Biochemical Pharmacology, Vol. 54, pp. 899–907, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00251-7

Inhibition of Proliferation and Apoptosis of Human and Rat T Lymphocytes by Curcumin, a Curry Pigment

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ABSTRACT. Curcumin (diferuoylmethane), the yellow pigment in the rhizome of tumeric (*Curcuma longa*), an ingredient of curry spice, is known to exhibit a variety of pharmacological effects including antitumor, antiinflammatory, and antiinfectious activities. Although its precise mode of action remains elusive, curcumin has been shown to suppress the activity of the AP-1 transcription factor in cells stimulated to proliferate. In this study, we observed that curcumin (50 μM) inhibited proliferation of rat thymocytes stimulated with concanavalin A (Con A) as well as that of human Jurkat lymphoblastoid cells in the logarithmic growth phase. The pigment also inhibited apoptosis in dexamethasone-treated rat thymocytes and in UV-irradiated Jurkat cells as judged by DNA ladder formation, cellular morphological changes, and flow cytometry analysis. The inhibition of apoptosis by curcumin in rat thymocytes was accompanied by partial suppression of AP-1 activity. Complete suppression of AP-1 activity was observed in Con A-treated, proliferating thymocytes. The capacity of curcumin to inhibit both cell growth and death strongly implies that these two biological processes share a common pathway at some point and that curcumin affects a common step, presumably involving a modulation of the AP-1 transcription factor. BIOCHEM PHARMACOL **54**;8:899–907, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. T lymphocytes; proliferation; apoptosis; AP-1; curcumin

Curcumin (diferuoylmethane) is the pigment in tumeric (*Curcuma longa*) widely used as a spice and responsible for the yellow color of curry. Curcumin exhibits a variety of pharmacological effects including antitumor, antiinflammatory, and antiinfectious activities. The anticarcinogenic properties of curcumin in animals have been demonstrated by its inhibition of both tumor initiation induced by benz[α]pyrene and 7,12-dimethylbenz[α]anthracene [1] and tumor promotion by phorbol esters [2, 3]. Recent results have indicated that dietary administration of curcumin significantly inhibits forestomach, duodenal, colon, and tongue carcinogenesis in mice and rats [4–6]. The anti-inflammatory action of curcumin has been shown both *in vivo* [7] and *in vitro* [8]. Although the exact mechanism underlying these effects of curcumin remains to be elucidated, the antioxidant characteristics of this compound are likely to be involved [9]. Several independent studies have shown that curcumin inhibits lipid peroxidation [10, 11] and free radical generation [12, 13] as well as possessing scavenging properties [14], thus serving to protect various cellular constituents, including DNA, from oxidative injury [15]. Inhibition of cell proliferation by curcumin could be explained by its capacity to inhibit diverse protein kinases, such as protein kinase C [16] and phosphorylase kinase [17]. The ability of curcumin to inhibit the growth of mouse 3T3 cells has been correlated with a decrease in epidermal growth factor receptor phosphorylation [18]. The pigment has also been shown to inhibit the expression of several proto-oncogenes, such as c-*jun*, c-*fos*, and c-*myc*, in mouse skin and c-*jun* in JB6 cells [19, 20]. The activity of the AP-1 transcription factor, which is the dimer composed of protein products of the c-*jun* and c-*fos* gene families, is also suppressed by curcumin in TPA§-stimulated mouse fibroblasts [21], transforming growth factor-β-treated osteoblasts [22], and tumor necrosis factor-treated human myeloid ML-1a cells [23].

It is believed that some early events in cell cycle progression are shared with programmed cell death (apoptosis) [24]. Indeed, the involvement of c-*fos* and c-*jun* not only in cell proliferation but also in cell death in prostate of the rat as well as in mouse lymphocytes and fibroblasts has been well documented [25–28]. In addition, the direct induction of the AP-1 transcription factor has recently

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Received 4 January 1996; accepted 3 May 1997.

§ *Abbreviations:* Con A, concanavalin A; DAPI, 4′,6′-diamidino-2-phenylindole; Dex, dexamethasone; PI, propidium iodide; ROS, reactive oxygen species; TPA, 12-O-tetradecanoylporbol-13-acetate.

been shown to occur in apoptotic cells [29–36]. These observations prompted us to investigate whether curcumin can inhibit apoptosis as well as proliferation and whether the inhibition of these two biological phenomena correlates with AP-1 suppression. Indeed, we show that curcumin not only inhibits proliferation of rat thymocytes and human Jurkat cells but also apoptosis of these cells. These two inhibitory activities of curcumin correlate with the suppression of AP-1 activity.

MATERIALS AND METHODS

Cell Culture

Thymocytes were obtained from the thymus of suckling Wistar albino Glaxo rats and resuspended at a density of 5×10^6 cells/mL in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Warsaw, Poland). Jurkat human lymphoblastoid T cells were routinely grown in the same medium and seeded at a density of 1×10^6 cells/mL every 2 or 3 days.

[^{3}H]Thymidine Incorporation Measurement

To measure proliferation, cells were seeded in 96-well plates in 200 μL of complete medium with or without one or more reagents (concanavalin A (Con A), curcumin) at a density of 5×10^6 cells/mL (rat thymocytes) or 2×10^6 cells/mL (Jurkat cells). Con A was obtained from Sigma-Aldrich (Poznan, Poland) and curcumin from Merck (Darmstadt, Germany). After 1 day of incubation, cells in each well were pulsed for 18 h with 4 μCi of [^{3}H]thymidine (specific activity, 27 Ci/mmol) (Amersham, Braunschweig, Germany) and collected with cell harvester (Skatron, Tranby, Norway); incorporated radioactivity was measured in a liquid scintillation counter (Beckman Instruments, Inc, Fullerton, CA, USA).

Apoptosis Measurements

To induce apoptosis, rat thymocytes were treated with 10^{-7} M dexamethasone (Dex) (Sigma-Aldrich) as described previously [29], and Jurkat cells were UV-irradiated at a fluency of 70 J/m^2 (254 nm) using UV Stratalinker (Stratagene, La Jolla, CA, USA). Apoptosis was monitored in several ways, i.e. by staining with Hoechst 33258 (Sigma-Aldrich) [37], DNA fragmentation [38], and flow cytometry analysis [39]. For measurement of DNA fragmentation, portions of 5×10^6 cells (thymocytes) or 2×10^6 (Jurkat cells) were washed and spun down. Pellets were resuspended in 20 μL of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM EDTA, 0.5% sodium lauryl sarcosinate, and 0.5 mg/mL proteinase K, and incubated at 50° for 1 h. Then, 10 μL of 0.5 mg/mL RNAse was added to each sample, and incubation was continued for a further hour at 50°. Samples were heated to 70°, and 10 μL of buffer containing 10 mM EDTA, 1% low-gelling-temperature agarose, 0.25% bromphenol blue, and 40% sucrose was mixed with each sample before loading into the dry wells of 1.8% agarose containing ethidium bromide. Electrophoresis was carried out in 2 mM EDTA, 80 mM Tris-phosphate (pH 8.0). All enzymes were purchased from Gibco, and other reagents were purchased from Sigma-Aldrich. 1 kbp and *Eco*RI DNA molecular weight markers were from Pharmacia Biotech (Vienna, Austria).

For Hoechst staining and flow cytometry analysis, cells were fixed in 70% ethanol according to Gong *et al.* [40]. Flow cytometry analysis was done after cell staining with 4′,6′-diamidino-2-phenylindole (DAPI) (1 μg/mL) and sulforhodamine (20 μg/mL) (Molecular Probes, Leiden, The Netherlands) at 4°. For each time point, 1×10^6 cells were stained. Cells were analyzed for DNA and protein content on FACS Vantage (Becton Dickinson, Heidelberg, Germany) using Cell-Quest software (Becton Dickinson). For propidium iodide staining, the viable cells were resuspended in the medium containing 20 μg/mL of this reagent (Molecular Probes).

Gel Shift Assay

Nuclear extracts for gel shift assays were typically obtained from 2×10^7 cells as described previously [29]. Briefly, cells were lysed in 10 mM HEPES, pH 7.9, containing 10 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mg/mL aprotinin. After 20 min of incubation at 4°, Nonidet P-40 was added to the sample to 0.4% (v/v) final concentration. Lysates were centrifuged for 30 s in an Eppendorf microfuge (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany), and the nuclear precipitates were resuspended in 20 mM HEPES, pH 7.9, containing 0.4 M NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mg/mL aprotinin. After 15 min of incubation at 2° with shaking, samples were centrifuged for 5 min at 14000 × *g*. The resulting supernatants, which represented the nuclear extracts, were stored at −70°. The protein content of the final extract was estimated according to Bradford [41].

The double-stranded oligonucleotide harboring the AP-1 (5′-CTAGTGATGAGTCAGCCGGATC-3′) consensus sequence was from a Stratagene gel shift kit. This AP-1 oligonucleotide probe was labeled with [^{32}P]αATP (Amersham) by terminal transferase (Boheringer-Mannheim, Mannheim, Germany) and purified on Nick columns purchased from Pharmacia Biotech. The binding reaction was carried out in 16 μL of mixture containing EMSA buffer (20 mM HEPES, pH 7.8, 0.2 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 10 mg/mL BSA, and 4% Ficoll), labeled probe (about 20,000 cpm), 3 μg of nuclear extract (3 μL), and 0.4 mg poly (dI-dC) as a nonspecific competitor. A 50-fold excess (1 μL) of cold AP-1 sequence was added as a specific competitor where necessary. After 20 min of incubation at room temperature, 1 μL of 0.1% bromphenol blue was added, and the samples were electrophoresed for 2 h through a 4% polyacrylamide gel (30:1 cross-linked) at 20 mAmps in a cold room. Finally, the gels were dried and exposed overnight to Hyperfilm-MP (Amersham) in the presence of intensifying screens.

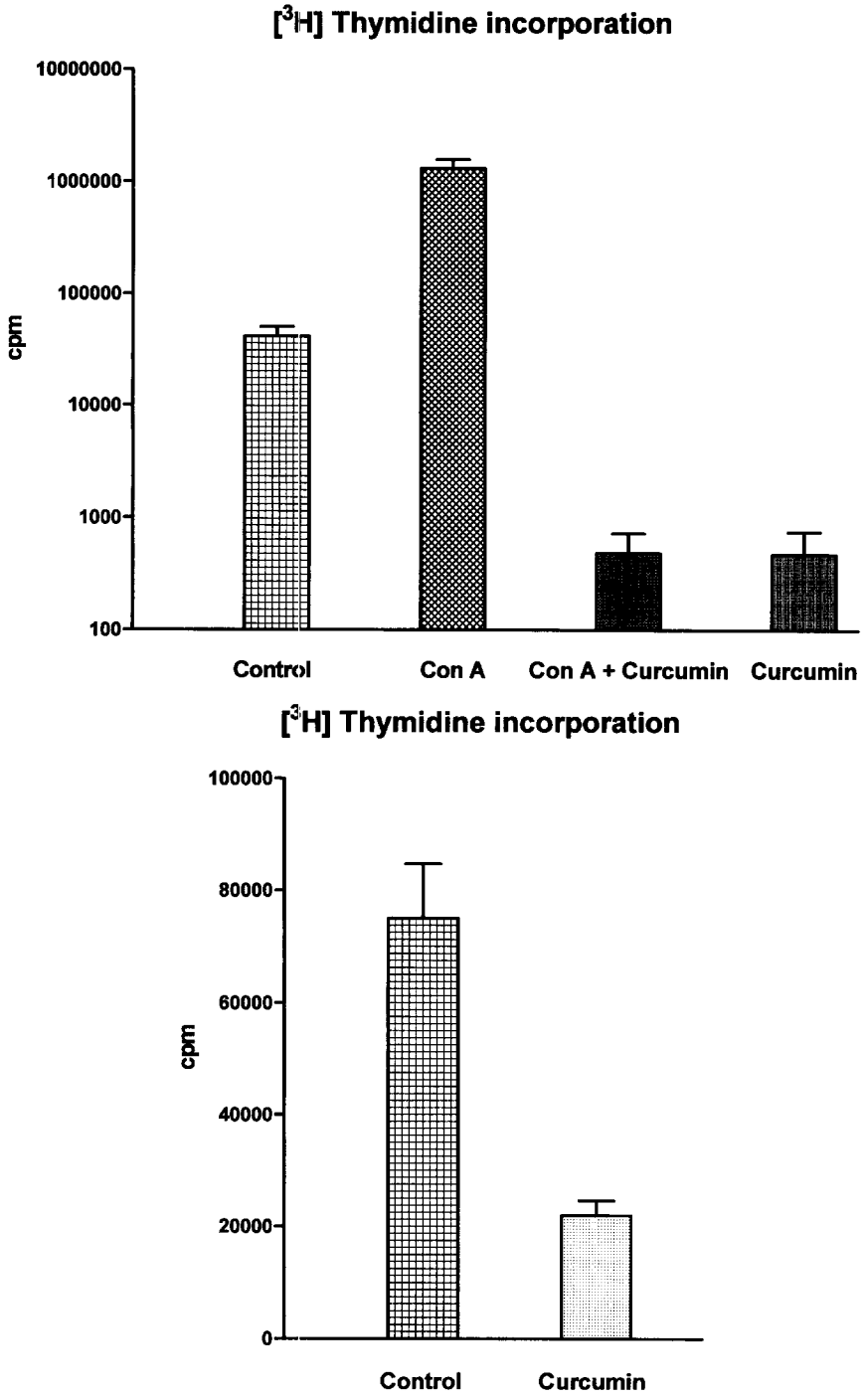

FIG. 1. [^{3}H]Thymidine incorporation into rat thymocytes (upper panel) and to Jurkat cells (lower panel), nontreated or treated as indicated. [^{3}H]Thymidine was added 24 h after thymocyte stimulation, with Con A or Jurkat cell cultivation for a further 18 h. Cells were collected by using a cell harvester, and radioactivity was measured in a liquid scintillation counter. The results are means ± SD from three separate experiments.

Statistical Evaluation

Statistical evaluation for [^{3}H]thymidine incorporation was performed by the Mann-Whitney test and for gel shifts by two-way analysis of variance (mixed design).

RESULTS

Curcumin Inhibits T Cell Proliferation

Rat thymocytes were stimulated to proliferate with Con A, and proliferation capacity was estimated by [^{3}H]thymidine incorporation into cellular DNA (Fig. 1, upper panel). Con A-stimulated thymocytes incorporated almost 30 times more radioactivity than did untreated control cells. Intriguingly, curcumin (50 μM) almost completely abolished [^{3}H]thymidine incorporation both in Con A-treated cells and nontreated cells, indicating that the pigment can inhibit not only the function of the cell population susceptible to Con A stimulation but also the subpopulation of the cells proliferating spontaneously. [^{3}H]Thymidine incorporation was also inhibited in logarithmically growing Jurkat cells following curcumin treatment. However, the extent of inhibition was much less, since curcumin-treated cells

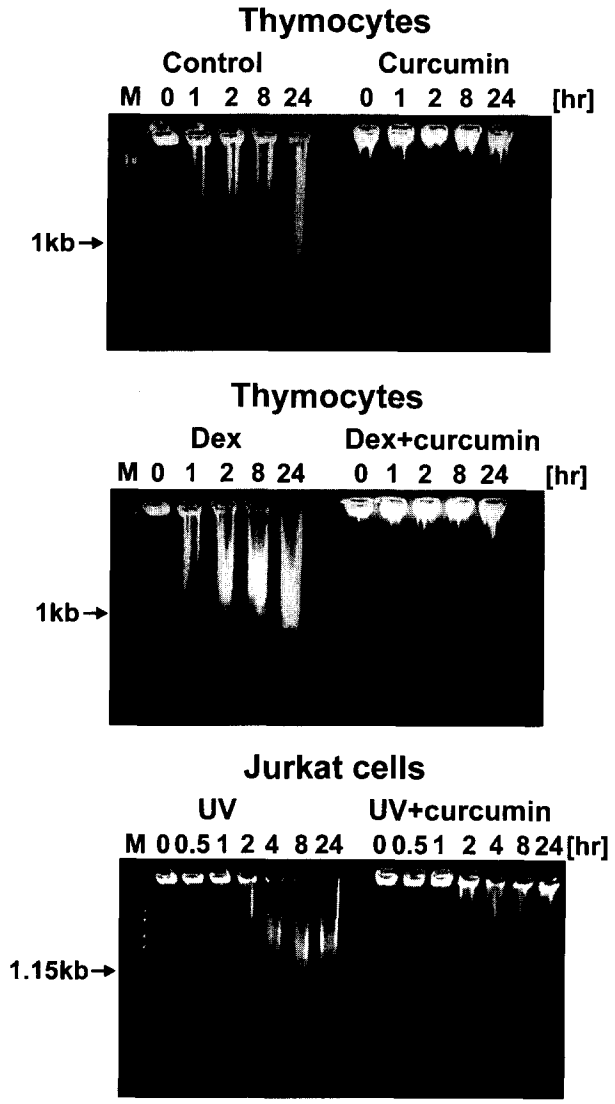

FIG. 2. Gel agarose electrophoresis of DNA isolated from control (upper) and Dex-treated (middle) rat thymocytes as well as UV-irradiated Jurkat cells (lower). Cells were cultivated without or with curcumin and collected at different time points after treatment, as indicated. The results are representative of at least five separate experiments. Line M, molecular weight marker.

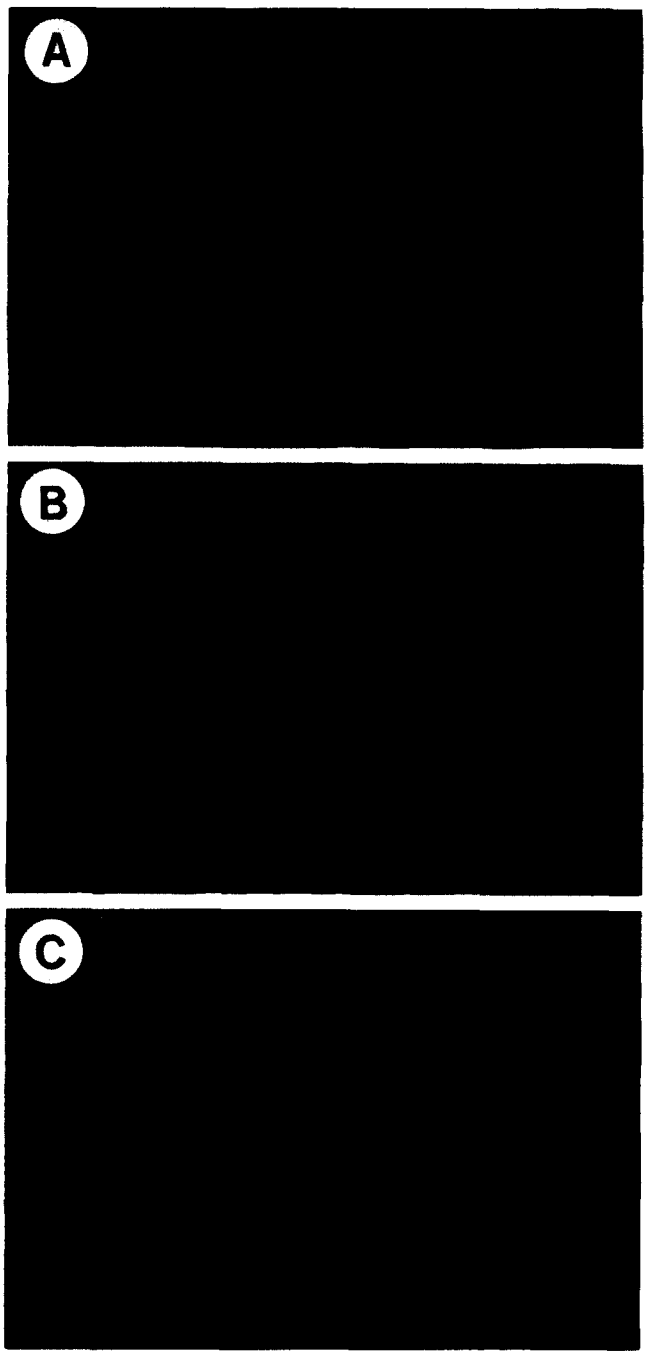

FIG. 3. Morphology of Jurkat cells. Cells were nontreated (A), UV-irradiated (B), or UV-irradiated and grown in the medium supplemented with curcumin (C). At an appropriate time after cultivation, cells were harvested and stained by Hoechst dye. Magnification, 400×.

incorporated [^{3}H]thymidine into their DNA at ca. 30% of the rate displayed by control cells (Fig. 1, lower panel; $p < 0.001$).

Curcumin Inhibits T Cell Apoptosis

Cell death of Dex-treated rat thymocytes and UV-irradiated Jurkat cells was characterized as apoptotic as judged by their DNA fragmentation (Fig. 2). In both cell types, the "DNA ladder" pattern usually appeared at 1 to 2 h after stimuli. Curcumin added to the medium together with Dex (thymocytes) or immediately after UV irradiation (Jurkat cells) at 50 μM concentration abolished DNA fragmentation, as was observed even 24 h after cell treatment. The DNA fragmentation observed in control, nontreated rat thymocytes suggested that existence of cells undergoing spontaneous apoptosis was also abolished by curcumin (Fig. 2, upper panel). Nevertheless, in UV-irradiated Jurkat cells some smears were observed from 2 to 24 h after curcumin treatment (Fig. 2, lower panel). Staining of UV-irradiated vital (not fixed) cells with propidium iodide (PI) showed that only 6% of cells were able to uptake this dye at 4 h after curcumin treatment. Supravital exposure to

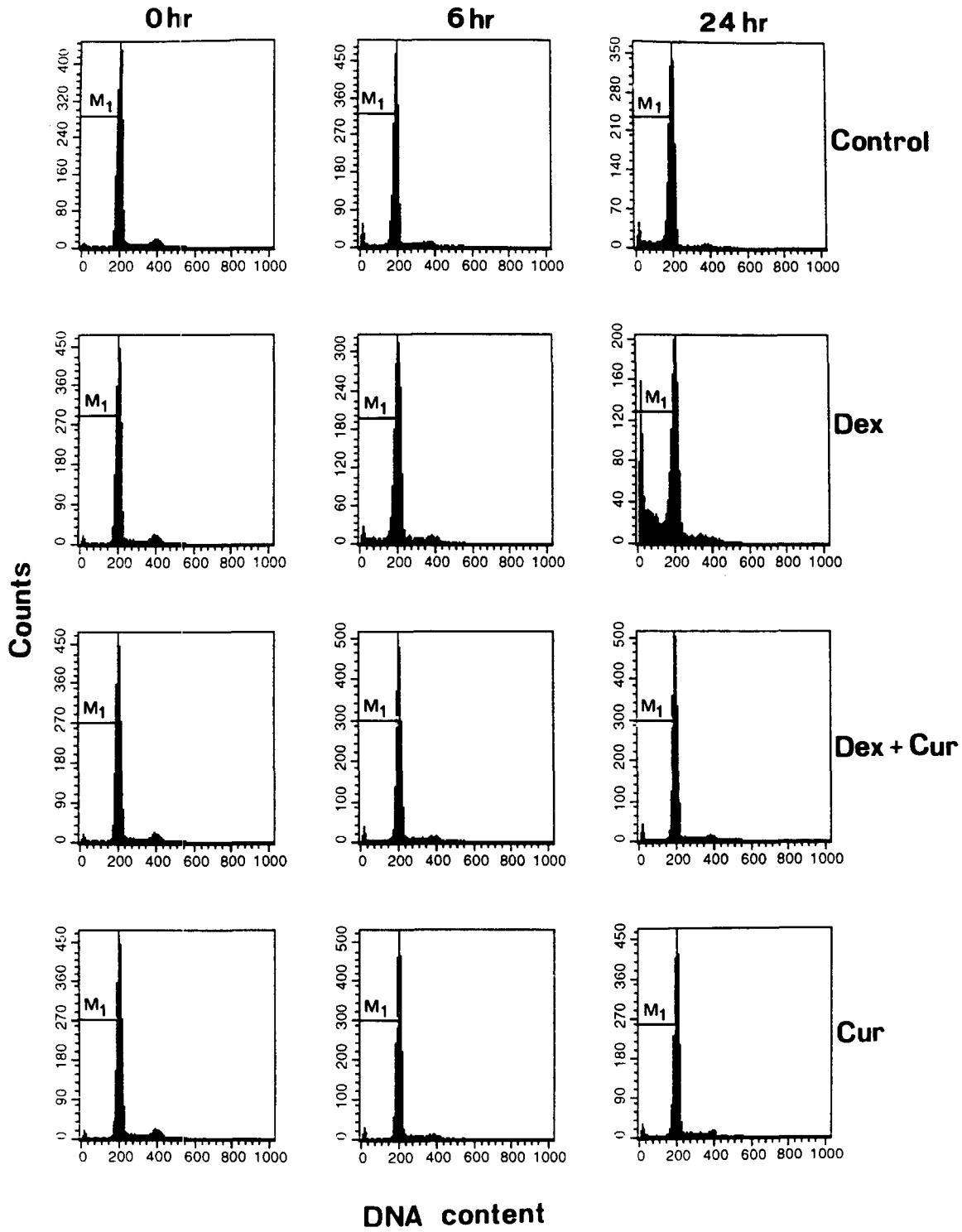

FIG. 4. Flow cytometry analysis of sub-G_1 fraction in rat thymocytes. Cells were nontreated (control), treated with Dex, treated with Dex and curcumin (Dex + Cur), or with curcumin alone (Cur). Thymocytes were stained with DAPI and sulforhodamine. Time after treatment is indicated. Line M1, sub-G_1. The results are representative of three separate experiments.

PI gives information about apoptosis even when DNA cleavage does not produce low molecular weight DNA fragments and is also capable of distinguishing necrotic cells [42]. Therefore, we can practically exclude the possibility of occurrence of any kind of death in the majority of cells at 4 h after UV and curcumin treatment.

Furthermore, morphological observation after Hoechst staining indicated that curcumin was able to protect cells against apoptosis. Figure 3 shows nontreated, control (panel A), and UV-irradiated Jurkat cells collected 4 h after apoptotic stimuli growing in the medium supplemented (panel C) or not (panel B) with curcumin. At 4 h after UV

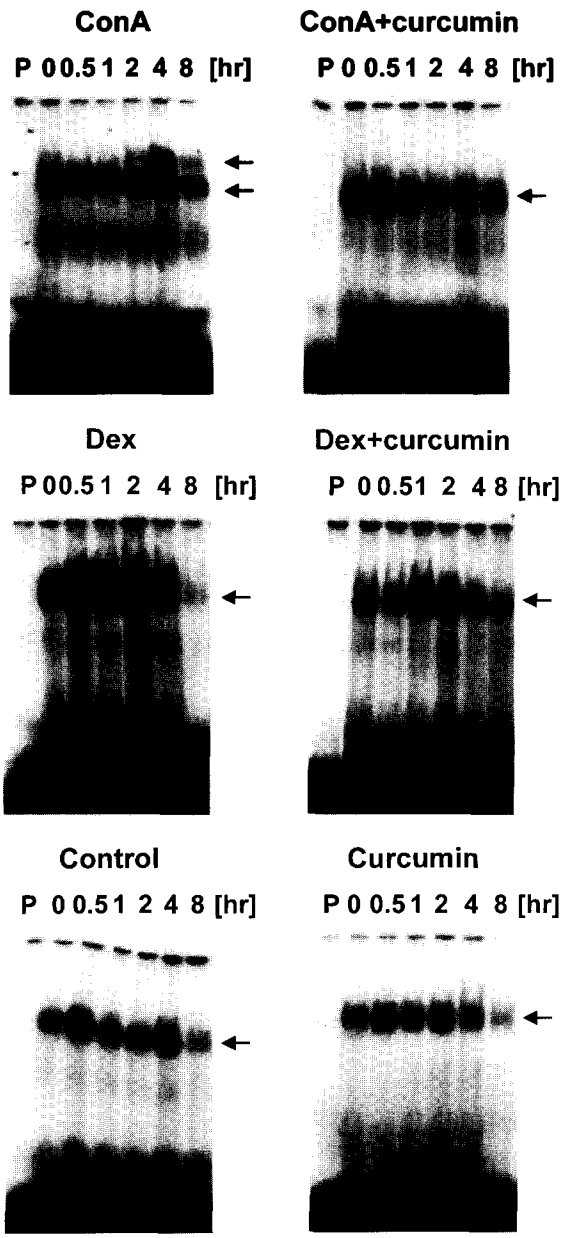

FIG. 5. The kinetics of AP-1 DNA binding activity in Con A-treated (upper panel), Dex-treated (middle panel), and control (lower panel) rat thymocytes growing in the medium without or with curcumin, as indicated. The AP-1 DNA binding activity was assessed by using the gel shift assay as described under "Materials and Methods". The binding activity to the AP-1 consensus sequence in nuclear extracts derived from rat thymocytes untreated (0 h and control) or treated with Con A, Dex, and curcumin for different times is shown. The P line shows probe. The results are representative of three separate experiments. The arrow shows the AP-1 specific band (for ConA-treated cells, two specific bands are visible). The AP-1 binding specificity was estimated in competition experiments using nonradioactive probe (not shown).

irradiation, the morphology of a large fraction of the cells can be described as apoptotic, but the majority of cells growing in the medium supplemented with curcumin showed normal morphology. Flow cytometry analysis showed that a short time after curcumin treatment (4 h) protection against apoptosis was almost complete, but at 24 h after curcumin treatment, approximately 40% of UV-irradiated Jurkat cells were found to be apoptotic in comparison with 70% found in UV-irradiated but not curcumin-treated cells.

Changes in DNA integrity are the hallmark of cellular apoptosis and can be assayed accurately and reproducibly by flow cytometry analysis using fluorochromes such as DAPI, which stains nuclear DNA, and sulforhodamine, which stains protein [39]. The nuclei in apoptotic cells show reduction in DNA stainability with DAPI, indicated by the appearance of a sub-G_1 peak on the DNA histogram. Figure 4 shows the flow cytometry analysis of rat thymocytes. In 24 h culture, ca. 27% in control and 50% in Dex-treated rat thymocytes showed the DNA degradation characteristic of apoptosis. On the other hand, cells treated with curcumin with or without Dex showed an apoptotic fraction not exceeding 8%. This strongly indicates the ability of curcumin to protect rat thymocytes against spontaneous and Dex-induced apoptosis.

Curcumin Suppresses Induction of AP-1 DNA Binding Activity in Proliferating and Apoptotic Rat Thymocytes

We previously documented the induction of AP-1 DNA binding activity in rat thymocytes treated either with Con A to stimulate proliferation or with Dex to induce apoptosis [29, 30]. Using the same method (i.e. the gel shift assay), we demonstrated here that curcumin was able to completely suppress AP-1 activation in Con A-treated rat thymocytes, which was observed at 4 h as an additional specific band (Figs. 5 and 6). In Dex-treated thymocytes, curcumin completely suppressed AP-1 induction at 0.5 h after treatment. At 1 and 2 h, only 30% suppression by curcumin was observed in Dex-treated thymocytes (Figs. 5 and 6). Nevertheless, in both cases the differences between the kinetics of treated and nontreated cells were statistically significant ($p < 0.01$ for Dex and $p < 0.05$ for ConA-stimulated) as judged by two-way analysis of variance. Curcumin alone had no effect on AP-1 activity (Fig. 5).

DISCUSSION

Curcumin is a potent inhibitor of cell growth in many cancerous cells [4–6]. In this paper, we documented that curcumin inhibited proliferation of Jurkat cells, which are human neoplastic lymphoid cells. We additionally showed that curcumin inhibited the growth of normal rat thymocytes stimulated to proliferate with lectin mitogen, Con A. Moreover, the growth inhibition was much more evident in normal cells mitogenically stimulated to proliferate than in logarithmically growing neoplastic lymphocytes (Fig. 1). One could expect that in addition to possessing growth inhibition capacity, curcumin would also be able to induce cell death, especially since [^{3}H]thymidine incorporation into curcumin-treated cells was much lower than in control cells (Fig. 1). Unexpectedly, curcumin protected cells against

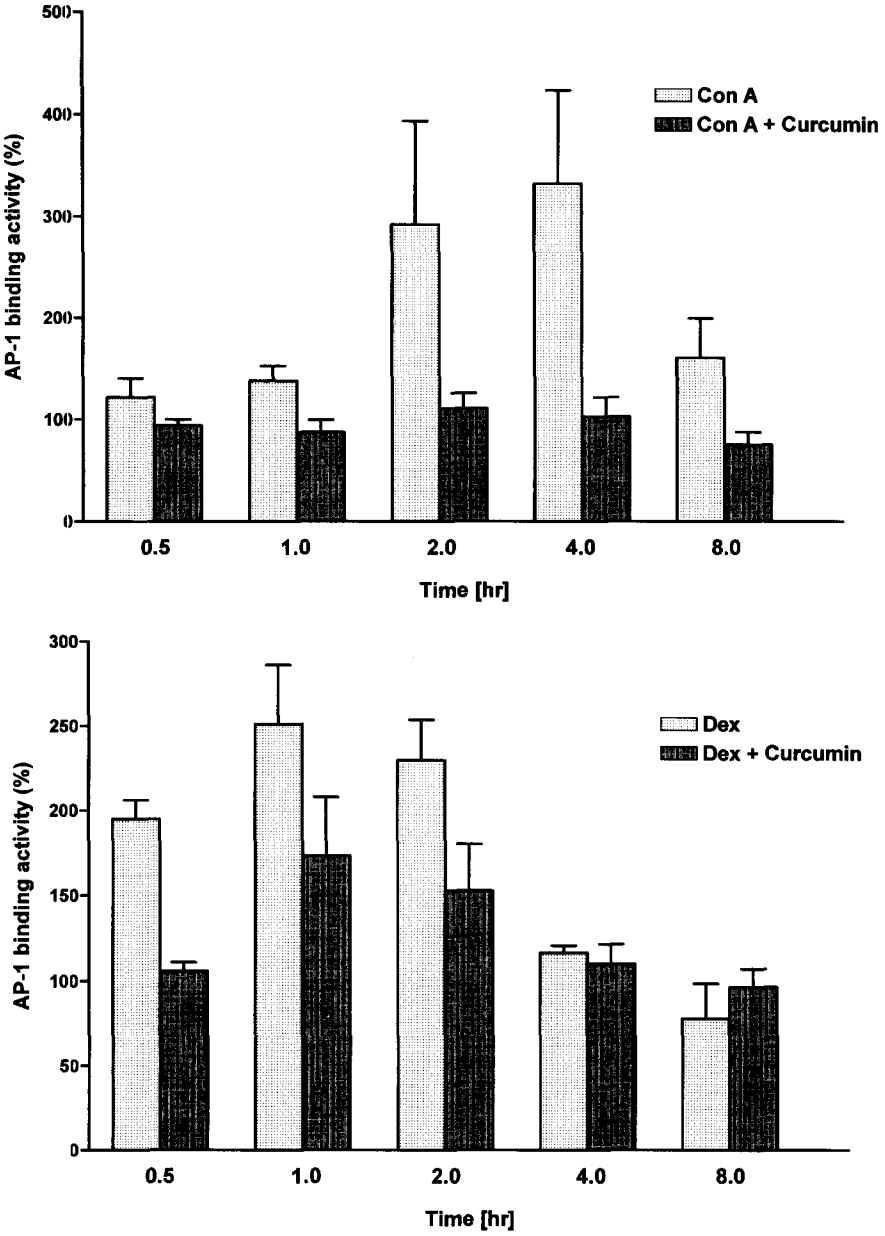

FIG. 6. The levels of binding activities to the AP-1 consensus sequence in nuclear extracts from Con A (upper) and Dex-treated rat thymocytes (lower), as assessed by densitometric scanning of autoradiograms. Values are expressed as percentages of basal levels of binding activity. The data represent means of values obtained in three separate experiments ± SD.

programmed cell death as judged by morphological observation, DNA fragmentation, and flow cytometry analysis. In particular, curcumin added to the cell medium together with Dex in the case of rat thymocytes and after UV irradiation in the case of Jurkat cells abolished DNA fragmentation to internucleosomal fragments (Fig. 2), protected morphology as is shown for Jurkat cells (Fig. 3), and lowered the apoptotic fraction of rat thymocytes to the level observed in the control cells (0 h) as measured by flow cytometry analysis (Fig. 4). Thus, curcumin inhibited not only proliferation but also apoptosis in rat and human lymphoid cells. Curcumin by itself did not induce DNA fragmentation in rat thymocytes even at 100 μM concentration (not shown), and the appearance of sub-G_1 fraction at 50 μM concentration was not observed (Fig. 4). Dissimilar to the rat thymocytes, at 24 h after curcumin treatment, approximately 40% of UV-irradiated Jurkat cells were found to be apoptotic, although internucleosomal DNA cleavage was not observed in these cells. These data suggest that in Jurkat cells curcumin can protect DNA cleavage to the internucleosomal but not to the high molecular weight DNA and that it can delay the process of apoptosis. This observation needs to be tested in further experiments (e.g. pulse field gel electrophoresis). It is noteworthy that curcumin is less effective either as proliferation or apoptosis inhibitor in neoplastic Jurkat cells than in primary thymocytes. Unfortunately, our data do not elucidate curcumin's apparently complex mechanism of action, although some

observations suggest that curcumin can interact with the processes common to proliferation and apoptosis. One of these could be the activity of the AP-1 transcription factor. AP-1 has been known to be activated not only in proliferating but also in apoptotic cells [29–36]. It has recently been shown that ceramide-induced growth inhibition and DNA fragmentation in HL60 cells were both prevented by treatment with curcumin. Moreover, this prevention was correlated with the specific inhibition of c-*jun* and AP-1 activation [43]. Our own observation revealed that curcumin inhibited AP-1 activation in apoptotic rat thymocytes as assessed by the gel shift assay, although this suppression was only partial in contrast to the complete suppression observed in proliferating thymocytes [Fig. 6].

Another possible mechanism of cell proliferation and cell death inhibition by curcumin merits particular attention. As curcumin possesses strong antioxidant and reactive oxygen species (ROS) scavenging properties, it may be expected to exert its inhibitory activity by influencing the cellular redox state, ROS detoxification, and inhibition of ROS generation. ROS involvement in cell death and cell proliferation in particular has strong experimental support [44, 45]. On the other hand, AP-1 activation is usually observed under antioxidant, but not under pro-oxidant conditions [46]. Thus, some additional experiments are necessary to clarify the role of curcumin as antioxidant and ROS scavenger in apoptosis.

The capacity of curcumin to inhibit either cell growth or cell death again stresses how these two somewhat contradictory phenomena are coupled with each other. One more possibility raised by our preliminary observation is that curcumin can influence the activity of acidic endonuclease, which was induced either in proliferating or in apoptotic HL60 cells.*

More recently, curcumin has been shown to inhibit HIV replication as well as integrase activity [47, 48]. It would be worthy to investigate whether curcumin is able to inhibit the apoptosis of HIV-infected and uninfected lymphocytes from HIV-positive patients.

The authors are deeply indebted to Prof. Zbigniew Darzynkiewicz (New York Medical College, Valhalla, New York, USA) for valuable remarks regarding flow cytometry analysis and to Prof. Malcolm Paterson (Cross Cancer Institute, Edmonton, Canada) for important methodological remarks as well as for fruitful discussion helpful in preparing this manuscript. These studies were supported in part by KBN Grant 6 P207 037 07.

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