Curcumin induces caspase-3-dependent apoptotic pathway but inhibits DNA fragmentation factor 40/caspase-activated DNase endonuclease in human Jurkat cells

Ewa Sikora,[1] Anna Bielak-Żmijewska,[1] Adriana Magalska,[1] Katarzyna Piwocka,[1] Grazyna Mosieniak,[1] Magdalena Kalinowska,[3] Piotr Widlak,[3] Iwona A. Cymerman,[2] and Janusz M. Bujnicki[2]

[1]Laboratory of Molecular Bases of Aging, Nencki Institute of Experimental Biology; [2]Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cellular Biology in Warsaw, Warsaw, Poland; and [3]Maria Sklodowska-Curie Cancer Center and Institute of Oncology, Branch in Gliwice, Gliwice, Poland

Abstract

Curcumin is a natural pigment that has been shown to induce cell death in many cancer cells; however, the death mode depends on the cell type and curcumin concentration. Here we show that, in Jurkat cells, 50 μmol/L curcumin severely lowers cell survival and induces initial stage of chromatin condensation. It also induces caspase-3, which is sufficient to cleave DNA fragmentation factor 45 [DFF45/inhibitor of caspase-activated DNase (ICAD)], the inhibitor of DFF40/CAD endonuclease. However, the release of DFF40/CAD from its inhibitor does not lead to oligonucleosomal DNA degradation in curcumin-treated cells. Moreover, curcumin treatment protects cells from UVC-induced oligonucleosomal DNA degradation. In biochemical experiments using recombinant DFF activated with caspase-3, we show that curcumin inhibits plasmid DNA and chromatin degradation although it does not prevent activation of DFF40/CAD endonuclease after its release from the inhibitor. Using DNA-binding assay, we show that curcumin does not disrupt the DNA-DFF40/CAD interaction. Instead, molecular modeling indicates that the inhibitory effect of curcumin on DFF40/CAD activity results from curcumin binding to the active center of DFF40/CAD endonuclease. [Mol Cancer Ther 2006;5(4):927–34]

Received 9/8/05; revised 12/19/05; accepted 2/15/06.

Grant support: Scholarship from the Postgraduate School of Molecular Medicine at the Medical University of Warsaw (I.A. Cymerman); European Molecular Biology Organization and Howard Hughes Medical Institute Young Investigator Program (J.M. Bujnicki); and Ministry of Science and Information Society and Technologies grant 3P05A10424 (P. Widlak).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Ewa Sikora, Laboratory of Molecular Bases of Aging, Nencki Institute of Experimental Biology, 3 Pasteura, 02-093 Warsaw, Poland. Phone: 4822-5892436; Fax: 11-48-228225342. E-mail: e.sikora@nencki.gov.pl

doi:10.1158/1535-7163.MCT-05-0360

Introduction

Curcumin (diferuloyl methane) is a naturally occurring yellow pigment derived from the rhizome of *Curcuma longa*, a plant which has been cultivated in Asia for centuries. Curcumin exhibits a variety of pharmacologic effects including anti-inflammatory, anti-infectious, and anticancer activities (1). The massive exposure of populations worldwide to curcumin and its many uses has led to studies aimed at elucidating the mechanism of its activities, in particular the anticancer activity. These culminated with several phase I human trials that have shown this compound to be well tolerated (2–5). Curcumin has been shown to inhibit tumor promotion of skin, oral, intestinal, and colon cancers in experimental animals. It has been also shown that curcumin can inhibit proliferation and/or induce cell death in *in vitro* experiments. The most common cell death mode on curcumin treatment seems to be apoptosis (reviewed in refs. 1, 6). Two major apoptotic pathways exist: the death receptor and the mitochondrial pathways (reviewed in refs. 7, 8). Multiple apoptotic stimuli trigger the activation of proteases called caspases, which in turn initiate and execute the apoptotic program (reviewed in ref. 9). One of the hallmarks of the terminal stages of apoptosis is internucleosomal DNA breakdown, which was first recognized by Wyllie et al. (10). Recent years have led to the discovery of two major apoptotic nucleases, termed DNA fragmentation factor (DFF) or caspase-activated DNase (CAD), and endonuclease G. Both endonucleases attack chromatin to yield 3-hydroxyl and 5-phosphate termini, first creating 50- to 300-kb cleavage products and then oligonucleosomal fragmentation, but these nucleases show different cellular locations and are regulated in fundamentally different ways. In nonapoptotic cells, DFF exists in the nucleus as a heterodimer, composed of a 45-kDa chaperone, an inhibitory subunit [DFF45; also called inhibitor of CAD (ICAD-L)], and a 40-kDa latent nuclease subunit (DFF40/CAD). Apoptotic activation of caspase-3 or caspase-7 results in the cleavage of DFF45/ICAD and the release of the DFF40/CAD nuclease that forms active homo-oligomers (reviewed in ref. 11). Although activation of the executorial caspases seems to be indispensable for realization of the apoptotic program, several forms of cell demise have been shown to be caspase independent or even accelerated by caspase inhibitors (reviewed in ref. 12).

Previously, we showed that curcumin induced death in Jurkat cells without oligonucleosomal DNA degradation typical for caspase-3-dependent apoptosis but with a morphology distinct from necrosis (13). In this article, we show that, unexpectedly, curcumin treatment does in fact activate caspase-3, which then cleaves its substrates, poly(ADP-ribose) polymerase 1 and DFF45/ICAD. However, the release of DFF40/CAD from its inhibitor does not lead to oligonucleosomal DNA degradation. We provide data indicating that curcumin induces the apoptotic pathway but, at the same time, protects cells against oligonucleosomal DNA degradation, apparently inhibiting the endonuclease activity of DFF40/CAD without blocking its binding to DNA.

Materials and Methods

Cell Culture and Treatments

Jurkat T-cell leukemia cells (DSMC ACC 282) were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, and antibiotics at 37°C in a humidified atmosphere of 5% CO_2. To induce apoptosis, cells were treated with curcumin (Merck, Darmstadt, Germany) or irradiated with UVC, or both as previously described (14).

DNA Content Analysis by Flow Cytometry

Cells were analyzed for DNA content by flow cytometry. One million cells were collected, washed, and suspended in Nicoletti buffer [0.1% sodium citrate (pH 7.4), 0.1% Triton X-100, and 50 μg/mL propidium iodide]. DNA content was determined on a flow cytometer (FACSCalibur, Becton Dickinson, Warsaw, Poland). The sub-G_1 fraction represents apoptotic cells; cellular debris was excluded from the analysis. The percentage of apoptotic cells induced in specific experimental conditions was calculated according the following formula: (percentage of induced apoptosis − percentage of spontaneous apoptosis) / (100 − percentage of spontaneous apoptosis) × 100%.

Cell Viability Measurement

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, Poznan, Poland) assay as described by the manufacturer. Cell viability was measured by propidium iodide assay (Sigma) as described by the manufacturer.

Cell Morphology

Morphologic observation was done after Hoechst 33258 staining (Molecular Probes, Eugene, OR). Cells (0.2×10^6-0.3×10^6) were centrifuged on cytospin, fixed with 70% ethanol, washed in PBS, and stained for 10 minutes with 1 μmol/L Hoechst 33258 dye. Samples were visualized by epifluorescence microscopy (Nikon, Tokyo, Japan) and images were acquired with a color CCD camera.

Caspase-3 Activation

The activation of caspase-3 was analyzed by flow cytometry using phycoerythrin-conjugated anti–active caspase-3 monoclonal antibody (BD PharMingen, Warsaw, Poland) according to the protocol of the manufacturer. For negative controls, cells were coincubated with caspase inhibitor Z-VAD-fmk (Bachem, St. Helens, United Kingdom) at 50 μmol/L.

Western Blotting

Cells were collected and washed twice with cold PBS. For cytoplasmic extracts, cells were incubated for 15 minutes on ice in lysis buffer composed of 0.5% NP40, 0.25 mol/L sucrose, 10 mmol/L KCl, 4 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L DTT, and 20 mmol/L Tris-HCl (pH 7.5), supplemented with a mixture of protease inhibitors (Complete, Roche Applied Science, Mannheim, Germany). After incubation, the cell lysates were centrifuged for 15 minutes at 10,000 × *g* and the resulting supernatant was referred to as the cytoplasmic extract. To obtain total cell proteins, cells were collected and washed twice with cold PBS, and then incubated for 15 minutes on ice in radioimmunoprecipitation assay lysis buffer composed of 1% Triton X-10, 150 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 7.5), 1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, supplemented with Complete. After incubation, cell lysates were centrifuged for 15 minutes at 10,000 × *g*.

Proteins (40 μg/lane) were separated on 12% SDS-PAGE and electrotrasferred onto nitrocellulose membrane (Hybond-C, Amersham Int., Little Chalfont, United Kingdom). Membranes were probed overnight at 4°C with rabbit polyclonal anti-DFF40 (1:500), anti-DFF45 (1:500), or mouse monoclonal anti–poly(ADP-ribose) polymerase 1 (1:500; BD Biosciences, Warsaw, Poland). Specific proteins were visualized with horseradish peroxidase–conjugated anti-immunoglobulin antibodies and the enhanced chemiluminescence reagent (Amersham Int.).

Purification and Activity Assay of Recombinant DFF40/DFF45

Hexa-His-tagged human DFF40 was coexpressed from a polycistronic vector with human DFF45. Hexa-His-tagged hamster caspase-3 was expressed in *E. coli* and purified as previously described (15) using Ni-NTA agarose (Qiagen, MA). DFF40/DFF45 heterodimer (0.5 pmol) was activated by incubation with caspase-3 (0.1 pmol) for 15 minutes at room temperature, and then caspase-3 was inhibited with 10 μmol/L Ac-DEVD-Cho. Nuclease activity was assayed as follows: 1 μg of plasmid DNA (4.2 kb) or 3 μg of chromatin substrate (nuclei purified from HeLa cells lysed with NP40) were incubated with activated DFF for 30 minutes at 33°C in a buffer consisting of 10 mmol/L KCl, 50 mmol/L NaCl, 4 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L DTT, and 20 mmol/L Tris-HCl (pH 7.5) in the presence or absence of curcumin. The reaction was stopped by adding EDTA, SDS, and proteinase K, and deproteinized samples were run on 1.5% agarose gels and stained with ethidium bromide.

Homology Modeling of Human DFF40/CAD and Docking of Ligands

A model of human DFF40/CAD was generated based on the optimal alignment to the template structure of the *Mus musculus* CAD nuclease (PDB code 1V0D). Homology modeling and validation of the model were carried out using the ''FRankenstein's monster'' approach (16), which was shown to be one of the most reliable methods for template-based protein modeling in the course of the last

two CASP competitions. The model of DFF40/CAD-DNA complex was created by superposition of the common spatial motif ''ββα-Me finger'' in DFF40/CAD and Vvn and I-PpoI nucleases, for which protein-DNA cocrystal structures are available. The fragment of the DNA molecule that exhibited similar conformation in Vvn and I-PpoI, together with the catalytic metal ion, was merged with the coordinates of the DFF40/CAD model. Docking of curcumin was done using the SURFLEX1.29 algorithm (17), which uses an idealized pose called a protomol. Both input molecules, the receptor (DFF40/CAD) and the ligand (curcumin), were protonated and the docking was run using the ''whole molecule'' approach with the ''none'' option for protomol definition. Docking was carried out for the DFF40/CAD structure without any ligands, with the metal ion, and with the metal ion and the DNA.

Nickel Affinity Pulldown Assay and DNA Binding Assay

One microgram of 6-His-DFF40/DFF45 heterodimer was activated with caspase-3 and bound to 50 μL of Ni-NTA agarose, and then incubated for 10 minutes at 20°C in the presence or absence of curcumin (100 μmol/L) in buffer consisting of 50 mmol/L NaCl, 1 mmol/L DTT, 0.01% Triton X-100, and 20 mmol/L Tris-HCl (pH 7.5). The beads were then washed five times with 1 mL of the same buffer to remove unbound curcumin. The nuclease was eluted with 0.25 mol/L imidazole and incubated with DNA for 30 minutes at 33°C in the presence of 4 mmol/L $MgCl_2$ to asses for its activity.

For DNA binding assay, 100 ng of ^{32}P-end-labeled linear plasmid DNA were incubated with 10 nmol of caspase-3-activated DFF in the presence of different amounts of curcumin for 15 minutes at 20°C in 20 μL of binding buffer consisting of 10 mmol/L KCl, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 20 mmol/L Tris-HCl (pH 7.5), 0.1% Triton X-100, 5% glycerol, and 0.25 mg/mL of bovine serum albumin. Reaction mixtures were then separated electrophoretically on 0.9% agarose (0.5× Tris-borate EDTA) gel, which was then dried and autoradiographed.

Results

Curcumin Induces Cell Death but Protects Human T Cells against Apoptotic DNA Fragmentation

Curcumin has been shown to induce apoptosis in many cancer cells (reviewed in ref. 6). However, in our hands, 50 μmol/L curcumin induced death in Jurkat cells without the oligonucleosomal DNA fragmentation and morphology typical for apoptotic cells (13, 18). Here we further analyze the influence of curcumin on DNA fragmentation in Jurkat cells. Data presented in Fig. 1A show that 8 and 24 hours after UVC irradiation, >25% of Jurkat cells revealed significant DNA fragmentation and were present in a sub-G_1 fraction. In contrast, the sub-G_1 fraction did not exceed 2% to 3% for curcumin-treated cells. Most interestingly, the sub-G_1 fraction of cells irradiated with UVC and then incubated in the presence of 50 μmol/L curcumin was very close to that of cells treated with curcumin alone. A similar inhibition of DNA fragmentation by curcumin was previously observed in UVC-treated Jurkat cells when assayed by gel electrophoresis (''DNA ladder''; ref. 18). Apoptotic chromatin condensation correlates temporally with DNA fragmentation and, often, its efficiency depends on the degree of internucleosomal cleavage (19). Accordingly, UVC-irradiated cells, but not curcumin- or UVC/curcumin–treated ones, revealed characteristic chromatin condensation and fragmentation, followed by formation of apoptotic bodies typical for apoptosis, thus confirming the inference from the above DNA content analysis (Fig. 1B).

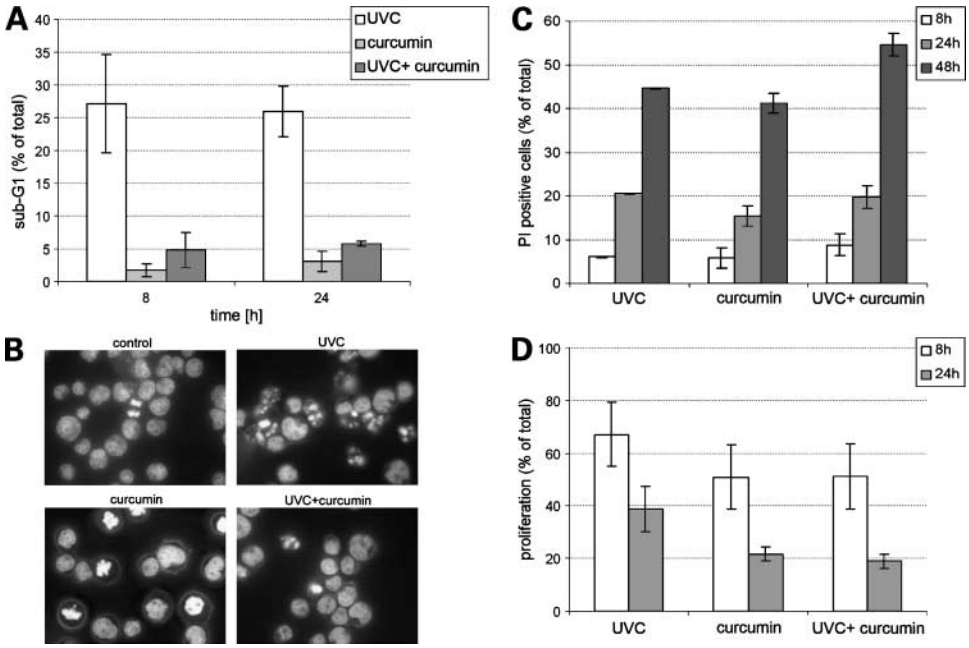

Figure 1. DNA fragmentation (**A**), chromatin condensation (**B**), viability index (**C**), and proliferation index (**D**) of Jurkat cells treated with curcumin, UVC irradiated, or treated with UVC/curcumin combination. **A**, DNA fragmentation was assessed by flow cytometry and sub-G_1 fraction was calculated. *Columns,* mean from four independent experiments; *bars,* SD. **B**, chromatin condensation was observed microscopically after staining of cells with Hoechst 33258. Cell viability (**C**) was measured by propidium iodide assay and cell proliferation (**D**) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values are in relation to untreated control. *Columns,* mean from four independent experiments; *bars,* SD.

Although curcumin apparently protected Jurkat cells against apoptotic DNA fragmentation, it did not prevent cell death. In fact, curcumin was a very effective cell death inducer. Data presented in Fig. 1C show that ~40% of Jurkat cells died during a 48-hour treatment with curcumin, which was tested using propidium iodide assay. More of dying cells could be observed after UVC or combined UVC/curcumin treatment whereas UVC irradiation alone was less efficient in inducing cell death. Curcumin was more effective than UVC in proliferation inhibition as was shown by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 1D).

Curcumin Induces Caspase-3 Activation followed by Cleavage of Its Substrates

In the majority of cells, the apoptotic oligonucleosomal DNA degradation results from the activity of the DFF40/CAD endonuclease. The nuclease is activated on caspase-3-catalyzed cleavage of its inhibitor DFF45/ICAD (20–22). Thus, bearing in mind the lack of oligonucleosomal fragmentation documented above, one could conclude that the cell death induced by curcumin alone or in combination with UVC irradiation should be caspase-3 independent. To verify this assumption, the presence of activated caspase-3 was analyzed using a flow cytometry method (Fig. 2A). Unexpectedly, the analysis revealed the presence of active caspase-3 in Jurkat cells treated with curcumin alone, UVC alone, or UVC irradiation followed by curcumin treatment. However, the percentage of caspase-3-positive cells was the lowest in curcumin-treated cells and was <30%, compared with 40% and 50% in UVC-irradiated and UVC/curcumin–treated cells, respectively. The additive effect of UVC/curcumin treatment on caspase-3-positive cells does reflect the proportion of dead cells (propidium iodide positive; Fig. 1C). The activity of caspase-3 was assessed in the same cells by detection of an 89-kDa fragment of poly(ADP-ribose) polymerase 1, a product of the poly (ADP-ribose) polymerase 1 cleavage by caspase-3 (Fig. 2B). The truncated form of poly(ADP-ribose) polymerase 1 could be detected in Jurkat cells both irradiated with UVC, either with or without curcumin postincubation, and treated with curcumin alone. This proved that curcumin not only does not prevent caspase-3 activation in UVC-irradiated cells but also induces caspase-3 activation by itself, although curcumin is clearly a weaker activator of caspase-3 than UVC.

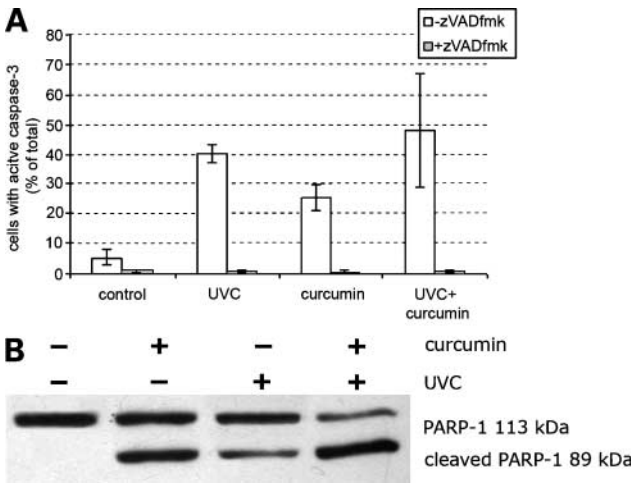

Figure 2. Activation of caspase-3 in Jurkat cells 8 h after UVC irradiation, treatment with 50 μmol/L curcumin, or UVC irradiation followed by curcumin treatment. The percentage of cells that contained active form of caspase-3 was measured by flow cytometry. **A**, data for cells cotreated with pan-caspase inhibitor Z-VAD-fmk. *Columns*, mean from four independent experiments; *bars*, SD. **B**, the truncated form of poly(ADP-ribose) polymerase 1 (*PARP-1*) was assessed in whole-cell lysates by Western blotting.

Curcumin Induces Degradation of DFF45/ICAD, Leading to the Formation of a Potentially Active DFF40/CAD Nuclease

In healthy nonapoptotic cells, the DFF40/CAD nuclease resides in the nucleus as a heterodimer with its inhibitor DFF45/ICAD (a 35-kDa splicing variant of DFF45, DFF35/ICAD-S, is present in the cytoplasm). Activation of caspase-3 results in the cleavage of DFF45/ICAD and release of DFF40/CAD, which then forms active homo-oligomers (15, 19, 23). Here we show that DFF40/CAD is present in Jurkat cells, and its total level remains essentially unchanged in cells treated with curcumin and is slightly elevated in UVC- or UVC/curcumin–treated cells, as measured by Western blotting in whole-cell lysates (Fig. 3A). In marked contrast to the level of DFF40/CAD, the level of DFF45/ICAD-L (and DFF35/ICAD-S as well) significantly decreased in UVC-irradiated or curcumin-treated Jurkat cells (Fig. 3B). As expected, the extent of DFF45/35 disappearance was proportional to the level of active caspase-3 (Fig. 2).

The homodimer of DFF40/CAD is the basic structural form of the active nuclease after its release from the inhibitor (23). Several lines of evidence, however, have shown that activated DFF40/CAD can also form larger homo-oligomers, which are resistant to extraction from nuclei of apoptotic cells (24, 25). To check whether DFF40/CAD forms such nuclear oligomers resistant to extraction, the levels of DFF40/CAD were measured in cytoplasmic extracts obtained by cell lysis with 0.5% NP40 with nuclei removed by centrifugation (Fig. 3C). As expected, DFF40/CAD was almost quantitatively extracted from nuclei of control cells. However, only a fraction of DFF40/CAD could be extracted from nuclei of cells with caspase-3 activated by either UVC irradiation, curcumin treatment, or UVC/curcumin treatment. This result further confirmed that curcumin does not prevent activation and oligomerization of the nuclease after its release from the inhibitor.

Curcumin Inhibits DFF40/CAD Endonuclease Activity by Direct Interaction with the Enzyme

We have shown above that proteolytic removal of the nuclease inhibitor does not lead to an induction of internucleosomal chromatin cleavage in curcumin-treated cells. This unexpected finding could be explained if one assumed that curcumin inhibits the activity of the nuclease following its activation by caspase-3. To test this possibility, we did experiments using recombinant human heterodimer

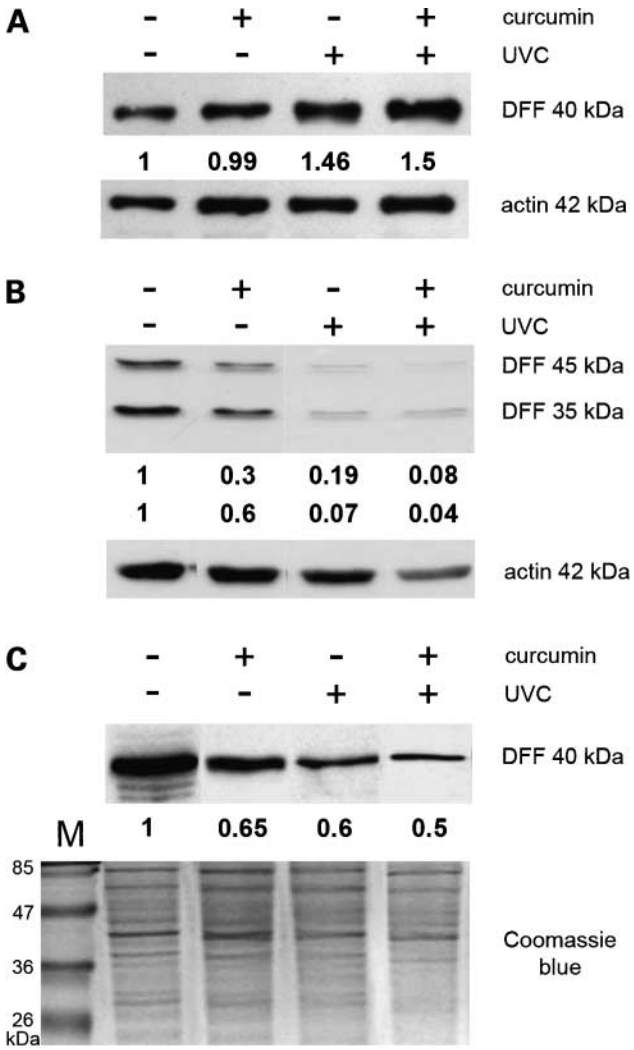

Figure 3. Content of DFF subunits in Jurkat cells 8 h after UVC irradiation, treatment with 50 μmol/L curcumin, or UVC irradiation followed by curcumin treatment. DFF40/CAD (**A**) and DFF45/ICAD (**B**) were assessed by Western blotting in whole-cell lysates. The content of DFF40/CAD was also assessed in cytoplasmic extracts (**C**). Quantification of data by densitometry is shown in each case.

DFF40/DFF45 (DFF hereafter; see Materials and Methods for detailed description). The caspase-activated DFF was incubated with either naked DNA or chromatin as substrates in the absence or in the presence of curcumin at 30 or 100 μmol/L (Fig. 4). The presence of 30 μmol/L curcumin reduced the nucleolytic activity of the enzyme severalfold with both substrates. At the higher (100 μmol/L) concentration, curcumin almost totally inhibited the cleavage of DNA and chromatin by the caspase-3-activated DFF.

We assumed that curcumin could affect DNA cleavage by DFF due to binding to either the enzyme or its substrates. Because we showed by spectrophotometrical analysis that curcumin did not bind to chromatin (not shown), we have analyzed by molecular modeling the possibility that curcumin binds to the endonuclease.

A Docking Study: Curcumin Binds to DFF40/CAD and Blocks the Active Site

DFF40/CAD consists of two domains: NTD, responsible for the interaction with DFF45/ICAD, and CTD, which takes part in the DNA binding and cleavage. CTD comprises the catalytic center, formed by the so-called ββα-Me-finger motif (26), and a helical extension, which may form the principal DNA-binding site (ref. 23, and this work). To identify the preferred binding site of curcumin on the DFF40/CAD surface, we generated a homology model of human CAD based on the crystal structure of the closely related murine enzyme (PDB code 1V0D; ref. 23) and carried out computational docking of curcumin (see Materials and Methods for technical details). All top-scoring conformations of curcumin were found to bind to the active site of DFF40/CAD, with two 2-methoxyphenol rings placed adjacent to the catalytic His260. Figure 5A shows the model of DFF40/CAD-DNA complex obtained by superposition with an enzyme-DNA complex of another ββα-Me-finger nuclease I-PpoI; it reveals the spatial and electrostatic compatibility of the extended grove on the protein surface with the proposed location of the substrate DNA. Figure 5B shows the top-scoring conformation of curcumin (score: $-\log(K_d) = 3.67$; penalty = −1.42) docked to the apo form of the enzyme, as calculated with the Surflex algorithm. This model suggests that curcumin inhibits the endonuclease activity of DFF/CAD by sterically blocking the active site and possibly the magnesium ligand binding to the active center of the endonuclease, thus protecting the DNA fragmentation. Pulldown type of experiment showing that nuclease preincubated with curcumin remains inhibited after removal of unbound drug, although indirectly, supports our notion that the enzyme activity is inhibited due to the curcumin binding (Fig. 6A).

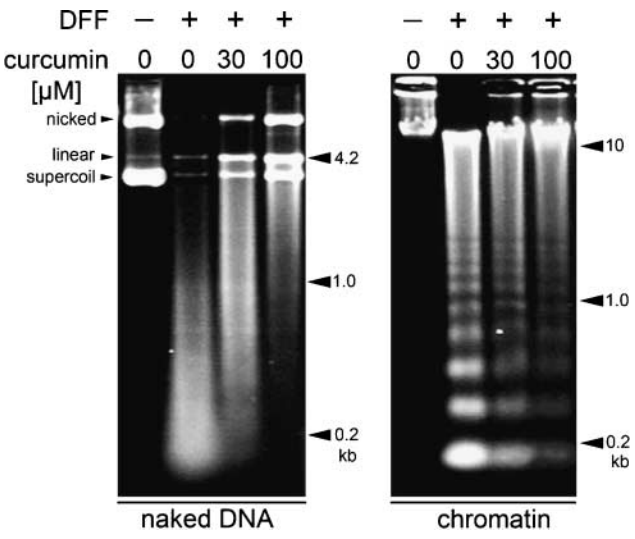

Figure 4. Curcumin inhibits DNA cleavage by DFF40/CAD. Caspase-activated recombinant heterodimer DFF40/DFF45 (*DFF*) was incubated with plasmid DNA (*left*) or chromatin of HeLa cell nuclei (*right*) in the absence or presence of 30 or 100 μmol/L curcumin. Positions of molecular weight markers are indicated.

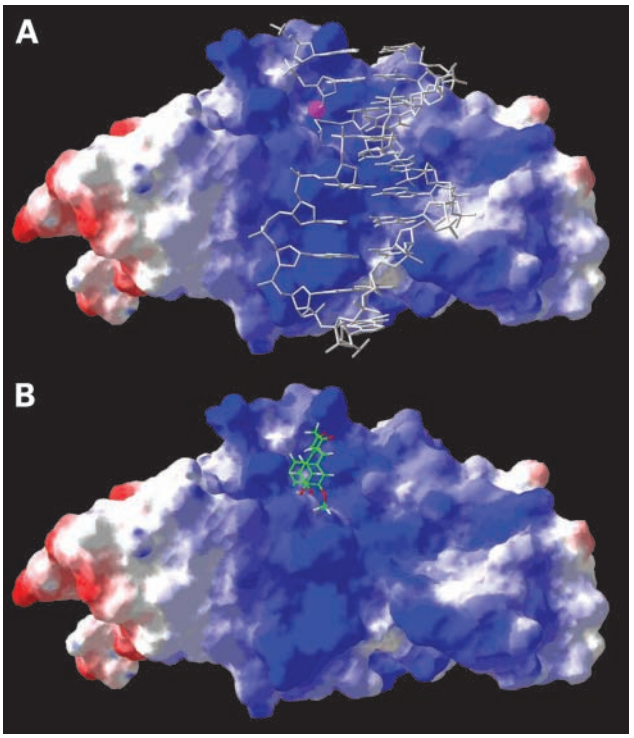

Figure 5. A theoretical model of human DFF40/CAD complexed with DNA and Mg^{2+} (**A**) and with curcumin (**B**). For the clarity of presentation, only the protein fragment comprising amino acids 128 to 327 from one monomer is shown. The protein molecular surface is colored according to the distribution of the electrostatic potential (*red,* −4 kT, to *blue,* +4 kT), indicating a large positively charged groove in the predicted DNA-binding site. DNA and curcumin molecules are shown in the wireframe representation (*gray* and *green*, respectively). The Mg^{2+} ion, indicating the active site, is shown as a magenta sphere.

Recently, it has been shown that DFF40/CAD can bind to DNA without cleaving it (27), which suggests that DFF40/CAD may bind the substrate using only a part of the binding surface. According to our model, curcumin does not block the entire DNA-binding site, but only the catalytic pocket (Fig. 5). We investigated if curcumin affects DNA binding using a gel electrophoresis band-shift assay. We found that curcumin does not affect the ability of caspase-activated DFF to bind the radioactive linear plasmid DNA (Fig. 6B). Thus, we postulate that curcumin does not interfere with DNA binding by DFF40/CAD but specifically prevents the interaction between the active site of DFF40/CAD and DNA, leading to inhibition of the enzyme.

Discussion

It clearly appears from published data that the effect of curcumin is cell type specific. Indeed, we have previously shown that 50 μmol/L curcumin induces characteristic apoptotic morphology and oligonucleosomal DNA degradation in HL-60 cells whereas many other human and rodent cells, either cancer or normal, show moderate morphologic changes and die without oligonucleosomal DNA fragmentation on the same treatment (28). Similarly, others have reported that curcumin used in the same concentration exerted different effects in various cells as far as ''DNA laddering'' is concerned (29).

Nevertheless, the variability of the curcumin-induced effects reported in the literature could also be attributable to different experimental conditions, of which curcumin concentration is the most important. Jurkat cells treated with curcumin at a concentration lower than 50 μmol/L revealed some sub-G_1 fraction (not shown), which was not detected in cells treated with 50 μmol/L curcumin. Accordingly, that concentration was chosen by us as the most effective one in protecting cells against DNA

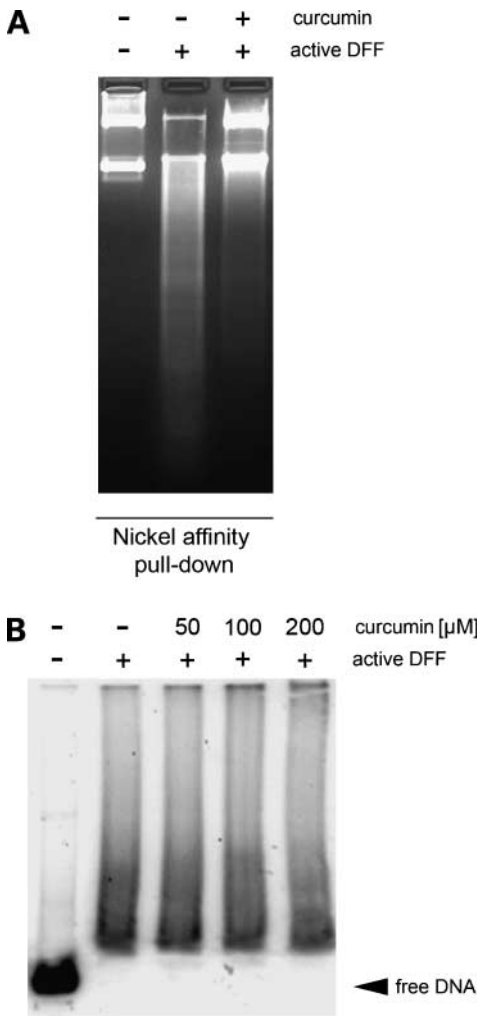

Figure 6. Curcumin binds to DFF but does not impair DNA binding by DFF. Caspase-activated DFF was incubated with 100 μmol/L curcumin, the nuclease, and nuclease-bound curcumin isolated by nickel-affinity pulldown, and then its activity was assayed (**A**). Caspase-activated recombinant heterodimer DFF40/DFF45 was incubated with linear plasmid in the absence or presence of curcumin in concentrations as indicated, and the protein-DNA complexes were separated by agarose electrophoresis (**B**).

oligonucleosomal fragmentation (18). In contrast, Somasundaran et al. (30) showed that even 10 μmol/L curcumin inhibited camptothecin-induced DNA fragmentation in MCF-7 cells. Curcumin-caused inhibition of apoptosis assessed as oligonucleosomal DNA degradation was observed for other apoptosis inducers and cell types as well. The effect was dose dependent, but even 1 μmol/L curcumin significantly blocked DNA fragmentation, by up to ~30% in the case of BT-474 cells treated with camptothecin (29).

In this article, we elucidated the mechanisms of the inhibitory activity of curcumin on DNA fragmentation. Despite the fact that curcumin itself induced caspase-3 activation and following DFF activation (or enhanced both events in UVC-irradiated cells), it protected against UVC-induced DNA fragmentation in Jurkat cells.

Biochemical experiments with recombinant human DFF proved that curcumin inhibited DNA degradation. However, the possibility that curcumin disrupts the binding of DFF40/CAD to DNA was excluded. Instead, we showed by molecular modeling that curcumin possibly blocks the magnesium ligand binding to the active center of the endonuclease.

The mechanism seems to be universal because curcumin prevented DNA fragmentation in Jurkat cells treated with staurosporine or etoposide (data not shown). In contrast, however, we found that treatment with 50 μmol/L curcumin induced oligonucleosomal DNA fragmentation in HL-60 cells (14). This can be explained by existence of a variety of cell death pathways in which DFF/CAD might not be the main executor of DNA fragmentation. Thus, it cannot be excluded that in HL60 cells, curcumin induces another nuclease, which triggers the oligonucleosomal fragmentation observed in such cells. However, in normal human T cells, we observed essentially the same effect like in Jurkat cells (i.e., caspase-3 activation without DNA degradation).[4]

In addition, the presented data show how important it is to exploit as many complementary tests as possible when studying the mechanism of apoptosis. Assessing only for DNA fragmentation (e.g., by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling, sub-G_1, or ''DNA laddering'') and related changes in nuclear morphology, which is common in the literature, may lead to apparently incorrect conclusions about the mechanism of cell death.

[4] A. Magalska, A. Brzezinska, A. Bielak-Żmijewska, K. Piwocka, G. Mosieniak, and E. Sikora, unpublished data.

References

1. Lin JK, Pan MH, Lin-Shiau SY. Recent studies on the biofunctions and biotransformations of curcumin. Biofactors 2000;13:153–8.

2. Cheng AL, Hsu CH, Lin JK, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 2001;21:2895–900.

3. Hsu CH, Chuang SE, Hergenhahn M, et al. Pre-clinical and early-phase clinical studies of curcumin as chemopreventive agent for endemic cancers in Taiwan. Gan To Kagaku Ryoho 2002;29:194–200.

4. Sharma RA, Euden SA, Platton SL, et al. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res 2004;10:6847–54.

5. Sharma RA, Gescher AJ, Steward WP. Curcumin: the story so far. Eur J Cancer 2005;41:1955–68.

6. Karunagaran D, Rashmi R, Kumar TR. Induction of apoptosis by curcumin and its implications for cancer therapy. Curr Cancer Drug Targets 2005;5:117–29.

7. Krammer PH. CD95's deadly mission in the immune system. Nature 2000;407:789–95.

8. Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med 2000;6:513–9.

9. Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 2004;5:897–907.

10. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251–306.

11. Widlak P, Garrard WT. Discovery, regulation, and action of the major apoptotic nucleases DFF40/CAD and endonuclease G. J Cell Biochem 2005;94:1078–87.

12. Nicotera P, Melino G. Regulation of the apoptosis-necrosis switch. Oncogene 2004;23:2757–65.

13. Piwocka K, Zablocki K, Wieckowski MR, et al. A novel apoptosis-like pathway, independent of mitochondria and caspases, induced by curcumin in human lymphoblastoid T (Jurkat) cells. Exp Cell Res 1999; 249:299–307.

14. Bielak-Zmijewska A, Piwocka K, Magalska A, Sikora E. P-glycoprotein expression does not change the apoptotic pathway induced by curcumin in HL-60 cells. Cancer Chemother Pharmacol 2004;53:179–85.

15. Liu X, Zou H, Widlak P, Garrard W, Wang X. Activation of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease). Oligomerization and direct interaction with histone H1. J Biol Chem 1999; 274:13836–40.

16. Kosinski J, Cymerman IA, Feder M, Kurowski MA, Sasin JM, Bujnicki JM. A ''FRankenstein's monster'' approach to comparative modeling: merging the finest fragments of Fold-Recognition models and iterative model refinement aided by 3D structure evaluation. Proteins 2003;53: 369–79.

17. Jain AN. Surflex: fully automatic flexible molecular docking using a molecular similarity-based search engine. J Med Chem 2003;46: 499–511.

18. Sikora E, Bielak-Zmijewska A, Piwocka K, Skierski J, Radziszewska E. Inhibition of proliferation and apoptosis of human and rat T lymphocytes by curcumin, a curry pigment. Biochem Pharmacol 1997; 54:899–907.

19. Widlak P, Palyvoda O, Kumala S, Garrard WT. Modeling apoptotic chromatin condensation in normal cell nuclei. Requirement for intranuclear mobility and actin involvement. J Biol Chem 2002;277: 21683–90.

20. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 1998;391:43–50.

21. Halenbeck R, MacDonald H, Roulston A, Chen TT, Conroy L, Williams LT. CPAN, a human nuclease regulated by the caspase-sensitive inhibitor DFF45. Curr Biol 1998;8:537–40.

22. Liu X, Li P, Widlak P, et al. The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc Natl Acad Sci U S A 1998;95:8461–6.

23. Woo EJ, Kim YG, Kim MS, et al. Structural mechanism for inactivation and activation of CAD/DFF40 in the apoptotic pathway. Mol Cell 2004;14: 531–9.

24. Widlak P, Lanuszewska J, Cary RB, Garrard WT. Subunit structures and stoichiometries of human DNA fragmentation factor proteins before and after induction of apoptosis. J Biol Chem 2003; 278:26915–22.

25. Lechardeur D, Xu M, Lukacs GL. Contrasting nuclear dynamics of the caspase-activated DNase (CAD) in dividing and apoptotic cells. J Cell Biol 2004;167:851–62.

26. Scholz SR, Korn C, Bujnicki JM, Gimadutdinow O, Pingoud A, Meiss

G. Experimental evidence for a ββα-Me-finger nuclease motif to represent the active site of the caspase-activated DNase. Biochemistry 2003;42: 9288 – 94.

27. Korn C, Scholz SR, Gimadutdinow O, Lurz R, Pingoud A, Meiss G. Interaction of DNA fragmentation factor (DFF) with DNA reveals an unprecedented mechanism for nuclease inhibition and suggests that DFF can be activated in a DNA-bound state. J Biol Chem 2005;280: 6005 – 15.

28. Bielak-Zmijewska A, Koronkiewicz M, Skierski J, Piwocka K, Radziszewska E, Sikora E. Effect of curcumin on the apoptosis of rodent and human nonproliferating and proliferating lymphoid cells. Nutr Cancer 2000;38:131 – 8.

29. Gautam SC, Xu YX, Pindolia KR, Janakiraman N, Chapman RA. Nonselective inhibition of proliferation of transformed and nontransformed cells by the anticancer agent curcumin (diferuloylmethane). Biochem Pharmacol 1998;55:1333 – 7.

30. Somasundaram S, Edmund NA, Moore DT, Small GW, Shi YY, Orlowski RZ. Dietary curcumin inhibits chemotherapy-induced apoptosis in models of human breast cancer. Cancer Res 2002;62: 3868 – 75.

Molecular Cancer Therapeutics

Curcumin induces caspase-3-dependent apoptotic pathway but inhibits DNA fragmentation factor 40/caspase-activated DNase endonuclease in human Jurkat cells

Ewa Sikora, Anna Bielak-Zmijewska, Adriana Magalska, et al.

Mol Cancer Ther 2006;5:927-934.