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The effect of fibrillar matrix architecture on tumor cell invasion of physically challenging environments

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ABSTRACT

Local invasion by and dissemination of cancer cells from a primary tumor are key initial steps of metastasis, the most lethal aspect of cancer. To study these processes *in vitro*, the invasion of cells from multicellular breast cancer aggregates embedded in three-dimensional (3D) extracellular matrix culture systems was studied. This work showed that in 3D fibrillar environments composed of collagen I, pore size – not the viscoelastic properties of the matrix – was the biophysical characteristic controlling breast cancer cell invasion efficiency. Furthermore, it was shown that fibrillar matrix architecture is a crucial factor that allows for efficient 3D invasion. In a 3D non-fibrillar environment composed of basement membrane extract (BME), invasion efficiency was greatly diminished, the mesenchymal individual mode of 3D invasion was abolished, and establishment of cell polarity and protrusions was compromised. These effects were seen even though the BME matrix has invasion permissive viscoelasticity and suitable adhesion ligands. The altered and limited invasive behavior observed in BME was rescued through introduction of fibrillar collagen into the non-fibrillar matrix. The biophysical cues of fibrillar collagen facilitated efficient invasion of sterically disadvantageous environments through assisting cell polarization and formation of stable cell protrusions. Finally, we suggest the composite matrices employed in this study consisting of fibrillar collagen I and BME in either a liquid-like or gelled state are suitable for a wide range of 3D cell studies, as these matrices combine fibrillar features that require cells to deploy integrin-dependent mechanotransduction machinery and a tunable non-fibrillar component that may require cells to adopt alternative migratory modes.

1. Introduction

Extracellular matrix (ECM) is a complex mixture of structural macromolecules and cytokines that regulates key cellular processes including polarization, differentiation, and proliferation through biochemical and biophysical cues. Altered ECM properties have been associated with numerous pathological conditions including atherosclerosis [1], fibrotic diseases [2–4], and cancer [5,6]. In all cases, changes to the ECM are not simply symptoms of disease but are contributors to the pathogenic process.

In cancer, alterations in ECM composition and organization have been implicated in the progression of malignant tumors from circumscribed masses to locally invading entities to eventual metastatic disease. While cancer progression is regulated by a complex interplay of genetic and epigenetic changes, evolving interactions between tumor cells and the surrounding ECM play a critical role in the metastatic cascade [7,8].

The importance of the local environment and changes to that environment have attracted particular attention among breast cancer researchers, as high breast tissue density has been associated with both higher risk of breast cancer and poor prognosis in patients [9,10]. High breast tissue density is due to higher than average amounts of the fibrillar structural protein collagen I, and elevated amounts of this protein – as well as fibrillar collagen III – in and around tumors have been demonstrated by both histology and genetic profiling [11–15].

ECM surrounding breast tumors differs not only in biochemical composition from that of normal tissue but also in physical properties, notably stiffness [16]. A recent study correlated the stiffness of and around breast tumors with their aggressiveness [17]. The effect of stiffness on cell spreading and migratory capacity has been well studied on two dimensional (2D) substrates [18,19]. Such studies have also been undertaken in more physiologically relevant three-dimensional (3D) environments, though isolating effects of stiffness from those of ligand availability and network architecture is a significant challenge in the 3D context [20–24].

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Beyond differences in composition and stiffness, the ECM surrounding invasive breast tumors also demonstrates changes in network architecture relative to normal ECM. Increased deposition of fibrillar collagens may lead to decreased porosity near the tumor. Moreover, cellular remodeling of fibrillar collagens may lead to heterogeneities in density and network organization. Indeed, architectural changes in ECM have been correlated with breast cancer prognosis. Alignment and orientation of collagen I fibers perpendicular to the tumor boundary has been associated with recurrence after surgical excision and metastasis, and breast cancer cells have been shown to migrate preferentially along radially aligned collagen fibers at the interface of the primary tumor and ECM [25,26].

In sum, accumulating evidence suggests that the presence and particular organization of fibrillar collagen I is a marker of and/or a causal factor in breast cancer invasion. Here, we dissect the contributions of biophysical properties of fibrillar collagen I to the invasion efficiency of breast cancer cells. In particular, the invasion of triple negative highly aggressive breast adenocarcinoma cells (MDA-MB-231) cultured as multicellular tumor spheroids (MTSs) in both 3D (cells immersed within a matrix) and pseudo-3D (cells placed atop a thick matrix) settings was studied. The multicellular invasion efficiency as reflected by invasive distance was analyzed. Invasive mode as reflected by cell polarization, establishment of protrusions, and ECM rearrangement on both the spheroid and single cell level was also investigated. The 3D matrices employed were composed of either fibrillar collagen I and/or non-fibrillar basement membrane extract (BME). The matrices were prepared so as to exhibit stiffnesses that varied over two orders of magnitude and displayed distinct network architectures, with varying porosity and fibrillar dimensions.

2. Materials and methods

2.1. Cell lines and reagents

MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). All cell culture reagents unless otherwise stated were obtained from Gibco (Grand Island, NY). Acid-solubilized (AS) rat tail collagen I was obtained from BD Biosciences (San Jose, CA) as an 8.5–9 mg/ml solution. Pepsin-treated (PT) bovine collagen I was obtained from Advanced BioMatrix (San Diego, CA) as a 5.9–6.1 mg/ml solution. Growth factor-reduced, phenol red-free basement membrane extract (BME)/Matrigel was obtained as an 8.9–9.4 mg/ml solution from BD Biosciences (San Jose, CA). 10× DMEM solution, sterile NaOH (1 N), and sodium bicarbonate solution (7.5%) were purchased from Sigma Aldrich (St. Louis, MO). Gibco 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (1 M) was obtained from Invitrogen (Carlsbad, CA). Aprotinin, leupeptin, pepstatin A, and E-64 were obtained from MP Biomedicals (Solon, OH). Triton-X, marimastat (BB-2516), and blebbistatin were obtained from EMD Millipore Chemicals (Billerica, MA). Alexa Fluor-conjugated phalloidin was obtained from Invitrogen Life Technologies (Grand Island, NY), and 1.0 mg/ml propidium iodide solution was obtained from Sigma Aldrich. 10% buffered formalin phosphate, acetone, and ethanol were obtained from Fisher Scientific (Pittsburgh, PA).

2.2. Cell culture and generation of multicellular tumor spheroids

Cells were cultured in growth medium consisting of 1× high glucose DMEM containing 10% (v/v) FBS, 1% (v/v) 100× penicillin/streptomycin solution, and 1% (v/v) 100× non-essential amino acids solution at 37 °C with 5% carbon dioxide. Cells were subcultured when 70–80% confluent. Multicellular tumor spheroids (MTSs) were formed using the Perfecta 3D Biomatrix hanging drop system (Neta Scientific, Hainesport, NJ). 30 μl drops of a 4×10^5 cells/ml cell suspension were placed in the wells of the hanging drop plate. Droplets were held in place by surface tension, and cells accumulated at the bottom of each droplet to form a spheroid, or MTS. Spheroids were allowed to form and grow for 6 days at 37 °C with 5% carbon dioxide.

2.3. Cell treatments

Blebbistatin was used to inhibit myosin II-phosphorylation and was added to the growth medium at 10–20 μM in 2D experiments. When blebbistatin was used for cells embedded in 3D matrices, the drug concentration in the overlaying medium was increased to achieve a 20 μM concentration within the 3D gel. Thus, 200 μl gels were overlaid with 50 μl of 100 μM blebbistatin containing growth medium. DMSO was used as the solvent control at equivalent concentrations. Inhibition of endogenous proteases for cells cultured in 3D environments was achieved through addition of a protease inhibitor cocktail as described in Wolf et al. [27]. Both the collagen solution and the growth medium added on top of the 3D collagen matrix were supplemented with 100 μM marimastat, 250 μM E-64, 100 μM pepstatin A, 2 μM leupeptin, and 2.2 μM aprotinin.

2.4. Preparation of cell free gels

Collagen gel solutions from 1.0 to 4.0 mg/ml were prepared by diluting the high-concentration collagen stock solutions. Appropriate amounts of collagen stock solution were prepared with 10% (v/v) 10× DMEM, 2.5% (v/v) HEPES buffer, 2.5% (v/v) sodium bicarbonate and distilled, deionized water. To prevent self-assembly of collagen monomers, all solutions were held and mixed at 4 °C. NaOH was added to adjust the pH to 7.4, and the gel was transferred immediately to the chosen gelation temperature (22 °C or 37 °C). Collagen solutions were allowed to gel for 45 min, which was sufficient to complete gelation at either temperature, and then transferred to the incubator at 37 °C.

BME gels were prepared by diluting the BME stock solution (8.9–9.4 mg/ml) with ice-cold 1× DMEM (serum free) to the required concentration. All steps were performed at 4 °C with pre-chilled solutions and instruments. Solutions were transferred immediately to an incubator at 37 °C and allowed to gel for 45 min.

To prepare collagen/BME composite gels, first 10× DMEM, HEPES buffer, and sodium bicarbonate were mixed. Then, the required amount of BME stock solution was added. This solution replaced a proportion of the H_2O that would be added in the equivalent pure collagen gel. Subsequently the collagen stock solution was added and the solution was brought to pH 7.4 by adding NaOH. After careful mixing, the solution was transferred to the chamber and gelled at 37 °C.

2.5. Preparation of cell-embedded gels

To prepare collagen gels loaded with a single MTS, collagen solution was prepared as described above. 200 μl of neutralized collagen solution was added to a chamber consisting of a 5 mm glass cylinder glued to a coverslip-bottom cell culture dish. A nylon mesh was placed on the inner circumference of the cylinder to anchor the collagen gel. The spheroid was added to the liquid collagen in 7 μl growth medium. The chamber was then transferred immediately to the desired temperature for gelation (45 min) and then to the 37 °C incubator. The collagen gels were overlaid with 40–50 μl growth medium after 2 h and surrounded by additional liquid to prevent drying during extended incubation periods. To prepare BME and collagen/BME composite matrices loaded with a single MTS, the chambers were first pre-coated with BME or a collagen/BME solution to prevent sedimentation of MTSs to the bottom of the chamber. The coating was gelled at 37 °C. Following this, the bulk volume of the matrix solution was added to the chamber and individual spheroids implanted as described above.

To prepare collagen gels loaded with dispersed cells, the collagen solution was prepared omitting the water and was neutralized at 4 °C. The water was substituted with ice-cold growth medium containing the desired number of cells. Subsequently 70–200 μl of cell-loaded collagen was added to the chamber and gelled at 22 °C or 37 °C as described above for MTS loaded collagen gels. After 2 h gels were overlaid with 40–50 μl growth medium. For preparation of BME gels with dispersed cells, part of the DMEM was substituted with medium containing the desired number of cells. After careful mixing of the cell-containing liquid component with the BME, the solution was added to the chamber and transferred to the 37 °C incubator. After 2 h gels were overlaid with 40–50 μl growth medium. To prepare cell-embedded composite matrices, the mixture was prepared omitting H_2O and substituted with cells suspended in serum-free medium that was added after the collagen/BME solution was brought to physiological pH. Gelling and overlay were performed as described for the BME gels.

2.6. Collagen contraction assay

Collagen solutions of different collagen concentration containing 5×10^5 MDA-MB-231 cells/ml were prepared, and 500 μl gels were cast onto the 23 mm coverslip bottom of FluroDishes (35 mm). Gels were polymerized for 60 min at either 22 °C or 37 °C, overlaid with 2 ml growth medium, and manually released from the glass bottom. Contraction was allowed for 24 h at normal incubation conditions. Gel contraction was documented using digital photography. Images were taken at $t = 0$ h (before gel release) and at $t = 24$ h, and gel area was measured. Contraction is expressed as a percentage decrease of gel area over 24 h. All conditions were tested in triplicate.

2.7. Immunocytochemistry

For immunocytochemical staining, dispersed cells were embedded in or plated on matrices of 100–200 μl. Cells were fixed in neutrally buffered 4% formalin solution for 20 min at 24 °C. After extensive washing and permeabilization with 0.5% Triton-X, samples were washed again with PBS to remove the detergent, fluorescently labeled phalloidin was added and the samples were incubated for 16–24 h at 4 °C. After extensive washing with PBS, samples were overlaid with PBS and immediately subjected to imaging.

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