Acta Biomaterialia 58 (2017) 122–135

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Decellularized tongue tissue as an *in vitro* model for studying tongue cancer and tongue regeneration

Long Zhao [a], Linxuan Huang [a], Shuyi Yu [a], Junheng Zheng [a], Hua Wang [b], Yan Zhang [a,*]

[a] Key Laboratory of Gene Engineering of the Ministry of Education, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou Higher Education Mega Center, Guangzhou 510006, PR China
[b] Department of Oral and Maxillofacial Surgery, Guanghua School of Stomotology, Hospital of Stomotology, Sun Yat-sen University, PR China

ARTICLE INFO

Article history:
Received 12 February 2017
Received in revised form 25 April 2017
Accepted 25 May 2017
Available online 7 June 2017

Keywords:
Extracellular matrix
Tongue
Oral squamous cell carcinoma
Cell movement
Tissue regeneration

ABSTRACT

The decellularization of tissues or organs provides an efficient strategy for preparing functional scaffolds for tissue engineering. The microstructures of native extracellular matrices and biochemical compositions retained in the decellularized matrices provide tissue-specific microenvironments for anchoring cells. Here, we report the tongue extracellular matrix (TEM), which showed favorable cytocompatibility for normal tongue-derived cells and tongue squamous cell carcinoma (TSCC) cells under static or stirring culture conditions. Our results show that TEM retained tongue-specific integrated microstructures and abundant matrix components, which offer mechanical support and spatial signals for regulating cell behavior and function. Reconstructed TSCC by TEM presented characteristics resembling clinical TSCC histopathology, suggesting the possibility for TSCC research. In addition, TEM might be capable of guiding tongue-derived cells to the niche, benefiting cell survival, proliferation and differentiation.

Statement of significance

In this study, we prepared decellularized tongue extracellular matrix (TEM) and evaluated the possibility for tongue squamous cell carcinoma (TSCC) research and tongue regeneration. TEM has six irreplaceable advantages: (1) tongue-specific intricate structures of TEM, which offer mechanical support for the cells; (2) abundant matrix components and spatial signals benefiting for cell attachment, survival, differentiation, and long-term viability of the highly functional phenotypes of tongue cells or TSCC cells; (3) reconstructed TSCC by TEM exhibited tumor heterogeneity, extremely resembling clinical TSCC histopathology; (4) ideal model to evaluate TSCC movement mode; (5) guiding tongue-derived cells to the site-appropriate niche; and (6) the possibility for static or stirred cell culture. These properties might be considered in TSCC research or tongue regeneration.

1. Introduction

The tongue is an important organ with numerous functions, such as articulation, deglutition, and tasting. Impairment of tongue function severely affects quality of life [1]. Tongue squamous cell carcinoma (TSCC) is the most common malignancy in the oral cavity, especially in people who drink alcohol and smoke tobacco [2]. While the incidence of tongue cancer has varied significantly worldwide, 275,000 new cases have been estimated per year [3]. Surgical resection is a preferred treatment strategy in the clinic; radical resection achieves a satisfactory 5-year overall survival rate [4]. However, radical resection of tongue cancer unavoidably obliterates part of the tongue, resulting in tongue dysfunction, which is inconvenient for speech and swallowing. There is no consensus on how to repair partial tongue defects after radical resection. Free flaps have been widely used in the reconstruction of tumor-related defects in the oral cavity; the most common flaps used in the clinic are the anterolateral tight flap, the pectoralis major and latissimus dorsi myocutaneous flap [5]. Unfortunately, these kinds of flaps are bulky and not always suitable for restoring the coordinated lingualis function because the structure of the lingualis is mutually staggered and complex. On the other hand, fundamental research on TSCC is limited and lacks the appropriate matrix for *in vitro* culture.

* Corresponding author at: School of Life Sciences, Sun Yat-sen University, Guangzhou Higher Education Mega Center, Guangzhou 510006, PR China.
E-mail address: zhang39@mail.sysu.edu.cn (Y. Zhang).

http://dx.doi.org/10.1016/j.actbio.2017.05.062
1742-7061/

The decellularization of tissues or organs has been investigated as a promising strategy to fabricate functional scaffolds for cell culture and transplantation [6]. Through decellularization using detergents and enzymes, cellular components are removed from the tissues, while extracellular matrix (ECM) and various kinds of proteins are retained. Various ECM components, including collagen, fibronectin and laminin in decellularized matrices provide a suitable microenvironment for cultured cells that is similar to that of native tissues and could promote the survival, proliferation, and differentiation of the cells. Additionally, the absence of cellular components allows the matrices to serve as functional scaffolds with minimal immunogenicity for transplantation.

Decellularized ECM has been tried in numerous types of tissues or organs recently, such as the heart [7–10], liver [11–14], lung [15–20] and kidney [21–23]. However, a relevant report has not been published about the tongue. In the present study, decellularized tongue extracellular matrix (TEM) was prepared efficiently and at low cost by physical, chemical and enzymatic treatment. The TSCC model constructed by TEM presented an acceptable simulation for TSCC behavior and development. Furthermore, our study demonstrated that TEM not only has good biocompatibility but also has an ability to guide the cells to the tissue-specific niche, which indicates that TEM might have a potential application in TSCC research and repairing partial tongue defects.

2. Materials and methods

2.1. Ethics

Paraffin-embedded TSCC specimen were prepared after surgical excision in the Department of Oral & Maxillofacial Surgery, Stomatological Hospital, Sun Yat-sen University. The Ethics Committee at Sun Yat-sen University approved the specimen collection, informed consents were obtained from TSCC patients. All animal work was performed in accordance with animal welfare act, institutional guidelines and approved by Institutional Animal Care and Use Committee, Sun Yat-sen University.

2.2. Preparation of TEM

The tongues were decellularized using a modified immersion method. The laboratory animals were from Sun Yat-sen University Laboratory Animal Center. Male C57-BL/6J mice (6–8 weeks), male large white pigs (40–50 weeks) and male wistar rats (6–8 weeks) were used in this study. Each tissue sample was collected from a single animal, at least three samples from mice, pigs or rats were respectively decellularized for TEM preparation. The laboratory animals were euthanized by CO_2 inhalation and then removed tongues in sterile conditions. The tongues of porcine and rat were cut into 1 cm^3 pieces. All decellularization steps were carried out with agitation at 4 °C in sterile conditions. First, the samples were frozen at −80 °C, thawed at room temperature for 3 cycles and then washed in ultrapure water for 12 h. The samples were then treated with 1 M NaCl (Sigma Aldrich) for 24 h, and washed in 2% Triton X-100 (Sigma Aldrich) for 24 h. After being treated with 5 mM $CaCl_2$ (Sigma Aldrich) and 5 mM $MgCl_2$ (Sigma) for 24 h, the samples were incubated with DNase (300 U/ml, Sigma) at 37 °C for 24 h and then washed in PBS for 1 day. The samples were then stored at 4 °C until use.

2.3. DNA quantification

Approximate 25 mg of native tongues (n = 3) and TEM (n = 3) were subjected to DNA extraction using DNeasy Blood & Tissue Kit (69506, QIAGEN) according to the manufacturer's instructions. A Nanodrop 1000 (Thermo Scientific, Wilmington, DE) was used to measure the amount of DNA by spectrophotometry.

2.4. Histological analyses, immunohistochemistry and immunofluorescence

Samples were fixed, sectioned and stained following protocol previously described [24]. Images were acquired using a Zeiss Imager Z1 fluorescence equipped with an AxioCam MRc5 digital CCD camera (Carl Zeiss Microimaging Inc, Oberkochen, Germany). Antibodies used in the study is shown in Supplementary Table 1.

2.5. Scanning electron microscopy and transmission electron microscopy

Native tongues or TEM were dried by using critical point drying apparatus (Samdri® PVT-3D, Tousimis, Rockville, MD) and coated with Au/Pd by using an ion sputtering apparatus (E-1010 Ion Sputter, Hitachi, Tokyo, Japan). Electron microscope images were taken by using a scanning electron microscopy (S-3400NII, Hitachi, Tokyo, Japan). For transmission electron microscopy, Samples (60-nm thin) were sliced using an ultramicrotome (UC6, Leica, Wetzlar, Germany) and dyed with 2% aqueous uranyl acetate for 15 min and 1% aqueous lead citrate for 10 min. Transmission electron microscope (JEM-1400, JEOL, Tokyo, Japan) was used to observe the results, pictures were taken by Olympus iTEM 5.0.

2.6. Atomic force microscopy measurement of elastic modulus

Atomic force microscopy (AFM, Dimension FastScan, Bruker, Germany) was used to measure elastic modulus E for the evaluation of sample stiffness. In this study, a 20 nm non-conductive silicon nitride tip and a rectangular cantilever (MCLT, Bruker, Germany) with resonant frequency of 15 kHz the spring constant of 0.02 Nm^{-1} were chosen for sensing. Tongue tissue and TEM were harvested and flatten fixed on glass slides, and then the height sensor, peak force error and DMT modulus were measured. The elastic modulus E was then analyzed by AFM NanoScope Analysis software (Bruker, Germany).

2.7. TEM proteomic analysis

Samples were prepared according to the protocol previously described [25]. Sample was loaded onto a precolumn and then analyzed by EASY-Spray column (15 cm by 75 μm, C_{18}, 3 μm) using nanoliquid chromatography (LC, Thermo Scientific EASY-nLC 1000 system, Wilmington, DE). A hybrid linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Elite; Thermo Scientific, Wilmington, DE) with a Nanospray Flex ion source was coupled to the outlet of the nano-LC. A full-scan MS was recorded with a resolution of 60,000 (at m/z 400), and the precursor ion scan was recorded over the m/z range of 350–1800. A database search was performed with Proteome Discoverer 1.3 using the SEQUEST program, and the results were matched in the UniProt complete mouse database (downloaded in April 2015 including 45,182 protein entries). Biological replicates (n = 2) were collected for the protein category of TEM measurements in this study.

To measure the protein abundance in the TEM, we adopted label-free quantification (LFQ) by an approach in MaxQuant (v1.5.5.1) [26]. The primary data were searched using MaxQuant in the complete mouse UniProt database, and then, the results were uploaded into Perseus (v1.5.5.3). Based on the data clustering, a heat map was created by using the maximum distance method. The classifications of proteins and pathways were carried out in PANTHER classification system (http://pantherdb.org/geneListAnalysis.do). The protein interaction relationships