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Optimization of optical and mechanical properties of Real Architecture for 3-Dimensional Tissue equivalents: Towards treatment of limbal epithelial stem cell deficiency

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ABSTRACT

Limbal epithelial stem cell (LESC) deficiency can cause blindness. Transplantation of cultured human limbal epithelial cells (hLE) on human amniotic membrane (HAM) can restore vision but clinical graft manufacture can be unreliable. We have developed a reliable and robust tissue equivalent (TE) alternative to HAM, Real Architecture for 3D Tissue (RAFT). Here, we aimed to optimize the optical and mechanical properties of RAFT TE for treatment of LESC deficiency in clinical application. The RAFT TE protocol is tunable; varying collagen concentration and volume produces differing RAFT TEs. These were compared with HAM samples taken from locations proximal and distal to the placental disc. Outcomes assessed were transparency, thickness, light transmission, tensile strength, ease of handling, degradation rates and suitability as substrate for hLE culture. Proximal HAM samples were thicker and stronger with poorer optical properties than distal HAM samples. RAFT TEs produced using higher amounts of collagen were thicker and stronger with poorer optical properties than those produced using lower amounts of collagen. The 'optimal' RAFT TE was thin, transparent but still handleable and was produced using 0.6 ml of 3 mg/ml collagen. Degradation rates of the 'optimal' RAFT TE and HAM were similar. hLE achieved confluency on 'optimal' RAFT TEs at comparable rates to HAM and cells expressed high levels of putative stem cell marker p63α. These findings support the use of RAFT TE for hLE transplantation towards treatment of LESC deficiency.

1. Introduction

The cornea is the uniquely transparent tissue located on the front of the eyeball that provides us with a window to the world. It is multilayered, consisting of an epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium [1]. The stroma accounts for the majority of the thickness of the cornea but remains optically transparent due its highly organized structure, comprising optimally spaced, orthogonally arranged lamellae of collagen fibrils. This arrangement is tightly controlled to ensure that the stroma remains transparent throughout life, so that visual acuity is maintained, which is the major functional requirement of corneal tissue [2].

A continuously renewed epithelial cell layer protects the underlying stroma from external damage. Sloughed epithelial cells are repopulated by the progeny of limbal epithelial stem cells (LESC) that are located in the limbus, the vascularized border between central cornea and conjunctiva [3,4]. However, if LESC are damaged or lost, this can no longer occur. Inflammation, vascularization and ingrowth of neighbouring conjunctival cells follow and can lead to loss of corneal transparency [5].

One treatment for LESC deficiency is transplantation of pre expanded human limbal epithelial cells (hLE) on a carrier, such as human amniotic membrane (HAM) [6–8]. Although often effective, clinical graft manufacture using HAM can be inconsistent, perhaps due to its inherent biological variability [8–10]. Additionally, intra donor variation also exists whereby HAM samples isolated from different locations display different physical properties [11,12]. Further drawbacks of HAM are that before use, it must be screened, which is costly, and supply can also be unreliable [13].

As a result, many have aimed to develop materials that could be used for transplantation of hLE in place of HAM. Criteria for such a material should include capability to support hLE expansion along with appropriate optical and mechanical properties (i.e. the material should be as transparent as possible, but also be strong enough

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to withstand transplantation onto the recipients' eye). Proposed materials range from naturally occurring materials such as fibrin, [14,15], fish scale collagen [16] and silk fibroin [17,18] to engineered polymers such as poly(lactide co glycolide) [19] and poly ε caprolactone [20].

Our approach is a tissue equivalent (TE), RAFT (Real Architecture for 3D Tissue), produced by gently wicking water away from Type 1 collagen hydrogels using hydrophilic porous absorbers [21]. Collagen is an attractive material for regenerative medicine applications as it is biocompatible, lowly immunogenic, can be remodelled by cells and is already used in numerous clinical applications (reviewed in [22]). We have previously shown that RAFT TEs can support hLE expansion and established RAFT TE as a good *in vitro* model of central cornea [23] and limbus [21]. The RAFT TE production process has also been through numerous iterations such that it is now reliable, robust and reproducible [21,23,24], which is desirable when developing tissue engineered products.

However, although promising in terms of biological function, we had not yet optimized RAFT TEs in terms of physical properties. Therefore, the aim of this study was to optimize the RAFT TE production process, which fortunately is tunable, to produce TEs with appropriate optical and mechanical properties for use in the treatment of LESC deficiency. RAFT TEs were compared to denuded HAM, which is a commonly used carrier for cultured hLE transplantation.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Life Technologies, Paisley, UK, unless stated otherwise.

2.2. Preparation of RAFT constructs

Bovine dermis Type I collagen (Koken, Tokyo, Japan) (8 parts) was mixed with 1 part 10x Minimum Essential Medium (MEM) (Invitrogen). Collagen was used either neat (3 mg/ml) or prediluted using 1 mM hydrochloric acid (HCl) to 2 mg/ml or 1 mg/ml. Sodium hydroxide (5 M) was added dropwise to neutralize the solution to achieve a pH between 7.2 and 7.4. Finally, 1 part 1x MEM was mixed in carefully. This mixture was left on ice for 30 min to allow any air bubbles to disperse. The neutralized collagen mixture was pipetted into individual wells of a 24 well plate (Greiner, Stonehouse, UK) in volumes of either 2.4 ml, 1.2 ml or 0.6 ml, and heated to 37 °C for 30 min so that fibrillogenesis occurred, and a hydrogel formed. The majority of the liquid was wicked away from the hydrogels to produce RAFT TEs by application of hydrophilic porous absorbers (TAP Biosystems, Royston, UK) to the surface of the hydrogels for 30 min at 37 °C as described previously [21]. RAFT TEs were stored at 4 °C in phosphate buffered saline (PBS) before analysis. (For clarity, the protocol for RAFT TE production in previous studies was 2.4 ml of 2 mg/ml collagen [21,23]).

2.3. Preparation of human amniotic membrane samples

HAM samples with appropriate research consent were obtained from the University Eye Hospital (Heinrich Heine Universität, Düsseldorf, Germany). Ethical permission for this study was obtained from the Research Ethics Committee (UK) (reference No. 10/H0106/57-11ETR10). HAM samples from areas proximal and distal to the placental disc were isolated from 3 donors. In a laminar flow hood, intact HAM was washed with PBS to remove blood, before being stripped from the chorion. HAM was stored at −80 °C in 1x antibiotic, antimycotic/Dulbecco's Modified Eagle Medium (100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone). Prior to use, HAM was defrosted in a 37 °C water bath and washed for 3 × 10 min in Hank's Balanced Salt Solution, once with agitation. HAM was oriented epithelial side up, and incubated with 0.25% trypsin ethylenediaminetetraacetic acid for 10 min and epithelium removed using cell scrapers. Denuded HAM was trephined using a 16 mm trephine (AngioTech, Vancouver, Canada) and stored in PBS in 24 well plates at 4 °C until analysis.

2.4. Subjective assessment of transparency

HAM and RAFT TE samples were placed over text (font: Cambria, size: 12), whilst still in a 24 well plate, still with 1 ml of PBS on top. Macroscopic photos were taken from a fixed distance using the same level of diffuse illumination.

2.5. Thickness measurements

The thickness of each RAFT TE and HAM sample was measured using optical coherence tomography (OCT). The PBS was aspirated and samples held in place between 2 glass coverslips. An OCT machine with anterior segment adaptor (HRA and OCT Spectralis, Heidelberg Engineering, Hemel Hempstead, UK) was used to image individual samples (10 line scans per sample). Images were opened using ImageJ software and scale calibrated. The line measurement tool was used to measure the thickness of the samples (all 10 scans per image were used for these measurements). OCT measurements were performed in triplicate for RAFT TEs and in duplicate for HAM samples within each experiment.

2.6. Transparency measurements

The PBS was aspirated from all samples and 14 masked observers assessed the transparency of each RAFT TE and HAM sample on 3 different days from a fixed distance using a standardized chart. Visual acuity of each masked observer was 20/20 or better with glasses or contact lens correction worn where required. An empty well of a 24 well plate was used as a control. The last line successfully read by each masked observer through control and test wells was recorded. When transparency of the sample was too poor to permit line 1 to be read, this was scored as 0 lines.

2.7. Light transmission measurements

Absorbance (400–700 nm) of RAFT TE and HAM samples with 1 ml of PBS on top was measured using a spectrophotometer (SAFIRE, Tecan, Reading, UK). A well containing 1 ml of PBS alone was used as a control. Absorbance readings were converted to percentage transmission using: $\%\ \text{transmission} = 10^{-\text{absorbance}} \times 100$ [25]. Absorbance readings were performed in duplicate for each RAFT TE or HAM sample within each experiment.

2.8. Mechanical property testing

2.8.1. Quantitative

RAFT TE and HAM samples were removed from PBS storage and cut into "dog bone" shapes as described previously [26], 4 mm wide and 10 mm long, using a scalpel. Each end of the samples was clamped between metal mesh grips (MeshDirect, Burslem, UK) and loaded into a custom made tensile strength testing device, similar to that described previously [26]. Samples were held in place and weights applied incrementally until failure (breakage). The load at which failure occurred was recorded (any samples that slipped, instead of breaking, were excluded from analysis). Break

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