Biomaterials 78 (2016) 50–61

Contents lists available at ScienceDirect

Biomaterials

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

Modelling the tumour microenvironment in long-term microencapsulated 3D co-cultures recapitulates phenotypic features of disease progression

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ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form
16 November 2015
Accepted 17 November 2015
Available online 19 November 2015

Keywords:
3D
Co-culture
Alginate microencapsulation
Stirred-tank bioreactors
Tumour microenvironment
Tumour progression

ABSTRACT

3D cell tumour models are generated mainly in non-scalable culture systems, using bioactive scaffolds. Many of these models fail to reflect the complex tumour microenvironment and do not allow long-term monitoring of tumour progression. To overcome these limitations, we have combined alginate microencapsulation with agitation-based culture systems, to recapitulate and monitor key aspects of the tumour microenvironment and disease progression. Aggregates of MCF-7 breast cancer cells were microencapsulated in alginate, either alone or in combination with human fibroblasts, then cultured for 15 days. In co-cultures, the fibroblasts arranged themselves around the tumour aggregates creating distinct epithelial and stromal compartments. The presence of fibroblasts resulted in secretion of pro-inflammatory cytokines and deposition of collagen in the stromal compartment. Tumour cells established cell–cell contacts and polarised around small lumina in the interior of the aggregates. Over the culture period, there was a reduction in oestrogen receptor and membranous E-cadherin alongside loss of cell polarity, increased collective cell migration and enhanced angiogenic potential in co-cultures. These phenotypic alterations, typical of advanced stages of cancer, were not observed in the mono-cultures of MCF-7 cells. The proposed model system constitutes a new tool to study tumour-stroma crosstalk, disease progression and drug resistance mechanisms.

1. Introduction

The tumour microenvironment is composed of cancer cells, fibroblasts, endothelial cells, immune cells and extracellular matrix (ECM), whose interactions are critical for tumour initiation and progression [1]. Tumour cells can induce a phenotypic change in healthy fibroblasts to become cancer associated fibroblasts (CAFs) with cancer-promoting properties such as secretion of matrix components (collagen and fibronectin), growth and inflammation factors [1]. Abnormal deposition of collagen has been associated with cancerous states due to increased matrix stiffness which is known to contribute to tumour cell dissemination [2]. Additionally, the activated stromal cells promote tumour progression by stimulating cancer cell proliferation and migration, and ultimately tumour metastasis [3]. Infiltrating stromal cells in the tumour are the main providers of matrix metalloproteinases (MMPs) that, through remodelling of ECM, release chemotactic agents and loosen the matrix contributing to tumour cell dissemination [4]. These changes are responsible for the recruitment of immune cells and for increasing chronic inflammation, which also contributes to tumour aggressiveness [5].

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http://dx.doi.org/10.1016/j.biomaterials.2015.11.030
0142-9612/

In an attempt to mimic the complexity of the tumour microenvironment, many *in vitro* models have been developed in the recent years [6]. In most of these models however, tumour cells are grown as monotypic cultures in two-dimensions (2D). In 2D, cells are not able to organize into tissue-like structures since they lack the tridimensionality (3D) bestowed by the surrounding microenvironment [7]. In contrast, heterotypic tumour aggregate 3D cultures enable tumour cells to establish cell–cell and cell-ECM interactions, which are important elements in tumour signalling and which modulate tumour responses to therapeutic agents [8]. However, tumour aggregates are mostly cultured in low-adherence conditions [9] or embedded in bioactive scaffolds such as collagen I or matrigel [6]. These scaffolds also have limitations, including batch-to-batch variation and an incomplete understanding of their impact on cell behaviour [10,11]. In contrast, hydrogels such as alginate present many advantages over bioactive scaffolds due to their inert properties, biocompatible gelation and ease of cell recovery. Hydrogels also provide the possibility of conjugation with defined adhesion ligands or delivery of specific biomolecules (growth factors, pro-angiogenic factors, amongst others) [12,13].

Alginates are polysaccharide hydrogels composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) obtained from particular brown algae species [13]. Alginate comprises 99% water, but still retains high plasticity and mechanical strength. Gelling occurs almost instantaneously by cross-linking with divalent ions, like Ca^{2+}, allowing for cell entrapment under physiological conditions and rapid cell recovery by gel dissolution [14]. Most cell lines are able to grow in non-functionalized alginates, despite the absence of cell adhesion sites [13]. Alginate microencapsulation has been used to investigate the effect of biomechanical forces exerted on tumour aggregates [15]. More recently, alginate microencapsulation and microfluidic devices have been used to study the interaction between different cell types [11,16]. However, these models have been generated in non-scalable culture systems, with no control of the physicochemical parameters and which allow end-point analysis only [17,18]. As a result, studies on the molecular mechanisms behind disease progression and drug resistance as well as high-throughput drug screening are performed in models that lack the complexity of human tumours and which do not allow continuous monitoring of the culture progression.

Herein, we describe a novel *in vitro* culture model system for long-term co-culture of tumour and stromal cells, based on the combination of alginate microencapsulation with suspension cultures in agitation-based culture systems. We used alginate as a scaffold for cell entrapment, not only due to its properties outlined above, but also to provide physical support and cell confinement, in a manner compatible with stirred-tank systems. This strategy provides a means of long-term culture of tumour cell aggregates either alone or in combination with fibroblasts, continuously monitored with non-destructive sampling. The developed model system can be transferred across several pathologies and will provide a new tool for characterization of disease progression and drug resistance mechanisms *in vitro*.

2. Materials and methods

2.1. 2D cell culture

MCF-7 cells transduced with the lentiviral vectors PGK-dsRED and pCDH-CMV-MCS-EF1-Puro, were kindly provided by Professor Cathrin Brisken (EPFL, Switzerland) within the scope of the PREDECT consortium. MCF-7 reporter cells were used as a complementary tool for live monitoring purposes. Cell expansion was performed in Dulbecco's Modified Eagle Medium (DMEM) with 25 mM Glucose, supplemented with 1% (v/v) penicillin-streptomycin, 4 mM Glutamax, 1 mM sodium pyruvate and 10% (v/v) fetal bovine serum (FBS). Cells were passaged twice weekly at a inoculum concentration of 1.5×10^4 cell/cm^2. Human Dermal Fibroblasts (HDF), from Innoprot, were passaged once weekly for up to 10 to 12 passages at a seeding density of 0.5×10^4 cell/cm^2, in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 1% (v/v) penicillin-streptomycin and 10% (v/v) FBS (all from Life Technologies). Both the MCF-7 cells and the HDFs were cultured in static culture systems, in an incubator at 37 °C with humidified atmosphere containing 5% CO_2 in air.

2.2. 3D cell culture

MCF-7 cells, non transduced and transduced with the lentiviral vectors described above, were inoculated as single cell suspensions (0.2×10^6 cell/mL) into 125 mL stirred-tank vessels with flat centred cap and angled side arms (Corning – http://catalog2.corning.com/LifeSciences/en-US/Shopping/ProductDetails.aspx?categoryname=&productid=4500-125%28Lifesciences%29) and cultured at 80 rpm, to induce cell aggregation. For alginate microencapsulation, tumour cell aggregates were collected from the stirred-tank vessels after 24 h of culture. Aggregates corresponding to approximately 25×10^6 tumour cells were dispersed in 3 mL of 1.1% (w/v) of Ultrapure Ca^{2+} MVG alginate (UP MVG NovaMatrix, Pronova Biomedical, Oslo, Norway) dissolved in NaCl 0.9% (w/v) solution either alone (mono-cultures) or together with HDFs, in a 1:1 ratio for approximately 50×10^6 total cells (co-cultures). Mono-cultures of HDFs were also microencapsulated (25×10^6 total cells) and used as controls. Microencapsulation was performed using an electrostatic bead generator (Nisco VarV1, Zurich, Switzerland), to produce beads of approximately 500 μm in diameter [19]. The alginate droplets were cross-linked in a 100 mM $CaCl_2$/10 mM HEPES (pH 7.4) solution for 10 min, further washed three times in a 0.9% (w/v) NaCl solution and finally equilibrated in culture medium before being transferred to stirred-tank vessels. The microencapsulated mono and co-cultures were kept in 125 mL stirred-tank vessels at 80 rpm, in a humidified incubator, with 5% CO_2 in air, for 15 days with 50% medium exchange every 3–4 days.

2.3. Cell viability

Cell viability was assessed using fluorescein diacetate (FDA; Sigma–Aldrich) at 10 μg/mL to label live cells, and To-PRO-3 iodite (LifeTechnologies) at 1 μM, for dead cells. Microencapsulated tumour aggregates and fibroblasts were incubated for 5 min at RT with the labels then visualized using a fluorescence microscope (DMI6000, Leica Microsystems GmbH, Wetzlar, Germany) or a spinning disk microscope (Andor Revolution Å~ D, Andor Technology PLC, Belfast, Northern Ireland).

2.4. Aggregate size

To measure aggregate size, alginate microcapsules were dissolved in a chelating solution (Sodium citrate 50 mM/Sodium chloride 100 mM), for 5 min at room temperature (RT), and washed twice with Phosphate-Buffered Saline (PBS; Life Technologies). Aggregates were imaged using a fluorescence microscope (DMI6000, Leica Microsystems GmbH, Wetzlar, Germany). Aggregate surface area was quantified using FIJI open source software (Rasband, WS, ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997–2012.), by applying automated threshold adjustment followed by the area measurement algorithm.

Statistical analysis was carried out using GraphPad Prism 5 software. Data is presented as mean ± SD from three independent