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Curcumin induces apoptosis in human breast cancer cells through p53-dependent Bax induction

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Abstract The aim of this study was to determine the mechanisms of curcumin-induced human breast cancer cell apoptosis. From quantitative image analysis data showing an increase in the percentage of cells with a sub-G0/G1 DNA content, we demonstrated curcumin-induced apoptosis in the breast cancer cell line MCF-7, in which expression of wild-type p53 could be induced. Apoptosis was accompanied by an increase in p53 level as well as its DNA-binding activity followed by Bax expression at the protein level. Further experiments using p53-null MDAH041 cell as well as low and high p53-expressing TR9-7 cell, in which p53 expression is under tight control of tetracycline, established that curcumin induced apoptosis in tumor cells via a p53-dependent pathway in which Bax is the downstream effector of p53. This property of curcumin suggests that this molecule could have a possible therapeutic potential in breast cancer patients. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Apoptosis; Breast cancer; Curcumin; p53; Bax; Bcl-2

1. Introduction

Curcumin (diferuloylmethane), a naturally occurring phytochemical responsible for the yellow color of the commonly used spice turmeric (*Curcuma longa* Linn), is receiving attention from cancer investigators because of its chemopreventive properties against human malignancies. The anti-carcinogenic properties of curcumin in animals have been demonstrated by its inhibition of tumor initiation induced both by various carcinogens and by phorbol esters [1–3]. Recent reports also demonstrated the anti-tumor property of curcumin in cancers of colon, forestomach, breast etc. [2–5]. Besides its anti-carcinogenic effects, curcumin has been reported to have a wide range of pharmacological properties including anti-inflammatory, anti-toxic and anti-oxidative [6–8]. The pharmacological safety of curcumin is demonstrated by its consumption for centuries at up to 100 mg/day by people in certain countries [9]. It has been found to reduce the number of cells with chromosomal aberrations but protects normal cells from chromatid breaks due to exposure to γ-radiation [10]. However, the mechanisms underlying these diverse effects of curcumin are not fully understood. Among the possibilities, regulation of an array of cellular biochemical processes such as inhibition of nitric oxide synthase, receptor tyrosine kinase and protein kinase C [3,11,12] activities and the alteration of transcriptional factors c-*jun*/AP-1 and nuclear factor κB, p53 by curcumin have been suggested [13,14]. Recently it has also been suggested that production of reactive oxygen intermediates may be the cause of tumor cell apoptosis as a result of curcumin treatment [15].

Mechanisms that suppress tumorigenesis often involve modulation of signal transduction pathways, leading to alterations in gene expression, arrest of cell cycle progression or apoptosis. Apoptosis is a mode of cell death used by multicellular organisms to eradicate cells in diverse physiological and pathological settings. Several studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents, including cisplatin, camptothecin, etoposide etc. There is accumulating evidence that the efficiency of anti-tumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis [16]. Recent evidence also shows that suppression of apoptosis by tumor-promoting agents in pre-neoplastic cells is thought to be an important mechanism in tumor promotion [17]. In this context, it is noteworthy that apoptosis-inducing ability seems to have become a primary factor in considering the efficiency of chemopreventive agents.

The tumor suppressor gene p53 is important in the etiology of cancer and is mutated, deleted, or rearranged in more than half of all human tumors [18]. p53 mediates either apoptosis or cell cycle arrest in response to DNA damage, thus acting as a molecular 'guardian of the genome' [19–21], each of which operates in a distinct context. This tumor suppressor protein not only controls the transition of cells from the G1 to S phase or G2 to M, but it is also capable of inducing programmed cell death [22]. In contrast, in normal cells, p53-mediated growth arrest prevents the replication of damaged DNA, reduces genetic instability and allows the cells to perform critical repair functions before progressing through the cell cycle [22–24], whereas apoptosis induced by p53 is necessary for eliminating aberrant cells. Bax, the pro-apoptotic member of the Bcl-2 family, has also been shown to be a p53 target and is up-regulated in a number of systems during p53-mediated apoptosis. On the other hand, the up-regulation of Bax expression and down-regulation of Bcl-2 have been demonstrated during apoptosis [19,25]. Recent observations not only implicated p53 signaling pathway in the transcriptional activation of Bax in apoptosis, but also in the alteration of the Bcl-xL (pro-proliferative member of the Bcl-2 family)/

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Abbreviations: DAPI, 4′,6′-diamidino-2-phenylindole

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Bax ratio [26], thereby indicating that cross-talk between these pro- and anti-apoptotic proteins is one of the important factors deciding the fate of a cell. However, the role of curcumin in regulating the balance between these pro- and anti-apoptotic factors is not yet revealed.

In the present study, we addressed the relevance of p53 and its downstream effectors, Bax and Bcl-xL, in curcumin-induced human breast cancer (MCF-7) cell apoptosis. We suggest that such a multi-marker analysis of apoptosis pathways could be useful for individualization of therapeutic strategies in the future.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), streptomycin, penicillin, tetracycline, insulin, L-glutamine, sodium pyruvate and non-essential amino acids were purchased from Gibco BRL (Gaithersburg, MD, USA). Curcumin, 4′,6′-diamidino-2-phenylindole (DAPI), T4 polynucleotide kinase and general reagents were purchased from Sigma (St. Louis, MO, USA). Polyclonal anti-Bcl-xL and anti-Bax, anti-α-actin antibodies, horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were obtained from Pharmingen (San Diego, CA, USA). Wild-type anti-p53 antibody (monoclonal) was obtained from Oncogene Science (Cambridge, MA, USA). The consensus p53 binding palindromic oligonucleotide (5′-GGACATGCCCGGGCATGTCC-3′) was synthesized by Operon Technologies (Alameda, CA, USA). [α-^{32}P]dATP (specific activity 3000 Ci/mmol) was purchased from BRIT (India). The remaining chemicals were purchased from local firms (India) and were of highest purity grade.

2.2. Cell culture

Human breast cancer cells (MCF-7) were obtained from NCCS, India. The MDAH041 post-crisis cell line was derived from the fibroblasts of a patient with Li–Fraumeni syndrome. There is a frameshift mutation of one p53 allele at codon 184 and the normal p53 allele has been lost during in vivo propagation [27]. TR9-7 cells, expressing wild-type p53 under the control of a tetracycline-regulated promoter, were derived from MDAH041 cells [28]. Cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum, insulin (0.1 U/ml), L-glutamine (2 mM), sodium pyruvate (100 μg/ml), non-essential amino acids (100 μM), antibiotics (100 μg/ml streptomycin and 50 U/ml penicillin) ± tetracycline (1 μg/ml) at 37°C in a humidified incubator containing 5% CO_2. Cells were trypsinized and sub-cultured at 1:3 ratio for routine maintenance and experiments.

2.3. Quantitative image analysis of cell cycle phase distribution

MCF-7, MDAH41 or TR9-7 cells were grown as monolayers on coverslips. After curcumin treatment the cells were fixed with chilled methanol and stained with DAPI (0.2 μg/ml for 20 min at room temperature). The cells on coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). A Leica model DM 900 fluorescent microscope was used to visualize the fluorescent images. The filter cube A was used to detect the signal from DAPI. Digital images were captured with a highly sensitive cool (−25°C) CCD camera (Princeton Instruments) controlled with Metamorph software (Universal Imaging) [29]. The exposure times were adjusted so that the brightest signal in the specimen gave less than 90% of the maximum linear range for the camera (a gray scale of 0–4096; 16 bit Images). Each captured image was corrected by subtracting the background image followed by shading correction (to correct the uneven signal collection across the field). The nuclear region of each cell was defined by image analysis of DAPI pictures. Then the fluorescence signal from individual nuclear regions was integrated using Metamorph image analysis features. The relative fluorescence data were transferred to Microsoft Excel and processed.

2.4. Western blotting

For Western blot analysis of p53, Bcl-xL and Bax an aliquot of 50 μg protein from the lysates of MCF-7 cells (untreated or curcumin-treated) was mixed with an equal volume of 2×SDS–PAGE sample buffer, heated at 100°C for 5 min and loaded onto a 10% SDS polyacrylamide gel. After electrophoresis the gel was transferred to a nitrocellulose membrane on a semidry transfer apparatus from Bio-Rad. The membrane was blocked with 3% non-fat milk in phosphate-buffered saline containing 0.05% Tween 20 prior to antibody treatments. The protein of interest was visualized by enhanced chemiluminescence. The blot was then stripped in buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 200 mM 2-mercaptoethanol) at 50°C for 30 min. After extensive washing, the blot was used again for probing the next molecule, beginning from the blocking.

2.5. Gel electrophoresis mobility shift assay

Gel electrophoresis mobility shift assay was done as described [30]. Double-stranded p53 protein-binding consensus oligonucleotide was end-labeled by T4 polynucleotide kinase. Protein–DNA complexes were separated by electrophoresis on 4% polyacrylamide gel electrophoresis. The supershift was induced with 0.2 μg/assay of anti-p53 antibody. Twenty-fold unlabeled oligonucleotide competitor was added at the same time as the radiolabeled probe was added to the reaction mixture. The gel was dried and exposed to a PhosphorImager screen for 90 min and quantitation was done using the Molecular Analysis program (Bio-Rad).

3. Results

3.1. Effect of curcumin on MCF-7 cell cycle progression

We employed quantitative image analysis techniques to appraise the effect of curcumin on the MCF-7 cell cycle phase distribution. Twenty-four hours of continuous exposure to different doses of curcumin resulted in cessation of cell growth followed by significant cell death. Optimum inhibition was obtained at a dose of 25 μM, beyond which no further decrease in cell number could be obtained. The LD_{50} of curcumin was 10 μM for MCF-7 cells (data not shown). It was observed that at 10 μM curcumin, the percentage of cells in G0/G1 phases decreased with a concomitant increase of cell in the sub-G0/G1 phase up to 24 h. After 24 h the percent of sub-G0/G1 phase cells decreases (Fig. 1). Phenotypically, apoptosis is characterized by cell shrinkage, chromatin compaction, nuclear blebbing and collapse of the nucleus into small intact fragments (apoptotic bodies) which were evident from the DAPI-stained fluorescent images of curcumin-treated MCF-7 nucleus (not shown).

3.2. Effect of curcumin on the expression of p53, Bcl-xL and Bax

p53 has been found to be importantly involved in apoptosis induced by a broad range of agents. We examined by Western blot analysis, using antibody directed against wild-type p53, whether curcumin has any effect on the expression of this pro-apoptotic protein in MCF-7 cells. Results in Fig. 2 shows that upon 10 μM curcumin treatment, p53 started increasing from as early as 4 h, reached a maximum level by 12 h and persisted up to 24 h. At 48 h of curcumin treatment the level of p53 was found to decrease to control level. The level of Bcl-xL was moderately high in MCF-7 cells and remained almost unaltered after curcumin treatment (Fig. 2A,B). On the other hand, the level of Bax increased significantly after 8 h and attained a peak at 24 h after curcumin treatment (Fig. 2A,B). The p53, Bcl-xL and Bax levels were measured by quantitative Western blot analysis after normalizing with α-actin content. Interestingly, it is to be noted that the expression of p53 precedes Bax expression in MCF-7 cells as a result of curcumin treatment. Since it is known that p53 can transactivate Bax expression [31], our observation leads to the possibility that curcumin induces MCF-7 cell apoptosis in a

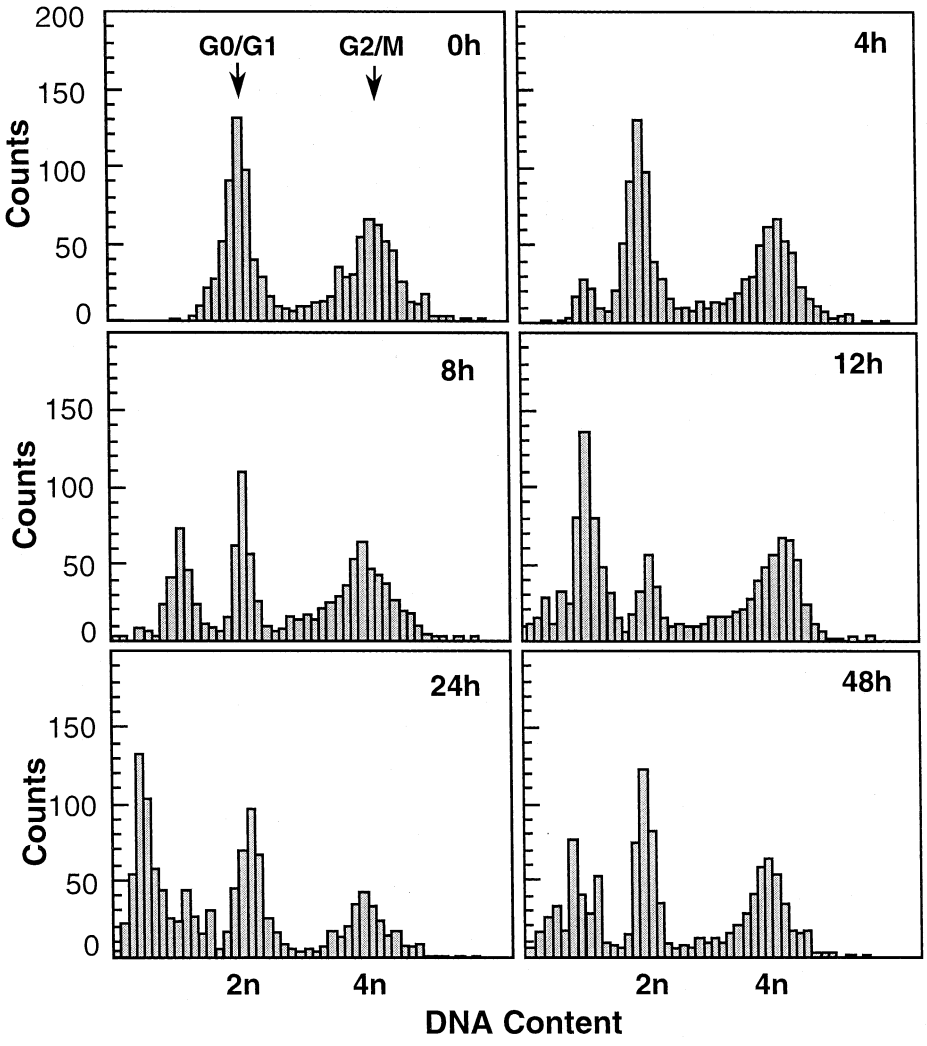

Fig. 1. Curcumin-induced cell cycle phase distribution of MCF-7 cells. Cell cycle phase distribution of MCF-7 nuclear DNA was determined by quantitative-image analysis as described in Section 2. At different times after curcumin treatment, cells were fixed and stained with DAPI and analyzed for DNA content by fluorescent microscopy. The normal DNA content was determined from '0 h' cells where the predominant peak corresponded to the normal G0/G1 content of DNA (2*n*). The DNA content in curcumin-treated cells was displayed in different panels indicated by their corresponding time of incubation. Less than 2*n* DNA content was regarded as sub-G0/G1 peak or apoptotic cells.

p53-dependent pathway in which Bax is a downstream effector molecule.

3.3. Role of curcumin in p53 DNA-binding activity in MCF-7 cells

Next, using a gel electrophoresis mobility shift assay, we examined the relative activity of p53 to bind its known cognate sequence. In nuclear extracts of curcumin-treated MCF-7 cells, a band corresponding to the p53–DNA complex was detected (Fig. 2C). The band intensity began to increase at 4 h, reached a maximum level by 24 h and then declined at 48 h after curcumin treatment. The specificity of this binding was confirmed by competition analysis with a 20-fold excess of unlabeled homologous oligonucleotide. The presence of p53 in the complex with retarded mobility in the gel was identified by further retardation of the band following incubation with p53 antibody (Fig. 2C). All these data indicate that MCF-7 cells express wild-type p53 protein and the DNA-binding activity of the same is induced by curcumin.

3.4. Involvement of p53 in curcumin-induced Bax expression

Since p53 expression was found to precede Bax expression, we hypothesized that curcumin induced Bax expression through the transactivation by p53. To test this hypothesis, we used p53-null (MDAH041) as well as tetracycline-regulated wild-type p53 expressing cell lines (TR9-7) originating from a post-crisis cell line derived from the fibroblasts of a patient with Li–Fraumeni syndrome [28]. No immunoreactivity of p53 could be found in p53-null MDAH041 cells even when the cell were treated with 10 μM curcumin for 24 h (Fig. 3A). In these cells the content of Bcl-xL remained unaltered in the case of curcumin treatment. Interestingly, curcumin treatment failed to show the appearance of Bax in this p53-null cell line (Fig. 3A). On the other hand, in tetracycline-regulated TR9-7 cells, expression of wild-type 53 was low and increased moderately with simultaneous increase in Bax when cells were treated with curcumin (Fig. 3A). However, the level of Bcl-xL expression is slightly inhibited by curcumin under this condition (Fig. 3A). Under the tetracycline-withdrawn condition,

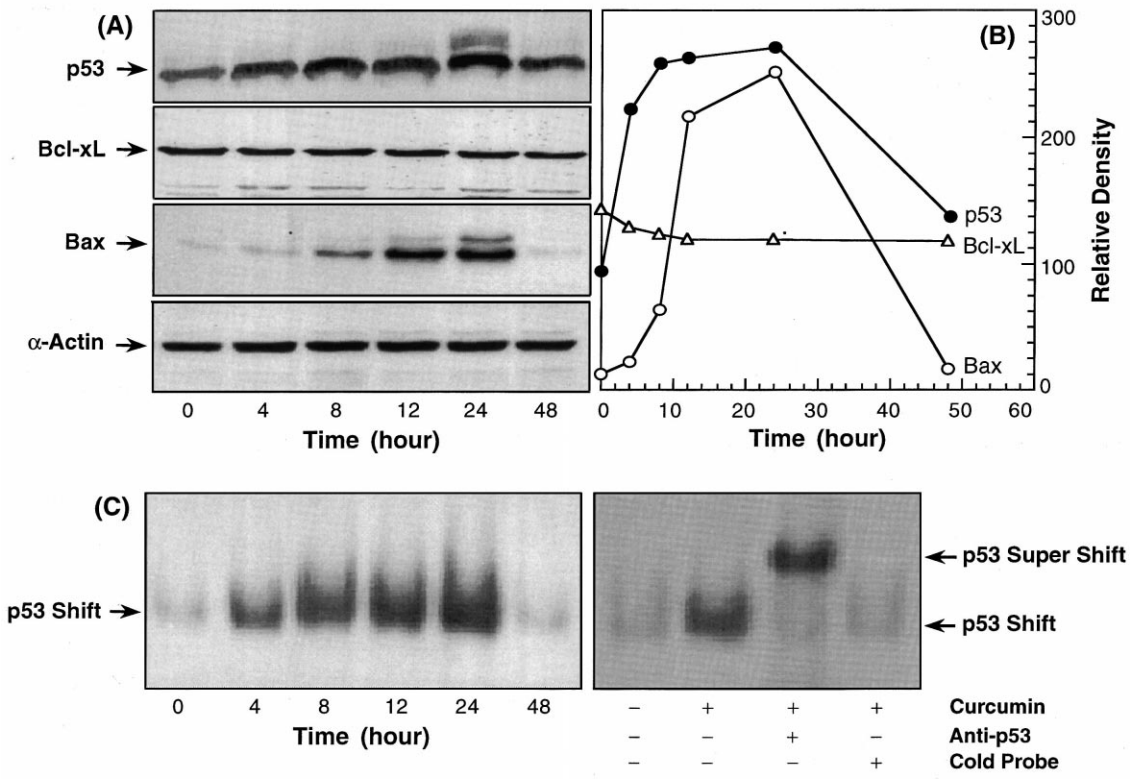

Fig. 2. Effect of curcumin on the expression of p53, Bcl-xL and Bax proteins and induction of p53 DNA-binding activity. MCF-7 cells were treated with 10 μM curcumin for different time intervals. A: Cell lysates were subjected to Western blot analysis for the determination of the expression levels of p53, Bcl-xL and Bax using specific antibodies. The blots were re-probed with anti-α-actin antibody to confirm equal protein loading. B: The relative densities of each band were expressed graphically by quantitative Western blot analysis after normalizing with α-actin band. C: Time-dependent induction of p53 DNA-binding activity in MCF-7 cells was determined by gel electrophoresis mobility shift assay. To induce the supershift, the reaction mixture was incubated with anti-p53 antibody and a 20-fold excess of non-radioactive p53 probe was used for the competition assay.

the expression of p53 was high and it was further elevated by curcumin treatment. Bax expression in these high p53-expressing cells showed a dramatic increase in case of curcumin treatment. In these cells the Bcl-xL level was low and remained unaltered after curcumin treatment (Fig. 3A).

To determine the relative DNA-binding activity of p53, nuclear extracts of curcumin-treated p53-null as well as low-p53 (in the presence of tetracycline) and high-p53 (in the absence of tetracycline) cells were used and gel electrophoresis mobility shift assay using the oligonucleotide of the p53-binding consensus sequence was employed. In nuclear extracts of p53-null MDAH041 cells, no appreciable p53 DNA-binding activity was observed. Even curcumin treatment could not increase the intensity of this band (Fig. 3B). In tetracycline-regulated low p53-expressing TR9-7 cells, a band corresponding to the p53–DNA complex was detected (Fig. 3B). The band intensity increased after curcumin treatment. In the case of high p53-expressing TR9-7 cells, p53 DNA-binding activity was already high and was further increased by curcumin pretreatment (Fig. 3B). All the above results suggest that curcumin potentiates p53 DNA-binding which in turn induces Bax expression.

3.5. Involvement of p53 in curcumin-induced apoptosis

To demonstrate that p53 is specifically involved in curcumin-induced apoptosis, we used the p53-null cell line (MDAH041) as well as tetracycline-regulated wild-type p53-expressing cell line (TR9-7). In p53-null MDAH041 cells, curcumin failed to induce apoptosis (Fig. 4). In TR9-7 cells, curcumin moderately induced apoptosis in low p53-expressing condition, i.e. in the presence of tetracycline, which down-regulates wild-type p53 expression. In fact, under this condition, only 23% of cells underwent apoptosis (Fig. 4). On the other hand, when TR9-7 cells were released from tetracycline-inhibited p53 expression, curcumin induced a high level of apoptosis. In this case, the number of cells containing hypoploid or $< 2n$ DNA increased as a result of curcumin treatment ($> 50\%$). All these results signify that curcumin asserts its apoptogenic effect in a p53-dependent manner.

4. Discussion

The concept that cancer can be prevented or certain diet-derived substances can postpone its onset is currently eliciting considerable interest. Curcumin has been demonstrated to inhibit tumor initiation induced by various carcinogens. However, the molecular events of curcumin action have not been studied in detail. The aim of this paper was thus to identify the events which may ultimately initiate the apoptotic cascade leading to cancer cell death as a result of curcumin treatment.

One of the most interesting questions in the p53 field is how a cell makes the decision to undergo growth arrest or apoptosis. It has been proposed that p53 may induce two sets of genes upon stress signals. One set mainly functions in cell growth control, such as p21/*Waf-1* and GADD45, and the other set acts on apoptosis, such as Bax [23]. In this study,

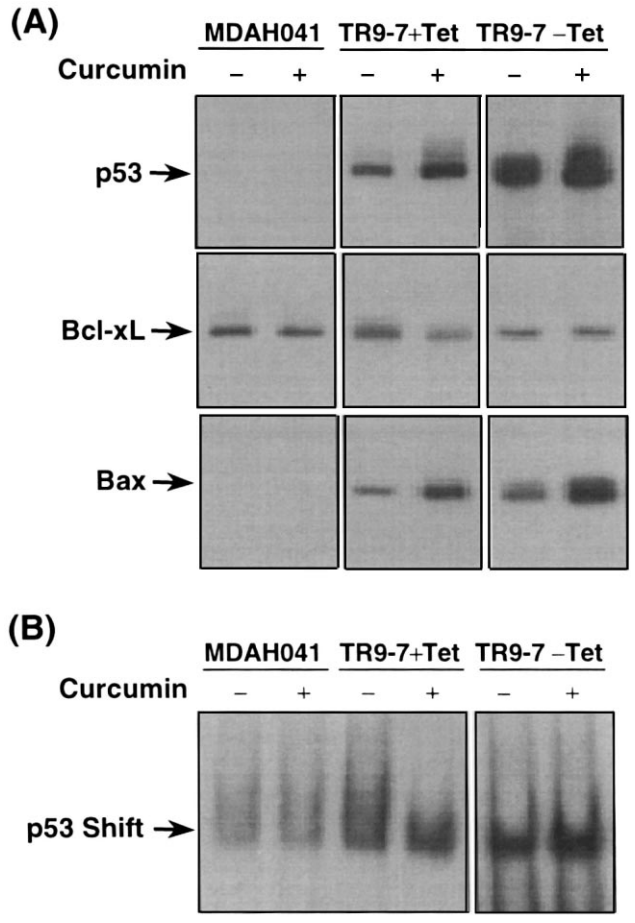

Fig. 3. Involvement of p53 in curcumin-induced Bax expression. p53-null MDAH041 cells, low p53-expressing TR9-7 cells (in the presence of tetracycline; +Tet) and high p53-expressing TR9-7 cells (in the absence of tetracycline; −Tet) were incubated with or without 10 μM curcumin for 24 h. A: p53, Bcl-xL and Bax levels were detected by enhanced chemiluminescence. B: Induction of p53 DNA-binding activity in p53-null MDAH041 cells, low p53-expressing (+Tet) and high p53-expressing (−Tet) TR9-7 cells was determined by gel electrophoresis mobility shift assay.

we observed that curcumin is capable of inducing apoptosis in breast carcinoma cell line MCF-7, in which expression of wild-type p53 can be induced. The apoptosis is accompanied by an increase in p53 level as well as its DNA-binding activity followed by Bax expression at the protein level. Further experiments using p53-null cells as well as TR9-7 cells, in which wild-type p53 expression is under tight control of tetracycline, established that curcumin induces apoptosis in tumor cells via a p53-dependent pathway in which Bax is the downstream effector of p53.

It is well recognized that whether a cell becomes committed to apoptosis partly depends upon the balance between proteins that mediate cell death, e.g. Bax, and proteins that promote cell viability, e.g. Bcl-2 or Bcl-xL [25,26], and the ratio of Bax to Bcl-2 or Bcl-xL appears to be a critical determinant of a cell's threshold for undergoing apoptosis [26]. Interestingly, wild-type p53 has been shown to be capable of both down-regulating the death suppressor Bcl-2 and up-regulating the death promoter Bax, thereby changing the Bcl-2/Bax ratio and disposing to programmed cell death [25,26]. Bax overexpression has recently been shown to enhance radiation-induced apoptosis, but only in the presence of functional p53 [31]. These observations clearly show that these oncoproteins act in a well-regulated concerted manner to decide the ultimate fate of any cell.

Keeping all this information in mind, we attempted to establish the relationship, if any, between p53 status, Bcl-2/Bax ratio, cell cycle arrest and apoptosis in curcumin-treated tumor cells. To achieve this goal we took three different approaches. In the first approach, we selected human breast carcinoma cells (MCF-7) in which wild-type p53 is constitutively expressed. In these cells, curcumin induced apoptosis with an increase in p53 level. Interestingly, the curcumin-induced increase in p53 expression precedes that of Bax thereby leading us to hypothesize that p53 transactivates Bax expression. In these cells the Bcl-xL level remained almost unchanged thereby shifting the Bcl-xL/Bax ratio towards apoptosis. That activation of the p53 signaling pathway may play a causative role in apoptosis by modulating the Bcl-xL/Bax ratio has also been reported by Gonzalez de Aguilar et al. [26]. All these observations point to the evidence for the implication of p53 signaling pathway in curcumin-induced tumor cell apoptosis and support the candidature of Bax as the downstream effector in this pathway.

To re-establish our findings, we took a second approach in which the p53-null cell line (MDAH041; which has a frameshift mutation in one p53 allele and the normal allele has been lost during in vivo propagation from fibroblasts of a post-crisis Li–Fraumeni patient [27,28]) was used as the experimental model. In these cells curcumin failed to induce either Bax expression or apoptosis. These results support our hypothesis that p53 is required for Bax expression as well as curcumin-induced apoptosis. To further confirm these observations, we took a third approach in which we used a well-controlled system to regulate wild-type p53 expression with tetracycline (TR9-7 cells; MDAH041 cells over-expressed with tetracycline-regulated wild-type p53). In the presence of the regulator tetracycline curcumin increased the p53 level moderately with a moderate elevation in Bax level. However, removal of tetracycline and treatment of curcumin resulted in a dramatic increase in p53 as well as Bax levels resulting in apoptosis. All these data strongly confirm that p53 signaling acting via Bax activation is essential for the apoptogenic effect of curcumin. These findings differ from those of Bush et al. [32] who showed that curcumin-induced apoptosis in human melanoma cells is independent of p53. In fact, melanoma cells rarely contain mutant p53 and hardly undergo apoptosis by wild-type p53 [33]. However, adenovirus-mediated transfer of p53 in these melanoma cells suppressed growth and induced apoptosis [33]. Involvement of p53 in curcumin-induced apoptosis of hepatoblastoma cells has also been documented [14]. All these reports together with our findings suggest that depending on the nature and condition of the concerned cell, and the circumstances involved, the downstream effector of any signaling agent that will come into play is determined. Thus, p53 signaling acting via Bax activation is essential for the apoptogenic effect of curcumin in our model system.

Over the years, cancer therapy had witnessed many exciting developments, but cure of cancer has still remained as complex as the disease itself, since the mechanisms of tumor killing are still not fully realized. Identification of individual components of signaling pathways leading to tumor cell death as well as targeted alteration of those molecules may be of im-

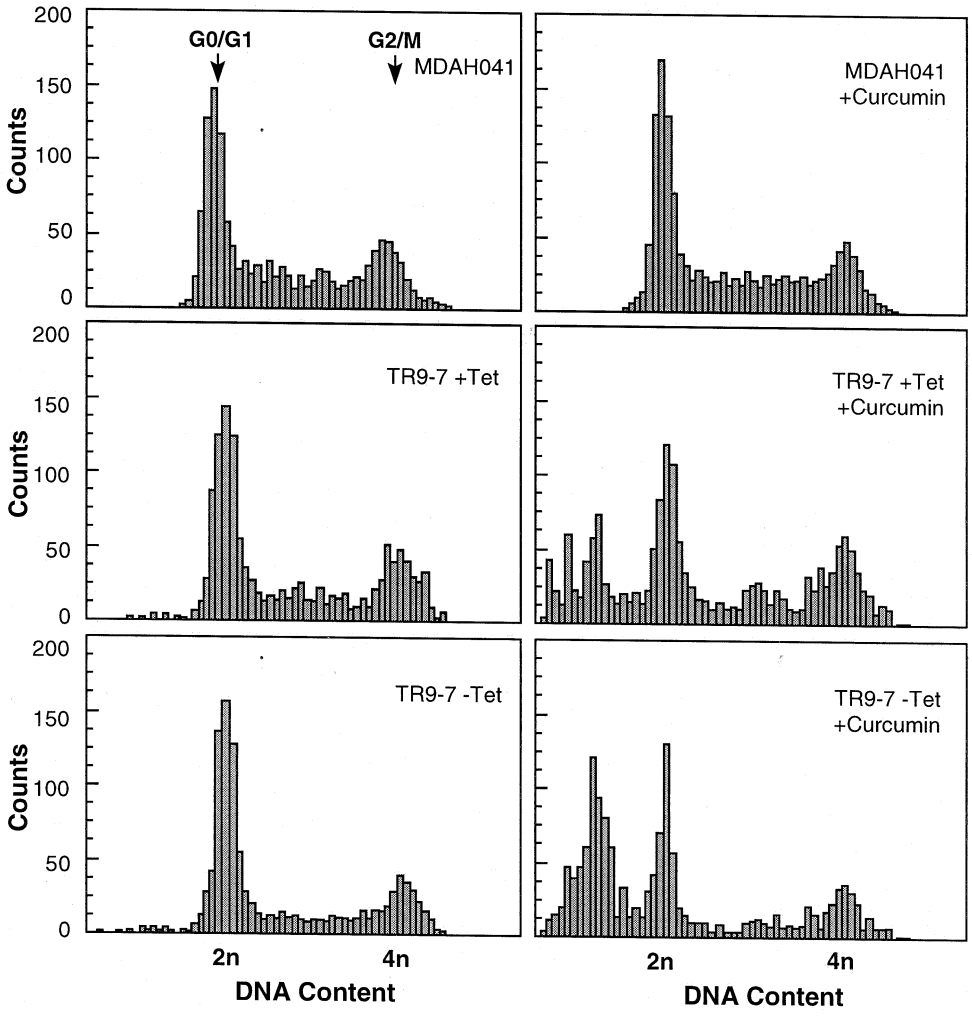

Fig. 4. Involvement of p53 in curcumin-induced apoptosis. p53-null MDAH041 cells, low p53-expressing TR9-7 cells (in the presence of tetracycline; +Tet) and high p53-expressing TR9-7 cells (in the absence of tetracycline; −Tet) were incubated with or without 10 μM curcumin for 24 h. Cell cycle analysis of nuclear DNA was determined by quantitative image analysis as described in Section 2. The normal DNA content was determined from curcumin-untreated cells (left panel in each case) where the predominant peak corresponded to the normal G0/G1 DNA content (2*n*). The DNA content in curcumin-treated cells is displayed in the right panel. Less than 2*n* DNA content was regarded as sub-G0/G1 peak or apoptotic cells.

mense help to selectively induce apoptosis in cancer cells. Knowledge acquired from this study will, therefore, lead us one step forward towards that goal.

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