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 ± 7.8 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\textsuperscript{TM} from Organogenesis, Inc.). LTEs have also been used extensively as \textit{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
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, Hydnone, Logan, UT), and 1% penicillin/streptomycin/amphotericin B (Invitrogen, Carlsbad, CA) at 37°C in humidified, 10% CO₂ conditions. Cells were harvested at 90% confluence 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), 150µg/ml (519µM) t-aspartic acid phosphate magnesium salt n-hydrate (Wako Pure Chemicals, Japan), and 1% penicillin/streptomycin/amphotericin 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 (4.5g/l) with t-glutamine and sodium pyruvate, Mediatech) and Ham’s F12 (Invitrogen) with the addition of 5µg/ml insulin (Sigma-Aldrich, St. Louis, MO), 5ng/ml selenious acid (Sigma-Aldrich), 10⁻⁴M ethanolamine (Sigma-Aldrich), 150µg/ml t-aspartic acid phosphate magnesium salt n-hydrate (Wako), 2.5ng/ml epidermal growth factor (BD Biosciences) in 5µg/ml human serum albumin (EMD Biosciences, San Diego, CA), 5ng/ml basic fibroblast growth factor (BD Biosciences), 1.0 × 10⁻⁵m dexamethasone (Sigma-Aldrich), 2 × 10⁻⁶M t-3,3’,5-triodothyronine (Sigma-Aldrich), 4 × 10⁻⁵M of Glutamax™ (Invitrogen), 1µg/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 Eldsade and Bard [16] by mixing 0.2 ml of collagen stock solution (5mg/ml of 5mM HCl-extracted rat tail tendon collagen in 5mM acetic acid, 0.05ml 5 × DMEM (Mediatech), 0.65ml DMEM (Mediatech) with cells, 0.1ml fetal bovine serum (FBS, ATCC), 150µg t-aspartic acid phosphate magnesium salt n-hydrate (Wako) and 1% penicillin/streptomycin/amphotericin B (Invitrogen) at room temperature. One milliliter of the resulting solution was added into each 24mm diameter well. The initial collagen concentration was 1.0mg/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 24mm diameter well. The initial fibrinogen concentration was 1.0mg/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 0.1ml fetal bovine serum (FBS, ATCC), 150µg/ml (519µM) t-aspartic acid phosphate magnesium salt n-hydrate (Wako Pure Chemicals, Japan), 1% penicillin/streptomycin/amphotericin B (Invitrogen).
porous inserts (0.4 μm TransWells™, Corning Life Sciences, Acton, MA) suspended above standard 6-well plates, and allowed to sit undisturbed at room temperature. After a 1-h period, 3 ml of the appropriate medium was carefully added below and 1 ml above each sample and the samples were incubated at 37 °C in humidified, 10% CO₂ conditions. Samples were fed every other day (3 ml below and 2 ml above each porous insert) with the appropriate medium for three weeks. Human penile skin was obtained through Analytical Biological Services Inc. (Wilmington, DE) and shipped cold in RPMI medium with antibiotics and subjected to biomechanical testing followed by biochemical analysis within 12 h of removal from the subject. The subdermal fatty layer was surgically removed prior to testing. Only the skin from the distal two-thirds of the tip was used. Four samples were tested in each group except for the human skin group which had seven samples.

2.5. Mechanical testing

After three weeks in culture, the samples were exposed to ddH₂O for one hour to lyse the cells and then equilibrated in phosphate buffered saline (PBS, Mediatech) for biomechanical testing. This treatment eliminates the intrinsic tension produced by the fibroblasts; it is not intended to decellularize the matrix. The thickness, failure tension, failure strain, and ultimate tensile strength (UTS) of the samples were determined using a custom tissue inflation device [18] that measures the displacement and pressure at which a sample bursts when inflated with PBS at a constant rate of 1 ml/min. The sample is circularly clamped at and inflated through a 1 cm diameter opening, thus causing the sample to form an approximately spherical cap before failing. The increasing pressure applied to the sample was measured by a pressure transducer (model PX102-025GV, Omega Engineering, Stamford, CT). The displacement of the center of the cap was measured with a laser displacement system (LDS-080, Keyence, Woodcliff Lake, NJ). The LSD-080 was also used to measure the thickness of each sample after being slightly compressed by a 1 cm diameter opening, thus causing the sample to form an approximately spherical cap before failing. The increasing pressure

where \( \lambda \) is the stretch ratio as the sample fails (unitless). For samples that exhibit a spherical-cap geometry, the average stretch ratio along a meridian can be estimated from the radius of curvature using the geometric relationship:

\[
\lambda = \frac{R \arcsin(a/R)}{a}.
\]

2.6. Biochemical analysis

Following biomechanical testing, the samples were weighed (wet weight), lyophilized overnight, and then weighed again (dry weight). Each lyophilized sample was solubilized in 1 ml of 0.5 M acetic acid and 1 mg/ml pepsin (Sigma-Aldrich) and incubated overnight at 20 °C with rotation. This extraction step was repeated twice more to achieve complete extraction of the acid and pepsin soluble fraction of collagen. The samples were then centrifuged at 14,000 rpm for 1 h at 15 °C, and the supernatant was combined with samples from the earlier two extractions and used for determining non-acid and pepsin extractable collagen content using the Sircol™ Assay (Biocolor, Belfast, N. Ireland). The Sircol™ Assay was used to quantify the content of intact collagen monomers in the solution according to manufacturer’s instructions. This colorimetric assay does not detect degraded collagen which we found to be approximately 5–10% of the total collagen content determined by the hydroxyproline assay (see methods below). Total non-collagenous protein content of each extract was determined with the Tp-Blue™ Total Protein Assay (Biocolor) according to manufacturer’s instructions. Total protein content was obtained by adding this value to the total amount of collagen obtained for each sample. The remaining pellets of each sample (which we term the “non-acid and pepsin extractable fraction” of the sample) were digested with Proteinase K (Invitrogen), 30 μg in 500 μl solution of 10 mM EDTA and 0.1 M sodium phosphate (pH 6.5) (Fisher) overnight at 60 °C. A 100 μl aliquot of the digest was used for digesting sulfated glycosaminoglycan and proteoglycan content (that does not include hyaluronic acid) with the Blycan™ Assay (Biocolor) according to manufacturer’s instructions. A 10 μl aliquot of the digest was then used to determine DNA content, and thus cell number (assuming standard 8 pg of DNA per cell), with Hoechst 33258 dye (Amersham Biosciences, Piscataway, NJ) on a DyNA Quant 200 fluorometer (Amersham Biosciences). Aliquots of the Proteinase K digests (100–200 μl) were used to determine the non-acid and pepsin extractable collagen content (insoluble collagen fraction) using the hydroxyproline assay. The hydroxyproline assay, based on previous methods [19,20] consists of hydrolyzing each sample in 6.0 M HCl for 16 h at 110 °C, followed by drying of the samples under vacuum, reconstituting to 2.0 ml with assay buffer (consisting of 5 g/l citric acid (Sigma-Aldrich), 1.2 ml/l glacial acetic acid (EMD Chemicals, Gibbstown, NJ), 12 g/l sodium acetate, and 3.4 g/l sodium hydroxide), mixing with 1.0 ml of chloramine-T reagent (made from 62 mM chloramine-T solution in 20.7% ddH₂O, 26% n-propanol and 53.3% of assay buffer) for 20 min at room temperature, adding 1.0 ml of freshly prepared dimethylaminobenzaldehyde reagent (made from 15 g of p-dimethylaminobenzaldehyde (Sigma-Aldrich) in 60 ml of n-propanol and 26 ml of 60% perchloric acid) and incubating each sample at 60 °C for 15 min, cooling each sample in tap water for 5 min, and measuring the absorbance of each sample at 550 nm within 45 min. Absorbance readings were correlated with collagen amount using a standard curve and a conversion factor of 10 μg collagen to 1 μg 4-hydroxyproline. The standard curve was created and the conversion factor determined with known amounts of trans-4-hydroxy-L-proline (Sigma-Aldrich) and rat-tail type I collagen, respectively. All reagents were from VWR (Bridgeport, NJ) unless otherwise noted.

2.7. Histology and transmission electron microscopy

One sample from each group was prepared for histological evaluation by fixing in 10% zinc formalin for 1 h, followed by washing and storing in 70% ethanol at 4 °C. The samples were embedded onto paraffin blocks,
sectioned into 10 μm thick sections, and stained with hematoxylin and eosin (H&E). The stained sections were imaged and photographed at 200 × with a Richardson RTM-3 Microscope (Richardson Technologies, Bolton, Ontario, Canada). One sample from each group (except **CDM and penile dermis) was fixed for transmission electron microscopy in 2% glutaraldehyde for 1 h, rinsed three times in sodium cacodylate buffer, fixed in osmium tetroxide for 10 min, and dehydrated in 10 min ethanol steps followed by two washes in propylene oxide (all from Electron Microscopy Sciences, Hatfield, PA). The samples were then embedded in epon-araldite resin (Electron Microscopy Sciences). Briefly, the samples were embedded in a 1:1 ratio of propylene oxide to epon araldite for 2 h, followed by embedding in 100% Epon Araldite for 3 h, and then transferring each sample to embedding molds with fresh 100% Epon Araldite and cured in a 60 °C oven overnight. 60–90 nm gold- and silver-colored sections were stained for 5 min with uranyl acetate saturated in 50% ethanol, followed by staining for 20 min in lead citrate according to the methods of Venable and Coggeshall [21]. The stained sections from each of the samples were then imaged with a Philips CM 10 transmission electron microscope (Philips, Eindhoven, Netherlands) for observation of cell, collagen, and matrix morphology. The average collagen fibril diameter and density were calculated from the average measured diameters and number of collagen fibrils per square micrometer from five randomly chosen fields taken at high magnification (57,000 × or 88,000 ×). The collagen diameters were measured at the thinnest point of cross-sectioned collagen fibrils with a 5 × magnified ruler. If a randomly chosen field fell on a grid, then another field continued to be randomly chosen until it fell solely on the sample.

2.7.1. Statistics

Mean values and standard deviations are presented in all tables and text. Statistical differences between groups were determined using one-way ANOVA with the Student–Newman–Keuls method of post hoc analysis for pairwise multiple comparisons (SigmaStat, Systat Software, Inc., Point Richmond, CA). Differences were considered significant with p < 0.05. Since most groups were significantly different for each metric, the groups that were not significantly different are indicated. Linear regression was performed to determine the correlation between measured selected physical and biochemical indices and adjusted R²-values were obtained to determine the degree of association between the independent and dependent variables (SigmaStat).

3. Results and discussion

Our first goal was to determine whether the ECM produced and assembled solely by hyperconfluent fibroblasts is mechanically superior to ECM generated by allowing cells to compact and remodel gels cast from purified solutions of collagen or fibrin. To reduce experimental variability, all groups were grown in parallel with the same number of fibroblasts from the same batch and fed with the same standard serum-supplemented medium at the same time. The CGs and fibrin gels started out at a thickness of 2.2 mm and contracted to a thickness 83 ± 5 and 218 ± 5 μm, respectively (Fig. 1a, b; Table 1). In contrast, the cell-produced LTEs started out at slightly more than a single cell layer thick and grew to 125 ± 6 μm, with the synthesized ECM organizing into several layers (Fig. 1c). After three weeks in culture in standard serum-supplemented medium, the CDMs were 30% and 70% stronger than the CGs and FGs, respectively (Table 1); these are the first published results for the physical properties of planar CDM. Although mechanical data from our custom biaxial inflation test and standard uniaxial tensile tests are not directly comparable, and the culture conditions and duration are somewhat different from other studies (e.g., the cell density was generally lower in previous studies and the duration shorter), the strength of our CGs and FGs are similar to results obtained by other researchers [4,22,23]. These results demonstrate that cell-produced ECM is stronger than reconstituted ECM and can be made within a relatively short period of time.

Fig. 1. Representative histomicrographs from (a) collagen gel (CG), (b) fibrin gel (FG), (c) cell-derived matrix (CDM), (d) CDM cultured in chemically defined media (**CDM), (e) CDM cultured in chemically defined media with only initial growth factor supplementation (**CDM), and (f) dermis of human penile skin (HPS) samples. Arrow in HPS section points to a portion of the epidermis. All gels and matrices were initially seeded with the same volume of solution containing two million cells/ml and their respective protein and media components. They were then cultured for three weeks in porous inserts fed from above and below every three days. The thickness in the micrographs is less than measured with the laser system due to shrinkage during histological processing. All micrographs taken at 200x on unstained sections using a Richardson Technologies Real-Time Microscope; scale bar = 100 μm.
Table 1
Results from mechanical and biochemical analysis of human penile skin and cell-produced matrices (CDMs and *CDMs), fibroblast-populated collagen gels and fibrin gels containing human foreskin fibroblasts

<table>
<thead>
<tr>
<th></th>
<th>Collagen gel&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fibrin gel&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CDM&lt;sup&gt;c&lt;/sup&gt;</th>
<th>*CDM&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Penile skin&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate tensile strength (kPa)</td>
<td>168 ± 39.3&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>133 ± 8.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>223 ± 9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>313 ± 8.7</td>
<td>713 ± 55.2</td>
</tr>
<tr>
<td>Thickness (μm)</td>
<td>83 ± 5 (2220 μm)</td>
<td>218 ± 5 (2220 μm)</td>
<td>125 ± 6 (~30 μm)</td>
<td>395 ± 6 (~30 μm)</td>
<td>651 ± 30 (NA)</td>
</tr>
<tr>
<td>Extensibility (mm/mm)</td>
<td>0.14 ± 0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.23 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.19 ± 0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.39 ± 0.04</td>
<td>0.53 ± 0.14</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>1.08 ± 0.03&lt;sup&gt;(1.0 mg)&lt;/sup&gt;</td>
<td>1.58 ± 0.04&lt;sup&gt;(1.0 mg)&lt;/sup&gt;</td>
<td>1.25 ± 0.06&lt;sup&gt;(1.0 mg)&lt;/sup&gt;</td>
<td>4.40 ± 0.08</td>
<td>6.74 ± 0.44</td>
</tr>
<tr>
<td>Collagen density (mg/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>7.7</td>
<td>7.7</td>
<td>7.7</td>
<td>7.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Collagen fibril density (fibrils/collagen fraction (%))</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>Collagen fibril diameter (nm)</td>
<td>52</td>
<td>52</td>
<td>52</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>Ultimate tensile strength (Pa/mg/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>Total collagen (mg)</td>
<td>0.99 ± 0.02&lt;sup&gt;(1.0 mg)&lt;/sup&gt;</td>
<td>0.51 ± 0.03</td>
<td>0.85 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.40 ± 0.04</td>
<td>3.98 ± 0.37</td>
</tr>
<tr>
<td>Collagen:total protein (%)</td>
<td>92 ± 1.1</td>
<td>32 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>68 ± 1.2</td>
<td>32 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59 ± 1.9</td>
</tr>
<tr>
<td>Non-acid and pepsin extractable collagen fraction (%)</td>
<td>1.5 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.3 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.3 ± 0.2</td>
<td>12.8 ± 0.5</td>
<td>15.7 ± 0.7</td>
</tr>
<tr>
<td>Collagen density (mg/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>26.5 ± 1.5</td>
<td>5.2 ± 0.2</td>
<td>15.1 ± 0.9</td>
<td>7.8 ± 0.2</td>
<td>13.5 ± 0.7</td>
</tr>
<tr>
<td>UTS/Collagen density (Pa/mg/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>6.4 ± 1.9</td>
<td>25.9 ± 2.4</td>
<td>14.5 ± 1.1</td>
<td>40.3 ± 0.4</td>
<td>52.9 ± 3.1</td>
</tr>
<tr>
<td>Collagen fibril diameter (nm)</td>
<td>52 ± 10</td>
<td>47 ± 5</td>
<td>46 ± 5</td>
<td>48 ± 5</td>
<td>—</td>
</tr>
<tr>
<td>Collagen fibril density (fibrils/μm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>81 ± 4</td>
<td>28 ± 4</td>
<td>79 ± 2</td>
<td>80 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>Total proteoglycans &amp; glycosaminoglycans (% Dry Weight)</td>
<td>2.7 ± 0.14</td>
<td>2.0 ± 0.02</td>
<td>1.7 ± 0.08</td>
<td>1.0 ± 0.02</td>
<td>0.86 ± 0.02</td>
</tr>
<tr>
<td>Dry weight/wet weight (%)</td>
<td>6.1 ± 0.3</td>
<td>5.0 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.7 ± 0.1</td>
<td>5.1 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>Cell number (millions)</td>
<td>2.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt; (2.0 million)</td>
<td>4.0 ± 0.1 (2.0 million)</td>
<td>2.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt; (2.0 million)</td>
<td>6.1 ± 0.1 (2.0 million)</td>
<td>9.8 ± 0.4 (N/A)</td>
</tr>
<tr>
<td>Cell density (10&lt;sup&gt;6&lt;/sup&gt; cells/cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>75 ± 6.1</td>
<td>40 ± 1.4</td>
<td>49 ± 4.4</td>
<td>34 ± 0.7</td>
<td>33 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Numbers indicate mean ± SD. Numbers in parentheses indicate approximate initial amount. Collagen fibril diameters and densities were measured in proximity to cell surfaces. As most groups were significantly different than all others for each metric, only the non-significant (p > 0.05, ANOVA with SNK post hoc test) values are indicated by superscripts (a = CG, b = FG, c = CDM, d = *CDM, e = Skin); e.g., for total collagen, all groups are different from each other except the CG and CDM groups (p = 0.4).

Our next goal was to develop a simple chemically defined medium that would promote fast growth of a strong and thick cell-produced LTE. Whereas chemically defined media have been formulated for increasing the proliferation of fibroblast monolayers [24,25] and for supporting the growth of fibroblasts and keratinocytes in dermal equivalents [26,27], our chemically defined medium was optimized for promoting high ECM synthesis by multilayered fibroblasts. This medium is similar to the medium used for the CG, FG and CDM groups described above except, in lieu of serum supplementation, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), dexamethasone, and t-3,3’,5-triiodothyronine, along with a few basic components necessary for cell growth (insulin, selenious acid, and a lipid precursor) present in serum [28] were added. EGF, in the co-presence of ascorbate, has been shown to promote growth and protein synthesis of cultured fibroblasts [29]; bFGF is a potent mitogenic and chemotactic factor for fibroblasts, regulating proliferation and ECM production [30,31]; dexamethasone has been reported to significantly increase fibroblast cell proliferation in serum-free media [24,25] by upregulating the PDGF-α receptor mRNA and protein [32] and promote actin stress fiber formation and increased fibronectin matrix accumulation that aid in the more effective organization of the ECM [33,34]; t-3,3’,5-triiodothyronine increases the rate of cell metabolism, while insulin increases the residence time of procollagen mRNA and upregulates latent TGF-β1 synthesis among its many other effects [35]. The growth factors were added fresh at each feeding (*CDM) or added only once to the stock medium at the start of the 3-week culture period in a small sub-group of samples (**CDM). For direct comparison between groups, all cells were passaged together in the same flasks with bovine serum prior until the start of the 3-week culture period for this study; for a completely serum-free product the chemically defined medium could be utilized from the cell isolation onward. These components (and possibly the lack of the numerous inhibitory components present in serum) stimulated a tripling in thickness, a 40% increase in the tensile strength, and a doubling of extensibility compared to the serum-supplemented CDM samples. The chemically defined media also resulted in a doubling of the cell number, a five-fold increase in the fraction of non-acid and non-pepsin extractable protein, and a 40% decrease in proteoglycan and glycosaminoglycan proportion compared to serum-supplemented CDM, resulting in values that more closely resembled native tissue. Interestingly, when CGs were fed with the chemically defined medium, the contractile forces within the CGs became so great that they detached from the wells and contracted into themselves within 24 h of the initial feeding. This excessive contraction prevented a comparison of the effects of the chemically defined medium and the serum-supplemented medium on the development of gels.

Our third goal was to compare the structure and biochemical composition between the five types of LTEs as well as with native human skin. We also determined how mechanisms of cell-mediated strengthening of ECM (see Table 1). For example, fibroblasts entrapped in purified collagen synthesize far fewer ECM proteins than in monolayer cultures, yet fibroblasts entrapped in purified...
fibrin retain their ability to produce ECM proteins [36].

Our data support these previous findings and furthermore demonstrate that fibroblasts in self-produced collagen-rich matrices produce an even greater amount of ECM proteins than in purified fibrin gels. It is interesting to note that, by three weeks, the fibroblasts in the cell-produced matrices synthesized a similar amount of total collagen as that initially cast into the CGs, yet the synthesized collagen did not appear to result in inhibition of collagen synthesis in these matrices. Inhibition of collagen synthesis may occur at collagen densities such as those in the CGs (>90%) whereas the presence of non-collagenous proteins in the CDM yielding only 68% collagen in the CDM and 32% in the *CDM appear to be sufficient to not inhibit further collagen production.

The net increase in total protein synthesized by the cells in the cell-produced matrices was significantly greater than the net increase in total protein in the CGs and fibrin gels (total protein minus initial protein cast into the gels; see Table 1). These values are somewhat higher but of the same magnitude as obtained by other researchers for gels and cell-produced matrices [4,6,9,22]. These compositional differences are likely due to a combination of factors including the substantially higher initial cell density of our cultures, the 80% greater concentration of L-ascorbate in the more stable form of L-ascorbic acid phosphate magnesium salt n-hydrate (1-week stability versus 24-h stability for L-ascorbate) [7], and the use of porous inserts that allowed the samples to be fed from two sides instead of just from above. In pilot studies, cell-produced matrices grown on porous inserts to allow increased diffusion of nutrients to the basal side of the samples were 50% thicker than samples grown on regular 6-well plates, yet the strength normalized to the thickness of the samples was equivalent (data not shown).

The strength of soft connective tissues, as well as LTEs such as CGs, is generally attributed to the collagen density and structural arrangement, average collagen fibril diameter, and density of collagen cross-links [22,37–39]. Observation by transmission electron microscopy (TEM) revealed that the diameters of the collagen fibrils in all samples were similar with the exception of the CGs. In the CGs, the collagen fibrils were almost 20% thicker than in the other samples suggesting that the majority of the collagen was mature fibrils from the original casting. However, there was a small presence of approximately 46 nm diameter fibrils in the CGs, presumably newly synthesized collagen (Fig. 2). The collagen fibril diameters measured in all groups fall within the range found in native soft connective tissue (40–100 nm) [22,37–39]. The collagen fibril density in proximity to the cell surface was similar for the cell-produced matrices and CGs, and was more than 2.5 times greater than for the fibrin gels. Interestingly, although the CGs in this study had the greatest collagen density and the largest collagen fibril diameters, they were significantly weaker than the cell-produced matrices grown under identical conditions.

The UTS/collagen density, a metric that is independent of the thickness of the samples and represents the strength of the constructs normalized per unit of collagen, was significantly greater for the cell-produced matrices than the CGs. The relatively high strength afforded by the collagen in the CDM could be due to a greater presence of intramolecular covalent cross-links between the collagen fibrils and/or more extensive bundling of the collagen fibrils into fibers in the cell-produced matrices than in the CGs [40]. Overall, the UTS of all specimens was found to correlate closely to the non-acid and pepsin extractable collagen density ($R^2 = 0.99$). As the name implies, the non-acid and pepsin extractable collagen fraction is the amount of collagen that was not solubilized by repeated 0.5 M acetic acid and pepsin (1 mg/ml) extraction steps at 60°C over a 2-day period. This fraction appears to represent highly cross-linked or bundled collagen that resists extraction, giving rise to a more resistant structure that leads to strengthening of the matrix. Thus, the strength of LTEs, as well as native skin (see below), is not just correlated with collagen density and fibril diameter, but more specifically to a very stable form of collagen represented by the non-acid and pepsin extractable collagen density in this study.

Human penile skin was used as a comparison of native soft connective tissue to the five different LTEs produced in this study since the fibroblasts used in making the LTEs were obtained from human foreskin. The cell-produced matrices developed with the chemically defined media most closely resembled penile skin in terms of the UTS/collagen density, non-acid and pepsin extractable collagen fraction, and relative proportion of proteoglycans and glycosaminoglycans. The *CDM was also similar to native tissue in that they had to be exposed to significantly higher temperatures during the pepsin as well as the Proteinase K extraction steps than the other groups before the samples began to disintegrate. The extensibility was significantly greater for the *CDMs than its serum-supplemented counterparts, yet significantly lower than for native skin.

An additional group was tested to determine the effect of adding the growth factors to the chemically defined media only once at the beginning of the study, rather than preparing fresh solutions for each feeding. This modification would allow for easier automation and decrease production costs. The activity of growth factors such as bFGF and EGF are known to have short half-lives in stock media, which is why they are generally added fresh at each feeding. Since EGF stimulates the synthesis of non-collagenous proteins but inhibits the transcription of type I collagen genes, and high bFGF concentrations favor increased cell proliferation over enhanced synthesis and strengthening of the ECM [41], we hypothesized that the high growth factor concentration in the first days of culture would result in the cell-produced matrix developing mostly in thickness by rapid cell proliferation and non-collagenous ECM production, followed by development in strength by increased collagen synthesis for the remainder of the growth period. The one-time addition of growth factors
at the start of the 3-week culture period resulted in a matrix with approximately one half of the thickness (0.23 mm) and cell number (3.6 million) of parallel samples for which the growth factors were added fresh at every feeding (*CDM) and resulted in a more than two-fold increase in strength (700 kPa) and relative collagen content (67%) but did not alter the extensibility (0.36), UTS/collagen density, or the fraction of collagen that was non-acid and pepsin extractable, possibly indicating that the increase in strength was due to the increased fraction of synthesized collagen in the **CDMs. Although these preliminary data are interesting, statistical comparisons of the **CDM data with data from other groups were not completed due to the low number of samples (n = 2 or 3 for each measurement) and corresponding low statistical power for this small subset. In pilot tests, adding fresh but lower concentrations of growth factors (e.g., 1 ng/ml for EGF) at each feeding did not reproduce the same results; in fact, the resulting matrices were too weak or thin to be measured indicating that relatively high concentrations of the growth factors are needed for rapid production of ECM proteins. Cell-produced matrices grown for longer periods (up to 6 weeks) further continued to increase in thickness and strength (data not shown). This preliminary data indicates that a stronger, more collagenous matrix is produced if growth factors are added into the stock solution which may be beneficial in commercial applications; however, the maximum thickness, total protein amount, and cellularity are only achieved by adding fresh growth factors at each feeding.

4. Conclusion

We report here on a new culture method for the rapid production of a strong natural matrix by human dermal fibroblasts for tissue engineering applications. The results of this study demonstrate that completely cell-produced ECM is mechanically superior to reconstituted ECM (e.g., fibroblast-populated collagen and fibrin gels). This strength is highly correlated to the fraction of very stable collagen.
The cell-produced matrices created in this study have potential in being used as soft connective tissue substitutes since they are produced solely from cells fed with a chemically defined medium that does not contain animal components (thus presenting a minimal likelihood of an antigenic response and disease transmission), and are biochemically more similar to native human connective tissue than currently used “living tissue equivalents” (e.g., fibroblast-populated collagen and fibrin gels). The rapid growth and lack of expensive and inherently variable serum contribute to the commercial viability of these cell-produced matrices as soft connective tissue substitutes and may open the door to mainstream acceptance. Due to the relative simplicity of the chemically defined medium developed for this study, these cell-produced matrices could also serve as in vitro biological models for the effects of nutritional components and pharmaceutical products on the growth and development of soft connective tissue. They may also be useful for studying numerous in vitro conditions and processes such as wound healing, connective tissue formation, fibrosis, and the development and interaction of different cells in a soft connective tissue environment. In summary, the ability to grow thick and strong cell-produced matrices in a relatively short period of time in chemically defined conditions enables the development of an attractive alternative to collagen gels, fibrin gels, and even native tissues for tissue replacement and studying many tissue processes in vitro.

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References

growth factor and ascorbate in cultured human skin fibroblasts. Eur J

growth factor (b-FGF) in the perimatrix of cholesteatoma. HNO

[31] Aktas G, Kayton R. Ultrastructural immunolocalization of basic
fibroblast growth factor in fibroblasts and extracellular matrix.

Dexamethasone activates expression of the PDGF-alpha receptor
and induces lung fibroblast proliferation. Am J Physiol 1998;

[33] Zoppi N, Ghinelli A, Gardella R, Barlati S, Colombi M. Effect of
dexamethasone on the assembly of the matrix of fibronectin and on
its receptors organization in Ehlers-Danlos syndrome skin fibro-

[34] Brenner KA, Corbett SA, Schwarzbauer JE. Regulation of fibro-
nectin matrix assembly by activated Ras in transformed cells.

[35] Ghahtary A, Tredget EE, Mi L, Yang L. Cellular response to latent
TGF-beta1 is facilitated by insulin-like growth factor-II/mannose-6-

[36] Clark RAF. Wound repair: overview and general considerations. In:
Clark RAF, editor. The molecular and cellular biology of wound

[37] Hukins DWL. Biomechanical properties of collagen. In: Weiss JB,
Jayson MIV, editors. Collagen in health and disease. Edinburgh:

[38] Roeder BA, Kokini K, Sturgis JE, Robinson JP, Voytik-Harbin SL.
Tensile mechanical properties of three-dimensional type I collagen
extracellular matrices with varied microstructure. J Biomech Eng

[39] Christiansen DL, Huang EK, Silver FH. Assembly of type I
collagen: fusion of fibril subunits and the influence of fibril
diameter on mechanical properties. Matrix Biol 2000;19(5):

[40] Parry DA. The molecular and fibrillar structure of collagen and its
relationship to the mechanical properties of connective tissue.

[41] Kurata S, Hata R. Epidermal growth factor inhibits transcription of
type I collagen genes and production of type I collagen in cultured
human skin fibroblasts in the presence and absence of l-ascorbic acid
266(15):9997–10003.