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Tissue engineered pre-vascularized buccal mucosa equivalents utilizing a primary triculture of epithelial cells, endothelial cells and fibroblasts☆

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ABSTRACT

Artificial generated buccal mucosa equivalents are a promising approach for the reconstruction of urethral defects. Limiting in this approach is a poor blood vessel supply after transplantation, resulting in increased morbidity and necrosis. We generated a pre-vascularized buccal mucosa equivalent in a triculture of primary buccal epithelial cells, fibroblasts and microvascular endothelial cells, using a native collagen membrane as a scaffold. A successful pre-vascularization and dense formation of capillary-like structures at superficial areas was demonstrated. The lumen size of pre-formed blood vessels corresponded to the capillary size *in vivo* (10–30 μm). Comparing native with a highly cross-linked collagen membrane we found a distinct higher formation of capillary-like structures on the native membrane, apparently caused by higher secretion of angiogenic factors such as PDGF, IL-8 and angiopoietin by the cells. These capillary-like structures became functional blood vessels through anastomosis with the host vasculature after implantation in nude mice. This *in vitro* method should result in an accelerated blood supply *to the biomaterial with cells after* transplantation and increase the succes rates of the implant material.

1. Introduction

In the field of regenerative urology the reconstruction of inherent or acquired urethra failures is an issue of great interest. State of the art for the reconstruction of this kind of defect is the utilization of autologous tissue. Many different approaches have been developed to find suitable donor tissue such as skin from the scrotum and penis or from extra genital areas such as the ureter, appendix, rump, extremities as well as mucosa from the foreskin, bladder or the gingiva [1–6]. Although autologous tissue transplants are widely used in this field, it has been found that transplants from certain donor sites were associated with complications after transplantation such as transplant shrinkage, stricture and formation of diverticula for example in the case of tissue from skin or bladder [7].

In the case of the good accessible skin tissue it could be shown that the transplantation resulted in post-operative contraction, constriction or swelling [5]. For bladder mucosa one of the biggest issues has been the difficult accessibility [6]. In contrast, buccal tissue has been shown to be suitable for urethra reconstruction due to its favourable morphological and mechanical properties. The

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favorable morphology of buccal mucosa is basically due to the epithelial cover layer and the fibrous layer underneath, the lamina propria. In general, the epithelium is a multilayer of a basal layer, spinous layer, granular layer and the superficial layer with a thickness of approximately 500 μm [8]. In areas with higher mechanical stress such as the palate, the superficial layer consists of keratinized cells. The lamina propria is a fibrous and good vascularized layer, which is situated underneath the epithelium and is crucial for the nutrient supply of the epithelium. It consists of type I and type III collagen, elastin and fibroblasts and thus plays an important role for the mechanical stability of the epithelial layer above [9,10].

Since the success of an operative urethra reconstruction is mainly determined by the size of adequate donor tissue for the tissue transfer [11], one of the main issues is the limited quantity of applicable donor tissue. Due to the described issues associated with autologous transplants, one promising scientific approach is the creation of artificial tissue equivalents *in vitro* via tissue engineering. It has been demonstrated that buccal tissue equivalents consisting of keratinocytes alone or of a co-culture with keratinocytes and fibroblasts could be cultivated on dermal matrices and then implanted as “full thickness oral mucosa” [10–13].

However, a limitation in such artificial tissue transplants is the missing vascularization which results in necrosis. This is one of the main reasons for an insufficient in-growth of the host vasculature and survival of the artificial transplants [14–16]. In order to overcome the problem of insufficient vascularization after transplantation, various strategies have been developed to promote blood vessel formation: (1) a direct application of endothelial cells on a biomaterial followed by the implantation, (2) addition of growth factors to the biomaterial, (3) direct injection of endothelial cells into the implantation site, in addition to others [17–19]. Nevertheless, a satisfactory result has as yet not been reached by this approach. As a solution to this problem, the formation of a capillary network *in vitro* appears to be the most advantageous at this point to accelerate the vascularization of a tissue engineered scaffold after transplantation [14].

The aim of the current study is the generation and cultivation of pre-vascularized buccal tissue equivalents based on a tri-culture of human gingival epithelial cells, fibroblasts and microvascular endothelial cells and the evaluation of the tissue equivalent integration *in vivo* using a mouse model. As a scaffold a native collagen membrane was utilized in order to generate a functional tissue equivalent with pre-formed capillary-like structures. Furthermore, the impact of collagen cross-linking on the formation of capillaries has been evaluated in a comparative study with the native and the highly cross-linked collagen membrane, and moreover the secretion of angiogenic factors has been analyzed *in vitro*. The *in vivo* experiments, using a nude mouse model, showed the functional connection of the pre-formed capillary-like structures within the generated tissue equivalents by the staining of a human specific endothelial cell marker CD31 and by the perfusion of the pre-formed vasculature with mouse erythrocytes 10 days after implantation [20]. It is anticipated that such a pre-vascularized construct will lead to an accelerated integration in the body and may result in an improved blood vessel supply associated with increased survival and higher success rates after transplantation in humans.

2. Materials and methods

2.1. Sample preparation

Native collagen membrane, i.e. processed collagen derived from natural source (Geistlich Bio-Gide®), and cross-linked collagen membrane (Geistlich Bio-Gide® Pro, Wolhusen, Switzerland), both porcine type I and III collagens, bilayered, including a cell occlusive and a rough side [21], were used (Fig. 1A). The 4X cross-linking reaction of the Geistlich Bio-Gide® Pro matrices was performed using ethyl(dimethylaminopropyl)carbodiimide/N-hydroxysuccinimid (EDC/NHS) chemistry. Prior to cell experiments, collagen membranes were punched in 6 mm diameter, 28.3 mm^2, using biopsy punches and stored sterile until used. In order to distinguish between the cell occlusive and rough side, samples were carefully marked. Immediately before cell seeding collagen membranes were rehydrated with PBS.

2.2. Cell isolation and cell culture

Epithelial cells and fibroblasts were isolated from human gingiva, obtained from patients who underwent surgery at the Department of Maxillofacial Surgery, University Medical Center Mainz, Germany. The study was performed in agreement with the Declaration of Helsinki and approved by local ethics committee (Landesärztekammer Rheinland-Pfalz, Mainz, Germany: 837.439.05). Informed consent was obtained from each patient.

Epithelial cell isolation procedure: Prior to isolation, gingiva samples were stepwise sterilized for 15 s in sterillium (Bode Chemie GmbH, Hamburg, Germany), 15 s in 70% ethanol and finally washed twice in sterile PBS. Then the tissue samples were punctured on the epithelial side using a cannula and incubated for 4 h in a 0.04% trypsin solution in DMEM at 37 °C. Afterwards the trypsin reaction was stopped using DMEM including 10% FCS (fetal calf serum) and the epithelial layer was carefully separated from the connective tissue. To isolate epithelial cells, the epithelial layer was cut into small tissue fragments, placed in a 6-well plate and covered with the epithelial cell-specific medium containing 60% DMEM (Dulbecco's modified eagle medium, PAA, Pasching, Austria), 30% DMEM/HAM's F12 (Gibco, Darmstadt, Germany), 10% FCS, 100 U/100 μg/ml Penicillin/Streptomycin, 2.5 μg/ml Fungizone (Gibco, Darmstadt, Germany), 22 μg/ml adenine, 7.4 ng/ml cholera toxin, 9 ng/ml EGF, 36 ng/ml hydrocortisone and 4.6 μg/ml insulin.

Fibroblast isolation procedure: To isolate fibroblasts, the connective tissue of the buccal mucosa was cut into small fragments (1×1 mm^2), transferred and cultivated in 25 cm^2 cell culture flasks using DMEM medium with 10% FCS, 100 U/100 μg/ml Penicillin/Streptomycin and 2.5 μg/ml Fungizone. After one week of cultivation cell media were changed at least every 3 days.

Microvascular endothelial cell isolation: Microvascular endothelial cells (human dermal microvascular endothelial cells, HDMEC) were isolated from human juvenile foreskin [22] and cultured in PC-Medium (Customer Formulation Medium, Promo Cell, Heidelberg, Germany) supplemented with 15% FCS, 10 μg/ml Na-heparin, 0.2 ng/ml bFGF, 100 U/100 μg/ml Penicillin/Streptomycin and 2.5 μg/ml Fungizone.

All cells were cultivated at 37 °C, 5% CO_2 and 95% relative humidity.

2.3. Cell cultivation of the co- and triculture

Co- and tricultures were generated by placing the collagen membrane (6 mm diameter, 28.3 mm^2) in cell culture inserts for 24 well plates (Netwell Insert No. 3477 from Corning Costar Corporation, Amsterdam, Netherlands). In order to evaluate the influence of the cell number on the formation of capillary-like structures different cell numbers of endothelial cells and fibroblasts (ratio 1:1) in coculture were seeded (2×10^5, 4×10^5, 8×10^5 each cell type/scaffold). Epithelial cells were seeded with a cell number of 2×10^5.

For the generation of the triculture, the endothelial cells were first seeded onto the rough side of the collagen membrane. After