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Tumorsphere assay provides more accurate prediction of in vivo responses to chemotherapeutics

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Abstract

Although the sphere culture system has been widely used in stem cell biology, its application for drug screening is limited due to lack of standardized, rapid analytical tools. To optimize sphere cultures for in vitro screening of drugs, we evaluated the properties of primary tumor cells growing as tumorspheres and compared their chemosensitivity to those of cells growing in monolayer. Most cells in tumorsphere cultures were quiescent whereas cells in monolayer culture had a high mitotic index. Moreover, doxorubicin showed better cytotoxicity than paclitaxel in the sphere cultures, but their efficacy was reversed in the monolayer cultures. Importantly, the response of cytotoxic outcomes for suspension cultures matched the in vivo response better than monolayer cultures, providing support for the use of short term suspension cultures of primary cells as a model for drug testing.

Keywords

Breast tumor cells; Chemoresistance; Doxorubicin; High-throughput screening; Paclitaxel; Quiescence; Sphere culture system; Tumorsphere

Introduction

Initial identification of anticancer agents is typically based on the ability to kill rapidly proliferating cells in vitro in high throughput screens. The efficacy of these agents is subsequently further screened in mouse xenograft models. This approach has been developed and widely accepted with the assumption that every cell within a tumor can proliferate to form new tumors (Greaves and Maley 2012). However, recent studies have proposed that growth potential is not equal for every cell, and that only some cells have the properties of cancer stem cells. Indeed, these cells may contribute to treatment failure and tumor recurrence (Al-Hajj et al. 2003; Tirino et al. 2013; Gupta et al. 2009). However, it is a challenge to develop a suitable in vitro culture system that includes the potential to identify heterogeneous responses in tumor cell populations. A method often used as a surrogate for maintaining cancer stem cells in vitro is to culture them as spheres in anchorage-independent conditions (Dontu et al. 2003). Originally established from an assay of neural stem cell activity and called “neurospheres” (Reynolds and Weiss 1992), this sphere assay has been adapted to the study of breast cancer cells to detect and propagate human breast cancer stem cells in several studies, under the moniker of “mammospheres” (note several assays are

termed mammosphere assays, some not suspension-independent) or “tumorspheres” (Dontu et al. 2003; Farnie et al. 2007; Grimshaw et al. 2008; Liu et al. 2007; Ponti et al. 2005). The ability to grow in suspension or anchorage-independence has been used for 50 years in cancer biology; this property is often typical of a sub-population of cells, and is clearly linked to tumorigenicity in vivo (Schwartz 1997).

In this paper, we describe a system that captures several key endpoints for drug screening. Firstly, high throughput capacity is made possible by the plating of only 10,000 dispersed tumor cells per 96 well. Since tumors can be grown as large (2 cm) masses, and harvested, singlized and frozen, there is a vast excess of number of cells for any given assay. We show that we can substitute out the mammosphere forming efficiency (MFE) assay for automatic counting methods (Shaw et al. 2012; Montales et al. 2012). The assay is run entirely with defined media components (no serum), to help their consistency. This study provides support for the relevance of this model for the screening of drugs aimed at the treatment of basaloid breast tumors. Presumably similar adaptation of other mouse model tumors will be useful for the study of other solid tumors.

Materials and methods

Mice

BALB/cJ (Jackson Labs, Bar Harbor, ME) mice were bred and maintained according to the guidelines set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, published by the U.S. Public Health Service. The Institutional Animal Care and Use Committee at the University of Wisconsin-Madison approved all experimental protocols. To obtain primary carcinogen-induced tumors, female *BALB/cJ* mice were administered 6×1 mg 7,12 dimethylbenz[*a*]anthracene (DMBA, Sigma, 0.2 ml/dose, ~250 mg/kg) by intragastric gavage once a week, starting at 12 weeks. After dissociation of primary DMBA-induced tumors, 10,000 cells were transplanted into cleared fat pads of 3 week-old *BALB/c* recipients to generate secondary tumors. Secondary tumors were used for both the in vitro and in vivo studies for testing chemotherapeutics (see Fig. 1a below).

Cell preparation

All reagents regarding to tumor cell preparation procedure were from Stem Cell Technologies Inc. (Vancouver, BC, Canada) unless otherwise specified. Tumors were chopped and digested for 1 h, 37 °C in Epicult-B supplemented with 5 % (v/v) fetal bovine serum, 300 U collagenase/ml and 100 U hyaluronidase/ml. After lysis of the red blood cells in NH_4Cl , tumor cells were dissociated by gentle pipetting for 1 min in 5 mg dispase II ml^{-1} plus 0.1 mg DNase I ml^{-1} (Roche). Single cells suspension was obtained by filtration through a 40 μm mesh.

Adherent culture in vitro

Cells were seeded into matrigel (BD Biosciences)-coated 8 well-chamber slides (Nalge Nunc International) or 96 well plates. Cells were maintained in DMEM/F12 supplemented with 10 % (v/v) FBS, (10 μg insulin/ml, 20 ng EGF/ml (R&D Systems, Minneapolis, MN)) and 100 U penicillin/streptomycin/ml.

Suspension sphere cultures in vitro

The protocol for the sphere culture was as previously described (Farnie et al. 2007) with modifications. Briefly, the freshly isolated cells or sorted cells were resuspended in serum-free MEGM (Cambrex BioScience, Verviers, Belgium), supplemented with 1:50 B27 (Sigma) and MEGM SingleQuot kit (Cambrex BioScience, Verviers, Belgium), which

contains aliquots of EGF, gentamicin/amphotericin-B, hydrocortisone, and insulin, and transferred to non-adherent 96 well plates.

Cell kinetic assay

Cells were plated from 5,000 to 100,000 cells/well into three 96 well plates; one under non-adherent conditions (see above), and the other two into monolayer cultures. To provide a zero reading in day 0, premixed cell proliferation reagent WST-1 (Roche, Indianapolis, IN) was added into one plate seeded with cells and after 4–5 h incubation, and the absorbance at 480 nm was measured according to the protocol provided by the manufacturer. The cultures of suspended cells were cultured for 1 week, while the other plate of adherent cultures was cultured for 4 days (almost to confluence), and then assayed using WST-1.

Immunofluorescent assay

Tumorspheres, dried briefly onto microscope slides, or cells grown in chamber slides, were fixed in ice-cold methanol and acetone for 2 and 4 min, respectively. Cell preparations were blocked in 10 % non-immune goat serum and stained with the following antibodies; rabbit anti-K5 (Covance, Madison, WI), rat anti-K8 (Troma-I) (Developmental Studies Hybridoma Bank, University of Iowa), and mouse anti-Brdu (Roche, Indianapolis, IN). Samples were incubated with anti-rabbit IgG-Pacific blue, anti-rat IgG-Alexa 546, and anti-mouse IgG-Alexa 488 secondary antibodies (All from Invitrogen, Carlsbad, CA) for 1 h at room temperature. Nuclei were counterstained with ToPro3 (Invitrogen) for 30 min prior to visualization using confocal microscopy.

In vitro response

To compare chemo-sensitivity of cells in the two culture systems, various concentrations of doxorubicin (DOX, Sigma) or paclitaxel (PTX, Sigma) (0.003–30 μ M) were added for the whole culture period and cell viability was measured at the endpoint. The absorbance of untreated cells was set to 1 and used to normalize the WST1 readings of treated cells. A zero value was assigned to the absorbance of cells treated with 10 μ M cycloheximide.

In vivo response: isograft models

10,000 cells were transplanted into cleared fat pads of 3-week old *BALB/c* mice (Badders et al. 2009). For the prevention study, doxorubicin (5 mg/kg) or paclitaxel (20 mg/kg) was administered by intraperitoneal injection once a week for 2 weeks, 1 week after surgery, and before any tumors appeared. The treatment cohort was administered with these drugs (once only, at the same doses) when tumors were palpable.

Results

Tumorspheres have a slow rate of cell division

To provide a more accurate reflection of hierarchal organization and heterogeneity that might be driven by cancer stem cells, we used carcinogen-induced primary tumor cells in this study. We have previously shown that DMBA-induced basaloid breast tumors are clearly heterogeneous. They comprise basal- and luminal-cell equivalents, and tumor initiating cell (TIC) potential is restricted to the basal-cell populations (Kim et al. 2012). This approach is more likely to be accurate than using immortal cultured cancer cell lines.

Carcinogen-induced primary tumors were obtained by administration of 6×1 mg DMBA to female *BALB/c* mice by intragastric gavage once a week, tumors were dissociated, and 10,000 single cells were transplanted into cleared fat pads to generate secondary tumors

(Fig. 1a, b). The tumor cells were maintained entirely in vivo through serial transplantation, and resultant tumors have a stable phenotype over several generations (Kim et al. 2012).

First, we cultured tumor cells in non-adherent culture condition for 7 days to test their ability to form tumorspheres. Carcinogen-induced primary tumor cells successfully form spheres, and the Immunostaining analysis of tumorspheres with lineage specific markers, K5 and K8, showed the maintenance of intact heterogeneity of DMBA-induced tumors (Fig. 1c). However, we determined that measuring their MFE was subjective, spheres ranged from 20 to over 150 μm , and may well comprise aggregates. Measuring an MFE is not conducive to high throughput screening. Thus, to develop a standard method for quantifying and analyzing sphere growth in non-adherent culture condition, we focused on the basic theory underlying the measurement of sphere forming ability which assumes that differentiated cells undergo anoikis when cultured in anchorage-independent conditions (Dontu et al. 2003). Since “spheres” or floating masses often form as an aggregate of dead and dying cells, we validated these cultures by staining for viability with Trypan Blue. Regardless of sphere size, tumorspheres contain >95 % viable cells, while most single cells that could not form spheres were not viable (Fig. 2a). This data suggests that measuring cell viability of cells grown in suspension culture can reflect on total number of cells within spheres. Thus, we utilized the premixed cell proliferation reagent, WST-1, to detect surviving cells in sphere culture system after 7 days, and measured absorbance at 420–480 nm using a 96 well plate reader.

Cells were plated from 2,500 to 50,000 cells/well into 96 well plates, either non-adherent plates, or regular tissue culture plates. We measured cell growth using WST-1 absorbance as a surrogate. When primary tumor cells were transferred to monolayer culture, they usually reached a stationary phase after 4–5 days of culture. Thus, the WST-1 absorbance for monolayer culture was measured after 4 days, whereas the cells grown as tumorspheres were maintained in suspension culture for 7 days (Fig. 2b). After 4 days of monolayer culture, or 1 week of tumor-sphere culture, total metabolism was assayed using WST-1 absorbance (Fig. 2b). The absorbance of uncultured cells was used as control at day 0. The overall WST1 readings of suspension cultures were at least two times less than monolayer culture cell counts, seeded with the same cell number. This slow cell growth rate in tumorspheres paralleled their mitotic index.

When tumorsphere cultures were labeled with BrdU for the entire cultural period (7 days), only 10–20 % of total cells in tumorspheres entered the cell cycle, while over 95 % of total cells in monolayer culture were BrdU-positive (Fig. 2c). All the cells divided at least once in monolayer culture (indicated as incorporation of BrdU), and the cultures became quickly confluent. In contrast, tumorsphere cultures grew slowly, indicated as an approximate threefold increase in overall absorbance over 7 days of culture. More interesting was the fact that the majority of cells in tumorspheres never divided (i.e. were BrdU-negative). Thus, only 10 % of cells were mitotic.

We calculate that if this mitotic subpopulation was continuously dividing approx. daily, the overall cell number would increase by sixfold over the course of 7 days. Therefore, the BrdU incorporation data we observe is fully consistent with the overall cell number measured by the WST1 assay. However, the growth pattern of cells in these cultures is very distinct from monolayer cultures, and provides a much more heterogeneous and challenging task for a drug assay. To be effective against cells growing in spheres, drugs would need to be able to disable or kill quiescent cells, alongside the mitotic cells, which are probably an easier target for drug inhibition.

Tumorspheres are relatively resistant to paclitaxel compared to monolayer cultures

Since quiescence may underlie chemo-resistance in tumors, we analyzed whether culturing cells as spheres affects their chemo-sensitivity. We chose drugs from classes of compounds widely used to treat breast cancer patients, namely paclitaxel (an anti-mitotic agent in the taxol class) and doxorubicin (adriamycin), a DNA intercalator (Gamucci et al. 2007; Sledge et al. 2003). To compare the cytotoxic effects of doxorubicin and paclitaxel on DMBA-induced tumor cells transferred to monolayer or sphere cultures, cells were exposed to compounds from 0.003 to 100 μM for the whole culture period, and cell viability was measured with WST-1 (Fig. 3a). The absorbance of untreated cells was used to normalize data (set to 1), while the absorbance of cells treated with 10 μM cycloheximide was set to zero.

Interestingly, different responses were observed in sphere cultures compared to monolayer cultures. Tumor cells cultured as a monolayer were highly sensitive to paclitaxel ($\text{IC}_{50} = 0.008 \mu\text{M}$) or doxorubicin ($\text{IC}_{50} = 0.1 \mu\text{M}$). In suspension, cells showed the similar IC_{50} for doxorubicin, but were highly resistant to paclitaxel, with an IC_{50} value of 1 μM . Thus, paclitaxel would be identified as a better cytotoxin from monolayer cultures, whereas doxorubicin emerges from the screen of sphere cultures (Fig. 3b, c).

Outcomes for tumor development in vivo match the response of sphere culture

Since there was a significant difference in chemosensitivity between tumorspheres and cells cultured in monolayers, we evaluated the response of isografted tumors growing in vivo to these same two drugs. We used two preclinical models that we dubbed the “prevention” model and the “treatment” model (Fig. 4a). For the prevention study, mice were injected with doxorubicin or paclitaxel before tumor re-growth and mice were palpated every 4 days. In the treatment study, mice that had developed 1 cm tumors (measured with calipers) were injected with these two compounds and tumor volumes were measured every day. Administration of paclitaxel failed to delay tumor initiation compared to the untreated group, while doxorubicin delayed tumor initiation and tumors appeared after approx. 1 month in the prevention study (Fig. 4b). Similar drug efficacy was observed in a treatment study. Tumor volumes decreased with treatment of doxorubicin, while tumors did not regress with the treatment of paclitaxel (Fig. 4c). These data indicate that these carcinogen-induced tumors were sensitive to doxorubicin treatment in both of the “prevention” model and the “treatment” model, matching the response of sphere cultures.

Discussion

In this study, we focused on evaluating properties of primary tumor cells growing in suspension compared to cells in monolayer, seeking to optimize sphere cultures as an in vitro screening method for drugs. Although the sphere-forming assay has been widely used in stem cell biology, the application of sphere-forming assays for high-throughput screening is limited due to formation of variable sizes of spheres and lack of a simple and well-established analytical tool. Generally, the size and number of spheres with manual counts are the final readout of sphere-forming assays. However, criteria regarding to which spheres qualify as large enough could have an influence on quantifying and analyzing sphere growth (Shaw et al. 2012). Moreover, a recent critical review of the neurosphere assay highlights that formation of spheres has been influenced by experimental variability including cell density and surface area of culture dish, and duration in culture before quantification (Pastrana et al. 2011). To provide a robust method for quantifying and analyzing sphere growth, we showed that tumorspheres contain >95 % viable cells (regardless of sphere size) and used the colorimetric WST1 assay. More interestingly, we found that most cells in a tumorsphere culture have a slow rate of cell division, while the same cells in monolayer

culture actively proliferate. Similar to this study, cells in G0/G1 phase accumulated in the sphere forming population of hepatoma cells compared to the same cells in 2D culture (Uchida et al. 2010). Since most cells in primary spheres survived but did not divide, it is clear that these are cell aggregates and not clonal spheres derived from stem cells. Indeed, this is often the case for sphere assays, though rarely disclosed (Pastrana et al. 2011). Thus, compounds identified from this assay may be able to target cells in a quiescent state, and we make no claims for targeting cancer stem cells specifically. Dividing cells are targeted by most current chemotherapeutics, although this quiescent cell population may play an important role in tumor regrowth and resistance. We propose therefore that this assay is a useful development.

Interestingly, although the sensitivity to doxorubicin was similar for both monolayer and suspension cultures, despite the difference in absolute growth rates, the suspended cells were highly resistant to the anti-mitotic spindle drug, paclitaxel. The molecular mechanism behind the insensitivity of suspension cultures to paclitaxel is not known, but may depend upon the relative impact of anti-microtubular drugs under these two different conditions (Rouzier et al. 2005). Many factors, seemingly unrelated to microtubule biology, also affect paclitaxel sensitivity (Rouzier et al. 2005; Blagosklonny and Fojo 1999). Interestingly, isografted tumors growing in vivo were more sensitive to doxorubicin treatment than paclitaxel, matching the response of sphere cultures. The correspondence of cytotoxic outcomes for suspension cultures with outcomes for tumor development in vivo suggest that spheres in a relatively quiescence state may be more suitable model for drug evaluation studies.

Moreover, the tumor cells described in this report are maintained entirely in vivo through serial transplantations. There is no period of culture adaptation and karyotypic rearrangement. This model provides a better reflection of the heterogeneity of tumors. It is simple to harvest 10,000 cells for each well of a 96 well plate, and the whole assay is only a week long. Furthermore, this model is ideal for the evaluation of the in vivo response to chemotherapeutics because parallel aliquots of cells can be inoculated at low cell numbers and can re-grow within 2–4 weeks after isografting to mammary fat pads (Kim et al. 2012). The tumors that regrow are similar to the primary tumor, and share many points of similarity with human triple negative basaloid tumors, including their heterogeneity, their transcriptomic program, their expression of key signaling molecules such as EGFR and the fact that the homologous DNA repair processes is intact. This last aspect is important, since this group of triple negative basaloid tumors appear to be the most refractive to typical radiation and chemotherapeutic treatments (Dent et al. 2007; Haffty et al. 2006).

In summary, we propose that the correspondence of cytotoxic outcomes for suspension cultures, with outcomes for tumor development in vivo, provides compelling justification for the use of these short-term cultures of explanted mouse tumor cells as a drug screening method.

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Fig. 1.

Sphere formation of carcinogen-induced primary tumor cells. **a** The experimental scheme for generating breast tumors in response to the administration of the carcinogen, DMBA, and for serial passage of tumor strains in vivo for assays. **b** DMBA-induced breast tumors are basaloid. Immunostaining of DMBA-induced tumors with anti-K5 (red) and anti-K8 (green). Scale bar 50 μ m. **c** Immunostaining of tumorspheres with anti-K5 (blue) and anti-K8 (red) exhibits the maintenance of intact heterogeneity of DMBA-induced tumors. Scale bar 50 μ m

Fig. 2.

Growth of cells transferred to monolayer or sphere cultures. **a** Viability of tumorspheres. Trypan blue staining reveals that most cells within spheres are viable while single cells are typically not viable and stain blue (*arrow head*). *Scale bar* 50 μ m. **b** Primary tumor cells were placed in monolayer or suspension culture, and their proliferation rate measured using WST-1 (times and conditions as indicated). **c** BrdU-incorporation: BrdU was incubated with the cells for the entire culture period (4 days for monolayer culture and 7 days for sphere culture), and then cells were stained with BrdU (*green*) and ToPro3 for DNA staining (*blue*). *Scale bar* 50 μ m

Fig. 3.

Different chemotherapeutic responses of tumor cells transferred to monolayer or sphere cultures. **a** The experimental scheme for testing chemotherapeutic efficacy in vitro. *PTX* paclitaxel; *DOX* doxorubicin. Compounds (0.003–30 μ M) were added to cultures for the whole culture period, and the cell viability/proliferation was measured with WST-1. Dose response curves of paclitaxel and doxorubicin in **b** monolayer cultures and **c** tumorsphere cultures

Fig. 4.

Evaluation of chemotherapeutic efficacies in a preclinical model. The in vivo cytotoxicity profiles matched chemotherapeutic response of tumor-sphere cultures better than monolayer culture. **a** The experimental scheme for testing chemotherapeutic efficacy in vivo, divided into two arms, one a prevention study, and the other a treatment study. **b** The efficacy for prevention of tumor growth was measured as days-tumor free. **c** For the treatment protocol, efficacy was measured by measuring tumor volumes