Curcumin sensitizes Epstein-Barr-immortalized lymphoblastoid cell lines to inorganic arsenic toxicity

MACARIO MARTÍNEZ-CASTILLO1, GABRIELA CRUZ-ROBLEDO1,2, ARACELI HERNÁNDEZ-ZAVALA1 and EMILIO J. CÓRDOVA2

1Section of Research and Postgraduate, Superior School of Medicine, National Institute Polytechnique, Casco de Santo Tomás, Mexico City 11350; 2Oncogenomics Consortium Laboratory, National Institute of Genomic Medicine, Clinic Research, Arenal Tepepan, Mexico City 14610, Mexico

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Correspondence to: Dr Emilio J. Córdova, Oncogenomics Consortium Laboratory, National Institute of Genomic Medicine, Clinic Research, 4,809 Periférico Sur, Arenal Tepepan, Mexico City 14610, Mexico
E-mail: ecordova@inmegen.gob.mx

Key words: curcumin, inorganic arsenic, Epstein-Barr virus, chemosensitizing

Abstract. Chronic exposure to inorganic arsenic (iAs) through contaminated drinking water is an important health problem in certain countries. The use of phytochemicals such as curcumin has recently emerged as an alternative strategy for preventing cellular damage caused by iAs. The Epstein-Barr virus (EBV) affects ~90% of the population and experimental evidence suggested that curcumin mediates cytotoxicity against EBV-infected cells. Due to the potential for an interaction of these factors, the aim of the present study was to evaluate the effect of this phytochemical on iAs-related toxicity in EBV-infected cells. Two independent EBV-immortalized human lymphoblastoid cell lines (LCLs) were used as the model. The cell lines were first incubated with increasing concentrations of curcumin or iAs for 24 and 15 h, respectively, to determine the individual effects of each exposure on cell death. In the next experiment, cell cultures were pre-incubated with 5 µM curcumin for 9 h prior to treatment with 10 µM iAs for 15 h, followed by evaluation of cell death and the cell cycle profile via flow cytometry. The results indicated that individual treatment with either curcumin or iAs induced cell death in a concentration-dependent manner. Furthermore, curcumin pre-treatment enhanced iAs-induced cell death and promoted cell cycle arrest in G1 phase. Taken together, these results suggested that curcumin sensitizes EBV-positive LCLs to the cytotoxic effects of iAs.

Introduction

Inorganic arsenic (iAs) is a natural pollutant found in the air and soil, as well as dissolved in groundwater. The main source of human exposure to iAs is through contaminated drinking water and the maximum allowed limit established by the World Health Organization for iAs in drinking water is 10 µg/l (1). However, an estimated 500 million individuals worldwide still are exposed to high concentrations of iAs, ranging from 400 to 25,000 µg/l (2). Chronic exposure to iAs through contaminated drinking water may lead to non-malignant disorders and to various types of cancer, including bladder, prostate, lung and skin (3). For this reason, the presence of this metalloid in drinking water has been recognized as a significant global health issue (4).

Recently, the use of natural compounds with chemopreventive properties has been explored as a way to reduce iAs-related cellular damage. Curcumin is a phytochemical with potent antioxidant properties against a variety of environmental and intracellular hazards, including iAs (5). Curcumin is consumed worldwide, mainly through using turmeric as a spice. This spice is common in traditional dishes of India, including curry, and is also used as a food colorant and preservative (6). In certain regions such as India, the population normally consumes around 60-100 mg of curcumin per day without any apparent adverse health effects (7). As a chemopreventive compound, curcumin reduces the generation of oxidative stress and DNA damage associated with iAs exposure in different cell types, including keratinocytes, lung cells and lymphocytes (8-10). Of note, the ability of curcumin to mitigate iAs-related DNA damage has been demonstrated in circulating lymphocytes obtained from individuals chronically exposed to iAs-contaminated drinking water (11,12).

Epstein-Barr virus (EBV) belongs to the γ-herpesvirus family and is among the most prevalent human viruses, affecting ~90% of the population worldwide (13). Although EBV infection has been associated with several human diseases, including infectious mononucleosis and Burkitt's lymphoma, in most cases, infected individuals remain asymptomatic (13,14). It is worth noting that in cellular models derived from gastric and nasopharyngeal carcinomas that are positive for EBV, curcuminoids have higher toxicity (15). Similarly, experimental treatment with curcumin promoted apoptosis in EBV-related Hodgkin's and Burkitt's lymphoma cells (16,17). Furthermore, curcumin promotes apoptosis in EBV-immortalized lymphoblastoid cell lines (LCLs) (18,19).

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These results suggest that EBV infection may influence the chemopreventive properties exerted by curcumin against iAs-induced toxicity. Thus, in the present study, two independent EBV-immortalized LCLs were used to evaluate whether the presence of EBV may modify the protective properties of curcumin against the cytostatic and cytotoxic effects of iAs. The results suggested that curcumin pre-treatment sensitized LCLs to iAs-associated toxicity, induced a slight proportion of G1 phase arrest and promoted cell death.

Materials and methods

Cell culture and reagents. The lymphoblastoid-derived cell lines CL-45 and CL-49 had been generated by EBV immortalization of peripheral blood mononuclear cells obtained from non-related healthy Mexican-mestizo donors, as described in previous studies by our group (20,21). The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% L-alanyl-L-glutamine (GlutaMax 100x), 1% non-essential amino acids and 1% antibiotic-antimycotic (10,000 U/ml penicillin and 10 mg/ml streptomycin). All cell culture reagents were purchased from Gibco (Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. Sodium arsenite (NaAsO₂) as the iAs and curcumin were purchased from Sigma-Aldrich (Merck KGaA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck KGaA) or nuclease-free water (Sigma-Aldrich; Merck KGaA) to obtain 20 and 50 mM stock solutions, respectively. Curcumin and iAs stocks were protected from light and aliquots were maintained at -20°C until use.

Cell treatments. CL-49 cells were seeded at a density of 200,000 cells/ml in 4 ml of complete growth medium and allowed to grow for 24 h prior to incubation with different concentrations of curcumin (5, 10 or 15 µM) for 24 h or iAs (5, 10 or 20 µM) for 15 h. In the case of iAs, the incubation time was selected considering a 9 h pre-treatment with curcumin followed by the 15 h of iAs treatment, which corresponds to a total incubation time of 24 h, the approximate duration time of cell cycle in LCL. For time-response assays, CL-49 cells were treated with 5 µM curcumin for 12, 18 or 24 h. As a negative control, cell cultures were treated with the respective vehicles used with curcumin (0.1% DMSO) and iAs (0.1% H₂O) for 24 h in the concentration-response and time-response assays. For the pre-treatment assays, 8x10⁵ CL-45 and CL-49 cells were grown for 24 h and treated with 5 µM curcumin for 9 h. Subsequently, the culture medium with curcumin was removed and the cells were washed with 20 ml of 1X PBS. The cells were then re-cultured in fresh culture medium and treated with 10 µM iAs for a further 15 h. Similar washing and re-seeding protocols were used for the following individual treatments groups: i) In the vehicle control group, cell cultures were pre-treated with 0.1% DMSO for 9 h, followed by 15 h with 0.1% nuclease-free water; ii) curcumin treatment alone included incubation of cell lines with 5 µM curcumin for 9 h followed by 0.1% H₂O for 15 h; and iii) in the iAs-only group, LCLs were pretreated with 0.1% DMSO for 9 h and then incubated with 10 µM iAs for 15 h.

Cell death assay. After the treatments, LCLs were harvested and cell death levels determined using the LIVE/DEAD® Fixable Dead Cell Stain kit (Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. This assay is based on the use of a cell-impermeable fluorescent dye that reacts with the amines of cellular proteins. In viable cells, cell membrane integrity prevents cell entry of the fluorescent compound, resulting in its reaction with only the cell-surface amines and the production of a low-intensity staining. However, in cells with compromised membranes, i.e., dying cells, the fluorescent reagent reacts with amines inside the cell and on its surface, producing intense fluorescent staining. In brief, 8x10⁵ cells were resuspended in 1 ml of 1X PBS, stained with 1 µl of a 1:20 dilution of LIVE/DEAD red fluorescent reactive dye and incubated for 30 min at room temperature in the dark. The cell lines were then washed and resuspended in 1 ml 1X PBS with 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA). Samples were analyzed with a BD FACS/Aria III flow cytometer system (BD Biosciences, Inc.), capturing at least 10,000 events for each sample. Data analysis was performed using Flowing Software version 2.5.1 (Turku Centre for Biotechnology).

Cell cycle assay. To analyze cell cycle profile changes in the pre-treatment assays, cell lines were harvested, washed with 1X PBS and fixed overnight at -20°C with 1 ml of ice-cold 70% ethanol. After fixation, the cells were washed with 1 ml cold 1X PBS and resuspended in 250 µl of 1X PBS. The cellular suspension was then incubated with 0.5 mg/ml RNase A (Sigma-Aldrich; Merck KGaA) for 1 h at 37°C. Finally, cells were stained with 10 µg/ml propidium iodide (Sigma-Aldrich; Merck KGaA) for 60 min on ice. Samples were analyzed using the BD FACS/Aria III flow cytometer system (BD Biosciences, Inc.), capturing at least 20,000 events for each sample. Cell cycle histograms were generated and analyzed using ModFit LT 3.2 software (Verity Software House).

Statistical analysis. Values are expressed as the mean ± standard deviation. Differences between groups were analyzed by one-way analysis of variance followed by Tukey’s multiple-comparisons test. Statistical analysis was performed using GraphPad Prism 7 software (version 5.01; GraphPad Software, Inc.). All data analyses were performed using results from at least three independent biological experiments. P<0.05 was considered to indicate statistical significance.

Results

Cytotoxic effects of curcumin and arsenic in EBV-immortalized LCLs. Curcumin is a phytochemical with chemopreventive properties in normal cells but with cytotoxic effects in tumoral and immortalized cells. Thus, the cytotoxic effects of curcumin were evaluated in the EBV-immortalized lymphoblast cell line CL-49, including the measurement of the fluorescent intensity of the LIVE-DEAD viability dye through flow cytometry in concentration-response assays. A concentration-dependent increase was observed in the percentage of cell death after 24 h of incubation with 10 µM (20.1±2%) and 15 µM (45.8±8.1%) curcumin (Fig. 1A).
By contrast, treatment with 5 µM curcumin exhibited low toxicity compared with the vehicle control (7.9±1.4 vs. 4.8±0.9%; Fig. 1A). Accordingly, in time-response assays using 5 µM curcumin, in comparison with the vehicle control (4.6±1.7%), no significant increase in the percentage of cell death was obtained after incubation for 12 h (4.6±1.8%), 18 h (6.5±4.9%) or 24 h (6.1±2.2%) (Fig. 1B). These results suggested that 5 µM curcumin had no toxic effects on the CL-49 cell line.

The cytotoxic effect of iAs exposure for 15 h was then evaluated in CL-49 cells using concentration-response curves. This incubation time was selected based on the consideration of using a curcumin pre-treatment time of 9 h, which together with iAs incubation time for 15 h covers the LCL cell cycle of ~24 h. As expected, iAs treatment increased the levels of cell death in a concentration-dependent manner, starting at a concentration of as low as 5 µM (14.4±3.2%) and up to 20 µM (64.6±9.6%), which was the highest concentration used (Fig. 1C). The intermediate concentration of 10 µM iAs was used for further curcumin pre-treatment assays because this concentration may not be associated with massive necrotic cell death events, as may occur with a highly toxic concentration of 20 µM. In addition, the 10-µM concentration of iAs is equivalent to a high level of human exposure through iAs-contaminated drinking water (750 µg/l).

**Curcumin enhances arsenic-induced toxicity in EBV-immortalized lymphocytes.** To explore the effect of curcumin on iAs-induced toxicity on CL-49 cells, levels of cell death were analyzed after treatment with 10 µM iAs for...
15 h with or without pre-treatment with 5 µM curcumin for 9 h. The percentage of cell death increased from ~18% in the iAs-treated cultures in the absence of curcumin pre-treatment to almost 30% in CL-49 cells incubated with curcumin prior to iAs treatment (Fig. 2A and B). As expected, pre-treatment with 5 µM curcumin followed by incubation with iAs vehicle (H$_2$O) resulted in a non-significant increase in cellular toxicity in comparison with the vehicle control (5.6±0.8 vs. 2.5±0.8%; Fig. 2A and B). To rule out any cell line-specific effect on the drug-sensitizing capacity of curcumin, the same experimental conditions were applied in a second EBV-positive LCL (CL-45 cells) derived from an independent healthy donor. A previous study by our group suggested that treatment of CL-45 cells with 5 µM curcumin for 24 h did not significantly increase cell death (19). Similar to the effect on CL-49 cells, the CL-45 cell line treated with 10 µM iAs after incubation with curcumin vehicle (DMSO) exhibited lower levels of cell death than those observed in cell cultures treated with 5 µM curcumin prior to iAs vehicle (22.5±4.9 vs. 35.5±4.2%; Fig. 2C and D). These results suggested that curcumin sensitizes EBV-immortalized LCLs to iAs-related toxicity.

**Cytostatic effect of curcumin and arsenic on LCLs.** Next, alterations in cell cycle progression induced by curcumin sensitization to iAs toxicity were examined. As the cytotoxic effect of curcumin on CL-49 and CL-45 was similar and both cell lines were obtained from healthy individuals using the same strain of EBV, it was decided to evaluate the cytostatic
effects of curcumin only in CL-49 cells. The results suggested that treatment with 5 µM curcumin and iAs vehicle (H₂O), compared with the vehicle control, induced a significant accumulation of cells in G1 phase (59.2±3.8 vs. 51.8±2.3%), without any evident changes in the sub-G1 population (Fig. 3). In the group treated with iAs alone, no apparent effect on cell cycle progression was observed, while a significant increase in sub-G1 cells was detected in comparison with the vehicle group (17.1±4.2 vs. 1.6±0.6%; Fig. 3).

Of note, CL-49 cells treated with curcumin followed by iAs treatment exhibited a not significant increase in the number of cells in the G1 and S phases in comparison with curcumin vehicle and iAs treatment (G1: 56.5±4.9 vs. 52.5±2.3%; S: 35.4±5.1 vs. 33.7±2.1%; Fig. 3A and B). Consistent with the previous results for cell death, the sub-G1 phase levels in the curcumin + iAs group increased significantly with respect to cell cultures treated only with iAs (26.0±5.9 vs. 17.1±4.2%; Fig. 3C). These results indicated that curcumin may enhance iAs-related toxicity, possibly through cytostatic effects.

Discussion

Hydroarsenicism is a major health issue worldwide, as ~100 million individuals are chronically exposed to this pollutant (22). The natural compound curcumin has emerged as an important alternative strategy for diminishing the toxic effects caused by arsenic exposure (8,9,11,12,23). However, previous studies documented the cytotoxic effects of curcumin in EBV immortalized lymphoblastoid cell lines and in cell lines derived from tumors associated with chronic EBV infection (15-19). Considering the high prevalence of EBV infection in human populations, the use of this phytochemical as a chemo-preventive compound may be associated with different side effects on human health.

Thus, in the present study, EBV-immortalized lymphocytes were used as an experimental model to evaluate the effect of latent EBV infection on the chemopreventive properties of curcumin against iAs. First, a significant increase in CL-49 cell death was observed after individual treatment with curcumin or iAs, at concentrations ranging from 10 to
15 µM in the case of curcumin and from 5 to 20 µM for iAs. Previous studies have described the induction of cell death in EBV-immortalized LCLs treated with curcumin in the range of 15 to 20 µM (18,19). Furthermore, iAs-related induction of cell death by autophagy in LCLs and by apoptosis in EBV-positive Burkitt’s lymphoma cells has been described at similar concentrations (24,25).

When the protective effect of non-toxic concentrations of curcumin against iAs was evaluated in EBV-immortalized lymphocytes, higher levels of cell death were detected in cell cultures treated with curcumin prior to incubation with iAs than after treatment with iAs alone. This result is in clear contradiction with the chemopreventive effect exerted by curcumin against iAs-induced genotoxic damage and toxicity reported in different cellular and animal models (10,21,26-28). In addition, pre-treatment with curcumin enhanced iAs-related alterations in cell cycle progression, provoking a significant arrest in G1 and S phases and a reduction of cells in G2/M phase.

In the flow cytometry assays, the presence of a population of sub-G1 cells was observed after iAs treatment, with or without curcumin pre-treatment (Fig. 3A), which is indicative of apoptosis. iAs promotes genotoxic damage in LCLs and activates apoptosis with caspase processing in EBV-positive Burkitt’s lymphoma cells (25,29,30). However, it was reported that autophagy is the predominant type of cell death induced by iAs in human lymphocytes (24). Thus, further studies are necessary to conclusively determine the type of cell death induced by curcumin and iAs treatment in LCLs.

Previous studies also have described a delayed progression from S to M phase in circulating lymphocytes extracted from individuals chronically exposed to iAs through drinking water (31,32). Similarly, iAs concentrations ranging from 5 to 10 µM induce S-phase accumulation in cell lines derived from bladder and breast carcinomas and in myeloid leukemia cell lines after treatments for 12 to 24 h (33-36). Of note, iAs-treated cells exhibit high caspase activity (35).

In the case of curcumin, its anti-proliferative effects in EBV-immortalized LCLs have been reported to be predominantly exerted through causing G1-phase arrest. However, in cell lines derived from monocytic leukemia and gallbladder, breast and colorectal cancer cells, the phytochemical was observed to induce arrest in S phase (37-41). In addition, curcumin causes DNA damage in human lymphocytes (42,43) and activates apoptosis with phosphatidylserine exposure, cytochrome C release, poly(ADP-ribose) polymerase (PARP) cleavage and DNA damage in LCLs and EBV-positive B-lymphoma cell lines (17,18,44,45). Thus, in the current model, curcumin pretreatment increased the genotoxic properties of iAs promoting an initial arrest in the cell cycle, followed by induction of apoptosis.

The chemosensitizing effects of curcumin in EBV-positive cells may be explained by its capacity to modulate the EBV life cycle. Curcumin reduces levels of Epstein-Barr nuclear antigen 1, a critical protein for viral latency maintenance, promoting cell cycle arrest and apoptosis of EBV-nasopharyngeal carcinoma cells (46). Similarly, a previous study indicated that curcuminoids promote EBV lytic cycle reactivation, increasing the cell death of gastric and nasopharyngeal cell lines when combined with other lytic activators such as gemcitabine and valproic acid (15).

Of note, the concentration of curcumin used in the present study was significantly lower than that associated with adverse effects on human health. For instance, a study evaluating the effect of curcumin on DNA damage repair potential used an intervention of 500 mg (1.36 mM) twice a day for 3 months in 66 healthy volunteers without any apparent adverse effects on their health (12). Another study demonstrated the safety of ingesting increasing concentrations of a commercial curcumin formulation (C3 Complex™) of up to 12,000 mg (32.6 mM) in healthy individuals, revealing minimal toxicity that did not appear to be dose-related. The adverse health effects of the highest doses were headache and diarrhea, both classified as grade 1 according to National Cancer Institute, Common Toxicity Criteria version 2.0 (47).

The present study, as far as may be discerned, was the first to assess the chemosensitizing capacity of curcumin to iAs toxicity in LCLs. However, the present study had several limitations. For instance, the type of cell death (e.g. necrosis or apoptosis) observed after the different treatments (i.e. curcumin, iAs and curcumin plus iAs) was not characterized by using differential assays such as Annexin V/PI staining, caspase processing, PARP cleavage or DNA fragmentation. In addition, evaluation of the alteration of RNA or protein levels of cell cycle regulators was also lacking. This indicates the necessity of characterizing the specific type of cell death and the cellular mechanisms activated by curcumin pre-treatment before LCLs are exposed to iAs. In the same sense, the cell cycle alterations associated with the sensitizing effects of curcumin should be evaluated. Further studies are warranted to test the sensitizing effect of this phytochemical to iAs in primary lymphocytes and other EBV-infected cells.

In conclusion, the results of the present study indicated that curcumin pre-treatment sensitized EBV-positive LCLs to iAs toxicity. Thus, further studies analyzing the use of curcumin as a strategy for reducing iAs toxicity in individuals with chronic EBV infection are required.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.
Authors' contributions

Conceived and designed the experiments: EJC, AHZ and MMC. Performed the experiments: MMC and GCR. Wrote the manuscript: EJC, AHZ and MMC. Coordinated and facilitated the project: EJC. EJC and AHZ confirmed the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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