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A comparative study of skin cell activities in collagen and fibrin constructs

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ABSTRACT

Collagen and fibrin are widely used in tissue engineering due to their excellent biocompatibility and bioactivities that support in vivo tissue formation. These two hydrogels naturally present in different wound healing stages with different regulatory effects on cells, and both of them are mechanically weak in the reconstructed hydrogels. We conducted a comparative study by the growth of rat dermal fibroblasts or dermal fibroblasts and epidermal keratinocytes together in collagen and fibrin constructs respectively with and without the reinforcement of electrospun poly(lactic acid) nanofiber mesh. Cell proliferation, gel contraction and elastic modulus of the constructs were measured on the same gels at multiple time points during the 22 day culturing period using multiple non-destructive techniques. The results demonstrated considerably different cellular activities within the two types of constructs. Co-culturing keratinocytes with fibroblasts in the collagen constructs reduced the fibroblast proliferation, collagen contraction and mechanical strength at late culture point regardless of the presence of nanofibers. Co-culturing keratinocytes with fibroblasts in the fibrin constructs promoted fibroblast proliferation but exerted no influence on fibrin contraction and mechanical strength. The presence of nanofibers in the collagen and fibrin constructs played a favorable role on the fibroblast proliferation when keratinocytes were absent. Thus, this study exhibited new evidence of the strong cross-talk between keratinocytes and fibroblasts, which can be used to control fibroblast proliferation and construct contraction. This cross-talk activity is extracellular matrix-dependent in terms of the fibrous network morphology, density and strength.

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1. Introduction

Tissue engineering is an emerging multidisciplinary field involving biology, medicine and engineering to restore or regenerate tissue or organ function [1]. Tissue engineering consists of 3 core components: cell, scaffold and signaling molecule, that is generally referred to as the tissue engineering triad [2]. As one of the main components, scaffold serves as a template for cell delivery and support for tissue remodeling, fills voids and controls the release of signaling molecules. A good scaffold for tissue engineering skin should be biocompatible, biodegradable, support cell growth and tissue regeneration, assists appropriate contraction, and possesses similar mechanical and physical properties as the original skin [3–5]. In addition, it is also highly desirable that the scaffold is non-antigenic, non-toxic, readily available, has suitable microstructure, controllable degradation rate and can be stored for a long period of time.

The scaffold can be made of either natural or synthetic materials. The biggest advantage of natural materials is the excellent biocompatibility that supports cell bioactivities (e.g. attachment, migration, proliferation and differentiation), which in turn regulates and promotes tissue formation. Collagen and fibrin are two of the natural materials that have been widely used in tissue engineering for scaffold fabrication as they fulfill the majority of the desirable characteristic mentioned above. Collagen and fibrin can be easily tailored to form scaffolds that provide proper biological, chemical, structural and mechanical cues to the cells to guide tissue formation in vitro and in vivo [6,7].

Collagen is the major extracellular matrix protein of multiple tissues and organs. For example, approximately 70% of human skin extracellular matrices is collagen [8]. Collagen mainly resides in the dermis, providing mechanical strength to skin [9]. To date, more than 29 types of collagen consisted of no less than 46 distinct polypeptide chains have been identified [10,11]. Due to its excellent

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flexibility, collagen has been made into various forms and shapes, including tubes, sponges, sheets, foams, fleeces, nanofibers, and injectable viscous solutions for tissue engineering applications [12].

Fibrin is the matrix protein accumulated at wounds after injury to initiate hemostasis and healing [13]. Fibrin is formed via the polymerization of fibrinogen monomers in the presence of thrombin. The presence of fibrin as a transitional wound healing matrix during the healing process is crucial, as it has been found to promote haemostasis, angiogenesis, fibroblast proliferation and re-epithelialization, with a potential role in reducing wound contraction and risk of infection [13–16]. In addition, fibrin degradation products also have been found to play a profound role in wound healing by inducing fibroblast proliferation, extracellular matrix deposition and angiogenesis [17–19].

Collagen and fibrin have been widely used in skin tissue engineering to fabricate tissue-engineered skin substitutes. However, the two hydrogels have different gelation mechanisms. Fibrin network is initiated by thrombin-catalyzed cleavage of fibrinopeptides from fibrinogen to form fibrils. Collagen fibrils formation is through fibrillogenesis by self-assembly of triple-helical protocollagen molecules. Thus, the collagen fibers exhibit characteristic long bundling with twisted networks, whilst the fibers in the fibrin appear straighter and more individual [20]. Furthermore, collagen and fibrin gels have low mechanical properties initially, and collagen tends to contract, resulting in slower tissue regeneration and less favorable scar quality upon healing.

In contrast to natural materials, mechanical properties, microstructure and degradation time of synthetic polymers can be easily tailored and controlled to meet the requirement [21]. However, synthetic polymers lack cell-recognition signals. This undesirable characteristic can be altered via the addition of chemical functional groups on the polymer surface [22]. Another easier and probably more common alternative is the mixing of synthetic and natural materials. The combination of the advantages of both materials renders it more suitable for tissue engineering applications.

In this study, we intent to compare the regulatory effect of two hydrogels, collagen and fibrin, on skin regeneration and also the regulatory effect of keratinocytes on fibroblasts when grown in a different matrix environment. The poly(lactic acid) (PLA) nanofibers were incorporated into the collagen and fibrin constructs to improve their mechanical properties. We hypothesize that such a comparison study of the comprehensive matrix combination will provide a valuable insight for better selection of scaffolds in skin generation.

2. Materials and methods

2.1. Isolation and culture of murine epidermal keratinocytes and dermal fibroblasts

Murine dermal fibroblasts and epidermal keratinocytes were isolated using a method described previously [23,24]. In brief, the skin from 4–6 month-old Sprague-Dawley rats was cleaned from fats and hairs before cutting into 1–2 mm^2 pieces. The rats were killed by approved Schedule 1 methods, following guidelines from the UK Animals, Scientific procedures Act, 1986 and authorization from Keele Universities's local ethics committee. Then, the sample was digested with 0.6% (v/w) collagenase type I (Sigma, USA) at 37°C for 2–3 h under constant agitation, followed by 0.05% (w/v) trypsin-EDTA (TE; Lonza, Belgium) for 10 min to dislodge the cells. Isolated cells were cultured in Epilife medium (Gibco, UK) and F12:DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Lonza) at equal volume. The cells were cultured at 37°C and 5% CO_2. The medium was changed three times per week. Upon 80% confluence, fibroblasts were separated by exposing the culture to TE for 4 min. Separated fibroblasts were cultured with F12:DMEM medium supplemented with 15% FBS, whereas remaining keratinocytes were cultured with Epilife medium.

2.2. Electrospinning of PLA nanofibers

A 2% PLA solution was prepared by dissolving PLA (Sigma, UK) in chloroform (Sigma) and dimethylformamide (Sigma) in ratio 7:3. The process of electrospinning follows the established protocol [25]. In detail, the PLA solution was placed in a 10 ml glass syringe fitted with 18 G blunt end stainless steel needle. Random nanofibers were collected using round stainless steel wire ring of diameter 9 cm. Electrospinning was performed using the following processing parameters: ± 6 kV, 18 cm air gap, 0.025 ml/min flow rate and 0.200 ml volume. Collected nanofibers were air dried overnight and sterilized by UV radiation for 3 times, 90 s each, before use.

2.3. Preparation of collagen and fibrin constructs

Collagen construct**s** were prepared using 3.6 mg/ml rat tail collagen type I solution (BD Bioscience, USA). A total of 0.5×10^6 fibroblasts (F, labeled with PKH 2, green fluorescence (Sigma, UK)) were seeded per construct. The components of the final collagen constructs were 83.33% collagen type I solution, 10% 10× DMEM, 1.92% 1 N NaOH and 4.75% dH_2O. The final collagen concentration was 3 mg/ml. Collagen construct**s** were formed by placing 0.5 ml collagen mixture solution on top of a hollow filter paper ring of diameter 25 mm to prevent lateral contraction. To prepare collagen construct**s** with random nanofibers (NF), 0.1 ml of collagen gel mixture was used to form the base before random PLA nanofibers were placed on top of it and sealed with 0.4 ml collagen gel mixture (S1). The collagen mixture construct was incubated at 37°C for 45 min for complete gelation before F12:DMEM medium supplemented with 15% FBS, 1% antibiotic-antimycotic (AA; Gibco) and 50 μg/ml ascorbic acid was added.

Fibrin construct**s** were prepared using human plasma fibrinogen (Calbiochem, USA). The final fibrin construct**s** contained 5 mg/ml fibrinogen, 1 U/ml thrombin (Calbiochem) and 2 mg/ml aminocaproic acid (ACA, Sigma). Each fibrin construct consisted of 0.5 ml fibrin solution with 0.5×10^6 fibroblasts (labeled with PKH 2). Fibrin construct**s** were formed by placing 0.5 ml fibrin gel solution on top of a hollow filter paper ring. To prepare fibrin construct with NF, NF were placed on top of filter paper ring, followed by 0.5 ml fibrin solution. Fabricated construct**s** were incubated at 37°C for 1 h before F12:DMEM medium supplemented with 15% FBS, 1% AA, 50 μg/ml ascorbic acid and 2 mg/ml ACA was added. ACA is a lysine analog that promotes rapid dissociation of plasmin and is thus an inhibitor of fibrinolysis. A total of 1×10^5 keratinocytes (K; labeled with PKH 26, red fluorescence (Sigma)) were seeded on top of the collagen and fibrin construct**s** on day 2. Fabricated construct**s** were cultured at 37°C and 5% CO_2 with medium changed every 3 days.

For both hydrogels, four groups of samples have been constructed respectively as indicated in follows: NF^-K^-: constructs with fibroblasts but without nanofibers and keratinocytes; NF^-K^+: constructs with fibroblasts and keratinocytes but without nanofibers; NF^+K^-: constructs with fibroblasts and nanofibers but without keratinocytes and NF^+K^+: constructs with fibroblasts, keratinocytes and nanofibers

Labeling of fibroblasts with PKH 2 and keratinocyte with PKH 26 were performed according to manufacturer's recommendation. In brief, trypsinized cells were washed in serum-free medium before suspended in 300 μl of Diluent C and 300 μl of 4 μM PKH dye (for the staining of 6×10^6 cells). The cells were incubated in dark for 10 min and washed 3 times before mixing with the gels for construct fabrication.

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