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Curcumin-induced GADD153 gene up-regulation in human colon cancer cells

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Abstract:
Ingestion of plant products containing the phenolic phytochemical, curcumin, has been linked to lower incidences of colon cancer, suggesting that curcumin has cancer chemopreventive effects. Supporting this suggestion at the cellular level, apoptosis occurs in human colon cancer cells exposed to curcumin. However, the mechanism is unclear, prompting this investigation to further clarify the molecular effects of curcumin. HCT-116 colonocytes were incubated with 0-20 mM curcumin for 0-48 h. In concentration-dependent and time-dependent manners, curcumin induced DNA damage, resulting later in the appearance of cellular features characteristic of apoptosis. To identify a potential pro-apoptotic gene that could be responsive to the DNA damage in curcumin-treated cells, growth arrest and DNA damage-inducible gene 153 (GADD153) was considered. Curcumin increased GADD153 mRNA (and also protein) expression, which was prevented by actinomycin D and also by a broad protein kinase C inhibitor, but not by selective MAPK inhibitors. These findings suggest that curcumin-induced up-regulation of GADD153 mRNA expression was at the level of transcription, but apparently without depending on upstream MAPK. In determining the involvement of reactive oxygen species in mediating the effect of curcumin on GADD153, the antioxidants pyrrolidine dithiocarbamate and N-acetylcysteine (NAC), but neither α-tocopherol nor catalase, also blunted or prevented up-regulation of GADD153 mRNA expression caused by curcumin. Most noteworthy, when NAC was tested, it inhibited the DNA damage and apoptosis caused by curcumin. Because expression of GADD153 protein was detected before the appearance of apoptotic features, this observation raises the possibility that GADD153 protein might be important for curcumin-induced apoptosis.

Article:

INTRODUCTION

Diets rich in edible plant products, especially fruits, vegetables and herbs, provide substantial amounts of phenolic phytochemicals. These natural substances are thought to be potential cancer chemopreventive agents (1). A primary example would be curcumin. It is the principal bioactive curcuminoid found in the food spice, turmeric, which is produced commercially by processing the harvested rhizomes of the plant, Curcuma longa. It is believed that curcumin could be especially useful in preventing the development of colorectal cancer (2). However, at the cellular level, it is unclear how curcumin works as a cancer chemopreventive agent.

To elucidate a possible mechanism of curcumin, some previous studies have focused on the capability of curcumin to inhibit the growth or proliferation of human colon cancer cells. For example, in investigating the effects of curcumin on Lovo colon carcinoma cells, Chen et al. (3) reported that curcumin caused cell cycle arrest. Some evidence of apoptosis, namely, morphological changes and DNA fragmentation, was also found in the curcumin-treated cells. Moragoda et al. (4) corroborated these fundamental findings in working with HCT-116 colon cancer cells. Additionally, they showed that protein expression of cyclins D and E, but not cyclin B, was reduced in the curcumin-treated cells. The activity of the cyclindependent kinase, Cdc2, but not Cdk2, was induced. Furthermore, curcumin caused proteolytic cleavage of both poly-ADP-ribose polymerase and caspase-

3, induction of caspase-8 activity, and reduced expression of Bcl-XL. Apparently, the p53 gene and the WAF-1/CIP-1/p21 gene are not required for mediating apoptosis caused by curcumin in HCT116 cells (5). Therefore, there must be other critical genes, whose identities are unknown presently, involved in the induction of apoptosis by curcumin.

These above previous studies (3-5) have delineated events occurring relatively late after the cells had been treated with curcumin. The earlier events promoted by curcumin in colon cancer cells, i.e. prior to the actual initiation of apoptosis, are not entirely known. In particular, whether the expression of relevant early-response genes is changed has not been appreciably investigated. Hence, it is conceivable that curcumin may increase the expression of certain early-response genes that could consequently be important in mediating the initiation of apoptosis. One possibility would be the growth arrest and DNA damage-inducible genes (GADD).

The expression of GADD, but especially growth arrest and DNA damage-inducible gene 153 (GADD153), is often changed as an early response of cells that have been subjected to a stressful or potentially lethal environment that threatens to compromise cellular integrity. Situations that cause stress or malfunction in the endoplasmic reticulum have been reported to increase GADD153 gene expression (6). Additionally, GADD gene expression is up-regulated when cells are deprived of essential nutrients, including glucose (7), leucine (8), glutamine (9) and zinc (10). These experimental conditions most likely promote growth arrest as an initial effect. Exposing cells to DNA-damaging agents such as peroxynitrite (11), UV radiation (12) and anticancer drugs (13) increase GADD gene expression as well. These experimental conditions often promote cell death via apoptosis. GADD153, in particular, appears to have a direct role in initiating apoptosis, based on the occurrence of apoptosis in GADD153 expression vector-transfected cells (14). Several subsequent studies (13,15-17) have suggested that GADD153 triggers the critical early events leading to the initiation of apoptosis. Therefore, up-regulation of GADD153 gene expression is not recognized merely as a consequence of apoptosis.

To gain a better understanding of the molecular effects of curcumin on human colon cancer cells, the primary aim of the present study was to determine whether curcumin creates a cellular environment in HCT-116 colonocytes that can influence the expression of the GADD153 gene in causing cell death. Evidence is presented to support the concept that cur- cumin initially promotes cellular DNA damage, consequently stimulating the activation of GADD153 gene expression as a molecular event leading to apoptosis.

MATERIALS AND METHODS

Materials

HCT-116 human colon adenocarcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). PD098059 and SB203580 were bought from Calbiochem (San Diego, CA) and SP600125 from Tocris (Ellisville, MO). Curcumin and all other reagents were purchased from Sigma Chemical (St Louis, MO) unless otherwise stated.

Cell culture and treatment

HCT-116 colonocytes were propagated in McCoy's 5A medium (Sigma Chemical Co.) that was supplemented with 100 ml/l fetal bovine serum (BioWhittaker, Walkersville, MD), 2 mmol/l glutamine, 0.54 mmol/l fungizone, 100 000 U/l penicillin and 100 mg/l streptomycin (last four items from Atlanta Biologicals, Atlanta, GA). Upon reaching 70--80% confluency, the cells were exposed to 0-20 µM curcumin for 0--48 h depending on the experiment. In some experiments, the cells were co-incubated with 5 µg/ml actinomycin D (Act D) and curcumin, or exposed to 0-500 µM hydrogen peroxide (H_2O_2) when the cells were in either complete McCoy's 5A medium or just Hank's buffered saline solution (HBSS). In assessing possible effects of selective MAPK inhibitors and a general protein kinase C (PKC) inhibitor, cells were pre-incubated for 30 min with either PD098059 (50 µM), SB203580 (10 µM), SP600125 (10 µM) or staurosporine (0-2 µM), and then exposed to curcumin (10 µM) for 5 h. In assessing possible effects of free radical scavengers, cells were pre-treated with α-tocopherol (0-200 µM) for 24 h, pre-treated with 2500--10 000 U/ml catalase for 0.5 h, pre-

treated with 50 µM pyrrolidine dithiocarbamate (PDTC) or 20 mM N-acetylcysteine (NAC) for 2 h, prior to exposing these different cell samples to curcumin (10 µM) for 5 h.

Assessment of cellular DNA damage

Single-strand breaks in DNA were assessed in the cells by the comet assay, as described previously (18). Briefly, cells were suspended in 0.5% agarose gel, and aliquots of the suspension pipetted onto frosted glass slides, which had been pre-coated with 1% agarose gel. After allowing the cell/agarose suspension to congeal, the slides were then immersed in ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 300 mM NaOH, 10% DMSO and 1% Triton X-100) for 1 h. Next, the slides were equilibrated in alkaline buffer (300 mM NaOH, 1 mM EDTA) for 20 min, before being electrophoresed for 20 min at 20 V (300 mA). After electrophoresis, the slides were washed with a neutralization buffer (0.4 M Tris--Cl, pH 7.5) for 5 min and then stained with ethidium bromide. The slides were viewed on an Olympus BX-60 fluorescence microscope equipped with a SPOT digital camera. For each of the three replicate experiments, 50 nucleoids were scored on a scale of 0 (no DNA damage) to 4 (severe DNA damage) in regard to the appearance of a comet taillike shape, reflecting single-strand breaks in DNA. To calculate the total score, the individual scores for each of the 50 nucleoids were added in each experiment and then the three total 'comet scores' from the three replicate experiments averaged for presentation in the appropriate figure below.

To detect the presence of oxidatively modified DNA, immunocytofluorescence microscopy was performed. Cells were grown and treated with curcumin in LabTek chamber slides. After washing with phosphate-buffered saline (PBS), the cells were fixed with 4% formaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were treated with RNase (100 µg/ml) for 1 h at 37°C and proteinase K (10 µg/ml) for 10 min at room temperature. Cellular DNA was then denatured with 4 N HCl at room temperature. Blocking was achieved with 1% bovine serum albumin (BSA) in PBS for 3 h. The cell samples were incubated with 1:50 dilution of IF7 murine anti-8-oxodeoxyguanine monoclonal antibody (Trevigen) overnight at 4°C, followed by incubation at room temperature with 1:500 dilution of rabbit anti-murine IgG conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR). After mounting, the slides were viewed on the fluorescence microscope as before.

Annexin V-FITC binding assay

HCT-116 colonocytes that had been grown and treated with curcumin in chamber slides were washed twice with cold PBS. The washed cells were equilibrated for 10 min in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). After equilibration, the binding buffer was removed from the chamber slide. Then, fresh binding buffer but containing annexin V-Alexa Fluor 488 conjugate (Molecular Probes) and propidium iodide was pipetted into the chamber slide for 15 min of incubation. Slides were viewed immediately on the fluorescence microscope.

Staining of cellular DNA with 4',6-diamidino-2 -phenylindole (DAPI)

HCT-116 colonocytes were washed three times and re-suspended in PBS. Aliquots of the cell suspension were pipetted onto lysine-coated glass slides for centrifugation in a Stat Spin Cytofuge. To fix the cells, slides were immersed in 4% formaldehyde solution in PBS for 25 min and placed in 70% ethanol for 5 h at -20°C. Before staining, the slides were washed three times with deionized water. Then, they were immersed in 1 mg/ml DAPI for 10 min for eventual viewing on the fluorescence microscope. For each experiment performed in triplicate, 100 randomly selected cells were scored either for the absence or presence of DNA condensation and fragmentation.

TUNEL assay

HCT-116 colonocytes were fixed as above. The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay was performed using a kit along with a set of instructions from Promega (Madison, WI). The samples were examined by fluorescence microscopy as before. For each experiment performed in triplicate, 100 randomly selected cells were scored for either the absence or presence of green fluorescence (TUNEL-labeling).

Determination of GADD153 mRNA expression

Total RNA was isolated from the cells using a Qiagen RNeasy Mini kit. The expression levels of GADD153 mRNA were determined by multiplex relative RT-PCR analysis of total RNA using a Qiagen OneStep RT-PCR kit and gene- specific primers. The GADD153 PCR primer sequences (19) were: sense, 5'-GCACCTCCCAGAGCCCTCACTCTCC-3' and antisense, 5'-GTCTACTCCAAGCCTTCCCCCTGCG-3'. The Β-actin QuantumRNA primer/competimer sets (Ambion, Austin, TX) were utilized to generate the internal standard. The RT-PCR conditions were 30 min at 50°C followed by 15 min at 95°C (RT), then 0.5 min at 95°C, 0.5 min at 60°C, and 1 min at 72°C (PCR) for 25 cycles. The resulting cDNA products were separated by 2% agarose gel electrophoresis with ethidium bromide staining. For the target gene (GADD153), the PCR product size was 422 bp. For the internal control (β-actin), the PCR product size was 294 bp.

Western immunoblotting analysis of GADD153 protein expression

After scraping, the cells were harvested and washed by centrifugation (500 g for 5 min) in PBS. The cell pellets were sonicated in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and Roche Complete Protease Inhibitor Cocktail, pH 7.4) and left on ice for 30 min. Next, the samples were centrifuged at 16 000 g for 20 min at 4°C, and the supernatant was saved. The protein concentration of the supernatant was determined with a BCA protein assay kit (Pierce).

Whole cell lysate supernatant (50 mg protein) was electrophoresed using Novex NuPAGE mini-gels (4-12% Bis--Tris) before blotting the gel to nitro-cellulose membrane. Blocking was performed for 1 h at 25°C in blocking buffer consisting of 5% skim milk powder in TBST (20 mM Tris-HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.4). The membrane was incubated overnight at 4°C with GADD153 rabbit polyclonal R-20 antibody (Santa Cruz) in blocking buffer (1:5000). After washing in TBST, the membrane was then incubated for 2 h at 25°C with goat anti-rabbit IgG/HRP conjugate (Santa Cruz) in blocking buffer (1:100 000). Finally, after washing again in TBST, the membrane was placed in a plastic pouch for incubation with Pierce Super-Signal WestFemto Maximum Sensitivity Substrate for 5 min before analysis (Kodak digital science™ image station 440 CF). Afterwards, the membrane was kept in the plastic pouch overnight at 25°C, before re-probing for β-actin as a control for sample loading. The membrane was washed with TBST and incubated for 2 h at 25°C with mouse monoclonal anti-β-actin antibody (Sigma) in blocking buffer (1:50 000). After incubation for 1 h at 25°C with HRP-conjugated goat polyclonal anti-mouse IgG (Santa Cruz) in blocking buffer (1:100 000), the membrane was washed in TBST and processed for analysis as before.

Immunocytofluorescence determination of GADD153 protein expression

Cells in LabTek chamber slides were fixed with 4% formaldehyde in PBS at 25°C for 15 min and permeabilized with 0.2% Triton X-100 in PBS at 4°C for 10 min. The slides were blocked with 1% BSA in PBS at 25°C for 3 h. The cells were then incubated overnight at 4°C with 1:500 dilution of R-20 rabbit polyclonal anti-GADD153 antibody (Santa Cruz) in the blocking buffer. Finally, the cells were incubated with 1:1000 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes) in the blocking buffer. The cells were counterstained with propidium iodide. After mounting, the slides were viewed on the fluorescence microscope as before.

RESULTS

Increased DNA damage in HCT-116 colonocytes exposed to curcumin

To determine if curcumin promotes single-strand breaks in the DNA of HCT-116 colonocytes, the cells were exposed to curcumin and the extent of DNA damage assessed by the comet assay. As shown in Figure 1A, the representative nucleoids of control cells (0 μM curcumin) were uniformly spherical in shape, reflecting the absence of any DNA damage. In contrast, with only 1 h of exposure to curcumin, the representative nucleoids of curcumin-treated cells (10 and 20 μM) resembled 'comets', reflecting the presence of significant DNA damage as evidenced by the typical 'comet tail' whose overall span is proportional to the extent of DNA

damage. After scoring of the nucleoids to attain comet scores (Figure 1B), it was found that curcumin promoted single- strand DNA breaks in essentially a time-dependent and concentration-dependent manner.

To evaluate the possibility that the DNA damage caused by curcumin could be the result of reactive oxygen species, an immunochemical method was performed to find out if 8- oxodeoxyguanine is present in curcumin treated HCT-116 colonocytes. This modified base is recognized as a marker of oxidative DNA damage as would be caused by reactive oxygen species. Hence, the existence of 8-oxodeoxyguanine can be determined by its immunoreactivity with an antibody specific for 8-oxodeoxyguanine, which is readily visualized by fluorescence microscopy. As can be seen in Figure 1C, treating HCT-116 colonocytes with curcumin resulted in cells with fluorescence images indicative of the presence of 8-oxodeoxyguanine, and hence, oxidative DNA damage.

Cellular features characteristic of apoptosis in HCT-116 colonocytes exposed to curcumin

As shown in Figure 2A, many of the cells in curcumin-treated samples had rounded up after 12 h. Subsequently, after 48 h, most of the cells had general morphology consistent with apoptosis, i.e. cell shrinkage, membrane blebbing, and

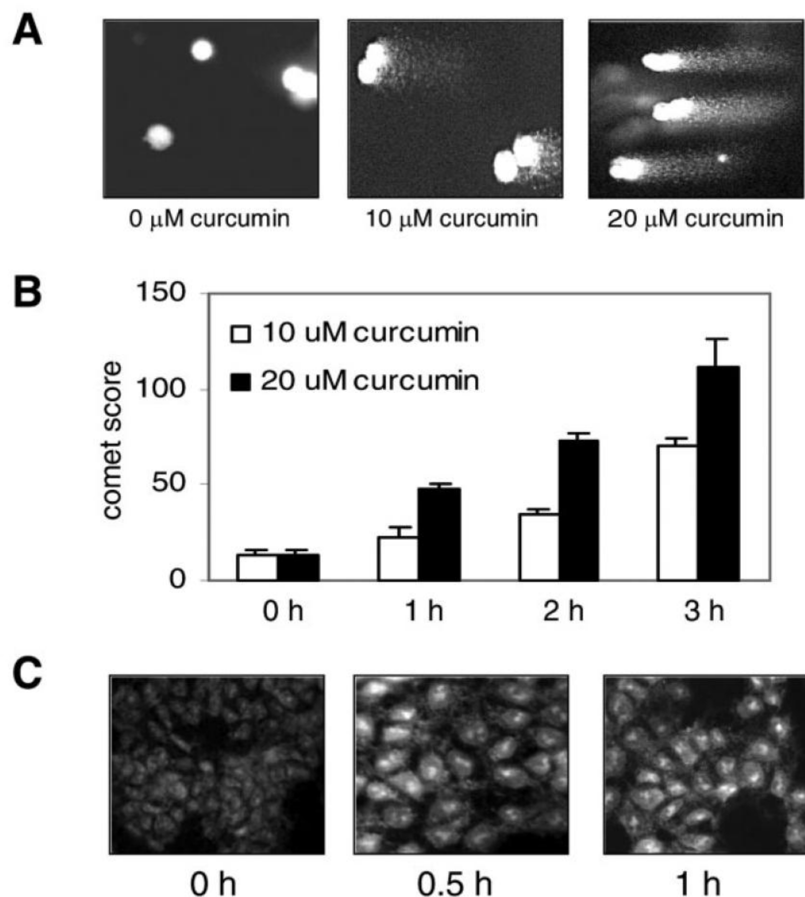

Fig. 1. Induction of DNA damage in HCT-116 colonocytes by curcumin. The cells were incubated with 0–20 μM curcumin for 0–3 h. Then, the comet assay was performed to assess the relative extent of single-strand breaks in DNA. The photos (**A**) show representative results of experiments where cells were incubated with curcumin for only 1 h. More complete results including scoring of comets are also provided (**B**). Values are the average ± SEM, $n = 3$. In other experiments, cells were incubated with 10 μM curcumin for 0–1 h. Then, immunocytofluorescence microscopy (**C**) was performed on the cells using a murine anti-8-oxodeoxyguanine monoclonal antibody along with rabbit anti-murine IgG conjugated to Alexa Fluor 488.

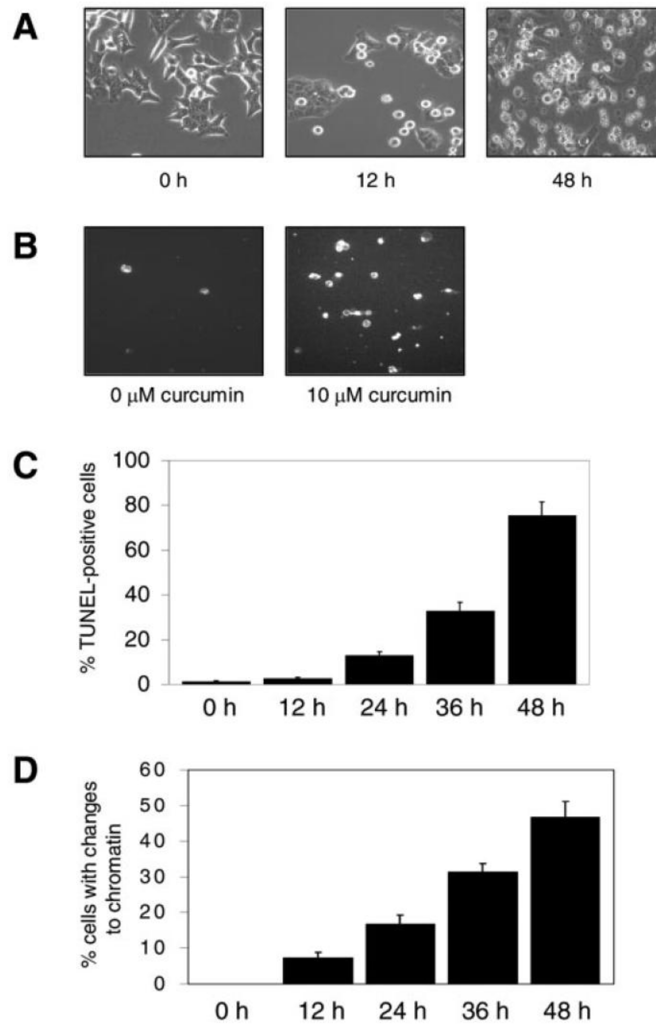

Fig. 2. Evidence of apoptosis in HCT-116 colonocytes exposed to curcumin. (**A**) Cells were incubated with 10 μM curcumin, and photos taken at 0-, 12- and 48-h time points. (**B**) Cells were incubated with 10 μM curcumin for 24 h, and then, annexin V-Fluor 488 binding determined. Additionally, cells were incubated with 10 μM curcumin for 0–48 h. Then, the TUNEL assay (**C**) and DAPI staining (**D**) performed to detect the percentage of cells with DNA degradation and chromatin condensation/fragmentation, respectively. Values are the average ± SEM, $n = 3$.

apoptotic bodies. To determine if translocation of plasma membrane phosphatidylserine (a known marker of the early stages of apoptosis) occurred in curcumin-treated HCT-116 colonocytes, the cells were exposed to 10 μM curcumin for 24 h. Then, the standard annexin V-Alexa Fluor 488 binding assay was performed (Figure 2B). A greater number of curcumin-treated cells than control cells bound the annexin probe, giving them a brilliant green fluorescence at the cell surface. Furthermore, more cells eventually died (red fluorescence) after exposure to curcumin. Therefore, these findings indicate that a significant number of HCT-116 colonocytes were in the early stages of apoptosis after 24 h of exposure to curcumin.

To determine if DNA degradation (a known marker of the late stages of apoptosis) occurred in curcumin-treated HCT116 colonocytes, we exposed the cells to 10 μM curcumin for 48 h, and then, the standard TUNEL assay was performed to detect any DNA degradation (Figure 2C). The effect of curcumin was time-dependent. After 24 h of exposure of the cells to curcumin, an increase in the number of cells with DNA degradation started to become noticeable. With continued time of exposure (36 and 48 h), curcumin caused additional DNA degradation incrementally. Likewise, DAPI staining of cell nuclei revealed that curcumin increased the numbers of cells having chromatin condensation and fragmentation with increasing time of incubation with the cells (Figure 2D).

Effect of curcumin and H_2O_2 on GADD153 mRNA expression in HCT-116 colonocytes

Exposing HCT-116 colonocytes to curcumin increased GADD153 mRNA expression, as determined by multiplex relative RT-PCR analysis using gene-specific primers for GADD153 and the internal control, β-actin (Figure 3A). It can be seen that GADD153 mRNA was constitutively expressed in control cells (0 µM curcumin), as was the expected β-actin mRNA. After cells were exposed to curcumin (particularly at 10 µM), GADD153 mRNA increased noticeably. Moreover, the effect of curcumin began to become noticeable after only 2 h of exposure to curcumin (Figure 3B).

To get an indication of whether curcumin increases GADD153 mRNA at the level of transcription, a standard experiment using the RNA synthesis inhibitor, Act D, was performed. Cells were treated with 10 µM curcumin and 5 µg/ml Act D for 5 h and multiplex relative RT-PCR analysis was then performed (Figure 3C). As can be seen, curcumin increased GADD153 mRNA in the cells (lane 3), when compared with control cells (lane 1). When cells were co-treated with curcumin and Act D, the increased expression of GADD153 mRNA caused by curcumin was prevented (lane 4). Therefore, the data suggest that curcumin-induced GADD153 mRNA up-regulation was due largely to increased synthesis, and not stability, of the transcript.

The previous data (Figure 1C) suggested that curcumin promoted the formation of reactive oxygen species, based on the detection of oxidatively modified DNA in curcumintreated cells. The GADD153 gene is often turned on when there is a stressful environment (11-13), as would be expected if reactive oxygen species are generated in cells exposed to curcumin. Therefore, an experiment was next performed to compare the effect of curcumin with that of H_2O_2, which is recognized as a reactive oxygen species, on GADD153 mRNA expression in HCT-116 colonocytes (Figure 3D). Using cells in complete McCoy's 5A culture media, curcumin (lane 2) as before and H_2O_2 but only at the highest concentration tested (lane 5) increased GADD153 mRNA expression. However, using cells in HBSS, which is devoid of the endogenous antioxidants found in the complete McCoy's 5A culture media, markedly lower concentrations of H_2O_2 (lanes 6 and 7) were able to increase GADD153 mRNA expression.

Induction of GADD153 protein expression by curcumin in HCT-116 colonocytes

Changes in mRNA expression do not always translate to changes in protein expression. To determine whether the changes in GADD153 mRNA levels produced corresponding increases in GADD153 protein levels, western-immunoblotting analysis was first performed on curcumin-treated cells (Figure 4A). As can be seen, no GADD153 protein expression was detectable in untreated control cells (lane 1). But, after being treated with curcumin, GADD153 protein expression started to become apparent (faint band with an apparent molecular weight of 27 kDa) after only 2 h and increased considerably with time of incubation. The effect of curcumin on GADD153

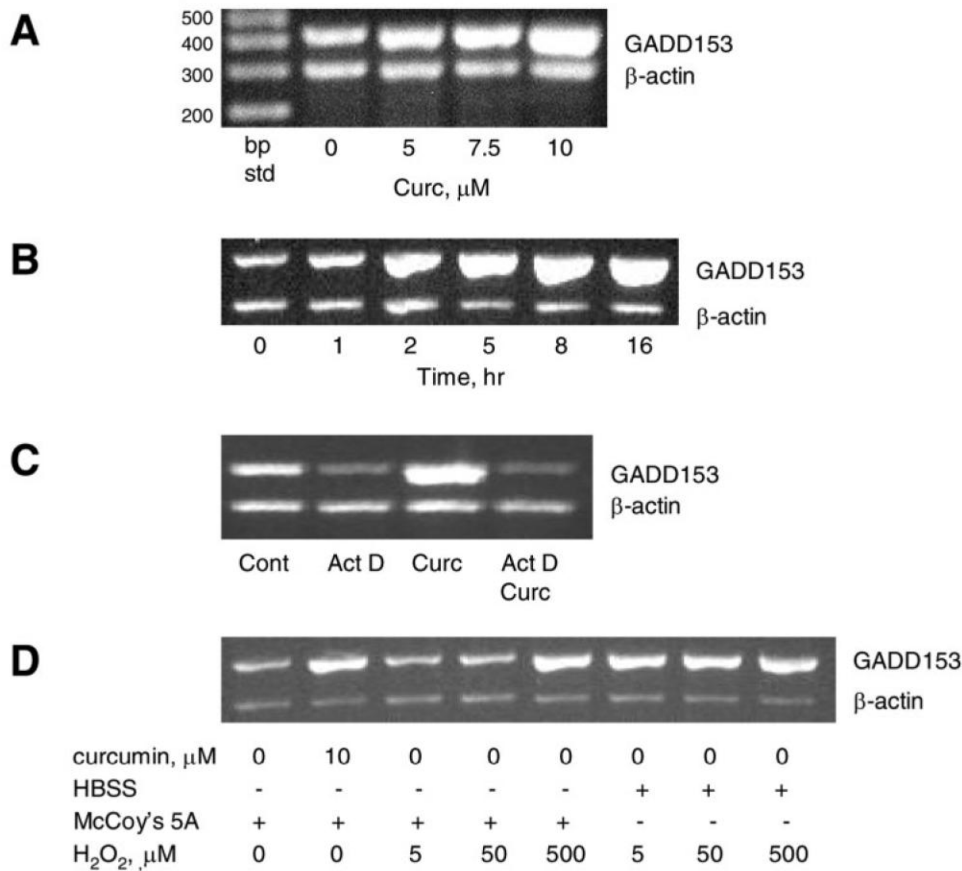

Fig. 3. Up-regulation of GADD153 mRNA expression by curcumin in HCT-116 colonocytes. The cells were incubated with 0–10 μM curcumin for 5 h (**A**), or with just 10 μM curcumin for 0–16 h (**B**). Additionally, cells were incubated with 10 μM curcumin in the absence and presence of 5 μg/ml Act D for 5 h (**C**). In a comparative experiment (**D**), the cells covered with either McCoy's 5A complete culture medium or HBSS were incubated with either curcumin or H_2O_2 for 1 h. After incubation, total RNA was isolated for subsequent multiplex relative RT–PCR analysis using gene-specific primers for the target gene, GADD153, and also the internal control gene, β-actin. The results are representational of three different experiments.

protein expression was confirmed by immunocytofluorescence microscopy (Figure 4B). When counterstained with propidium iodide and viewed with a rhodamine filter, it can be seen that the number of cells in the untreated and curcumin-treated samples are similar. Using the fluoroscein filter to view the same samples, it can be seen that the curcumin-treated cells expressed GADD153 protein in clear contrast to the untreated cells. Thus, over time, GADD153 protein expression paralleled GADD153 mRNA expression, suggesting that newly synthesized GADD153 mRNA was rapidly translated into GADD153 protein. On the other hand, the pre-existing or basal GADD153 mRNA could have been translated as well to partly account for the induction of GADD153 protein expression by curcumin.

Effect of protein kinase inhibitors on curcumin-induced up-regulation of GADD153 mRNA expression in HCT-116 colonocytes

MAPK have been reported to be involved in up-regulation of GADD153 mRNA expression (11). To determine if MAPK were involved in increasing the expression of GADD153 mRNA as caused by curcumin, HCT-116 colonocytes were pre-treated with selective chemical inhibitors of MAPK and then exposed to curcumin. ERK inhibitor PD098059, p38 inhibitor SB203580 and JNK inhibitor SP600125 did not attenuate curcumin-induced up-regulation of GADD153 mRNA (Figure 5A). It is noteworthy that higher concentrations of SP600125 per se actually increased GADD153 mRNA expression and caused apoptosis (data not shown).

To determine if PKC is important in curcumin-induced up- regulation of GADD153 mRNA expression, cells were pretreated with staurosporine (a general PKC inhibitor) before exposing them to curcumin. Staurosporine prevented the increase in GADD153 mRNA expression caused by curcumin (Figure 5B). As can be seen, curcumin markedly increased GADD153 mRNA expression (lane 2). Pre-treatment of cells with staurosporine

prevented the effect of curcumin on GADD153 mRNA expression in a concentration-dependent manner (lanes 5-7).

Effect offree radical scavengers or antioxidants on curcumininduced up-regulation of GADD153 mRNA and protein expression in HCT-116 colonocytes

Oxidative stress promotes GADD gene activation (11), suggesting an involvement of reactive oxygen species. There is evidence that curcumin promotes formation of ROS (20). Hence, an experiment was performed to determine if free radical scavengers or antioxidants could prevent curcumin-induced up-regulation of GADD153 mRNA expression. Unexpectedly, neither the lipophilic antioxidant, α-tocopherol (Figure 5C), nor the H_2O_2-scavenging enzyme, catalase

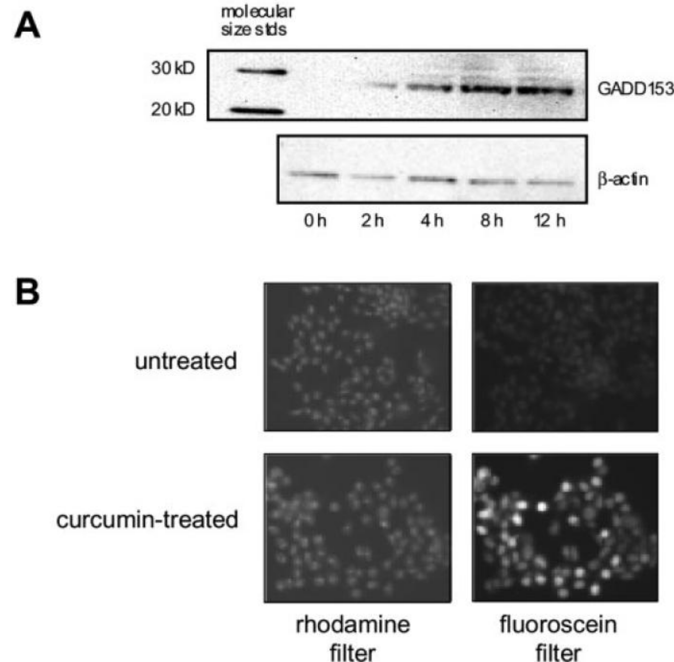

Fig. 4. Induction of GADD153 protein expression by curcumin in HCT-116 colonocytes. **(A)** Cells were exposed to 10 μM curcumin for 0–12 h. Then, whole cell lysates were prepared for subsequent western-immunoblotting analysis of GADD153 protein and also β-actin. **(B)** Cells were exposed to 10 μM curcumin for a single time period of 12 h. Then, immunocytofluorescence microscopy was performed on the cells using a rabbit anti-GADD153 polyclonal antibody along with goat anti-rabbit IgG conjugated to Alexa Fluor 488. Counterstaining was achieved with propidium iodide (PI). The green fluorescence of Alexa Fluor 488 and red fluorescence of PI were viewed with fluoroscein and rhodamine filters, respectively.

(Figure 5D), was able to attenuate curcumin-induced up-regulation of GADD153 mRNA expression.

In seeming contrast, PDTC, which is a known chelator of metal ions that promote the generation of reactive oxygen species, inhibited to a noticeable extent curcumin-induced up-regulation of GADD153 mRNA expression (Figure 5E, left panel, lane 4). On the other hand, at the concentration tested, PDTC per se seemed to increase GADD153 mRNA expression to a slight extent (Figure 5E, left panel, lane 3). NAC, which is known to increase intracellular concentrations of the antioxidant glutathione (21), was able to prevent the increased GADD153 mRNA expression caused by curcumin (Figure 5E, right panel, lane 4). Moreover, NAC by itself did not affect basal expression of GADD153 mRNA (Figure 5E, right panel, lane 3). When western immunoblotting analysis was performed to determine GADD153 protein expression in some of the above cell samples, it was found that staurosporine, PDTC and NAC prevented induction of GADD153 protein caused by curcumin (Figure 5F).

Most noteworthy, in selecting NAC for further study, NAC inhibited curcumin from causing DNA damage as assessed by the comet assay (Figure 6A), DNA degradation as assessed by the TUNEL assay (Figure 6B) and chromatin condensation and fragmentation as detected by DAPI staining (Figure 6C).

DISCUSSION

There is considerable support for curcumin as a potential cancer chemopreventive agent (22), based largely on three general observations. First, curcumin is known to modulate the activities of certain enzymes involved in the bioactivation and disposition of chemical carcinogens (23). Secondly, curcumin can inhibit signal transduction events critical for proliferation of cancer cells (24). Thirdly, of relevance to the present study, curcumin can induce apoptosis in cancer cells, including those of colonic origin (3,4).

However, from another perspective, the effects of curcumin in vitro can be contradictory depending on the experimental conditions. In examining the effect of curcumin on DNA, paradoxical findings have been reported previously. Because it is well known that curcumin has antioxidant properties, it is not surprising that curcumin inhibited H_2O_2/ferric nitrilotriacetate-induced damage to purified calf thymus DNA (25). With intact cells, curcumin inhibited radiation-induced damage to DNA in human skin fibroblasts and blood lymphocytes (26), as well as H_2O_2-induced damage to human peripheral blood lymphocytes (27). However, in the presence of Cu^{2+}, curcumin had the opposite effect in that it actually induced damage to calf thymus DNA (28,29). In doing so, it was further found that curcumin directly generated superoxide anion and H_2O_2, and when copper was present, hydroxyl radical as well. It has also been reported that curcumin, or a metabolite produced by the action of cytochrome P-450 isozymes in the presence of metal ions, induced damage to calf thymus DNA (30).

In the present study, curcumin caused DNA damage in the form of single-strand breaks in HCT-116 colonocytes. Despite the reported findings (28,29) that curcumin can generate reactive oxygen species at least under a set of defined experimental conditions, it is not unequivocally known if curcumin generated reactive oxygen species to cause damage to DNA in HCT116 colonocytes. Nevertheless, there are two lines of current evidence to partially support the view that curcumin did induce oxidative stress in causing the cellular DNA damage. First, HCT-116 colonocytes had immunoreactivity with an antibody specific for 8-oxodeoxyguanosine, which is a modified base residue known to be present in DNA that has been attacked by reactive oxygen species. This finding is consistent with that in a recent study (29), where it was reported that curcumin caused the formation of 8-oxodeoxyguanosine in calf thymus DNA as determined by HPLC analysis. Secondly, curcumin-induced DNA damage was inhibited by pre-treating colonocytes with the free radical scavenger or antioxidant, NAC. This finding is consistent with the results of our earlier study (31) where single-strand breaks in DNA caused by curcumin in Jurkat T-lymphocytes was prevented by an antioxidant. The damaging effect of curcumin on DNA can perhaps be further explained by the observation that some phenolic phytochemicals act as pro-oxidants under cell culture conditions (32). That is, certain phenolic phytochemicals generate H_2O_2, which in the presence of trace metal ions would subsequently generate hydroxyl radicals that actually damage cellular DNA. In any event, the detection of significant DNA damage, before the appearance of any clear signs of apoptosis, in curcumin-treated HCT-116 colonocytes raises the possibility that the DNA damage could have been the critical event that somehow set off the chain of events leading to apoptosis.

Evidence of apoptosis was found later after the DNA damage had occurred in HCT-116 colonocytes exposed to curcumin. More specifically, the annexin V binding assay, DAPI staining and TUNEL assay positively identified markers of apoptosis. The present findings corroborate and expand

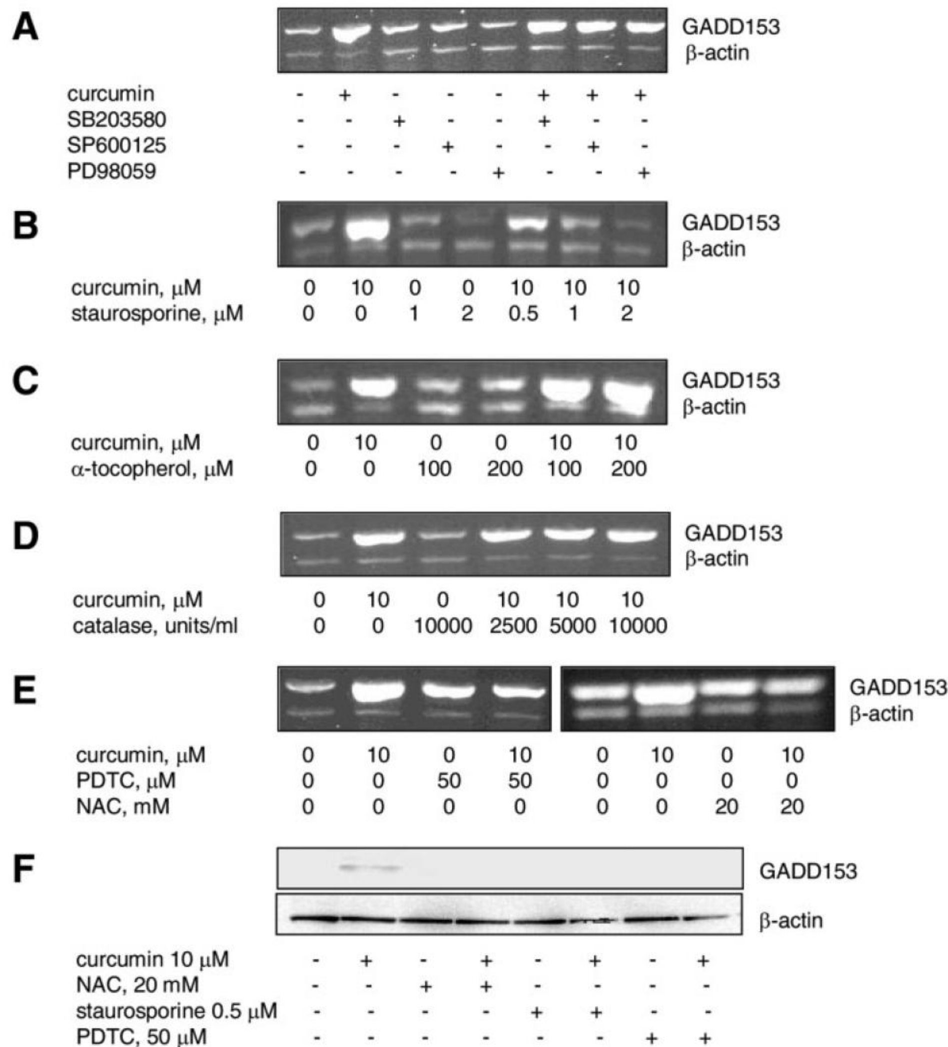

Fig. 5. Effect of protein kinase inhibitors and free radical scavengers on curcumin-induced up-regulation of GADD153 mRNA and protein expression in HCT-116 colonocytes. In testing selective MAPK inhibitors (**A**), cells were pre-treated for 0.5 h with either PD098059 (50 μM), SB203580 (10 μM) or SP600125 (10 μM), and then treated with curcumin (10 μM) for 5 h. In testing a general PKC inhibitor (**B**), cells were pre-treated with staurosporine for 0.5 h and then exposed to curcumin for 5 h. In testing free radical scavengers, cells were pre-treated with α-tocopherol (0–200 μM) for 24 h (**C**), pre-treated with 2500–10 000 U/ml catalase for 0.5 h (**D**), pre-treated with 50 μM PDTC or 20 mM NAC for 2 h (**E**), prior to exposing these different cell samples to curcumin (10 μM) for 5 h. Afterwards, multiplex relative RT–PCR analysis was then performed using gene-specific primers for the target gene, GADD153, and the internal control gene, β-actin (A–E). For certain samples, whole cell lysates were prepared for subsequent western-immunoblotting analysis of GADD153 protein and also β-actin (**F**). The results are representational of three different experiments.

similar findings by Moragoda and coworkers (4), who earlier reported that curcumin induced features characteristic of apoptosis in HCT-116 colonocytes. Concerning the cell death pathway, they also found activation of caspases, including caspase-3 that is the main executioner of cells (33). Furthermore, there was reduced expression of Bcl-XL, which is a known anti-apoptotic gene (34). Hence, down-regulation of Bcl-XL supposedly helped mediate the curcumin-treated colonocytes to undergo apoptosis. On the other hand, it is conceivable that curcumin increased the expression of other relevant genes, such as certain early-response genes, that could have also been potentially important in promoting apoptosis.

As the central novel finding in the present study, curcumin increased expression of the GADD153 gene, which has been acknowledged as a pro-apoptotic gene (13-17), in HCT-116 colonocytes. Because the GADD153 gene is typically induced in response to cellular DNA damage, it is suggested that curcumin-induced up-regulation of GADD153 mRNA expression was an early transcriptional response to the DNA damage caused by curcumin. The ability of Act D to essentially prevent curcumin-induced up-regulation of GADD153 mRNA expression supports the view that the effect of curcumin on GADD153 mRNA was due primarily to increased transcription rather than greater mRNA stability. However, other inducers of GADD153 gene expression increase GADD153

mRNA levels in distinct ways. For example, depriving cells of leucine (8) increased GADD153 mRNA expression that was attributed to both increased transcription and mRNA stability. Glutamine deprivation also induced GADD153 mRNA expression (9), but the primary way was by mRNA stabilization.

Insights into a possible molecular cascade leading to curcumin-induced GADD153 gene activation can be extrapolated from other studies. When p38/SAPK2 was activated by anisomycin in Jurkat T-lymphocytes, the levels of GADD153 transcript increased, but this effect was nullified with a

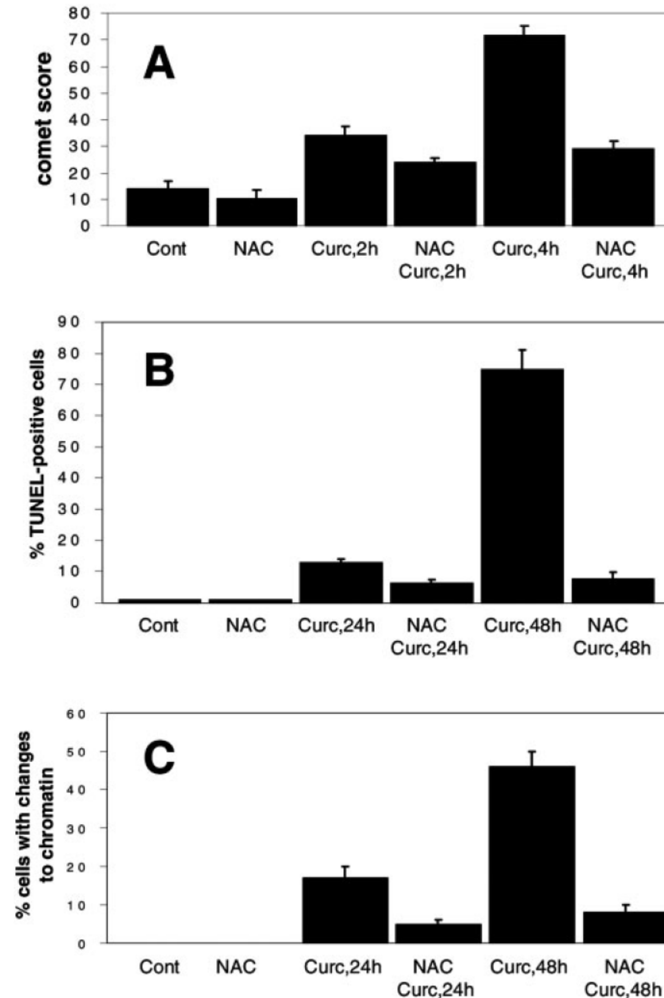

Fig. 6. Impact of NAC on HCT-116 colonocytes exposed to curcumin. Cells were pre-treated with 20 mM NAC for 2 h and then treated with 10 μM curcumin for 0–48 h. Afterwards, DNA damage was assessed with the comet assay (**A**), DNA degradation (associated with apoptosis) determined by the TUNEL assay (**B**), and chromatin condensation/fragmentation detected after DAPI staining (**C**).

selective chemical inhibitor of p38/SAPK2 (35). Exposing HCT-116 human colon adenocarcinoma cells to UV radiation, which promoted the formation of reactive oxygen species, induced transcriptional activation of GADD45 that involved ERK and JNK signaling based on the findings that a selective chemical inhibitor of ERK and a dominant negative mutant JNK1 expression vector blunted reporter construct activation (36). Hence, because GADD45 is a member of the GADD gene family, GADD153 would probably also be activated by the UV radiation, which causes oxidative DNA damage. In some cases of apoptosis, MAPK are required to increase gene expression of GADD genes, including GADD153, and to subsequently mediate apoptosis (37).

Collectively, the above studies would suggest that MAPK and reactive oxygen species could be involved in curcumin-induced up-regulation of GADD153 mRNA expression. This possibility is further suggested by a

recent study showing that curcumin caused activation of the stress-response, redox-sensitive heme oxygenase-1 gene, which is involved in MAPK signaling (38). Moreover, it has been reported that curcumin can promote the formation of reactive oxygen species (28,29). In the present study, negative results were obtained in assessing the potential of selective MAPK inhibitors to attenuate the effect of curcumin on GADD 153 mRNA expression in HCT-116 colonocytes. More specifically, three selective MAPK inhibitors (PD098059, SB203580 and SP600125) did not prevent or noticeably attentuate the increased GADD153 mRNA expression caused by curcumin. Therefore, these findings suggest that curcumin-induced up-regulation of GADD153 mRNA expression did not involve MAPK. As such, it seems that other protein kinases could be important. Along this line and despite its capability to also induce cell cycle arrest and apoptosis (39), the general PKC inhibitor, staurosporine, did prevent curcumin-induced GADD153 mRNA up-regulation. However, the role of PKC in curcumin-induced GADD153 gene activation remains unclear and will require further investigation. As a step in this direction, the involvement of specific PKC isoforms needs to be examined to help resolve the problem.

Regarding whether reactive oxygen species may be involved in curcumin-induced up-regulation of GADD153 mRNA expression in HCT-116 colonocytes, exposing the cells to H_2O_2 was found to up-regulate GADD153 mRNA expression. Hence, based on this finding and on studies reporting that curcumin can promote the formation of reactive oxygen species (28,29), it is conceivable that curcumin could have up- regulated GADD153 mRNA expression in HCT-116 colonocytes through H_2O_2. Whether curcumin would have a similar effect in vivo is open to speculation in light of few studies examining potential modulation of GADD153 gene expression by environmental factors at the whole organismic level. One study, however, found that GADD153 gene expression was up-regulated in the livers of mice fed alcohol (40), a substance that is known to induce liver damage associated with oxidative stress. Evidence of apoptosis was also found in liver specimens from the alcohol-fed mice. It is unlikely that curcumin would behave similarly as ethanol under such experimental conditions. In fact, curcumin is known to protect against hepatoxicity caused by hepatotoxic substances (41,42). Apparently, in other experimental situations specifically related to cancer chemoprevention, curcumin can promote death of cancer cells but not normal cells. For example, ingestion of curcumin by mice with familial adenomatous polyposis resulted in a reduction of intestinal tumors in the animals, in association with increased apoptosis in enterocytes of the intestinal tissue examined (43). Cellular apoptosis was increased in tumors of nude mice, which developed as a result of implantation with prostate cancer cells, when the animals were given curcumin in their diets (44). Unfortunately, in these two previous studies (43,44), the possible involvement of apoptotic genes in the stimulation of apoptosis in the curcumin-fed mice was not investigated. In particular, it would have been worthwhile to have examined GADD153.

If reactive oxygen species were actually involved in curcumin-induced up-regulation of GADD153 mRNA expression, free radical scavengers or antioxidants would be expected to blunt or prevent the effect of curcumin. As such, scavengers of reactive oxygen species would have been anticipated to prevent the effect of curcumin on GADD153 mRNA expression. Along this line, NAC, but not α-tocopherol and catalase, prevented curcumin-induced up-regulation of GADD153 mRNA expression. Therefore, the involvement of reactive oxygen species in curcumin-induced up-regulation of GADD153 mRNA expression remains somewhat unclear.

Since NAC is known to markedly increase intracellular concentrations of reduced glutathione (21), an effect not likely matched by α-tocopherol and catalase, this observation suggests that bolstering the concentrations of reduced glutathione (GSH) relative to oxidized glutathione (GSSG), as opposed to overall antioxidant stores, in the cells could be more important in counteracting the effects of curcumin. The intracellular GSH:GSSG ratio is known to influence the expression of redox-sensitive genes (45). A ratio favoring GSH, as expected with NAC, could help protect the cells against a stressful situation. Another major finding in the present study was the ability of NAC to inhibit DNA damage and the subsequent apoptosis both caused by curcumin in HCT-116 colonocytes.

Although the full significance of up-regulation of GADD gene expression in curcumin-treated cells is not known, the effect of curcumin on GADD153 in particular might contribute to the capacity of curcumin to induce apoptosis. Taken collectively, the current findings can be interpreted to propose a temporal sequence of

events regarding the effects of curcumin on HCT-116 colonocytes. The data suggest that curcumin first induces cellular DNA damage within 1 h. As a prompt consequence, GADD153 gene transcription is increased, and the newly synthesized GADD153 mRNA rapidly translated into GADD153 protein. However, it is probable that pre-existing or basal GADD153 transcript also becomes rapidly translated. As such, this could explain why GADD153 protein expression essentially paralleled GADD153 mRNA expression during the first few hours after initially treating cells with curcumin. In any event, the GADD153 protein synthesized then supposedly was later involved in mediating cell death, with early signs of apoptosis being widely detected after 24 h and late signs of apoptosis being widely detected at 36-48 h. There is substantial recognition that GADD153 has a role in triggering apoptosis (13-17). Therefore, it is conceivable that the expression of GADD153 protein, which preceded the appearance of recognizable features of apoptosis, in curcumin-treated HCT116 colonocytes could be involved in triggering apoptosis in these cells as well. This is not to say, however, that other apoptosis-related genes or their protein products are unimportant in the apoptosis induced by curcumin. Future studies are needed to determine whether GADD153 protein has a direct role in initiating apoptosis in HCT-116 colonocytes exposed to curcumin.

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