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Stiffening and unfolding of early deposited-fibronectin increase proangiogenic factor secretion by breast cancer-associated stromal cells

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ABSTRACT

Fibronectin (Fn) forms a fibrillar network that controls cell behavior in both physiological and diseased conditions including cancer. Indeed, breast cancer-associated stromal cells not only increase the quantity of deposited Fn but also modify its conformation. However, (i) the interplay between mechanical and conformational properties of early tumor-associated Fn networks and (ii) its effect on tumor vascularization remain unclear. Here, we first used the Surface Forces Apparatus to reveal that 3T3-L1 pre-adipocytes exposed to tumor-secreted factors generate a stiffer Fn matrix relative to control cells. We then show that this early matrix stiffening correlates with increased molecular unfolding in Fn fibers, as determined by Förster Resonance Energy Transfer. Finally, we assessed the resulting changes in adhesion and proangiogenic factor (VEGF) secretion of newly seeded 3T3-L1s, and we examined altered integrin specificity as a potential mechanism of modified cell–matrix interactions through integrin blockers. Our data indicate that tumor-conditioned Fn decreases adhesion while enhancing VEGF secretion by pre-adipocytes, and that an integrin switch is responsible for such changes. Collectively, our findings suggest that simultaneous stiffening and unfolding of initially deposited tumor-conditioned Fn alters both adhesion and proangiogenic behavior of surrounding stromal cells, likely promoting vascularization and growth of the breast tumor. This work enhances our knowledge of cell – Fn matrix interactions that may be exploited for other biomaterials-based applications, including advanced tissue engineering approaches.

1. Introduction

Varied physicochemical properties of the extracellular matrix (ECM), a dynamic and complex fibrillar network, modulate cellular behavior. In tumors, the ECM is primarily generated by cancer-associated cells (e.g. fibroblasts and adipogenic precursors) and contributes to sustained tumor growth and survival [1–8]. It exhibits numerous altered materials properties relative to normal ECM including variations in protein composition, structure, and rigidity. In fact, analysis of tumorous ECMs revealed differences in collagen I deposition relative to normal ECMs as suggested by elevated quantities, reorganization, crosslinking, and stiffness of collagen [4,9–13]. Moreover, fibronectin (Fn) might be responsible for additional ECM structural alterations, as indicated by the presence of highly stretched and unfolded Fn fibers in tumor-associated matrices [14,15]. It is important to recognize that tumor-associated Fn and collagen alterations are functionally linked since Fn (i) is essential for the deposition of collagen I in ECMs [4,16–18] and (ii) is also used as an indicator for increased tumor aggressiveness [19]. Nevertheless, a clear correlation between structural, conformational, and mechanical properties of the tumorous ECM network and the role of Fn in this process has not been established. This correlation has been hindered partly by the intrinsic complex composition of the ECM, and by the lack of analytical tools that

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permit simultaneous assessment of ECM materials properties from the matrix/cellular to the molecular scale. Indeed, both collagen and Fn fibers are present in mature ECM and likely synergize to modulate the bulk properties of the tumor ECM [17,20]. Additionally, there is a lack of materials science tools to separately assess morphology and mechanics of native (uncrosslinked) ECM at both matrix/cellular and molecular scales under physiologically relevant conditions.

Altered materials properties of the tumor ECM are clinically relevant as they promote tumor malignancy via direct effects on tumor cells [8] and indirectly by enhancing the formation of new blood vessels (angiogenesis) [4,9–13]. In fact, altered ECM can enhance angiogenesis either by increasing the activity of surrounding endothelial cells [14,15] or by stimulating the secretion of proangiogenic factors (e.g. vascular endothelial growth factor [VEGF]) from cancer-associated fibroblasts [4,16–18]. However, the specific ECM properties and associated mechanisms responsible for the proangiogenic capability of tumor-associated cells remain unclear.

Here, we integrated a set of physical sciences tools with cancer biology to: (i) characterize the mechanics, conformation, and topology of tumor-associated Fn matrices at both the matrix and molecular scales, and (ii) correlate these materials properties with adhesion and proangiogenic factor (VEGF) secretion of adipose stromal cells. Our results revealed that tumor-conditioned Fn matrices were stiffer and more unfolded than control matrices, and that these dysregulated matrices contributed to enhanced VEGF secretion by stromal cells.

2. Materials and methods

2.1. Cell culture

As an *in vitro* model of cancer-associated stromal cells, we utilized tumor-associated 3T3-L1 preadipocytes (ATCC, VA). Tumor soluble factors (TSF) from an aggressive metastatic breast cancer line, MDA-MB231 cells (ATCC, VA), were collected to mimic paracrine signaling between a tumor and its surrounding microenvironment. After exposing 3T3-L1s to TSF for 3 days, the preconditioned cells were detached and cultured on mica substrates for 24 h. Afterwards, culture systems were decellularized [19,21] and the resulting cell-free matrices were used for parallel mechanical, topological, and conformational characterization.

2.2. Surface forces apparatus

The Surface Forces Apparatus (SFA) (SurForce LLC, CA) is an interferometry-based technique that uses fringes of equal chromatic order (FECO) to quantify the absolute surface separation between two reflecting surfaces, with nm resolution, while both normal (adhesion) and lateral (friction) forces can be measured. This technique is extensively described in Refs. [17,20,22,23]. Briefly, in our study, the lower surface was mounted on a double cantilever spring of known elastic constant while the upper surface was connected to a step motor to apply normal load on the lower surface. A white light source was directed through two SFA surfaces (silica discs) previously glued with semi-reflective silvered mica, building an optical interferometer. The resulting interference FECO were directed towards the entrance slit of a photo-spectrometer (Princeton Instruments, NJ) and recorded with a CCD camera (Princeton Instruments, NJ) for further FECO analysis. The acquisition software used was LightField v4.0 (Princeton Instruments, NJ).

2.3. Substrate preparation for SFA characterization

Muscovite mica (S&J Trading, Australia) is a negatively charged, hydrophilic aluminosilicate that is used as preliminary substrate in all SFA experiments. To obtain transparent, uniform, and atomically smooth mica surfaces, we cleaved mica into 1 cm^2 sections of 2–5 μm in thickness and metallized them with 55 nm of silver to make them semi-reflective. The mica sections (silver side facing down) were then glued with UV curing glue (E = 1.034 GPa, product 61) (Norland, NJ) onto semi-cylindrical silica discs of 10 mm in diameter and 20 mm of curvature radius (ESCO Products, NJ). All preparation steps were performed in a laminar flow cabinet to minimize particulate contamination. Each SFA experiment requires a pair of discs glued with mica sections cut from the same sheet to ensure equal mica thicknesses on both upper and lower discs. Customized PDMS chambers containing cell culture media were used to house the lower discs during the 24 h matrix deposition process while the upper discs (bare mica), used as indenters during force measurements, were kept clean and stored in a desiccator until needed.

2.4. Force curve acquisition and elastic measurements via SFA

Upper and lower SFA cylindrical discs were mounted in a crossed axis configuration to ensure a well-defined circular contact junction. The lower disc holding the ECM was mounted on a 980 N/m spring and the upper disc (bare mica) was used to indent ECM, as depicted in Fig. 1A. The SFA stainless steel chamber was filled with 75 mL of warm (37 °C) PBS to keep the ECM in physiological conditions during mechanical characterization, and the entire system was allowed to equilibrate at 37 °C for 1 h. Each ECM was then probed at 4 different locations (approximately 500 μm apart) and each location was indented 3 consecutive times. The system was allowed to equilibrate for 30 min between each indentation and 15 min between locations. Approach (In) and retraction (Out) measurements (force runs) were performed in quasi-static conditions (at a constant speed of 0.5 μm/min) to minimize viscous effects. During force runs, FECO were acquired at a rate of 3 frames per second and post-processed with Matlab R2012b (MathWorks, MA) to yield force–distance profiles. These profiles were further analyzed to extract the compressive elastic moduli using Hertzian contact mechanics between a sphere and an elastic half-space proposed by Johnson [8,24], equation (1) (see Results).

2.5. Creep testing via SFA

Samples were prepared and mounted in the SFA as described in the previous section. However, the lower surface was mounted onto a more compliant spring (k = 676 N/m) and the ECM samples were indented instantaneously (rather than quasi-statically) by applying increasing step-loads of approximately 3.7 mN (indentation approximately 5 μm) using the SFA fine micrometer, resulting in forces that correspond to $F_1 = F'_1 = 3.7$ mN, $F_2 = F'_2 = 7.4$ mN, and $F'_3 = 11.1$ mN. Changes in ECM indentation depth (creep) were then monitored over 1800 s by following the shift of the FECO fringes.

2.6. FRET labeling of fibronectin

Alexa Fluor 488 succinimydyl ester (donor fluorophores) and Alexa Fluor 546 maleimide (acceptor fluorophores) (Invitrogen, CA) were used to label Fn for intramolecular Förster Resonance Energy Transfer (FRET) as previously described by Baneyx et al. [25] and Smith et al. [26]. Fn concentrations and labeling ratios between donors and acceptors were determined using a DU®730 UV–Vis spectrophotometer (Beckman, IN) at 280 nm, 495 nm, and 556 nm. FRET calibration of labeled Fn was first carried out in denaturant solution by varying guanidine hydrochloride concentrations between 0 and 4 M to obtain acceptor/donor intensity ratios (I_A/I_D), termed FRET ratios, as a function of protein denaturation. Additional FRET calibration of Fn embedded in fibers was performed via a custom-made strain device and used to correlate Fn fiber FRET ratios with fiber uniaxial strain, as described in Refs. [27,28].

2.7. Cell seeding and sample decellularization

3T3-L1 (ATCC, VA) preadipocytes (passages 4–10) were preconditioned for 3 days in either α-MEM culture medium (Control) or α-MEM medium containing normalized TSF. After this preconditioning period, cells were trypsinized and used for parallel SFA and FRET experiments.

Both flat mica sections (culture area: 64–81 mm^2/well) and curved mica surfaces (mounted on SFA discs, culture area: 80 mm^2/disc) in PDMS chambers were first incubated with human plasma Fn (Life Technologies, NY) at a concentration of 30 μg/mL in phosphate buffered saline (PBS) for 60 min at room temperature to facilitate cell adhesion. After rinsing 3 times with PBS, a concentrated cell solution comprised 2×10^4 preconditioned 3T3-L1s (Control or Tumor) was seeded on the mica substrates. After 20 min of cell adhesion, 400 μL of exogenous Fn (50 μg/mL) low serum (1% fetal bovine serum (FBS)) was added. For FRET experiments, the exogenous Fn consisted of 90% unlabeled Fn (unFN) and 10% FRET-labeled Fn to prevent intermolecular FRET. For SFA experiments, only unFN was used.

After culturing at 37 °C and 5% CO_2 for 24 h, cultures were decellularized via a modified Cukierman protocol [21] that included deoxycholic acid incubation and extra wash steps, and left unfixed in PBS. Further samples were fixed for 1 h at 4 °C, and washed three times with PBS for immunostaining and morphology studies.

2.8. FRET data acquisition

Samples were imaged with a Zeiss 710 confocal microscope (Zeiss, Munich, Germany) using the C-apochromat water-immersion 40×/1.2 objective, a pinhole of 2 AU, a 488 nm laser set at 10% power, and a pixel dwell time of 6.3 μs to acquire 16-bit z-stack images spaced 2 μm apart. FRET-Fn fluorescence was simultaneously collected for the donor fluorophores in the PMT1 channel (514–526 nm) and for the acceptor fluorophores in the PMT2 channel (566–578 nm), in addition of brightfield imaging. Donor and acceptor z-stack images were analyzed pixel by pixel with a customized Matlab code to generate false color FRET ratio (I_A/I_D) images and FRET histograms for each image. Individual FRET z-stack images were stacked in ImageJ (NIH) and reconstructed in Volocity (PerkinElmer, Inc., MA) [26,29,30].