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Fibroblast Morphogenesis on 3D Collagen Matrices: The Balance Between Cell Clustering and Cell Migration

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Abstract

Fibroblast clusters have been observed in tissues under a variety of circumstances: in fibrosis and scar, in the formation of hair follicle dermal papilla, and as part of the general process of mesenchymal condensation that takes place during development. Cell clustering has been shown to depend on features of the extracellular matrix, growth factor environment, and mechanisms to stabilize cell-cell interactions. In vitro studies have shown that increasing the potential for cell-cell adhesion relative to cell-substrate adhesion promotes cell clustering. Experimental models to study fibroblast clustering have utilized centrifugation, hanging drops, and substrata with poorly adhesive, soft and mechanically unstable properties. In this review, we summarize work on a new, highly tractable, cell clustering research model in which human fibroblasts are incubated on the surfaces of collagen matrices. Fibroblast clustering occurs under procontractile growth factor conditions (e.g., serum or the serum lipid agonist lysophosphatidic acid) but not under promigratory growth factor conditions (e.g., platelet-derived growth factor) and can be reversed by switching growth factor environments. Cell contraction plays a dual role in clustering to bring cells closer together and to stimulate cells to organize fibronectin into a fibrillar matrix. Binding of fibroblasts to a shared fibronectin fibrillar matrix stabilizes clusters, and fragmentation of the fibrillar matrix occurs when growth factor conditions are switched to promote cell dispersal.

Keywords

Cell clustering; cell aggregation; cell contraction; cell migration; fibronectin; adherens junctions; 3D-collagen matrix; tissue morphogenesis

Introduction

Composition, organization and physiological function of multicellular organisms depend in part on the presence of connective tissues, which are observed as early in Metazoan evolution as Porifera [1]. Connective tissues exhibit remarkable diversity ranging from blood to fibrous connective tissue to cartilage to bone. In fibrous connective tissue, cells interact with a non-cellular component called extracellular matrix (ECM), composed of glycoproteins, proteoglycans, and glycosaminoglycans. ECM exhibits a dual function in fibrous connective tissue as structural organizer and as physiological regulator of cell behavior including diverse properties such as proliferation, differentiation, and migration.

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Cell-ECM interactions are active and iterative in the sense that cellular responses to ECM result in chemical and physical remodeling of the matrix, which in turn influences subsequent cell behavior [2–4].

Type I collagen is the major ECM component of fibrous connective tissue. Type I collagen matrices containing fibroblasts and other cell types have been used as an in vitro model of connective tissue to learn about cell physiology and biomechanics in a 3D tissue-like environment [5–10]. Recently, we and others began to study fibroblasts cultured on the surfaces of 3D collagen matrices as a new platform to investigate morphogenic cell clustering and dispersion [11–13].

Diverse terms have been used to describe multicellular aggregates including cell clusters, spheroids, and microtissues. Given their stability, cell clusters can in a sense be treated as biomaterials [14] and used to produce more complex higher order structures such as tissue cubes and tubes incorporating homotypic and heterotypic cellular interactions [14–17]. In this review, we will summarize briefly the phenomenon of cell clustering and then describe in more detail findings regarding the molecular mechanism of clustering by fibroblasts.

Different Types of Cell Clusters

Cell clustering phenomena first were studied to better understand tissue organization. Dissociated cells from early chick embryos were shown to re-associate into tissue-specific arrangements [18], giving rise to the hypothesis of tissue morphogenesis based on differential cell-cell adhesion [19]. Cell-cell adhesive interactions mediated by adherens junctions were believed to provide the specificity underlying the differential adhesion hypothesis [20, 21].

During embryonic development, cell clustering known as mesenchymal condensation represents a pivotal developmental stage that precedes tissue-specific differential gene expression [22–26]. Directional cell migration and cell adhesion both influence cluster formation [22]. For instance, undifferentiated mesenchymal cells can be stimulated to migrate to the site of skeletal formation by release of chemotactic factors from the overlaying epithelium [26]. Mesenchymal cells form adherens junctions with N-cadherins [27] and cell-cell interactions also are stabilized by fibronectin (FN) and integrins [28, 29]. After mesenchymal cells aggregate, cartilage and bone formation begin [23].

Skin appendage formation represents another important example of mesenchymal condensation [30]. In the case of hair follicles, the cluster of specialized fibroblasts known as the dermal papilla provides the inductive signals that drive skin epidermal cells to undergo differentiation into hair follicle cells [31–33]. 3D organization of dermal papilla cells is believed to be essential for hair follicle induction, and expression of markers associated with induction occurs preferentially in cells cultured on poorly adhesive substrates under conditions in which the cells cluster [34]. On conventional surfaces to which cells attach well, they do not cluster and expression of dermal papilla specific markers is lost [35, 36]. Fibronectin also is believed to be important for dermal papilla organization based on its localization in vivo [37] and in vitro [38], and addition of exogenous FN promotes cell clustering and formation of dermal papilla structures [39].

Another type of cell clustering, distinct from mesenchymal condensation, occurs during wound repair. During repair, fibroblasts are recruited to the wound site from three different sources: the local fibroblast population, local epithelial-mesenchymal transition, and extravasation of circulating fibrocytes [40–42] These cells differentiate into myofibroblasts and organize into a network interconnected by adherens junctions and stabilized by extracellular matrix-integrin interactions [43–46]. Contraction of the myofibroblast network

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contributes to wound closure and can be a major cause of scarring [47–50]. Nodules of highly contractile, aggregated fibroblasts occur in hypertrophic scars [51–53]. In idiopathic pulmonary fibrosis, clusters of myofibroblasts are known as fibroblastic foci [54–57]. Fibroblast spheroids formed in vitro have been reported to produce and release proinflammatory mediators [58].

Solid tumors historically were described as three-dimensional cell aggregates [59]. However, unlike the clustering phenomena discussed so far, unregulated cell proliferation is responsible for primary tumor formation [60], and it is the loss of normal cell-cell interactions that results in tumor cell invasiveness into surrounding tissues [61, 62]. In the case of ovarian tumors, spheroids of tumor cells can be released from the primary tumor and attach at secondary sites where they are invasive [63, 64].

Mechanisms of Fibroblast Clustering

Cell clustering has been shown to depend on features of the extracellular matrix, growth factor environment, and mechanisms to stabilize cell-cell interactions. In general, conditions that increase the possibility for cell-cell adhesion relative to cell-substrate adhesion promote cell clustering. Approaches to influence this balance include cell growth on poorly adhesive substrata, cell centrifugation, and hanging drop culture [16, 21, 35, 58, 65–67]. Formation of cell clusters on soft polyacrylamide gels [68, 69] was found to be relate to the ability of cells to displace the gel substrate sufficiently so as pull themselves closer together. Cell clustering on mechanically unstable substrata [70] was suggested to depend on cell-mediated detachment of the ECM coating the matrix in which the cells subsequently became enmeshed.

Our laboratory has been particularly interested in the behavior of fibroblasts interacting with 3D collagen matrices. Since 3D collagen matrices are softer (<0.1 kPa) [71, 72] than the softest polyacrylamide substrates (> 500 kPa) [68, 69], we anticipated that fibroblasts cultured on the surfaces of soft collagen matrices also would undergo clustering. In addition, however, we wanted to learn if cell-clustering depended on the growth factor environment. That is, fibroblasts interacting with collagen matrices exhibit markedly different behavior under what we refer to as promigratory and procontractile conditions. Promigratory conditions occur in the presence of growth factors (e.g., platelet-derived growth factor, PDGF) that activate Rac and stimulate protrusion of fibroblast dendritic extensions; whereas procontractile conditions occur in the presence of growth factors (e.g., serum, or lysophosphatidic acid, LPA) that activate Rho and stimulate retraction of dendritic cell extensions [4]. Most previous cell clustering experiments such as those on soft polyacrylamide substrates had been carried out in medium containing serum, i.e., procontractile.

We found that fibroblasts cultured on 1 mg/ml 3D collagen matrices exhibited cell clustering according to the growth factor environment and collagen matrix density [11, 13]. Figure 1 illustrates some key features of our findings. After overnight incubation of human fibroblasts on collagen matrices in fetal bovine serum (FBS)-containing medium (procontractile conditions), cells were well spread (actin) and organized into clusters (Hoechst nuclear stain). However, if the incubations were carried out in PDGF-containing medium (promigratory conditions), then cells were well spread but remained dispersed. In either case, collagen matrix remodeling occurred, which resulted in a marked decrease in matrix height (>70%) as described previously [77]. Increasing the collagen concentration from 1 to 4 mg/ml, which increases substrate stiffness and also decreases spacing between potential collagen adhesion sites [72], resulted in decreased cell clustering [11],.

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Time-lapse microscopy showed that cell-cell encounters occurred continuously under promigratory conditions. Therefore, collagen matrix remodeling and cell-cell encounters were not sufficient for cell clustering to occur. The results suggested that procontractile conditions were necessary for cell interactions that stabilized cell clusters, and these morphogenic changes were reversible. That is, switching from procontractile to promigratory conditions resulted in cluster dispersion as cell migrated away, whereas switching from promigratory to procontractile conditions resulted in cell clustering [11].

Fibroblast adherens junctions mediated by N-cadherin play diverse roles in fibrous connective tissues [78]. As already discussed, a variety of evidence has implicated adherens junctions in cell clustering. Adherens junctions can be observed along the boundaries of corneal fibroblasts that develop cell-cell interactions when cultured on fibrin matrices or fibrin-coated 2D substrates [79]. Transformed fibroblasts expressing low levels of cadherins were shown to form loose rather than compact clusters [80]. Similarly, we observed that oncogenic Ras transformed fibroblasts lost their ability to form adherens junction [76] and were unable to form cell clusters on collagen matrices [11]. Therefore, we anticipated that adherens junctions might be required for stabilizing cell-cell interactions necessary for fibroblast cluster formation. However, while siRNA silencing of N-cadherin in human fibroblasts interfered with the ability of cells to form adherens junctions, the cells still were able to form cell clusters (unpublished observation).

In addition to adherens junctions, various studies have shown that fibronectin and its receptor integrin α5β1 play a role in formation and compaction of cell clusters [21, 28, 58, 65, 66]. Moreover, FN-null mouse embryo fibroblasts cultured on collagen matrices can utilize exogenously added FN to form a fibrillar FN matrix in which cells spread and proliferate [12]. Also, exogenous FN also can promote compaction of cell spheroids [21] and formation of dermal papilla [39]. Conversely, interfering with FN matrix organization using a 70kDa FN fragment that blocks fibril formation was observed to prevent cell clustering [12, 65]. Therefore, we tested the possibility that organization of FN into a fibrillar matrix also played a role in human fibroblast clustering on collagen matrices [13].

As shown in Figure 1, fibronectin matrix assembly occurred under procontractile conditions that resulted in cell clustering but not under promigratory conditions. Selected frames from time-lapse videos shown in Figure 2 model the events that occur during cell clustering under procontractile conditions. After initial cell attachment to the collagen matrix (Figure 2A), cell spreading begins. As cells attempt to spread, they reorganize and align the collagen matrix resulting in the appearance of tension lines between cells, and the cells pull closer to each other (Figure 2B). At this time, organization of fibronectin into a fibrillar matrix (detected using rhodamine-labeled fibronectin) can be observed along cell extensions and associated with small cell clusters (Figure 2C).

Initial cell spreading establishes preferential paths of future cell protrusion [81, 82]. The resulting positive feedback promotes alignment of cell extensions and radial organization of collagen fibrils under tension. After several hours, most cells have spread in elongated morphology and formation of cell clusters becomes evident (Figure 2D). The extensive fibrillar FN matrix associated with cell clusters (Figure 2E) appears by confocal microscopy to be localized primarily beneath and along their margins [13].

The dynamics of cell clustering and FN matrix organization suggested that clustering requires cells to bind to a shared FN matrix. Consistent with this idea, blocking FN fibrillar matrix formation by inhibiting Rho kinase and myosin II activity [83, 84] prevented cell clustering. Also, blocking FN using antibodies or inhibiting or interfering with expression of integrin α5β1 receptors prevented FN matrix formation and cell clustering [13]. Therefore,

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cell contraction was required not only to bring cells closer together [68, 69], but also to organize FN into a fibrillar matrix. Cell-cell encounters and collagen matrix remodeling that occur under promigratory conditions are not sufficient to promote cell clustering.

Dispersion of Cell Clusters

Epithelial cell morphogenesis provided the major initial focus of studies on mechanisms of cell clustering. Loss of epithelial morphogenic stability as a result of malignant transformation has provided the predominant focus of studies on cell dispersion. Factors contributing to the invasiveness of epithelial cells into the surrounding stromal connective tissue (epithelial-mesenchymal transition), include loss of normal cadherin-mediate cell-cell adhesions, changes in integrins, and upregulation of matrix metalloproteinases [60, 61, 85–88]

Relatively little is known about reversibility of mesenchymal cell clusters. However, the model system described in Figure 1 offers unique opportunities to study this phenomenon. The observation that fragmentation of FN matrix occurs during disruption of cell clusters and cell dispersion suggests that the dispersal process requires more than just PDGF-activation of cell migration. In 2D culture, fibronectin matrix can retard cell migration, which can be reversed by matrix metalloproteinase-dependent degradation of the FN matrix [89]. In preliminary experiments we found that broad spectrum metalloproteinase inhibitors GM6001 and BB-94 added to pre-clustered fibroblasts on collagen matrices blocked subsequent fibroblast dispersion and FN matrix fragmentation stimulated by switching to promigratory conditions. Cell clusters and their fibronectin matrix remain intact.

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Future perspectives

Future work regarding cell clustering on 3D collagen matrices can be understood from three different perspectives: mechanism, physiological consequences, and potential usefulness as a research model. In terms of mechanism, formation of cell clusters appears to be a consequence of the changing balance between cell-cell and cell-substrate adhesive properties and migratory conditions. Much remains to be learned about the effects of the growth factor environment, stabilization of cell-cell interactions, and factors controlling cluster dispersion. In terms of physiological consequences, fibroblast clustering results in a potentially important 'gain in function" such as occurs when cell differentiation follows mesenchymal stem cell condensation and hair follicle development follows dermal papilla formation. The physiological consequences also can be potentially negative such as production and release of proinflammatory mediators following formation of fibroblast spheroids. Fibroblast clustering also might be viewed as analogous to formation of the interconnected myofibroblast network responsible for stromal fibrosis. In this case, understanding the failure of oncogenic Ras transformed fibroblasts to undergo clustering might provide insights into the independence of invasive, tumor-derived mesenchymal cells from the interconnected myofibroblast network of the tumor stroma. Finally, several features make fibroblast clustering on collagen matrices a highly tractable research model for investigating the mechanism and physiological consequences of clustering including the ease of measuring clustering and modifying experimental clustering conditions and the possibility to study cluster dispersion. Research on the fibroblast-collagen clustering model offers a new approach to understanding differential fibroblast morphogenic responses in relationship to substratum biochemistry and biomechanics and should provide new insights into tissue organization and advance the field of tissue engineering.

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Highlights

- Fibroblasts clusters occur under diverse physiological and pathological conditions.
- Cluster formation depends interplay between ECM and the growth factor environment.
- Soft substrates and procontractile growth factors promote fibroblast clustering.
- Clustering depends on organization of a fibronectin fibrillar matrix scaffold.
- Fragmentation of the FN fibrillar matrix occurs during cell dispersal from clusters.

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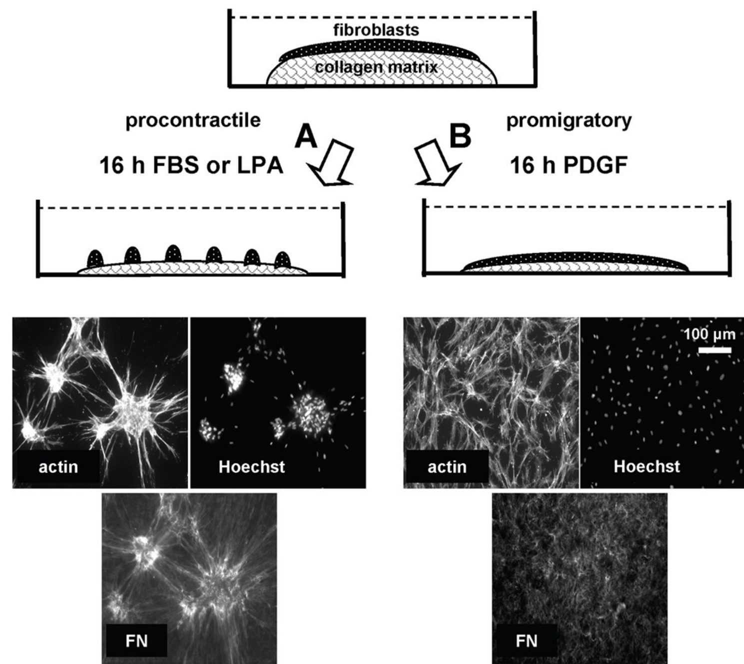

Figure 1.

Model to study fibroblast clustering and dispersion.

Human fibroblasts cultured on 3D collagen matrices form clusters under procontractile growth factor conditions (A) but migrate as individuals without clustering under promigratory growth conditions (B). Contraction plays a dual role in cell clustering, bringing cells closer together and stimulating fibronectin fibrillar matrix organization. See text for other details.

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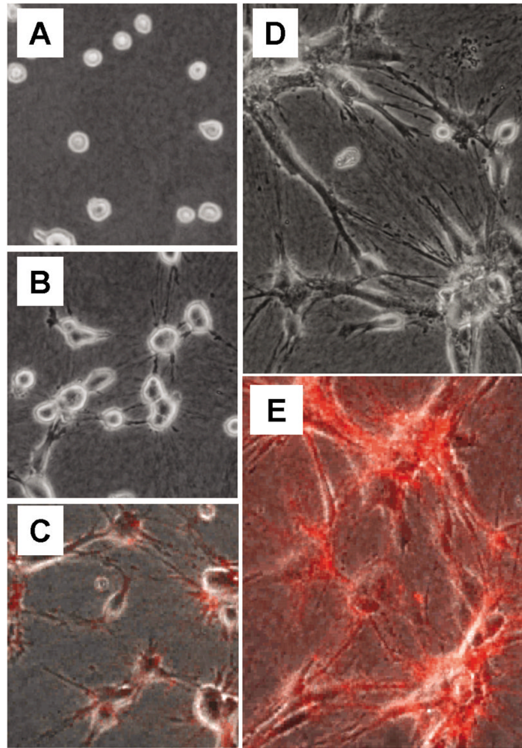

Figure 2.

Stages in fibroblast clustering.

(A) Initial cell attachment to the collagen matrix. (B) As fibroblasts begin to spread, they reorganize and align the collagen matrix resulting in the appearance of tension lines in the collagen between cells, and the cells pull closer to each other. (C) Fibronectin can be seen to organize along cell extensions and associate with small cell clusters. (D) By 6–8 hours, most cells have spread in elongated morphology and formation of large cell clusters becomes evident. (E) At this time, an extensive fibrillar matrix can be observed associated with the cell clusters. Images A, C, and E were selected from Supplemental Video #2 [11]. Images B and D were selected from Supplemental Video #2 [13]. See text for other details.