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Dual effect of curcumin targets reactive oxygen species, adenosine triphosphate contents and intermediate steps of mitochondria-mediated apoptosis in lung cancer cell lines

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ABSTRACT

Exposure to arsenic is one of the major causes of lung cancer due to production of Reactive Oxygen Species (ROS). Herbal medicine is a new approach used for prevention or treatment of cancers. Among various herbal compounds, a lot of attention has been paid to curcumin, as antioxidant, anti-proliferative, anti-carcinogenic and anti-tumor and pro-apoptotic properties of curcumin have been well studied. In the present study, we investigated the effects of curcumin on lung cancer cell lines and arsenic-treated lung cancer cell lines, originated from different stages of lung cancer development. Here, we measured ROS generation and caspase 3/7 activity for both curcumin-treated cell lines and those co-treated with arsenic and curcumin. Then, we studied lipid peroxidation, intracellular ATP content, and cytochrome c release to further investigate how ROS generation and curcumin exert synergistic effects and direct cells toward apoptosis. According to our data, curcumin has a dual effect on ROS generation which is dependent on specific concentration as a threshold and seems to induce apoptosis by two different mechanisms. Moreover, for the first time we report that curcumin delays the drop in ATP levels in these cell lines and hence provides required energy for apoptosis process. Furthermore, western blot analysis reveals that release of cytochrome c is highest when ATP begins to drop in the presence of curcumin. To sum it up, it seems that curcumin is strong candidate for prevention or treatment of lung cancer, especially at stage 2.

1. Introduction

Lung cancer is one of the major health issues and is among the top 20 causes of death in the entire world, occurring in both genders and at different ages (El-Telbany and Ma, 2012; Ferlay et al., 2010; Kastner et al., 2012; Wu et al., 2010). After cardiovascular diseases and motor vehicle accidents, cancers are the most widespread causes of death in Iran, among which lung cancer is the second one (Saadat et al., 2014). Lung cancers are divided into two major types: Small Cell Lung Cancer (SCLC) responsible for approximately 15% and Non-Small Cell Lung Cancer (NSCLC) accounts for almost the rest of 85% of all lung cancers (Cruz et al., 2011). Tobacco smoke, exposure to Radon gas, asbestos and chemicals such as chromium, nickel and arsenic, air pollution and some dietary supplements, family history of lung cancer and genetic predisposition of individuals are the most important risk factors known to be responsible for initiation and development of lung cancer (Corbett et al., 2007; Du et al., 1998; Hosseini et al., 2009). Over the past years, lots of attention have been paid to harmful metalloids such as arsenic due to their carcinogenic effects in humans (Tchounwou et al., 2012; Valko et al., 2005).

Arsenic is a metal largely found in the environment and different occupational settings (Andrade et al., 2015). Arsenic exists as organic and inorganic forms and applied in glass industry, production of semiconductor and agriculture and may enter human bodies via contaminated drinking water. It has frequently been reported that arsenic is a harmful compound whose toxicity is dependent on the oxidative status. Arsenic exerts its toxicity through the ability to cause multiple malignant tumors, cancers and internal diseases at high dosage in bladder, skin and especially lung because of the presence of high partial pressure of oxygen and excretion of dimethylarsine in lung (Flora et al., 2007a). Alternatively, Reactive Oxygen Species (ROS) produced by arsenic include hydrogen peroxide, superoxide anion, singlet oxygen and hydroxyl radicals are able to damage cellular DNA and protein and this is another mechanism through which arsenic direct cells

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toward cancer development which in turn leads to apoptosis (Flora et al., 2007b; Li et al., 2001; Sharma et al., 2012).

Over the decades different approaches have been used for prevention or treatment of lung cancer (Lev-Ari et al., 2006; Oxnard et al., 2013). In recent years herbal medicine has received much attention because it is applicable for chronic diseases, inexpensive and widely available (Packer et al., 2004). Curcumin (diferuloylmethane), an active ingredient and yellow pigment extracted from the rhizoma of turmeric (*Curcuma longa*) has antioxidant, anti-proliferative, anti-carcinogenic and anti-tumor properties in different types of cell lines and animals (Anand et al., 2008; Lev-Ari et al., 2014). It has been demonstrated that curcumin is an appropriate compound to remarkably prevent or attenuate the production of ROS and neutralize harmful free radicals (Shehzad and Lee, 2013; Thayyullathil et al., 2008). These characteristics may persuade us to consider curcumin as a suitable and potential candidate for lung cancer treatment. Therefore, in the current study, we further investigated the effects of ROS generation through arsenic treatment and how curcumin would behave on lung cancer cell lines and evaluated its effects on the reduction of ROS generation. Moreover, we measured lipid peroxidation (LPO) and adenosine triphosphate (ATP) content of the arsenic-treated cell lines in the presence and absence of curcumin and analyzed the impact of these effects on cytochrome c release and apoptosis induction. We here used three common types of NSCLC cell lines including adenocarcinomas, squamous cell carcinomas and large cell carcinomas and the synergic effects of co-treatment of arsenic and curcumin were analyzed on each cell line.

2. Material and method

2.1. Human normal and NSCLC cell lines

The A549 non-small cell lung cancer cell line was purchased from Iranian Biological Resource Center and NCI-H520, NCI-H810 and CCD-19Lu cell lines were obtained from American Type Culture Collection (ATCC). NSCLC cell lines were cultured in DMEM (Dulbecco's Modified Eagle's Medium, GIBCO, USA) medium supplemented with 2 mM L-glutamine (GIBCO–BRL, Basel, Switzerland) and 10% fetal bovine serum (FBS), 100 Units/ml penicillin and 100 μg/ml streptomycin, purchased from GIBCO BRL (Invitrogen, Grand Island, NY,USA) and incubated at 37 °C, under 5% CO_2 and 95% air. Stocked cells were plated to a culture flask or a 6-well plate at a density of 3.0×10^5 cells per ml prior to each experiment.

2.2. Optimum time and concentration for curcumin and arsenic

At the first step we examined the optimum concentrations for both arsenic and curcumin. Furthermore, the optimum times for apoptosis detection were determined. For this, first at a proposed constant concentration of arsenic, ROS generation was detected at different times after treatment in normal human lung cell line. Then after, different concentrations of arsenic were used to specify the optimum concentration by measuring the caspase 3/7 activity in normal human lung cell line. The same procedure was performed for the optimum time and concentration of curcumin in apoptosis induction.

2.3. Cells treatment with curcumin and co-treatment with arsenic and ROS detection

The number of 3.0×10^5 cells per ml of all the four cell lines were plated on 6-well plates and treated with 30 μM curcumin at the time of medium change. Then Abcam's Cellular Reactive Oxygen Species Detection Assay Kit (Cambridge, UK) was used according to manufacturer to quantify the ROS production. To evaluate the ability of curcumin in apoptosis induction, caspase 3/7 activity was assayed. In another experiment, cells were first treated with 10 μM arsenic (Arsenic (III) oxide, Sigma-Aldrich, Germany) and after measuring the ROS generation, cells were co-treated with 30 μM curcumin. Again, caspase 3/7 activity was assayed.

2.4. Caspase-3/7 assay

The caspase-Glo 3/7 luminescent assay was used to measure caspase 3/7 activity (promega) as an indicative of apoptosis in the curcumin-treated and lead/curcumin-cotreated cells in triplicate. Cell lysate prepared by the cytosolic fractionation method with equal protein concentration was subjected to detect caspase-3/7 activity as described by manufacturer. For this, Cells were harvested and washed with PBS, pelleted by centrifugation then resuspended in 400 ml of hypotonic buffer A (10 mM HEPES–KOH pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM Na-EDTA, 68 mM sucrose, and 1 mM PMSF). Subsequently, the cells swelled and burst on ice for 15 min, and then mixed for 30–45 s by vortex. The cell debris was pelleted by centrifugation at 4 °C, 14,600 g for 30 s. For luciferase assay, the supernatant of cell lysate was used for luciferase assay. The activity was immediately recorded upon addition of 20 ml of luciferase assay complex (25 mM Tris, 10 mM $MgSO_4$, 1 mM luciferin (Resem BV, Netherlands), and 2 mM ATP (Roche)) to 20 ml of cell lysate using a luminometer (Berthold Detection System, Germany) as reported earlier (Azad et al., 2015).

2.5. Lipid peroxidation (LPO) assay

All the four cell lines were seeded at a density of 3×10^5 cells in 6-well culture plates. The cells were grown under an atmosphere of 95% and 5% CO_2 at 37 °C, to 80% confluence. The medium was aspirated from the cell monolayer and treated with 10 μM arsenic trioxide. The experiment was carried out in triplicates. After 24 h incubation, the cells were collected and resuspended in 1 ml of PBS, and were washed three times with PBS pH 7.4. The LPO assay was performed by LPO (MDA) Assay Kit according to the manufacture protocol (Sigma-Aldrich, Germany).

2.6. ATP assay

ATP was measured in three cancerous cell lines by luciferin–luciferase system. ATP concentration is directly proportional to the amount of light generated by the reaction of ATP with recombinant luciferase. Sensitivity was augmented by addition of the D-luciferin to the luciferase. 50 μl of cells, lysed with TCA 10% (tricoloroaceticacid) and neutralized with KOH 1 M and diluted with HEPES buffer 100 mM pH 7.8 (1:64), added to a cuvette containing 10 μl luciferin (sigma), 10 μl $MgSO_4$, 10 μl luciferase (1 mg/ml). The peak light efflux from cuvette to which either known ATP standards or samples are added was determined using a luminometer (Sirius tube Luminometer, Berthold Detection System, Germany). ATP standard curve was obtained on the day of experiment.

2.7. Western blotting

Western blot analysis was performed to evaluate the presence of cytochrome c in cytosol. Cells were lysed in Cell Culture Lysis Reagent (CCLR) buffer containing 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1% (v/v) Triton X-100, and 10% (v/v) glycerol. 100 μl of CCLR buffer was added to harvested cells and the mix was stored at −80 °C. Standard