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Self-Organizing Tissue-Engineered Constructs in Collagen Hydrogels

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Abstract

A novel self-organizing behavior of cellularized gels composed of collagen type 1 that may have utility for tissue engineering is described. Depending on the starting geometry of the tissue culture well, toroidal rings of cells or hollow spheroids were prompted to form autonomously when cells were seeded onto the top of gels and the gels released from attachment to the culture well 12 to 24 h after seeding. Cells within toroids assumed distinct patterns of alignment not seen in control gels in which cells had been mixed in. In control gels, cells formed complex three-dimensional arrangements and assumed relatively higher levels of heterogeneity in expression of the fibronectin splice variant ED-A—a marker of epithelial mesenchymal transformation. The tissue-like constructs resulting from this novel self-organizing behavior may have uses in wound healing and regenerative medicine, as well as building blocks for the iterative assembly of synthetic biological structures.

Keywords

collagen; tissue engineering; self-organizing; epithelial-mesenchymal transformation

Introduction

Hydrogels composed of collagen are frequently used as three-dimensional (3D) scaffolds for cultured cells (Huynh et al., 1999; Cen et al., 2008; Glowacki & Mizuno, 2008; Pang & Greisler, 2010; Pedraza et al., 2010). Among its advantages, preparation of the gel is straightforward; cells can be mixed directly in, enhancing the uniformity of encapsulation (Thevenot et al., 2008); and the clarity of the gel aids in immunolabeling and microscopy (Agarwal et al., 2001; Levitz et al., 2010). These 3D scaffolds are also amenable to experimental screens of cell migration, proliferation, death, angiogenesis, and tissue

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differentiation (Tonnesen et al., 2000; Hansen et al., 2006; Mercado-Pimentel & Runyan, 2007). In one widely used assay, contraction of cellularized collagen gels is utilized as a biomechanical index of interactions between fibroblasts and the matrix (Carver et al., 1995; Feng et al., 2003; Ngo et al., 2006; Wilson et al., 2009).

Collagen hydrogels are also of significant interest to regenerative medicine (Patino et al., 2002; Badylak et al., 2009; Hunt & Grover, 2010). Tissue-engineered constructs composed of collagen are well tolerated immunologically when implanted in the body (Ford, 1986; Macleod et al., 2005; de Castro Brás et al., 2010). Moreover, gels placed in living tissues are remodeled over time by enzymes such as matrix metallo-proteases, enhancing the potential for integration of implants with surrounding tissue. Collagen is also used clinically in neurosurgery as a replacement for the outer meningeal membrane of the brain (Zerris et al., 2007) and has shown efficacy for the treatment of burns and slow healing skin wounds (Falanga & Sabolinski, 1999) and in reparation of damaged vocal cords (Luu et al., 2007; Kimura et al., 2010).

In this communication we describe a novel self-organizing behavior of cellularized gels containing collagen type 1 that may have utility for tissue engineering. The method involves placing cells on the top of low-density collagen gels constrained by attachment to a tissue culture well, release of the gel 12 to 24 h later, and further culture of the gel. Depending on the starting geometry of the culture well, self-organizing tissue-like units, including toroids and hollow spheroids, form autonomously. Conceivably, these tissue-like structures could provide building blocks to iterate more elaborate tissue-engineered constructs, such as synthetic blood vessels.

Materials and Methods

Cell Culture

Rabbit lens epithelial cells (RLEC) N/N1003A (Reddan et al., 1986) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 8% rabbit serum (RS; Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin G (Sigma), and 100 µg/mL streptomycin (Sigma). Cells were cultured until 80–90% confluent, at which time they were trypsinized and counted. N/N1003A cells are an undifferentiated lens epithelial cell line derived from a newborn rabbit. Rat epicardial mesothelioma cells (REC) kindly provided by Dr. Hoda Eid were cultured in DMEM high glucose (SH3028501 Hyclone) supplemented with 10% FBS, 2 mM alanyl-glutamine (Sigma A8185), 100 µg/mL Primocin™ (Fisher Scientific NC9141851; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C, 5% CO₂. Every 3 days they were dissociated with trypsin-EDTA 0.05% (Fisher Scientific SN3023601) and subcultured at 2.5×10^5 cells/mL. RECs are a multipotent cell line derived from epicardial progenitors in the embryonic rat heart (Eid et al., 1992; Wada et al., 2003). Human mesenchymal stem cells (SCR108 Chemicon International, Inc., Temecula, CA, USA) derived from human bone marrow have been validated for expression of CD44, CD90, and STRO-1 and for the absence of CD14 (leukocytes), CD19 (B-lymphocytes), and CD146 (endothelium) markers. The cells were cultured at 1×10^6 cells/T75 in mesenchymal stem cell (MSC) expansion medium (SCR105 Chemicon International) at 37°C, 5% CO₂. At 80% confluence cells were dissociated with Accutase (A11105-01 Invitrogen, Carlsbad, CA,

USA) and subcultured at 2×10^6 cells/T75 in MSC expansion medium, supplemented with 100 µg/mL Primocin™ (Fisher Scientific NC9141851).

Substrate Preparation

Collagen gels were prepared in 24 well culture dishes (Nalge Nunc International, Rochester, NY, USA). A neutral collagen stock solution was prepared by combining 0.2N HEPES pH 9.0, 10× minimum essential medium (Sigma) and bovine dermal collagen type 1 (Cohesion Technology, Palo Alto, CA, USA) (1:1:8; v/v/v) and placed on ice. The ice-cold collagen mixture was then added to the wells at 1 mL per well.

Contraction Assay

Contraction assays were carried out to determine the ability of RLEC to contract collagen gels in the presence or absence of αCT 1 peptide, reverse control peptide (Hunter et al., 2005; Rhett et al., 2008, 2011), or TGF-β (R&D Systems, Minneapolis, MN, USA). Assays were carried out in 24-well plates. Contraction gels consisted of a 1:1 mixture of collagen stock solution and RLEC resuspended to a final concentration of 1.0×10^5 cells/mL, or an equal volume of media. Cells were either mixed in the collagen mixture, or plated directly on top of polymerized gels. Prior to plating, cells were treated with the αCT 1 peptide (180 µM), control peptide (150 µM), or TGF-β (30 ng/mL) and incubated for 45 min at 37°C. Gels were allowed to polymerize for 1 h at 37°C. RLEC media were added to each gel and incubated for 24–72 h. In additional experiments, gels were allowed to polymerize after which RLEC (1.0×10^6 cells/mL) were plated on top of collagen gels and incubated for 24–72 h. Gels were photographed at various time intervals and contraction was measured, using image pro-plus software, as either percent of control or average area contracted. For the different well geometries, cell types and treatments all assays were repeated in three or more independent experiments.

Immunohistochemistry

Gels were fixed in 2% paraformaldehyde for 30 min at room temperature and washed three times with PBS/0.01M glycine/0.1% Triton-X-100. Next, gels were incubated in 5% BSA/PBS to block nonspecific staining. Primary antibody staining was diluted in 1% BSA/PBS, (IST-9) ED-A splice-variant of fibronectin (FN) (Abcam, Cambridge, MA, USA), ZO-1 (Zymed Laboratories, San Francisco, CA, USA), or CX43 (Sigma) 1:100 and incubated at 37°C for 1 h. Gels were washed three times in 1% BSA/PBS. Gels were then incubated in secondary antibody (goat anti-rabbit Cy⁵ or donkey anti-mouse Texas Red) 1:100 for 1 h at 37°C. Gels were rinsed three times in PBS and incubated in Phalloidin-488 diluted 1:50 in PBS for 2 h. Finally, gels were stained with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) diluted 1:5,000 in PBS for 15 min. Gels were washed three times in PBS and mounted in DABCO. Images were captured using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Results

Generation of Toroids from Lens Epithelial Cells *In Vitro*

The lens epithelial cell line N/N1003A (LECs) can be activated to undergo an epithelial mesenchymal transformation (EMT) and differentiate into a myofibroblastic phenotype (DuRant et al., 2002). Dispersed LECs (100,000–1 million) were placed on top of a 15 mm diameter, 5 mm deep gel comprised of 1% type 1 collagen in a 24 well circular culture well. Following culture for 24 h, the gels were released from attachment to the plastic sides of the well. After a further 24 h, LECs were observed repeatedly to form into distinct, uniform rings within the gel (Fig. 1A). The formation of these ring-shaped or toroidal tissue-like structures was not observed when LECs were premixed into liquid collagen prior to gelation (Fig. 1C).

Irrespective of whether LECs were placed on top or mixed into the collagen matrix, contraction of the gel proceeded with time (Figs. 1B,C, respectively). TGF- β 1 is a cytokine that enhances EMT and myofibroblast differentiation of LECs (Lee & Joo, 1999; Saika et al., 2009). Placement of 1 ng/mL TGF- β 1 in the culture media after the initial 24 h of culture resulted in LEC toroids of consistently smaller diameter and increased contraction of the gel compared to non-TGF- β 1 treated wells (Fig. 1B). Conversely, toroids differentiated in the presence of a peptide mimetic based on the C-terminal of the gap junction protein connexin43- α CT1 (Palatinus et al., 2010; Rhett et al., 2011), tended to be larger in diameter than control toroids and displayed lower levels of gel contraction (Supplementary Fig. 1).

In previous studies we have shown that α CT1 has effects on skin and cardiac wound healing (Ghatnekar et al., 2009; O'Quinn et al., 2011; Palatinus et al., 2011), including on myofibroblast differentiation (Soder et al., 2009)

Increased gel contraction was also observed for gels containing mixed-in LECs, a response to TGF- β 1 that has been reported previously in this assay for various cells types (Potts & Runyan, 1989; Carver et al., 1995; Ikuno & Kazlauskas, 2002; Watanabe et al., 2006), though again no toroid of LECs formed in gels with cells mixed in (Fig. 1D). Cotreatment with α CT1 peptide did not inhibit the enhanced gel contraction prompted by TGF- β 1.

Cells Form Aligned Patterns within LEC Toroids

After 48 h of culture, gels containing LEC toroids or premixed LECs were fixed in 2% paraformaldehyde and labeled/immunolabeled for ED-A (splice variant of FN) phalloidin-488 and DAPI (Fig. 2). In gels containing mixed-in cells, LECs were randomly distributed within gels, showing no particular orientation with respect to each other (Fig. 2D). By contrast, gels seeded on top with LECs were found to assume distinct radial alignments within the toroid (Fig. 2A). It was also noted that LECs mixed into the collagen demonstrated much higher between-cell variability in immunolabeling patterns than cells aligned in toroids (Fig. 2D). Specifically, it was observed that a quarter to a third of LECs in mixed-in gels were unlabeled for ED-A (asterisk on Fig. 2D), whereas the remaining cells intensely labeled for this mesenchymal marker. By comparison, nearly all cells in toroids were uniformly positive for ED-A (Fig. 2B).

Generation of Spheroids from LECs *In Vitro*

Next, we sought to determine what would happen if the geometry of the culture well in which the gel was polymerized was changed. Dispersed LECs were thus either put on top of or mixed into a 5 mm deep 1% collagen gels in a two well chamber slide (Lab-Tek™) square-sided culture wells, cultured for 24 h and then released from constraint as outlined for circular gels (Figs. 3A–3J). For cells mixed in, the gel retained a flattened and roughly square shape that slowly contracted over a 48 h period following release (Figs. 3A–3E). As was the case for circular gels (Fig. 2D), mixed-in cells in square gels assumed mesenchymal phenotype and were randomly oriented with respect to each other.

Square gels on which LECs were placed on top demonstrated a remarkable morphological progression over the 48 h following release from constraint by attachment to the well sides (Figs. 3F–3J). In contrast to square gels with mixed-in LECs, the corners of these gels lifted symmetrically like petals on a flower, progressively folding the square into a spheroidal shape (Fig. 3J).

Immunolabeling with ED-A, phalloidin-488, and DAPI of square gels 2 to 6 h after release from the well sides indicated evidence of significant levels of organization of the LECs, with cells of mesenchymal phenotype showing striking alignment and extension at the corners of square gels (Figs. 3K, 3L). The distinct alignment of LECs in the corners of the square presumably reflected patterns of mechanical force being generated by the cells that subsequently contributed to the folding of the gel into a spheroid.

Spheroids appeared to be hollow and have apertures at the top, as might have been anticipated from the manner in which they had been folded up during the 48 h time course. The edges could be seen lifting off the bottom of the slide moving upward to fuse (Fig. 4A insert). We thus took the opportunity to determine whether spheroids could be filled with the soluble, fluorescent dye fluorescein isothiocyanate (FITC). Remarkably, FITC dye was not observed to leak out over a 20–30 min period from the hollow spheroid formed by the folded gel (Figs. 4A, 4B). This suggested that the LEC-generated forces that led to spheroid formation were also sufficient to maintain a seal that inhibited visible dye leakage.

Generation of Toroids from Other Cell Types *In Vitro*

In a final step, we sought determine whether cell types other than rabbit LECs responded to the simple method for generating toroids within collagen hydrogels. A few different types of cells were tested including chick, rat, and human bone marrow mesenchymal stem cells (BMSCs) and REC cells—a multipotent cell line derived from epicardial progenitors in the embryonic rat heart (Eid et al., 1992; Wada et al., 2003). Independent of which of the four cells was tested, when dispersed cells were placed on top of a 1% collagen gel, tissue-like toroidal structures were observed (Fig. 5).

Examples of toroids generated *in vitro* from REC and human BMSCs are provided in Figures 5A, 5C, 5E, and 5G. As was the case with LECs, the other cell types showed radial alignment within the toroid and demonstrated relatively uniform phenotype as assessed by immunolabeling for markers of EMT (ZO-1, snail, alpha-smooth muscle actin). Again, for cells mixed into the gel, no distinct geometries were observed to form (Figs. 5B, 5D, 5F,

5H). Mixed-in cells also showed random arrangements within the matrix and demonstrated higher levels of cell-to-cell variability in phenotyping markers than when cells were placed on top of the gel.

Discussion

A method is described using hydrogels composed of collagen type 1 that enables repeatable generation of tissue-like structures *in vitro* from cultured cells. Depending on whether the tissue culture well in which the gel was polymerized had a circular or square geometry, cells formed into ring-like toroidal or a hollow spheroidal construct, respectively. In what follows, we discuss observations that led to the development of the method, the mechanisms that may contribute to the apparent self-organizing behavior, and potential uses for these cellularized collagen constructs in tissue engineering, wound healing, and regenerative medicine.

The method outlined in this article arose serendipitously from a modification of the well-known gel contraction assay (Carver et al., 1995). LECs can be activated by mechanical stress to undergo an EMT and differentiate into myofibroblasts (Lee & Joo, 1999; Saika et al., 2009). Collagen type 1 is also a known activator of EMT (Hay & Zuk, 1995; Shintani et al., 2008; Medici & Nawshad, 2010). We hypothesized that by seeding LECs at high density on the top of collagen hydrogels, the prospect of a coordinated EMT following contact with the collagen substrate might be enhanced. Contraction of the gel was noted when LECs were seeded on top of the gel. However, the most notable aspect of the modified protocol was the autonomous formation of distinct cellular geometries within the gels.

An unanswered question is the mechanism prompting the formation of the toroidal and spheroidal geometries. One contributory factor, EMT, has been mentioned. When dispersed LECs were mixed directly into gels, cells assumed heterogeneous mesenchyme-like phenotypes at random orientations in the collagen scaffold (e.g., Fig. 2D). However, when placed on top of the gel, there is an increased opportunity for cells to settle and form intercellular junctions with each other and attach to the substrate prior to moving into the matrix.

A coordinate EMT-like process may explain why cells placed on top of the gel showed increased alignment within toroids. This may also be a factor in the increased regularity of phenotype that was observed when cells were seeded on the top of gels, as compared to mixed into the collagen matrix. Coordinated migration and differentiation of interconnected cells is a natural phenomenon that occurs subsequent to EMT *in vivo* (Baum et al., 2008), particularly during embryonic development. The method we describe *in vitro* leading to toroid formation may be mimicking certain assignments of EMT in synchronizing the cellular activities and gene expression patterns. Coordinate patterns of force generation have long been recognized as factors in self-organizing behaviors by cells such as fibroblasts (Harris et al., 1984; Stopak et al., 1985).

An additional factor contributing to the distinctive arrangements that the cells assume when placed on top of gels may be the fluidity of polymerized collagen. When fluorescent (FITC) 10 μ m microbeads were mixed in and gels kept at 37°C (i.e., tissue culture incubator

temperature), slow convectional flow of the matrix over time was observed (data not shown). These slow patterns of flow were seen independent of whether cells were added to the gel or not. Convectional movements of the collagen matrix are unlikely to contribute to increased cell alignment and phenotype uniformity. However, such en-masse movements of the matrix seem likely to vary with the shape of the well and thus may influence aspects of the organization that cells assume in gels.

Figure 6 illustrates two uses that are envisaged for toroids generated by the method described in this article. The first is to utilize toroids as “building blocks” to engineer more complex structures (Fig. 6, top). In organ printing, cells or cellular aggregates are progressively layered to iterate complex tissue-like structures (Mironov et al., 2003). Stacking of toroids in an organ printing-like process might provide a convenient method for elaborating a tubular construct. As cells self-organize into aligned orientations, toroids may have advantages over randomly aggregated cells as a “building block” for engineering contractile biological structures.

This ability to adjust diameter size of tissue-engineered blood vessels is a very basic requirement for potential clinic applications. We have shown that toroid diameter, and hence the width any potential tubular structure resulting from combining toroids, can be altered. The three variables examined thus far include alteration of the EMT efficacy (TGF- β 1), well size and shape, and assignments of the Cx43 C-terminus (Supplementary Fig. 1). Other variables to test would include the starting cells type used. Induced progenitor stem cells (iPS cells) would represent one interesting prospect for future study.

A second use for toroids is envisaged in wound healing and more broadly regenerative medicine (Fig. 6, bottom). We have already tested this idea by placing toroids composed of BMSCs into excisional wounds on rats (J.D. Potts & R.G. Gourdie, in preparation). Relative to nontreated controls and controls containing gels with BMSCs mixed in, wounds receiving BMSC toroids closed at faster rates and showed reduction in scar tissue. BMSCs have already been reported to have efficacy in preclinical models of wound healing in healthy and diabetic animal models (McFarlin et al., 2006; Badillo et al., 2007; Kwon et al., 2008). Priming stem cells with an EMT-like step *in vitro* prior to use in diseased or injured tissues represents an interesting avenue for ongoing work.

Conclusion

Body organs display complex shapes, surfaces, and internal structures including blood vessels. Coupled to morphological complexity, the component tissues of organs possess dynamic contractile and mechanically responsive elements. The recapitulation of naturally occurring complexities in shape, histoarchitecture, and biomechanical functionality in the replacement or repair of biological structure is a holy grail for tissue engineering. Harnessing self-organizing processes such as those described in this article may provide one path to reaching this goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A, B: Toroids formed from following seeding of LECs on the top of collagen gels. Control gels are shown in panel **A**, and in panel **B**, the gels were treated with 30 ng/mL TGF- β 1 over a 24 h period following release from the well sides. Note the toroids are smaller in diameter and gels are more contracted following exposure to TGF- β 1. **C, D:** Examples in which LECs were mixed in with the collagen gel as in a standard gel contraction assay. Control gels are shown in panel **A**, and in panel **C**, the gels were treated with 30 ng TGF- β 1 following release are shown in panels **B** and **D**. Scale bar = 5 mm.

Figure 2.

A: A toroid formed after LECs were seeded on top of a collagen gel. **B:** A segment of toroid is shown at higher magnification following immunolabeling for ED-A, phalloidin-488, and DAPI. Note the radial alignment of cells in the toroid. **C:** Example of a collagen gel in which the LECs were mixed in. **D:** Detail within a mixed-in collagen gel immunolabeled for ED-A, phalloidin-488, and DAPI. Note that some of LECs in the control gel are unlabeled for the mesenchymal marker ED-A (asterisk). Scale bars = **A, C** (5 mm), **C, D** (10 μ m).

Figure 3.

A–E: LECs mixed into a square collagen gel contract and retain a roughly square shape over 48 h after release from constraint. **F–J:** By contrast, when LECs are seeded onto the top, the gel folds into a distinctive spheroid over a 48 h time course. **K:** Phalloidin labeling of an “on top” square gel 6 h after release from constraint. **L:** Detail of LECs in corner of square gel (asterisk on K) immunolabeled for ED-A, phalloidin-488, and DAPI. Note the highly aligned cells in the gel corner. Scale bars = **A–J** (5 mm), **K** (10 mm), **J** (100 μ m).

Figure 4.

A: Spheroid formed in a square well. An aperture is apparent on the top of the folded gel.

Inset: Side view of spheroid that has had its corners opened up. **B:** FITC dye is retained within a folded spheroid if loaded in through the aperture. Scale bars = **A** (7.5 mm), **B** (17.5 mm).

Figure 5. Rat epicardial (REC) cells (**A–D**) and human bone marrow stem (BMS) cells (**E–H**) migrate to form toroidal rings when layered onto polymerized collagen and allowed to progress through an EMT (**A, C, E, G**), but remain relatively uniformly dispersed within the matrix when mixed into the gels before polymerization (**B, D, F, H**).

Figure 6.

Hypothetical examples of how contractile toroids could be used (A) to be assembled iteratively into the branched tubular scaffold of an artificial blood vessel or (B) to enhance wound closure.