Journal of Biotechnology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Impact of the spheroid model complexity on drug response

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ARTICLE INFO

Article history:
Received 27 August 2014
Received in revised form 16 February 2015
Accepted 24 February 2015
Available online xxx

Keywords:
Spheroid model
Drug screening
Cancer cell line
Human tumor tissue

ABSTRACT

Pharmaceutical investigators are searching for preclinical models closely resembling the original cancer and predicting clinical outcome. This study compares drug response of three *in vitro* 3D-drug screening models with different complexity.

Tumor cell line spheroids were generated from the cell lines Caco-2, DLD-1, COLO 205, HT-29 and HCT-116, and treated with clinically relevant combination therapies, namely 5-FU/oxaliplatin (FO), 5-FU/irinotecan (FI) and the molecular drugs Cetuximab, Trastuzumab, Vorinostat and Everolimus. Treatment results were compared with spheroids originated from tumor cell lines (Caco-2, DLD-1) co-cultured with stromal cells (PBMCs, cancer-associated fibroblasts of colorectal origin) and spheroids directly prepared from colon cancer tissues.

Different microenvironment compositions altered the tumor cell line spheroid response patterns. Adding PBMCs increased resistance to FO treatment by 10–15% in Caco-2 and DLD-1 spheroids but decreased resistance to FI by 16% in DLD-1 spheroids. Fibroblast co-cultures decreased resistance to FI in Caco-2 spheroids by 38% but had no impact on FO. Treatment of colon cancer tissue spheroids revealed three distinct response pattern subgroups not detectable in 3D cell lines models.

The cancer tissue spheroid model mimics both tumor characteristics and the stromal microenvironment and therefore is an invaluable screening model for pharmaceutical drug development.

1. Introduction

Preclinical cancer models predicting clinical treatment outcome are urgently required in early drug development. Currently much effort is being spent on the development of refined preclinical models, *in vitro* as well as *in vivo*, to bridge the gap between successful preclinical studies and success in clinical trials.

One of the key players impacting drug efficacy is the tumor microenvironment (Hanahan and Weinberg, 2011). A number of factors such as hypoxia and acidosis, complex interactions between cancer cells and different stromal cell types as well as the extracellular matrix and various soluble factors have been associated with both drug resistance and sensitization. In addition, the tumor microenvironment has been previously associated with changes in biomarker expression on cancer cells, which is important for patient stratification in targeted therapy. Stromal cell types that are interesting especially with regard to treatment efficacy include the tumor endothelial cells, the tumor infiltrating lymphocytes (TILs), the tumor associated macrophages (TAMs) and the tumor associated fibroblasts (TAFs, CAFs). All these cell types are targets of new treatment strategies aimed at the tumor stroma (Correia and Bissell, 2012; Hanahan and Coussens, 2012; Zhang and Liu, 2013; Santoni et al., 2013; McMillin et al., 2013; Kyi and Postow, 2014).

The complexity, heterogeneity, plasticity and diversity of the human tumor microenvironment cannot be replicated in preclinical 2D tumor cell line models, leaving these inaccurate for deductions regarding clinical response. Similarly, despite the remarkable progress in generation of humanized mouse models, mouse biology does not allow for an authentic model of human tumors to be generated (Das Thakur et al., 2014; Hayes et al., 2014; Malaney et al., 2014).

Abbreviations: FO, 5-FU+oxaliplatin; FI, 5-FU+irinotecan; FOC/FIC, FO/FI+Cetuximab; TILs, tumor infiltrating lymphocytes; TAMs, tumor associated macrophages; TAFs/CAFs, tumor/cancer associated fibroblasts; CRC, colorectal cancer.

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http://dx.doi.org/10.1016/j.jbiotec.2015.02.029

The increasing research on the different cellular subtypes in the tumor micromilieu, which communicate directly and indirectly with the cancer cells, has led to the development of more complex preclinical *in vitro* models in early drug development (Alépée et al., 2014; Lovitt et al., 2014; Xu et al., 2014).

Using the example of human colorectal cancer in the present study, 3D spheroid models of different complexity regarding cellular composition were generated and the impact of clinically relevant drug combinations, targeted and non-targeted, was evaluated in each model.

2. Materials and methods

2.1. Cell lines and culture conditions

Five colon cancer cell lines were used for the initial experiments, namely Caco-2 (ATCC Nr. HTB-37), DLD-1 (ATCC Nr. CCL-221), COLO 205 (ATCC Nr. CCL-222), HT-29 (ATCC Nr. HTB-38) and HCT-116 (ATCC Nr. CCL-247). Cell lines were cultivated in RPMI 1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% FBS (PAN Biotech, Aidenbach, Germany) and 2 mM L-Glutamin (PAN Biotech, Aidenbach, Germany). Primary cancer associated fibroblasts of colorectal origin were cultured in MEM (PAN Biotech, Aidenbach, Germany) supplemented with 20% FBS and 0.26 μM Amphotericin B (Biochrom, Berlin, Germany). DMEM/F12 medium (PAN Biotech, Aidenbach, Germany) supplemented with 10% FBS (PAN Biotech, Aidenbach, Germany), 2× MEM non-essential amino acid solution (PAN Biotech, Aidenbach, Germany), 2× MEM vitamin solution (PAN Biotech, Aidenbach, Germany), 0.14 mM Ampicillin (ratiopharm, Ulm, Germany), 0.26 μM Amphotericin B (Biochrom, Berlin, Germany), 7.54 μM Ciprofloxacin (Fresenius Kabi, Bad Homburg, Germany), 50 μg/ml Gentamicin (ratiopharm, Ulm, Germany) and 0.29 mM Metronidazol (B. Braun Melsungen, Melsungen, Germany) was used for primary colorectal cancer cells isolated from tissue samples. All cells were incubated under standard culture conditions (37 °C, 5% CO_2). Expansion and passaging of adherent cells were performed by detachment with a 0.05% trypsin/0.53 mM EDTA solution (PAN, Aidenbach, Germany). To determine cell number and cell viability, 0.4% trypan blue solution (Sigma–Aldrich, Steinheim, Germany) was used in a ratio of 1:1 for the trypan blue exclusion test.

2.2. Tissue and blood samples

The study was conducted according to the Declaration of Helsinki Principles and was approved by the local ethics committee. Tissue and blood samples were obtained with the informed consent of the three healthy donors (blood) and the 16 cancer patients (tissue). Samples were handled and stored according to standard bio banking guidelines with special focus on short ischemic times of less than 30 min. Tissue samples were used for fibroblast isolation as well as spheroid preparation and peripheral blood mononuclear cells (PBMCs) were isolated from donated blood samples.

2.3. Isolation of PBMCs

PBMCs were isolated according to the standard Ficoll density gradient centrifugation method (Mallone et al., 2011). Briefly, lithium heparin blood samples (15 ml) were filtered through a 100 μm cell strainer and the strainer washed once using DPBS (PAN Biotech, Aidenbach, Germany) to obtain a final volume of 30 ml. Two separate tubes were prepared with Ficoll-Paque Plus (GE Healthcare, Little Chalfont, UK) and overlaid with the blood/DPBS mixture in a 1:1 ratio. Following centrifugation (30 min, 497 g, 21 °C) the interphase was transferred to a new tube and washed with primary cell culture medium. After determination of the viability the isolated PBMCs were resuspended in primary cell culture medium and used for homotypic and co-culture experiments.

2.4. Isolation of primary cancer-associated fibroblasts of colorectal origin

Primary human fibroblasts were isolated similar to Herrera et al. (2013b). Briefly, fresh primary colorectal tumor samples were mechanically and enzymatically digested using collagenase (Sigma–Aldrich, Steinheim, Germany). The single cell suspension was transferred into 1% sterile gelatin biocoated cell culture flasks and cultured under standard conditions as described above. The medium was renewed twice a week to remove tissue debris. For experiments fibroblasts between passages three and eight were used.

2.5. Spheroid preparation

2.5.1. The tumor cell line spheroid model

Spheroids were generated as previously described (Mayer et al., 2001). Briefly, monolayer cultures of tumor cell lines were allowed to reach a minimal confluency of 90% for spheroid culture. The viability and the cell number of the cell suspensions used for spheroid culture were assessed. Only cell suspensions with a viability of at least 90% were used for spheroid culture. For spheroid formation, 5×10^4 vital cells per well in either 100 μl or 50 μl per well were seeded in a 96 well microtiter plate and cultured for 48 h at standard culture conditions as mentioned above.

2.5.2. 3D co-culture of tumor cell lines with stromal cells

For co-culture experiments with fibroblasts isolated from primary colorectal cancer samples, a cell suspension consisting of an equal number of tumor cells and primary cancer-associated fibroblasts of colorectal origin were used for spheroid formation. Vital cells were seeded at a density of 5×10^4 per well.

Co-culture experiments with PBMCs were performed using homotypic tumor cell line spheroids. 5×10^4 freshly isolated PBMCs were added per well to pre-formed spheroids 48 h after tumor cell seeding simultaneously with the drugs used for the experiments.

2.5.3. Heterotypic spheroids from cancer tissue

Surgical tissue resectates were dissected and cancer tissue samples provided by pathologists of the University Hospital LMU, Germany. After macroscopic removal of the non-tumorous tissue and the performance of several washing steps, the tumor tissue was mechanically and enzymatically digested with Liberase TM (Roche Applied Science, Basel, Switzerland) according to the manufacturer's protocol in FBS free medium until the extracellular matrix was almost entirely digested and all cell types were isolated to obtain a single cell suspension. The digestion progress was microscopically monitored every 30 min. The enzymatic reaction was terminated by adding 10% FBS to the cell suspension. Most importantly, all the different cell types given in the individual cancer tissue were retained. No cell depletion or enrichment step was performed. The cell suspension was washed twice with primary cell culture medium as described above and the cell number and viability assessed before the cells were directly used for spheroid formation. A minimal vitality of 80% was required for spheroid culture (Gaedtke et al., 2007).

2.6. Cancer therapy

After 48 h of spheroid formation, chemotherapeutic agents and molecular drugs were administered to the spheroids in clinically relevant combinations at the peak plasma concentrations

Please cite this article in press as: Hoffmann, O.I., et al., Impact of the spheroid model complexity on drug response. J. Biotechnol. (2015), http://dx.doi.org/10.1016/j.jbiotec.2015.02.029