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Regeneration of the oesophageal muscle layer from oesophagus acellular matrix scaffold using adipose-derived stem cells

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ABSTRACT

This study explored the feasibility of constructing a tissue engineered muscle layer in the oesophagus using oesophageal acellular matrix (OAM) scaffolds and human aortic smooth muscle cells (hASMCs) or human adipose-derived stem cells (hASCs). The second objective was to investigate the effect of hypoxic preconditioning of seeding cells on cell viability and migration depth. Our results demonstrated that hASMCs and hASCs could attach and adhere to the decellularized OAM scaffold and survive and proliferate for at least 7 days depending on the growth conditions. This indicates adipose-derived stem cells (ASCs) have the potential to substitute for smooth muscle cells (SMCs) in the construction of tissue engineered oesophageal muscle layers.

1. Introduction

Generating a functional artificial oesophagus using the tissue engineering (TE) approach as an appropriate substitute might be a preferred solution for the repair of diseased or injured oesophagus [1]. Numerous attempts using natural or synthetic biomaterial as an exogenous scaffold to support the epithelial cells when constructing tissue engineered oesophagus have been made [2,3]. Smooth muscle constitutes a critical layer of the oesophagus and generating a smooth muscle layer is a prerequisite for successfully reconstructing the whole oesophagus.

Although smooth muscle cells (SMCs) can be obtained from autologous tissue, the proliferation capability of mature differentiated SMCs is limited. Moreover, they rapidly lose their contractile ability during in vitro expansion. These disadvantages make it necessary to explore an alternative cell source for oesophageal TE. ASC, an adipose-derived stem cell, has been widely employed due to its easy accessibility, abundant number, and differentiation capability along multiple lineages. Adipose-derived stem cells (ASCs) have been successfully differentiated into functional SMCs with transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-4 (BMP4) [4]. The successful use of ASCs in other tissue engineered organs such as urinary bladder, small diameter elastic blood vessel and tendon suggests the potential as a cell source in engineered oesophageal muscle layer [5–7].

SMCs are not terminally differentiated and are capable of switching between a synthetic phenotype and a contractile phenotype. When contractile phenotypic SMCs are cultured in vitro, it will lose the contractile properties over time [8]. Hypoxia has been shown to regulate ASC differentiation, influencing chondrogenesis and osteogenesis [9]. Our previous results suggested that both expression of SMC-specific markers and contractile properties were enhanced when ASCs were differentiated in 5% O_2 compared to 20% O_2 [10]. Conditioned medium from hypoxic ASCs was shown to enhance the migration ability of human dermal fibroblasts and wound healing [11].

With regards to framework for cells to grow, synthetic polymers have been used as a substrate to support epithelial cells or SMCs in tissue engineered oesophagus [12]. However, one major issue of these synthetic materials is their biologically inert surface impeding the integrity of cells and polymers [13]. Recently, acellular matrix scaffolds from a variety of tissues such as acellular porcine aorta matrix [14], gastric acellular matrix [15], and porcine

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acellular small intestinal submucosa (SIS) [16] have been widely utilized as a scaffold for cell repopulation. In comparison with these grafts, an oesophageal acellular matrix (OAM) scaffold has significant advantages as it has similar composition, micro-architecture, biomechanical properties and biochemical cues to the native oesophagus, providing a 3-D morphology with appropriate microenvironment, which is vital in directing cells to generate appropriate cellular responses in the structure and function regeneration [17].

Marzaro et al. indicated that acellular matrix implant seeded with SMC presented a larger degree of SMCs ingrowth and less inflammatory response compared to acellular matrix alone 3 weeks after surgery in porcine animal model [18]. Likewise, the conclusion from Badylak's group showed that extracellular matrix (ECM) bioscaffolds seeded with autologous muscle tissue, but not ECM alone, can facilitate the *in situ* reconstitution of oesophagus tissue [19]. Therefore, a combination of appropriate cells and scaffold should be a superior approach to repopulate the scaffold. However, the use of undifferentiated stem cells or smooth muscle differentiated from stem cells for such a purpose is not well characterized. From our previous work, ASCs differentiated in 5% oxygen might exhibit greater migration capability in a decellularized oesophagus and increase the potential for reconstructing the muscle layers. The purpose of this study was to explore the adhesive and migratory properties, as well as the differentiation potential of ASCs and smooth muscle cells seeded on an OAM in growth conditions supporting either proliferation or differentiation and preconditioned either at 5% or 20% oxygen concentrations.

2. Materials and methods

2.1. Production of OAM

Decellularized porcine OAM were produced at Northwick Park Institute for Medical Research (NPIMR) using an in house developed protocol. Oesophagi were harvested from pigs from unrelated studies undergoing termination at NPIMR. All animal surgery and handling was performed in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 following ethical approval from NPIMR. Oesophagi were harvested from eleven Large-White/Landrace crossbreed pigs under standard operating conditions. After euthanasia by anaesthetic overdose, the oesophagi were harvested and stored at −20 °C for a minimum of 24 h. Each oesophagus was initially frozen for 24 h and then completely thawed at room temperature. The entire decellularization process was carried out using a perfusion system with 1% Penicillin/Streptomycin in all solutions.

Briefly, the thawed tissue was perfused with 50 nmol/l latrunculin B (Sigma-Aldrich, UK) in high glucose Dulbecco's modified Eagle's medium (DMEM) for 2 h at 37 °C. All further steps were performed at room temperature. Each scaffold was washed via perfusion with distilled water twice for 15 min between each perfusion steps. The tissue was then perfused in 0.6 mol/l potassium chloride for 2 h, followed by 1.0 mol/l potassium iodide for 2 h and then left to wash overnight in distilled water. The following morning the potassium chloride and potassium iodide incubations steps were repeated, followed by incubation in 1 kU/ml DNase I (Sigma-Aldrich, UK) for 3 h. The decellularization protocol was concluded with an intensive wash using sterile water over 48–72 h.

2.2. Histological evaluation of OAM

Samples were fixed for a minimum of 24 h in 10% neutral buffered formalin solution at room temperature. They were dehydrated in graded alcohol and embedded in paraffin and sectioned at 5 μm. Sections were stained with haematoxylin and eosin (H&E), picrosirius red and Miller's elastin stains.

2.3. DNA analysis of OAM

The GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich, UK) was used for DNA extraction and quantification following the manufacturer's instructions. In brief, 25 mg of minced wet tissue of fresh and decellularized tissue were placed in a micro-centrifuge tube with proteinase K and incubated in a water bath at 55 °C for 4 h with vortexing at 30 min intervals. Complete digestion was confirmed macroscopically and the samples were then subjected to a ribonuclease solution at room temperature for 2 min. The samples were incubated with lysis reagents from the DNA extraction assay kit at 70 °C for 10 min. The lysates were loaded into prepared columns for binding DNA. After several washing steps to remove contaminates, the DNA was finally eluted in 200 μl of a Tris-ethylene diaminetetra acetic acid solution. The absorbance was read at 260 nm and 280 nm using a self-masking quartz micro-cuvette and a spectrophotometer (Helios Alpha, Thermo Fisher Scientific, Loughborough, UK) and the absolute amount of DNA per milligram of tissue was calculated.

2.4. Glycosaminoglycan (GAG) quantification

The Blyscan GAG assay kit (Biocolor, Carrickfergus, Northern Ireland) was used to quantify sulphated glycosaminoglycan (sGAG) content of fresh and decellularized samples. In brief, 50 mg of minced wet tissue was placed in a micro-centrifuge tube and incubated with 1 ml of papain digestion buffer at 65 °C for 18 h. Aliquots of each sample were mixed with 1, 9-dimethyl-methylene blue dye and reagents from the GAG assay kit. The absorbance at 656 nm was measured with a spectrophotometer and the absolute GAG content calculated by comparing to a plot of standards.

2.5. Culture and differentiation of SMCs

HASMCs and culture media were purchased from Cascade Biologics (CA, USA) unless otherwise stated. HASMCs were cultured in medium 231 supplemented with smooth muscle growth supplement (SMGS), 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.05 mg/ml gentamicin at a density of 5×10^3 cells/cm^2. When reaching 80% confluence, the hASMCs were passaged or cryopreserved for further experiments. Human ASMCs were differentiated in medium 231 supplemented with SMDS. The culture medium was changed twice weekly.

For assessment of cell morphology, phase contrast images were acquired using an Olympus CKX41 microscope (Olympus Life Science) with a PixeLINK PL-A782 camera.

2.6. Isolation, culture and differentiation of ASCs

Adipose tissue was obtained from a healthy 52-year-old male patient, from an elective liposuction procedure at the Grymer Privat Hospital (Arhus, Denmark). The patient gave informed consent and protocols were approved by the regional committee on research ethics of Northern Jutland, Denmark. Human ASCs were isolated as previously described with a slight modification [22]. To obtain SMCs from ASCs, ASCs were induced with 5 ng/ml TGF-β1 and 2.5 ng/ml BMP4 (R&D systems, Minneapolis, MN, USA) for 1 week. The culture medium was changed twice a week.

2.7. Seeding

In preparation for seeding, sterilized scaffold was splayed open

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