Enhancing the Three-Dimensional Structure of Adherent Gingival Fibroblasts and Spheroids via a Fibrous Protein-Based Hydrogel Cover

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Key Words
Extracellular matrix · Three-dimensional cell rearrangement · Adherent fibroblast · Spheroid fusion · Fibrous protein hydrogel · Tissue modeling

Abstract
Tissue engineering-based therapies rely on the delivery of monolayered fibroblasts on two-dimensional polystyrene-coated and extracellular matrix (ECM) surfaces to regenerate connective tissues. However, this approach may fail to mimic their three-dimensional (3D) native architecture and function. We hypothesize that ECM fibrous proteins, which direct the migration of cells in vivo, may attach and guide polystyrene- and Matrigel™-ECM (M-ECM)-adherent fibroblasts to rearrangement into large multicellular macrostructures with the ability to proliferate. Gingival monolayered fibroblasts and their derived spheroids were added and adhered to tissue culture polystyrene and M-ECM surfaces. The cells were covered with a layer of collagen1 hydrogel combined with vitronectin, fibronectin or fibrin, or 10% M-ECM. The development of 3D cell constructs was characterized by epifluorescence and confocal scanning microscope image analysis. The ECM turnover and the proliferative capabilities of the fibroblasts were determined via gene expression profiling of collagen1, fibronectin, matrix metalloproteinase/metallopeptidase 2, Nanog, and SRY (sex-determining region Y)-box2 (Sox2). Expression of the Sox2 protein was followed by immunostaining. The collagen1 protein had the strongest effect on monolayered and spheroid cell rearrangements, forming large spherical shapes and fused 3D macrostructures. The addition of fibrin protein was typically required to achieve a similar effect on M-ECM-adherent monolayered fibroblasts. The spheroid fusion process was followed by an increase in cell density and the formation of tight clusters. The fused spheroids continued to maintain their intracellular ECM turnover and proliferation capacities. Collagen1 is a valuable component in the rearrangement of adherent fibroblast monolayers and spheroids. Fibroblast spheroids should preferably be used as basic building blocks to assemble multicellular connective tissue-like macrostructures.

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Abbreviations used in this paper

| 2D | 2-dimensional |
| 3D | 3-dimensional |
| DPBS | Dulbecco’s phosphate-buffered saline |
| ECM | extracellular matrix |
| Fibro | fibronectin |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| M-ECM | Matrigel™-extracellular matrix |
| MMP2 | matrix metalloproteinase/metallopeptidase 2 |
| Sox2 | SRY (sex-determining region Y)-box2 |
| TCPS | tissue culture polystyrene |
| Vitro | vitronectin |

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Introduction

Gingival fibroblasts, a major component of the connective tissue of the gingiva, play a critical role in the wound-healing process of oral soft tissues. They are responsible for the initial steps in the regeneration and replacement of injured tissue with healthy tissue through the secretion and regulation of supportive extracellular matrix (ECM) components, growth factors, and inflammatory mediators [Olczyk et al., 2014]. Additionally, the cells continue to develop the dermal layer by enhancing vascularization and initiating epithelialization [Pastar et al., 2014]. Fibroblasts possess the ability to adhere in vivo to the ECM and migrate toward the damaged area through interactions with local proteins such as collagen, fibronectin, and vitronectin [Clark et al., 2003; Somanath et al., 2007]. These interactions result in the formation of a three-dimensional (3D) environment which allows cell-cell and cell-ECM interactions and generates mechanical forces and chemical signals that control cell movement and tissue structure [Rozario and DeSimone, 2010; Knight and Przyborsky, 2014]. Collagen1 is the most abundant ECM protein with the ability to induce cell migration in healthy developed tissues [Wolf et al., 2009]. It also shares a similar role with fibrin during the migration of fibroblasts towards coagulated fibrin, which forms blood clots in injured tissues to initiate the formation of connective tissue [Brown et al., 1993; Laurens et al., 2006]. Fibronectin and vitronectin glycoproteins have also been reported to assist in the attachment of cells to ECM components via the RGD (arginyl-glycyl-aspartic acid) sequence and to affect cell movement in various conditions [Clark et al., 2003; Zhou et al., 2003; Madsen et al., 2007].

There exist two major tissue-engineering approaches based on cell delivery: (a) direct placement of cells on the wounded tissue and (b) in vitro cultivation of cells on a biomaterial scaffold or allograft prior to their in vivo administration [Khademhosseini et al., 2006]. However, the current use of two-dimensional (2D) or pseudo 3D scaffolds for the culture of cell monolayers is an inadequate platform to expand cells in 3 dimensions prior to their implantation. The 2D cultivation conditions of the deposited cells induce the development of cell monolayers with modified gene expression and regulation profiles which are far from conveying the real properties of the cells in native tissue [Knight and Przyborsky, 2014]. Scaffold-based techniques are facing challenges because they limit the initial interactions with the native surrounding tissue. The exposure to local signaling mediators (e.g. cytokines and chemokines) and the interactions with resident adjacent cells are restricted. Other issues involve triggered inflammatory responses, incompatible scaffold components, and a limited ability to obtain a uniform cell distribution within the scaffold [Discher et al., 2009; Jakab et al., 2010]. These difficulties led to the development of a scaffold-free approach in which individual cells organize themselves (self-assemble) into multicellular sub-units called spheroids. These cell aggregates are considered to be more attractive than individual cells as building block candidates for tissue construction. They form their own ECM components and have a unique 3D architecture that more closely resembles the structural complexity and physiological environment of real tissue. Spheroids increase the interactions with ECM components and better replicate the mechanical and biochemical signals present in vivo [Bajaj et al., 2014; Dissanyaka et al., 2014]. Moreover, the spontaneous generation of spheroids demonstrates an inherent tissue fusion process in which 2 or more distinct cell populations make contact and coalesce. The scaffold-free approach is currently used in tissue regeneration, biofabrication, and bioprinting techniques [Sun et al., 2005; Mironov et al., 2009; Gattazzo et al., 2014; Murphy and Atala, 2014].

Our goal in this study is to determine whether the native ECM fibrous protein collagen1 has an impact on the directed migration and rearrangement of Matrigel™-ECM (M-ECM)- and tissue culture polystyrene (TCPS)-adherent fibroblast monolayers and their complex aggregates (spheroids) to form 3D macroconstructs. Specifically, we are interested in determining whether the developed constructs: (a) can expand in all 3 dimensions (x, y, and z), (b) structurally resemble the tissue-like architecture characterized by highly dense cell clusters, and (c) support ECM turnover and cell proliferation.

To test our hypothesis, cells were adhered to TCPS and M-ECM surfaces and then covered with ECM-guiding fibrous proteins such as collagen1 alone or collagen1 combined with proteins that have similar characteristics such as fibronectin, vitronectin, and fibrin. The coverage effect of the proteins embedded in hydrogel was tested on monolayered gingival fibroblasts and their aggregated form. Ten-percent M-ECM, which contains a collection of ECM components secreted from Engelbreth-Holm-Swarm mouse sarcoma cells, was selected for this purpose as a positive control cover [Greiling and Clark, 1997; Achilli et al., 2012], and the standard culture medium was used as a negative control. The 3D structural rearrangements of the adherent fibroblasts and their derived spheroids were followed and analyzed using a stereomicroscope, inverted epifluorescence, and laser scanning

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DOI: 10.1159/000446821
confocal microscopes to view the vertical and horizontal development of the structures. Cell density was calculated by interpretation of the z-stack images via a custom algorithm to analyze the cell density distribution in the developed macroconstructs. The proliferation potential of the fibroblast spheroid macroconstructs was examined by immunocytochemistry staining of the pluripotency SRY (sex-determining region Y)-box2 (Sox2) protein and RT-PCR analysis of the genes which play a role in the support and maintenance of the cell-native ECM and its self-renewal.

Materials and Methods

Cell Culture and Spheroid Formation

Mouse gingival fibroblasts (ESK-1) were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% Pen/Strep at 37°C and 5% CO₂. When the cells reached about 80% confluence, the cultures were split at 1:50 ratios. 3D cell cultures were prepared by coating a 48-well plate (Greiner) with 100 μl M-ECM (Corning) and incubating for 30 min at 37°C. The cells were washed twice with Dulbecco’s phosphate-buffered saline (DPBS; Invitrogen) and incubated with Dispase (Corning) diluted 1:400 with DPBS. The suspension was incubated at 37°C and 5% CO₂ for 2–3 h. The adherent cells were covered with 10% M-ECM within the hydrogel and incubated for 14 days [Lee et al., 2007]. The culture medium was replaced twice a week with a fresh medium. The ESK-1 cells were obtained as a generous gift from Dr. John R. Klein (University of Texas Health Science Center) [Jones et al., 2010].

Spheroid Dissociation

At 14 days, mature fibroblast-spheroid cultures were washed twice with Dulbecco’s phosphate-buffered saline (DPBS; Invitrogen) and incubated with Dispase (Corning) diluted 1:400 with DPBS. The suspension was incubated at 37°C and 5% CO₂ for 2–3 h and centrifuged at 340 g. Embedded and separated spheroids were treated with trypsin-EDTA (Invitrogen) and direct cell counts were taken using a hemocytometer. Fibroblast monolayers and dissociated spheroids were seeded on uncoated and M-ECM-coated TCPS surfaces and adhered to the surface for 2–3 h at 37°C and 5% CO₂. The cells were covered with 200 μl of 10% M-ECM or the tested fibrous protein-based hydrogel and incubated for 14 days. The culture medium was replaced with a fresh medium twice a week.

Collagen1, Fibronectin, and Vitronectin Hydrogel Covers

3D collagen1 gels were prepared according to the manufacturer’s instructions as follows: 30 μl of 1 M NaOH were added to 200 μl of 4 mg/ml rat tail collagen type 1 (Advanced Biomatrix). Fresh DMEM (800 μl) was added to the collagen1 gel. Vitronectin or fibronectin was added to the collagen1 gel at a final concentration of 100 μg/ml, correspondingly. Fibrin gels were formed by mixing 150 μl fibrinogen with 3 μl of 12 U/ml thrombin and then added to 0.75 ml medium, which was subdivided into 3 wells.

Cell Staining

Gingival fibroblasts and their derived spheroid 3D cultures were washed twice with DPBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences). After permeabilization with 0.1% Triton X-100 for 5 min, cell nuclei and F-actin were stained with 0.1 μm phalloidin and 1 μg/ml Hoechst for 30 min (Molecular Probes), respectively. For immunocytochemistry, the cells were washed twice with 0.1% bovine serum albumin (Invitrogen) and blocked for 45 min with 10% normal donkey serum, 0.3% Triton X-100, and 1% bovine serum albumin. Then, the cells were incubated with a monoclonal anti-mouse Sox2 antibody (MAB2018; R&D Systems) diluted 1:50 for 18 h at 4°C (R&D Systems). Next, the cells were washed twice and incubated with donkey anti-mouse IgG NorthernLights™ NL557-conjugated antibody (NL009; R&D Systems) diluted 1:100 for 1 h at room temperature, followed by subsequent washing steps and incubation with 14.3 mM DAPI for 5 min. Finally, the cells were washed twice and kept in DPBS at 4°C before being analyzed.

Microscopy

The intact 3D structures of the multicellular layers obtained from culturing individual cells and their resulting spheroids were analyzed under a stereomicroscope (Leica) with 2 light sources at a constant magnification. The diameter of the structures was measured using Image-Pro software (Media Cybernetics). Immunostained cells were analyzed using an inverted Axio Vert.A1 fluorescence microscope (Carl Zeiss). DAPI and RFP channels were used to detect the nuclei and F-actin or Sox2, respectively. The images obtained in each channel were combined into a single image representing both fluorescence channels. z-stacks of the 3D constructs, covered by the gelatinous protein, were collected using laser scanning confocal microscopy (Leica; 3.5-μm intervals and 2 fluorescence channels). 3D structures were created from stacking images taken with the laser scanning confocal microscope, and analyzed using ImageJ software (V.1.48; NIH). Full-thickness measurements of the intact fibroblasts and fused spheroid structures were performed by stitching a 4 × 4 grid of 20 sliced images along the z-axis (motorized inverted Eclipse Ti-E epifluorescence microscope; Nikon). The thickness was measured along the x-z and y-z planes (NIS-Elements software; Nikon). The heights of the multicellular constructs were measured by recording the number of gradients needed to move from the plane of focus at the lowest edge of the construct to its peak [Sevilla et al., 2010].

Digital Image Analysis of 3D Spheroids

Cell density was estimated based on analysis of the z-stacks of fluorescent images. The images were collected in a DAPI channel using a laser confocal scanning microscope and analyzed using a custom-written computer code. The following assumptions and steps were made and taken to write the code: (a) raw images were converted into a 3D numerical array (NumPy array); (b) a 100-micron Gaussian blur was applied to increase the intensity of the cells, followed by use of a multidimensional image processing kit (‘ndimage’) to produce a smooth 3D distribution; (c) the scattering was averaged along the area at which the cells interacted with the surface; (d) the resulting 2D scattering was then rescaled to obtain lowest and highest values denoted by 0 and 1, respectively; (e) using the plotting algorithm ‘Matplotlib’, the data was plotted and color coded in the form of a heat map, and (f) regions with a higher value close to 1 implied a higher concentration of fluorophores within the range of a 100-μm radius [Kitchin, 2008; McKinney, 2012; Rey-Villamizar et al., 2014]. The following link includes 2 repository files that contain the custom algorithm: https://github.com/aefitimia/3D-Cell-Counting.
RT-PCR Analysis

Total RNA was extracted from spheroids embedded in 3D cultures using an RNeasy Fibrous Tissue Mini Kit (Qiagen) and quantified using a Nanodrop ND-100 spectrophotometer (NanoDrop Technologies). cDNA was generated using a SuperScript III cDNA First-Strand Synthesis Kit (Invitrogen) according to the manufacturer’s instructions, and the yield was quantified by optical density measurements. PCR analysis were carried out via a standard technique using Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer’s instructions, and the yield was quantified by optical density measurements. PCR analysis were carried out via a standard technique using Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer’s instructions, and the yield was quantified by optical density measurements. Growth and self-renewal mechanisms. The sequences are as follows: mouse Col1α1 (forward: 5′gccagagacatcctgaa3′; reverse: 5′tgtcaacggtcgggaataca3′), mouse Col1α2 (forward: 5′ggagtgggaggaagaggtaa3′), mouse Sox2 (forward: 5′tgcaagaactctcctccattc3′; reverse: 5′ggagtgggaggaagaggtaa3′), mouse fibronectin (forward: 5′cagctgtgtcttgagaagatta3′; reverse: 5′cctccaggtggttccaa3′), and mouse Nanog (forward: 5′tgtaactctgccaaat3′; reverse: 5′ttccagtggttttccaa3′). The integrity and equal loading of cDNA reactions were checked by quantification of GAPDH. The PCR analysis was repeated at least 3 times for each RNA sample.

Statistical Analysis

3D structure diameter and height measurements were expressed as mean values of at least 3 separate experiments performed in triplicate. The SD was typically identified and added. Statistical comparisons were performed using a 1-way analysis of variance (ANOVA) followed by a 2-tailed Student’s t test for unpaired samples. \( p < 0.05 \) was considered statistically significant. The statistical analysis of the construct thickness and boxplots was computed in R (version 3.2.1).

Results

Collagen1 Hydrogel Cover Triggers 3D Rearrangement of TCPS-Adherent Fibroblasts

Gingival fibroblasts attached to TCPS did not develop 3D structures when covered with 10% M-ECM and continued growing to form cell monolayers (fig. 1A, 2A, C) as if they were growing in a standard medium (data not shown). However, when the cells were covered with collagen1 hydrogel, 3D-like spherical structures developed after 14 days. The constructs were filled with fibroblasts (fig. 1A, 2B, D). The addition of fibronectin or vitronectin proteins to collagen1 hydrogel did not change the shape or the average diameter (2.03 ± 0.19 and 2.7 ± 0.45 mm, respectively) of the spherical constructs compared to the addition of collagen1 alone (2.3 ± 0.36 mm; fig. 2E). Also, the thickness of the constructs was unaffected by the addition of fibronectin or vitronectin (433.33 ± 57.7 and 490.25 ± 65.9 μm, respectively) and it was comparable to the thickness achieved with the collagen1 treatment (448.15 ± 62.7 μm; fig. 2F).

Addition of Fibrin Augments the Clustering of Fibroblasts Adhered to M-ECM

Gingival fibroblasts that adhered to M-ECM surfaces developed distinctive 3D structures when covered with 10% M-ECM or collagen1 hydrogel. The latter caused cells to rearrange into branched cell aggregates (spheroids) with diverse orientations (fig. 1B, 3A, B). On the contrary, collagen1-covered cells developed a continuous ring-shaped structure with thin walls (fig. 1B, 3C, D). The diameter and thickness of the constructs in the collagen1 system (3.07 ± 0.15 mm and 147.03 ± 43.2 μm, respectively) did not significantly change in fibronectin- (3.22 ± 0.11 mm and 140.12 ± 44.1 μm, respectively) and/or vitronectin-supplemented systems (3.37 ± 0.21 mm and 150.78 ± 44.9 μm, respectively; fig. 3G, H). Although the average diameter of the ring-shaped constructs seemed to be larger than that of the spherical ones obtained in the TCPS-adherent cells covered with collagen1, the thickness of these structures showed the opposite results (fig. 1A, B).

Since the collagen1 hydrogel cover failed to reorganize M-ECM-adherent fibroblasts into spherically shaped constructs, an additional component was required to augment the clustering of the fibroblasts. In the presence of a collagen1/fibrin mixed gel, however, the fibroblasts clustered to create a thick-wall ring-shaped structure. Although the diameter of this construct was smaller than that of the one obtained when covering with collagen1 alone, the cells almost completely filled the entire open central space of the ring-shaped structure (fig. 1B, 3E, F). The resultant 3D structure resembled more closely the one that was obtained from TCPS-adherent fibroblasts covered with collagen1 with a similar diameter (2.5 ± 0.48 mm; fig. 3G). Collagen1/fibrin treatment nearly doubled the thickness of the construct (288.33 ± 63.3 μm; \( p = 0.044 \)) in comparison to the structures formed by treatment with collagen1 alone (fig. 3H). The development of small-sized 3D structures by monolayered fibroblasts led us to the assumption that larger cell aggregates will elaborate larger tissue-like macrostructures under the same treatment.

Collagen1 Coverage Induces and Directs the Self-Assembly of M-ECM- and TCPS-Adherent Spheroids

Fusion of fibroblast spheroids occurred to a greater extent under collagen1 hydrogel (fig. 1C, 4A, B, G, H) and...
10% M-ECM (fig. 1C, 4C, D, I, J) coverage compared to the standard culture medium control, where the fusion process was barely detected (fig. 4E, F, K, L). The best assembly was developed under collagen1, where the spheroid shape was completely integrated to form a large-scale dense 3D macroconstruct (fig. 4B, H). Different levels of integration were noticed between spheroids covered with 10% M-ECM versus collagen1 hydrogel. Early phases of TCPS- and M-ECM-adherent spheroid fusion were detected in 10% M-ECM (fig. 4D, J). In both cases, the greatest thickness of the integrated macroconstructs was detected when the spheroids were covered with collagen1 (mean values: 763.6 ± 300.4 and 438.7 ± 147.9 μm, respectively; p = 0.015 and p < 0.001, correspondingly). Fused spheroids with lower values were revealed when the integration was induced by 10% M-ECM coverage (215.3 ± 117.9 and 298.5 ± 94.7 μm, respectively; p = 0.042 and p = 0.015, correspondingly; fig. 4M, N). The increase in the variance of thickness for both integrated macroconstructs (covered with collagen1) corresponded with the lack of a uniform thickness along the z-plane. That was particularly evident for M-ECM-adherent spheroids. M-ECM-adherent cell monolayers and spheroids covered with collagen1 yielded drastically different results. The spheroids assembled into...
structures with a thickness approximately 2 times greater than that of the monolayered cells. The thickness of the macroconstructs was even greater than that obtained via rearrangement of the monolayered cells covered with the mixed collagen1/fibrin hydrogel. However, similar thicknesses were noticed between constructs and spherical structures developed by TCPS-adherent spheroids and monolayered fibroblasts, respectively.

The Spheroid Fusion Process Is Followed by Cell Rearrangement into Highly Dense Clusters

Individual spheroids were highly dense in the center (fig. 5A); however, the cell density decreased as we moved away and reached the peripheral area (fig. 5B; the dotted arrows point in the direction of the density gradient). Fusion of M-ECM- and TCPS-adherent spheroids (fig. 5C, E, G, I) was followed by early stages of cellular rearrangement. It involved the formation of tightly connected 3D cell clusters with minimum intercellular spaces. These rearrangements occurred in a specific area located on the opposite side of the spheroid fusion (attachment) site (fig. 5C, E, G, I, indicated by the dotted arrows). This feature was not observed in individual spheroids cultured in the standard media (data not shown).

The cellular rearrangement area was demonstrated by z-stack confocal microscopy images that were analyzed...
using a custom algorithm. The rearrangement was clearly visualized by the appearance of red-stained patches (fig. 5C, E, H, J) in the analyzed integrated structures (fig. 5C, E, G, I, indicated by the bold arrows). Although the reorganization was observed in spheroids covered with both collagen1 and 10% M-ECM, it was more extensive with collagen1 treatment.

The cell density gradient in the fused structures was evaluated using heat maps obtained from software analysis, particularly in spheroids adhered to M-ECM. The constructs included a high-intensity area (evidenced by a red color; score 5.0–8.0) that changed gradually into a low-intensity area (indicated by a blue color; score 1.0–1.6) as we moved away from the cellular rearrangement location towards the spheroid fusion zone (fig. 5D, F, H, J).

The next step, after investigating the spheroid fusion and cell rearrangement process, was to determine whether the fibroblast clusters were capable of maintaining their ECM regeneration and pluripotency capacity.

The Fusion Process Is Functionally Characterized by a Continuous Expression of the Genes Involved in ECM Turnover and Cell Replication

The consistency in the expression patterns of genes associated with ECM turnover along with cell proliferation and self-renewal mechanisms was evaluated by comparing their expression profiles in the following 3 different physiological conditions of spheroids: (a) mature spheroids developed in M-ECM 3D cultures, (b) culture-dissociated spheroids, and (c) recultured spheroids on M-ECM-coat-
Fig. 4. Collagen1 directs the self-assembly and fusion of TCPS- and M-ECM-adherent spheroids into integrated constructs with an increased thickness. Top-view photographs (A, C, E, G, I, K; low magnification) and fluorescent microscopy images (B, D, F, H, J, L; high magnification) of fused constructs formed by M-ECM- (A–F) and TCPS- (G–L) attached spheroids covered with collagen1 hydrogel (A, B, G, H), 10% M-ECM (C, D, I, J), and media (E, F, K, L). Red: F-actin; blue: nuclei. The thickness of the integrated constructs formed by M-ECM- (M) and TCPS- (N) adherent spheroids covered with collagen1 and 10% M-ECM covering hydrogels vs. non-fused spheroids incubated in the media.
ed plates covered with 10% M-ECM for 14 days. The expression levels of the main structural ECM proteins such as collagen1 (α1 and α2) and fibronectin, as well as pluripotency cell markers such as Sox2 and Nanog, were found to be consistent in all of the physiological conditions. The expression level of the ECM turnover metalloproteinase (MMP2) decreased drastically in the dissociated spheroids. However, it was upregulated again in the fused macroconstructs (fig. 6A).

In conjunction with Sox2 gene expression, we followed the protein expression of Sox2 in M-ECM- and TCPS-adherent spheroid macroconstructs covered with collagen1 (fig. 6B, C) and 10% M-ECM (fig. 6D, E) hydrogels. The results were compared to those in spheroids grown in standard culture media (fig. 6F, G). The fusion process did not hinder the ability of the fibroblasts to express the pluripotency marker. However, the majority of the fibroblasts in the nonfused spheroids cultured in the control media stopped expressing the protein.

**Discussion**

Guided arrangement of endothelial cells and mesenchymal stem cells into a 3D tissue-like architecture has been explored in a considerable number of studies [Sun et al., 2005; Ghibaudo et al., 2009; Gjorevski et al., 2014]. The purpose of these studies has been to closely mimic the spatial distribution of gingival fibroblasts and their physiological and functional properties found in the native underlying connective tissue. A closer spatial 3D resemblance to native tissue will increase their chances of adapting to the surrounding tissue after implantation [Achilli et al., 2012; Bajaj et al., 2014; Dissanayaka et al.,...
In this study, we found that collagen1 is the major protein that enables cell rearrangement of TCPS-adherent fibroblast into complete, cell-loaded, spherical structures. However, the same effect was difficult to achieve in M-ECM-adherent fibroblasts. The cells were rearranged into thin-wall ring-shaped structures. The addition of fibrin [Wolf et al., 2009; Feng et al., 2013] was required to fill the inner opened space of the ring and increase its thickness. Supplementation of collagen1 hydrogels with fibronectin and vitronectin had no effect on ring texture or thickness. The responsiveness of the TCPS-adherent cells to collagen1 without the need for fibrin may reflect the nature of cell-surface versus cell-protein interactions. The strong interactions between proteins present in M-ECM and fibroblasts influenced their attachment and migration capabilities, which resulted in the creation of smaller 3D constructs. In contrast, cells on the TCPS surface created larger structures because of the weak interaction between cells and the surface [Gumbiner, 1996; Zeiger et al., 2013]. Nevertheless, the addition of fibrin to the collagen1 covering gels allowed constructs to expand vertically while decreasing their diameter.

Fig. 6. Fused spheroids retain their regenerative capacity. RT-PCR gene expression profile of GAPDH, col1a1, col1a2, fibronectin (Fibro), MMP2, Sox2, and Nanog encoding genes in gingival fibroblasts of mature, dissociated, and fused (recultured) spheroids (A).

Immunostaining analysis of Sox 2 protein expression in M-ECM- (B, D, F) and TCPS- (C, E, G) adherent spheroids covered with collagen1 hydrogel (B, C), 10% M-ECM (D, E), and the standard control media (F, G). Orange: Sox2; blue: nuclei.

Matrigel™ is known to contain a repertoire of secreted ECM components other than collagen1, such as laminin and collagen4 [Kleinman and Martin, 2005]. However, 10% M-ECM coverage failed to induce TCPS-adherent monolayered fibroblasts to develop into 3D structures. In contrast, 10% M-ECM coverage directed M-ECM-adherent monolayered cells to form elongated branch-like structures without demonstrating organized and oriented growth. This finding accentuates the important role of collagen1 not only in guiding cell movement but also in reorganizing them into a consistently arranged multicellular layer. This property represents an essential early phase in the regeneration of a new tissue [Bajaj et al., 2014].
The limited size of the investigated structures encouraged us to look for spheroid-based units, which are structurally more complex and capable of fusing and forming larger 3D macroconstructs.

Overall, our study indicated that collagen1 and fibrin were the most efficient proteins in directing the fusion and self-assembly of M-ECM- and TCPS-adherent spheroids in hydrogels. The integrated constructs obtained by the ECM-adherent spheroids increased their size and thickness in comparison to the structures developed by monolayered fibroblasts. Although mature spheroids were developed and adapted to M-ECM cultures, the effect of the 10% M-ECM covering gel on their assembly and fusion was less pronounced than the effect of a collagen1 hydrogel cover. Fused spheroids triggered cell rearrangement into a tissue-like texture by clustering cells into stacks with tight intercellular connections. This rearrangement appeared in distinct areas of the integrated structures, opposite the fusion (attachment) site. Some studies have referred to this process as a stage of tissue maturation and a remodeling process [Hajdu et al., 2010]. The same process was not detected in the separated spheroids (grown in the control media), and it seemed to develop gradually as we moved away from the fusion site. Our results suggest that spheroid fusion may be an important process for triggering cell reorganization and tissue remodeling. The latter process may depend on the gradient concentration of certain signaling mediators possibly released by the fused spheroids. Cell clustering is a very important property in rigid scaffolds with a poor ability to accommodate tissue remodeling [Jakab et al., 2010]. The integrated constructs of both M-ECM- and TCPS-adherent spheroids continued to retain their pluripotency and proliferation potentials by expressing the marker Sox2 [Fong et al., 2008; Arnold et al., 2011], which makes them good candidates as building blocks for tissue regeneration. Interestingly, the majority of the fibroblasts, in the separated spheroids that were not fused, did not express the self-renewal marker. The reasons for this could be related to the dynamics of the actual fusion process or the chemical and mechanical changes involved in going from the aqueous to the gel milieu. Future research will further clarify these points. The integrated spheroids continued to express consistently major genes involved in degradation, replacement, and regeneration of the cells’ ECM as well as cell pluripotency and self-renewal mechanisms. The inconsistent expression of MMP2 turnover protein in the dissociated spheroids may indicate the initial process of differentiation, since the expression of MMP2 is related to cell proliferation [Henderson et al., 2007]. However, the expression in the fused spheroids strengthened their capabilities to proliferate and self-renew.

Conclusion

In this study we investigated the effect of covering polystyrene- and M-ECM-adherent fibroblast monolayers and spheroids with ECM fibrous protein (mainly collagen1 and fibrin) hydrogels to induce cell rearrangement into large 3D tissue-like multicellular constructs. The results indicated that: (a) collagen1 hydrogel coverage elicits the rearrangement of cells and spheroids into fused spherically shaped constructs; (b) a combined collagen1 and fibrin hydrogel was required to achieve a similar effect on M-ECM-adherent cells; (c) fused spheroids yielded large constructs with an increased cell density and tight intercellular connections, and (d) fused spheroids retained their maintenance and propagation. These findings suggest that the use of gingival fibroblast spheroids as basic building blocks to form larger functional integrated macroconstructs is a more attractive alternative than the use of monolayered cells given their poor forming capabilities. Further in vivo testing of spheroid-based treatments to regenerate tissues and heal open wounds should be carried out in order to evaluate the efficiency and viability of this technique for optimization of tissue regeneration.

Acknowledgements

This study was supported by the American Dental Association Foundation (ADAF). We would like to thank Dr. John R. Klein for the gingival fibroblasts strain ESK-1 and Dr. D. Skrtic for contributing to this paper.

Disclosure Statement

The authors indicate no potential conflicts of interest.

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