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Dendrosomal curcumin significantly suppresses cancer cell proliferation *in vitro* and *in vivo*

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ABSTRACT

Curcumin, the main compound of spice turmeric, is one of the natural products that has been shown to possess effective anti-cancer properties. However, the absorption efficacy of curcumin is too low to make dramatic results in therapy. Therefore, we based the main aim of this study on improving the bioavailability of curcumin taking advantage of dendrosome nanoparticles; and subsequently evaluating *in vitro* and *in vivo* anti-tumor properties of dendrosomal curcumin. *In vitro* studies were carried out utilizing A431 and WEHI-164 cell lines and mouse embryonic normal fibroblasts. Our data revealed that dendrosomal curcumin not only exhibits a much higher bioavailability than void curcumin ($P<0.05$) but also inhibits the proliferation of cancer cells ($P<0.01$) in a time- and dose-dependent manner that could be ascribed to the induction of apoptosis. However, dendrosome did not indicate any toxic effect on different types of cell lines. For *in vivo* studies, BALB/c tumor-bearing mice were treated with dendrosomal curcumin, void curcumin, dendrosome and PBS. The results indicated that dendrosomal curcumin reduces significantly the tumor size in comparison with void curcumin and control samples ($P<0.05$). Furthermore, in animals treated with dendrosomal curcumin a longer survival was observed ($P<0.01$). We also found that the mice treated with dendrosomal curcumin, showed a significant increase in splenocyte proliferation and IFN-γ production as well as a significant decrease in IL-4 production. This can be a proof of anti-tumor immunity caused by dendrosomal curcumin. The findings demonstrate that dendrosomal curcumin offers a great potential to be a promising anti-cancer therapeutic agent.

1. Introduction

Cancer is defined as an intricate progressive multistep disease resulting from deregulation of several genes in different cellular pathways that lead to transformation of normal cells to malignant ones. Despite recent intensive attempts in understanding the molecular basis of tumor cells as well as successful targeted therapies for a limited number of tumor types, cancer remains to be one of the main causes of death worldwide [1]. On the basis of this idea and numerous epidemiological findings that diet plays a major role in cancer prevention, recent efforts have focused on traditional medicine (e.g. spices used in dietary) as a rich source of effective phytochemicals and their potential profits in treatment of chronic diseases especially cancer [1,2]. Curcumin is the flavoring agent of turmeric powder (*Curcuma longa*) with various therapeutic properties especially anti-tumor activity without any side effects on normal cells [3]. Its anti-tumor activities embrace cancer growth inhibition and apoptosis induction by modulating different signal transduction pathways *in vitro* and *in vivo* [4]. In spite of these tempting attributes of curcumin, insolubility of the compound in aqueous solutions, actually the main reason for its poor bioavailability *in vivo*, has limited its exploitation as therapeutic agent [5]. To overcome this problem and increase the solubility of curcumin, numerous approaches have been taken by using adjuvants, liposomes and more recently nanoparticles [6–9]. However, there has not been found any perfect formulation yet.

Here, we improved the solubility of curcumin thereby its anti-cancerous property by employing dendrosome nanoparticles *in vitro* and *in vivo*. Dendrosome is a neutral, amphipathic and biodegradable nano-material synthesized previously by our group and its capability has been shown in safely delivering genes into different cell lines [10,11]. Our data for the first time reveals that dendrosome not only boosts the solubility of curcumin and its uptake in cell lines but also increases its toxicity on cancer cells rather than healthy ones.

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Meanwhile, our *in vivo* work with fibrosarcoma models in BALB/c mice suggests that dendrosomal curcumin compound suppresses tumor growth, coupled with induction of immune response against cancer cells.

2. Materials and methods

2.1. In vitro study

2.1.1. Cells and reagents

Three cell lines including BALB/c mouse fibrosarcoma cell line WEHI-164, normal mouse embryonic fibroblast cell line MEF (both from Pasteur Institute, Tehran, Iran) and human epidermoidzcarcinoma cell line A431 (ATCC-Nr. CRL-1255) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) containing 10% fetal bovine serum (FBS; GIBCO, USA) at 37 °C in a humidified atmosphere of 5% CO2. Curcumin was purchased from Sigma-Aldrich Company, USA. Dendrosome nanoparticle specified Den O400, a nonionic biodegradable denderic glycol ester (MW: 590 Da, HLB: 12.5 Mh/M, hydroxyl value: 95 mg KOH/g and acid value: mg KOH/g) was a gift from Institute of Biochemistry and Biophysics, University of Tehran, Iran [10,11].

2.1.2. Dendrosomal curcumin preparation

Different weight/weight ratios of dendrosome/curcumin (dendrosomal curcumin) ranging from 50:1 to 10:1 were tested before settling an appropriate proportion of 25:1. Briefly, curcumin was dissolved in various amounts of liquid dendrosome and checked for absorbance spectra by UV spectrophotometery (TECAN, Switzerland). Then, the appropriate mixture of dendrosome and curcumin was evaluated for excitation/emission value in comparison with curcumin dissolved in PBS and 1% methanol as control samples. Subsequently, dendrosomal curcumin was sterilized by 0.22 μm filter and aliquots of this solution were stored in a dark and place and 4 °C.

2.1.3. Uptake kinetics of dendrosomal curcumin versus void curcumin

In order to determine the uptake kinetics of dendrosomal curcumin by different cell lines, the cells were treated with the same amount of curcumin for increasing periods of time. Cells were harvested in 3 cm petri dishes and allowed to grow overnight. Then, the petri dishes were treated with 12 μM dendrosomal curcumin (containing 12 μM curcumin) as well as void curcumin for 0 to 7 h. The cells were detached and transferred to FACS tubes for analysis in a flow cytometer (BD Bioscience FACS, USA). At least 10 000 events were used for each measurement. The peak measured in each sample was compared to a non-treated sample as a control, determining the relative fluorescence intensity per cell count.

To evaluate whether dendrosomal curcumin is attached to the cell membrane or absorbed into cells, its cellular localization was evaluated by fluorescence microscopy after treating with 12 μM dendrosomal curcumin and void curcumin in various incubation times. Each experiment was repeated at least three times.

2.1.4. Cell viability assay

Cell viability was measured by MTT (3-[4, 5-dimethylthiazol-2-yl] 2,5-iphenyltetrazolium bromide) assay according to the manufacturer's instructions (Sigma-Aldrich, USA). Briefly, WEHI-164, A431 and MEF cells were seeded onto 96-well plates and allowed to adhere and grow overnight in 200 μl medium. The cells were then incubated with fresh medium containing serial concentrations (0 to 100 μM) of curcumin in the form of dendrosomal curcumin and void curcumin solutions for 12, 24 and 48 h. Void dendrosome was also employed as negative control. Afterward, 20 μl of 5 mg/ml MTT was added to each well and incubated for additional 4 h at 37 °C followed by addition of 200 μl of di-methyl sulfoxide (DMSO). The relative cell viability was determined at 540 nm by a 96-well plate reader (TECAN, Switzerland) and the concentration at which cell growth was inhibited by 50% (IC50) was determined by *standard curve method* [12]. Each experiment was carried out in triplicate wells and repeated at least three times.

2.1.5. Apoptosis assay

2.1.5.1. Annexin V/FITC staining. Annexin V staining was performed by Annexin V-FITC kit (Miltenyi Biotech, Germany) to ascertain the mode of cell death. According to the manufacturer's manual, 2 cell lines including WEHI-164 and MEF were incubated with 12 μM of dendrosomal curcumin and void curcumin for 12, 24 and 48 h as mentioned above. Cell pellet was washed 2 times with binding buffer followed by incubating the cells with 10 μl of annexin V-FITC in a dark place. After 15 min, cells were washed with 1 ml of binding buffer and centrifuged. Finally, cell pellet was dissolved in 500 μl of binding buffer and 5 μl of PI solution was added immediately prior to analysis by flow cytometry. The experiment was repeated three times.

2.1.5.2. PARP cleavage assay. PARP protein cleavage, as an indicator of apoptosis, was investigated using western blotting analysis. Briefly, cells were treated with different amounts of dendrosomal curcumin for 24 h and processed for protein extraction. Equal amounts of the proteins (1.3 mg/ml) determined by Bradford assay on the lysate of 5×10^5 A431 cells were fractionated on 10% SDS-PAGE gels and transferred into a polyvinylidene difluoride membrane. After washing, the membrane was blocked with 10% skim milk at room temperature for 1 h and incubated with PARP monoclonal antibody (1:1000, Cell Signaling, USA) at 4 °C overnight. β-actin antibody was also used as internal control (Santa Cruz, USA). After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-goat antibody (1:1000, Santa Cruz, USA) at room temperature for 1 h. After washing, the immunoreactive bands were detected by 0.5 mg/ml diaminobenzidine (DAB) (Sigma-Aldrich, Germany) and 0.1% H2O2 in PBS.

2.2. In vivo study

2.2.1. Tumor models

Female inbred BALB/c mice at 6–8 weeks of age were purchased from Pasteur Institute, Tehran, Iran, and kept under the standard conditions. Animal care and treatment was conducted in conformity with the guideline of Animal Care and Research Committee of Tarbiat Modares University, which is in compliance with the *Guide for the Care and Use of Laboratory Animals* [13]. A total of 1×10^6 WEHI-164 cells/200 μl of PBS in logarithmic growth phase were injected subcutaneously (s.c.) into the right flank of BALB/c mice. Once tumor mass became established on day 12, animals were randomized in 4 groups ($n=8$ per group) including two test groups receiving intraperitoneally 12.5 mg/kg bw (body weight) of dendrosomal curcumin and void curcumin for three weeks as well as 2 control groups obtaining dendrosome and PBS. Tumors volume (mm^3) was measured 3 times per week using a digital vernier caliper (Mitutoyo) until sacrificing mice on day 35 post injection and calculated by the formula: $\text{Length}\times\text{width}2\times\pi/6$ [14]. On the other hand, the changes in median survival time (% IMST) were calculated by the formula: $\text{IMST}=[(T-C)/C]\times100$, where T is median survival time of treated group and C that of control group [14]. The institutional review board (IRB) of Tarbiat Modares University approved the procedure of survival study in tumor-bearing mice.

2.2.2. Splenocytes proliferation index

At the end of treatment, mice ($n=4$ per group) were sacrificed followed by spleen isolation. According to the previous work [15], spleen tissues were washed twice with PBS, homogenized and passed through a 100 μm filter to obtain a single cell suspension. Viable splenocytes were separated by density gradient centrifugation over Ficoll/