Tissue and Cell 48 (2016) 133–143

Contents lists available at ScienceDirect

Tissue and Cell

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

Effects of decellularized matrices derived from periodontal ligament stem cells and SHED on the adhesion, proliferation and osteogenic differentiation of human dental pulp stem cells *in vitro*

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ARTICLE INFO

Article history:
Received 26 November 2015
Received in revised form
21 December 2015
Accepted 21 December 2015
Available online 25 December 2015

Keywords:
Dental pulp
Extracellular matrix
Focal adhesion
Mitosis
Osteogenesis

ABSTRACT

A major bottleneck to the therapeutic applications of dental pulp stem cells (DPSC) are their limited proliferative capacity *ex vivo* and tendency to undergo senescence. This may be partly due to the sub-optimal *in vitro* culture milieu, which could be improved by an appropriate extracellular matrix substratum. This study therefore examined decellularized matrix (DECM) from stem cells derived from human exfoliated deciduous teeth (SHED) and periodontal ligament stem cells (PDLSC), as potential substrata for DPSC culture. Both SHED-DECM and PDLSC-DECM promoted rapid adhesion and spreading of newly-seeded DPSC compared to bare polystyrene (TCPS), with vinculin immunocytochemistry showing expression of more focal adhesions by newly-adherent DPSC cultured on DECM *versus* TCPS. Culture of DPSC on SHED-DECM and PDLSC-DECM yielded higher proliferation of cell numbers compared to TCPS. The qRT-PCR data showed significantly higher expression of nestin by DPSC cultured on DECM *versus* the TCPS control. Osteogenic differentiation of DPSC was enhanced by culturing on PDLSC-DECM and SHED-DECM *versus* TCPS, as demonstrated by alizarin red S staining for mineralized calcium deposition, alkaline phosphatase assay and qRT-PCR analysis of key osteogenic marker expression. Hence, both SHED-DECM and PDLSC-DECM could enhance the *ex vivo* culture of DPSC under both non-inducing and osteogenic-inducing conditions.

1. Introduction

In recent years, numerous studies have demonstrated that dental pulp stem cells (DPSC) have promising therapeutic applications not only in regenerative dentistry (Aurrekoetxea et al., 2015; Cao et al., 2015), but can also be utilized for tissue engineering, repair and regeneration of a variety of non-dental and non-oral tissues including bone (Kwon et al., 2015), cartilage (Rizk and Rabie, 2013), blood vessels (Li et al., 2014), cardiac muscle (Gandia et al., 2008), brain (Yamagata et al., 2013) and spinal cord (Yamamoto et al., 2014). Amongst the major advantages of DPSC are that these cells are readily available and easily isolated from biological waste routinely produced during dental treatment, and that the transplantation of autologous DPSC within the same patient can circumvent the immunological barrier in cell-based therapy (Aimetti et al., 2014; Tatullo et al., 2015).

Nevertheless, a major bottleneck to the therapeutic applications of DPSC is the limited proliferative capacity of these cells and their tendency to undergo senescence after a limited number of passages within *ex vivo* culture (Wu et al., 2015). Only a relatively small quantity of autologous DPSC can be isolated from each individual patient, and it is often necessary to carry out extensive *ex vivo* expansion of these cells to attain sufficient numbers of cells required for successful transplantation or tissue engineering. Additionally, *ex vivo* culture may also be necessary for induction of DPSC into desired lineages for therapeutic applications.

The major technical challenge faced in the *ex vivo* culture of DPSC is that the 2D *in vitro* culture milieu is often sub-optimal and differs considerably from the physiological 3D microenvironment of the stem cell niche *in vivo*, which may in turn compromise the proliferative and differentiation capacity of these adult stem cells. One potential strategy to overcome this deficiency may be to utilize an appropriate extracellular matrix (ECM) substratum for the *ex vivo* culture of DPSC. Physiologically *in vivo*, cells are naturally surrounded by extracellular matrix within the stem cell niche, which is composed of a diverse array of high molecular weight

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http://dx.doi.org/10.1016/j.tice.2015.12.004
0040-8166/

structural proteins (*i.e.* collagen, laminin and fibronection), proteoglycans and glycosaminoglycans, as well as growth factors (Walters and Gentleman, 2015). Indeed, it is well-known that ECM-cellular interactions play key roles in orchestrating development and influencing the lineage fate of adult stem cells, in addition to regulating tissue homeostasis and repair (Walters and Gentleman, 2015).

Nevertheless, most commercially-available extracellular matrix coatings for *in vitro* culture such as Matrigel® are often derived from animal sources or cancer cell lines, *i.e.* Matrigel® being derived from Engelbeth-Holm swarm mouse sarcoma (Orkin et al., 1977). This in turns poses a major technical barrier and regulatory hurdle for clinical therapy, because the culture of human cells on such commercially-available products would invariably lead to the adhesion of either xenogenic or tumorigenic proteins on the cell membrane, which could in turn provoke an immunological reaction upon transplantation within the patient, even if these cells were autologous in origin. Hence, it is imperative to look for an alternative source of human-derived ECM substrata for the *ex vivo* culture of DPSC.

One promising source of such material could be other dental and oral stem cells that are also readily available and easily isolated from biological waste routinely produced by dental treatment. This study will therefore attempt to extract decellularized matrix (DECM) from stem cells derived from human exfoliated deciduous teeth (SHED) and periodontal ligament stem cells (PDLSC), and utilize these as substrata for the *ex vivo* culture of DPSC under both non-inducing and osteogenic-inducing conditions. The adhesion, proliferation and osteogenic differentiation of *ex vivo* cultured DPSC on these DECM would be assessed and compared by various techniques including immunocytochemistry, WST-8 assay, qRT-PCR, alizarin red S staining and alkaline phosphatase assay.

2. Materials and methods

2.1. Cells, reagents, culture media, supplements and labware

Human DPSC (Cat No. DP003F) and SHED (Cat No. DP004F) were obtained from AllCells LLC. (Alameda, CA, USA). Human PDLSC were obtained as a gift from the School of Stomatology of Fujian Medical School (Fujian, China) and were isolated as previously described (Seo et al., 2004). Unless otherwise stated, all chemical reagents were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA), all culture media and associated culture supplements were obtained from Life Technologies Inc. (Carlsbad, CA, USA), while all labware were obtained from Becton-Dickinson Inc. (Franklin Lakes, NJ, USA).

2.2. Cell culture

The culture milieu utilized for expansion culture of DPSC, SHED and PDLSC was identical, and comprised of α-minimum essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin antibiotic solution. Fresh culture media was replenished every 3–4 days. For routine sub-culture, 0.5% (w/v) Trypsin-EDTA was used to dissociate confluent monolayers. A humidified 5% CO_2 incubator set at 37 °C was utilized for all cell cultures. All subsequent experimental protocols involving human DPSC, PDLSC and SHED were approved by the Institutional Review Board (IRB) of the University of Hong Kong.

2.3. Derivation of decellularized matrices

DECM were derived from confluent cell sheets of SHED and PDLSC (passage 5–10) according to previously published protocol (Decaris et al., 2012). Briefly, SHED and PDLSC were cultured to confluency within 6-well dishes, in the presence of 50 μg/ml of L-ascorbic acid for at least 1 week prior to DECM derivation. The confluent cell sheets were rinsed with PBS and treated with PBS containing 20 mM NH_4OH and 0.5% Triton X-100 for 5 min at 37 °C. DECM remaining in the wells were then rinsed again with PBS and subjected to DNAse treatment for 1 h at 37 °C, prior to being rinsed in deionized water and subjected to air-drying. The DECM thus obtained from the confluent cell sheets within 6-well plates were stored at 4 °C prior to being utilized in subsequent experiments.

2.4. Immunohistochemistry for detection of collagen and fibronectin expression on the decellularized matrices

SHED and PDLSC were seeded at a density of 20,000 cells/cm^2 on glass cover slips placed within 6-well culture plates and cultured to confluence in the presence of 50 μg/ml of L-ascorbic acid for at least 1 week, prior to derivation of DECM, as previously described. The cover slips with DECM were initially blocked in PBS supplemented with 10% (v/v) FBS for 2 h. This was followed by washing three times in PBS and incubation at room temperature for 2 h with primary antibodies specific for collagen (Cat. No. SC-8784-R, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or fibronectin (Cat No. FBN11, ThermoFisher Scientific Inc., Waltham, MA, USA), which were diluted in 1% (w/v) BSA/PBS at appropriate dilution factors, according to the manufacturer's instructions. Subsequently, the cover slips with DECM were then washed in 1% (w/v) BSA/PBS and incubated in the dark for 2 h at room temperature with either goat anti-rabbit secondary antibody that was conjugated to TRITC (Cat No. ab6718, Abcam Inc., Cambridge, UK) or goat anti-mouse secondary antibody that was conjugated to Alexa fluor 488® (Cat No. ab150117, Abcam Inc., Cambridge, UK). The secondary antibodies were diluted in 1% (w/v) BSA/PBS at appropriate dilution factors, according to the manufacturer's instructions. Following another wash with 1% (w/v) BSA/PBS, the cover-slips with DECM were mounted on glass slides with FluorSave® mounting reagent (Merck Millipore Inc., Darmstadt, Germany), prior to imaging under an Olympus IX81 confocal fluorescent microscope (Olympus Inc., Tokyo, Japan) with the appropriate excitation/emission wavelength for Alexa fluor 488® and TRITC.

2.5. Quantification of total protein, collagen and glycosaminoglycan contents of the decellularized matrices

The total protein contents of PDLSC-DECM and SHED-DECM were determined by utilizing a bicinchoninic acid (BCA) protein assay kit (Cat No. 23225, ThermoFisher Scientific Inc., Waltham, MA, USA), following the manufacturer's protocol. Briefly, the DECM within each well of a 6-well plate was incubated with 0.5 ml of assay reagent for 2 h at 37 °C, under mild agitation. Absorbance readings were then measured at 570 nm, and used to calculate the corresponding protein concentrations from a standard curve.

The collagen contents of PDLSC-DECM and SHED-DECM were determined by colorimetric analysis utilizing a hydroxyproline assay kit (Cat No. MAK008, Sigma–Aldrich Inc., St. Louis, MO, USA), following the manufacturer's protocol. Briefly, the DECM were scraped from the surface of the 6-well plates in deionized H_2O and hydrolyzed in 12 M HCl at 120 °C for 3 h. Subsequently, 100 μL of supernatant was then mixed with 200 μL of assay reagent in duplicates, and incubated at 60 °C for 24 h, followed by transfer of 200 μL of the sample reaction mixture into a 96-well plate for absorbance readings at 560 nm. Subsequently, the hydroxyproline concentrations were determined from the corresponding absorbance readings *via* a standard curve. The collagen contents of PDLSC-DECM and SHED-DECM were then computed based on an assumption of a collagen-to-hydroxyproline ratio of 10:1 (w/w).