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Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells

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1 The possible mechanisms of the antiproliferative and apoptotic effects of curcumin (diferuloylmethane), a polyphenol in the spice turmeric, on vascular smooth muscle cells were studied in rat aortic smooth muscle cell line (A7r5).

2 The proliferative response was determined from the uptake of [^{3}H]-thymidine. Curcumin (10^{-6}–10^{-4} M) inhibited serum-stimulated [^{3}H]-thymidine incorporation of both A7r5 cells and rabbit cultured vascular smooth muscle cells in a concentration-dependent manner. Cell viability, as determined by the trypan blue dye exclusion method, was unaffected by curcumin at the concentration range 10^{-6} to 10^{-5} M in A7r5 cells. However, the number of viable cells after 10^{-4} M curcumin treatment was less than the basal value (2×10^5 cells).

3 To analyse the various stages of the cell cycle, [^{3}H]-thymidine incorporation into DNA was determined every 3 h. After stimulation with foetal calf serum, quiescent A7r5 cells started DNA synthesis in 9 to 12 h (G_1/S phase), then reached a maximum at 15 to 18 h (S phase). Curcumin (10^{-6}–10^{-4} M) added during either the G_1/S phase or S phase significantly inhibited [^{3}H]-thymidine incorporation.

4 Following curcumin (10^{-6}–10^{-4} M) treatment, cell cycle analysis utilizing flow cytometry of propidium iodide stained cells revealed a G_0/G_1 arrest and a reduction in the percentage of cells in S phase. Curcumin at 10^{-4} M also induced cell apoptosis. It is suggested that curcumin arrested cell proliferation and induced cell apoptosis, and hence reduced the [^{3}H]-thymidine incorporation.

5 The apoptotic effect of 10^{-4} M curcumin was also demonstrated by haematoxylin-eosin staining, TdT-mediated dUTP nick end labelling (TUNEL), and DNA laddering. Curcumin (10^{-4} M) induced cell shrinkage, chromatin condensation, and DNA fragmentation.

6 The membranous protein tyrosine kinase activity stimulated by serum in A7r5 cells was significantly reduced by curcumin at the concentration range 10^{-5} to 10^{-4} M. On the other hand, the cytosolic protein kinase C activity stimulated by phorbol ester was reduced by 10^{-4} M curcumin, but unaffected by lower concentrations (10^{-6}–10^{-5} M).

7 The levels of *c-myc*, *p53* and *bcl-2* mRNA were analysed using a reverse transcription-polymerase chain reaction (RT-PCR) technique. The level of *c-myc* mRNA was significantly reduced by curcumin (10^{-5}–10^{-4} M) treatment. And, the level of *bcl-2* mRNA was significantly reduced by 10^{-4} M curcumin. However, the alteration of the *p53* mRNA level by curcumin (10^{-5}–10^{-4} M) treatment did not achieve significance. The effects of curcumin on the levels of *c-myc* and *bcl-2* mRNA were then confirmed by Northern blotting.

8 Our results demonstrate that curcumin inhibited cell proliferation, arrested the cell cycle progression and induced cell apoptosis in vascular smooth muscle cells. Curcumin may be useful as a template for the development of drugs to prevent the pathological changes of atherosclerosis and post-angioplasty restenosis. Our results suggest that the antiproliferative effect of curcumin may partly be mediated through inhibition of protein tyrosine kinase activity and *c-myc* mRNA expression. And, the apoptotic effect may partly be mediated through inhibition of protein tyrosine kinase activity, protein kinase C activity, *c-myc* mRNA expression and *bcl-2* mRNA expression.

Keywords: Antiproliferative effect; cell cycle; apoptosis; curcumin; smooth muscle cell (vascular); protein tyrosine kinase; protein kinase C; *c-myc*; *p53*; *bcl-2*

Introduction

Atherosclerosis and post-angioplasty restenosis are characterized by the abnormal accumulation of vascular smooth muscle cells, inflammatory cells and extracellular matrix proteins (Lundergan *et al*., 1991; Ross, 1993). We have previously shown that some plant phenols, curcumin (diferuloylmethane) from *Curcuma longa*, esculetin (6,7-dihydroxycoumarin) from *Artemisia scoparia*, and baicalein (5,6,7-trihydroxyflavone) from *Scutellaria baicalensis*, exhibited antiproliferative effects in cultured vascular smooth muscle cells (Huang *et al*., 1992; 1993; 1994a,b). Curcumin has been demonstrated to inhibit the proliferative responses of both rabbit vascular smooth muscle cells and human blood mononuclear cells (Huang *et al*., 1992). Curcumin may be useful for the study and treatment of the pathological changes of atherosclerosis and restenosis. Turmeric, the powdered rhizome from the root of the plant *Curcuma longa* has long been used as a spice in foods and as a naturally occurring medicine for the treatment of inflammatory diseases. Curcumin, a major phenolic antioxidant and anti-inflammatory agent in turmeric has been demonstrated to be an effective inhibitor of tumour production (Lu *et al*., 1994; Tanaka *et al*., 1994; Stoner & Mukhtar, 1995). Recently, we

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found that curcumin inhibited cell proliferation, arrested cell cycle progression and induced cell apoptosis in vascular smooth muscle cells (SMC). *In vivo*, vascular SMC are apparently arrested in the quiescent phase (G_0). Following serum-stimulation, quiescent cells progress through the checkpoint of the G_1 phase and regulatory factors required for initiation of DNA replication are sequentially expressed and/or activated. Then, cells progress through the S phase for DNA replication. We found that accumulation of the cells in the G_0/G_1 phase of the cell cycle accompanied by cell apoptosis occurs in vascular SMC treated with 10^{-4} M curcumin. Apoptosis (programmed cell death) plays a critical role in both the normal development and the pathology of a wide variety of tissues, is characterized by cytoplasmic shrinkage, nuclear condensation and DNA fragmentation (Jacobson *et al.*, 1997; Nagata, 1997). In recent years, cell cycle and apoptosis regulation gained wide attention as a possible future means of eliminating excessively proliferative cells.

The transmission of signals from the plasma membrane to the nucleus involves a number of different pathways. The antiproliferative effects of esculetin and baicalein have been shown by us to be mediated through the inhibition of protein tyrosine kinase activity and growth factor gene expression (Huang *et al.*, 1993; 1994a). Several studies have demonstrated that protein tyrosine kinase and protein kinase C are involved in the regulation of the cell cycle progression and cell apoptosis (Lucas *et al.*, 1994; Simon *et al.*, 1995; Lavin *et al.*, 1996). It seemed of interest to investigate the effects of curcumin on signal transduction pathways. Some proto-oncogenes, *bcl-2* and *c-myc*, and tumour suppressor gene, *p53*, have been shown to regulate cell proliferation and/or apoptosis (Hale *et al.*, 1996). In this study, the molecular mechanisms of the antiproliferative and apoptotic effects of curcumin in vascular smooth muscle cells were investigated to determine whether the transduction signals and/or genes expression are involved.

Methods

Measurement of cell proliferation

A7r5 cells (rat aortic smooth muscle cells) were grown in Dulbecco's modified Eagle's medium (supplemented with 10% v/v foetal calf serum, 100 u ml^{-1} penicillin, and 1 μg ml^{-1} streptomycin). The proliferative responses of vascular smooth muscle cells stimulated by serum were determined by the uptake of tritiated thymidine (Huang *et al.*, 1992). Before all the experiments, confluent smooth muscle cells were rendered quiescent by culturing for 48 h (with one medium change after 24 h) in 0.5% v/v foetal calf serum (FCS) instead of 10%. In A7r5 cells (10^4 cells/well), 10% v/v FCS and the test compound were added to the medium 16 h before [^{3}H]-thymidine was added. Four hours after addition of [^{3}H]-thymidine, the cells were harvested and the [^{3}H]-thymidine incorporated into cells was counted.

Each experiment was performed in triplicate and repeated 5 to 6 times. The inhibitory activity of the test compound is expressed as percentage of the control value stimulated by serum without test compound. The concentration evoking 50% maximal inhibition (IC_{50}) was calculated for each experiment.

Cell viability was determined with the trypan blue dye exclusion method. After addition of the test compound for 20 h, cells were harvested from the dishes using a 0.1% w/v trypsin solution, and the viability was examined by the trypan blue dye exclusion test. The number of viable cells was estimated by microscopic cell counting using a haemocytometer.

Chronological analysis of DNA synthesis

DNA synthesis was determined by the incorporation of [^{3}H]-thymidine. [^{3}H]-Thymidine (0.2 μCi/well) was added 3 h before the termination of the experiment. Vascular SMC were synchronized in the G_0 phase by serum depletion for 48 h. They were allowed to enter the cell cycle by stimulation with 10% v/v foetal calf serum at 0 h. The [^{3}H]-thymidine incorporated to cells was measured every 3 h thereafter to examine the relationship between the time duration after serum stimulation and DNA synthesis in SMC. After stimulation with serum, quiescent SMC started DNA synthesis. The effect of test compound at various stages of the cell cycle was determined by administering it only transiently for limited periods of time at specific time points. When the cultures underwent transient incubation with the test compound, they were thoroughly washed with serum-free DMEM after the incubation. The medium was subsequently changed to DMEM containing 10% v/v FCS without the test compound until the termination of the experiment. The radioactivity was then measured (18 h for A7r5).

Flow cytometric analysis

Cells were synchronized at the G_0 phase by serum depletion for 48 h. Then, the cells were washed and incubated in fresh medium containing 10% v/v FCS to allow the progression through the cell cycle. At various time periods after release from the quiescent state, cells were analysed for cell cycle distribution by flow cytometry (Sherwood & Schimke, 1995). After various treatments, A7r5 cells (10^6 cells ml^{-1}) were treated with trypsin and RNase and then stained with propidium iodide (CycleTEST plus DNA reagent kit, Becton Dickinson). The samples were analysed using FACScan (Becton Dickinson) and the data were collected, stored and analysed by use of ModFit software. Each experiment was repeated 4 to 5 times.

Morphological evaluation by haematoxylin-eosin staining

Cells were stained with haematoxylin-eosin and examined using light microscopy. Apoptotic cells were identified according to the following criteria: cell shrinkage, chromatin condensation and formation of apoptotic body.

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay for apoptosis

Incorporation of biotinylated nucleotides at free 3′-OH ends of DNA in cultured cells was modified from the method described by Gavrieli *et al.* (1992). After treatment with test compound, cells were plated on slides, air dried, and fixed with ice-cold 95% v/v ethanol. The slides were rehydrated in PBS and incubated in 3% v/v H_2O_2 in water for 5 min to block endogenous peroxidase. The specimens were then covered with terminal deoxynucleotidyl transferase (TdT) (0.3 eu μl^{-1}) and biotinylated dUTP (10^{-3} M) in TdT buffer containing 3×10^{-2} M Tris-HCl (pH 7.2), 1.4×10^{-1} M sodium cacodylate, and 10^{-3} M cobalt chloride for 1 h at 37°C. The reaction was terminated by Tris-buffered saline for 5 min at room temperature. The slides were covered with avidin-peroxidase complex for 30 min and peroxidase was visualized using

3,3′-diaminobenzidine for 10 min. Counterstaining was performed with 5% w/v methyl green for 5 min. Stained cells were analysed under light microscope. In each experiment, the negative control received only the label solution without the terminal transferase, and the positive control was exposed, before the TUNEL reaction, to DNase I (1 μg ml^{-1} in 4×10^{-2} M Tris-HCl, 6×10^{-3} M $MgCl_2$, pH 7.5) for 10 min at room temperature.

DNA laddering

Cleavage of DNA into oligonucleosomal fragments, recognizable as a DNA ladder when electrophoresed on an agarose gel, is usually considered as the biochemical hallmark of apoptosis. After treatment with test compound, cells (10^7 cells/sample) were washed with PBS and lysed in cell lysis buffer containing Tris, EDTA and sodium dodecyl sulphate. After the addition of RNase A (0.6 u ml^{-1}), the mixture was incubated at 37°C for 30 min. Protein precipitation solution (ammonium acetate) was added to the samples to eliminate the contamination of proteins and centrifuged at 2,000 *g* for 10 min. Cell lysates were treated with 100% isopropanol to precipitate DNA. The DNA pellet was washed with 70% v/v ethanol and dissolved in DNA hydration buffer containing Tris and EDTA. The concentration of DNA was determined at 260 nm by spectrophotometry. Twenty micrograms of DNA was electrophoresed on a 1% w/v agarose gel containing 0.5 μg ml^{-1} ethidium bromide. The bands of DNA fragmentation were photographed under u.v. light and analysed using an image analyser (Winstar, Taiwan).

Measurement of protein tyrosine kinase activity

The membranous protein tyrosine kinase activity of A7r5 cells was measured as previously described (Huang *et al*., 1993). A7r5 cells were lysed in ice-cold extraction buffer, containing 10^{-3} M Tris-HCl (pH 7.4), 10^{-3} M $MgCl_2$, 10^{-3}M EDTA, 2.5×10^{-1} M sucrose, 10^{-3} M dithiothreitol, 50 kiu aprotinin and 10^{-3} M phenylmethyl-sulphonylfluoride, by a sonicator. The suspension was centrifuged first at 800 *g* for 10 min and then at 100,000 *g* for 1 h. The final pellet was resuspended in solubilizing buffer containing 5×10^{-2} M Tris-HCl (pH 7.5), 2×10^{-2} M $Mg(C_2H_3O_2)_2$, 5×10^{-3} M NaF, 2×10^{-4} M EDTA, 8×10^{-4} M EGTA, 10^{-3} M dithiothreitol and 3×10^{-5} M Na_3VO_4 and re-sonicated. After a 1 h incubation on ice, the homogenate was centrifuged at 100,000 *g* for 1 h. The supernatant thus obtained was the membrane fraction. The protein tyrosine kinase activity in the resultant supernatant was assayed with a protein tyrosine kinase assay kit (Gibco) by incubating with the synthetic peptide substrate, RR-SRC (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly), in the presence of [γ-^{32}P]-ATP, and activating enzyme with 10% v/v FCS. After removal of protein by acid precipitation, labelled peptide was bound to a phosphocellulose disc and phosphorylation of the peptide was measured by a scintillation counter. The protein content was determined according to the method of Lowry *et al*. (1951). The results are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}.

Measurement of protein kinase C activity

The protein kinase C activity of A7r5 cells was measured as previously described (Huang *et al*., 1993). A7r5 cells were lysed in ice-cold buffer A, containing 2×10^{-2} M Tris-HCl (pH 7.5), 2×10^{-3} M EDTA, 5×10^{-4} M EGTA, 3×10^{-1} M sucrose, 2×10^{-3} M phenylmethylsulphonylfluoride and 2.3×10^{-5} M leupeptin, by a sonicator. After centrifugation at 2,500 *g* for 5 min, the supernatant was centrifuged at 100,000 *g* for 1 h, and the resulting supernatant was retained as the cytosol fraction. Afer ultracentrifugation, the pellets were washed with buffer B (buffer A without sucrose) by centrifugation (12,000 *g*, 15 min), resuspended in buffer B with 1% v/v Triton X-100, and resonicated. After a 1 h incubation at 4°C, the homogenate was centrifuged at 100,000 g for 1 h, and the supernatant thus obtained was the membrane fraction. Protein kinase C activity was measured with a protein kinase C assay kit (Amersham) by a modification of a mixed micelle assay (Hannun *et al*., 1985), activating the enzyme with 39 μM phorbol-12-myristate-13-acetate. After removal of protein by acid precipitation, labelled peptide was bound to a phosphocellulose disc and phosphorylation of the peptide was measured by a scintillation counter. The protein content was determined according to the method of Lowry *et al*. (1951). The results are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}.

RT-PCR analysis of c-myc*,* p53 *and* bcl-2 *mRNA expression*

The expression of *c-myc*, *p53* and *bcl-2* mRNA was analysed by the reverse transcription-polymerase chain reaction (RT-PCR) technique (Wang *et al*., 1989; Huang *et al*., 1994a). Before the RNA extraction, confluent smooth muscle cells were rendered quiescent by culturing for 48 h in 0.5% w/v FCS instead of 10%. Then, test compound together with 10% v/v FCS was added to the medium and incubated for indicated time period. Total cellular RNA was prepared by acid guanidinium thiocyanate extraction (Chomczynski & Sacchi, 1987). RNA was then reverse transcribed into cDNA. A 20 μl reverse transcription reaction mixture containing 1 μg of total cellular RNA, 1 × PCR buffer (10 M Tris-HCl, pH 8.3, 5×10^{-2} M KCl), 3×10^{-4} M deoxynucleoside triphosphates (dNTPs), 1 unit of RNase inhibitor, 2.5×10^{-6} M of oligo(dT)$_{16}$ and 10 units of M-MLV reverse transcriptase was incubated at 42°C for 1 h, heated to 95°C for 5 min, and then quick-chilled at 5°C for 5 min. PCR was performed at a final concentration of 1 × PCR buffer/0.5 μM each 3′ and 5′ primers/2.5 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer) in a total volume of 100 μl. The mixture was amplified for 35 cycles with the Perkin-Elmer thermal cycler (GeneAmp PCR System 9600). The amplification profile involved denaturation at 95°C for 15 s, annealing and extension at 65°C for 15 s. Primers for *c-myc*, *p53* and *bcl-2* were used to generate 479, 371, and 235 bp fragments, respectively. The GAPDH primer (452 bp) was used as the internal standard. In each experiment, the negative control without reverse transcriptase was performed. Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The gel was photographed with Polaroid type 667 film, and the digitized images were analysed using image analyser (Winstar LV1, Taiwan). The signal intensity of test gene was normalized to their respective GAPDH signal intensity and expressed in arbitrary units. Samples were further confirmed by Northern blotting using standard methods (Sambrook *et al*., 1989).

In a preliminary experiment, various aliquots taken from the RT reaction were used in PCR for analysis of the test transcripts. The amounts of cDNA produced were proportional to the inputs of the RT-PCR products in the range 0.5 to 5 μl. Therefore, in subsequent experiments, the amounts of reverse-transcriptase products taken for PCR amplification of the test transcripts were within the linear ranges, in order to

ensure that the amounts of cDNA produced truly reflect the levels of mRNA in the original samples.

Materials

Curcumin (diferuloylmethane) (Figure 1) and ferulic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Rat aortic smooth muscle cell line (A7r5 cells) were purchased from the American Type Culture Collection (Rockville, MD). Rabbit cultured vascular smooth muscle cells were isolated from their rabbit thoracic aorta and cultured as previously described (Huang *et al*., 1992). Foetal calf serum, penicillin, streptomycin, and Dulbecco's modified Eagle's Medium (DMEM) were purchased from Gibco Lab. (Grand Island, NY). [Methyl-^{3}H]-thymidine (5 Ci mmol^{-1}) and [γ-^{32}P]-ATP (5000 Ci mmol^{-1}) were purchased from Amersham Co. (Buckinghamshire, U.K.). Terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Avidin-peroxidase complex was purchased from Vector Lab., Inc. (Burlingame, CA). Aprotinin, leupeptin, phenylmethylsulphonylfluoride, dithiothreitol, 3,3′-diaminobenzidine, genistein (4′,5,7-trihydroxyisoflavone), and H-7 (1-(5-isoquinoline-sulphonyl)-2-methylpiperazine-2HCl) were purchased from Sigma Chemical Co. (St. Louis, MO). The cycleTEST plus DNA reagent kit was purchased from the Becton Dickinson Co. (San Jose, CA). The DNA isolation kit was purchased from Gentra systems, Inc. (Research Triangle Park, NC). Moloney murine leukaemia virus (M-MLV) reverse transcriptase and *AmpliTaq* DNA polymerase were purchased from Perkin-Elmer (Norwalk, CT). Primer for *bcl-2* (235 bp) was purchased from Maxim Biotech, Inc. (So. San Francisco, CA). Primers for *c-myc* (479 bp), *p53* (371 bp) and GAPDH (452 bp) were purchased from Clontech Lab., Inc. (Palo Alto, CA).

Statistical analysis

The data are expressed as mean ± s.e.mean. P values less than 0.05 were considered statistically significant (ANOVA and Student's t test).

Results

Antiproliferative effects

Cell proliferation was studied by measuring the incorporation of [^{3}H]-thymidine into DNA in cells. The control value of [^{3}H]-thymidine incorporation in A7r5 cells (10^4 cells/well) stimulated by serum without test compound was 14,595 ± 2,187 c.p.m./well. Exposure of A7r5 cells to curcumin (10^{-6}–10^{-4} M), inhibited the serum-stimulated [^{3}H]-thymidine incorporation in a concentration-dependent manner. The IC_{50} and maximal suppression values of curcumin and ferulic acid are shown in Table 1 ($n=5$) and compared with the previous results from rabbit cultured vascular smooth muscles ($n=6$) (Huang *et al*., 1992). The activities of test compounds in two cell types were comparable. Ferulic acid was a much less effective inhibitor than curcumin in both cell types.

Cell viability, as determined by the trypan blue dye exclusion method, was not affected by curcumin and ferulic acid at the concentrations lower than 10^{-4} M (Figure 2) ($n=5$). As shown in Figure 2, the numbers of viable cells after curcumin (10^{-6}–10^{-5} M) and ferulic acid (10^{-6}–10^{-4} M) treatment were more than the basal value (2×10^5 cells). However, 10^{-4} M curcumin significantly reduced the cell viability.

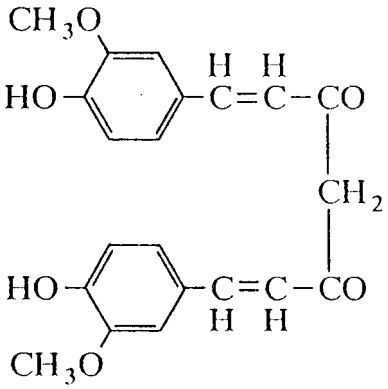

Figure 1 Structure of curcumin (diferuloylmethane).

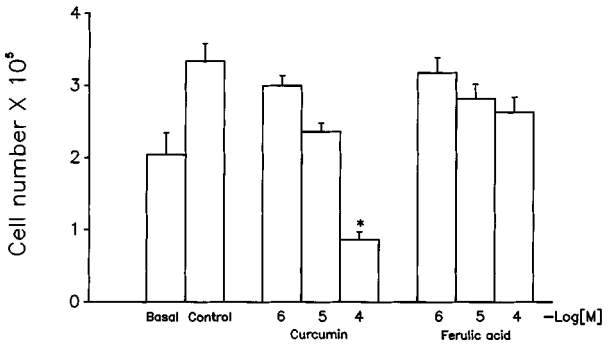

Figure 2 Effects of curcumin and ferulic acid on A7r5 cell growth. Quiescent A7r5 cells (basal level, 2×10^5 cells) were stimulated with serum. After addition of serum for 20 h in the absence (control level) or presence of test compound, cells were harvested and the viability was examined by trypan blue dye exclusion test. The number of viable cells was estimated by using a haemocytometer. Each column represents the mean ± s.e.mean ($n=5$). *$P<0.05$, compared to the cell number in basal condition (basal level).

Table 1 Antiproliferative activities of curcumin and ferulic acid on A7r5 cells and rabbit vascular smooth muscle cells

	A7r5 cells		*Rabbit vascular SMC*	
	IC_{50} ($\times10^{-6}$ M)	*Max. suppression at 10^{-4} M (%)*	*IC_{50} ($\times10^{-6}$ M)*	*Max. suppression at 10^{-4} M (%)*
Curcumin	6.4 ± 0.6	100.0 ± 1.6	23.3 ± 0.7	100.0 ± 0.0
Ferulic acid	344.2 ± 108.3	63.5 ± 1.9	433.7 ± 256.0	43.5 ± 7.6

Values are means ± s.e.mean of 5 to 6 triplicate experiments. The inhibitory effects of curcumin and ferulic acid were tested on serum-stimulated [^{3}H]-thymidine incorporation in A7r5 cells and compared to results in rabbit vascular smooth muscle cells. The inhibitory activities are expressed as percentages of serum-stimulated control values without test compound (% of control). The control values of cells untreated with test compound were 14,595 ± 2,187 and 10,921 ± 2,739 c.p.m./well in A7r5 cells and rabbit vascular smooth muscle cells, respectively.

Effects at various stages of cell cycle

In order to examine the relationship between time duration after serum-stimulation and DNA synthesis in vascular smooth muscle cells, quiescent cultures were stimulated with serum and [^{3}H]-thymidine incorporation was measured every 3 h thereafter. Incorporation of radioactivity in A7r5 cells did not increase in the first 6 h, followed by an increase from 9 h to 18 h when it reached a plateau (Figure 3). The time period was 9 to 12 h for the G_1/S phase and 15 to 18 h for the S phase in A7r5 cells.

We attempted to find out the point(s) of action of the test compound in more detail by administering it for limited periods of time at specific time points. When the test compound was added at the G_1/S phase (9 h after serum-stimulation) and administered for 3 h, curcumin (10^{-6} 10^{-4} M) significantly inhibited [^{3}H]-thymidine incorporation (50.0 ± 2.1 and 70.2 ± 3.7% inhibition for 10^{-5} and 10^{-4} M, respectively) (Figure 4a). When test compound was added at the S phase (15 h after serum-stimulation) and administered for 3 h, curcumin (10^{-6}–10^{-4} M) also significantly inhibited [^{3}H]-thymidine incorporation (46.0 ± 4.3 and 63.9 ± 1.6% inhibition for 10^{-5} and 10^{-4} M, respectively) (Figure 4b).

Cell cycle analysis by flow cytometry

The effect of the test compound on the cell cycle in A7r5 cells was further analysed with the flow cytometry technique (Figure 5). Most cells were synchronized at the G_0 phase in the basal condition after 48 h of serum depletion (Figure 5a). When the synchronized cells were stimulated with serum, the higher percentage of cells at the S phase represents the proliferative response stimulated by serum (Figure 5b). Figure 6 shows the percentages of cells in the G_0/G_1 phase (Figure 6a) and S phase (Figure 6b) after serum-stimulation in the absence and presence of curcumin (10^{-6}–10^{-4} M). In Figure 6b, the cells started to progress to the S phase 9 h after serum-stimulation in the control cells without test compound. The peak ratio of cells in the S phase was observed 15 h after serum-stimulation. Curcumin (10^{-5}–10^{-4} M) treatment significantly arrested cells in the G_0/G_1 phase (Figures 6a and 5d, e) and reduced the percentage of cells in the S phase (Figures 6b and 5d, e). The results obtained with flow cytometry were comparable to those with [^{3}H]-thymidine incorporation.

Apoptosis

Curcumin induced cell apoptosis in A7r5 cells at 10^{-4} M, but not at concentrations lower than 10^{-4} M. The apoptosis induced by curcumin was characterized by flow cytometry,

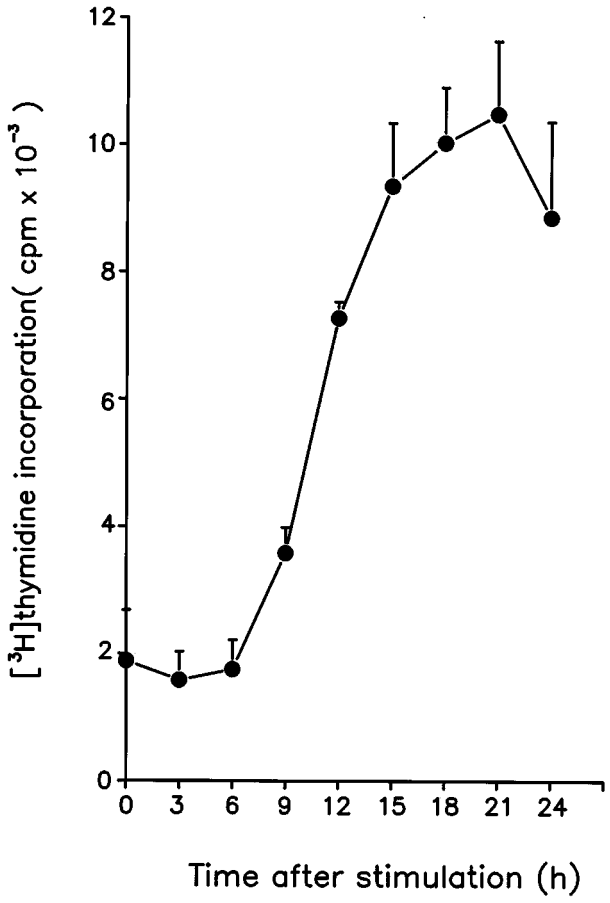

Figure 3 [^{3}H]-Thymidine incorporation of A7r5 cells stimulated with serum as a function of incubation time. Cells were synchronized in the G_0 phase by serum depletion for 48 h. They were allowed to enter the cell cycle by stimulation with serum at 0 h. The radioactivity incorporated was measured at the indicated times of incubation subsequent to 3 h exposure to [^{3}H]-thymidine. Each point represents the mean and vertical lines show s.e.mean ($n=6$).

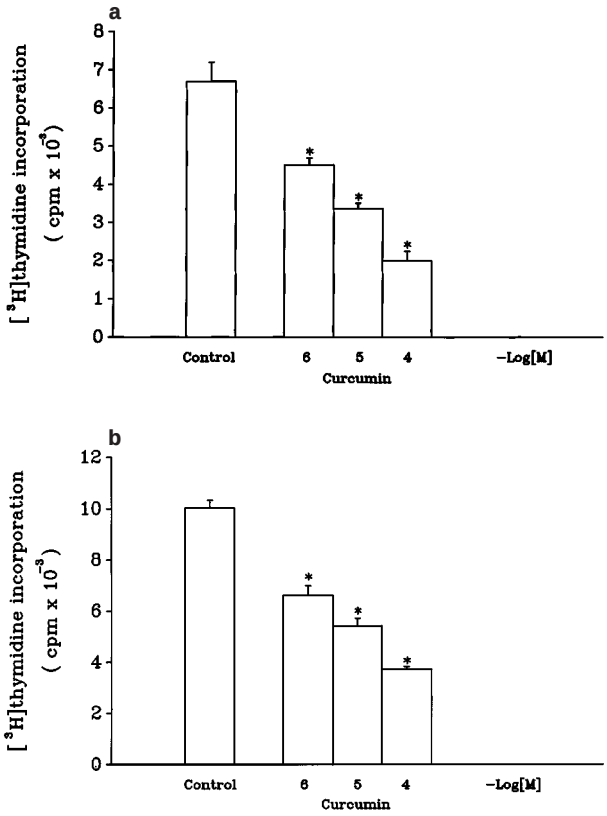

Figure 4 Effect of curcumin on [^{3}H]-thymidine incorporation of A7r5 cells administered in (a) the G_1/S phase and (b) the S phase. A7r5 cells were synchronized in the G_0 phase by serum depletion for 48 h. They were allowed to enter the cell cycle by stimulation with serum at 0 h (control value). Curcumin (10^{-6}–10^{-4} M) and [^{3}H]-thymidine were added at (a) 9 h (G_1/S phase) and (b) 15 h (S phase) after serum-stimulation for 3 h. The medium was subsequently changed to DMEM without the test compound. The radioactivity was then measured at 18 h. Each column represents the mean $\pm$ s.e.mean ($n=5$).

haematoxylin-eosin staining, TdT-mediated dUTP nick end labelling (TUNEL) analysis, and DNA laddering.

By using flow cytometric analysis, the percentage of cells in an apoptotic region (Ap, sub-G_0/G_1 peak, subdiploid peak) increased after 10^{-4} M curcumin treatment for 24 h ($25.7 \pm 5.2\%$) (Figure 5e).

By haematoxylin-eosin staining, the characterized morphological changes of A7r5 cells treated with curcumin were examined (Figure 7). When exposed to 10^{-4} M curcumin for 20 h, some cells shrank and retracted from neighbouring cells (Figure 7c). Cytoplasm condensation was observed, but few blebbed apoptotic bodies were found. Some surviving cells also showed certain degrees of morphological change, with an elongated and bipolar appearance.

The TUNEL procedure stains nuclei that contain nicked DNA, a characteristic exhibited by cells in the early stages of apoptotic cell death (Figure 8). A7r5 cells untreated and treated with 10^{-5} M curcumin were negative for TUNEL staining (Figure 8a, b). The cells which had nuclei that were not stained with TUNEL (TUNEL-negative, green-coloured) were visualized by counterstaining with methyl green. However, a large number of TUNEL-positive nuclei were detected in cells after treatment with 10^{-4} M curcumin for 20 h (Figure 8c). In the TUNEL-positive (brown-coloured) cells, the stain localized in the cell nuclei rather than in their cytoplasm.

The degradation of DNA into a specific pattern of fragments is a characteristic feature of apoptosis. These fragments were visualized by agarose gel electrophoresis as a DNA ladder consisting of multimers of approximately 200 base pairs. The DNA fragmentation associated with apoptosis is characterized by cleavage of the DNA at regular intervals, as opposed to the random fragmentation associated with necrosis. After treatment of A7r5 cells with 10^{-4} M curcumin for 20 h, the genomic DNA from cells was subjected to agarose gel electrophoresis. A clear DNA fragmentation ladder was found in ethidium bromide-stained gel (Figure 9, lane 3). By contrast, the pattern of fragmentation was not visible in cells untreated or treated with lower concentration of curcumin ($< 10^{-4}$ M) ($n = 3$) (Figure 9, lanes 1 and 2).

Effect on protein tyrosine kinase activity

The membranous protein tyrosine kinase activity in serum-stimulated A7r5 cells was 3.2 ± 0.2 pmol mg^{-1} protein min^{-1} over the basal activity ($n = 20$) in A7r5 cells. The serum-

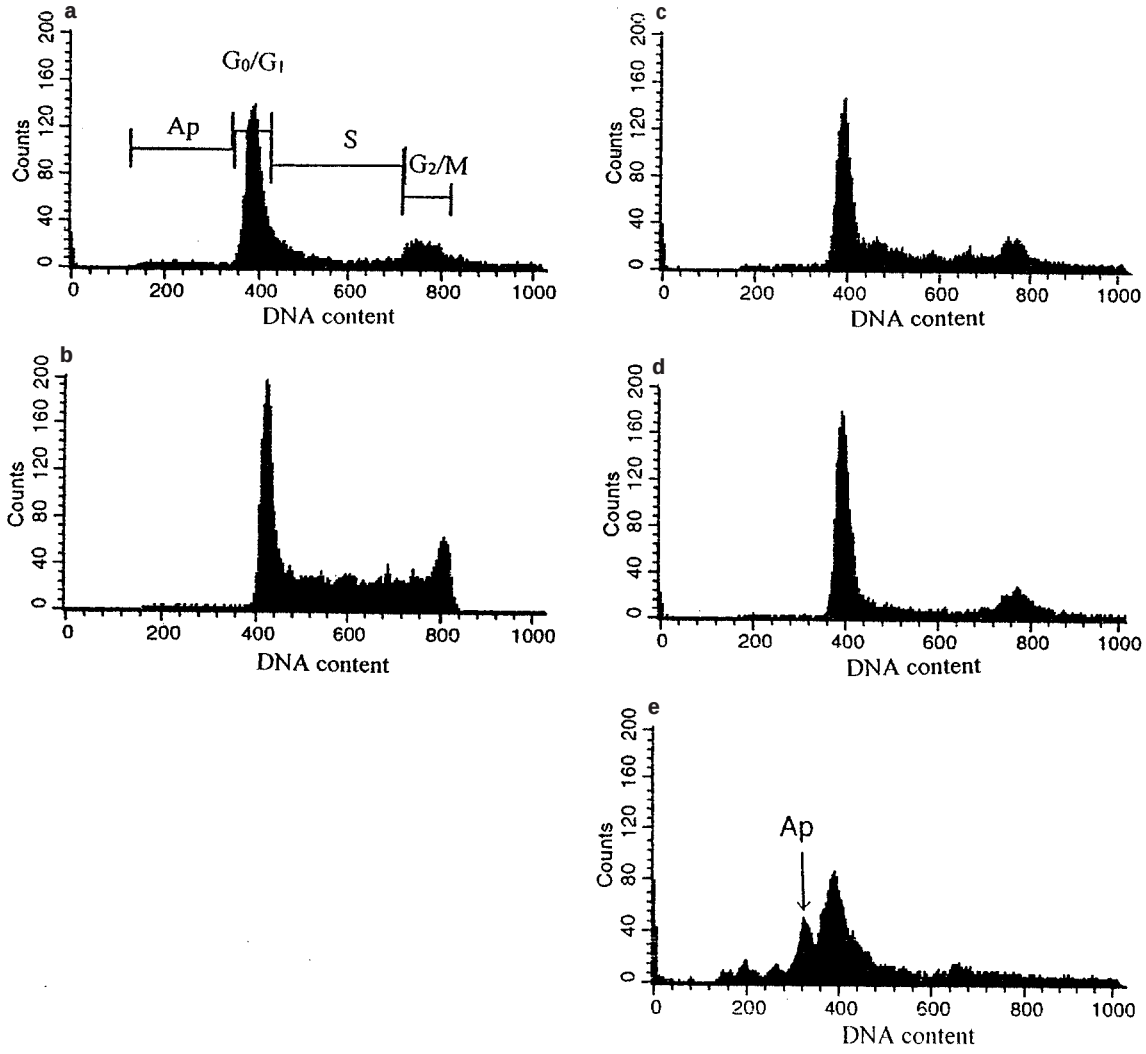

Figure 5 Cell cycle analysis of A7r5 cells by flow cytometry. Area definition of DNA histogram: apoptotic area (Ap, sub-G_0/G_1 peak), G_0/G_1 phase, S phase, and G_2/M phase. A7r5 cells (a) during quiescent basal state, (b) stimulated with serum untreated, and treated with (c) 10^{-6} M, (d) 10^{-5} M and (e) 10^{-4} M curcumin were analysed by flow cytometer with propidium iodide staining. Figures shown are the representative histograms from five experiments.

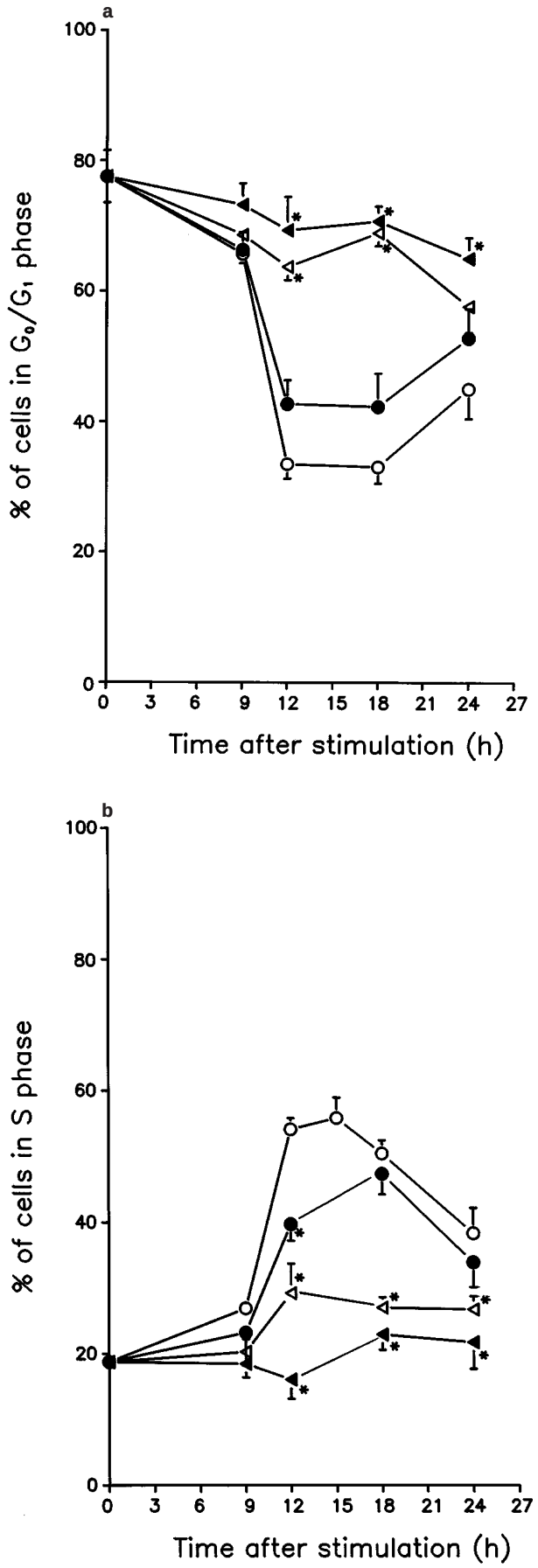

Figure 6 Effects of curcumin on the percentages of cells at (a) the G_0/G_1 phase and (b) the S phase. A7r5 cells were synchronized at the G_0 phase by serum depletion for 48 h. Cells were then stimulated by serum at 0 h in the absence (open circles) or presence of 10^{-6} M (solid circles), 10^{-5} M (open triangles) or 10^{-4} M (solid triangles) curcumin. The DNA content of A7r5 cells in the cell cycle were analysed using flow cytometry with propidium iodide staining. The percentage of cells at different stages of the cell cycle were analysed by Modfit software (Becton Dickinson). Curcumin (10^{-5}–10^{-4} M) treatment significantly arrested cells in the G_0/G_1 phase and reduced the percentage of cells in the S phase. Each point represents the mean and vertical lines show s.e.mean ($n=5$). *$P<0.05$, compared to the serum-stimulated control without curcumin.

stimulated protein tyrosine kinase activity was significantly reduced by curcumin at the concentration range of 10^{-5} to 10^{-4} M (37.5±9.3 and 43.7±6.3% inhibition for 10^{-5} and 10^{-4} M, respectively) ($n=5$) (Figure 10). In our assay system, 10^{-5} M genistein, a protein tyrosine kinase inhibitor, inhibited protein tyrosine kinase activity by 43.6±6.2% ($n=5$).

Effect on protein kinase C activity

The cytosolic protein kinase C activity of A7r5 cells was 22.0±1.4 pmol mg^{-1} protein min^{-1} ($n=16$) in the presence of 39 μM phorbol-12-myristate-13-acetate. The protein kinase C activity was reduced on incubation with 10^{-4} M curcumin by 29.1±8.2% but unaffected by concentrations lower than 10^{-4} M (Figure 11) ($n=5$). In our assay system, 10^{-5} M H-7, a protein kinase C inhibitor, inhibited protein kinase C activity by 55.5±5.0%.

Effects on c-myc, p53 *and* bcl-2 *mRNA expression*

The mRNA levels of *c-myc*, *p53* and *bcl-2* in serum-stimulated A7r5 cells untreated and treated with curcumin were measured using the RT-PCR technique. All three genes were expressed in A7r5 cells in the presence of serum. In a preliminary experiment, the inhibitory effect of curcumin on the expression of *c-myc* mRNA peaked at 2 h. And, the inhibitory effect on the expression of *p53* and *bcl-2* mRNA peaked at 4 h. The signal intensities of test genes and GAPDH were quantified by an image analyser, and changes in the signal intensities of test genes relative to GAPDH are shown (bottom of Figure 12). The effects of curcumin (10^{-5}–10^{-4} M) on the levels of *c-myc*, *p53* and *bcl-2* mRNA are shown in Figure 12. The level of *c-myc* mRNA 2 h after the addition of curcumin (10^{-5}–10^{-4} M) was significantly reduced (47.2±14.1 and 61.7±7.8% inhibition for 10^{-5} and 10^{-4} M, respectively) ($n=5$). The level of *bcl-2* mRNA 4 h after the addition of 10^{-4} M curcumin was also significantly reduced (60.7±4.2% inhibition) ($n=5$). However, the level of *p53* mRNA was not significantly affected by curcumin (10^{-5}–10^{-4} M) ($n=5$). The effects of curcumin on the levels of *c-myc* and *bcl-2* mRNA were then confirmed by Northern blotting using standard methods (Sambrook *et al.*, 1989).

Discussion

The present results demonstrated for the first time that curcumin arrested cell cycle progression and induced cell apoptosis in vascular smooth muscle cell line, A7r5. Curcumin (diferuloylmethane) was much more effective in inhibiting the serum-stimulated cell proliferation than ferulic acid. Thus, the antiproliferative activity of curcumin is not mediated by the release of ferulic acid, but may be caused by the characteristics of the diferuloylmethane molecule itself. We have previously

shown that curcumin inhibited the proliferative responses in rabbit vascular smooth muscle cells and human blood mononuclear cells (Huang *et al*., 1992). Atherosclerosis and post-angioplasty restenosis are characterized by the abnormal accumulation of vascular smooth muscle cells, inflammatory cells and extracellular matrix proteins (Lundergan *et al*., 1991; Ross, 1993). Therefore, curcumin may be useful in the study and treatment of atherosclerosis and restenosis. Similarly, the effects of curcumin on cell cycle progression have been demonstrated *in vivo* in that it attenuated pharmacologically-induced preneoplastic/neoplastic lesion formation in mouse and rat tissues (Lu *et al*., 1994; Tanaka *et al*., 1994; Stoner & Mukhtar, 1995). However, 2.5×10^{-6} M curcumin, a concen-

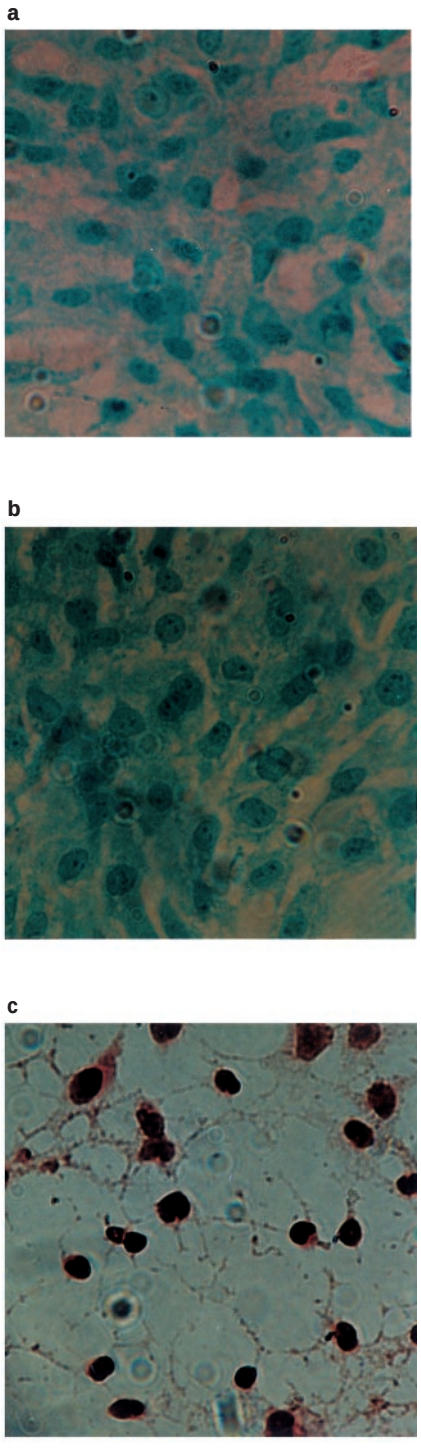

Figure 7 Morphology of A7r5 cells stained with haematoxylin and eosin. Exponentially growing A7r5 cells (a) untreated and (b) treated with 10^{-5} or (c) 10^{-4} M curcumin were cultured in cover slides for 20 h. Cells were then fixed and stained with haematoxylin and eosin. The fragmented or condensed nuclei were observed in 10^{-4} M curcumin-treated cells. Bar = 30 μm. Similar results were obtained in three independent experiments.

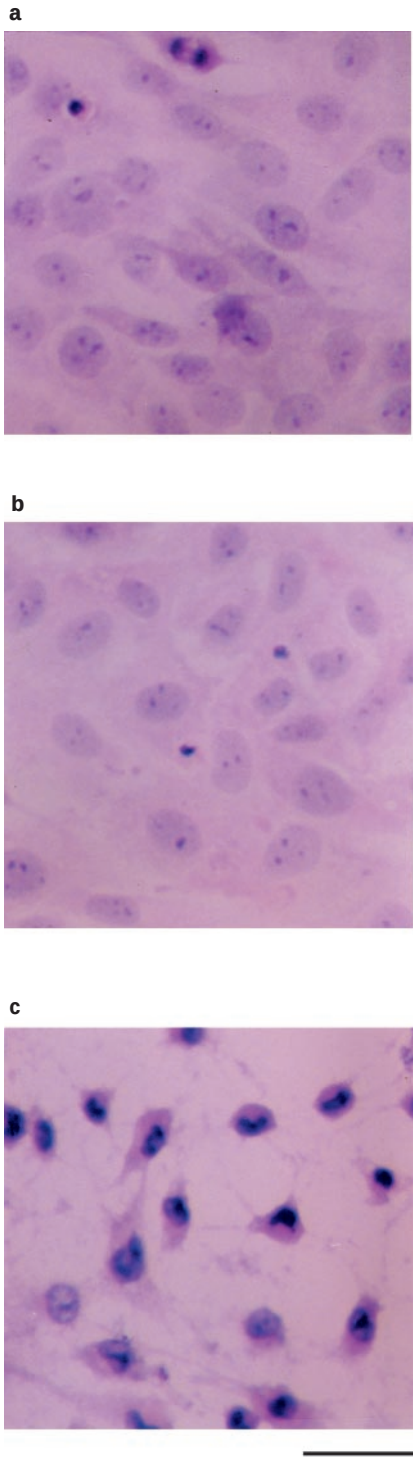

Figure 8 TUNEL analysis of A7r5 cells treated with curcumin. A7r5 cells (a) untreated and (b) treated with 10^{-5} or (c) 10^{-4} M curcumin were plated in slides for 20 h. Cells were then fixed and stained by TUNEL procedure with the enzyme TdT and biotin-conjugated dUTP. TUNEL-positive cells were visualized with a peroxidase-substrate system showing brown nuclei, and counterstaining with methyl green revealed green-blue nuclei. None of the nuclei is positively labelled for DNA fragmentation in untreated and 10^{-5} M curcumin-treated cells; whereas, a large proportion of the nuclei are TUNEL-positive in 10^{-4} M curcumin-treated cells. Bar = 30 μm. Similar results were obtained in three independent experiments.

tration close to the 6.4×10^{-6} M, the IC_{50} demonstrated for the antiproliferative effect of this molecule in the present study, was devoid of any antimitogenic activity in epidermal cells (Lu *et al*., 1994). These results demonstrate the different sensitivity of various cell types to curcumin. In addition to its antiproliferative and anti-inflammatory effects, curcumin also exhibits potent antioxidant activity (Sharma, 1976). As such, antioxidants (probucol, α-tocopherol, and ascorbic acid) have been studied in the treatment of advanced atherosclerosis. They have also been shown experimentally to have positive effects and to be useful therapeutic adjuncts to angioplasty (Carew *et al*., 1987; Gey *et al*., 1987). Antioxidants have been shown to inhibit low density lipoprotein (LDL) oxidation and the subsequent degradation of the oxidized-LDL by human monocyte-derived macrophages (Mangiapane *et al*., 1992). However, the *in vitro* antiproliferative effect of ascorbyl palmitate, a lipid soluble derivative of ascorbic acid, in cell lines was found to be much less potent than curcumin (Huang *et al*., 1992). It seems likely that the antioxidative property of curcumin may not be the major mechanism for its anti-proliferative effect in the present study. Curcumin should have actions, other than the antioxidative effect, leading to the inhibition of cell proliferation.

In vivo, vascular smooth muscle cells are apparently arrested in the quiescent phase (G_0). Following serum-stimulation, quiescent cells progress through the checkpoint of the G_1 phase and regulatory factors required for initiation of DNA replication are sequentially expressed and/or activated. Then, cells progress through the S phase for DNA replication. Recent advances in our understanding of the cell cycle reveal how the fidelity of the normal checkpoint controls can be abrogated by specific genetic changes or kinase auto-phosphorylation by some oncoproteins (Pines, 1994; Carlos, 1995). Compounds which prevent cell cycle progression through the G_1 phase into the S phase of the cell cycle can be used as negative regulators of cell proliferation in proliferative diseases such as cancer, atherosclerosis and restenosis. In the present study, curcumin (10^{-6}–10^{-4} M) added during either the G_1/S phase or S phase significantly inhibited the [^{3}H]-thymidine incorporation in A7r5 cells. By using flow cytometry technique with propidium iodide (PI) staining, curcumin was found to arrest cells in the G_0/G_1 phase and reduce the percentage of cells in the S phase.

Apoptosis, a form of programmed cell death involved in tissue morphogenesis and homeostasis, is characterized by

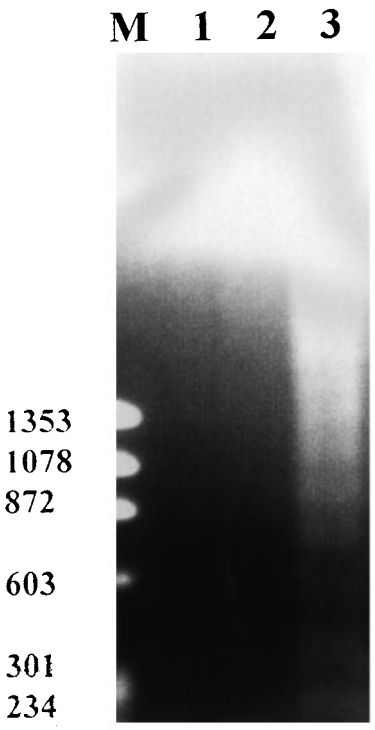

Figure 9 Electrophoresis of fragmented DNA in A7r5 cells treated with curcumin. Genomic DNA was isolated from cells untreated (control) and treated with curcumin for 20 h. DNA fragmentation was evaluated by electrophoresis in agarose gel containing ethidium bromide and photographed under u.v. light. A typical DNA ladder was detected in cells treated with 10^{-4} M curcumin. Lane M, φX174/*Hae*III DNA size marker; lane 1, untreated control; lane 2, treated with 10^{-5} M curcumin; lane 3, treated with 10^{-4} M curcumin. Similar results were obtained in three independent experiments.

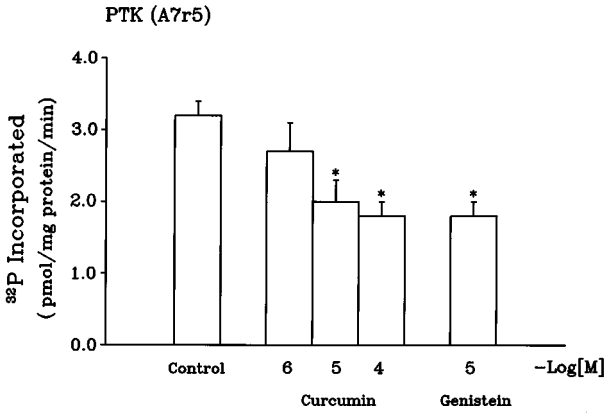

Figure 10 Effect of curcumin on membranous protein tyrosine kinase activity in A7r5 cells. Subcellular fractions were prepared as indicated in Methods. The membranous protein tyrosine kinase was stimulated by serum in the absence (control level) or presence of test compound. The effect of test compound was compared to that of 10^{-5} M genistein (a protein tyrosine kinase inhibitor). Protein tyrosine kinase activities are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}. Each column represents the mean ± s.e.mean ($n = 5$). $^*P < 0.05$, when compared to the control value without test compound.

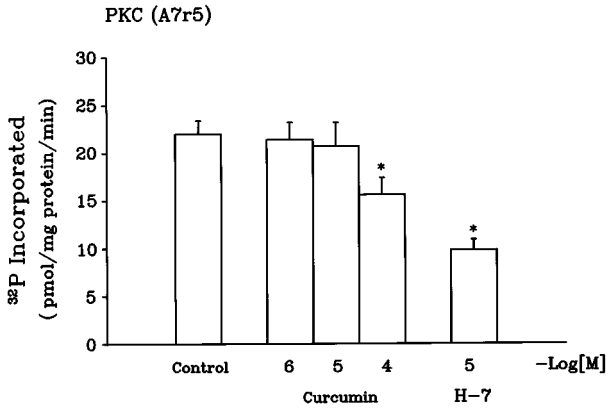

Figure 11 Effect of curcumin on protein kinase C activity in A7r5 cells. Subcellular fractions were prepared as indicated in Methods. The cytosolic protein kinase C was stimulated by phorbol ester in the absence (control level) or presence of test compound. The effect of test compound was compared to that of 10^{-5} M H-7 (a protein kinase C inhibitor). Protein kinase C activities are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}. Each column represents the mean ± s.e.mean ($n = 5$). $^*P < 0.05$, when compared to the control value without test compound.

cytoplasmic shrinkage, nuclear condensation and DNA fragmentation. In addition to inhibiting the cell growth, curcumin at 10^{-4} M induced cell apoptosis as observed by using flow cytometry, DNA laddering and TUNEL staining. The curcumin-induced apoptosis may in part contribute to the reduction of [^{3}H]-thymidine incorporation at high concentration (10^{-4} M). By contrast, a low concentration of curcumin (5×10^{-7} M) has been shown to prevent ceramide-induced growth inhibition and DNA fragmentation (Sawai *et al.*, 1995). Recent evidence suggests that the failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune diseases and viral infections. In addition, a wide number of diseases characterized by cell loss, such as neurodegenerative disorders, AIDS (acquired immunodeficiency syndrome) and osteoporosis, may result from accelerated rates of physiological cell death. Specific therapies designed to enhance or decrease the susceptibility of individual

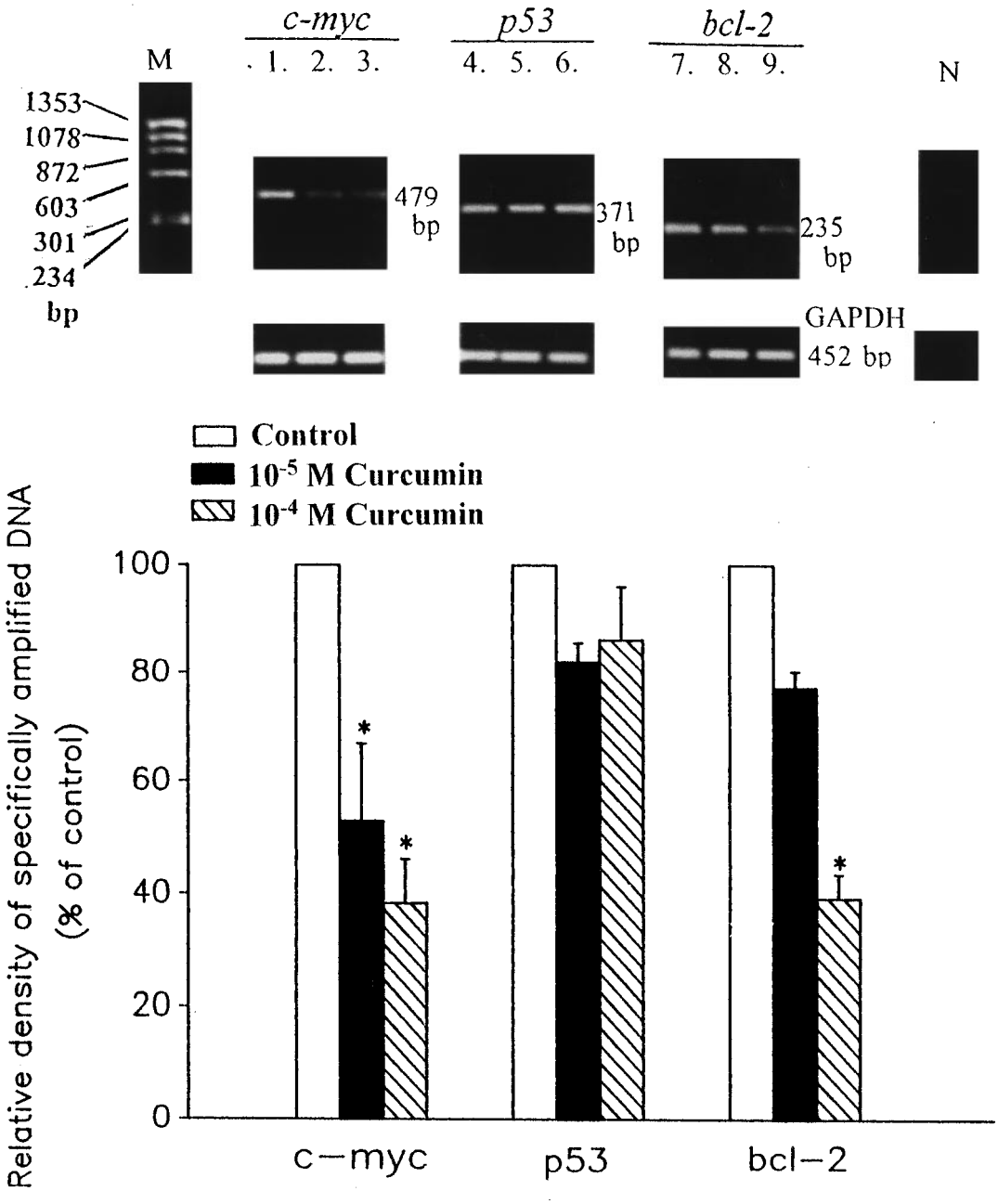

Figure 12 Effects of curcumin on *c-myc*, *p53* and *bcl-2* mRNA levels in A7r5 cells. Quiescent A7r5 cells were stimulated by serum in the absence and presence of test compound for the indicated time periods. The serum-stimulated test gene mRNA levels of untreated cells (control) and those treated with 10^{-5} M or 10^{-4} M curcumin were analysed by RT-PCR amplification as indicated in Methods. Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The signal intensities of test genes and GAPDH were quantified by an image analyser, and changes in the signal intensities of test genes relative to GAPDH were calculated. Results are expressed in arbitrary units as compared to the mRNA level in the control level without test compound (bottom) ($n=5$). On the top of each panel, corresponding electrophoretic patterns of PCR products are shown. Lane M, φX174/*Hae*III DNA size marker; lane 1, 4, 7, control without curcumin; lane 2, 5, 8, 10^{-5} M curcumin-treated; lane 3, 6, 9, 10^{-4} M curcumin-treated. *c-myc* mRNA level: lane 1, 2, 3; *p53* mRNA level: lane 4, 5, 6; *bcl-2* mRNA level: lane 7, 8, 9; lane N, negative control, no RT. Each column represents the mean$\pm$s.e.mean ($n=5$). *$P<0.05$, when compared to the control value without test compound.

cell types to undergo apoptosis could form the basis for treatment of a variety of human diseases (Thompson, 1995).

There are many factors, such as growth factors, protein tyrosine kinases, transcriptional factors, protein phosphatase, protein kinase, oncosuppressor genes, oncogenes and cyclins, involved in the control of the cell cycle (Pines, 1994). Our results further show that curcumin may inhibit the transduction signals leading to DNA synthesis. Several studies have demonstrated that protein tyrosine kinase and protein kinase C are involved in the regulation of the cell cycle progression and cell apoptosis (Lucas *et al*., 1994; Simon *et al*., 1995; Lavin *et al*., 1996). There are some signalling pathways for growth factors and mitogens (Powis & Kozikowski, 1991). The receptors for growth factors such as platelet-derived growth factor and epidermal growth factor are protein tyrosine kinase that, when activated by ligand binding, phosphorylate themselves as well as other proteins to affect the processes in the nucleus directly linked to cell proliferation. Tyrosine kinase activity has also been found to be associated with other growth factors and oncoproteins (Bishop, 1983). The activation of the protein tyrosine kinase promotes cell cycle entry and progression through the G_1 phase (Debra *et al*., 1993; Fantl *et al*., 1993). Some protein tyrosine kinase inhibitors, such as genistein and herbimycin A, have been shown to inhibit proliferation and induce accumulation of cells in the G_0/G_1 phase of the cell cycle (Yao & Scott, 1993; Luba *et al*., 1994). Genistein may also induce apoptosis through inhibition of tyrosine kinase and topoisomerase II. The present results show that curcumin may inhibit the proliferative responses through inhibiting transduction signals leading to DNA synthesis. The membranous protein tyrosine kinase activity of A7r5 cells was significantly reduced by curcumin in the concentration range 10^{-5} to 10^{-4} M, indicating that curcumin may act partly through inhibition of protein tyrosine kinase to arrest cell cycle progression and induce cell apoptosis. On the other hand, peptides such as vasopressin and bombesin partly act through cell surface receptors, coupled to a guanine nucleotide binding protein to activate a specific phospholipase C that hydrolyzes phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate and diacyglycerol. Diacyglycerol then activates protein kinase C (Powis & Kozikowski, 1991). It has also been reported that the protein kinase C inhibitor, H-7, blocks the G_1/S transition in A65 cells (Miyamoto *et al*., 1993). Curcumin has been demonstrated to inhibit phorbol ester-induced protein kinase C activity in mouse fibroblast cells (NIH3T3) (Liu *et al*., 1993). In our study, the cytosolic protein kinase C activity was significantly reduced by direct incubation with curcumin at 10^{-4} M, but not 10^{-5} M. Thus, the inhibition of protein kinase C activity may in part contribute to the apoptotic effect, but not antiproliferative effect, of curcumin in vascular smooth muscle cells. It is now evident that some signal transduction pathways are common to stimuli that lead to mitogenic and apoptotic responses. Some of the protein kinases pathways lead to activation of transcription factors and the subsequent induction of genes involved in the process of cell proliferation or cell death (Lavin *et al*., 1996).

Some oncogenes and oncosuppressor genes play a major role in cell cycle progression and cell apoptosis. The signals inducing apoptosis are varied and the same signals can induce differentiation and proliferation in other situations. Some genes with established roles in the regulation of proliferation and differentation are also important in controlling apoptosis. Several of these are proto-oncogenes, e.g. *c-myc*, or tumour suppressor genes, e.g. *p53*. However, some pathways appear to be of particular significance in the control of cell death. One of these is the gene family related to the proto-oncogene *bcl-2* (Hale *et al*., 1996). Kakar & Roy (1994) have shown that curcumin protects against phorbol ester-mediated promotion of mouse skin cancers by modulating the expression of proto-oncogenes, *c-fos*, *c-jun* and *c-myc*. We examined the effect of curcumin on *c-myc*, *p53* and *bcl-2* gene expression by measuring the mRNA levels using a reverse transcription-polymerase chain reaction (RT-PCR) technique. The level of *c-myc* mRNA was significantly reduced by curcumin at 10^{-5} to 10^{-4} M and that of *bcl-2* mRNA was significantly reduced at 10^{-4} M only. However, the alteration of the *p53* mRNA level by curcumin did not achieve significance. Thus, alteration in gene expression may partly contribute to the antiproliferative and apoptotic effects of curcumin. In mammalian cells, Myc is a central regulator of cell proliferation and links external signals to the cell cycle machinery. Myc also induces cells to undergo apoptosis, unless specific signals provided either by cytokines or by oncogenes block the apoptotic pathway. Recent progress sheds light both on the factors regulating the function and expression of Myc and on the downstream targets in the cell cycle. Together, these findings suggest the existence of a novel signal transduction pathway regulating both apoptosis and proliferation (Desbarats *et al*., 1996). The *p53* tumour suppressor gene controls cellular growth after DNA damage through mechanisms involving growth arrest at the G_1 phase of the cell cycle and apoptosis (Velculescu & El-Deiry, 1996; Yonish-Rouach, 1996). The outcome—a G_1 arrest or apoptosis—will depend on a complex network of regulatory signals. The decrease of *c-myc* expression induced by curcumin may in part contribute to the loss of some essential *myc*-associated transcripts necessary for cell proliferation and survival. However, curcumin did not significantly affect *p53* mRNA expression, indicating that curcumin-induced apoptosis may not be mediated through an increase of *p53*. Our results also demonstrate that the level of *bcl-2* mRNA was reduced by 10^{-4} M curcumin, but not 10^{-5} M. Evidence suggests that *bcl-2* can prevent the characteristic internucleosomal DNA fragmentation and cellular morphological changes. Apoptosis induced by 10^{-4} M curcumin may partly be mediated through *bcl-2* gene deregulation.

In summary, we found that curcumin inhibited cell proliferation, arrested cell cycle progression, and induced cell apoptosis in rat aortic smooth muscle cell line (A7r5). Curcumin may be useful for the study and treatment of the pathological changes of atherosclerosis and restenosis. Curcumin-treatment may arrest the cell cycle progression and induce cell apoptosis to reduce thymidine incorporation. Our results suggest that the antiproliferative effect of curcumin (10^{-5} M) may partly be mediated through inhibition of protein tyrosine kinase activity and *c-myc* mRNA expression. In addition, the apoptotic effect of curcumin (10^{-4} M) may partly be mediated through inhibition of protein tyrosine kinase activity, protein kinase C activity, *c-myc* mRNA expression, and *bcl-2* mRNA expression. For treatment of atherosclerosis and restenosis, the dose range of antiproliferative or apoptotic-inducing agent which can slow down the overdrive response of cell division without a deleterious effect on the stability of atherosclerotic plaque should be used.

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