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Spatial development of gingival fibroblasts and dental pulp cells: Effect of extracellular matrix

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ABSTRACT

Cells sensing changes in their microenvironmental stiffness and composition alter their responses, accordingly. This study determines whether gingival fibroblasts (GFs) and dental pulp mesenchymal stem cells (DPMSCs) support the formation of continuous layers in vitro by mimicking the stiffness and protein composition of their native extracellular matrix (ECM). Immortalized cells were incubated with (i) 0–100% Matrigel-ECM (M-ECM) for 7-28d, and with (ii) collagen and fibrin matrices for 14d. Cultures were analyzed by phase-contrast, fluorescence and confocal microscopies. The diameters and surface areas were measured via ImageJ. Self-renewal markers were detected by RT-PCR and immunocytochemistry assays. GFs and DPMSCs developed spheroids interconnected by elongated cell bundles or layers, respectively, expressing the self-renewal markers. Increased matrix stiffness resulted in spheroids replacement by the interconnecting cells/layers. Both cells required 100% M-ECM to reduce their spheroid diameter. However, it reduced the surface area of the interconnecting layers. Those differences led to extended, spindle-shaped GFs vs. compact, ring-shaped DPMSCs constructs. Collagen and fibrin matrices developed continuous layers of tightly connected cells vs. distinctive scattered cell aggregates, respectively. The ability of GFs and DPMSCs to create tissue-like multicellular layers at various matrix conditions may be imprinted by cells' adaptation to mechanical forces and composition in vivo.

1. Introduction

Tissues, composed of cells and extracellular matrix (ECM), possess elastic and viscous properties (Bronshtein et al., 2013; Xing et al., 2015). ECM provides chemical and mechanical stimuli to influence cell behavior (Gattazzo et al., 2014). Most cells adhere to ECM proteins and fibers via concentrated and clustered trans-membrane adhesion receptors, integrins (Alberts et al., 2002; Lodish et al., 2000; Dike and Farmer, 1988). On a stiffer substrate/matrix, cells enhance the formation of organized cytoskeleton and make adhesion points more contractile (Discher et al., 2005). Compared to a culture surface such as polystyrene, cells cultivated in a traditional 2D milieu are in a highly non-physiological mechanical environment (Baker and Chen, 2012; Griffith and Swartz, 2006). In contrast, ECM-based gels, such as fibrin, collagen and Matrigel, provide a required physiological stiffness. Comparative studies in 2D and 3D matrices accentuate the importance of matrix dimensionality (Wells, 2008) in regulating cell behavior.

Cells respond to physiological and mechanical properties of the microenvironment (Gattazzo et al., 2014; Griffith and Swartz, 2006) by altering proliferation, growth and survival, motility and migration, adhesion and contractility, cell-cell and cell-matrix interactions as well as tissue remodeling (Edmondson et al., 2014). Stiffness, i.e., the ability to resist deformation is measured by the solid elastic modulus (E) (McKee et al., 2011). ECM-associated cells sense stiffness of their microenvironment and consequently change their morphology and migration behavior (Cavo et al., 2016). Fibroblasts change their spreading behavior and motility when plated on substrates with different E values (Solon et al., 2007). Mechanical and physical features of the extracellular environment dramatically affect cell shape and cellular regulatory mechanisms (Rhee et al., 2007). Contrary to cells attached to the rigid surface, in 3D environments, cells extend to engage integrins or entan-

Abbreviations: ADAF, American Dental Association Foundation; ANOVA, analysis of variance; ECM, extracellular matrix; DAPI, 4′,6-diamidino-2-phenylindole; cDNA, complementary DNA; DPBS, Dulbecco's phosphate-buffered saline; DPMSC, dental pulp mesenchymal stem cell; GF, gingival fibroblasts; M-ECM, matrigel-ECM; LSCM, laser-scanning confocal microscope; Oct 4, octamer-binding transcription factor 4; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; Sox2, SRY (sex determining region Y)-box 2.

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gle with matrix fibrils yielding integrin-independent mechanical interactions (Rhee et al., 2007; Jiang and Grinnell, 2005). A balance between the adhesion and traction forces governs cell migration on 2D surfaces. However, matrix stiffness is a key factor that influences cell movement in 3D. When matrix ligands and cell integrin receptors are held constant, maximal cell movement shifts to less stiff matrices (Zaman et al., 2006).

Integrins, the heterodimeric transmembrane glycoprotein receptors for the ECM ligands are the primary sensors for mechanical forces caused by adhesion. They regulate downstream signaling and transduce the forces in response to matrix stiffness by binding ECM proteins on the exterior side of the cells and the cytoskeleton on the interior side (Schwartz, 2010). The most commonly used in vitro 3D matrix models are the basement membrane extract (Matrigel), collagen and fibrin gels. Matrigel is used as a reconstituted basement membrane gel containing polymerized extract from Engelbroth-Holm-Swarm mouse sarcoma and it is mostly composed of collagen IV, laminin, entactin, and proteoglycans. Matrigel binds with a variety of integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_{10}\beta_1$. Type I Collagen, considered a major component of the connective tissue, exists in vivo as fibers and it is primarily connected to the integrin receptor $\alpha_2\beta_1$. Fibrin, formed by thrombin cleavage of fibrinogen, is typically used as a matrix in wound healing. Fibrin is considered a self-polymerized matrix protein similarly to collagen. However, its fibers are thinner, shorter and straighter than collagen fibers. Compared to collagen1, fibrin adheres to different integrins ($\alpha_5\beta_1$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$) (Hakkinen et al., 2011).

The tension state of cell-matrix interactions is generally recognized as an important factor that determines the morphology of fibroblasts and provides a mechanism for their interconnectivity in 3D architectural arrangements. Formation of the extensions with a microtubule core and actin rich tips associated with the initial cell spreading and the polarization process at later stages have been reported (Rhee et al., 2007). On the other hand, the branching morphology of mesenchymal stem cells (MSCs), different from the spherical morphology of the derived murine cells, is linked to the culturing in soft vs. stiffer gels (MacQueen et al., 2013).

Dental pulp is a soft connective tissue, including large and various numbers of cells with major functions in dentin production and maintenance of its health. The pulp tissue is very vulnerable to external insults such as mechanical trauma, chemical irritation and/or microbial invasion. Engineering and regenerating pulp is considered a difficult task for bioengineers/dental researchers (Huang, 2009). On the other hand, gingival connective tissue, the *lamina propria*, is generally much stiffer and dense than dental pulp and it is composed of thick protein fiber bundles and cells (mostly fibroblasts). The predominantly collagenous fibers enhance tissue rigidity to withstand the mastication forces and protect against external threats such as chemicals and microbial challenges (Chen et al., 2015). Biofabrication of new oral or dental tissue-like layers by cell implantation requires deep understanding of not only the surrounding extracellular matrix composition but also of the mechanical interplay between the cells and the matrix. In this study, we assess the in vitro ability of gingival fibroblasts (GFs) and dental pulp mesenchymal stem cells (DPMSCs) to develop the continuous multicellular layers by culturing the cells in (i) ECM-Matrigel (M-ECM; makes the culture stiffer and more viscous to the cells) and in (ii) type I collagen and fibrin. The specific objective was to determine the effect of ECM environmental parameters (stiffness and composition) on the spatial behavior of cells from different connective tissues. Findings that these cells indeed sense, identify and respond to ECM composition and mechanical challenges, i.e., preserve functions typically seen under similar conditions in vivo, may have a significant impact on developing a new platform for the regeneration and biofabrication of gingival and dental pulp connective tissues involving local cell implantation.

2. Materials and methods

2.1. Cell culturing

Immortalized mouse GFs and dental papilla mesenchymal stem cells (DPMSCs) were cultured in DMEM and α-MEM media (Invitrogen, San Diego, CA), respectively. Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/mL of penicillin and 100 μg/mL of streptomycin. At approx. 80% confluence, GF and DPMSC cultures were split at 1:100 and 1:50 ratios, respectively. Since DPMSCs contain the G418-resistance gene they were incubated with 600 μg/mL of G418 (Sigma-Aldrich, St. Louis, MO). All incubations were performed in a 5% CO_2 humidified incubator at 37 °C.

2.2. 3D culturing

3D cell cultures were prepared by coating 48-well plate (Greiner bio-one, Monroe, NC) surface with 100 μL of Matrigel™-ECM (Corning, Tewksbury, MA) and incubating for 30 min at 37 °C. Cells were seeded on the solidified M-ECM (1.7×104/well) and incubated for 2 h. The adherent cells were split into three groups and received different treatments; group1 was covered with 100 μL of medium only (coating culture), group 2 was covered with medium containing 10% M-ECM (on-top culture) and group 3 was covered with 100% M-ECM (embedded culture). Cells were also seeded on uncoated plate and covered with similar volume of medium (2D control culture). All the cultures were finally added with 100 μL of media after 30 min of incubation (Fig. 1A, B) (Kaufman et al., 2016). The culture medium was replenished twice weekly. The cultures representing each cell type were incubated for 7d, 14d and 21 d. The on-top cultures were also incubated for 28 days.

3D collagen gels were prepared according to the manufacturer instructions. 30 μL of 1 M NaOH was added to 200 μL of 4 mg/mL of rat-tail type I collagen (Advanced Biomatrix, Carlsbad, CA), mixed and then combined with 800 μL of fresh medium. Fibrin gels were produced by mixing 150 μL of fibrinogen with 3 μL of thrombin (12 U/mL; Sigma Aldrich) and then combining the mixture with 0.75 mL media. The fibrinogen/thrombin/media mixture was subdivided into three wells.

2.3. Cell staining

3D cell cultures were washed twice with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). After 5 min permeabilization with 0.1% Triton X-100 (Alfa Aesar, Ward Hill, MA), nuclei and F-actin were stained with 0.1 μM phalloidin and 1 μg/mL Hoechst for 30 min (Molecular Probes, Eugene, OR), respectively.

For immunostaining, cells were first washed twice with 0.1% bovine serum albumin (BSA; Invitrogen) and then blocked for 45 min with 10% normal donkey serum (GeneTex, Irvine, CA), 0.3% Triton X-100 and 1% BSA. GFs and DPMSCs were then incubated overnight at 4 °C with 10 μg/mL of mouse anti-human/mouse Sox2 (MAB2018, R&D Systems, Minneapolis, MN). DPMSCs were also incubated with rat anti human/mouse Oct4 (MAB1759, R&D Systems) monoclonal antibodies. Following the incubation, cells were first washed twice and incubated for 1 h at 22 °C with 1:200 diluted either donkey anti-mouse Immunoglobulin G (IgG; NL009, R&D Systems) for GFs and DPSCs or goat anti-rat IgG (NL013, R&D Systems) NorthernLights™ NL557-conjugated polyclonal antibodies