RESEARCH ARTICLE
Bull Biotechnol (2023) 4(2):49-53
https://doi.org/10.51539/biotech.1358300 Journal homepage: www.dergipark.org.tr/biotech

Bulletin of Biotechnology

The effect of curcumin on the necroptosis signaling pathway in colon cancer cells

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Received : 11/09/2023
Accepted : 26/10/2023

Abstract: Colon cancer is the one of the most common types of cancer in humans. A sedentary lifestyle, increasing obesity and the consumption of food additives favor the development and occurrence of colon cancer. It is emphasized that curcumin, a yellow compound isolated from the turmeric plant, is important in preventing cancer. Studies have shown that curcumin has an anticancer effect by driving cancer cells into apoptosis, but studies showing its effect on necroptosis are inconclusive. Necroptosis is a form of programmed cell death mediated by RIP proteins and has been shown to play an important role in cancer. This study aims to determine the effect of curcumin on the necroptosis signaling pathway. For this purpose, HT-29 and HCT-116 colon cancer cells were cultured and exposed to different concentrations of curcumin and MTT experiments were performed to determine the effect on cell viability. The expression levels of RIPK1, RIPK3, and MLKL genes, which are markers of necroptosis, were analyzed by real-time PCR. It was found that the expression level of RIPK1, RIPK3, and MLKL genes significantly increased after exposure of HT -29 cells to 50 μM curcumin. Moreover, the expression of RIPK1 and MLKL genes increased in HCT-116 cells after curcumin administration. Consequently, the current data clearly suggest that curcumin is a prominent driver of necroptotic signaling-mediated colon cancer cell death.

Keywords: Colon cancer; Curcumin; Necroptosis; RIPK1; RIPK3; MLKL

1 Introduction

Cancer is the second-leading public health problem worldwide after cardiovascular diseases. Among the other cancer types, the incidence and mortality of colon cancer is quite high. In addition, studies have shown that colon cancer is mainly characterized by the accumulation of genetic and epigenetic changes (Marley et al. 2016). According to 2020 Global cancer statistics, it was reported that 1.1 million people were diagnosed with colon cancer and 0.58 million people died due to colon cancer (Sung et al. 2021). Also, sedentary lifestyle and increased obesity, low consumption of fibrous food sources and consumption of industrial foods with additives increase the development of colon cancer. Furthermore, according to the World Health Organization, 75-80% of the world's population consumes medicinal plants as their primary source of health care (Verma and Singh 2008). The main reason for this appears to be the anticancer activities of these plant species. In addition, it has been reported that the risk of developing lung, stomach, mouth, pharynx, esophagus, colon, and rectum cancers is inversely proportional to fruit and vegetable consumption.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a polyphenolic compound isolated from the rhizome of Curcuma longa Linn. Accumulating mass of indication suggests that curcumin has antioxidant, anti-inflammatory, anti-bacterial, anti-diabetic, and anti-cancer activities (Selvam et al. 2019). Curcumin has shown to have significant impact on many signaling pathways in cancer cells, which controls various cellular activities. The anticancer activities of curcumin have also been shown in colon cancer cells (Selvam et al. 2019). Curcumin has been demonstrated to show anti-cancer activity in HT-19 colon cancer cells by suppressing colony formation, cell viability, and DLEC1 promoter methylation (Guo et al. 2015). In addition, curcumin has been shown to suppress the level of ATG5 (autophagy related 5) protein in HCT-116 cells, leading to the suppression of autophagosome formation, cellular senescence, and cell cycle arrest (Mosieniak et al.

2012). In addition, studies have shown that curcumin has anticancer activity in other colon cancer cells such as Caco-2, HCT-15, and SW620 (Selvam et al. 2019). However, the effects of curcumin on the necroptosis pathway have not yet been demonstrated and remain elusive.

It is evident that apoptosis acts as a natural barrier against the development of cancer (Hanahan and Weinberg 2011). Also, resistance to apoptosis is an important hallmark of cancer and is often responsible for both tumorigenesis and drug resistance (Hanahan and Weinberg 2011; Johnstone et al. 2002). In addition to studies to overcome resistance to apoptosis, it is of great interest to develop approaches to induce non-apoptotic forms of programmed cell death as alternative therapeutics in cancer (Gong et al. 2019). One of these non-canonical cell death mechanisms is necroptosis. Necroptosis a distinct form of cell death mechanism because it resembles apoptosis by the presence of a signal transduction pathway and necrosis by cell morphology. Also, necroptosis is a caspase-independent cell death mechanisms and has been shown to be associated with the pathobiology of various diseases including cancer. Holler et al. were the first to report RIPK1 (receptor-interacting protein [RIP] kinase 1) as a member of necroptotic signaling pathway (Holler et al. 2000). Activation of RIP1 induces either apoptosis or necroptosis. Necroptotic signaling is initiated when RIP1 phosphorylates RIP3 to induce the activation of MLKL (mixed lineage kinase domain-like protein) (Cho et al. 2009; He et al. 2009; Sun et al. 2012). MLKL is then oligomerized and transported to the plasma membrane, thereby leading to the execution of necroptosis, resulting in necrotic plasma membrane permeability. Subsequently, necroptotic cell death occurs, characterized by swelling of the cell and loss of cell and organelle integrity (Cai et al. 2014; Sun et al. 2012).

It is well documented that curcumin has a wide range of biological activities, especially the anti-cancer activity of curcumin attracts more attention. However, there are few studies demonstrating the effect of curcumin on the necroptosis pathway in colorectal cancer cells. Accordingly, here we aimed to investigate the effect of curcumin on the necroptosis pathway in colon cancer cells.

2 Materials and Method

2.1 Cell culture

HT-29 and HCT-116 colon cancer cells were used for this study. Cells were cultured at 37 ºC in a carbon dioxide incubator containing 5% CO^2 and 95% air. HT-29 and HCT-116 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Confluent cells were removed with trypsin and centrifuged at 1500 rpm for 5 minutes. After centrifugation, the supernatant was removed, and the cells were homogenized in the medium.

2.2 Cell viability experiments

Cells were plated in 96-well plates at a concentration of 30,000 cells/ml and incubated for 24 hours. After 24 hours, the medium was removed and washed with 1X PBS (phosphate buffered saline). A 400 µM master stock solution was prepared by dissolving powdered curcumin with dimethyl sulfoxide (DMSO). Serial dilutions were then prepared from the main stock and HT-29 and HCT-116 cells were treated with the different concentrations of 200-100-50-25-12.5-6.25-0 µM curcumin and incubated for 24 hours. Following 24 hours of incubation, supernatants were discarded, and cells were rinsed with 1X PBS. Then, cells were treated with 1 mg/ml MTT (methylthiazolyldiphenyl-tetrazolium bromide) solution and incubated for 45-60 min at 37 °C. After the incubation, the supernatant was withdrawn, and the blue-violet formazan particles were dissolved with DMSO and read in a microplate reader at a wavelength of 570 nm and half maximum inhibitory concentrations were determined accordingly.

2.3 Colony forming assay

Colony formation experiments were carried out to determine the effect of curcumin on colony formation. First, cells were seeded at 1000 cells per well in 12-well plates and incubated for 24 hours. The cells were then exposed to curcumin and kept in the incubator for about 2 weeks. The experiment was terminated when there were approximately 50 cells in each colony. Colonies were then visualized by incubating 1 ml of 0.5% Crystal Violet dye solution at room temperature for 20 minutes. After incubation, wells were rinsed with distilled water until the colonies were visible and the plate was turned upside down and allowed to dry.

2.4 Gene expression analysis

RNA isolation

For gene expression analysis, total RNA was first isolated from cells using the GeneJET RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's recommendations. To determine the concentrations of the RNA samples, the RNAs were then measured in a NanoDrop 1000 (Thermo Fisher, USA) spectrophotometer and immediately placed in the refrigerator at -80 °C.

cDNA synthesis

The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used for single-stranded cDNA synthesis from isolated RNA samples. The mixture prepared according to the recommended procedure was exposed to thermal conditions at 25°C for 5 minutes, at 42°C for 60 minutes, and then at 70°C for 5 minutes. The synthesized cDNA was immediately placed on ice and stored at -80°C.

Real-Time PCR

Specific primers for the RIPK1, RIPK3, MLKL, and GAPDH genes were designed to determine their expression levels (Bozgeyik et al. 2023). RealQ Plus 2x Master Mix Green Kit (Amplicon, Denmark) was used to determine gene expressions. The prepared PCR mixtures were subjected to thermal conditions at 95 °C for 15 minutes (1 cycle), at 95 °C for 15 seconds, at 60°C for 30 seconds, and at 72 °C for 30 seconds (40 cycles). At the end of each reaction, melting curve analysis was performed between 55 and 95°C. After the reaction, the Ct value was determined for each sample at an appropriate threshold value. Gene expression level was determined according to the formula $2^{-\Delta Ct}$ ($-\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{references gene}}$).

3 Results

3.1 The effect of curcumin on cell viability and colony forming capabilities of colon cancer cells

To determine the antiproliferative activity of curcumin on HT-29 and HCT-116 colon cancer cells, we used MTT cell viability assay. A dose-dependent effect on cell viability was determined according to the increasing concentrations of curcumin. The minimum inhibitory concentration was determined to be 50 µM in both HT-29 and HCT-116 colon cancer cells (Fig 1). In addition, after the effective dose of curcumin was determined, its effect on colony formation was determined. Moreover, in consistent with the cell viability analysis, colony forming capabilities of colon cancer cells were determined to be dramatically reduced in cells treated with curcumin (Fig 2).

3.2 The effect of curcumin on the necroptosis pathway

To determine the effect of necroptosis in cells exposed to curcumin, the expression level of RIPK1, RIPK3 and MLKL genes was determined by Real-Time PCR. After exposure of HCT-116 cells with 50 µM curcumin, expression levels of RIPK1 ($p=0.0032$) and MLKL ($p=0.0008$) genes were found to be significantly increased compared to control. However, because RIPK3 expression was absent in HCT-116 cells, no change was observed (Fig 3). In addition, expression levels of RIPK1 ($p<0.0001$), RIPK3 ($p<0.0001$) and MLKL ($p=0.0006$) genes were found to increase after exposure of HT-19 cells to 50 µM curcumin (Fig 4). This change in the expression levels of RIPK1, RIPK3, and MLKL genes suggests that curcumin may be an important flavonoid in regulating the necroptosis pathway.

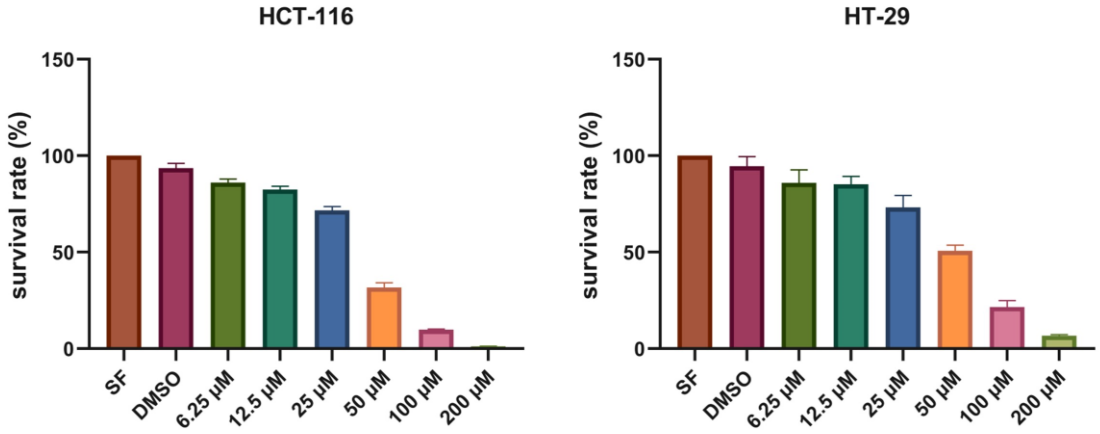

Fig 1. Effect of curcumin on cell viability at different concentrations in HCT-116 and HT-29 colon cancer cells.

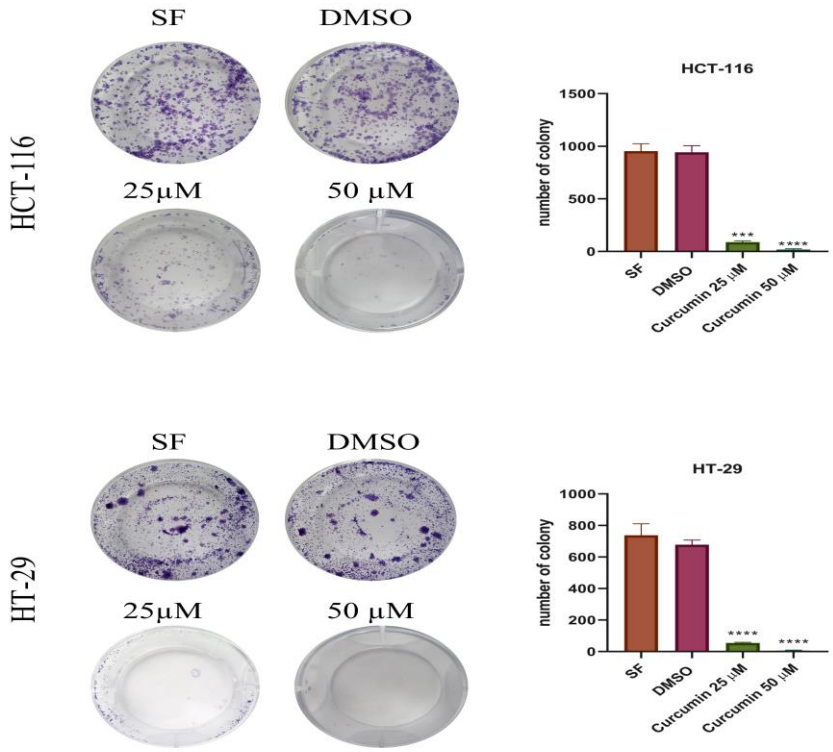

Fig 2. Effect of curcumin on colony forming capabilities of colon cancer cells.

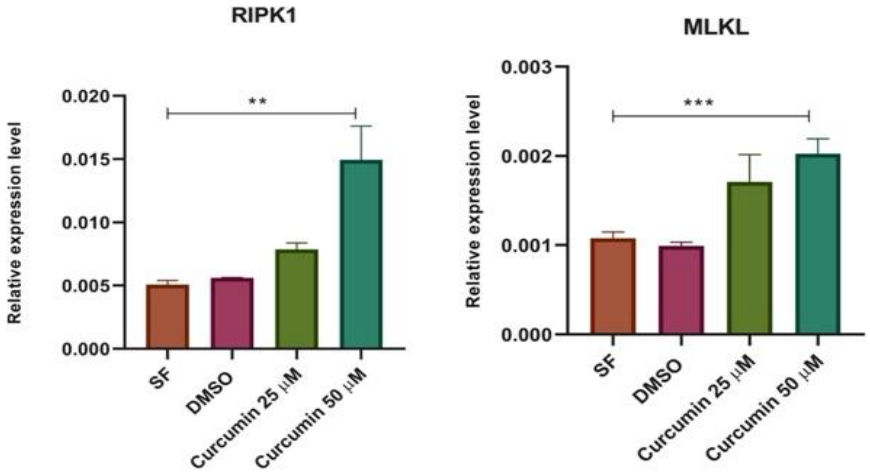

Fig 3. Gene expression changes of RIPK1 and MLKL genes following curcumin administration in HCT-116 cells. ** p<0.01, *** p<0.001.

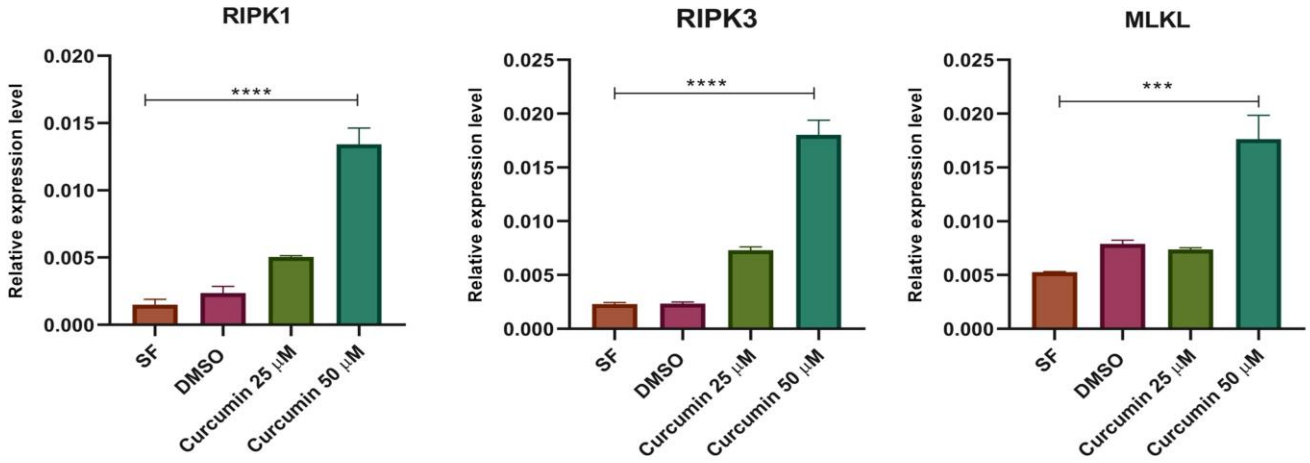

Fig 4. Gene expression changes of RIPK1, RIPK3 and MLKL following curcumin administration in HT-29 cells. *** p<0.001, **** p<0.0001.

4 Discussion

In the present study, necroptotic death of colon cancer cells were found to be significantly induced by curcumin administration. Notably, expression level of RIPK1, RIPK3 and MLKL genes, which are important necroptosis markers, increased significantly when HT-29 colon cancer cell was exposed to curcumin compared to the control group. In addition, curcumin was found to interfere with the proliferation of colon cancer cells by activating the necroptosis signaling pathway in these cells, strongly suggesting that curcumin is a significant regulator of necroptotic cell death mechanism.

Curcumin, which is extracted from the plant Curcuma longa, is known to have numerous biological and pharmacological activities. Curcumin has been reported to stimulate cell death pathways such as apoptosis, autophagy, and pyroptosis in many different cancer cells. In a study by Blakemore et al., curcumin was shown to disrupt the cell cycle progression by inducing G2/M cell cycle arrest in various colon cancer cells (Blakemore et al. 2013). In addition, curcumin treatment has been shown to cause abnormal mitotic spindle formation and DNA damage. In addition, curcumin has been shown to dose-dependently suppress cell proliferation and induce p53-mediated apoptosis in HT-29 and HCT-116 cells (Watson et al. 2010), increase the expression of pro-apoptotic genes such as BAD, BAX, and decrease the expression of anti-apoptotic BCL2 (Guo et al. 2013; Rana et al. 2015). Another biological feature of curcumin is its effect on epithelial mesenchymal transition and invasion and metastasis of cancer cells. In both in vivo and in vitro studies, it has been reported that curcumin has anti-metastatic activity in colon cancer cells by causing downregulation of the expression of the transcription factor Sp-1 and the cell adhesion component FAK and upregulation of the epithelial marker E-cadherin (Chen et al. 2013). In a study by Chen et al., it was reported that N-cadherin, Vimentin, Wnt3a, Snail1, Twist genes were downregulated in SW480 colon cancer cells depending on curcumin concentration (Chen et al. 2020). Despite this wide range of biological activities, studies showing the effect of curcumin on the necroptosis pathway in colon cancer cells are still unclear. In a study by Lee et al, curcumin was shown to target both apoptosis and necroptosis by causing DNA damage, increased reactive oxygen species production, and mitochondrial dysfunction in PC -3 prostate cancer cells (Lee et al. 2021). In HUVECs, a curcumin analog has also shown a necroptotic effect and induction of cell death by the curcumin analog could be partially reversed by administration of Necrostatins (Liu et al. 2019).

5 Conclusion

Remarkably, in our study, curcumin was shown to stimulate necroptosis by increasing the expression of RIPK1, RIPK3, and MLKL genes, especially in HT -29 colon cancer cells. In conclusion, present findings strongly indicates that curcumin is a significant driver of colon cancer cell death mediated by necroptotic signaling.

Acknowledgements

This work was funded by The Scientific & Technological Research Council of Turkey (TUBITAK) (Grant No: 1919B012202271).

Authors' contributions: SH: Methodology, Data curation, Writing - review & editing ACT: Methodology, Writing - review & editing IB: Methodology, Data curation, Writing - original draft, Writing - review & editing HB: Methodology, Writing - review & editing. EB: Conceptualization, Supervision, Methodology, Data curation, Writing - original draft, Writing - review & editing.

Conflict of interest disclosure: The authors of this study declare that they have no conflict of interest.

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