AP-1 is also inhibited by curcumin [14, 15]. Many of the inhibitory effects of curcumin are believed to result from its inhibition of signal transduction pathways dependent upon serine/threonine protein kinase as well as protein tyrosine kinase.

In most studies, the inhibition of signal transduction and cell proliferation has been studied using transformed cell lines. The effect of curcumin on the proliferation of nontransformed hematopoietic and nonhematopoietic cells has not been established. In this paper, we demonstrated that the antiproliferative effect of curcumin is nonselective since both normal and transformed cells were inhibited identically by curcumin at all concentrations of curcumin tested. Furthermore, cell death that occurred at higher concentrations of curcumin in both normal and transformed cells was not always due to apoptosis.

MATERIALS AND METHODS

Cell Lines

Leukemia cell lines P210 (myeloid), C1.18.4 (myeloma), L1210 (lymphoid), U937 (monocytic), and HL-60 (promyelocytic) were maintained in RPMI-1640 (Life Technologies, Inc.) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), 2 mM of glutamine and 10% FBS. Nontransformed fresh BM or BM cells obtained from mice treated with 5-FU 72 hr earlier (5-FU-BM) and 32Dc13 myeloid cell lines were maintained in IMDM supplemented with 10% FBS and 30% culture supernatant conditioned by Wehi-B cells containing interleukin-3 and other growth factors. Nontransformed fibroblastic cell lines NIH 3T3, 3T3-C2, CCD-974 Sk (normal skin), and +/+-LDA.11 (BM stroma) were maintained in DMEM supplemented with 10% FBS and 2 mM of glutamine. All cultures were maintained by passaging twice a week.

* Corresponding author: S. C. Gautam, Ph.D., Division of Hematology/Oncology, K13, Henry Ford Hospital, 2799 West Grand Blvd., Detroit, MI 48202. Tel. (313) 874-6998; FAX (313) 874-3770.

† *Abbreviations:* NF-κB, nuclear factor kappa B; AP-1, activation protein 1; IMDM, Iscove's modified Dulbecco's medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BM, bone marrow; and 5-FU, 5-fluorouracil.

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[^{3}H]Thymidine Incorporation Assay

To determine the effect of curcumin on the proliferation of cells, 5×10^4 leukemia/hematopoietic progenitor cells or 2×10^4 fibroblastic cells/100 μL of culture medium were seeded into each well of a 96-well microtiter tissue culture plate. The stock solution of curcumin in DMSO (250 mM) was appropriately diluted in warm culture medium and added to cells in aliquots of 100 μL to obtain a final concentration of curcumin ranging from 6.25 to 50 μM in triplicate wells. Cultures were incubated for 24 hr at 37° and 5% CO_2. [^{3}H]Thymidine (0.5 μCi; sp. act. 74 Ci/mmol) was added to each well, and cultures were incubated for an additional 16 hr. Cultures were harvested with an automatic cell harvester (Skatron) using distilled water. The amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer.

Clonal Assay

The effect of curcumin on cell proliferation was also measured in clonogenic assays. For this purpose, cells were first treated with curcumin at 6.25 to 50 μM for 5 hr at 37°. Cells were washed three times and resuspended in 0.3% agar in tissue culture medium at 2×10^4 cells/mL. One milliliter of cell suspension was placed in each 35-mm petri dish in triplicates for each concentration of curcumin. After 10 days of incubation at 37° the number of colonies developed in each petri dish was counted under an inverted microscope.

DNA Fragmentation

To determine whether curcumin induces apoptosis, 6×10^6 cells were treated with curcumin for 5 hr. Cells were washed with PBS and then lysed in cold lysis solution (5 mM of Tris, pH 7.4, 20 mM of EDTA, 0.5% Triton X-100) for 20 min. Cell lysates were centrifuged at 27,000 *g* for 15 min, and DNA was extracted from the aqueous phase with phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) containing 0.1% hydroxyquinoline. DNA was precipitated with 0.3 M of sodium acetate and 2 vol. of cold 100% ethanol. DNA samples were fractionated on 1.2% agarose gel in Tris-acetate (0.04 M of Tris-acetate, 0.001 M of EDTA) electrophoresis buffer. Following fractionation of DNA, gels were treated with RNase (20 μg/mL) for 3 hr and stained with ethidium bromide.

RESULTS AND DISCUSSION

The antiproliferative effect of curcumin on transformed and nontransformed cells was measured in [^{3}H]thymidine incorporation assays and clonal assays. Results presented in panels A–C of Fig. 1 show the effect of curcumin on the proliferation of leukemic cells (A), nontransformed hematopoietic progenitors (B), or fibroblastic cells (C). In all experiments, 6.25 μM of curcumin caused slight to moderate inhibition of the proliferation of leukemia hematopoietic cell types (Fig. 1, A and B) but not fibroblasts (Fig. 1C). The inhibition of proliferation of all cell types, whether transformed or nontransformed, progressively increased with increasing concentrations of curcumin from 12.5 to 50 μM. Complete inhibition of proliferation varied between 25 and 50 μM of curcumin. Overall, leukemia/hematopoietic progenitor cells were more sensitive to curcumin than fibroblastic cells. Bulk tissue culture experiments demonstrated a significant loss of cells (50–60%) in cultures at 50 μM of curcumin. Cell losses in cultures at lower concentration of curcumin were minimal (<10%) (data not shown). Thus, contrary to the previously reported antiproliferative effect of curcumin on transformed cells only, our results demonstrate that at 25 μM and higher concentrations, curcumin inhibited the proliferation of both transformed and nontransformed cells equally. The inhibitory effect of curcumin on the proliferation of cells was not due to DMSO used for dissolving curcumin, since equivalent concentrations of DMSO alone had no effect on cell viability (trypan blue dye exclusion) or cell proliferation ([^{3}H]thymidine incorporation) (data not shown).

To determine whether the inhibitory effect of curcumin is irreversible or reversible, cells were treated with curcumin at 6.25 to 25 μM (leukemia/hematopoietic cells) or at 12.5 to 50 μM (fibroblasts) for 3 hr. After incubation, cells were washed three times in PBS and resuspended in culture medium. Cells were added to the wells of a microtiter tissue culture plate, and the incorporation of [^{3}H]thymidine was measured as described before. The results are presented in panels D–F of Fig. 1. With the exception of human promyelocytic cells (HL-60), the antiproliferative effect of curcumin on other transformed cells was not reversed by removing curcumin from the cultures (Fig. 1D). The proliferation of HL-60 cells was fully restored after removal of curcumin. Similarly, proliferation of nontransformed hematopoietic progenitor cells (Fig. 1E) remained suppressed following the removal of curcumin. Partial recovery in proliferation of fibroblastic cells NIH 3T3 and CCD-974 Sk but not BM stroma was also seen. 3T3-C2 cells showed recovery when treated at 25 but not at 50 μM of curcumin. These data suggest that most cell types, both transformed and non-transformed, are irreversibly affected by curcumin. In instances where proliferation was restored, the recovery ranged from complete (HL-60) to partial (NIH 3T3, CCD-974 Sk).

Much like the inhibition of cell proliferation by curcumin observed in [^{3}H]thymidine incorporation assays, the number of colonies formed by transformed (P210, U937) and nontransformed (5FU-BM) cells treated with curcumin decreased significantly in a concentration-dependent manner (Fig. 2). The maximum suppression of clonal growth by curcumin was at a 25–50-μM concentration of curcumin. These results corroborate the antiproliferative effect of curcumin on both transformed and nontransformed cells previously observed in short-term [^{3}H]thymidine incorporation assays.

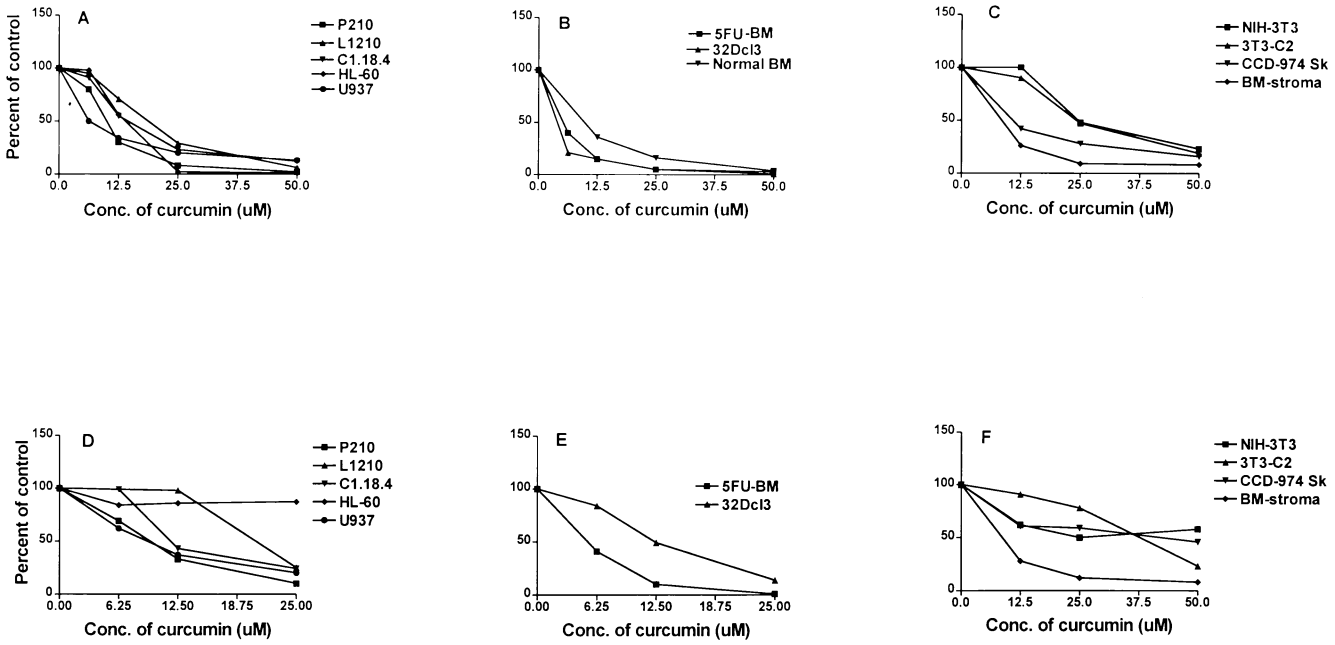

FIG. 1. Effect of curcumin on the incorporation of [^{3}H]thymidine by transformed and non-transformed cells. Leukemia cells (5×10^4) (A), 5×10^4 hematopoietic progenitor cells (B), or 2×10^4 fibroblastic cells (C) were seeded into each well of a 96-well microtiter plate in 0.2 mL of tissue culture medium containing different concentrations of curcumin as indicated. Alternatively, cells were first treated with curcumin for 3 hr and washed three times before seeding into microtiter plates (D, E, and F). Cultures were incubated at 37° and 5% CO_2 for 24 hr and pulsed with [^{3}H]thymidine (0.5 μCi/well) for 8–12 hr. [^{3}H]Thymidine incorporation was measured by liquid scintillation spectrometry. Data are presented as percent of control and represent the means of three to four different experiments. The incorporation of [^{3}H]thymidine in control cells in the absence of curcumin was as follows: (A) P210 = 11.5×10^3 cpm, L1210 = 103.5×10^3 cpm, C1.18.4 = 65.8×10^3 cpm, HL-60 = 34.1×10^3 cpm, and U937 = 76×10^3 cpm. (B) 5FU-BM = 2.4×10^3 cpm, 32Dcl3 = 30.4×10^3 cpm, and normal BM = 21.2×10^3 cpm. (C) NIH-3T3 = 13.2×10^3 cpm, 3T3-C2 = 52.8×10^3 cpm, CCD-974 Sk = 5×10^3 cpm, and BM-stroma = 43.8×10^3 cpm. (D) P210 = 60.4×10^3 cpm, L1210 = 149.7×10^3 cpm, C1.18.4 = 62×10^3 cpm, HL-60 = 46.6×10^3 cpm, and U937 = 74.2×10^3 cpm. (E) 5FU-BM = 18×10^3 cpm, and 32Dcl3 = 65.2×10^3 cpm. (F) NIH-3T3 = 6.3×10^3 cpm, 3T3-C2 = 62×10^3 cpm, CCD-974 Sk = 1.6×10^3 cpm, and BM-stroma = 49.9×10^3 cpm.

Curcumin has been shown to induce apoptosis in transformed cells, but not in nontransformed primary fibroblasts [16]. Therefore, we next determined whether curcumin inhibition of cell proliferation and loss of cells in cultures at a high concentration of curcumin (50 μM) is associated with programmed cell death. Figure 3 (A and B) demonstrates that although curcumin at 50 μM inhibited proliferation of all cell types tested in this study, inhibition of cell proliferation was not always associated with the induction of apoptosis. Among the leukemic cells, curcumin induced apoptosis in C1.18.4 and P210 cells (Fig. 3, lanes 2 and 4) but not in L1210 (lane 6), U937 or Wehi-B cells (not shown). On the other hand, whereas 32Dc13 cells, a nontransformed hematopoietic progenitor cell line, underwent apoptosis following treatment with curcumin (Fig. 3B lane 8), none of the fibroblastic cells showed nucleosomal DNA fragmentation (Fig. 3B, lanes 2, 4, and 6). These data indicate that not all cell types, transformed or nontransformed, that are inhibited by curcumin follow apoptosis as a mode of cell death.

Previous studies on the toxicity of long-term feeding of curcumin (8–60 weeks) to dogs, guinea pigs, or monkeys have indicated no evidence of histopathological changes or any teratogenic or carcinogenic effects [17]. In contrast, curcumin has been shown to chemoprevent carcinogenesis [9, 10] and inhibit the growth and metastasis of tumor cells [6, 8, 18]. In a recent report, curcumin was shown to inhibit the proliferation and cell cycle progression of human umbilical vein endothelial cells [19]. The inhibition of proliferation of endothelial cells was associated with a significant loss in thymidine kinase (TK) activity. Curcumin has also been shown to inhibit protein kinase C (PKC) [20] and phosphorylase kinase [21], which are believed to be involved in the regulation of cell proliferation and growth [21, 22]. The antiproliferative effect of curcumin on various transformed and nontransformed cells examined in the present study may have resulted, at least in part, from the inhibition of TK, PKC and/or phosphorylase kinase activity. Additional studies are required to determine whether differences in the susceptibility of the hematopoietic versus fibroblastic cells to the antiproliferative activity of curcumin at equimolar concentrations, as observed in our experiments, are related to the varying levels of suppression of various kinases.

Very little is known about the metabolism and pharmacology of curcumin, primarily because of poor absorption following its administration orally or intraperitoneally. The principal metabolites of curcumin are glucuronide conju-

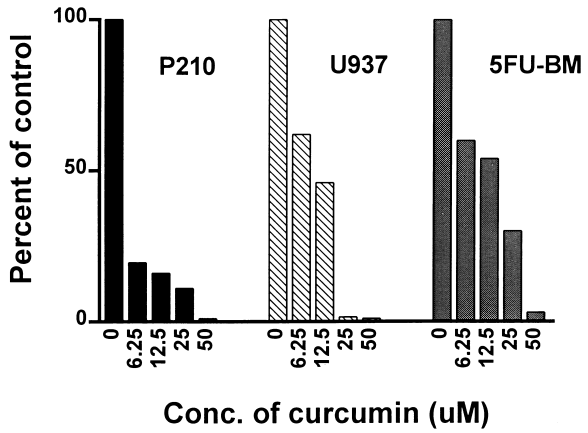

FIG. 2. Effect of curcumin on clonal growth of leukemia and hematopoietic progenitor cells. Leukemia cells (P210 and U937) or hematopoietic progenitor cells (5FU-BM) were treated with curcumin at concentrations as indicated for 3 hr. Cells were washed three times and resuspended at 2 × 10^4 cells/mL in 0.3% agar dissolved in tissue culture medium. One milliliter of each cell suspension was placed in a 35-mm petri dish in triplicates and incubated at 37° and 5% CO_2. The number of colonies formed was determined on day 10. Data represent the means of two to three experiments and are shown as percent of the control values. The numbers of colonies formed in control cultures (0 μM of curcumin) were: P210 = 1050 (976–1140), U937 = 1022 (960–1080), and 5FU-BM = 577 (556–610).

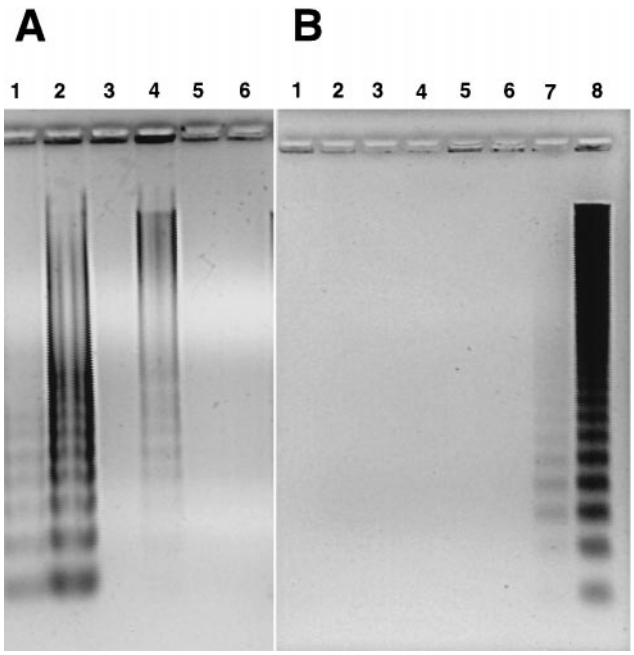

FIG. 3. Agarose gel electrophoresis of DNA fragments from cells treated with curcumin. DNA was isolated from C1.18.4, P210, or L1210 leukemia cells (A: lanes 1 and 2; 3 and 4; and 5 and 6, respectively), fibroblastic cell lines +/+-LDA.11, NIH-3T3, 3T3-C2 (B: lanes 1 and 2; 3 and 4; 5 and 6, respectively) or 32Dcl3 progenitor cells (B: lanes 7 and 8) that were either untreated (A: lanes 1, 3, and 5; B: lanes 1, 3, 5, and 7) or treated with 50 μM of curcumin for 5 hr (A: lanes 2, 4, and 6; B: lanes 2, 4, 6, and 8). Oligonucleosomal length DNA fragments were separated by agar gel electrophoresis. Similar results were obtained in three separate experiments.

gates of tetra- and hexahydrocurcumin secreted primarily in bile. Little or no unchanged curcumin is found in blood [23]. Whether inhibition of cell proliferation by curcumin *in vitro* is mediated directly by the parent compound or indirectly via one of its metabolites also remains to be determined. The resistance of fibroblastic cell types to apoptosis following treatment with curcumin might be attributable to the abnormal expression of specific proteins that regulate apoptosis. For example, inappropriate expression of Bc12 oncogene product, which provides survival advantage (antiapoptosis), or wild-type p53 protein, which promotes cell death (proapoptosis), especially in response to DNA-damaging events such as exposure to chemotherapeutic agents, could account for resistance of fibroblastic cells to the induction of programmed cell death by curcumin. Further investigations of the mechanisms that determine the susceptibility or resistance of various cell types to curcumin may provide critical information for developing curcumin-based novel anti-inflammatory or anticancer therapies.

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