

Curcumin differentially affects cell cycle and cell death in acute and chronic myeloid leukemia cells

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Received August 2, 2017; Accepted January 24, 2018

DOI: 10.3892/ol.2018.8112

Abstract. Curcumin is a phytochemical with potent anti-neoplastic properties. The antitumoral effects of curcumin in cells derived from chronic or acute myeloid leukemia have been already described. However, a comparative study of the cytostatic and cytotoxic effects of curcumin on chronic and acute myeloid leukemia cells has not yet been performed. In the present study, the cellular effects of curcumin on cell lines derived from chronic or acute myeloid leukemia were examined. Dose and time-response assays were performed with curcumin on HL-60 and K562 cells. Cell viability was evaluated with trypan blue exclusion test and cell death by flow cytometry using a fluorescent molecular probe. A cell cycle profile was analyzed, and protein markers of cell cycle progression and cell death were investigated. In the present study, the K562 cells showed a higher sensitivity to the cytostatic and cytotoxic effects of curcumin compared with HL-60. In addition, curcumin induced G1 phase arrest in HL-60 cells and G2/M phase arrest in K562 cells. Furthermore, curcumin-related cell death in HL-60 was associated with the processed forms of caspases-9 and -3 proteins, whereas in K562 cells, both the processed and the unprocessed forms were present. Accordingly, activity of these caspases was significantly higher in HL-60 cells compared with that in K562. In conclusion,

curcumin elicits different cellular mechanisms in chronic or acute myeloid leukemia cells and the powerful antitumoral effect was more potent in K562 compared with HL-60 cells.

Introduction

Curcumin is an important bioactive compound derived from the rhizome of the plant *Curcuma longa* Linn, which has been used in traditional medicine for centuries. This phytochemical has demonstrated potent antineoplastic activities in various animal and cellular models of cancer. For example, curcumin exerts powerful antiproliferative effects in cell lines derived from colon, and lung cancers (1,2). Similarly, curcumin is able to reduce the development of breast, lung, and hepatic tumors in various mouse models (3,4). Importantly, numerous human clinical trials have also demonstrated the safety and efficacy of curcumin as an adjuvant for cancer therapy (5,6).

Curcumin features natural anticancer properties and delivers potent cytostatic and cytotoxic effects against neoplastic cells. Notably, however, curcumin's cytotoxic effects, including cell cycle arrest and cell death, are specific to the genetic background of the tumor-derived cells. For example, curcumin exposure induced a cell cycle arrest in the G1 phase in prostate and breast cancer-derived cell lines (7,8); whereas, it induced a G2/M arrest in glioma and colorectal cancer cells (9,10). Likewise, curcumin exposure is able to promote different mechanisms of cell death depending on the tumor cell type. For example, curcumin treatment of prostate, colon, and lung cancer cells causes apoptosis (11-13), while causing mitotic catastrophe in pancreatic and esophageal tumor-derived cells (14,15). Furthermore, curcumin exposure was related to autophagy in melanoma and hepatocellular carcinoma cell lines (16,17).

In hematological malignancies, previous reports have shown the potential of curcumin to provoke a reduction in

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Key words: curcumin, chemoprevention, leukemia, K562, HL-60, cell cycle, apoptosis

cellular proliferation and an increase of cell death in cell lines derived from chronic and acute myeloid leukemia (18-22). However, controversial results have been obtained from these studies. For example, Sarkar *et al* (19) and Zhang *et al* (20) demonstrated that curcumin induced a cell cycle arrest at the G2/M phase in the acute myeloid leukemia-derived cell line HL-60; whereas in the same cell line, Liu *et al* (21) and Chen *et al* (22) reported a curcumin-related cell cycle arrest in G1 phase. In addition, previous reports showed that treatment of the chronic myeloid leukemia-derived cell line K562 with curcumin promoted apoptosis through the activation of caspases-9 and -3 (23,24). Another study found that curcumin activates both apoptosis and autophagy in K562 cells (25).

It is worth noting that a comparative study investigating the different cytotoxic and cytostatic effects of curcumin on cell lines derived from chronic or acute myeloid leukemia cells has not been carried-out. Therefore, in the present study, we compared the cytostatic and cytotoxic effects of curcumin on both K562 and HL-60 cell lines that are derived from chronic and acute myeloid leukemia cells, respectively. Our results illustrate that curcumin activates different mechanisms for cell cycle arrest and cell death in each type of leukemic cells. In HL-60 cells, curcumin caused a cell cycle arrest in G1 and displayed classical apoptosis, involving activation of caspases-9 and -3. In contrast, in K562 cells, curcumin induced a G2/M arrest, followed by a cell death process similar to mitotic catastrophe, with partial activation of caspases-9 and -3.

Materials and methods

Cell cultures. The cell lines derived from chronic myeloid leukemia (K562) and from acute promyelocytic leukemia (HL-60) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cell cultures were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin and 1% (v/v) non-essential amino acids (Gibco, Grand Island, NY, USA). Additionally, medium for HL-60 cells was supplemented with 2 mM GlutaMAX (Gibco, Grand Island, NY, USA). Cell cultures were maintained in an incubator at 37°C with 95% humidity and 5% of CO₂. Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at 30 mM. Stock solution was kept at -20°C and protected from light until use.

Cellular treatment. To compare the cytostatic and cytotoxic effects of curcumin on HL-60 and K562 cell lines, 2.5x10⁵ cells/ml from each cell line were grown for 24 h, after which they were incubated with 5, 10, 15, 20 or 30 µM of curcumin for 24 h (dose-response assays) or with 30 µM of curcumin for 6, 12, 18, or 24 h (time-response assays). DMSO 0.1% (v/v) (curcumin vehicle) treatment for 24 h was used as a control culture in both cell lines.

Cell viability assays. After incubation with the indicated treatment, cells were washed once with phosphate-buffered saline (PBS; 1X) and the number of viable cells was determined with the trypan blue exclusion test by direct counting of cells on a light microscope Olympus CKX41 (Olympus, Miami, FL,

USA). Results were expressed as a percentage relative to the respective control culture (which was set=100% of viability).

Cell death assays. Cell death was measured with the LIVE/DEAD[®] Fixable Dead Cell Stain Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). This method uses red fluorescent reactive dye to stain the cells with damaged membranes. As a positive control of cell death, both cell lines were treated with 40 mJ/cm² of UV irradiation for 2 min in a *cross-linker* GS Gene linker-UV chamber (Bio-Rad, Hercules, CA, USA) and recovered 24 h post-irradiation. After the indicated treatments, cells were pelleted, washed with PBS 1X, and stained with 1 µl of fluorescent reactive dye from the LIVE/DEAD[®] Stain Kit. Cell suspensions were protected from light and incubated on ice for 30 min. Data was captured using the FACSCalibur flow cytometer system (Beckman Coulter, San Jose, CA, USA). At least 20,000 events were recorded for each sample. Data analysis was performed with the Summit Software (version 5.1.0.5563; Beckman Coulter).

Cell cycle assays. The K562 and HL-60 cells exposed to dose- and time-response assays, were pelleted, washed with cold-PBS 1X, and fixed overnight at -20°C with 1 ml of ice-cold 70% ethanol. Pellets were resuspended in 250 µl of PBS 1X and treated with RNase A (0.5 mg/ml) for 1 h at 37°C. Then, the cell suspensions were incubated on ice with 10 µg/ml of propidium iodide (Sigma-Aldrich) for 30 min in the dark. DNA content was measured using a FACSCalibur flow cytometer (Beckman Coulter), and cell cycle analysis performed with ModFit LT[™] software (version 4.1; Verity Software House, Topsham, ME., USA). At least 20,000 events were recorded for each sample.

Western blot analysis. To obtain total protein extracts, cell cultures treated with curcumin were collected and lysed with ProteoJET[™] mammalian cell lysis reagent (Fermentas, Waltham, MA, USA), following the manufacturer's instructions. Whole-cell extracts (30 µg) were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane (Perkin-Elmer, Boston, MA, USA), blocked with 5% non-fat milk in TBS-Tween-20 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), and incubated overnight at 4°C with specific primary antibodies used at a dilution of 1:1,000. Primary antibodies used were rabbit anti-human polyclonal caspase-9, rabbit anti-human polyclonal caspase-3 and rabbit anti-human polyclonal PARP (Cell Signaling Technology, Inc., Boston, MA, USA; cat. nos. 9502, 9662 and 9542, respectively). Additionally, we used a mouse anti-human monoclonal BubR1, goat anti-human polyclonal p-Chk1 (Ser 345), mouse anti-human monoclonal Chk1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. nos. sc-47744, sc-17922 and sc-8408) and rabbit anti-human monoclonal Securin (GeneTex, Inc., Irvine, CA, USA; cat. no. GTX62173). Membranes were washed twice with TBS-Tween-20 and incubated at room temperature for 90 min with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Invitrogen, Camarillo, CA, USA; cat. nos. 62-6520 and 65-6120, respectively) at a dilution of 1:5,000. Signals were detected using SuperSignal West Pico Maximum Sensitivity Substrate (Thermo Fisher Scientific,

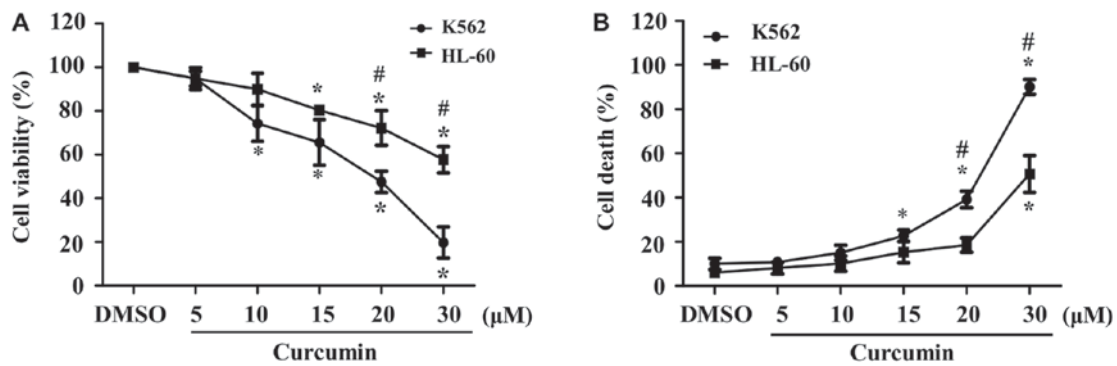


Figure 1. Effects of curcumin on growth and death in K562 and HL-60 cells. (A) Cell viability of K562 and HL-60 cell lines after 24 h of treatment with the indicated concentrations of curcumin. (B) Percentage of cell death in K562 and HL-60 after treatment with various concentrations of curcumin for 24 h. The mean \pm SD are shown. * $P < 0.05$ vs. the respective cell line control (DMSO); # $P < 0.05$ between K562 and HL-60 cells. Data are representative of at least 3 independent experiments.

Rockford, IL, USA). Membranes were reblotted and incubated with mouse anti-human monoclonal β -actin (1:500) (US Biological, Swampscott, MA, USA; cat. no. A0760-40) to account for well-to-well variation in loading.

Caspases activity assay. Activity of caspases-9 and -3 was evaluated in cell cultures treated with 30 μ M of curcumin for 24 h using specific fluorescence. Briefly, after treatment, cell lines were pelleted, washed, and lysed in lysis buffer (50 mM HEPES, 5 mM DTT and 1% Triton X-100) for 30 min. Cellular extracts were clarified by centrifugation at 16,000 \times g for 15 min at 4°C, and 100 μ g of proteins were incubated with 2 ml of caspase specific reaction buffer and 1 μ M of a fluorescent substrate to either caspase-3 (Ac-DEVD-AMC) or caspase-9 (Ac-LEDH-AFC) from Sigma-Aldrich at 32°C for 24 h, in the presence or absence of 2 μ M of specific inhibitors for caspase-3 (Ac-DEVD-CHO) or caspase-9 (Ac-LEDH-CHO), obtained from Enzo Life Sciences (Lausen, Switzerland). Caspase activity was measured as a function of fluorescence signal produced by the cleavage of synthetic substrate. Activity of caspases was determined by subtracting the fluorescence value obtained in the presence of caspase inhibitor from the value of total fluorescence.

Statistical analysis. Results are expressed as means \pm standard deviation (SD) of at least three independent experiments. Differences between control and curcumin treatments were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$. Statistical analysis was carried out using the GraphPad Prism 5 Software (version 5.01; Hearne Scientific, Pty Ltd., Melbourne, Australia).

Results

K562 cell line exhibits a higher susceptibility than HL-60 to curcumin effects. To determine the effect of curcumin on cell viability in acute (HL-60) and chronic (K562) myeloid leukemia-derived cell lines, both were treated for 24 h with increasing doses (5 to 30 μ M) of the phytochemical. Although we observed a dose-dependent reduction in cellular viability in both cell lines, the percentage of viable K562 cells decreased

to <80% at doses as low as 10 μ M, whereas in HL-60 cells, a similar reduction of the percentage of viability was observed until 20 μ M. Moreover, cellular viability at the highest dose of curcumin was significantly lower in K562 than in HL-60 cells (20 vs. 60%, respectively) (Fig. 1A). These data indicate that the K562 cell line is more sensitive to curcumin than HL-60 cells.

In addition, we evaluated cytotoxic properties of curcumin in K562 and HL-60 cells exposed to different concentrations of the phytochemical for 24 h. As expected, we observed a dose-dependent increase in the percentage of cell death in both cell cultures, which started at lower doses of the phytochemical in K562, in comparison with HL-60 cells (15 vs. 30 μ M, respectively). Similar to cell viability, the percentage of cell death at the highest dose of curcumin was significantly higher in K562 than in HL-60 cells (90 vs. 50%, respectively) (Fig. 1B).

Curcumin produces different effects on cell cycle progression in K562 and HL-60 cell lines. We next investigated the effect of curcumin on cell cycle progression in K562 and HL-60 cell lines in time-response assays, using 20 μ M of the phytochemical to avoid the excessively toxic effects observed at higher doses. We found an increase in the percentage of K562 cells at the G2/M phase in a time-dependent manner; whereas the HL-60 cell line had a small level of cells accumulated in G1 phase, which may suggest the presence of a G1 arrest (Fig. 2A and B). These observations indicate that HL-60 and K562 cells were affected by curcumin at different points in cell cycle progression.

To further analyze the cytostatic effect of curcumin in K562 and HL-60 cells, we co-incubated cell cultures with curcumin (20 μ M) and nocodazole (200 nM), an effective inhibitor of microtubule polymerization and a potent inducer of mitotic arrest. In the case of K562 cells, co-incubation of nocodazole with curcumin caused a higher accumulation of cells in G2/M (79.8%), than those observed in the individual curcumin treatment (44.3%) (Fig. 2C). On the other hand, HL-60 cultures incubated simultaneously with nocodazole and curcumin showed no significant difference in the number of cells in the G2/M phase (21.4%), in comparison with those treated with curcumin (22.8%) (Fig. 2D).

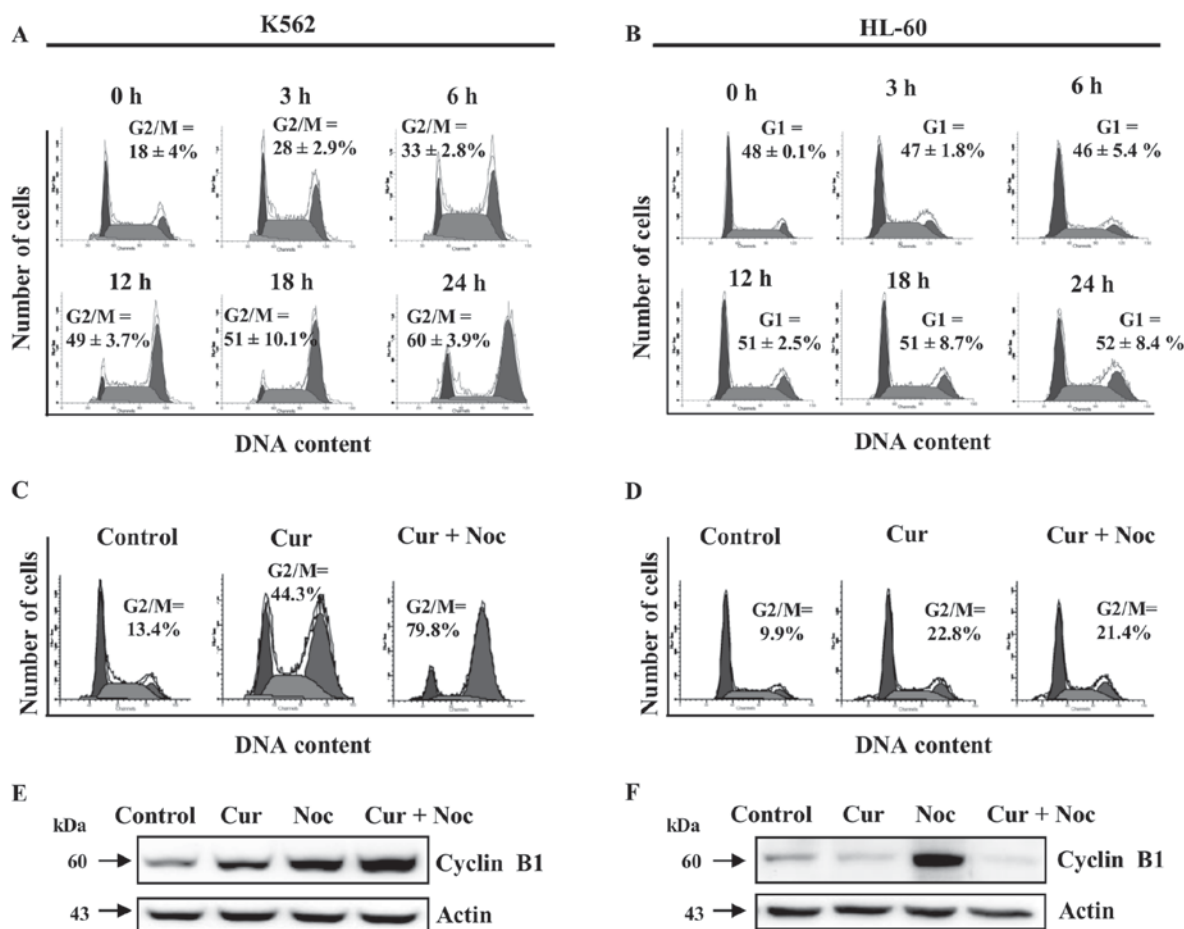


Figure 2. Curcumin differentially alters cell cycle progression in chronic or acute myeloid leukemia cells. (A) K562 and (B) HL-60 cells were treated with curcumin 20 μ M for different time points. Cells were fixed and stained with propidium iodide for analysis of cell cycle distribution. (C) K562 and (D) HL-60 cells were treated independently with 20 μ M of curcumin (Cur) or with 20 μ M of curcumin plus 200 nM of nocodazole (Cur+Noc) by 24 h. Cells were fixed and stained with propidium iodide for analysis of cell cycle distribution. Protein levels of cyclin B1 after the indicated treatment in (E) K562 and (F) HL-60 cells are shown. Actin was used as loading control. Control and time 0 cultures were treated with vehicle (DMSO) for 24 h. Data are representative of at least 3 independent experiments.

We also evaluated the protein levels of cyclin B1, which is normally a high abundance protein in the G2/M transition phase. In K562 cells, treatment with curcumin or nocodazole alone induced a significant accumulation of cyclin B1, whereas co-incubation produced a synergistic effect (Fig. 2E). In contrast, in HL-60 cells protein levels of cyclin B1 were increased only with the nocodazole treatment (Fig. 2F). Taken together, these data indicate that curcumin is able to induce a cell cycle arrest in both K562 and HL-60 cells, but at different phases of the cell cycle.

Curcumin-induced activation of caspases is defective in K562 but complete in HL-60 cells. Additionally, we analyzed the activation of caspases-9 and -3 in response to curcumin treatment as markers of apoptotic cell death. Using a curcumin concentration of 30 μ M in a time-response assay, the low molecular weight protein fragments of the processed forms of caspases-9 and -3 were observed, as well as the cleavage fragment of PARP, the caspase-3 substrate, in both K562 and HL-60 cells (Fig. 3A and B). However, in the K562 cell line, the unprocessed forms of caspases-9, -3, and PARP (47, 35 and 116 kDa, respectively) were still present up to the end of the incubation time (Fig. 3A; upper band in each panel). In

sharp contrast, in HL-60 cells the unprocessed forms of the two caspases, and PARP protein were no longer observable after 18 h of treatment with curcumin 30 μ M (Fig. 3B). In accordance with these data, activity of caspase-9 and -3 after incubation with 30 μ M of curcumin for 24 h was significantly lower in K562 as compared to the HL-60 cell line (Fig. 3C). These data suggest that caspases activation is an essential step in curcumin-induced cell death in HL-60 cells, but not necessarily in K562.

Curcumin triggers a defective DNA damage checkpoint at G2 phase in K562 cells. Curcumin is a known inducer of DNA damage in different types of tumoral cells (26,27). Thus, we analyzed in K562 cells, the activation of the DNA damage checkpoints in G2 and mitosis in response to curcumin, by detecting the phosphorylated status of the checkpoint proteins Chk1, Securin and BubR1. We observed no activation by phosphorylation in Ser-345 of the G2 checkpoint regulator Chk1 during the entire exposure to curcumin (Fig. 4A). In sharp contrast, the level of the phosphorylated and active forms of the mitotic checkpoint regulators Securin and BubR1 increased in a time-dependent manner after curcumin exposure (Fig. 4B).

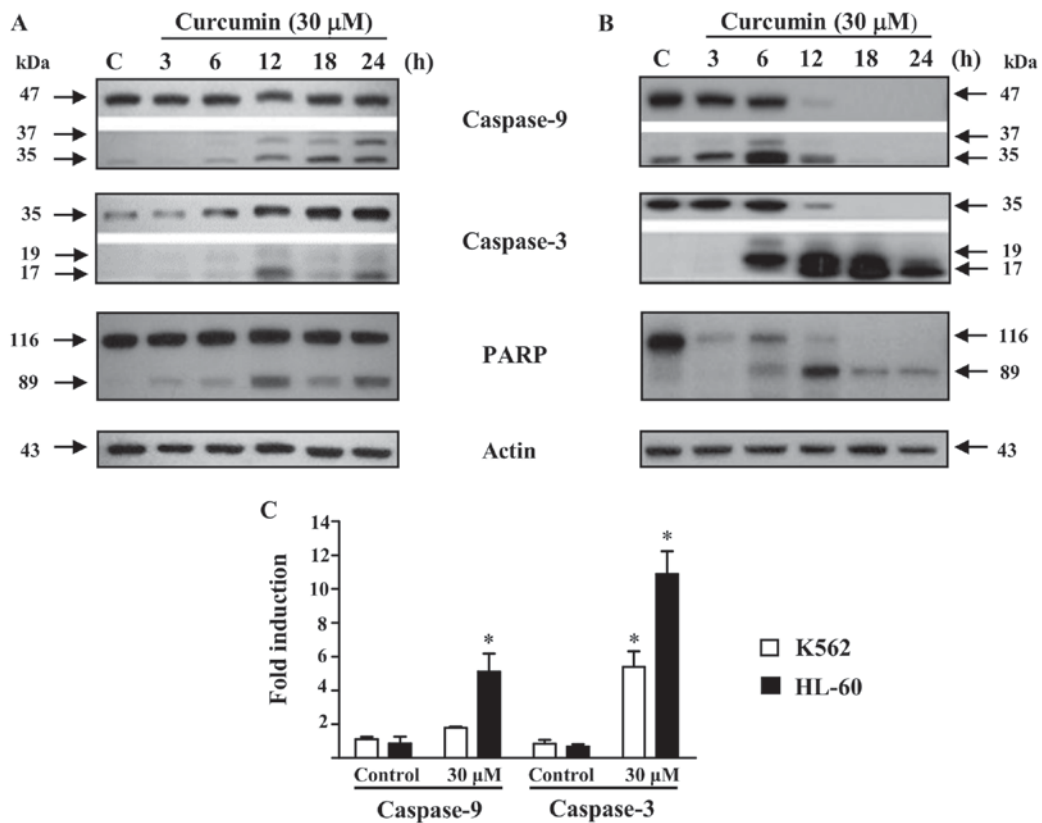


Figure 3. Curcumin induced differential activation of caspases in chronic or acute myeloid leukemia cells. Western blot analysis of caspases and PARP in (A) K562 and (B) HL-60 cells after different times of treatment with 30 μ M curcumin. Actin protein was utilized as loading control. Control cultures were treated with vehicle (DMSO) for 24 h. C=Control. Data are representative of at least 3 independent experiments. (C) Cell cultures were treated with 30 μ M curcumin for 24 h and activity of caspase-9 and -3 was evaluated in K562 and HL-60. Values are represented as the mean \pm standard deviation of at least 3 independent experiments. * P <0.05 vs. the respective cell line control (DMSO).

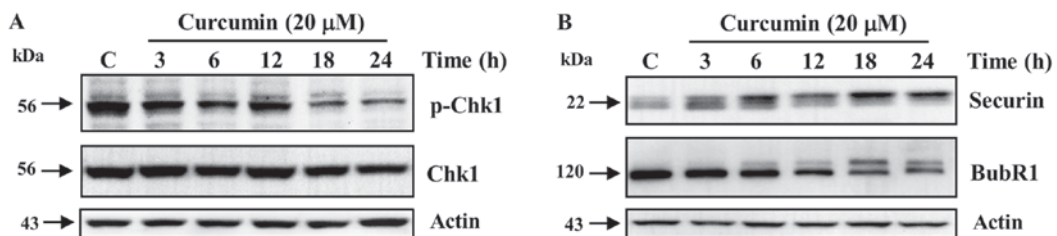


Figure 4. Curcumin exposure promoted a defective DNA damage checkpoint at G2 phase in chronic myeloid leukemia cells. K562 cells were incubated with curcumin 20 μ M for different time points. (A) Protein levels of total and phosphorylated Chk1 (p-Chk1) after incubation with curcumin. (B) Phosphorylation status of Securin and BubR1 protein in response to curcumin incubation. Actin was used as loading control. Control cultures were treated with vehicle (DMSO) for 24 h. Data are representative of at least 3 independent experiments.

Discussion

Curcumin is a natural bioactive compound which exerts antiproliferative and pro-apoptotic effects in a wide variety of cellular and animal cancer models. In fact, curcumin is being used as a radio and chemo-sensitizer in different types of cancer (28-30). However, several studies have shown that curcumin elicits different responses at the level of cell cycle regulation and cell death, depending upon the dose, time of exposure, and, perhaps most importantly, the tumor cell type. In this sense, the effect of curcumin on the regulation of cell cycle and in cell lines derived from acute and chronic myeloid leukemia (HL-60 and K562 cell lines, respectively) is highly controversial (19,25). Moreover, the cytostatic effects of this

phytochemical on cell lines derived from chronic myeloid leukemia have been poorly studied until now. This highlights the necessity of comparative studies assessing the cytostatic and cytotoxic effects of curcumin in these two types of myeloid leukemia cells.

In the case of the acute myeloid leukemia-derived cell line HL-60, previous reports have demonstrated the capacity of curcumin to induce a cell cycle arrest in G1 phase, followed by caspase-dependent induction of apoptosis (21,31). In addition, an independent study reported that G1 arrest induced by curcumin in HL-60 cells was dependent on the pro-oxidant properties of curcumin (30). In contrast, other studies described a G2/M arrest in HL-60 cells exposed to curcumin (19,20). Our assays, using simultaneous treatment

with nocodazole and curcumin (mitotic-trap), clearly demonstrated that the phytochemical induced a G1 arrest in the HL-60 cell line. In agreement with our present findings using this mitotic-trap method, curcumin exposure was also shown to induce a G1 arrest in other acute myeloid leukemia-derived cell lines such as MV4-11, KG1a, and Kasumi-1 (32). We have no clear explanation for the discrepancy between our findings and those describing G2/M arrest (19,20), but it is important to note that the curcumin concentration used in the previous study was lower (10 μ M) than that used in our present study.

Regarding chronic myeloid leukemia-derived cell line K562, few studies have analyzed the cytostatic effects of curcumin. However, in agreement with the curcumin-induced G2/M arrest we observed in the present study, in a mouse lymphoblastoid model overexpressing the BCR-ABL chimeric protein, curcumin exposure also induced a G2/M arrest (33). Notably, arsenic trioxide, a potent pro-oxidant compound used as a therapy in acute myeloid leukemia, also induced a G2/M arrest in K562 cells, whereas in leukemia-derived cell lines lacking the *BCR-ABL* fusion gene (e.g. HL-60 and U937) induced a G1 arrest (34). These data indicate that the underlying cellular mechanisms, which are responsible for curcumin-induced cell cycle arrest in HL-60 and K562 cells are different, and could be related to the presence of the *BCR-ABL* fusion.

We also observed differential effects of curcumin on cell death between HL-60 and K562 cells. In accordance with previous reports, we found that HL-60 cells exposed to curcumin showed induction of caspase-dependent apoptosis as a late event. Nevertheless, in the case of K562 cells, we found that curcumin-related cell death occurred at the G2/M transition phase, after a partial activation of caspases-9 and -3. This may suggest the presence of an alteration in the regulation of apoptosis in K562 cells that avoids full activation of caspases, and thereby triggers an alternative form of cell death. Furthermore, in a recent study, we have demonstrated that curcumin-induced cell death in K562 was produced by mitotic catastrophe (35). In contrast to our data, other studies have reported a curcumin-related activation of caspases-3 and -9, followed by apoptosis in K562 cells (23,24). It is important to note that a previous report demonstrated that curcumin exposure is able to induce apoptosis, through the activation of caspase-9 and -3, but also to promote autophagy, by the induction of Beclin1 and LC3 proteins (25).

Several previously published experiments highlight the generation of reactive oxygen species and the alteration of tubulin metabolism as the two main mechanisms underlying curcumin effects on regulation of cell cycle progression and cell death (36-38). Our results suggest that HL-60 cells may be able to respond to the pro-oxidant properties of curcumin, which induces cell cycle arrest at G1, followed by classical apoptosis, with full activation of caspases. On the other hand, in K562 cells, which may be not proficient at inducing cell cycle arrest at G1 and classical apoptosis, curcumin's effects are likely due to its ability to disrupt tubulin metabolism, which induces a G2/M arrest and cell death with partial activation of caspases. It is worth to mention that in a previous study (35), we demonstrated the phosphorylation of histone H3 in Ser-10 in K562 cells, after curcumin (20 μ M for 24 h) or nocodazole (200 nM for 24 h) exposure, indicating the arrest of K562 cells in mitosis. Considering that progression from G2 to mitosis

is promoted by the dephosphorylation of T14/Y15 at Cdc2 by CDC25C phosphatase, we speculate that in K562 cells, these residues are dephosphorylated in the treatment with curcumin. In order to test this hypothesis, in future studies we will investigate the cellular proteins involved in the regulation of cyclin B/Cdc2 activity in response to curcumin in chronic myeloid leukemia cells.

Supporting the hypothesis that K562 cells are not able to induce cell cycle arrest in response to curcumin-related DNA damage, we observed a lack of activation of the DNA damage checkpoint protein Chk1 in the presence of this phytochemical, together with the activation by phosphorylation of the mitotic checkpoint proteins BubR1 and Securin. These data suggest that in K562 the DNA damage checkpoint in G2 is not functioning correctly in response to curcumin, allowing cells with DNA damage to progress into mitosis, where the checkpoint machinery arrest the cell cycle. To further understand cellular response to curcumin exposure in both types of leukemia it would be necessary to evaluate the participation of key molecules in DNA damage response and cell cycle regulation. An important limitation of the present study is the absence of experimental data using markers of DNA damage such as phosphorylation of histone 2AX (γ -H2AX).

In summary, these data represent the first side-by-side comparison regarding the effects of curcumin on two leukemic cell lines, K562 and HL-60 cells. Our results clearly demonstrated that curcumin exposure arrest cell cycle at G1 in HL-60 cells and at G2/M in K562 cells. We also observed that the cellular mechanisms underlying curcumin's effects on cell death regulation in chronic and acute myeloid leukemia-derived cell lines were mechanistically distinct. The HL-60 cells appeared to be dependent upon caspase for curcumin-induced cell death, while the K562 cells underwent cell death in a caspase-independent manner. We consider that these data could help to further analyze the particular alterations in the mechanisms of cell cycle arrest and cell death in each type of leukemia.

Acknowledgements

The authors would like to thank Dr Jose Luis Cruz-Colin (National Institute of Genomic Medicine, Clinic Research, Mexico City, Mexico) for his valuable technical assistance for cell culture support and Dr Raúl Bonilla Moreno (Department of Molecular Biomedicine, Center of Studies and Advance Research, Mexico City, Mexico) for expert technical assistance. This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT) grants CB-2014-01-243587, 128686, CINVESTAV and INMEGEN. Work was also performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

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