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Biomechanical and biochemical characteristics of a human fibroblast-produced and remodeled matrix

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Abstract

We report on a culture method for the rapid production of a strong and thick natural matrix by human cells for tissue engineering applications. Dermal fibroblasts were cultured for three weeks at high density on porous substrates in serum-containing or chemically defined media. The mechanical and biochemical properties of the resulting cell-derived matrix (CDM) were compared to those of standard fibroblast-populated collagen and fibrin gels and native human skin. We found that the ultimate tensile strength of CDM cultured in our chemically defined media (313 ± 8.7 kPa) is significantly greater than for collagen gels (168 ± 39.3 kPa), fibrin gels (133 ± 8.0 kPa) and CDM cultured with serum (223 ± 9.0 kPa), but less than native skin (713 ± 55.2 kPa). In addition to the biomechanics, this *CDM is also biochemically more similar to native skin than the collagen and fibrin gels in terms of all parameters measured. As *CDM is produced by human cells in a chemically defined culture medium and is mechanically robust, it may be a viable living tissue equivalent for many connective tissue replacement applications requiring initial mechanical stability yet a high degree of biocompatibility.

Keywords: Extracellular matrix (ECM); Soft tissue biomechanics; Mechanical properties; Connective tissue; Wound healing

1. Introduction

Millions of surgical procedures are performed each year for tissue loss, amounting to a cost of billions of dollars per year in the United States alone [1]. Current treatments include tissue transfer from a healthy site in the same or another individual, use of medical devices to support the function of the lost tissue, and pharmacologic supplementation of the metabolic products of the lost tissue. Problems with these current treatments include limited number of tissue and organ donors, potential tissue complications such as imperfect matches and dependence on immunosuppressants, limited durability of mechanical devices, and the inconvenience and complexity of prolonged pharmacologic supplementation [2].

To address these problems, tissue engineering approaches to create living tissue equivalents (LTEs), notably cell-seeded collagen and fibrin gels, have gained considerable attention as replacements for lost or damaged connective tissue (e.g., Apligraf™ from Organogenesis, Inc.). LTEs have also been used extensively as *in vitro* wound healing models as well as systems for studying tissue remodeling (for review see Grinnell [3]). LTEs have several advantages over synthetic alternatives including being a natural cell substrate, allowing cellularity to be achieved directly, and being conducive to cell spreading and extracellular matrix (ECM) formation [4]. An LTE is traditionally made by mixing cells with a soluble biopolymer solution (e.g., collagen, fibrin, glycosaminoglycans, and/or proteoglycans) that sets into a gel by modifying the solution, pH, and/or temperature conditions. The cells

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partially rearrange, degrade, and contract the biopolymer scaffold over the next few days as well as synthesize new proteins throughout the culture period. The major drawbacks of these gel-based LTEs is that they are not completely synthesized and arranged by the cells themselves, i.e., they are still "artificially" made, and they are weak relative to native connective tissue. Tissue equivalents of high strength are in high demand since current shortages of load-bearing tissue have created a need for man-made tissues that can withstand *in vivo* mechanical forces [5]. An additional drawback of reconstituted biopolymer gels is the presence of animal components, namely the biopolymer itself and bovine serum that may produce unforeseen adverse reactions in patients.

Over the last two decades, methods for creating LTEs that are completely produced by cells *in vitro* have been developed [6–10]. This advance has allowed the *in vitro* study of tissue formation solely from cells without the confounding effects of an exogenous scaffold. Several research groups have demonstrated that, given a permissive environment, fibroblasts can be induced to produce a thin, three-dimensional sheet of ECM material *in vitro* over time [6–10]. These cell-produced matrices (also know as cell-derived matrix, CDM [11]) are composed in large part by supermolecularly organized collagen [6,7], appear to more closely approximate native tissue than "reconstituted" biopolymers described above [8,9], and support significantly enhanced cell adhesion, migration, proliferation, and acquisition of *in vivo*-like morphology than reconstituted ECM [11]. The multilayered fibroblasts in this cell-produced environment assume a synthetic phenotype characterized by high ECM accumulation, fibrillar fibronectin organization, and the formation of actin stress fibers and "3D matrix adhesions" [11,12]. Burst tests performed on laminated CDM tubes and uniaxial tests on thin strands of CDM provide a preliminary indication that CDMs may be strong compared to collagen gels and approach native tissue physical properties [8,13]. However, CDMs have lacked the mainstream acceptance that reconstituted gels have enjoyed in large part due to the extended culture duration necessary to create even thin constructs, generally in the order of months [14], whereas collagen and fibrin gels can be developed in only a few days [15].

The goal of this work is to develop a method for generating strong and thick cell-produced LTEs free from animal components within a relatively short culture period. Such a cell-produced LTE would be an attractive alternative to reconstituted gels and matrices produced using animal serum for tissue engineering applications and for *in vitro* wound healing studies. Here we compare the biochemical and biomechanical characteristics of cell-produced and cell-remodeled (reconstituted) matrices created in parallel by culturing fibroblasts for three weeks in collagen gels, fibrin gels, or three different media formulations permissive to the production of ECM.

2. Methods

2.1. Cells

Human neonatal foreskin fibroblasts (HFFs, American Type Culture Collection (ATCC), Manassas, VA) were cultured in T-300 tissue culture flasks (BD Biosciences, Bedford, MA) with high glucose Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% bovine calf serum (BCS, Hyclone, Logan, UT), and 1% penicillin/streptomycin/ampothericin B (Invitrogen, Carlsbad, CA) at 37 °C in humidified, 10% CO_2 conditions. Cells were harvested at 90% confluency with a 10 min application of 0.25% trypsin/0.05% EDTA solution (Mediatech). Two million passage five (p5) cells were used for each sample in all experiments.

2.2. Standard serum-supplemented medium

The standard serum-supplemented medium used for the collagen gels (CG), fibrin gels (FG), and CDM groups consisted of DMEM with 10% fetal bovine serum (FBS, ATCC), 150 μg/ml (519 μM) L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemicals, Japan), and 1% penicillin/streptomycin/ampothericin B (Invitrogen).

2.3. Chemically defined medium

The chemically defined medium used for the *CDM and **CDM groups consisted of a 3:1 ratio of DMEM (high glucose (4.5 g/l) with L-glutamine and sodium pyruvate, Mediatech) and Ham's F12 (Invitrogen) with the addition of 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 5 ng/ml selenious acid (Sigma-Aldrich), 10^{-4} M ethanolamine (Sigma-Aldrich), 150 μg/ml L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako), 2.5 ng/ml epidermal growth factor (BD Biosciences) in 5 μg/ml human serum albumin (EMD Biosciences, San Diego, CA), 5 ng/ml basic fibroblast growth factor (BD Biosciences), 1.0×10^{-7} M dexamethasone (Sigma-Aldrich), 2×10^{-10} M L-3,3′,5-triiodothyronine (Sigma-Aldrich), 4×10^{-3} M of Glutamax™ (Invitrogen), 1 μg/ml Glutathione (reduced) (Sigma-Aldrich), and 1% penicillin/streptomycin/amphotericin B (Invitrogen). Growth factors were added fresh at each feeding (*CDM), except for a small subset of samples where the growth factors were added into the entire stock medium at the start of the experiment (**CDM). Due to the low number of **CDM samples for many measurements ($n = 2$ or 3), statistics for this group are not presented.

2.4. Collagen gel, fibrin gel, CDM, and skin preparation

Fibroblast-populated CGs were prepared according to the methods of Elsdale and Bard [16] by mixing 0.2 ml of collagen stock solution (5 mg/ml of 5 mM HCl-extracted rat tail tendon collagen in 5 mM acetic acid), 0.05 ml 5× DMEM (Mediatech), 0.65 ml DMEM (Mediatech) with cells, 0.1 ml fetal bovine serum (FBS, ATCC), 150 μg L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako) and 1% penicillin/streptomycin/ampothericin B (Invitrogen) at room temperature. One milliliter of the resulting solution was added into each 24 mm diameter well. The initial collagen concentration was 1.0 mg/ml, and the initial cell concentration was 2 million cells/ml in 10% FBS. Fibroblast-populated FGs were prepared based on the methods of Grinnell and colleagues [17]. Briefly, HFFs in standard serum-supplemented medium were added to a fibrinogen (Sigma-Aldrich F4753 type IV) solution. One-milliliter samples were mixed with 4 units of bovine thrombin (Sigma-Aldrich T7513) at room temperature. One milliliter of the resulting solution was added into each 24 mm diameter well. The initial fibrinogen concentration was 1.0 mg/ml, and the initial cell concentration was 2 million cells/ml in 10% FBS. CDMs were prepared by mixing 2 million, passage 5 HFFs with standard serum-supplemented medium or the chemically defined medium at room temperature into a final volume of 1 ml per sample. The 1 ml samples of CGs, FGs, and cells were pipetted onto 24 mm diameter,