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The role of collagen reorganization on mammary epithelial morphogenesis in a 3D culture model

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ABSTRACT

Collagen-based three-dimensional (3D) *in vitro* models that recapitulate the structural and functional context of normal and malignant tissues provide a relevant surrogate to animal models in the study of developmental and carcinogenic processes. Human breast epithelial MCF10A cells embedded in a collagen gel formed both acinar and tubular structures only when the gel was detached (floating) from the cell culture plate's well, and allowed to be contracted by the cells. Epithelial phenotype depended upon the time and the location within the gel, as ducts formed exclusively on the upper layer of the gel while ductal branching occurred earlier in the central area of the gel, and gradually progressed toward the periphery. The addition of fibroblasts accelerated tubulogenesis. MCF10A cells facilitated the organization of thick collagen fibers packed into large bundles at the tip of the ducts and parallel to the direction of ductal elongation. In gels that were not detached from the well's wall, MCF10A cells organized in monolayer and collagen fibers were aligned along the axis of outstretched sprouts stemming from those cellular aggregates. Partial gel release induced uniaxial tubulogenesis associated with orderly aligned collagen fibers. These results suggest that proper collagen organization is necessary for epithelial morphogenesis to occur, and that biomechanical interactions between fibers and cells mediated duct formation, elongation and branching.

1. Introduction

The breast is a branched tubulo-alveolar gland with ducts formed by an epithelium supported by a stroma that contains an extracellular matrix (ECM), fibroblasts, immune cells, adipocytes, and blood and lymphatic vessels. Some of the ECM components include type I collagen, laminins, type IV collagen, fibronectin, and other macromolecules [1–3]. Stromal–epithelial interactions mediate mammary gland organogenesis during normal and neoplastic development [4–6].

In order to dissect the mechanisms that mediate stromal–epithelial interactions, defined three-dimensional (3D) models were developed [7–11]. The introduction of reconstituted basement membrane (rBM), a complex mixture of macromolecules secreted by a mouse chondrosarcoma [12], has facilitated the study of interactions between breast epithelial cells and BM components. Primary epithelial cells and established epithelial cell lines cultured in rBM mostly form acini, that together with ducts are the most common structures in a resting breast [7,13,14]. However, since most breast cancers are of ductal origin [15], experimental surrogate 3D models should include ductal as well as alveolar structures.

Collagen type I is the most abundant of the ECM components in normal and malignant breast [16]. Increased mammographic density, due in part to the abundance of collagen type I, has been shown to increase breast cancer risk in women [17]. In addition, increased collagen density has been associated with breast cancer initiation and progression in animal studies [18,19]. However, in 3D cultures, the role of collagen in epithelial morphogenesis is still being debated. Primary cultures of mouse mammary epithelial and myoepithelial cells grown in floating collagen gels formed structures resembling the alveoli present *in vivo* [20]. The human breast cell lines T47D and MCF10A formed acini and ducts when cultured in floating collagen gels, but only formed undifferentiated cellular sheets in attached gels [21,22]. In contrast, the human breast

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epithelial cell line HB2 formed only acini in collagen type I gels; however, when HB2 cells were co-cultured with various fibroblast cell lines or fibroblast-conditioned media branching tubular structures developed [23]. Thus, it is clear that the type of epithelial structure formed is influenced by various factors, including the epithelial cell types, presence of fibroblasts, and the biochemical and physical properties of the gel in which they are embedded.

Collagen gels have also been used to explore the role of the focal adhesion kinase pathway (FAK) on epithelial morphogenesis through transduction of mechanical stimuli. It has been suggested that acini and ducts occurred only in matrices compliant enough for the cells to contract them [22]. Disruption of the intracellular Rho kinase signal downstream of the integrin receptor has been shown to inhibit tubulogenesis and block collagen contraction [22]. This suggests that cell-adhesion receptors actively participate in collagen reorganization [22,24]. Collagen fibers bundle into large diameter fascicles through lateral alignment; this process is crucial in determining strain-transfer properties in 3D collagen-based matrices [25,26]. However, the role of collagen restructuring in epithelial tubulogenesis and branching, and the associated biomechanical interactions remain unknown.

In this study, we characterized the matrix composition and organization that allow the formation of mammary structures resembling those found *in vivo*. We used floating, attached and partially detached collagen gel models. Our results indicate that epithelial morphogenesis in 3D collagen gels is spatio-temporally regulated and that collagen reorganization may play a role in epithelial tubulogenesis and branching.

2. Materials and methods

2.1. Chemicals and cell culture reagents

Hydrocortisone, cholera toxin, insulin, methyl salicylate, and carmine were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, and penicillin–streptomycin solution were obtained from Gibco/Invitrogen (Carlsbad, CA). Equine serum and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT). Bovine type I collagen was purchased from Organogenesis (Canton, MA). Epidermal growth factor (EGF) and formalin were obtained from Fisher Scientific (Atlanta, GA).

2.2. Cell maintenance

All cells used in these experiments were maintained and expanded in cell culture plastic flasks (Corning, Corning, NY). Non-tumorigenic human breast epithelial cell line MCF10A was grown in DMEM/F12 containing 5% equine serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 0.1 mg/mL cholera toxin, 10 mg/mL insulin, and 1% penicillin–streptomycin solution. Human breast fibroblasts obtained from reduction mammoplasties (heretofore RMF, Sciencell) were grown in DMEM containing 10% FBS. All cells were incubated at 37 °C and 6% carbon dioxide. For all 3D cultures, a combined medium (one part of MCF10A cells medium and one part of RMF medium) was used. The combined medium was previously tested in cell culture flasks containing each cell type alone to ensure proper growth and behavior of cells.

2.3. 3D cell culture

The pH of type I collagen was neutralized using 75 mg/ml sodium bicarbonate (Lonza) (37.4 μL sodium bicarbonate were used to neutralize 1 ml collagen). The final collagen concentration was 0.72 mg/mL in Eagle's MEM containing 200 mM L-glutamine and 10% FBS. In co-cultures, MCF10A cells and RMF were seeded in a 3:1 ratio using 150,000 MCF10A cells and 50,000 RMF. This ratio matches the *in vivo* numbers of epithelial cells to fibroblasts. The same cell number was seeded for each cell type cultured independently. Cells were suspended in 1.5 mL collagen and seeded into 12-well plates. The gels were allowed to solidify for 30 min at 37 °C before adding culture medium onto each well. The gels were either a) left attached to the plastic well (these gels will be referred as attached gels), or b) detached from the sides and the bottom of the well immediately after solidification (floating gels). Cultures were maintained for 1–4 weeks, and the medium was changed every 2–3 days.

2.4. Matrix contraction measurement

The time of congealing was chosen as the time of experiment initiation ($t = 0$). Gel diameter was measured at the 2nd, 3rd, 4th, 6th, 10th, and 14th day during the culture period. Contraction was calculated by measuring the area covered by the gel.

2.5. Gel processing

On the day of harvest, the gels were cut into two pieces. One piece was fixed overnight in 10% phosphate-buffered formalin, paraffin-embedded, and used for histological analysis. Another piece was whole-mounted onto a slide, fixed, stained, and used for analysis by bright field, polarized light, and confocal microscopy.

2.6. Whole mount staining

The whole-mounted gels were placed onto a glass slide, fixed overnight, transferred to 70% ethanol, and stained with carmine alum overnight following a protocol described by Ref. [27] with modifications. After staining, the whole mounts were dehydrated in 70%, 95%, and 100% ethanol, cleared in xylene, and mounted with Permount (Fisher Scientific).

2.7. Confocal microscopy

Whole-mounted gels were analyzed using a Zeiss LSM 510 system. The HeNE 633 nm/5 mW laser was used for data acquisition due to the autofluorescence of carmine dye at this wavelength. Alveolar and ductal structures in collagen gels were scanned with a 20X objective lens, and 8-bit-depth images with a resolution of up to 1044 × 1044 pixels were taken. The data were three-dimensionally reconstructed using Zeiss software.

2.8. Immunohistochemical analysis

Hematoxylin and eosin staining was performed as previously described [13]. Formalin-fixed, paraffin-embedded sections of the collagen gels were stained with specific antibodies for laminin 5 (1:200 dilution, Chemicon, USA, CA), E-cadherin (1:150 dilution, Novocastra, UK), sialomucin (1:800 dilution, Abcam, USA, MA), and collagen type IV (1:50 dilution, Dako, DM). An antigen-retrieval method using microwave pretreatment and 0.01 M sodium citrate buffer (pH 6.0) was used. The antigen–antibody reaction was visualized using the streptavidin–peroxidase complex, with diaminobenzidine tetrahydrochloride (Sigma–Aldrich) as the chromogen. Counterstaining was performed with Harris' hematoxylin. Images were captured using a Zeiss Axioscope 2 plus microscope (Carl Zeiss MicroImaging, Thornwood, NY).

2.9. Picrosirius red staining

Paraffin-embedded sections or whole gels were stained using picrosirius red solution (0.1% sirius red diluted in saturated picric acid). Briefly, the samples were first rehydrated by sequential incubations in three xylene solutions (5 min each), decreasing concentrations of ethanol (3 min each), and water (3 min). Next, the sections were incubated in picrosirius red solution for 1 h (overnight in the case of whole mounts) and dehydrated by incubation in increasing ethanol concentrations and three xylene solutions.

2.10. Polarized light microscopy

The picrosirius red stained sections and whole mounts were observed using a Zeiss Axioscope 2 plus microscope equipped with a polarizer and an analyzer. The polarized transmitted light was captured by a color camera and visualized using the Axio Imager system (Zeiss). The optical parameters and camera exposure time were kept constant between samples of the same experiment.

3. Results

3.1. Collagen contraction of floating and attached gels

3.1.1. Floating gels

At day 1, the gel area covering the entire well was approximately 4 cm^2. The onset of gel contraction in MCF10A mono-cultures was observed on the third day; the gel area was reduced up to 85% of the initial value within the first 10 days of culture (Fig. 1A). Collagen gels containing only RMF began contracting after one week in culture and progressed at a slower rate than that observed in MCF10A mono-cultures. When MCF10A cells were co-cultured with RMF, collagen contraction began on the second day of culture but