Acta Biomaterialia xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

A physiologically relevant 3D collagen-based scaffold–neuroblastoma cell system exhibits chemosensitivity similar to orthotopic xenograft models

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ARTICLE INFO

Article history:
Received 23 November 2017
Received in revised form 2 February 2018
Accepted 5 February 2018
Available online xxxx

Keywords:
Neuroblastoma
Cisplatin
miRNA
Scaffolds
Xenografts

ABSTRACT

3D scaffold-based *in vitro* cell culturing is a recent technological advancement in cancer research bridging the gap between conventional 2D culture and *in vivo* tumours. The main challenge in treating neuroblastoma, a paediatric cancer of the sympathetic nervous system, is to combat tumour metastasis and resistance to multiple chemotherapeutic drugs. The aim of this study was to establish a physiologically relevant 3D neuroblastoma tissue-engineered system and explore its therapeutic relevance. Two neuroblastoma cell lines, chemotherapeutic sensitive Kelly and chemotherapeutic resistant KellyCis83 were cultured in a 3D *in vitro* model on two collagen-based scaffolds containing either glycosaminoglycan (Coll-GAG) or nanohydroxyapatite (Coll-nHA) and compared to 2D cell culture and an orthotopic murine model. Both neuroblastoma cell lines actively infiltrated the scaffolds and proliferated displaying >100-fold increased resistance to cisplatin treatment when compared to 2D cultures, exhibiting chemosensitivity similar to orthotopic xenograft *in vivo* models. This model demonstrated its applicability to validate miRNA-based gene delivery. The efficacy of liposomes bearing miRNA mimics uptake and gene knockdown was similar in both 2D and 3D *in vitro* culturing models highlighting the proof-of-principle for the applicability of 3D collagen-based scaffolds cell system for validation of miRNA function. Collectively, this data shows the successful development and characterisation of a physiologically relevant, scaffold-based 3D tissue-engineered neuroblastoma cell model, strongly supporting its value in the evaluation of chemotherapeutics, targeted therapies and investigation of neuroblastoma pathogenesis. While neuroblastoma is the specific disease being focused upon, the platform may have multi-functionality beyond this tumour type.

Statement of Significance

Traditional 2D cell cultures do not completely capture the 3D architecture of cells and extracellular matrix contributing to a gap in our understanding of mammalian biology at the tissue level and may explain some of the discrepancies between *in vitro* and *in vivo* results. Here, we demonstrated the successful development and characterisation of a physiologically relevant, scaffold-based 3D tissue-engineered neuroblastoma cell model, strongly supporting its value in the evaluation of chemotherapeutics, targeted therapies and investigation of neuroblastoma pathogenesis. The ability to test drugs in this reproducible and controllable tissue-engineered model system will help reduce the attrition rate of the drug development process and lead to more effective and tailored therapies. Importantly, such 3D cell models help to reduce and replace animals for pre-clinical research addressing the principles of the 3Rs.

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https://doi.org/10.1016/j.actbio.2018.02.004
1742-7061/

1. Introduction

In the native microenvironment, tumour cells are surrounded by three-dimensional (3D) essential physical scaffolding called extracellular matrix (ECM). The ECM composition is shaped by proteoglycans and fibrous proteins that are secreted locally by cells and remain closely connected [1]. The ECM shapes cellular architecture and maintains tissue homeostasis. The tumour ECM can influence disease progression, patient prognosis and response to treatment [2–4]. A major challenge today is to distinguish the relative contributions of structural, molecular and microenvironmental changes to cancer progression. Traditional 2D cultures do not completely capture the 3D architecture of cells and ECM leading to a gap in our understanding of mammalian biology at the tissue level and may explain some of the discrepancies between *in vitro* and *in vivo* results [5–8] leading to only 1 in 10 drugs reaching clinical trials and approval by the FDA [9].

3D scaffold-based *in vitro* cell culturing is a new innovative approach in cancer research to bridge the gap between conventional 2D culture and *in vivo* tumours [5–7]. The use of scaffold-based cell culturing would help to reduce and/or replace animals for pre-clinical research aligning with the guiding principles for the care and use of animals in biomedical research known as the 3Rs (Replacement, Reduction and Refinement [10]) and increase the potential for a strong economic impact and patient benefit. The use of such 3D culture systems allows for the precise manipulation of cell and ECM components of the microenvironment. The analysis of their contribution to the structure and function of a cell or tissue is vital in our understanding of disease progression and discovery of new effective drugs [6,8,9,11].

Scaffolds provide a 3D structural matrix which offers the necessary support for cells to proliferate, migrate, differentiate, deposit ECM and respond to stimuli, similar to *in vivo* biological systems. Collagen is a very attractive material for tissue-engineering and regenerative medicine applications because of its natural occurrence in the human body. Collagen triggers the driving force underlying the cell adhesion, migration, chemotaxis, and tissue development as well as contributes to its mechanical properties. 3D collagen-based scaffolds can be specifically engineered to mimic the intrinsic physiological conditions and have controllable and adaptable properties that facilitate the infiltration of cells and nutrients while being biocompatible, biodegradable and non-toxic [12–16]. Originally developed for regenerative medicine applications with a particular focus on bone repair [13,14,16–18], they are increasingly being used for disease modelling and drug screening [6,16,19–21]. The use of such scaffolds to culture cells in a 3D environment *in vitro* is gaining greater recognition as a technologically progressive platform for further investigation of disease mechanisms and pathology in an environment that partially mimics a solid tumour microenvironment [5,7,14,17,19,22–24].

Neuroblastoma is a highly aggressive paediatric solid tumour arising from the sympathetic nervous system, which accounts for approximately 15% of all childhood cancer deaths [25–27]. The disease displays certain clinical, genomic and transcriptomic alterations that contribute to a highly heterogeneous clinical behaviour and are associated with very unfavourable outcomes in paediatric patients [28–34]. Results from EUROCARE 5 analysing the survival of almost 60,000 children with cancer identified no improvements in survival for children with aggressive disease (e.g. high-risk neuroblastoma) despite an intensive induction treatment [35]. Almost 20% of children with the aggressive disease do not respond at all, and up to 50% of children that do respond experience disease recurrence with metastatic foci resistant to multiple drugs [25,26,36]. The main challenge in treating high-risk neuroblastoma is to combat tumour metastasis and development of resistance to multiple chemotherapeutic drugs thus emphasising an imminent need for new treatment options. Current neuroblastoma studies employ either 2D cell culture systems, murine models or alternatively a mix of both, increasing the risk of inconsistencies between these two research models [27,37], and thus highlighting the limited translational efficacy of the results obtained in 2D models and requiring new pre-clinical models.

In order to establish and characterise physiologically relevant tissue-engineered 3D neuroblastoma *in vitro* models capable of recapitulating elements of a native tumour tissue microenvironment, we aimed to examine cisplatin sensitive and resistant neuroblastoma cells on different collagen-based scaffolds, collagen-glycosaminoglycan (Coll-GAG) and collagen-nanohydroxyapatite (Coll-nHA). If successful, this model could be used further to gain insights into mechanisms of disease pathogenesis and improve the translational efficacy between results obtained *in vitro*, *in vivo* and in the clinic. Both Coll-GAG and Coll-nHA scaffolds have been successfully used to study primary tumour microenvironment in breast cancer [19] and metastasis to bone in prostate cancer [23], respectively. Therefore, they may represent attractive matrices for modelling of metastatic neuroblastoma as bone marrow (70.5%) and bone (55.7%) are the most common sites for metastases [38]. Both types of scaffolds have controllable physical and biological properties and consist of a porous, collagen-based layer fabricated using freeze-drying techniques that were originally developed and extensively studied for bone tissue engineering applications [12,14–17,39,40]. GAGs, negatively charged carbohydrates, are commonly found in the ECM involved in cell attachment, migration, proliferation and differentiation [15]. Nano-hydroxyapatites (nHAs), a calcium phosphate, are common elements of the mineral composition of the human bone tissues and extensively used as a biocompatible material for the bone replacement and regeneration [41]. Coll-nHA scaffolds have been extensively characterised for their biocompatibility, toxicity and the osteoconductive and osteoinductive features [12,14,16,17]. Thus GAGs and nHA are attractive composites for reconstructing primary and metastatic bone/bone marrow tumour microenvironment.

Having characterised the cell response to chemotherapeutic in the proposed 3D *in vitro* cell model, we then used this model to evaluate miRNA-mediated gene regulation. MiRNAs are a class of small, noncoding RNAs that regulate gene expression at translational level [42–44]. These molecules control the expression of a great variety of genes driving cell cycle, migration, differentiation, development, apoptosis, and metabolism [33,43,45,46]. Numerous studies describe dysregulation of miRNA expression in tumours, including neuroblastoma emphasising their potential in the generation of new drugs for therapeutic intervention. In neuroblastoma, some miRNA were found to be over- or under expressed demonstrating their complex role as either "oncomirs" or tumour-suppressors, respectively [29,33,46–48]. These functions can be exploited to repair signaling pathways essential for normal cellular function and block those upregulated in the pathological conditions. Unsurprisingly, the development of miRNA therapeutics is under extensive investigation by several companies for the variety of health conditions, including cancer [49–51]. Here, we explored the 3D scaffold-based *in vitro* cell culturing platform for neuroblastoma, however, it may have multi-functionality beyond this tumour type.

2. Materials and methods

2.1. Scaffold fabrication

Collagen-glycosaminoglycan (Coll-GAG) and composite collagen-nHA scaffolds (Coll-nHA) were manufactured as described previously [12,15,16]. Briefly, collagen slurry (0.5% (w/v)) was fabricated by blending fibrillary collagen I (Integra Life Sciences, Inc.) with 0.05 M acetic acid.

Please cite this article in press as: C. Curtin et al., A physiologically relevant 3D collagen-based scaffold–neuroblastoma cell system exhibits chemosensitivity similar to orthotopic xenograft models, Acta Biomater. (2018), https://doi.org/10.1016/j.actbio.2018.02.004