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ORIGINAL ARTICLE

Evaluation of Anticancer Effects of Curcumin on Multicellular Breast Cancer Spheroids

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OBJECTIVE
The anticancer properties of curcumin were determined in several studies, but on breast cancer stem cells (BCSCs) derived from a heterogeneous population of tumor cells, these effects have not been previously reported. This study aimed to evaluate these effects.

METHODS
After the development of an animal model of breast cancer, a heterogeneous population of breast cancer cells were isolated from the tumor mass. Spheroid formation as a reliable *in vitro* assay to assess the presence of BCSCs was conducted among these cells. The cytotoxic activity of curcumin on multicellular breast cancer spheroids was assessed by MTT assays. Induction of apoptosis was measured by Annexin V-propidium iodide (pi) flow cytometric analysis.

RESULTS
The curcumin has potent cytotoxic and apoptotic effects on breast cancer spheroids. Although, compared with monolayer, breast cancer cells are more resistant to apoptosis when cultured as multicellular spheroids.

CONCLUSION
This is the first report of the anticancer effects of curcumin on breast cancer stem like cells. Compared to many anti-cancer drugs and compounds, which have very limited ability to fight cancer stem cells, curcumin is a good candidate to combat BCSCs.

Keywords: Cancer stem cells; curcumin; sphere formation; triple-negative breast cancer.

Introduction

Despite many years of clinical research, statistics showed the number of diagnosed cases continues to rise and breast cancer remains the most common malignancy among ladies around the world.[1] The challenging characteristics of breast cancer such as metastasis, recurrence, and reduced overall survival are caused by breast cancer stem cells (BCSCs).[2] Accordingly, eradication of BCSCs is likely to be of clinical importance.[3] Multicellular tumor spheroids or *in vitro* three-dimensional (3D) culture systems are widely used models in tumor research.[4] Nowadays, multicellular tumor spheroids are the main candidate models in tumor research due to preserving the biological characteristics of original tumors better than conventional two-dimensional (2D) monolayer cultures.[5] These spheroids are purposed for the enrichment of

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cancer stem cells (CSCs) or cells with stem cell-related characteristics.[6]

Herbal extracts and their active components are a promising candidate for new treatment strategies against a variety of diseases included malignancies.[7] Curcuma genus has been extensively used in traditional medicine for treating several diseases.[8] One of the constituents of Curcuma species is curcuminoids including curcumin.[9] Antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer effects are the main biological activities of these phytochemicals. Among all, its anticancer potential has been the most described and remains under investigation.[10] Curcumin is believed to show its impact on cell growth and invasion of breast cancer through inducing apoptosis by regulating the expression of apoptosis-related genes, cell cycle arrest at the G2M phase and late S phase.[11-13] Studies have demonstrated that curcumin alone or combined with other drugs presents anticancer activities against malignant cell lines.[14]

Several studies have been focused on anticancer activities of curcumin but there is a lack of enough information on the effects of curcumin on multicellular breast cancer spheroids. In the present study, we are aimed to elucidate the anticancer properties such as cytotoxicity and apoptotic effects of curcumin on breast cancer multicellular tumor spheroids which are a great candidate on representing the native tumor microenvironment.

Materials and Methods

Cell Culture

Murine 4T1 cell line was obtained from the cell bank of Pasteur Institute of Iran (C604). These breast cancer cells were cultivated in high glucose Dulbecco's Modified Eagle's Medium (DMEM) in the presence of 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin (all from Gibco, USA) in humidified atmosphere of 5% CO_2 at 37°C.

Induction of Syngeneic Animal Model of Breast Cancer

As described in previous works[15] for tumor induction in female BALB/c mice, 4T1 cells were subcutaneously injected to the flank (or the right hind limb) of the mice (105 cells suspended in 100 µL phosphate-buffered saline [PBS]) using an insulin syringe. All animal experiments were in compliance with the relevant laws, and this study was approved by the Ethics Committee of Shahrood University of Medical Sciences (registration number: IR.SHMU.REC.1398.109).

Preparation of Heterogeneous Population of Breast Cancer Cells

CSCs are a small subset of the cancer cells among a heterogeneous population of cancer cells. Accordingly, for the first step, it is necessary to dissociate a tumor tissue sample into a single cell suspension to be able to isolate CSCs from the rest of the cancer cells. In the present research for isolation of heterogeneous population of tumor cells, primary tumor of cancerous mice was excised after 20 days of tumor induction in mice, and surface blood was removed by rinsing it in PBS. After mincing with scissors, fragments were placed to 50 ml conical tube. For enzymatic digestion, primary tumor was digested in 10 mg/ml collagenase type IV at 37°C for 75 min on a platform rocker. All enzymes were purchased from Sigma (St. Louis, MO, USA). The digested tumor filtered through 70 um cell strainers and washed with PBS. In the next step, washed cells were resuspended in medium containing 10% FBS, 100 U/ml penicillin, and 100 ug/ml streptomycin (all from Gibco, USA). Ultimately, the cells were cultured at 37°C in 5% CO_2 and passaged 2 times.

Cytotoxic Effect of Curcumin on Breast Cancer Spheroids

Suspension of heterogeneous population of cancer cells (isolated in previous steps) was prepared and cultured as described below:

1. For 2D monolayer, cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plates and cultured in high glucose DMEM containing 10% FBS and 2% penicillin-streptomycin (all from Gibco, USA) in humidified atmosphere of 5% CO_2 at 37°C. After 24 h incubation, cell culture medium was exchanged with complete medium supplemented with different concentrations of curcumin (5, 10, 15, 20, and 30 µM). Following 48 h incubation at 37°C, medium was removed and 50 ml of MTT solution at 5 mg/ml (Sigma) was added to the cultures and the incubation continued for a further 4 h period, after which 150 ml of dimethyl sulfoxide (DMSO) was added. Formed formazan crystals were allowed to dissolve for 30 min before measuring the optical density at 570 nm using CYTATION/5 imaging reader (Bio-Tek Instrument, USA). Finally, cell viability was expressed as percent compared to control wells according to the following equation:

$$Cell\ viability\ (\%) = \frac{(absorbance\ of\ treated\ well)}{(absorbance\ of\ control\ well)} \times 100$$

In this equation, blank means culture medium without cells and control means culture medium with cells. This experiment was performed in triplicate.

2. To form 3D spheroids, cells were seeded at a density of 1×10^4 cells/well in a 96-well Ultra-Low Attachment microplate and cultured in spheroid forming media comprised high glucose DMEM containing 0.5% FBS and 2% penicillin-streptomycin (all from Gibco, USA) in humidified atmosphere of 5% CO_2 at 37°C and incubating them for 6 days. The resulting tumor spheroids were treated for 48 h with different concentrations of curcumin (10, 25, 50, 75, and 100 μM) and MTT assay was performed as explained above.

Apoptosis Assay

For apoptosis assay, similar to previous step, 2D monolayer and 3D spheroids of breast cancer cells were prepared and treated for 48 h with IC50 concentrations of curcumin. Cell apoptosis assays were carried out with the use of MabTag's Annexin-V Apoptosis Detection Kit, according to the manufacturer protocol.

Statistical Analysis

Results are expressed as the mean ± standard deviation. Data were analyzed with GraphPad Prism statistical software 6.0 (GraphPad Software, La Jolla, CA, USA) using paired samples t-test. $P<0.05$ was considered statistically significant.

Results

Isolation of Heterogeneous Population of Tumor Cells

Most breast cancer cell lines have undergone changes in their function and genome due to multiple passages and manipulations.[16] Hence, we decided to use the heterogeneous population of primary tumor cells to create spheroid. For this, animal model of triple-negative breast cancer was generated (Fig. 1a). When tumor mass is palpable (20 days following tumor induction in BALB/c mice), tumor mass was removed aseptically (Fig. 1b). H and E staining and pathological confirmation were performed on tumor tissues (Fig. 1b). Heterogeneous population of tumor cells was isolated from tumor mass with enzymatical and mechanical digestion (Fig. 1c).

Multicellular Breast Cancer Spheroids Formation

For spheroid formation among heterogeneous population of tumor cells, we used non-adherent 96 well plates. As shown in Figure 1, after 6 days, the spheroids formed in the well (Fig. 1d). At this stage, the spheroids were ready for treatment with the curcumin.

Cytotoxic Effects of Curcumin against Multicellular Breast Cancer Spheroids

To determine the growth inhibitory activity of curcumin on breast cancer cells, heterogeneous population of tumor cells were treated with different concentrations of curcumin for 24 h, 48 h, and 72 h and cells viability was measured by MTT assay. The initial results indicated that cytotoxic effect was better analyzable qualitatively and statistically after 48 h. Therefore, the MTT assay was only done in dose-dependent manner. Exposing heterogeneous population of primary tumor cells to curcumin resulted in a significant decrease in cells viability in a dose-dependent manner ($p<0.05$), (Fig. 2). The IC50 value was considered as the concentration of the curcumin that caused a 50% decrease in cell viability relative to the negative control which was constituted by cell culture and DMSO without the curcumin. For 2D monolayer of tumor cells, IC50 value was found to be 10 uM by MTT assay. Compared with 2D monolayer, breast cancer cells are more resistant to cytotoxic effects of curcumin when cultured as multi-

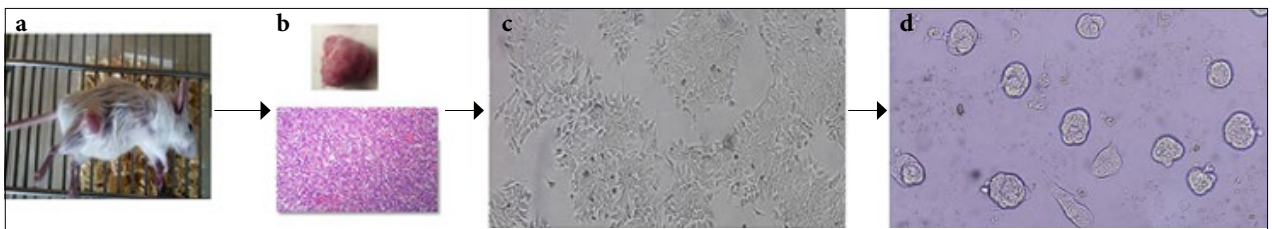

Fig. 1. Primary tumor cells isolation and multicellular breast cancer spheroids generation. (a) Animal model of triple-negative breast cancer was generated after 20 days of tumor induction in Balb/c mice. (b) Primary tumor isolation and H&E staining of tumor tissue were performed. (c) Primary tumor cell extraction was performed on primary tumor tissues. (d) Multicellular breast cancer spheroids generated from heterogeneous population of tumor cells in special culture condition after 6 days.

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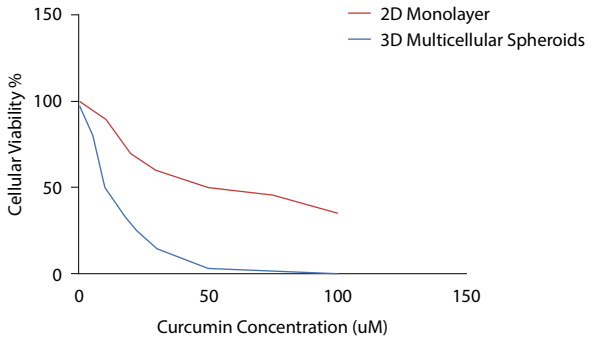

Fig. 2. Breast cancer cells viability in 2D monolayer and 3D multicellular spheroids under different concentration of curcumin. MTT assay results quantifying the viability of cells under different treatment conditions. Untreated cells were used as the control. Exposing heterogeneous population of primary tumor cells to curcumin resulted in a significant decrease in cells viability in a dose-dependent manner. IC_{50} value was found to be 10 μM for these cells in 2D monolayer format. Breast cancer cells in 3D culture were are more resistance to curcumin and IC_{50} value was found to be 50 μM for these cells in 3D multicellular spheroid format.

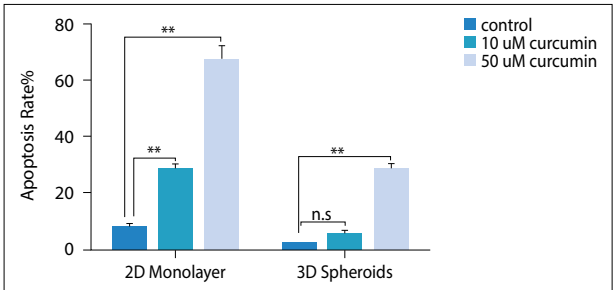

Fig. 3. Curcumin regulates apoptosis in breast cancer cells both in 2D monolayer and 3D multicellular spheroids. Breast cancer cells in 2D and 3D culture were treated with IC50 concentrations of curcumin (10 um and 50 um) for 48 h. Cell apoptosis was analyzed by flow cytometry. Compared with 2D monolayer, breast cancer cells are more resistant to apoptosis when cultured as multicellular spheroids. The level of apoptosis was 28% in 10 um concentration of curcumin in 2D conditions but this level was 5% in spheroids containing cancer stem cells (**p<0.005).

cellular 3D spheroids. IC50 value was found to be 60 uM by MTT assay for tumor cells presented in multicellular 3D spheroids. These IC50 concentrations were selected for all further mechanistic studies.

Apoptotic Effects of Curcumin

To determine the apoptotic effects of the curcumin, we used annexin test. For this purpose, both the heterogeneous population of tumor cells in 2D conditions and the population of cancer stem cells located in spheroids were treated with a concentration of IC50 concentrations of curcumin. The results of Annexin V/PI staining for curcumin after 48 h are shown in Figure 3. The curcumin could be induced apoptosis in breast cancer cells both in 2D and 3D condition but result indicated that breast tumor cells are significantly more resistant to apoptosis in 3D multicellular spheroids.

Discussion

Results of this study demonstrate that curcumin has apoptotic and cytotoxic effects against multicellular breast cancer spheroids. After preparation and isolation of a heterogeneous population of tumor cells, spheroid formation was conducted among these cells. The MTT assay results showed that in a dose-dependent manner, there was a significant decrease in cells viability. It should be noted that the cytotoxic effects of the curcumin on the spheroids were significantly reduced compared to 2D culture conditions. The apoptotic effects of curcumin were evaluated by the annexin test which showed apoptosis induction in both 2D conditions and the population of cancer stem cells located in spheroids, but the level of apoptosis was significantly lower in spheroids.

Curcumin showed cytotoxic and apoptotic potential against breast cancer in other studies as well. Several mechanisms such as inhibition of oncogene protein expression, stem-like properties, regulating the EMT process, cell cycle arrest, and interaction with oncogenic and tumor-suppressive miRNAs underlie curcumin cytotoxic effects.[13,17-19]

Most cytotoxicity assays are designed to evaluate anticancer drug effects on classic 2D cultures. Consequently, many cell characteristics and dynamic nature of tumor microenvironments are usually lost so 3D models are more predictive than monolayers in 2D cultures.[20,21] Similar to our findings, Abuelba et al.[22] also reported slight cytotoxic reduction in 3D breast cancer model compared to 2D condition. They worked on metastatic breast adenocarcinoma cell line (MDA-MB-231) and concluded that 3D tumor cell culture systems appear to be the ideal environment for *in vitro* assays regarding anticancer drug effects on cell viability.

Most 3D culture models are supposing tumor cell seeding on polymer scaffolds or cell embedding in hydrogels while some models are speculating adher-

ent cell ability to cluster in suspension or on low adherence surface. In a recent work, for achieving a 3D environment, researcher used encapsulation of cells in alginate hydrogel. The results of this study showed that curcumin in 3D culture conditions causes mortality in breast cancer cells (MCF-7).[23] Because of cell behavior in cell clusters is essential to further development of the tumor microenvironment, in our work, we used spheroids for 3D culture of breast cancer cells.

Evaluation of human MCF-7 breast cancer cells seeded in 2 and 3D culture systems confirmed that curcumin significantly decreased MCF-7 cells viability in dose- and time-dependent manners in 2 and 3D systems. However, cell viability in 2D cultures was significantly lower compared to 3D cultures; most probably due to cell clustering effect that may prevent curcumin penetration to inner cells in spheroids.[24] In this work, researchers used human MCF-7 breast cancer cell line. Most breast cancer cell lines have undergone changes in their function and genome due to multiple passages and manipulations.[16] Accordingly, in our work, we use the heterogeneous population of primary tumor cells to create spheroid.

It should be noted that curcumin effectiveness has been limited due to low bioavailability.[25] Several studies have been conducted to increase curcumin effectiveness.[26] To boost the bioavailability of this chemical component, various approaches have been undertaken like using adjuvant, liposomal curcumin, nanoparticles, phospholipid complex, and structural analogs of curcumin.[25]

In case of clinical use, since cancer is still one of the leading causes of death in the world, there is an increasing demand of new therapeutic interventions.[27] According to Sen et al.[28] study, curcumin is a potent chemosensitizer that improves the therapeutic index of widely used anti-cancer drugs. Therefore, it can be developed into an adjuvant chemotherapeutic drug. Xiong et al.[29] designed a dual-drug codelivery system which consisted of PTX (a US Food and Drug Administration approved chemotherapeutic drug for the treatment of breast cancer) and curcumin. The results of this *in vivo* study suggest that dual drugs could be a potential system for the treatment of breast cancers. Ferguson and Orlando also reported that addition of curcumin during 5-fluorouracil (an antimetabolite that inhibits cell proliferation) therapy enhanced the chemotherapeutic effectiveness by protecting normal cells from reduced viability and consequently permitting higher dosing or longer treatment times.[30]

Conclusion

The outcomes of this study clearly show that curcumin is capable of inducing apoptosis and has cytotoxic effects on both 2D and 3D breast cancer models. Up-to-date findings on curcumin anti-cancer properties suggest that it could open a new era in cancer treatment but further investigations are obligate.[31]

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Conflict of Interest: The author declares that they have no competing interest.

Ethics Committee Approval: This study was approved by the Shahrood University of Medical Sciences Ethics Committee (No: IR.SHMU.REC.1400.027, Date: 08/05/2021).

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