Biomaterials 35 (2014) 105–112

Contents lists available at ScienceDirect

Biomaterials

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

The effect of mechanical extension stimulation combined with epithelial cell sorting on outcomes of implanted tissue-engineered muscular urethras

Qiang Fu [a,**], Chen-Liang Deng [b], Ren-Yan Zhao [a], Ying Wang [a], Yilin Cao [c,d,e,*]

[a] Department of Urology, Shanghai 6th People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, PR China
[b] Department of Plastic and Reconstructive Surgery, Shanghai 6th People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, PR China
[c] Department of Plastic and Reconstructive Surgery, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, PR China
[d] National Tissue Engineering Center of China, Shanghai 200240, PR China
[e] Shanghai Key Laboratory of Tissue Engineering, Shanghai 200011, PR China

ARTICLE INFO

Article history:
Received 23 July 2013
Accepted 20 September 2013
Available online 17 October 2013

Keywords:
Urethral defect
Tissue engineering
Oral mucosal epithelial cell
Adipose-derived stem cells
Bioreactor

ABSTRACT

Urethral defects are common and frequent disorders and are difficult to treat. Simple natural or synthetic materials do not provide a satisfactory curative solution for long urethral defects, and urethroplasty with large areas of autologous tissues is limited and might interfere with wound healing. In this study, adipose-derived stem cells were used. These cells can be derived from a wide range of sources, have extensive expansion capability, and were combined with oral mucosal epithelial cells to solve the problem of finding seeding cell sources for producing the tissue-engineered urethras. We also used the synthetic biodegradable polymer poly-glycolic acid (PGA) as a scaffold material to overcome issues such as potential pathogen infections derived from natural materials (such as de-vascular stents or animal-derived collagen) and differing diameters. Furthermore, we used a bioreactor to construct a tissue-engineered epithelial–muscular lumen with a double-layer structure (the epithelial lining and the muscle layer). Through these steps, we used an epithelial–muscular lumen built *in vitro* to repair defects in a canine urethral defect model (1 cm). Canine urethral reconstruction was successfully achieved based on image analysis and histological techniques at different time points. This study provides a basis for the clinical application of tissue engineering of an epithelial–muscular lumen.

1. Introduction

Urethral defects are a common and disturbing clinical occurrence that usually arises secondary to urinary injury [1,2], tumor resection, infection or malformations [3]. Insufficient urethra rebuilding normally results in stricture and uroschesis that heavily impairs the quality of patients' life.

Traditional urethral reconstruction is limited by the shortage of an ideal repair tissue, but the rapidly expanding tissue-engineering technology provides tremendous opportunities to obtain satisfactory urethral tissue [4–7]. For example, an acellular bladder matrix achieved a high success rate in repairing anterior urethral strictures [8]. Oral epithelial- and muscle-derived cells have been used to construct a two-layered engineered urethra [9], and currently, tissue-engineered autologous urethras in the clinic have been successfully applied [10].

However, some limitations still exist in currently used urethral repair strategies and tissue-engineering techniques [11–13]. The important points of these strategies include avoiding urethral strictures and obtaining enough seed cells [13]. Reconstructive engineered urethras with a well-developed smooth muscle layer represent a good solution for urethral strictures, but smooth muscle cells (SMCs) are difficult to isolate and have lower proliferative ability; therefore, stem cells from different sources need to be differentiated into smooth muscle cells. During the recruitment of these cells, the use of adipose-derived stem cells (ADSCs) is favored because obtaining sufficient numbers of adipose stem cells is easy and minimizes donor site damage [14]. ADSCs have been proven to

* Corresponding author. Present address: Department of Plastic and Reconstructive Surgery, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, PR China. Tel.: +8621 63089563; fax: +8621 63087768
** Corresponding author. Department of Urology, Shanghai 6th People's Hospital, Shanghai Jiao Tong University, 600 Yishan Road, Shanghai 200233, PR China. Tel.: +86021 64369181; fax: +86021 64701361.
E-mail addresses: jamesqfu@aliyun.com (Q. Fu), yilincao@gmail.com, yilincao@yahoo.com (Y. Cao).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.09.067

be a promising source of SMCs [15,16], as both chemical and mechanical stimulation can trigger ADSCs' differentiation into SMCs [16,17]. Recently, accumulating evidence has demonstrated that mechanical stimulation is important for maintaining the proper biomechanical function of tissues such as bone, cartilage, muscle and smooth muscle [18–20]. Mechanical stimulation has also been proven to be effective in initiating the differentiation of ADSCs into functional smooth muscle tissue [16,21–23].

A urethral stricture is generated by fibrogenesis; thus, not only a well-developed muscular layer but also a vital urethral epithelium is crucial for the prevention of this disorder. Therefore, it is essential to build a robust epithelium in the engineered urethra. Primary oral mucosal epithelial cells are a promising source of seed cells for the reconstruction of tissue-engineered epithelial-cell sheets [8,24–26], but their proliferation and differentiation potential is impaired by their complex composition [27]. Many cell lineages and cells at different differentiated states are present in primary epithelial cells, and many cells among them have relatively low stemness [27,28]. Therefore, sorting epithelial stem cells from primary oral mucosal epithelial cells using flow cytometry might be a potential strategy to improve the stemness of primary epithelial cells.

To obtain a well-developed muscular tissue-engineered urethra, we combined *in vitro* mechanical stimulation of ADSCs with primary epithelial seed cell sorting. We found that regular mechanical extension can induce ADSCs to differentiate into a muscular layer in a poly-glycolic acid (PGA) scaffold, and this muscular layer is superior to non-mechanical extension-induced control smooth muscle tissue. Furthermore, sorting of primary epithelial cells can increase their proliferation, survival ability and β1 integrin expression.

2. Materials and methods

2.1. Primary cell isolation and culture

Primary oral mucosal epithelial cells were obtained from beagle dogs. After general anesthesia, small pieces of oral mucous membrane were excised from the bucca of the dogs. The harvested oral mucosa was washed three times each with 0.25% chloromycetin solution and PBS, cut into small pieces and digested with Dispase-II (2.5 U/ml) for 15 h at 4 °C. The digestion was terminated by the addition of 10% fetal bovine serum (FBS). The suspended cells were filtered through a cell strainer before cultivation. The primary oral mucosal epithelial cells were cultured in keratinocyte serum-free medium (K-SFM, Gibco company, USA) supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF). Primary cells at passage 1–3 were used in the experiments.

After general anesthesia, primary ADSCs were obtained from fat tissue, which was isolated from the groin. The fat tissue was washed three times with 0.25% chloromycetin solution and PBS, then digested with 0.01% collagenase I for 1 h at 37 °C. The digested cells were collected after filtration through a cell strainer. The primary ADSCs were maintained in Dulbecco's modified Eagle's (DMEM) supplemented with 10% FBS, and primary ADSCs at passage 1 were used in the subsequent experiments.

All cells were cultured at 37 °C at a humidified atmosphere with 5% CO_2.

2.2. Flow cytometer sorting of primary oral mucosal epithelial cells

An RPE-tagged rat anti-dog integrin α6 antibody and a FITC-tagged mouse anti-dog CD71 antibody were added to primary epithelial cells (1×10^6/ml) for 30 min at 4 °C, then washed three times using PBS containing 4% FBS. After centrifugation at 1000 rpm for 5 min, the supernatant was removed, and the precipitate was resuspended in 200 μl cold PBS containing 4% FBS. The fluorescence intensity was detected by a flow cytometer (EPICS® ALTRA™, Beckman Coulter, US). The integrin α6 (positive)/CD71(negative) cells were separated by fluorescence-activated cell sorting, as a minor subpopulation of the oral mucosal epithelial cells.

2.3. Design of a mechanical extension bioreactor

To conduct mechanical extension stimulation, we first designed and manufactured a bioreactor that was composed of a peristaltic pump, a reaction chamber, a fluid collection bottle and connection ducts (Fig. 1A and B). After filling the ducts and chambers with culture medium, the engineered PGA tubes were connected to ducts in the reaction chamber (Fig. 1C). The mechanical extension was rhythmical and was produced by a peristaltic pump at 75 times/min, with a pressure of 0.02 Mpa. Before the engineered muscular urethras were produced, the bioreactor was sterilized and placed in a CO_2 incubator.

2.4. Two-layer epithelial–muscular urethras preparation

The scaffold was tube of fibrous PGA mesh of bulk density 50 mg cm^{-3}, porosity 95%, and fiber diameter 12–15 μm (Albany International Research, Mansfield, USA). The tube diameter was 5 mm, the tube length was 20 mm and the tube thickness was 1.6 mm. PGA scaffold was placed in an incubation chamber containing 20 ml DMEM added 10% FBS. ADSCs (2×10^7) were suspended in DMEM containing 10% FBS and seeded in the PGA tubes. ADSCs and epithelial cells were loaded on scaffold by layered seeding technology. These tubes were rotated 90° every 15 min. 20 ml DMEM

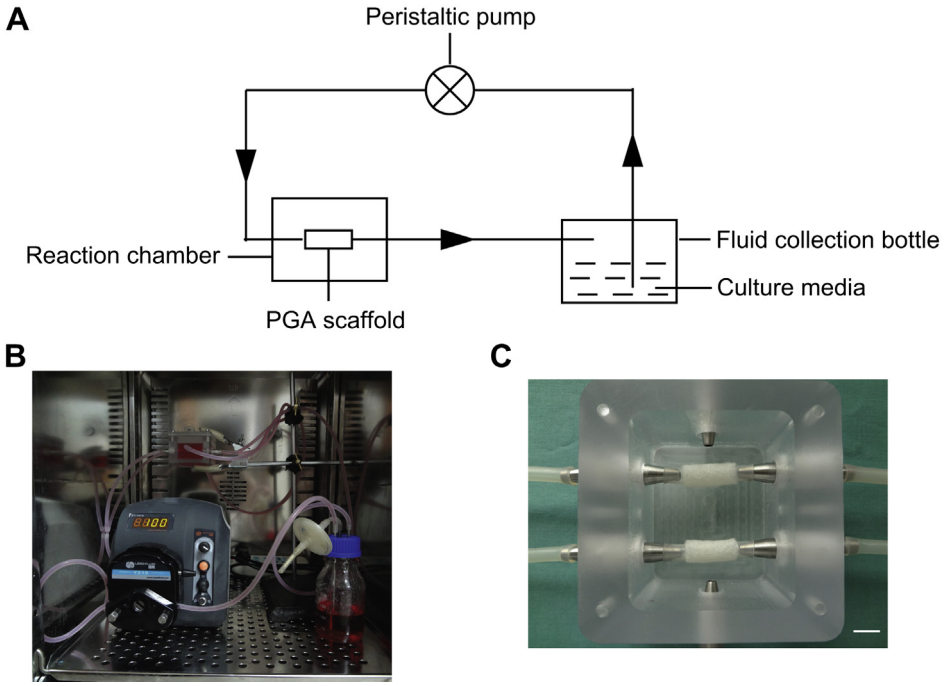

Fig. 1. Illustrations of the mechanical extension bioreactor. (A) Schematic diagram of the mechanical extension bioreactor. (B) An image of the mechanical extension bioreactor in an incubator. (C) An image of the reaction chamber; two PGA scaffolds are connected via ducts. Scale bar = 5 mm.