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Mitochondrial and Nuclear DNA Damage Induced by Curcumin in Human Hepatoma G2 Cells

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Curcumin is extensively used as a spice and pigment and has anticarcinogenic effects that could be linked to its antioxidant properties. However, some studies suggest that this natural compound possesses both pro- and antioxidative effects. In this study, we found that curcumin induced DNA damage to both the mitochondrial and nuclear genomes in human hepatoma G2 cells. Using quantitative polymerase chain reaction and immunocytochemistry staining of 8-hydroxydeoxyguanosine, we demonstrated that curcumin induced dose-dependent damage in both the mitochondrial and nuclear genomes and that the mitochondrial damage was more extensive. Nuclear DNA fragments were also evident in comet assays. The mechanism underlies the elevated level of reactive oxygen species and lipid peroxidation generated by curcumin. The lack of DNA damage at low doses suggested that low levels of curcumin does not induce DNA damage and may play an antioxidant role in carcinogenesis. But at high doses, we found that curcumin imposed oxidative stress and damaged DNA. These data reinforce the hypothesis that curcumin plays a conflicting dual role in carcinogenesis. Also, the extensive mitochondrial DNA damage might be an initial event triggering curcumin-induced cell death.

Key Words: **curcumin; DNA damage; mitochondrial DNA; nuclear DNA; quantitative polymerase chain reaction; HepG2 cells.**

Cancer mortality rates have risen throughout most of the past century and into the new millennium (Greenlee *et al.*, 2000). Cancer is already the leading cause of death in some countries (Tattersall and Thomas, 1999). This observation has engendered much research activity aimed at identifying cancer chemopreventive agents, especially naturally occurring compounds derived from the diet, which have the advantage of being relatively nontoxic (Jobin *et al.*, 1999). However, limited scientific evidence regarding the effectiveness of these natural derivatives in conjunction with a lack of mechanistic understanding of their actions have prevented their entry into the mainstream of medical care.

Curcumin (diferuloylmethane or 1,7-bis (4-hydroxy-3-methoxyphenol)-1,6-heptadiene-3,5-dione) is a natural compound present in turmeric, a rhizome of the plant *Curcuma longa* Linn. It is extensively used as a dietary spice and pigment in Asian cooking and also as an herbal medicine for inflammatory diseases. In certain countries, curcumin was consumed in the diet in amounts in excess of 100 mg/day without any side effects (Ammon and Wahl, 1991). Also in some other countries, up to 4 g per adult/day appears to lower the incidence rate of colorectal cancer (Sharma *et al.*, 2001). Further studies show that curcumin prevents cancer in many tissues of mice and rats and has been associated with regression of established solid malignancies in humans (Kuttan *et al.*, 1987; Plummer *et al.*, 2001). However, the mechanisms of action for curcumin are not well understood. Contradictory results have been obtained. For example, the antioxidant properties of curcumin are well established. In cellular experiments, curcumin suppressed the generation of reactive oxygen species (ROS) and protected against DNA damage induced by benz(a)pyren or H_2O_2 (Polasa *et al.*, 2004). On the other hand, curcumin can apparently act as a prooxidant. In human peripheral blood lymphocytes, curcumin itself resulted in ROS that damage DNA (Kelly *et al.*, 2001).

However, with regard to DNA damage, few studies have looked into the effect of curcumin on mitochondrial DNA (mtDNA). The 16-kb mitochondrial genome has been fully sequenced and encodes for only 13 proteins involved in the electron transport chain of oxidative phosphorylation (Linnane *et al.*, 1989). Depletion of the mtDNA reduces oxidative phosphorylation, decreases ATP synthesis, and induces dysfunction of the mitochondria. Mutations in mtDNA have been associated with aging, carcinogenic processes, and with several diseases such as neuronal degeneration and cardiovascular disease (Bandy and Davison, 1990). The mtDNA is also more susceptible to ROS compared to nuclear DNA (nDNA) and consequently is more prone to oxidative injury than the nDNA (Onuki *et al.*, 2004; Yakes and Van Houten, 1997).

The purpose of this study was to examine the effects of curcumin on both mtDNA and nDNA in human hepatoma G2 (HepG2) cells, a cell line that retains many characteristics

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TABLE 1
Comet Assay for DNA Damage in HepG2 Cells Treated with Curcumin

Curcumin (μg/ml)	Tail DNA (%)	Tail length (μm)	Tail moment (μm)
0	10.5 ± 1.4	38.1 ± 3.2	4.0 ± 0.5
2.5	18.7 ± 3.9*	71.5 ± 8.2**	13.3 ± 2.1**
5	27.3 ± 6.3**	107.3 ± 9.4**	29.2 ± 3.8**
10	39.1 ± 10.2**	151.4 ± 12.3**	59.1 ± 8.7**
20	65.1 ± 6.8**	232.7 ± 18.7**	151.0 ± 11.3**
40	87.0 ± 9.2**	413.0 ± 25.9**	334.2 ± 31.4**

*$p < 0.05$ versus control (0 μg/ml curcumin); **$p < 0.01$ versus control.

of hepatocytes such as the activities of phase I and phase II enzymes that play key roles in the activation and detoxification of DNA-reactive carcinogens. Therefore, HepG2 cells reflect the metabolism of xenobiotics in the human body better than other metabolically incompetent cells (Mersch-Sundermann *et al*., 2004), and they are a relevant *in vitro* model to evaluate the possible mitochondrial toxicity of newly developed drugs in liver cells and to examine a drug's interaction with liver functions (Sassa *et al*., 1987).

We are the first to evaluate the damage to nDNA and mtDNA induced by curcumin in the metabolically competent HepG2 cells. Using quantitative polymerase chain reaction (QPCR) and immunocytochemistry staining of 8-hydroxydeoxyguanosine (8-OHdG), we found that curcumin induced damage to both the mitochondrial and nuclear genomes in a dose-dependent manner and that the damage to mtDNA appeared more extensive than that of nDNA. The alkaline comet assay was used to determine nDNA fragmentation. As the conceivable cause of DNA damage, the level of ROS and lipid peroxidation were analyzed. Our data suggested that low doses of curcumin did not damage DNA, and at the low doses, curcumin could play an antioxidant role in carcinogenesis. But at high doses, curcumin itself imposed oxidative stress and damaged DNA. This study helps support the hypothesis that curcumin may play a conflicting dual role in carcinogenesis according to its doses. We also concluded that the extensive mtDNA damage may be an initial event triggering the observed curcumin-induced cell death.

MATERIALS AND METHODS

Cell culture and reagents. HepG2 cells (American Type Culture Collection (ATCC) HB-8065) were obtained from Peking Union Medical College (Peking, China) and cultured in minimum essential Eagle's medium (ATCC) containing 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 units/ml, Gibco), and streptomycin (100 μg/ml, Gibco). Curcumin was purchased from Xi'an Chongxin Natural Additives Co. Ltd. (Xi'an, China; purity > 95.6%). Curcumin was prepared as a 4-mg/ml stock solution in dimethyl sulfoxide (DMSO, Sigma, Louis, MO) and stored at −20°C. For each experiment, curcumin was diluted with cell culture medium to the concentration indicated with a final DMSO concentration of 1% (vol/vol). Two vehicles of culture medium with or without 1% DMSO were included in all subsequent experiments. In each experiment there was no difference between these controls; thus, we usually employed culture medium with 1% DMSO as the control for comparison.

Cell viability assay. Cell viability was assessed by the methyl thiazol tetrazolium bromide (MTT) assay as described previously (Mosmann, 1983). HepG2 cells were plated in a 96-well microtiter plate at a density of 1×10^4 cells per well in a final volume of 100 μl modified Eagle medium (MEM). The cells were treated with curcumin (2.5, 5, 10, 20, and 40 μg/ml) for 24, 48, and 72 h. After treatment, the cells were incubated with MTT solution (5 mg/ml) for 2 h at 37°C. The formazan crystals formed were dissolved in DMSO at 37°C for 1 h in the dark, and the absorbance was read at 595 nm in a microplate reader (BIO-RAD Model 3550).

DNA extraction and QPCR. HepG2 cells (1×10^6) were plated 24 h before treatment. Monolayer cultures were exposed to the indicated concentrations of curcumin for 2 h at 37°C. Cells were harvested immediately by trypsin (0.25% trypsin in phosphate-buffered saline [PBS] with 0.02% ethylenediaminetetraacetic acid [EDTA], Gibco). High–molecular weight DNA was isolated with the QIAamp DNA Micro kit (Qiagen, Hilden, Germany) as described by the manufacturer. DNA isolated by this technique has been shown to be suitable for QPCR (Onuki *et al.*, 2004). The DNA quantity was determined at 260 nm using an ultrospectrophotometer (Ultrospec II, LKB Biochrom, Cambridge, UK). The DNA samples were adjusted to a concentration of 0.5 mg/ml.

By examining a wide variety of DNA-damaging agents, QPCR assay has been proved to be quite robust for detecting both mtDNA and nDNA damage (Mandavilli *et al.*, 2005; Velsor *et al.*, 2004). This technique is based on the premise that DNA lesions, including oxidative damage such as strand breaks, base modifications, and abasic sites, will block the progression of the polymerase, resulting in a decrease in amplification of a target sequence (Yakes *et al.*, 1996), and only those DNA templates that do not contain polymerase-blocking lesions will be amplified. Thus, amplification is inversely proportional to DNA damage: the more lesions on the target DNA, the less the amplification. QPCR was performed as described by Yakes and Van Houten (1997), briefly in 50 μl volumes with the LA PCR Kit Ver. 2.1 (Takara, Dalian, China) using GeneAmp PCR System 2400 (Perkin-Elmer, Wellesley, MA). The reaction mixtures contained 2 μl template DNA, 5 μl 10 × LA PCR Buffer (Mg^{2+} plus), 8 μl deoxynucleoside triphosphate (dNTP) mixture, 1 μl of each primer, and 0.5 μl TaKaRa LA Taq. The primer nucleotide sequences were as follows: for the 16.2-kb fragment of the mitochondrial genome, 5′-TGAGGCCAAATATCATTCTGAGGGGC-3′ (RH1065) and 5′-TTTCATCATGCGGAGATGTTGGATGG-3′ (RH1066) (Yakes and Van Houten, 1997); and for the 17.5-kb flanking region of the β-globin gene (GenBank data base accession number, NT 009237), 5′-ACATGATTAGCAAAAGGGCCTAGCTTGGACTCAGA-3′ (bases 783–818), and 5′-TGCACCTGCTCTGTGATTATGACTATCCCACAGTC-3′ (bases 18357–18391).

The PCR was initiated at 75°C with hot-start addition of the polymerase and allowed to undergo the following thermocycler profile: an initial denaturation for 1 min at 94°C followed by 30 cycles of 98°C denaturation for 20 s and 68°C annealing and extension for 15 min. A final extension at 72°C was performed for 10 min. To ensure quantitative conditions, control reactions containing 50% control templates were included with each amplification. The reaction products were electrophoresed on a 0.8% agarose/TBE (Tris-borate-EDTA) gel containing ethidium bromide (1 μg/ml) at 120 V for 60 min. The amplified products were visualized on gels and quantitated with LabWorks (ver 4.6, UVP, BioImaging Systems, Upland, CA). Amplification of treated samples was then compared with controls, and the relative amplification was calculated. DNA lesion frequencies were also calculated as follows (Yakes and Van Houten, 1997). The amplification of damaged samples (A_D) was normalized to the amplification of nondamaged controls (A_O), resulting in a relative amplification ratio. Assuming a random distribution of lesions and using the Poisson equation [$f(x) = e^{-\lambda}\lambda^x/x!$, where λ is the average lesion frequency] for the control templates (i.e., the zero class; $x = 0$), the average lesion frequency per DNA strand was determined as $\lambda = \ln A_D/A_O$.

Immunocytochemistry staining for 8-OHdG. An immunoperoxidase method using a monoclonal 8-OHdG antibody has been developed for detection and quantitation of oxidative damage in single cells (Yarborough *et al.*, 1996). Using this method we detected 8-OHdG in both mtDNA and nDNA *in situ*, eliminating the need for isolation of DNA (Xu *et al.*, 1999). Exponentially growing cells were seeded onto coverslips in 12-well tissue culture plates at 1×10^5 cells and cultured for 24 h. Cells were treated with curcumin for 2 h at 37°C. H_2O_2 (20μM) was used as a positive control. After treatment, cells were rinsed twice with PBS and fixed with cold acetone for 10 min. To avoid detecting 8-OHdG incorporated into RNA, the fixed cultures were treated with RNase (100 μg/ml) for 1 h at 37°C. DNA was denatured for 5 min at 4°C and treated with 0.1% Triton X-100 for 5 min at 4°C. To block nonspecific antibody binding sites, 10% normal horse serum was used, and the cells were incubated with the primary antibody (JaICA, Fukuroi, Japan) in PBS (1:200) at 4°C overnight. Subsequently, using Ultrasensitive Streptavidin-peroxidase Kit (Maixin-Bio, Fujian, China), the cells were rinsed and biotin-conjugated secondary antibody was added for 30 min at room temperature, rinsed with PBS three times and streptavidin-peroxidase for 10 min at room temperature. Diaminobenzidene was applied as color presentation (3–10 min). For measurement of 8-OHdG of mtDNA, the cells were counterstained for nuclei with hematoxylin. The images were taken by microscope (Olympus BX-51, Omachi, Japan), and multiparameter image analysis software Image-pro plus 4.5.1 was used to quantify the staining intensity from 50 randomly selected cells per group per experiment. Staining data represent the average absorbance multiplied by 1000.

Comet assay. To detect cellular DNA damage as single-strand breaks, comet assay was performed as described by Singh and Stephens (1997). HepG2 cells (1×10^6 cells) were suspended in 2 ml MEM and incubated with curcumin (0, 2.5, 5, 10, 20, and 40 μg/ml) at 37°C for 1 h. H_2O_2 (20μM) was used as a positive control. After washing twice with PBS, the cells were suspended in 1 ml PBS. To avoid artifacts resulting from necrotic and apoptotic cells, the cell suspensions (50 μl) were mixed with Hoechst 33342 (8 μg/ml) and propidium iodide (50 μg/ml). After incubation in the dark for 15 min, the necrosis and apoptosis were identified under a fluorescent microscope (U-MWU2 filters). Only cell suspensions with no apoptotic cells and cell viabilities > 90% were used for determination of DNA fragments. Twenty microliters of the cell suspension was mixed with 160 μl of 0.6% low-melting agarose and placed on frosted slides prelayered with 1% regular agarose. After solidification of low-melting agarose, the slides were immersed in lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris, pH 10, and 1% sodium sarcosinate with 1% Triton X-100) at 4°C for 1 h. After the lysis, the slides were placed in alkaline solution (1mM Na_2EDTA and 300mM NaOH, pH 13) for 20 min to allow DNA unwinding, and then they were electrophoresed for 30 min at 200 mA. Cells were neutralized using 0.4M Tris (pH 7.5) and stained with 50 μl ethidium bromide (20 μg/ml). Slides were viewed at ×400 magnification using fluorescent microscopy with an excitation filter of 549 nm and barrier filter of 590 nm. Comets were quantitatively analyzed using Comet Assay Software Project casp-1.2.2 (University of Wroclaw, Poland). Each treatment was carried out in duplicate, and 100 randomly selected comets from two microscope slides were analyzed.

Measurement of intracellular ROS. The production of ROS was measured using the 2,7-dichlorofluorescein diacetate (DCFH-DA) method (Sohn *et al.*, 2005). DCFH-DA penetrates the cells and is hydrolyzed by intracellular esterases to the nonfluorescent DCFH, which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. Myhre *et al.* (2003) reported that DCFH-DA was more sensitive to H_2O_2 than other ROS. As curcumin is known to possess fluorescent properties, all our ROS experiments were corrected with the appropriate control sample containing only curcumin. HepG2 cells (5×10^5) were suspended in 2 ml medium and were incubated with curcumin (0, 2.5, 5, 10, 20, and 40 μg/ml) at 37°C for 1 h. H_2O_2 (20μM) was used as a positive control. Cells were washed twice with cold PBS, suspended in PBS at 5×10^5 cells/ml, and loaded with DCFH-DA at a final concentration of 5μM and incubated for 40 min at 37°C in the dark. The fluorescent intensity of the cell suspensions was detected using a fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan). Excitation and emission wavelengths were 485 and 550 nm, respectively. The results were expressed as fluorescent intensity per 1×10^6 cells.

Lipid peroxidation assay. Lipid peroxidation was determined by measurement of thiobarbituric acid–reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides (Leal *et al.*, 2005). After exposure to increasing concentrations of curcumin for 2 h, the cells (5×10^4) were lysed with Triton X-100. The lysed cells (250 μl) were placed into glass tubes and incubated in a water bath at 37°C for 1 h and followed by the addition of 400 μl 35% perchloric acid. Mixtures were centrifuged at 5000 × g for 10 min at room temperature, and 200 μl of 1.2% thiobarbituric acid was added to the supernatants (600 μl) and then placed in a boiling water bath for 30 min. After cooling, the absorbance was measured at 535 nm with BIO-RAD microplate reader Model 3550.

Statistical analysis. Results are expressed as means and SDs. Statistical analyses were performed with Student's *t*-test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Curcumin Inhibits the Growth of HepG2 Cells in a Concentration- and Time-Dependent Manner

The growth inhibition by curcumin in HepG2 cells was observed after incubation for 24 h, and the maximum growth inhibitory effects with curcumin were observed at 72 h (Fig. 1). Curcumin presented strong cytotoxic effects at concentrations above 20 μg/ml. The resulting growth curves showed that the inhibition was concentration and time dependent. The IC_{50} (inhibitory concentration 50%) values for 24, 48, and 72 h were 31.29 ± 0.90 μg/ml, 22.36 ± 1.45 μg/ml, and 16.43 ± 1.81 μg/ml, respectively.

Curcumin Leads to Dose-Dependent DNA Damage to both mtDNA and nDNA Detected by QPCR

QPCR was performed to detect polymerase-blocking lesions, such as abasic sites, strand breaks, and several damaged bases,

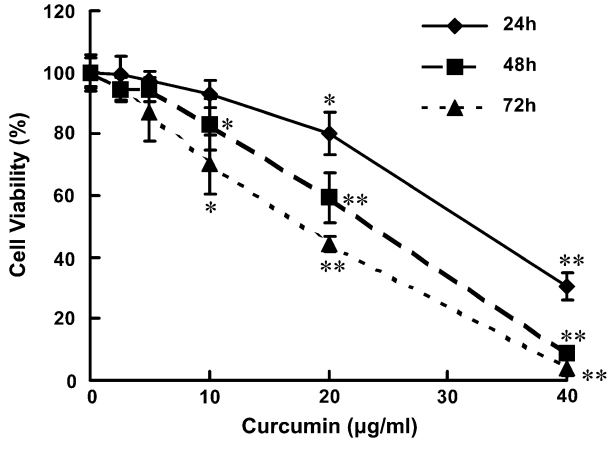

FIG. 1. Cytotoxicity in HepG2 cells after exposures to increasing concentrations of curcumin for 24, 48, and 72 h. Cytotoxicity was determined by MTT reduction and expressed as percent growth inhibition of controls. Each point is mean ± SD of three independent experiments (*$p < 0.05$ vs. control; **$p < 0.01$ vs. control).

including those generated by oxidative and alkylative damage. Thus, any decreased amplification of a damaged DNA template should be due to the blocking of Taq polymerase by DNA lesions and not the exhaustion of a critical reagent. For this to be the case, amplifications must be performed at a cycle number that is within the exponential phase of the PCR. This ensures that other components of the reaction, such as dNTPs, primers, or Taq polymerase, are not limiting. To further ensure that the limiting factor for amplification was nondamaged template availability, the amount of nondamaged template was titrated from 0.0625 to 1.0 μg to ensure that amplification signal was linearly related to the amount of nondamaged template (data not shown).

As shown in Figure 2, a 2-h exposure of HepG2 cells to curcumin led to a dose-dependent decrease in the amplification of both mtDNA and nDNA. Reduction in amplification of the mitochondrial genome occurred at significantly lower doses of curcumin (5 μg/ml) than for the nDNA (20 μg/ml curcumin). Damage to both genomes appeared to plateau at a higher concentration of curcumin. These data suggested that the curcumin-induced damage to mtDNA was more extensive than its damage to nDNA.

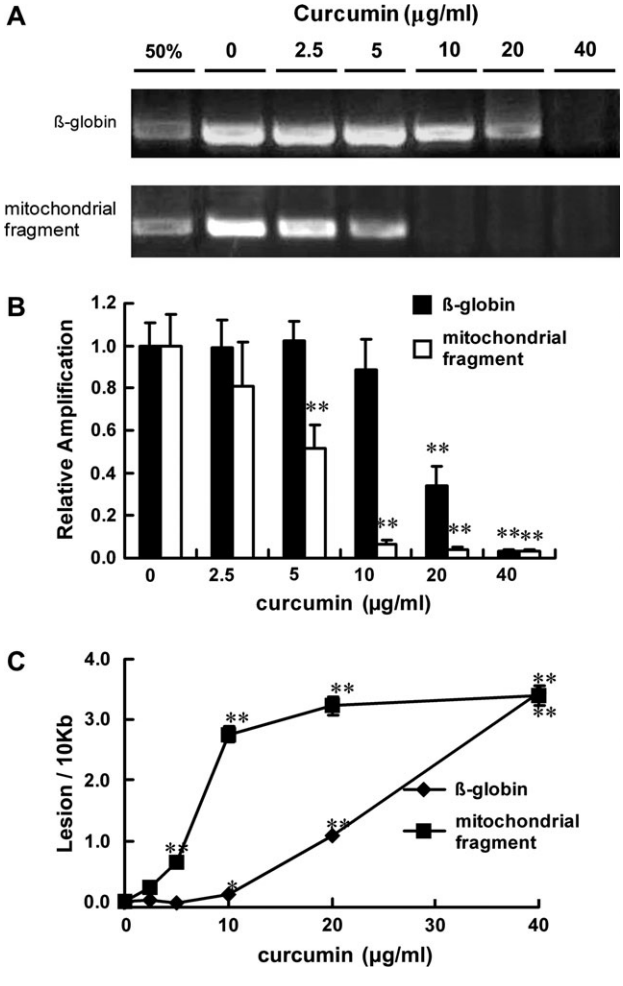

FIG. 2. QPCR amplification of the 17.5-kb β-globin fragment and the 16.2-kb fragment of the mitochondrial genome in HepG2 cells treated with increasing concentrations of curcumin for 2 h. The total DNA was extracted, and QPCR was performed using specific primers. The amplified DNA was analyzed on 0.8% agarose gel (A). Relative amplification (B) and lesion frequency (C) were analyzed. Data are the means ± SDs of three experiments (*$p < 0.05$ vs. control; **$p < 0.01$ vs. control).

8-OHdG Content Increased in Both mtDNA and nDNA at All Tested Concentrations

8-OHdG is the most widely measured oxidative damage to mtDNA as well as nDNA (Hayakava *et al*., 1992; Richter, 1995). In the present study, we employed an immunocytochemical approach using a monoclonal antibody against 8-OHdG to detect the 8-OHdG *in situ* in nuclei for nDNA damage and in the cytoplasm for mtDNA damage. H_2O_2 treatment to cells was used as a positive control for 8-OHdG formation. Our results showed that H_2O_2 treatment caused a significant increase ($p < 0.01$) in the staining intensity of 8-OHdG in HepG2 cells (data not shown). Figure 3 shows representative immunocytochemical staining for 8-OHdG in HepG2 cells with or without curcumin treatment. These results indicated that 8-OHdG staining was positive in both the nDNA and the mtDNA at all tested curcumin concentrations. As curcumin concentration increased, the staining intensity of 8-OHdG increased more in the mtDNA than in the nDNA, giving a 15-fold increase in 8-OHdG staining in mtDNA between 40 μg/ml and control compared to a 6-fold increase in nDNA staining. In agreement with our QPCR data, these results showed that the oxidative damage was more significant in mtDNA than in nDNA of HepG2 cells treated with curcumin.

Curcumin Induced nDNA Strand Breaks in HepG2 Cells

Comet assay, also called single-cell gel electrophoresis, allows detection of DNA fragments resulting from a wide variety of DNA damage (Uhl *et al.*, 2000). Using costaining with propidium iodide and Hoechst 33342, cell viability and any apoptosis were measured at the same time. In all groups, no apoptotic cells were observed, and the cell viabilities were more than 90% (data not shown). Thus, all cells observed were used for determining DNA damage. In the positive control group (H_2O_2 treated), a significant increase in tail moment (105.1 ± 5.8 μm, $p < 0.01$) was observed. As shown in Figure 4, curcumin increased the DNA migration in a dose-dependent manner. The presence of comet tails was evident after HepG2 cells were incubated with only 2.5 μg/ml curcumin, and the comet tail moment values increased by 9.3 μm (from 4 μm for the negative controls to 13.3 μm). More pronounced comets were detected in cells that had been incubated with 5 μg/ml curcumin (tail moment: 29.2 μm). Within the concentrations ranging from 10 to 40 μg/ml, there was a significant increase in the comet tail moment values from 59.1 to 334 μm, exceeding over 10, 30, and 80 times, compared to the nontreated cells. HepG2 cells that had been incubated with 40 μg/ml

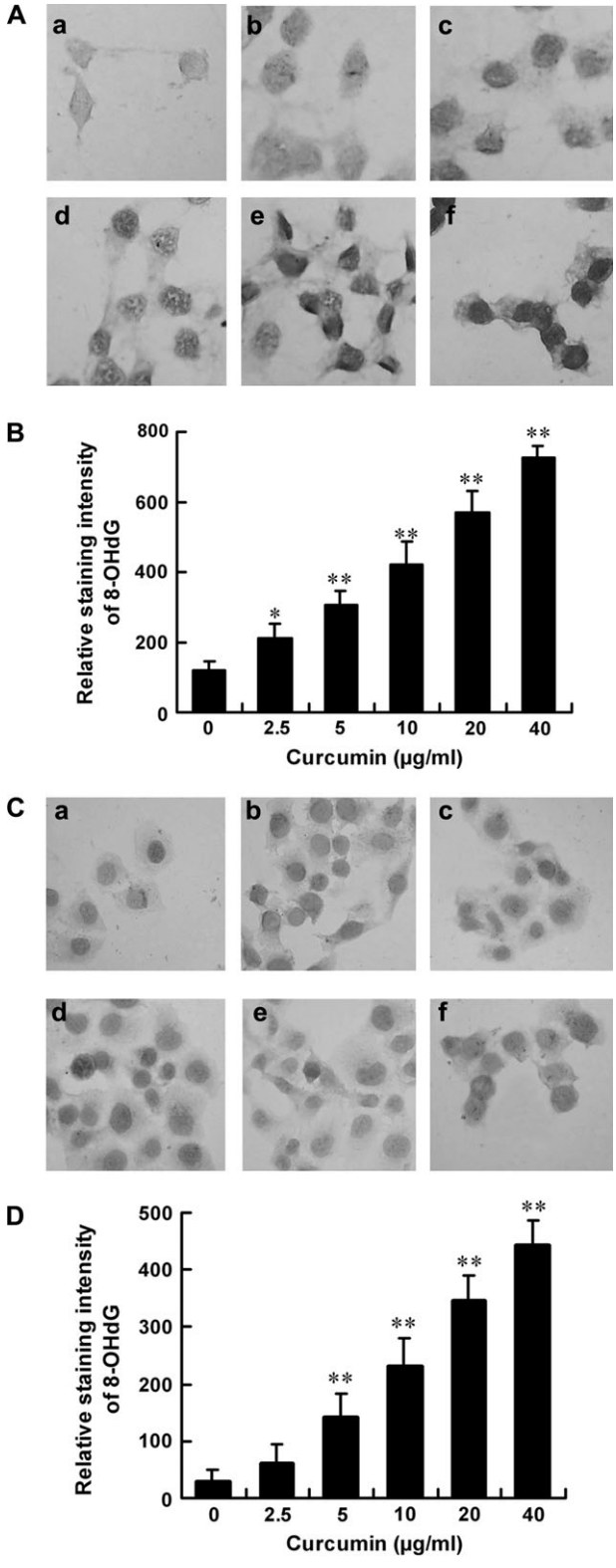

FIG. 3. Immunohistochemistry staining of 8-OHdG in HepG2 cells following a 2-h incubation with (a) 0 μg/ml, (b) 2.5 μg/ml, (c) 5 μg/ml, (d) 10 μg/ml, (e) 20 μg/ml, and (f) 40 μg/ml curcumin in nDNA (A) and mtDNA (C). The staining intensity of 8-OHdG was detected in HepG2 cells (B) and the cytoplasm (D) at all concentrations. Each figure is a typical representation of the 50 cells observed in at least three areas per group in three independent experiments; magnification: ×400 (*$p < 0.05$ vs. control; **$p < 0.01$ vs. control).

curcumin showed comets without heads and only tails, indicating much more extensive DNA damage by single-strand breaks (Table 1).

ROS Generated in HepG2 Cells Treated with High Concentrations of Curcumin

In this study, we employed DCFH-DA to measure intracellular generation of ROS. As a positive control, HepG2 cells were treated with H_2O_2. The DCF fluorescence intensity of this H_2O_2-treated group increased nearly threefold compared with the untreated control (data not shown). There was no increase in intracellular level of ROS after the 1-h incubation with 2.5 μg/ml curcumin ($p > 0.05$) compared to untreated. When the concentrations of curcumin were raised to 5 and 10 μg/ml, the level of ROS was significantly elevated ($p < 0.05$). The DCF fluorescence intensity increased further when the HepG2 cells were treated with 20 and 40 μg/ml curcumin ($p < 0.001$) compared to untreated. This dose response demonstrated that curcumin had a strong effect on ROS production (Fig. 5).

Treatment with Curcumin Increased Lipid Peroxidation in HepG2 Cells

Lipid peroxidation is the result of interactions between free radicals of diverse origins and unsaturated fatty acids and lipids. In this study, lipid peroxidation was determined by measurement of TBARS. In the positive control group (H_2O_2 treated), the TBARS absorbance increased twofold (data not shown). An increase in TBARS formation did not occur at the lower concentrations of curcumin (2.5 and 5 μg/ml). But TBARS formation significantly increased at higher concentrations of curcumin compared to negative control ($p < 0.01$; Fig. 6).

DISCUSSION

This is the first report of curcumin inducing DNA damage in both nuclear and mitochondrial genomes in HepG2 cells. HepG2 cells are suitable for detecting cytotoxic and genotoxic substances owing to their endogenous expression of a variety of antioxidant and xenobiotic-metabolizing enzymes. They are of great relevance to be a tool for chemical risk assessment (Mersch-Sundermann *et al.*, 2004).

The results of QPCR demonstrated that curcumin led to DNA damage in both the nuclear and mitochondrial genomes in a dose-dependent manner. Curcumin-induced mtDNA damage appeared more extensive than that of the nDNA.

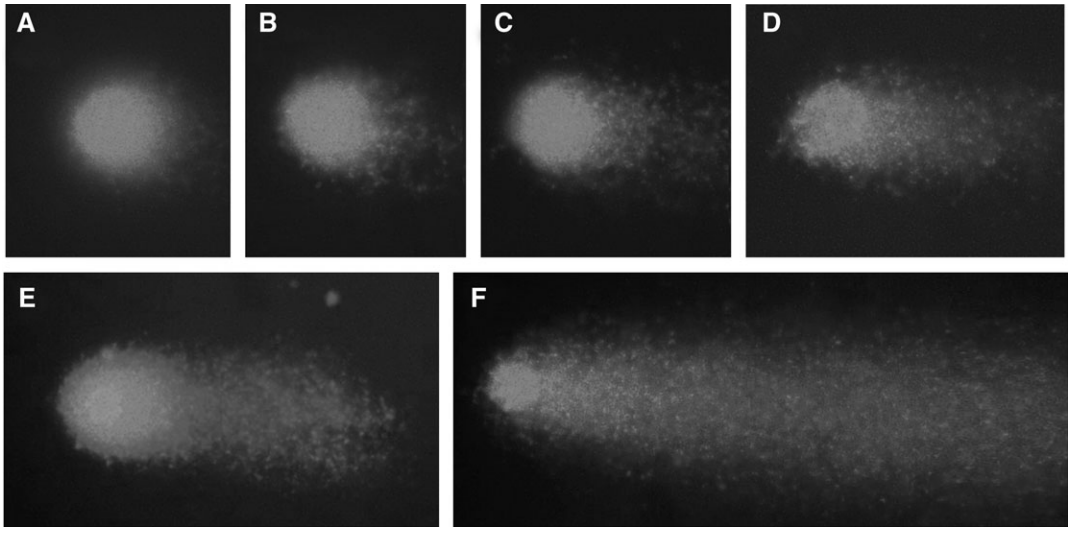

FIG. 4. Comet assays for DNA fragments in HepG2 cells exposed to (A) 0 μg/ml, (B) 2.5 μg/ml, (C) 5 μg/ml, (D) 10 μg/ml, (E) 20 μg/ml, and (F) 40 μg/ml curcumin for 1 h. Each figure represents a typical comet tail of the 100 observed cells from two slides in each experiment; magnification: ×400.

As a marker of oxidative DNA damage, 8-OHdG was detected using immunocytochemistry staining in both mtDNA and nDNA. 8-OHdG, constituting 10% of the total base adducts, is just one out of many lesions produced by ROS in DNA. However, it is the most widely used method to detect mtDNA oxidative modification because 8-OHdG is usually more abundant in oxidative damage in mtDNA than in nDNA (Hayakava *et al.*, 1992). The data of our study showed that 8-OHdG increased more significantly in the mtDNA than that of the nDNA, consistent with our QPCR results.

The vulnerability of the mitochondrial genome to oxidative damage could be due to many factors, including: (1) the absence of histones or DNA-binding proteins (Shoffner and Wallace, 1994); (2) having only a very basic repair mechanism (Croteau *et al.*, 1999); (3) genes consisting only of exons without introns; (4) replicating rapidly without a significant proofreading system; and (5) a mutation rate 10–100 times higher than that of nDNA (Kao *et al.*, 1998).

It is also known that mtDNA is more prone to oxidative damage as it is in closer contact to the ROS produced in the mitochondria. Lipids within the mitochondrial membrane contain components of the electron transport chain, and lipid peroxidation has been shown to be one potential source of continued DNA damage (Hruazkewycz, 1988). Secondary ROS reactions have the potential of overwhelming the repair

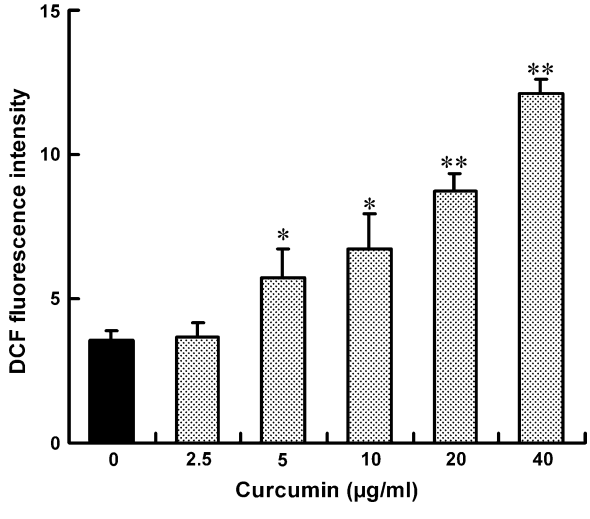

FIG. 5. ROS formation measured by DCFH-DA in HepG2 cells incubated with or without curcumin for 1 h. Data are mean fluorescent intensity ± SD calculated from three independent experiments (*$p < 0.05$ vs. control; **$p < 0.01$ vs. control).

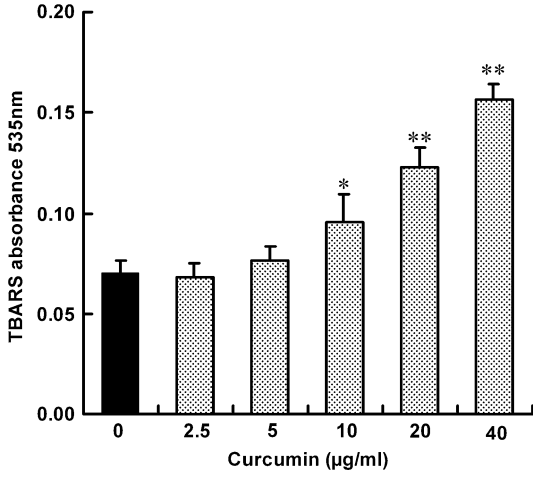

FIG. 6. TBARS formation in HepG2 cells after a 2-h exposure to curcumin. Data are means ± SDs of three independent experiments (*$p < 0.05$ vs. control; **$p < 0.01$ vs. control).

capacity of the mitochondrion and can lead to persistent DNA damage.

Therefore, we investigated the level of ROS and lipid peroxidation in HepG2 cells treated with curcumin. Our results provided support for the role of ROS and secondary ROS produced by lipid peroxidation in mtDNA damage. It inferred that ROS and lipid peroxidation generated directly or indirectly by curcumin underlies the mechanism of curcumin-induced DNA damage.

Comet assays also clearly showed that curcumin at high dosages can induce nDNA damage in HepG2 cells. Our data are in agreement with results obtained in gastric mucosa cells and human peripheral blood lymphocytes (Blasiak *et al*., 1999).

The damage to mtDNA is potentially more important than the damage to nDNA (Liang and Godley, 2003) because the genes coded by the mitochondrial genome are all expressed while nDNA contains a large amount of nontranscribed sequence. At the same time, mtDNA, unlike nDNA, is continuously replicated. Also, the mitochondrial genome codes essential components of the oxidative phosphorylation machinery, providing energy for every cell function. Hence, mtDNA damage potentially causes more adverse effects on cellular functions than does nDNA damage.

There are several hypotheses stating that mtDNA mutations could amplify the nDNA lesions, increase the probability of oncogene activation or tumor suppressor genes inactivation, and contribute to cellular aging and promotion of cancer (Bandy and Davison, 1990). So, curcumin-induced DNA damage in mtDNA and nDNA could both contribute to the carcinogenic process.

Curcumin is usually considered as a potent antioxidant, primarily because it is able to inhibit ROS generation and decrease lipid peroxidation (Chan *et al.*, 2005). In our study, curcumin at low doses had no effect on DNA damage, which does not contradict its observed antioxidant effect. However, we did find that curcumin at higher doses did cause oxidative DNA damage. Consequently, curcumin has both pro- and antioxidative effect according to doses.

Thus, it is important to know the concentration of the real-life exposure. Curcumin exhibits low oral bioavailability in rodents, and absorbed curcumin undergoes rapid first-pass metabolism and excretion in the bile (Sharma *et al*., 2005). In a clinical study of high-dose oral curcumin performed by Cheng *et al.* (2001), the 8-g/day dose resulted in a peak serum concentration of 1.77 ± 1.87μM. In another study, coadministration of piperine, primarily found in the fruit of the pepper vine *Piper nigrum* and also found in other vegetables and spices such as hot jalapeno peppers, may increase the systemic bioavailability of curcumin (Shoba *et al*., 1998). In the same study, coingestion of curcumin with 20 mg of the pepper constituent 1-piperoylpiperidine appeared to increase curcumin's bioavailability by 2000%. Combining the data from the two studies, the peak serum concentration of curcumin may be about 35.4μM (about 13 μg/ml). Also, although curcumin was rapidly biotransformed, the metabolites may retain the pharmacologic properties of curcumin. So the relatively low concentrations of curcumin in serum or tissues may not necessarily reflect the total biologic activity of oral curcumin. All these data indicate that the effect of DNA damage we observed *in vitro* could occur *in vivo*. Consuming high doses of curcumin should be avoided, and the coingestion of piperine-containing foods with curcumin should be limited.

In summary, the findings of the DNA damage induced by curcumin in a dose-dependent manner lead us to hypothesize that curcumin may play a dual role in carcinogenesis. The extensive curcumin-induced mtDNA damage shed light on a possible additional mechanism of cytotoxicity by curcumin. It is very important to establish the circumstances of the oxidative mode of curcumin, and the bioactivities of curcumin at low dose need further study.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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