Genetics & Applications

Institute for Genetic Engineering and Biotechnology University of Sarajevo

Research article Open access

Effects of curcumin and luteolin on viability and cell death induction in NFS-60 cell line

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DOI: 10.31383/ga.vol2iss2pp43-50

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Received
November, 2018
Accepted
November, 2018
Published
December, 2018

Abstract

Inducing cell death in tumor cells has been recognized as a promising strategy in curing tumors. Concurrently, interest has been rising for demanding and extensive clinical trials based on the effects of natural products, especially those with long-known usage in folk medicine. Aiming to contribute to the overall knowledge of antitumour potential of curcumin and luteolin, we analyzed the effect of their concentration gradient (5, 10 and 20 μM) on cell death induction in NFS-60 cell line, using Trypan blue exclusion assay and TransDetect® Annexin V-EGFP/PI assay. The results show that both tested agents induce cell death, especially in higher concentrations, but further investigations are needed to elucidate the mechanisms behind it.

Keywords

Bioflavonoids, apoptosis, necrosis, cytotoxicity, murine leukemia

Introduction

Being one of the leading causes of mortality and morbidity, cancer is in the focus of intense research. One of the main features of tumor cells is their ability to avoid apoptosis either by the loss of pro-apoptotic signals or by enhancing the activity of anti-apoptotic pathways that contribute to tumor growth (Hanahan et al., 2000). Apoptosis is a mechanism of programmed cell death which is activated when reparative mechanisms of the cell cannot restore the damaged genetic material. It is also a part of normal development and morphogenesis (Nikoletopoulou et al., 2013) while, in adult organisims, it represents a physiological process of mainting constant cell number (Cooper et al., 2004; Haverić et al., 2018a). A series of controlled biochemical reactions cause changes in the morphology of a cell driving the process towards the cell death. Apoptosis prevents replication and propagation of a fraction of cells that contain unreparable DNA, whose replication could otherwise lead to the development of cancer (Haverić et al., 2018a).

There are two main cell pathways of apoptosis: intrinsic (mitochondrial) activated by cellular stress

and extrinsic initiated by the activation of death receptors. Mitochondrial pathway includes activation of pro-apoptotic BH3-only members of Bcl-2 family that bind to anti-apoptotic Bcl-2 and Bcl-xL proteins resulting in the release of pro-apoptotic proteins Bax and/or Bak. However, the BH3-only proteins are also crucial for death receptor mediated apoptosis as well thus presenting essential initiators of both apoptotic patways (Shamas-Din et al., 2010). Extrinsic triggering of apoptosis occurs through the cell surface death receptors such as TNFα (*tumor necrosis factor α*), Fas (*apoptosis antigen 1 – APO1 or CD95*), TRAIL (*TNF related apoptosis inducing ligand*).

Patological necrosis, as another type of cell death, as opposed to apoptosis, presents an unordered response to cellular trauma. However, necrosis may also occur in a highly programmed manner (necroptosis), sometimes triggered by the same death signals that initiate apoptosis (Laster et al., 1988; Vercammen et al., 1998; Jouan-Lanhouet et al., 2012), especially when the apoptosis is blocked (Los et al., 2002).

Induction of apoptosis represents a favored strategy imployed by chemotherapeutic and chemopreventive treatments of tumors. Substances that possess the ability to block or inhibit the proliferation of tumor cells are considered potential anti-carcinogenics. It is a well-established fact that plants contain a wide variety of active substances, mostly polyphenols, that have the ability to induce apoptosis in order to eliminate cancer cells (Mukherjee Nee Chakraborty et al., 2006). They are the products of secondary metabolism of plants and are present in human diet thus contributing their considerable positive biological attributes (Bravo, 1998; Manach et al., 2004; Tsao, 2010).

Polyhenols are very potent antioxidants and have an important role in the prevention of degenerative diseases connected with oxidative stress, such as cancer, neurodegenerative and cardiovascular diseases (Manach et al., 2004; Tsao, 2010). They also affect the activity of cell enzymes and receptors, which, in turn, affect numerous cell pathways (Middleton et al., 2000). An abundance of the published data single out luteolin and curcumin as polyphenols with high anticancerogenic potential (Agarwal et al., 2003; Xagorari et al., 2002; Mukherjee Nee Chakraborty et al., 2006; Singh et al., 2006; Ju et al., 2007; Lin et al., 2008).

Luteolin

Luteolin is a polyphenol belonging to flavonoids, a large group of plant secondary metabolites, characterized by a specific chemical structure: two phenyl and one heterocyclic ring (Harborne & Williams, 2000). Luteolin itself has hydroxyl groups in positions 3', 4', 5 and 7, which, together with a double bond in position 2, are considered the most important parts of its structure as they are responsible for its various biochemical and biological activities. Luteolin's antioxidative, antiinflammatory and anticancerogenic activities are expressed through scavenging the free radicals (Lin et al., 2008), activation of numerous pro-apoptotic pathways (Horinaka et al., 2005; Ju et al., 2007), estrogenic activity (Zand et al., 2000), endotoxin supression (Kotanidou et al., 2002), inhibition of cancer metabolism (Kim et al., 2005) and many other activities. It is believed that luteolin's pharmacological properties are interconnected: for example, its antinflammatory effects are linked with its anticancerogenic function (Lin et al., 2008). Various research conducted on numerous cell lines have demonstrated luteolin-related apoptosis induction without adverse effects on healthy cells. However, the mechanisms of apoptosis induction and the cellular pathway activation by luteolin vary considerably among the different cell lines (Ko et al., 2002; Chen et al., 2018).

Curcumin

Curcumin is natural, yellow-orange phytopolyphenolic pigment that was first isolated in 1815 from the rhizomes of *Curcuma longa* Linnaeus (turmeric) (Gupta et al., 2012). It belongs to the diarylheptanoid class of polyphenols consisting of two aromatic rings linked through linear seven-carbon aliphatic chain. Curcumin has three important functional groups: two o-methoxy phenolic groups, two enone moieties and 1.3- keto-enol moiety (Priyadarsini, 2013).

For thousands of years, in traditional Indian, Chinese and Arabic medicine curcumin has been commonly used as an anti-inflammatory agent for treatment of wide variety of ailments. In recent years, it has been reported that beside its anti-inflammatory effect, curcumin possesses: antibacterial, antifungal, antiviral,

antioxidant, anti-inflammatory and anticarcinogenic activities (Aggarwal et al., 2003; Mukherjee nee Chakraborty et al., 2006; Singh et al., 2006; Epstein et al., 2010; Kunnumakkara et al., 2017). Such a wide variety of curcumin's activities is facilitated by its ability to act as an effective scavenger of reactive oxygen species (ROS) and to alter gene expression and activity of specific proteins, such as NF-κB i AP-1, or others involved in intrinsic and extrinsic pathways of apoptosis (Singh et al., 2006; Reuter et al., 2008). Curcumin was found to induce apoptosis in a variety of tumor cells including leukemia, colon cancer, prostate cancer, breast cancer and others (Mukherjee nee Chakraborty et al., 2006).

Here presented study aimed to analyse pro-apoptotic activity of luteolin and curcumin in NFS-60 cell line and their effects on cell viability.

Materials and methods

NFS-60 cell line

Analysis of citotoxic potential of curcumin and luteolin was performed on murine myeloblastic leukemia cell line NFS-60. This cell line was established after infection of (NFS x DBA/2) F1 adult mice *Mus musculus* Linnaeus, 1758, with Cas-Br-M murine leukemia ecotropic retrovirus (Holmes et al., 1985).

NFS 60 cell line was cultured at 37°C in a 5%CO_2 atmosphere with 95% humidity. RPMI 1640 medium was supplemented with L-glutamine, fetal bovine serum (FBS), antibiotics and 5% filtered supernatant of adherent cell line 5637 (ATCC® HTB-9™) (human urinary bladder epithelial carcinoma). All culture regents were obtained from Sigma-Aldrich Co. (St. Louis, MO). In order to grow, NFS-60 require interleukin 3 (interleukin-3, IL-3) or macrophage colony stimulating factor (M-CSF), the latter is produced by 5637 cell line and contained in supernatant (Myers et al., 1984; Morioka et al., 1989).

Culture treatments

Powdered luteolin (Phytolab GmbH & Co., Germany) and curcumin (Sigma-Aldrich Co., St. Louis, MO) were dissolved in DMSO and after 24 hours of cultivation period were added to the cultures to achieve final concentrations: 5, 10 or 20 μM. Apoptosis inducer, 5-fluorouracil, was used as positive control, while only DMSO treated culture served as negative control. Cultures were harvested after additional 48 hours of cultivation.

Trypan blue exclusion assay

Cytotoxic effects of curcumin and luteolin on NFS-60 cell line were analyzed by trypan blue dye exclusion assay. After the ending of incubation period the cells were harvested by centrifugation at 800 rpm for 5 minutes. The supernatant was discarded; the cells were re-suspended and mixed with trypan blue dye (1:1).

Within 10 minutes timeframe, viable and non-viable cells were counted in *Neubauer* hemocytometer and viability index calculated as (No. of viable cells/total No. of viable + non-viable cells) x 100. Significance of differences in cell viability between control and treated cultures was tested using proportion analysis in WINKS 4.5 Proffessional software (TexaSoft, SAD).

Screening of cell death

In order to evaluate the potential of curcumin and luteolin to induce processes leading to cell death in tumor NFS-60 cells, the frequencies of early and late apoptosis as well as necrosis were analysed using TransDetect® Annexin V-EGFP/PI assay, as a rapid and sensitive method for evaluation of cell death (Vashishtha et al., 1998; Peng & Zhao, 2009). Treated cultures were incubated with appropriate binding dyes (Baskic et al., 2006), slides prepared and analyzed within one hour in dark conditions using Olympus BX51 epifluorescence microscope. At least 200 cells per each treatment and controls were counted. Visual distinction of different stages of cell death was performed according to the manufacturer instructions. Differences between treatments were tested using proportion comparison in WINKS 4.5 Professional software.

Results and Discussion

Annexin V-EGFP/PI staining enables reliable distinction between early and late apoptosis as well as necrosis (Figure 1). Calculated percentages of cell viability and observed absolute frequncies of various stages of cell death in the control and treated cultures are presented in tables 1 and 2.

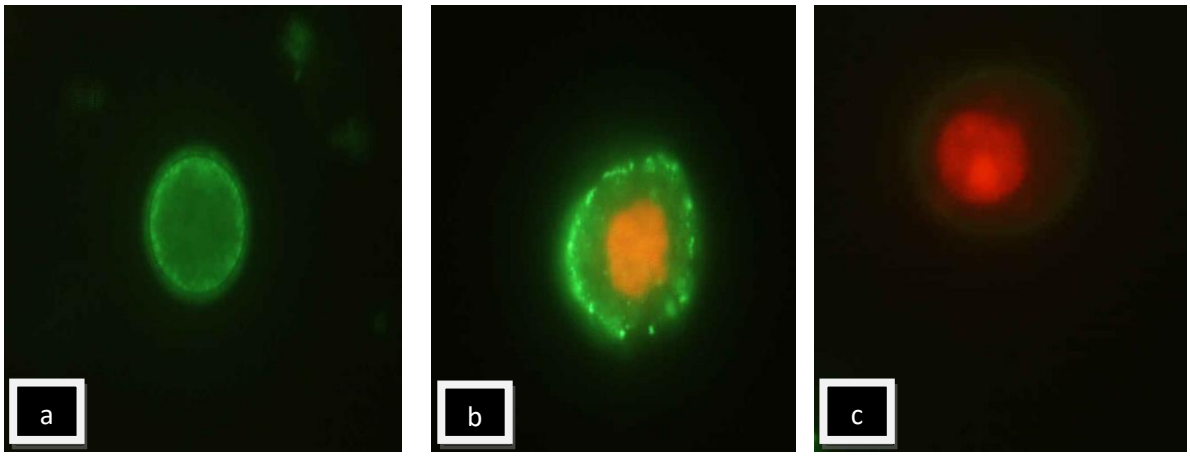

Figure 1. Fluorescent detection of cell death stages: a) early apoptosis b) late apoptosis; c) necrosis.

Proportion analysis revealed that both tested agents induce significant changes in percentages of viabile cells. The 5 μM concentration of curcumin increases cell culture viability while cell viability was reduced in 10 and 20 μM curcumin treatments (Figure 2). Increase in cell culture viability after luteolin treatment was registered for 5 and 10 μM concentrations while the highest applied concentration significantly reduced cell viability compared to negative control (Figure 2). The most significant reduction in NFS-60 cells viability was to 7.4% in 20 μM curcumin treatment. This finding is in accordance with previously reported antitumor activity of curcumin (Nelson et al., 2017).

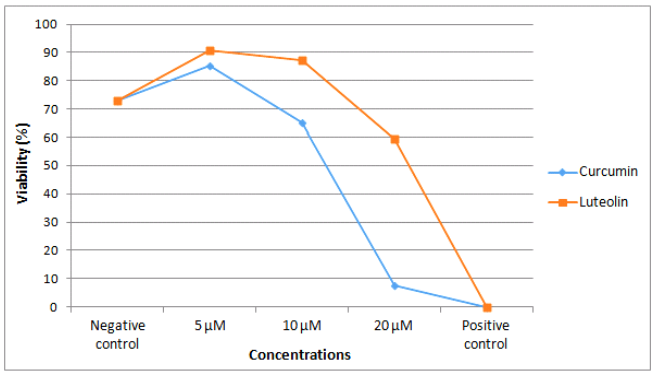

Figure 2. Cell viability in curcumin and luteolin treatments

Annexin V-EGFP/PI assay has shown that most of the observed cells are in early apoptosis, both in the control and in curcumin treated cultures. Late apoptosis and necrosis are more frequent in positive than in negative controls (Table 1 and 2). Curcumin treatment in all tested concentrations significantly reduced frequencies of early apoptosis in comparison to negative and positive control. Frequencies of early apoptosis, although lower in comparison to negative control, increased with the curcumin concentration increase. Frequencies of late apoptosis were significantly higher in all curcumin treatments compared to controls but their frequency decreased with the increase of curcumin concentration. Late apoptosis in curcumin treatments may be related to curcumin potential to accelerate apoptosis processes in different tumor cells. Analysis of quantitative cellular uptake of curcumin, analysed by absorption and fluorescence spectroscopy in normal and cancer cells, revealed significantly higher uptake of curcumin in cancer cells (Kunwar et al., 2008).

Tumor cells are even more sensitive to curcumin due to the lower registered levels of glutathione, important intracellular radical scavenger. Reduction of glutathione results in increased production of reactive oxygen species (ROS) that faciliate curcumin-induced apoptosis in tumor cells (Syng-ai et al., 2004). Unlike normal cells, nuclear factor – kappa B (NF-kB) is activated in the most of tumor cells. NF-κB activity promotes tumor cells proliferation, suppresses apoptosis, attracts angiogenesis, and facilitates distant metastasis (Xia et al., 2014). It has been shown that curcumin decreases activity of NF-kB and associated proteins that results in activation of apoptotic pathways in cancer cells (Gupta et al., 2012; Qadir et al., 2016). Apoptosis induction in tumor cells was the most efficient in curcumin concentrations of 20-25 μM and longer treatment periods while higher concentrations increased ratio of necrotic cells (Kuo et al., 1996; Syng-ai et al., 2004; Moustapha et al.,2015).

Table 1. Cell viability and stages of cellular death frequencies in curcumin treatment

		Negative control	Curcumin concentration 5 μM	10 μM	20 μM	Positive control
Viability (%)		73	85.2*	65.3*	7.4*	0
Nonviable cells (%)	Early apoptosis	90	47*	59.5*	61.5*	81
	Late apoptosis	8.5	41*	40.5*	38*	14.5
	Necrosis	1.5	12*	0**	0.5**	4.5

**p<0.05 in comparison to positive and negative control*
***p<0.05 in comparison to positive control*

Table 2. Cell viability and stages of cellular death frequencies in luteolin treatment

		Negative control	Luteolin concentration 5 μM	10 μM	20 μM	Positive control
Viability (%)		73	90.8*	87.3*	59.4*	0
Nonviable cells (%)	Early apoptosis	90	78.5*	80.5*	68***	81
	Late apoptosis	8.5	21.5*	19*	32***	14.5
	Necrosis	1.5	0**	0.5**	0**	4.5

**p<0.05 in comparison to negative control*
***p<0.05 in comparison to positive control*
**** p<0.05 in comparison to positive and negative control*

Genotoxic and cytotoxic effects of different curcumin concentrations (1, 2, 4, and 8 mM) in normal human lymphocytes culture were not recorded (Haverić et al., 2018b). However, curcumin citotoxicity and apoptosis induction has been also reported in some normal cells (Nelson et al., 2017).

The evidences for extrinsic apoptotic pathway activation are rare (Duvoix et al., 2003). Most of the studies are focused on mechanisms of curcumin-mediated apoptosis induction and show that curcumin acts pro-apoptoticaly through the mitochondrial pathway. It is activated by the accumulation of ROS, thus affecting decreased activity of anti-apoptotic members of Bcl-2 protein family associated with suppression of NF-kB and increased activity of pro-apoptotic Bax protein followed by the loss of mitochondrial outer membrane potential, release of cytochrome c in citosol and increased caspase activity (Syng-ai et al., 2004; Pae et al., 2007). Parallely, inhibitors of apoptosis, such as AKT and related proteins are inactivated (Hussain et al., 2006).

Analyses of luteolin effects on apoptosis and necrosis induction in NFS-60 cell line have shown significantly lower frequencies of early apoptosis and higher frequencies of late apoptosis for 5 and 10 μM concentrations compared to negative control and for 20 μM concentration compared to both controls. Necrosis were less frequent in all luteolin treatments and significantly lower compared to positive control. Luteolin potential to induce both the intrinsic and the extrinsic pathways of apoptosis is proven. Some of the effects are: higher expresion of death receptor 5 (Horinaka et al., 2005), activation of JNK (Shi et al., 2007), inhibition of topoisomerase I (Chowdhury et al., 2002), suprression of NF-kB (Ju et al., 2007), cell survival pathways or apoptosis inhibitors and anti-apoptotic proteins (Lin et al., 2008). The higher frequency of late apoptosis determined in NFS-60 cell line confirms luteolin as apoptosis inductor, although less efficient than 5-Fluorouracil. This is most likely due to the 5-FU direct incorporation in nucleic acids (Longley et al., 2003). Analysis conducted in HL-60 cell line have shown that apoptosis induction is directly related to luteolin concentration and time of incubation (Cheng et al., 2005). Research of Chen et al. (2018) in THP-1 cell

line show that increase in luteolin concentration results in decrease of cell viability while decrease of cell viability is not registered for normal monoclonal peripheral blood cells. Our previous research have also shown that luteolin in concentration of 50 μM protects and inhibits genotoxic damage in normal human lymphocytes culture (Hadžić et al., 2015). In the conditions of our experiment, curcumin and luteolin show different effects in the concentration of 10 μM, with curcumin decreasing and luteolin increasing NFS-60 cell culture viability, while in the lowest concentration (5 μM), both tested agents increase and in the highest concentration (20 μM) decrease cell culture viability.

Conclusions

As effects of luteolin and curcumin on induction of cell death in NFS-60 cell line have not been previously studied, this work presents a contribution to deeper understanding of their antitumor activity. Both curcumin and luteolin induce apoptosis in tested concentrations in NFS-60 cell line although further investigations are needed to elucidate the exact mechanisms involved.

Acknowledgement

This research has been supported by Federal Ministry of Science and Education of Federation of Bosnia and Herzegovina (grant No: 05-39-3087-9/16). We are also grateful to our colleague Dr. Nataša Skoko from International Centre for Genetic Engineering and Biotechnology (ICGEB) for providing initial NFS-60 cell line sample that enabled realization of this research.

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