Gene expression profiling identifies activating transcription factor 3 as a novel contributor to the proapoptotic effect of curcumin

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Abstract

The antitumor effect of curcumin (diferuloylmethane) is well established. However, there have been no unbiased studies to identify novel molecular targets of this compound. We therefore undertook a gene expression profiling study to identify novel targets of curcumin. A cDNA array comprised of 12,625 probes was used to compare total RNA extracted from curcumin-treated and -untreated MDA-1986 cells for differential gene expression. We identified 202 up-regulated mRNAs and 505 transcripts decreased ≥2-fold. The proapoptotic activating transcription factor 3 (ATF3) was induced >4-fold. Two negative regulators of growth control [antagonizer of myc transcriptional activity (Mad) and p27kip1] were induced 68- and 3-fold, respectively. Additionally, two dual-activity phosphatases (CL 100 and MKP-5), which inactivate the c-*jun*-NH_2-kinases, showed augmented expression, coinciding with reduced expression of the upstream activators of c-jun-NH_2-kinase (MEKK and MKK4). Of the repressed genes, the expression of Frizzled-1 (Wnt receptor) was most strongly attenuated (8-fold). Additionally, two genes implicated in growth control (*K-sam*, encoding the keratinocyte growth factor receptor, and *HER3*) as well as the E2F-5 transcription factor, which regulates genes controlling cell proliferation, also showed down-regulated expression. Considering its role in apoptosis, we determined the contribution of ATF3 to the antitumor effect of curcumin. Curcumin-treated MDA-1986 cells showed a rapid, dose-dependent increase in ATF3/mRNA protein. Moreover, expression of an exogenous ATF3 cDNA synergized with curcumin in inducing apoptosis. Thus, we have identified several putative, novel molecular targets of curcumin and showed that one, ATF3, contributes to the proapoptotic effects of this compound. [Mol Cancer Ther 2005; 4(2):233–41]

Introduction

Extensive research has previously documented the anticancer effect of curcumin (diferuloylmethane, a polyphenol derived from the tumeric plant) in experimental systems. In animals, curcumin possesses a wide range of antitumor effects, including the inhibition of angiogenesis, induction of chemosensitivity, and chemoprevention (1). This polyphenol achieves these therapeutic effects both by suppressing proliferation and inducing apoptosis in a variety of tumor systems, including leukemia, colon, basal cell carcinoma, and melanoma (1).

Several studies to date have investigated the mechanisms of action of curcumin. Curcumin induces the sequential activation of caspase 8-BID cleavage-cytochrome *c* release, caspase 3 activation, and poly(ADP-ribose) polymerase cleavage thereby leading to DNA fragmentation and cell death (1). Additionally, curcumin promotes apoptosis via down-regulating antiapoptotic proteins, including bcl-2 and bcl-xl (1). At the signaling level, curcumin inhibits the kinase activities of the epidermal growth factor receptor as well as HER-2/*neu* and the synthesis of the interleukins 8 and 10 cytokines in monocytes and macrophages is blocked by this agent (1). Finally, at the transcriptional level, activator protein (AP-1)–dependent gene expression, often elevated in cancer (2, 3), is countered by the polyphenol (4, 5) by way of inhibiting DNA binding of the Fos-Jun complex and possibly also through inhibition of the c-jun-NH_2-kinase (JNK) activity (1). Similarly, binding of the early growth response-1 gene product (*Egr-1*) to its consensus sequence is antagonized by this agent in endothelial cells (1). Curcumin also is a potent inhibitor of NFκB activation (6) by inhibiting the kinase (IκBα kinase) that induces IκBα phosphorylation (1).

Whereas these aforementioned studies have identified several potential molecular targets of curcumin, to date, no unbiased search has been undertaken to discover novel targets. Consequently, we undertook a gene expression profiling study to identify novel mediators of curcumin. Our studies revealed altered expression of a variety of mRNAs including activating transcription factor 3 (ATF3). We were particularly intrigued by the latter observation since this transcription factor has previously been reported to induce apoptosis (7, 8) in

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several diverse systems. The expression profiling studies were corroborated by both Western and Northern blotting that revealed elevated ATF3 protein and mRNA respectively in response to curcumin treatment. Moreover, transient transfection of ATF3 into head and neck cancer cells synergized with curcumin to induce apoptosis. Thus, we have identified a novel mediator of the anticancer effect of curcumin.

Materials and Methods

Cell Culture

The following squamous cell carcinoma cell lines were used: MDA-1986 cells, (derived from cervical nodal metastases of tongue cancer), FADU (poorly differentiated, hypopharynx), SCC4 (poorly differentiated, oral cavity), HN5 (moderately differentiated, tongue), and 14A (floor of mouth). All cells were cultured in MoCoys 5A medium supplemented with 10% fetal bovine serum. Curcumin (LKT Laboratories, Minneapolis, MN) was dissolved in DMSO at a concentration of 50 mmol/L and added to the cultures as indicated.

RNA Preparation

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Purified total RNA was washed with 75% alcohol, air-dried, and dissolved in DEPC-treated water.

cDNA Array

Preconfluent MDA-1986 cells were treated with or without 50 μmol/L curcumin for 8 hours, total RNA extracted and analyzed for differential gene expression using the HGU95Av2 array (Affymetrix, Santa Clara, CA) array comprised of 12,625 probes.

Northern Blotting

Total cellular RNA was resolved in a 1% agarose-formaldehyde gel and transferred to nylon membrane by capillary action using 20× SSC [1× SSC = 150 mmol/L NaCl and 15 mmol/L sodium citrate (pH 7.4)]. The blot was probed at 65°C with a random primed, [^{32}P]-labeled 0.52-kb cDNA fragment specific for human ATF3 mRNA. Stringencies were done with 0.1× SSC and 0.1% SDS at 65°C.

Western Blotting

Cells were washed with PBS and lysed in modified radioimmune precipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na3VO4, 1 mmol/L NaF, and 1× proteatinase inhibitor (Roche Molecular Biochemicals, Indianapolis, IN)] on ice for 30 minutes. Cell lysates were clarified by centrifugation, and 80 μg of protein resolved by SDS-polyacrylamide gel (12%) electrophoresis. Proteins were transferred to a nitrocellulose membrane, and the filter then blocked with 5% milk, and incubated with an 0.2 μg/mL of an anti-ATF3 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After extensive washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (Perkin-Elmer, Boston, MA).

Apoptosis Assays

Transfections were done on slide chambers using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instruction. Specifically, 10^5 cells were plated onto slide chambers and cultured overnight. The following day, a mixture of 0.8 μg of plasmid pCG/ATF3 expressing ATF3 cDNA or its empty vector pCG (gifts from Dr. T. Hai, Ohio State University, Ohio) with 2.5 μL of LipofectAMINE 2000 was added to the chambers. After an additional 24 hours, the cells were treated with 50 μmol/L of curcumin or solvent DMSO for 8 hours. The cells were then washed and subjected to immunostaining for ATF3.

For the terminal deoxynucleotidyl transferase–mediated nick labeling (TUNEL) assays, MDA-1986 cells (1.5×10^5) were transfected with 0.8 μg ATF3, or its vector pCG, using LipofectAMINE 2000. After 24 hours, the cells were treated with or without 50 μmol/L curcumin for 8 hours. The cells were then fixed in 4% paraformaldehyde at room temperature for 10 minutes followed by treatment with 0.2% Triton X-100 at room temperature for 15 minutes. After blocking with a PBS solution containing 5% normal horse serum and 1% normal mouse serum for 30 minutes, the slides were subsequently incubated with an ATF3 antibody (4 μg/mL, C-19, Santa Cruz Biotechnology) overnight at 4°C, followed by an Alex Fluor 594-conjugated anti-rabbit secondary antibody for 1 hour at room temperature. The slides were washed with PBS, and apoptotic cells labeled with the Dead End Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions.

Reporter Assays

MDA-1986 cells were seeded in 24-well plates at a density of 1.5×10^5 per well. The following day, cells were transfected with 0.6 μg of a luciferase reporter driven by nothing (pGL3) or by the ATF3 promoter (9). After 24 hours, curcumin (final concentration, 50 μmol/L), or an equivalent amount of carrier, was added and the cells incubated for 4 hours. The cells were then lysed and 20 μL of lysates assayed for luciferase activity.

Chromatin Immunoprecipitation Assays

This method was done as described previously (10) with modifications. Briefly, MDA-1986 cells were treated with 50 μmol/L curcumin or carrier (DMSO) for 3.5 hours and treated with 1% formaldehyde for 10 minutes at 30°C followed by incubation in 0.125 mol/L glycine for 5 minutes. The cells were washed and scraped in PBS, and subsequently washed with solution 1 [10 mmol/L HEPES (pH 7.5), 10 mmol/L EDTA, 0.5 mmol/L EGTA, and 0.75% Triton X-100] and solution 2 [10 mmol/L HEPES (pH 7.5), 200 mmol/L NaCl, 1 mmol/L EDTA, and 0.5 mmol/L EGTA]. Cell pellets were then resuspended in lysis buffer [150 mmol/L NaCl, 25 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, and 0.5%

deoxycholate] and sonicated. To pull down the polymerase II-bound chromatin, cleared cell lysates were incubated overnight with 1 μg anti-polymerase II (N-20, Santa Cruz Biotechnology) or a corresponding amount of immunoglobulin G, and the immunoprecipitates captured with protein A-agarose. The agarose beads were washed once only with radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP40, and 1 mmol/L EDTA], high salt buffer (radioimmunoprecipitation assay buffer with 500 mmol/L NaCl), LiCl wash buffer [50 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, 250 mmol/L LiCl, 1% NP40, and 0.5% deoxycholate], and twice with TE buffer. The beads were then treated with 50 μg/mL RNase A for 1 hour (37°C) and 0.25 μg/mL proteinase K for 4 hours (37°C) and finally incubated at 65°C overnight. DNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in TE buffer for quantitation by real-time PCR (10). The primers used for real-time PCR were 5′-TGGCAACACGGAGTAAACGA-3′ and 5′-AGAGAAGAGAGCTGTGCAGTGC-3′, amplifying a region spanning −75/+42 of the *ATF3* gene (relative to transcription start site). The PCR conditions and calculations were as described previously (10).

Results

Towards identifying novel targets of curcumin, MDA-1986 cells were treated with, or without, 50 μmol/L curcumin for 8 hours, total RNA extracted and analyzed for differences using the HGU95Av2 Affymetrix gene chip comprised of 12,625 cDNA probes. We identified 202 mRNAs up-regulated ≥2-fold, whereas conversely 505 transcripts were decreased ≥2-fold in response to the curcumin treatment.

Expression Profiling Identification of Novel Curcumin Targets Up-Regulated in Their Expression

Of the genes up-regulated by curcumin, several were of potential interest as mediators of curcumin action for two reasons: (a) their recognized role in growth control, apoptosis, and tumor progression and (b) with few exceptions, most gene targets of curcumin, described previously, are down-regulated. Thus, we first examined mRNAs induced by curcumin and these data are illustrated in Table 1. Of interest was the strong (19.6-fold induction) up-regulation of the dual phosphatase CL100 that inactivates the JNK subgroup of mitogen-activated protein kinases (MAPK; ref. 11). Similarly, we also observed an induction of MKP-5, (albeit less potent) a distinct dual specificity phosphatase also capable of inactivating both JNK and p38 MAPKs (12). These MAPK may exert both proapoptotic and antiapoptotic functions (13) and in case of the latter, their inactivation might very well contribute to the antitumor effect of curcumin.

Unexpectedly, we also observed elevated c-Fos and c-Jun transcripts, which was somewhat surprising considering previous observations that curcumin prevents the phorbol ester-dependent increase in mRNA encoding these transcription factors in mouse skin (1, 14). Nevertheless, AP-1-target genes may show a net repression if the increased amount of these transcription factors is offset by diminished *trans*-activating activity of these DNA-binding proteins (15, 16).

The 2.6-fold induction of the cyclin dependent kinase inhibitor p21 (WAF1/Cip1) as well as the 3.2-fold elevation in the p27kip1 mRNA amounts were also noteworthy considering the role of these proteins as inhibitors of cell proliferation (17). Indeed, these findings may be pertinent to the pronounced antiproliferative effects of curcumin evident in several diverse systems (1). The finding of curcumin-induced p27kip1 has not been reported prior although an increased amount of p21 transcript has been shown in prostate cancer. Although a 2-fold induction of c-Myc mRNA was also evident in curcumin-treated cells, any growth stimulatory effect of this proto-oncogene would be more than offset by the dramatic

Table 1. Genes regulated in expression by curcumin

Description	Fold increase
Human mRNA encoding the c-*myc* oncogene	2
Homo sapiens Kruppel-like zinc finger protein Zf9 mRNA	2
HUMMKK3A *H. sapiens* MKK3 mRNA	2
HUMPAIA Human, *plasminogen activator inhibitor-1* gene	2
H. sapiens mRNA for dual-specificity phospatase MKP-5	2
H. sapiens H4/e gene for H4 histone/cds	2.3
Human-soluble CD44 (CD44) mRNA, with exon v9 extension	2.5
Human mRNA for Arg-Serpin (plasminogen activator-inhibitor 2)	2.5
H. sapiens mRNA for histone H2B	2.5
Human mRNA for MKK3b	2.6
Human wild-type p53 activated fragment-1 (WAF1) mRNA	2.6
Human cyclin-dependent kinase inhibitor p27kip1 mRNA	3.2
Transferrin (human, liver, mRNA)	3.2
Human c-jun proto-oncogene (JUN)	3.2
H. sapiens mRNA for urokinase plasminogen activator receptor/cds	3.5
Human mRNA for stromelysin	3.5
Human ATF3 mRNA	4.6
Human mRNA for metalloproteinaise stromelysin-2	4.9
Human cellular oncogene c-*fos*	13.9
HSCL100 *H. sapiens* CL100 mRNA for protein tyrosine phosphatase	19.6
H. sapiens antagonizer of *myc* transcriptional activity (Mad) mRNA	68.5

NOTE: Total RNA was extracted from MDA-1986 squamous cell carcinoma cells treated with or without 50 μmol/L curcumin for 8 hours. Gene expression profiling was performed using the HGU95Av2 array (Affymetrix). Genes increased in their expression >2-fold are indicated together with the respective fold change.

(68.5-fold) increase in the Mad (antagonizer of myc transcriptional activity) which interferes with the transcriptional activation and proliferation-promoting functions of Myc-Max heterodimers (18).

We were somewhat surprised that the expression of both the urokinase plasminogen activator receptor as well as stromelysin were increased by the diferuloylmethane since these gene products promote proteolysis an event associated with tumor progression. It may very well be that their increased expression reflects *trans*-activation through AP-1 motifs in their respective promoters (19) as a consequence of higher levels of c-Jun and c-Fos achieved with curcumin. However, we also noted a parallel increase in the steady state amount of plasminogen activator inhibitor 2 and this induction could very well counter increased urokinase-dependent proteolysis (20) achieved as a consequence of a larger amount of the urokinase plasminogen activator receptor.

Expression Profiling Identification of Novel Curcumin Targets Attenuated in Their Expression

Of all the mRNAs down-regulated by curcumin (Table 2), the frizzled-1 transcript was the most dramatically attenuated (8-fold decrease). This observation was of particular interest since frizzled-1 represents one of the transmembrane-spanning G-protein-coupled receptors for the Wnt ligand (21). Indeed, the role of the Wnt pathway in Tcf/Lef-responsive gene expression and tumor progression is well established (22).

We also observed reductions in the amount of mRNA encoding two growth factor receptors: the human keratinocyte growth factor receptor (K-sam) and the human epidermal growth factor receptor (HER3). Indeed, ligand-dependent heterodimerization between HER2 and other HER family members including HER3 activates the HER2 signaling pathway previously implicated in tumor progression (23. Importantly, overexpression of the HER3 is also correlated with poor tumor prognosis. Likewise, keratinocyte growth factor–dependent growth stimulation is mediated through its corresponding receptor encoded by the *K-sam* gene (24) and amplification of the latter is associated with human gastric cancer progression (25).

Curcumin also suppressed E2F-5 mRNA amounts >2-fold. E2F is a family of heterodimeric transcription factors composed of EWF-like and DP-like subunits which regulate specific genes controlling cell proliferation (26). Indeed, studies have shown an essential role of the retinoblastoma/E2F pathway in the passage of cells through the G_1 phase of the cell cycle. Further Western blotting has revealed a marked up-regulation in E2F-5 levels in skin tumors when compared with normal epidermis. Thus, E2F-5 may represent a novel pharmacologic target of curcumin leading to growth inhibition.

The gene expression profiling studies also revealed reductions in the mRNAs encoding both MTK1 (also known as MEKK4) and MKK4 both involved in the activation of the JNK/p38 MAPK signaling pathways (27). As discussed in the previous section, JNK/p38 may protect cells from proapoptotic stimuli and antagonism of these upstream activators by curcumin could culminate in increased cell death. Repression of the genes encoding these transcripts were of particular interest considering the coordinated increases in phosphatases (CL100 and MKP-5) which could serve to reinforce the inactivation of these MAPK.

We were somewhat surprised by the observation that curcumin decreased >3-fold the amount of mRNA encoding both the tumor necrosis factor receptor-1 associated protein as well as the tumor necrosis factor–related apoptosis inducing ligand. Both molecules are well recognized as playing critical roles in execution of the apoptosis program (28).

Validation of ATF3 Up-Regulated Expression

We were particularly interested in the expression profiling data indicating that ATF3 was increased by curcumin for two reasons. First, in the most part, the biological targets of curcumin to date are suppressed (in expression and/or activity) by this pharmacologic agent. Second, ATF3 has previously been implicated in programmed cell death raising the possibility that this transcription factor represents a novel mediator of the

Table 2. Genes regulated in expression by curcumin

Descriptions	Fold decrease
Homo sapiens mRNA for frizzled-1	8
H. sapiens forkhead protein FREAC-2 mRNA	5.7
HUMMKK4A *H. sapiens* MKK4 mRNA	4.3
Human *APC* gene mRNA	4.3
Human fibroblast growth factor receptor (K-sam) mRNA	4.3
Human tumor necrosis factor–related apoptosis inducing ligand mRNA	3.7
Human epidermal growth factor receptor (HER3) mRNA	3.5
Human Ets transcription factors NERF-1a and NERF-1b (NERF-1a,b) mRNA	3.2
H. sapiens tumor necrosis factor receptor-1 associated protein mRNA	3
Nuclear transcription factor Y, α (CCAAT-Binding transcription factor subunit B, CBF-B)	2.8
H. sapiens adenyl cyclase type IX mRNA	2.6
H. sapiens transcription factor SL1 mRNA	2.5
Human transcription factor ERF-1 mRNA	2.5
Human α-5 collagen type IV (COL4A5) mRNA	2.3
H. sapiens mRNA for phosphoinositide 3-kinase/cds	2.3
Human transcription factor E2F-5 mRNA	2.1
Human protein tyrosine phosphatase 1E (PTP1E) mRNA	2.1
H. sapiens mad protein homologue (hMAD-3) mRNA	2.1
H. sapiens MEKK4 (MTK1) mRNA	2

NOTE: Total RNA was extracted from MDA-1986 squamous cell carcinoma cells treated with or without 50 μmol/L curcumin for 8 hours. Gene expression profiling was performed using the HGU95Av2 array (Affymetrix). Genes decreased in their expression >2-fold are indicated together with the respective fold change.

antitumor effect of curcumin. Thus, we next undertook experiments to validate our expression profiling data. Curcumin caused a dose-dependent increase in the amount of ATF3 protein (Fig. 1A) with a concentration of 50 μmol/L causing over a 5-fold induction. This increase reflected enhanced steady-state mRNA levels (Fig. 1B) as evident in Northern blotting. Moreover, the increased ATF3 mRNA level was due, at least in part, to elevated ATF3 promoter activity as apparent in transient transfection assays (Fig. 1C) and a >3-fold increase in the recruitment of RNA polymerase II to the endogenous ATF3 gene (Fig. 1E). More importantly, Western blotting showed a very rapid increase in the amount of ATF3 protein in response to curcumin treatment (Fig. 2A) consistent with a causal role of this transcription factor in apoptosis. Thus, the protein amount was increased after a 4-hour treatment and was subsequently maintained up to 24 hours. The increased amount of ATF3 protein was a consequence of elevated steady-state mRNA amounts as evident by Northern blotting experiments (Fig. 2B). Again, the increase in ATF3 mRNA was evident at the earliest time point (4 hours) and was maintained through 24 hours of curcumin treatment.

Curcumin Increases ATF3 Protein Levels in Various Head and Neck Cancer Cell Lines

To determine if the increase in ATF3 expression achieved with curcumin was evident with other head and neck

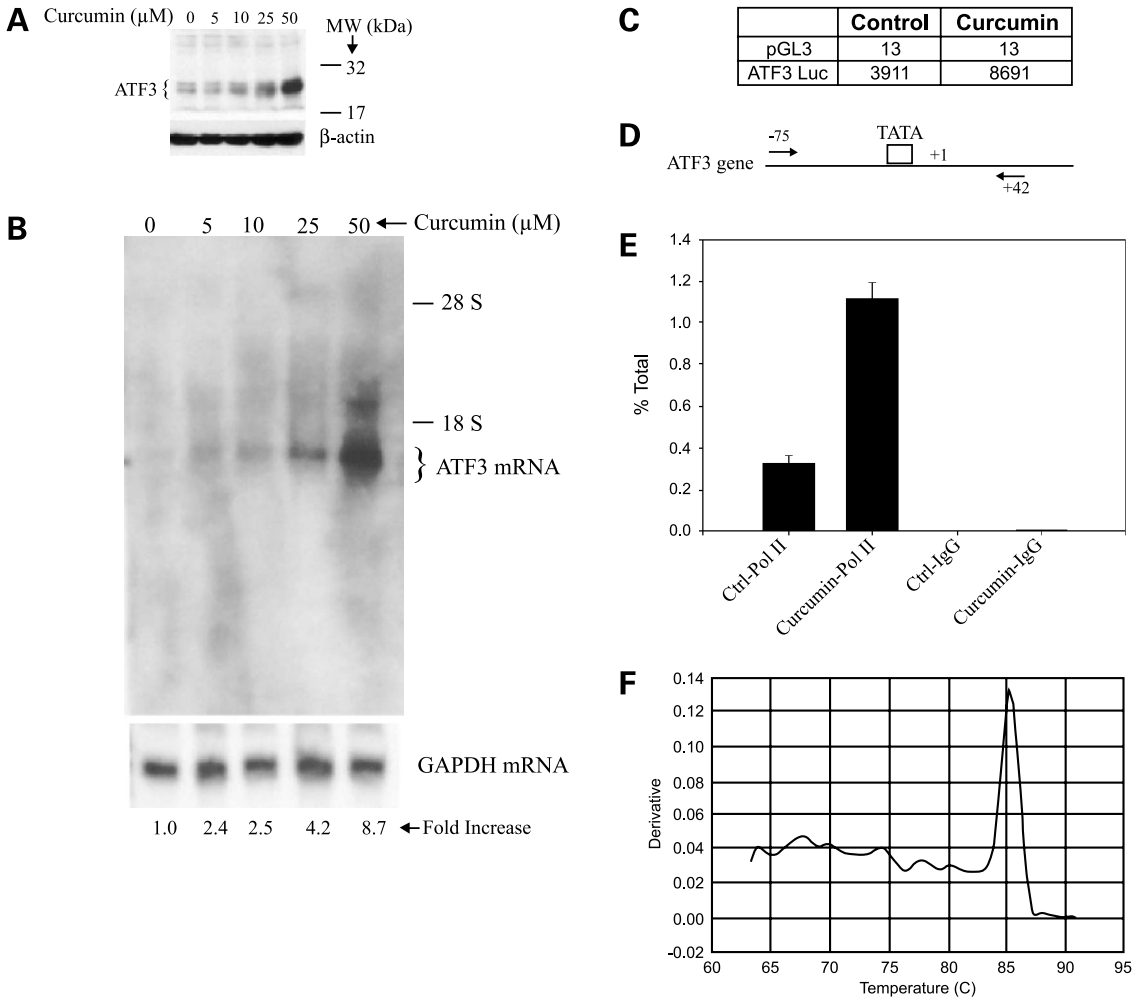

Figure 1. Curcumin increased ATF3 expression in a dose-dependent manner. MDA-1986 cells were treated with varying concentration of curcumin for 4 h, total RNA extracted and subjected either to Western blotting (**A**) or to Northern blotting (**B**) for ATF3 protein and mRNA, respectively. For the Northern blotting, cDNAs specific for ATF3 and GAPDH were employed. Fold changes in the ATF3 signal were determined by densitometric analysis. The experiment was repeated at least once. **C,** MDA-1986 cells were transfected with either a promoterless reporter (pGL3) or a luciferase reporter driven by the ATF3 promoter. After 24 h, curcumin (50 μmol/L final concentration), or carrier (*Control*), were added and the cells were incubated for an additional 4 h. Subsequently, cell lysates (equivalent protein) were assayed for luciferase activity. Average luciferase values of duplicate experiments. **D – F,** MDA-1986 cells were treated with curcumin (50 μmol/L) or DMSO (*Ctrl*) for 3.5 h after which DNA proteins were cross-linked with formaldehyde and subjected to chromatin immunoprecipitation using an anti-RNA polymerase II antibody (*Pol II*) or IgG. Reaction products were quantified by real-time PCR (**E**) using the indicated primers (**D**) complementary with the ATF3 gene. *Columns,* average of three separate determinations; *bars,* ±SD. After analysis, a melting curve (**F**) was done to confirm the amplification of a single product.

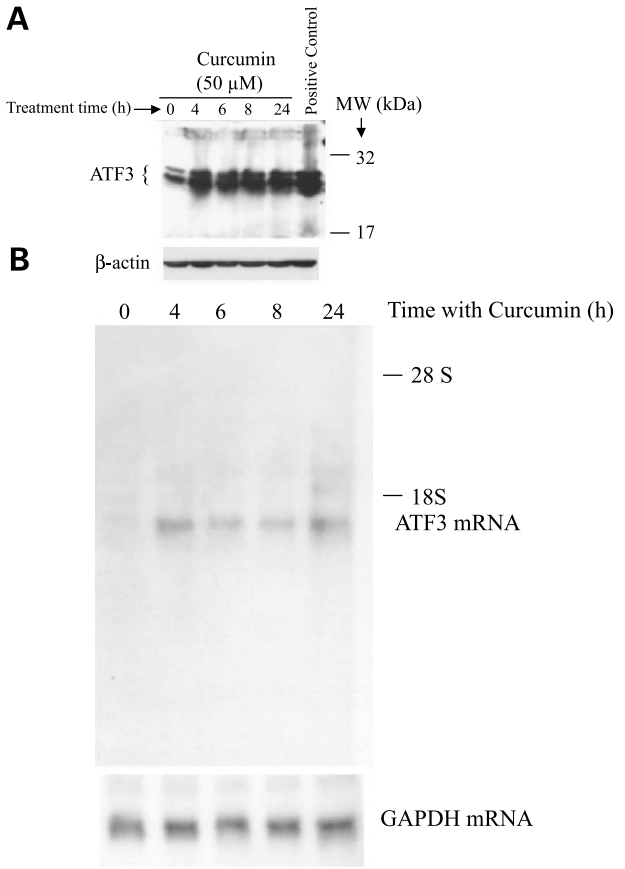

Figure 2. Curcumin increased ATF3 expression in a time-dependent manner. Western (**A**) and Northern (**B**) blotting of the time-dependent increase in ATF3 protein/mRNA, respectively. Conditions were as described in Fig. 1 legend with the exception that a concentration of 50 μmol/L curcumin was used. *Positive control* in **A** is HCT 116 cell lysate. Typical of duplicate experiments.

cancer cell lines, we analyzed the level of this protein in other cell lines treated with, or without, this agent. Western blotting (Fig. 3) clearly showed a dramatic increase in ATF3 protein in SCC4 and 14A cells with a more modest increase evident with FADU and the HN5 cells lines. Nevertheless, these data convincingly show that ATF3 expression is elevated by curcumin in some of the different types of squamous cell carcinoma cells present in head and neck tumors.

ATF3 Enhances the Proapoptotic Effect of Curcumin

The increase in ATF3 expression brought about by curcumin could either (a) have a causal role in the apoptotic response or (b) alternatively, be a consequence of programmed cell death. To distinguish between these possibilities, MDA-1986 cells were transiently transfected with a vector encoding ATF3 and treated with or without curcumin. In the absence of the expression vector or curcumin treatment, very few cells were apoptotic as evident by condensed, distorted nuclei (Fig. 4A). Somewhat surprisingly, expression of ATF3 had only a minimal effect on this low apoptotic rate (Fig. 4B). Curcumin treatment of MDA-1986 cells in the absence of exogenous ATF3 expression did however increase apoptosis with ~20% of the cells showing condensed, distorted nuclei (Fig. 4B). More dramatically, exogenous ATF3 expression synergized with curcumin in that apoptotic rate was further increased with close to 50% of the cells showing evidence of nuclear fragmentation (Fig. 4B). ATF3 expression was clearly evident in curcumin-treated cells undergoing apoptosis (Fig. 4A, *arrows*), whereas there was little evidence of nuclear fragmentation in MDA-1986 cells made to express the transcription factor only. Interestingly, ATF3 localization in the apoptotic cells was both nuclear and cytoplasmic in the apoptotic cells possibly reflecting leakage from the nuclear compartment as part of programmed cell death. To corroborate these data, we conducted parallel experiments using TUNEL staining to measure apoptosis. Again, ATF3 expression alone had little effect on apoptosis (Fig. 4D), whereas curcumin treatment by itself increased the number of TUNEL-positive cells from ~1% to 15%. However, curcumin treatment of ATF3-transfected MDA-1986 cells yielded a much higher apoptotic rate (~50%) compared with cells made to express ATF3 by itself (~15%). Altogether, these data suggest that ATF3 accelerates curcumin-dependent apoptosis but, by itself, is insufficient to bring about programmed cell death.

Discussion

The efficacy of curcumin as an antitumor compound in experimental models is well established and several molecular targets of curcumin have previously been identified. However, these studies have focused on a particular target and as of yet, no unbiased investigation has been carried out to identify novel targets of this agent. Using an mRNA expression profiling approach, we report herein the identification of several potential new targets of curcumin.

One of the genes up-regulated by curcumin is *ATF3* and we were intrigued by the possibility of a contributory role of this transcription factor in the anticancer effect of curcumin for two reasons. First, most studies to date have shown reduction in the activity and/or

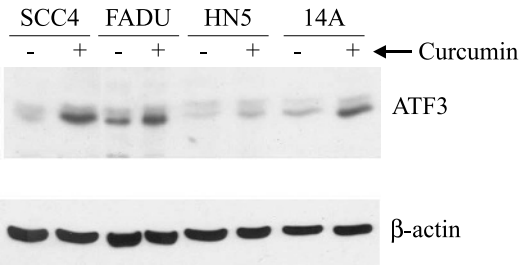

Figure 3. Curcumin increased ATF3 protein levels in various head and neck cancer cell lines. The indicated cell lines were treated with or without 50 μmol/L curcumin for 4 h and equal protein analyzed by Western blotting for ATF3 protein levels. Loading equality was checked by reprobing the blot with an anti-β-actin antibody.

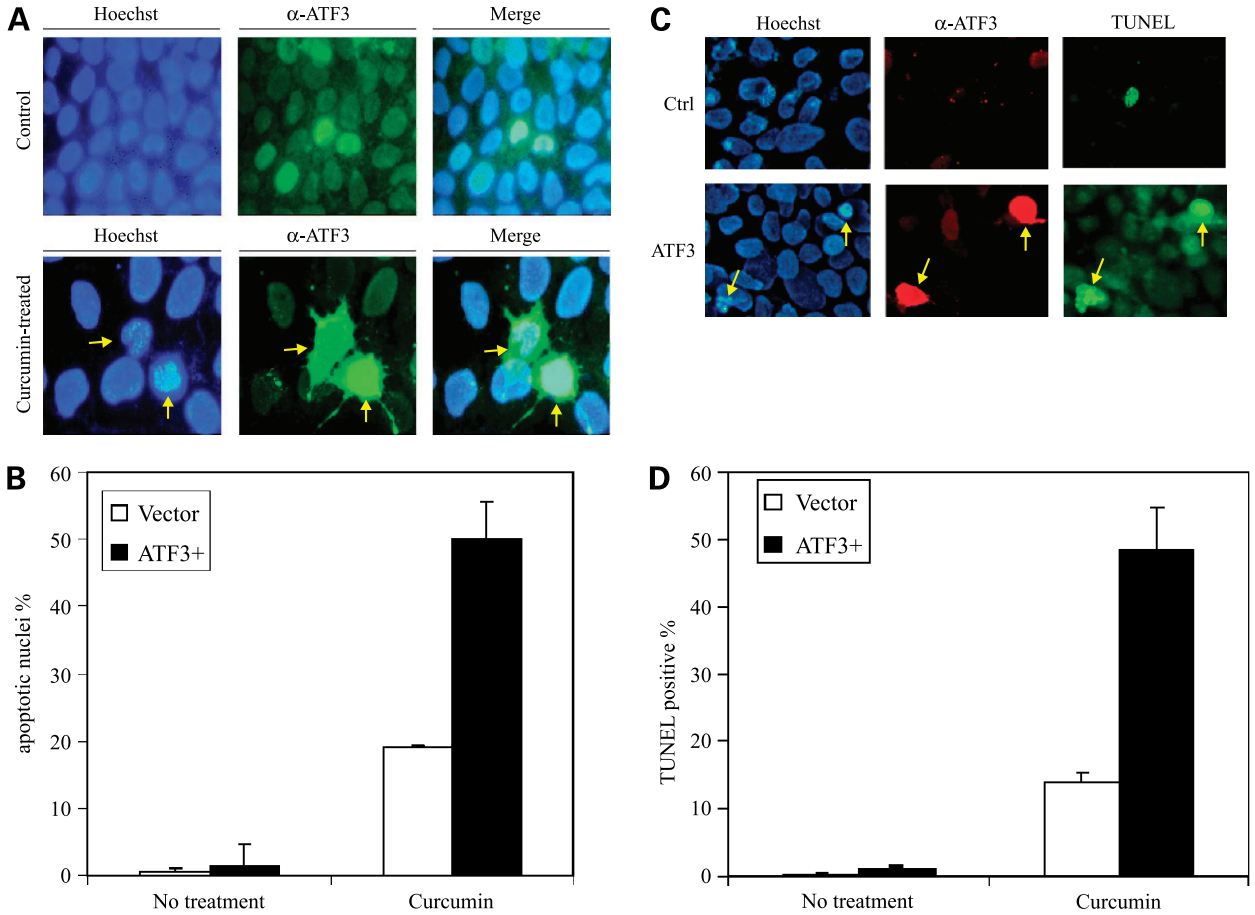

Figure 4. ATF3 accelerates the curcumin-dependent apoptosis of MDA-1986 cells. **A,** MDA-1986 cells were transfected with the pCG/ATF3 or empty vector. After 24 h, the cells were treated with or without 50 μmol/L curcumin for 8 h. The cells were then fixed, incubated sequentially with an anti-ATF3 antibody and an Alex 488-conjugated secondary antibody and counterstained with Hoechst 22345, and viewed for fluorescence. Apoptotic cells were identified as cells with condensed, disrupted nuclei (*arrow*, Hoechst staining). **B,** apoptotic cells in at least eight random fields or in the ATF3-positive staining population were counted. The percentage of apoptotic nuclei was calculated with the following formula: 100 × apoptotic cells number / total counted cell number. At least 400 apoptotic cells were counted for the ATF3-positive staining groups. **C** and **D,** MDA-1986 cells were transfected and treated as described for **A**. The cells were then immunostained with an ATF3 antibody and subsequently subjected to the TUNEL assay. ATF3-positive cells were visualized as red, whereas apoptotic cells were labeled green. **D,** apoptotic (TUNEL positive) cells were counted from either randomly selected microscopic fields, or from ATF3-positive cells (*red*). At least 300 ATF3-positive cells were counted.

expression of curcumin targets, whereas ATF3 was clearly up-regulated in its expression. Second, ATF3 has previously been reported to promote apoptosis (7, 8) thus bringing up the possibility that it plays a causal role in the therapeutic effect of curcumin. Indeed, we provide evidence herein that this transcription factor mediates in part the anticancer effect of curcumin by accelerating the proapoptotic rate of this agent.

How does ATF3 promote apoptosis? A previous study by Mashima et al. (8) might shed light onto this process. In that report, it was shown that ATF3 was strongly induced by camptothecin and its nuclear translocation coincided with caspase activation. Interestingly, an expression vector encoding an ATF3 mutant that was unable to bind DNA failed to accelerate the drug-induced apoptosis. Equally important, and similar to our study, ATF3 alone was insufficient to induce apoptosis but synergized with camptothecin to cause programmed cell death. Indeed, our data would suggest that curcumin-dependent apoptosis is achieved through a complex mechanism in which ATF3 is a contributing factor. Indeed, this supposition is given support by our finding that ATF3 expression alone was insufficient to induce apoptosis. Another possibility relating to the mechanism by which ATF3 promotes apoptosis is that the transcription factor may down-regulate antiapoptotic genes such as *bcl-2* and *bcl-xl* (1).

How is ATF3 expression increased by curcumin? It is well accepted that, with few exceptions, ATF3 mRNA levels are kept relatively low in most cells (29) but increased in response to a variety of stimuli including stress and growth factors such as fibroblast growth factor, epidermal growth factor, and hepatocyte growth factor (30). Some of these cues may then lead to mitogen-activated protein kinase kinase (MEKK) activation culminating in

increased ATF3 promoter activity (29) probably via activation of the intermediate JNK (7). However, two separate observations would argue against the relevance of MEKK pathway leading to ATF3 induction. First, we observed curcumin-dependent reductions in the mRNAs encoding the upstream activators MEKK4/MKK4 of JNK. Second, curcumin treatment also yielded a parallel induction of two dual activity phosphatases (CL100, MKP-5) which inactivate this MAPK.

Whereas we have identified several putative new targets for curcumin that might contribute to the antitumor effects of this agent, with the exception of ATF3 where a cause-effect was shown, we cannot exclude the possibility that the altered expression of these other genes represents an accompaniment rather than a causal effect of this agent. Clearly, experiments to distinguish between these two possibilities are necessary before assigning a role of these other putative genes as mediators of the antitumor effect of curcumin.

Although curcumin modulated the steady-state mRNA levels of several genes, the possibility that some of these effects reflect the ability of this agent to inhibit the COP9 signalosome (31–33) cannot be excluded. Several studies have shown that the COP9 signalosome regulates the degradation of a variety of proteins including transcription factors such as p53 and c-Jun via the ubiquitin pathway (31, 33, 34). Thus, altered protein levels of these DNA-binding proteins might then, consequently, regulate the expression or stability of some of the mRNAs identified in the current study. Indeed, for ATF3, this regulatory mechanism might explain why the increase (8.7-fold) in steady state ATF3 mRNA levels was larger than the more modest (3-fold) enrichment of RNA polymerase II at the ATF3 promoter.

We were somewhat surprised as to the increase in c-Fos and c-Jun mRNA levels achieved with curcumin. In contrast, Kakar et al. (14) reported that curcumin inhibited phorbol ester-induced expression of c-Fos, c-Jun, and c-Myc mRNAs in mouse skin. Two possible explanations may resolve these differences. First, it may be that whereas the phorbol ester-inducible c-Jun and c-Fos levels are antagonized by curcumin, constitutive levels (as evident in the current study) are modulated in the opposite direction. A second possibility is that the difference in results is a consequence of the two separate organ systems used in the two studies as well as the comparison between normal and neoplastic cells. Notwithstanding these considerations, increased c-Jun and c-Fos mRNA levels may not necessarily lead to a higher AP-1-dependent gene expression because the DNA binding of AP-1 protein complexes is interfered with by curcumin (35).

Although we have identified multiple, potential novel targets of curcumin, it must also be recognized that the antitumor benefit of curcumin, at least in head and neck cancer xenografts (36), may also be mediated through already known targets. As an example, curcumin also inhibits cyclooxygenase-2 activity (1). Considering that the mRNA and protein levels of this enzyme are elevated some 100-fold in head and neck cancer (37, 38) and that high levels of cyclooxygenase-2 predict shortened survival times of these patients (39), it is very plausible that the therapeutic benefit of this agent, at least in an experimental setting, is achieved in part via a block of this prostaglandin-synthesizing enzyme.

In conclusion, using an unbiased approach, we have identified several putative novel mediators of the anticancer effect of curcumin. Importantly, of these various targets, we have shown that one (ATF3) is causally involved in the antitumor effect of curcumin and does so by accelerating the apoptotic rate. Thus, ATF3 adds to a short list of curcumin-induced proteins that contributes to the antitumor effect of the polyphenol.

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