Three-dimensional chitosan scaffold-based MCF-7 cell culture for the determination of the cytotoxicity of tamoxifen

Harpreet K Dhiman\textsuperscript{a, b}, Alok R Ray\textsuperscript{a, b}, Amulya K Panda\textsuperscript{c,}\textsuperscript{*}

\textsuperscript{a} All India Institute of Medical Sciences, New Delhi 110029, India
\textsuperscript{b} National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India

Abstract

Three-dimensional (3D) culture of cancer cell lines has long been advocated as a better model of the malignant phenotype that is most closely related to tumorigenicity in vivo. Moreover, new drug development requires simple in vitro models that resemble the in vivo situation more in order to select active drugs against solid tumours and to decrease the use of experimental animals. A biodegradable, biocompatible and non-toxic polymer chitosan was employed for 3D culture of MCF-7 cell lines. Cells grown on chitosan scaffold produce more lactate from glucose in comparison to that secreted by cells grown on tissue culture plate, thus indicating the suitability of chitosan scaffold as an in vitro model resembling cancer tissue growth in vivo. Cytotoxic effect of tamoxifen at different concentrations was evaluated for MCF-7 breast cancer cell lines grown on tissue culture plate as well as on 3D chitosan scaffold. At a tamoxifen concentration of 10^{-6} M, 50\% reduction in cell growth was observed in tissue culture plate-grown cells where 15\% reduction in cell growth was observed when cells were grown in chitosan scaffold. Higher tamoxifen concentrations were required to achieve comparable cytostatic action in 3D culture, supporting the fact that 3D culture is a better model for the cytotoxic evaluation of anticancer drugs in vitro. Carbohydrate metabolism of MCF-7 cells in terms of glucose utilization and lactate production in 3D and monolayer culture were unaffected by tamoxifen treatment. Cathepsin D activity, an autocrine growth factor in breast cancer cells was monitored in all experiments. In 3D culture, addition of tamoxifen promoted cathepsin D secretion but inhibited its uptake by cells. Growth of cells in 3D chitosan scaffold indicated that action of tamoxifen on estrogen positive cancer cells is also mediated through inhibition of cathepsin D uptake from the culture medium.

Keywords: Chitosan scaffold; MCF-7 cell lines; Three dimensional culture; Tamoxifen; Cathepsin D

1. Introduction

Three-dimensional (3D) culture of animal cells on polymeric scaffold-based extracellular matrix is a better alternative model for understanding cell metabolism [1]. In vitro animal cell growth in 3D promotes normal epithelial polarity and differentiation [2], cells move and divide more quickly and have a characteristically asymmetric shape compared with that of cells in living tissue [3]. 3D culture of cancer cells allows to explore many basic questions related to cancer biology, as receptors for growth factors which play an important role in tumour development are expressed in different ways in comparison to the standard tissue culture plates [4,5]. For breast cancer, 3D culture provides a model system for understanding the regulation of cancer cell proliferation and for evaluation of different anticancer drugs [6,7]. The factors that control proliferation of breast cancer cell lines are complex and not yet well defined [8] and there are reports that breast cancer cells shows partial differentiation when grown in 3D culture [9]. Even though extensive work has been reported using 3D culture for understanding tissue architecture [6,10], very little has been published on the use of 3D culture as an in vitro model for the cytotoxic evaluation of anticancer drugs. There is a substantial amount of evidence that cells growing in 3D culture are more resistant to cytotoxic agents than cells in monolayer or
dispersed culture. Many studies have demonstrated an elevated level of drug resistance of spheroids culture compared with cells in monolayers [11]. Initially investigators attributed drug resistance of spheroids to poor diffusion of the drugs to interior cells but now it has been proved that only 3D culture accounts for drug resistance rather than mere inaccessibility to nutrients [12,13].

Factors which control the proliferation of breast cancer cell lines are not yet well defined as the growth is under the complex control of both estrogen and autocrine and paracrine growth factors [14]. Regulation of cellular proliferation by estrogen and growth factors [14,15], as well as growth inhibition by non-steroidal antiestrogen [16,17] is mediated by estrogen receptor. Tamoxifen is the most widely used drug in breast cancer therapy acts through these receptors [18-20]. The presence of estrogen receptors in most breast cancer cells thus makes them susceptible to growth inhibition by tamoxifen [21]. It is generally believed that tamoxifen has antiestrogenic activity and competes with estrogen receptors to inhibit cell growth [19]. Extensive studies have been undertaken using estrogen-receptor-positive MCF-7 cell lines to understand the molecular mechanism of tamoxifen action. With experiments carried out in monolayer culture in static T-flasks, where growth is limited not only due to space but also due to the build-up of local concentrations of inhibitory by-products of the cellular metabolism, a true picture of cellular proliferation and the mechanism of tamoxifen action thereof is not obtained. Apart from this, the two-dimensional (2D) culture does not provide information about the kinetics of the many growth factors of secretion and uptake, which are important for survival of cancer cells. Cathepsin D is an aspartyl protease abnormally secreted by breast cancer cells [22]. In breast cancer cell lines, only pro-cathepsin D is secreted [23] and shows pepstatin-inhibitable enzymatic activities at an acidic pH [24]. Cathepsin D activity has been reported to be associated with increased disease occurrence, metastasis and increased mortality, and may have a prognostic value to patients with breast cancer [25,26]. It has been demonstrated that the secreted pro-cathepsin D is an autocrine mitogen [23] and its over expression in vitro increases the cell proliferation [27]. Recent reports reveal that cathepsin D cleaves chemokines and other inflammatory proteins in breast cancer cells and it down regulation by antisense gene transfer inhibits tumour growth [28,29].

Recently we have reported the growth of MCF-7 cell lines on 3D porous chitosan scaffold [30]. Our hypothesis was that in vitro grown cells in 3D scaffold will provide better information on metabolic activities of breast cancer cells than the 2D culture. In this we report, we use 3D chitosan scaffold-based MCF-7 cell culture for the evaluation of cytotoxic effect of tamoxifen. Growth profiles of MCF-7 cell line, glucose uptake rate and lactate production rate during the growth on 3D scaffold was determined to provide information on primary metabolic activities. Modulation of cathepsin D enzymatic activity of MCF-7 cells by tamoxifen in 3D culture was also examined and compared with that of cell growth in the tissue culture plates.

2. Materials and methods

2.1. Materials

Chitin, from prawn shells, was obtained from the Central Institute of Fisheries Technology, Cochin, India. RPMI medium, and fetal calf serum was from Gibco BRL, USA. Trypsin EDTA, Trypan blue, Crystal violet, haemoglobin, glucose and lactate kit were from Sigma Chemicals (St. Louis, USA). Cathepsin D and Pepstatin A were also obtained from Sigma chemicals. Tamoxifen powder was a gift from Dabur India Limited, New Delhi. All other chemicals were of an analytical grade.

2.2. Preparation and selection of chitosan for 3D matrix

Chitosan was prepared from chitin by the deacetylation process using 50% (W/W) sodium hydroxide solution at 110°C for 5h [30]. After this treatment, flakes separated from the alkali layer were extensively washed with MQ water to remove the traces of alkali. The resulting flakes were dried in a vacuum oven at 50°C for 72h. Chitosan flakes were dissolved in 1% aqueous acetic acid solution and filtered through a sintered glass filter. Chitosan was precipitated from the resulting solution with 10% aqueous sodium hydroxide solution. The precipitate was washed several times with MQ water to remove the traces of alkali. The chitosan flakes were purified using the method described by Muzzarelli et al. [31] by Soxhlet extraction with methanol, MQ water, petroleum ether and acetone in that order, each for 24 h. Finally, the chitosan powder was dried in vacuum oven at 50°C for 20 h and stored in a desiccator. Chitosan matrix having an 80.9% degree of deacetylation was used for preparation of scaffold for 3D culture of MCF-7 cell lines; the detailed characterization has been described elsewhere [30]. Briefly, chitosan scaffold was prepared by precipitation of 1% chitosan solution in 0.1M NaOH. The scaffold was washed extensively with MQ water, and then transferred to a freezing bath and lyophilized [32]. The dried chitosan scaffold was sterilized by autoclaving in PBS for 15min at 121°C and used for the MCF-7 cell culture.
2.3. MCF-7 cell culture in T-flask

MCF-7 breast cancer cells were maintained in RPMI-1640 supplemented with 10% FCS in a T-flask in an incubator kept at 37°C and 5% CO₂ [30]. Inoculum concentration was 1 x 10^5 cells/ml for all experiments involving cell culture or tamoxifen evaluation. Cell viability was determined by trypan blue staining after dislodging the cells from the flask by trypsinization and cell number was counted using haemocytometer. Glucose and lactate concentrations in the culture supernatants were determined by enzymatic assay kits (Sigma chemicals, USA) [30].

2.4. MCF-7 culture on chitosan scaffold

Sterile chitosan scaffold were incubated in RPMI-1640 supplemented with 50% FCS for 3h and used for cell culture. Serum-treated scaffold was centrifuged at 800 rpm for 10 min, the supernatant was discarded and scaffold inoculated with MCF-7 breast cancer cells. Cell concentration of 1 x 10^5 cells/mg polymer scaffold/ml media were used in all experiments on the chitosan polymer matrix. A total of 2 mg of polymer scaffold and 2 ml of medium containing different concentrations of tamoxifen were taken in 15 ml sterile tubes. The cells were allowed to attach in static condition in CO₂ incubator for 1 h. After cell attachment, the scaffold were transferred to a 6-well plate and kept at 37°C in a humidified 5% CO₂ environmental incubator. Cell enumeration was done every day for 6 days. For cell enumeration, 1 ml of scaffold sample was taken out in a centrifuge tube, and centrifuged at 800 rpm for 15 min. The supernatant media was kept for glucose and lactate estimation. Cells were detached from the scaffold by using 1 ml of 0.1% (w/v) crystal violet in PBS containing 0.1 M citric acid. The sample was incubated at 37°C for 60 min. The suspension was then sheared with a Pasteur pipette to detach the nuclei from the micro-carriers. The nuclei were then counted on a haemocytometer.

2.5. Tamoxifen inhibition studies on MCF-7 cells

Tamoxifen stock solution (10^-4 M) was made in cell culture grade ethyl alcohol and was diluted in RPMI medium to get the desirable concentration (10^-5 - 10^-6 M). An inhibition study was conducted for 6 days by adding different concentrations of tamoxifen from day 0 of the cell culture. MCF-7 cell concentration of 0.5 x 10^5 cells/ml were used as inoculum in all experiments on 6-well plate. Tamoxifen studies were conducted both on monolayer cultures and also on cells grown on chitosan scaffold. Cells were trypsinized and viable cells were counted using trypan blue dye on haemocytometer for monolayer culture. For scaffold culture, the cells were treated with 0.1% of crystal violet in 0.1M citric acid for one hour at 37°C and the cell number was counted in a haemocytometer.

2.6. Cathepsin D activity measurement

Cathepsin D enzymatic activities were monitored by the method used for determining activity in tissue extracts of breast cancer patients [33]. The assay was modified for the cell culture supernatant. The cell culture supernatant (100 ml) was added to 600 ml of 0.1 M sodium formate buffer (pH 3.3) that had been pre-incubated for 3 min at 37°C with 100 ml of 0.4% (w/v) acid-denatured hemoglobin. Parallel assays were done; 100 ml of sodium formate buffer was added to one mixture whereas 100 ml of Pepstatin A (1.6 mg/ml, in sodium formate buffer pH 3.3) was added to the other, to determine the pepstatin-A inhibitable protease activity. The reaction mixtures were incubated at 37°C for 40 min and the reaction was stopped by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid (TCA). The reaction mixture was then centrifuged at 6000 rpm for 10 min at 4°C and the absorbency of the supernatant fluid was determined at 280 nm. Differences in the absorbencies of the reaction mixture in the presence and absence of pepstatin-A represent pepstatin-A inhibitable Cathepsin D activity. Blanks for the absorbance determination were done as monitored above except that haemoglobin was added after stopping the reaction with the TCA.

2.7. Statistical analysis

All cell culture data are expressed as the mean ± standard deviation (SD) for n = 3. Statistical significance of the cell growth during tamoxifen inhibition experiments were evaluated using the students t-test at a 94% confidence level (p-value 0.09).

3. Results and discussion

3.1. Growth kinetics of MCF-7 cell lines

MCF-7 cells were cultured in T-25 flasks as well as on 3D chitosan scaffold [Tamoxifen inhibition studies were conducted on 2D culture (6-well plate) as well as 3D culture using 3D chitosan scaffold.]

3.1.1. MCF-7 cell growth on a 6-well plate (2D culture)

MCF-7 cell lines were cultured on a 6-well plate and samples were taken at 24-h intervals to monitor cell growth, glucose consumption, lactate production (Fig. 1) and Cathepsin D activity. Maximum cell concentration of 0.45 x 10^6 cell/ml was achieved on tissue culture plate on day 5 of the culture. There was a fall in glucose concentration due to glucose uptake by
cells with concomitant increase in lactate production. However, the amount of lactate produced in the tissue culture flask was low.

3.1.2. Mcf-7 cell growth on chitosan scaffold (3d culture)

MCF-7 cell lines were grown on chitosan scaffold and samples were taken at 24 h intervals to monitor cell growth, glucose consumption, lactate production (Fig. 2). The peak cell growth was observed on Day 5 on the chitosan scaffold. Maximum cell concentration of 0.6 x 10^6 cell/ml was achieved on the chitosan scaffold on 5 days of the culture. There was a fall in glucose concentration due to glucose uptake by cells. The synthetic and metabolic functions, excretion and proliferation of anchorage-dependent cells can only occur after adhesion to a suitable culture surface, which must be hydrophilic and correctly charged [34]. The growth profile observed in 3D cell culture of MCF-7 cell was higher than that achieved in the tissue culture plate. The doubling time of the MCF-7 cell lines was around 24 h. Growth in chitosan matrix was associated with high lactate production. The lactate yield from glucose was more than 2, a value which is close to the tumour metabolism in tissue. Growth pattern in 3D matrix indicates that chitosan scaffold is suitable for tissue-like growth of MCF-7 breast cancer cells in in vitro.

3.2. Tamoxifen effect on MCF-7 cell line

3.2.1. Dose response studies in 2D culture

Cell growth, glucose utilization and lactate production profiles varied when the cells were cultured in the presence of tamoxifen. The action of tamoxifen on cell growth was observed after 24 h of addition. Tamoxifen dose response against different concentrations of drug (expressed in molar concentration) was observed on MCF-7 cell line in 2D culture (Fig. 3). At 5 x 10^-8 M drug concentration, very little growth inhibition (6%) was observed. There was a linear increase in growth inhibition, with concomitant increase in tamoxifen concentration. At tamoxifen concentration of 1 x 10^-5 M, 50% growth inhibition of MCF-7 cells was observed on day 5 of the culture. Complete cessation of cell growth was observed at a concentration of 5 x 10^-5 M in 2D culture. Cell viability in tamoxifen-treated cells was high (90% of the cells were viable), indicating that tamoxifen action was cytostatic and complete cytostatic action was observed after 72 h of tamoxifen addition.

3.2.2. Dose response in 3d culture on chitosan

Tamoxifen-dose response studies were carried out at different concentrations while growing MCF-7 cell lines on 3D culture using the chitosan polymer scaffold (Fig. 4). The first signs of growth inhibition were observed at 1 x 10^-6 M drug concentration during the cell growth in chitosan scaffold: the reduction in cell growth was 25% as compared to 50% observed in tissue culture plates.
3.3. Comparison of tamoxifen effect on 2D vs. 3D cell culture

Cell inhibition studies carried out with different tamoxifen concentrations was compared for both 2D (6-well plate) and 3D cell cultures (Fig. 5). It was observed that in 2D cell culture, the IC50 value (drug concentration needed to reduce cell growth by half) for tamoxifen was 1 x 10⁻⁵M, whereas for 3D culture, the IC50 value was 1 x 10⁻⁶M (59% growth inhibition). The 3D scaffold-based culture has a ten-fold higher IC50 value than that of 2D culture because of which the 3D cell culture exhibits increased resistance to Tamoxifen when compared to 2D cell culture. Tamoxifen concentration of 5.0 x 10⁻⁵M results in almost two-fold inhibition in MCF-7 cell growth in 2D cell culture (68%) as compared to 37% on 3D culture. Monolayers partially mimic cell-cell interaction and growth physiology in the 2D structure but not the 3D structure of a solid tumour. Moreover, monolayer cultures are over simplistic, since they do not mimic the complex and heterogeneous properties of tumours. The metabolic parameters determined for each growth condition are summarized in Table 1 for 2D culture and Table 2 for 3D culture. Tamoxifen inhibits the glucose uptake and lactate production. The lactate yield (\(\text{mol Wate/g glucose}\)) experiments was found to be somewhat higher than that of the theoretical value of 2.0 mol/mol, indicating the involvement of pathways other than glycolysis. In 3D culture the lactate yield (\(\text{mol Wate/g glucose}\)) was high similar to that observed for tumour cells in vivo. Tamoxifen acts at the early stage of the cell cycle and tamoxifen-treated cells remain viable during the culture period.

3.4. Cathepsin D enzymatic activity of MCF-7 cell lines

In order to determine the role of secreted cathepsin D on cell growth and tamoxifen inhibition, cathepsin D activity was monitored in MCF-7 cell lines. The protolytic activity of the culture increases during the early growth phase and then decreases before maximum growth is obtained. High cell yields in the later stages of growth in micro-carrier cultures were associated with the uptake of secreted cathepsin D by the cells (Fig. 6). As cathepsin D has been reported to be taken up by the cells it is possible that secreted procathepsin D acts as a mitogen leading to cellular growth. In Tamoxifen treated cells, cathepsin D secretion is high but its uptake was low (Fig. 6). In all the cases, cathepsin D activity was highest before the maximum cell growth in micro-carrier culture. In presence of tamoxifen, the cathepsin D activity was not only higher than that obtained during normal growth but also the build-up of cathepsin D activity was high; probably was not taken up by the cells as an autocrine mitogen in presence of tamoxifen. In cell culture, the effect of tamoxifen is observed after 24h after addition, and inhibition of cathepsin D uptake is evident from Day 1, wherein higher cathepsin D concentrations can be observed in Tamoxifen treated MCF-7 cell line as compared to control. Similar trend is observed in 2D as well as 3D culture, with tamoxifen inhibiting the cathepsin D uptake during Day 0 to Day 6 of MCF-7 cell culture. Even though the exact role of the secreted cathepsin D and its effect on cell proliferation remain to be elucidated, the data shows that tamoxifen inhibits cathepsin D uptake in MCF-7 cell lines.
Yield coefficients of glucose consumption and lactate production during 2D culture of MCF-7 cell line with different concentrations of Tamoxifen

Table 1

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>Glucose consumption (mmol/l)</th>
<th>Lactate production (mmol/l)</th>
<th>( Y_{Lacate/Glucose} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control No drug</td>
<td>Drug conc. 1 x 10^{-5}M</td>
<td>Drug conc. 5x10^{-4}M</td>
<td>Control No drug</td>
</tr>
<tr>
<td>1</td>
<td>1.94</td>
<td>1.17</td>
<td>2.00</td>
</tr>
<tr>
<td>2</td>
<td>3.11</td>
<td>3.39</td>
<td>3.3</td>
</tr>
<tr>
<td>3</td>
<td>4.94</td>
<td>6.22</td>
<td>4.50</td>
</tr>
<tr>
<td>4</td>
<td>7.88</td>
<td>6.77</td>
<td>6.97</td>
</tr>
<tr>
<td>5</td>
<td>10.93</td>
<td>8.55</td>
<td>9.57</td>
</tr>
<tr>
<td>6</td>
<td>10.95</td>
<td>10.10</td>
<td>9.59</td>
</tr>
</tbody>
</table>

4. Discussion

3D growth of MCF-7 cells on chitosan matrix provided a better model for the evaluation of the cytotoxic effect of tamoxifen. In the cell culture, the cytotoxic effect of tamoxifen was observed after 24 h after addition (equivalent to one doubling time) and complete inhibition of cell growth, which takes 72 h. The high viability of tamoxifen-treated cells and its time-dependent growth arrest in cell culture indicate that the mechanism of tamoxifen action is at the early stages of cell cycle regulation in MCF-7 cell lines. Tamoxifen action has been reported to be correlated with accumulation of cells in G1 or early G0-G1 phases of the cell cycle [35]. This is also supported by the fact that the metabolic yield (\( Y_{Lactate/Glucose} \)) of tamoxifen-treated cells remains unaffected in comparison to that of normal cells indicating the action of tamoxifen in cellular events before the triggering of primary metabolism. Our observations support the current hypothesis of tamoxifen action, which involves the arrest of cell growth in early phases of the cell cycle.

Higher concentration of tamoxifen is needed to arrest the growth rate to a similar extent when grown in a 3D culture in a polymer scaffold as compared to that observed in tissue culture mode. Structural architecture in a 3D tissue model regulates differentiated cell functions through changes in cell shape, as well as increased cell-cell and cell-matrix interactions. These intrinsic changes in cell function profoundly affect the response of a tissue model on external agents. Therefore, cell growth on 3D chitosan scaffold, which mimics in vitro tissue model, exhibits a greater drug resistance to tamoxifen as a multicellular tumour than as a monolayer. This resistance was not a result of mass transport effects but was an intrinsic property of the cells themselves. 3D cultures are composed of proliferating cells, non-proliferating viable cells and necrotic...
many more advantages like low immunogenicity, works on growth arrest of estrogen positive cancer cells. With the recent report of the role of cathepsin D in mechanism of tamoxifen action is not known. Cathepsin mechanisms of tamoxifen action can be through the inhibition of cathepsin D uptake. This observation is in support of the cell cycle arrest mechanism of tamoxifen action. The exact mechanism of cathepsin D uptake and its role in triggering of the cellular responses and the mechanism of tamoxifen action is not known. Cathepsin D has been proposed as an autocrine mitogen for the breast cancer cell line as it is overexpressed, hyper-secreted and partly endocytosed by the same cells [36]. With the recent report of the role of cathepsin D in breast cancer [29] it is tempting to postulate that one of the mechanisms of tamoxifen inhibition of cancer cell growth is by the inhibition of cathepsin D uptake. Such information could have only been available due to the 3D culture of cells on chitosan scaffold which helped in understanding the kinetics of cathepsin D uptake in the cell culture model.

3. Conclusion

In conclusion, the 3D culture of MCF-7 cells on chitosan matrix provided a better understanding of its carbohydrate metabolism, cytotoxic effect of anticancer drug and more importantly provided for the first time information about the kinetics and uptake of cathepsin D in breast cancer cells. On chitosan scaffold, MCF-7 cells produced more lactate similar to that observed for tumour cells in vivo, thus have metabolism more close to that observed in tissues. Tamoxifen-induced cell growth retardation was low in 3D culture in comparison to the tissue culture growth at a similar drug concentrations. It was also observed that cathepsin D uptake is inhibited by tamoxifen in 3D culture of MCF-7 cells. As cathepsin D is an autocrine mitogen for breast cancer cells, it can be concluded that one of the ways by which tamoxifen works on growth arrest of estrogen positive cancer cells is by the inhibition of cathepsin D uptake during cell growth. This information was available due to better analysis of the cells metabolic activities during 3D chitosan scaffold-based cell culture. Chitosan offers many more advantages like low immunogenicity, biodegradability and biocompatibility to be used as 3D scaffold for tissue engineering purposes. Such 3D tissue growth model thus can be used not only for evaluating the anticancer activities of new drugs but also can provide information about the regulation of both autocrine and paracrine growth factors that control cancer cell growth.

Acknowledgements

Authors are thankful to Dr. Sandip K Basu, Director, National Institute of Immunology, New Delhi for providing cell culture facility and encouragement to initiate work in the area of biomaterial and tissue engineering.

References


