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Curcumin homing to the nucleolus: mechanism for initiation of an apoptotic program

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Abstract

Curcumin is a plant-derived polyphenol that displays antitumor properties. Incubation of cultured SF-767 glioma cells with curcumin gave rise to intense intranuclear foci of curcumin fluorescence. *In vitro* studies revealed that nuclear homing by curcumin is not a result of DNA/chromatin binding. On the other hand, curcumin fluorescence colocalized with nucleophosmin, a nucleolus marker protein. To determine the temporal relationship between curcumin-induced apoptosis and nucleolar homing, confocal live cell imaging was performed. The data show that curcumin localization to the nucleolus occurs prior to cell surface exposure of phosphatidylserine. In studies of the mechanism of curcumin-induced apoptosis in SF-767 cells, its effect on the subcellular location of $p14^{ARF}$ was determined. Whereas $p14^{ARF}$ was confined to the nucleolus in untreated cells, 2 h following incubation with curcumin, it displayed a diffuse nuclear distribution. Given the role of nuclear $p14^{ARF}$ in binding the E3 ubiquitin ligase, mouse double minute 2 homolog (MDM2), the effect of curcumin treatment on cellular levels of the tumor suppressor protein, p53, was examined. Between 2 and 4 h following curcumin treatment, p53 levels increased with maximum levels reached by 8 h. Thus, curcumin homing to the nucleolus induces redistribution of $p14^{ARF}$ to the nucleoplasm where interaction with MDM2 leads to stabilization of p53, with subsequent initiation of apoptosis.

Keywords: Curcumin; Nucleolus; Apoptosis; $p14^{ARF}$; p53; Confocal fluorescence microscopy

1. Introduction

Curcumin is a yellow-colored polyphenol abundant in rhizomes of the plant, *Curcuma longa*. As an ingredient of the Indian spice, turmeric, health benefits of curcumin have been described for centuries. More recently, research efforts have provided compelling evidence that curcumin possesses intrinsic anticancer properties and can induce apoptosis both *in vitro* and *in vivo* [1–6]. Curcumin exerts pleiotropic effects by modulating the activity of transcription factors, growth factors and cytokines [7]. Intriguingly, although curcumin has been shown to induce cytotoxic effects in multiple tumor cell lines, normal cells appear to be largely spared from cytotoxicity [7–9].

In an effort to overcome problems associated with water insolubility and poor bioavailability, Ghosh et al. [10] described a tripartite, nanoscale delivery vehicle for curcumin, termed nanodisks (ND) [11,12]. In studies with cultured lymphoma cells, curcumin-ND displayed an enhanced ability to induce apoptosis compared to free curcumin [13]. Subsequent studies of the effect of curcumin-ND on cultured SF-767 glioblastoma cells included examination of its intracellular itinerary following uptake [14]. Confocal fluorescence microscopy analysis of intrinsic curcumin fluorescence after a 4-h incubation with cultured SF-767 cells revealed the presence of bright intranuclear foci of curcumin fluorescence. Insofar as the nuclear space is comprised of distinct subcompartments that harbor proteins involved in cell proliferation, cell cycle arrest and apoptosis [15,16], curcumin homing to discrete subnuclear sites is of great interest. To characterize this homing phenomenon, the intranuclear site of curcumin fluorescence intensity has been determined and the temporal relationship between nucleolar homing and apoptosis examined. The results reveal that curcumin-induced apoptosis of SF-767 cells correlates with redistribution of $p14^{ARF}$ from the nucleolus to the nucleoplasm, an event that leads to stabilization of the tumor suppressor protein, p53 [17]. Knowledge of the biochemical and cell biological basis underlying curcumin-induced apoptosis should increase opportunities for therapeutic exploitation of its anticancer properties.

2. Materials and methods

2.1. Reagents

Curcumin was obtained from Cayman Chemical and used without further purification. Calf thymus DNA was from Sigma Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-nucleophosmin, Alexa Fluor 647 goat anti-mouse IgG and Hoechst 33342 were from Life Technologies Corp. (Carlsbad, CA, USA). Monoclonal anti-p53 (clone DO-1) was from Sigma Aldrich. Rabbit polyclonal anti-$p14^{ARF}$, mouse monoclonal ß-actin and DyLight 405-conjugated goat anti-rabbit IgG were from Novus Biologicals (Littleton, CO, USA). Western Lightning Plus-ECL, obtained from PerkinElmer, Inc. (Waltham, MA, USA) was used for immunoblot analysis. Biotin-conjugated annexin V, Alexa Fluor 350-labeled streptavidin and propidium iodide (PI) were components of the Vybrant Apoptosis Assay Kit obtained from Life Technologies Corp. Goat anti-mouse HRP-conjugated secondary, NE-PER nuclear and cytoplasmic extraction reagents, Halt protease inhibitor cocktail and Restore Western blot stripping

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buffer were from Thermo Scientific (Rockford, IL, USA). HRP-conjugated goat anti-rabbit IgG secondary antibody was from Vector laboratories (Burlingame, CA, USA).

2.2. Curcumin incubations with calf thymus DNA

A stock solution of 4.5 mg/ml calf thymus DNA was prepared in water by gently inverting the sample overnight at 4°C. The DNA concentration was determined using a NanoDrop instrument. For spectrophotometric studies, a 25-mM curcumin stock solution, prepared in absolute ethanol, was used. Prior to fluorescence or absorbance measurements, a 2.5-mM curcumin working solution was prepared from the stock using 20 mM sodium phosphate, pH 7.0, 50 mM NaCl [18]. The absorbance and fluorescence properties of curcumin were determined in the absence and presence of increasing concentrations of calf thymus DNA. Ultraviolet–visible (UV/Vis) absorbance spectra were obtained on a Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). Samples were scanned from 350 to 500 nm. Fluorescence spectra were obtained on a Perkin-Elmer LS 50B luminescence spectrometer. Samples were excited at 420 nm and emission monitored from 480 to 600 nm (2.5-nm slit width).

2.3. Cell culture studies

SF-767 glioblastoma cells were obtained from Dr. Trudy M. Forte (Children's Hospital Oakland Research Institute) and maintained in high glucose DMEM (Thermo Scientific Hyclone) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. HepG2 cells were cultured in DMEM supplemented with 10% FBS. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For cell incubation studies, a 20-mg/ml curcumin stock solution (in DMSO) was diluted 100-fold with 20 mM sodium phosphate, pH7.4, 150 mM NaCl [phosphate-buffered saline (PBS)] immediately before use.

2.4. Colocalization study of curcumin and PI

To investigate curcumin interaction with cellular nucleic acid, SF-767 cells were incubated with 20 μM curcumin (as curcumin-ND [7]) for 24 h, washed with PBS, fixed with methanol/acetone (1:1 vol/vol) for 5 min and stained with 1 μg/ml PI for 15 min. Coverslips were mounted on a glass slide using Vectashield Mounting Medium (Vector Laboratories) and sealed. Confocal images were acquired on a Zeiss LSM710 microscope with a 63×, 1.4NA oil objective with a pinhole of 90 μm. Z-stack recordings of 0.566-μm-thick overlapping images were obtained. Image processing, deconvolution, 3D reconstruction and colocalization analysis were performed with Huygens Essential and Bitplane Imaris Suite package of Scientific Volume Imaging. The ImarisColoc module was used for colocalization analysis. This module uses an iterative procedure to determine an intensity threshold (0–255-pixel intensity scale) for the green (curcumin) and red (PI) fluorescent labels. The region of interest was defined using red as the mask channel. In conjunction with the automatic threshold, the region of interest was used to obtain colocalization data represented as a two-dimensional histogram.

2.5. Nuclear protein extraction

HepG2 cells were harvested with trypsin-EDTA, centrifuged at 500×*g* for 5 min, and the cell pellet was washed with PBS. Nuclear proteins were extracted using the NE-PER reagent according to the manufacturer's instructions. Total protein was quantified using the BCA Protein Assay (Thermo Scientific) using bovine serum albumin (BSA) as standard.

2.6. Live imaging confocal fluorescence microscopy

SF-767 cells were seeded onto 35-mm glass bottom microwell dishes (MatTek Corporation, Ashland, MA, USA) and incubated with 20 μM curcumin. After 4 or 24 h, cells were rinsed with annexin V binding buffer (Vybrant Apoptosis Assay kit component) and incubated with biotin-conjugated annexin V followed by Alexa Fluor 350 streptavidin solution. Cells were co-stained with PI and images acquired on a Zeiss LSM710 microscope with a C-Apochromat 63×, 1.20-W Korr M27 water objective. Some photobleaching occurred since no antifade reagent was used.

2.7. Immunoblot analysis

SF-767 cells, seeded onto 12 well plates at a density of 5×10^5 cells/ml, were left untreated or incubated with 20 μM curcumin. At indicated time points, cells were lysed with RIPA buffer [50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Triton X-100 and 1× Halt protease inhibitor cocktail]. Ten micrograms total cell protein from each lysate was separated on a 4% to 20% acrylamide SDS gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% (wt/vol) nonfat milk. Target proteins were visualized using the ECL detection system after incubation with anti-p53 (1:5000), anti-$p14^{ARF}$ (1:1000), anti-MDM2 (1:200) and anti-ß-actin (1:4000) antibodies. Goat anti-mouse and anti-rabbit HRP-conjugated secondary antibody was used at 1:10,000 and 1:2000 dilutions, respectively. The experiment was performed three times.

2.8. Confocal fluorescence microscopy

Cells were plated overnight on coverslips in 12-well plates at a density of 1.5×10^5 cells/ml in a final volume of 1 ml. Cells were incubated with 20 μM curcumin for specified times at 37°C. Subsequently, the coverslips were washed, and the cells were fixed with 4 % paraformaldehyde for 15 min on ice and washed with PBS containing 1% BSA. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature followed by three washes with PBS plus 1% BSA. Nucleophosmin and $p14^{ARF}$ were detected after incubation with anti-nucleophosmin or anti-$p14^{ARF}$ (both diluted 1:50) for 2 h at room temperature. Cells were washed and incubated with either Alexa Fluor 647-conjugated anti-mouse IgG (1:150 dilution for nucleophosmin) or DyLight 405-conjugated anti-rabbit IgG (1:200 for $p14^{ARF}$) for 1 h. In some cases, samples were stained with Hoechst 33342 before mounting with Vectashield for image acquisition on a Zeiss LSM710 confocal microscope.

3. Results

Recently, Ghosh and Ryan [14] studied curcumin uptake by cultured glioblastoma cells using curcumin-ND as the delivery vehicle. Investigation of curcumin's intracellular itinerary revealed a time-dependent accumulation of this polyphenol in the nucleus. To assess whether this phenomenon occurs in other cell types and if it may be specific to the mode of curcumin delivery, SF-767 and HepG2 cells were incubated with free curcumin. As seen in Fig. 1, after 24-h incubation, both cell types displayed distinct intranuclear foci of curcumin fluorescence. Given that curcumin fluorescence is concentrated in these discrete intranuclear sites, as opposed to a generalized dispersion, experiments were conducted to characterize the apparent homing process, identify the intranuclear site(s) of accumulation and examine the biological significance of this phenomenon.

3.1. In vitro DNA binding studies

Others have reported that curcumin binds the minor groove of DNA, albeit weakly [18,19]. Thus, it is conceivable that nuclear homing by curcumin is driven by a specific interaction with nucleic acid. Although this would also be consistent with an ability of curcumin to adopt a planar molecular structure, incubations with calf thymus DNA had little effect on either the UV/Vis absorbance spectrum or the fluorescence emission properties of curcumin (Fig. 2). On the basis of these data, it may be concluded that curcumin does not bind DNA *in vitro*.

3.2. Colocalization studies with PI

A possible explanation for results obtained upon incubation of curcumin with calf thymus DNA is that interaction with nucleic acid requires other components or features present only in intact chromatin. To explore this, SF-767 cells were incubated with curcumin for 24 h, fixed and stained with the fluorescent DNA intercalating dye, PI. Merged confocal fluorescence microscopy images provided no evidence of fluorescence colocalization between curcumin and PI (Fig. 3). The Z-stack recording of overlapping images spanning the entire cell volume clearly shows that the intranuclear foci of curcumin fluorescence (green) are distinct from sites of PI fluorescence (red). A two-dimensional intensity histogram encompassing the entire Z-stack indicates that, at no place within the cell, do curcumin and PI colocalize.

3.3. Curcumin homing to the nucleolus

The apparent lack of curcumin binding to DNA/chromatin prompted further probing of the intranuclear site(s) of intense curcumin fluorescence. Considering the size, shape and appearance of the fluorescent foci, it was hypothesized that curcumin homes to the nucleolus. To examine this, curcumin treated SF-767 and HepG2 cells were fixed and probed with an antibody directed against nucleophosmin, a nucleolus marker protein [20]. Confocal microscopy