Hyaluronan Mediated Adhesion: The Structure of the Pericellular Hyaluronan Coat and Its Role in Cell-Matrix Adhesion

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Abstract

A fully hydrated pericellular hyaluronan coat was visualized around RCJ-P chondrocytes and A6 epithelial cells using environmental scanning electron microscopy. This layer, in the two cell lines, was hyaluronidase-sensitive and reached an average thickness of 4.4±0.7 μm and 2.2±0.4 μm, respectively. We show that freshly-plated chondrocytes establish "soft contacts" with the substrate, mediated through this coat. Light microscopy indicated that these cells drift under shear stress of 6.5 dyne/cm^2, leaving behind hyaluronan-rich "foot prints" on the surface. This suggests that chondrocytes are surrounded by a gel-like coat composed of a multi-layer of entangled hyaluronan molecules. In contrast, attached A6 cells do not drift under similar shear stress and remain firmly anchored to the surface, suggesting that the pericellular coat around them consists of a single hyaluronan layer. Following the early hyaluronan-mediated adhesion, discussed here, cells establish stable integrin-mediated focal adhesions.

Keywords

Hyaluronan, ESEM, adhesion, pericellular coat

Introduction

Hyaluronan forms a highly hydrated pericellular coat around many cell types, including chondrocytes (1,2), fibrosarcoma (3) and smooth muscle cells (4). Due to the carboxyl group of the glucuronic acid moieties, hyaluronan is highly negatively charged at physiological pH, and behaves, in solution, as a polyelectrolyte, forming a viscous gel. Hyaluronan was shown to contribute to the structural and physiological properties of tissues and to cell behavior during tissue assembly and remodeling (5). It also regulates a variety of cellular processes including cell adhesion, migration and proliferation. Cell surface-bound hyaluronan mediates early stages of adhesion of different cells to extracellular surfaces. Thus, early stages of attachment of A6 epithelial cells to a variety of surfaces are resistant to inhibition by the integrin-specific RGD peptide, or to cytoskeleton-disrupting drugs, yet they can be inhibited by pretreatment of the cells with hyaluronidase (6,7). This adhesive interaction was shown to occur at a time-scale of seconds, and to promote the formation of focal adhesions, on a slower time-scale (8).

In order to understand the mechanism of hyaluronan-mediated ECM adhesion, it is important to establish the physical properties of the hyaluronan coat, including its thickness, density and stability. The major difficulty in visualizing the hyaluronan layer arises from its highly hydrated nature and poor antigenicity. Dehydration of such samples, a mandatory step in most conventional (non cryo-) electron microscopy techniques, inevitably destroys the hydrated pericellular coat, converting it into dispersed fibers, thus inducing a distorted view of the cell surface. In this study we have used environmental scanning electron microscopy (ESEM) to

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visualize the hyaluronan layer, and subjected freshly plated chondrocytes and A6 cells to shear stress, to test the mechanical properties of the hyaluronan-mediated attachment.

Materials and Methods

Cell culture

Cultured cells used in this study include: RCJ-P chondrocytes (rat chondrocytes from fetal calvaria, batch 15.01.98; Prochon Biotech LTD., Israel) and A6 cells (epithelial cells from *Xenopus Laevis*, ATCC.CCL 102).

Preparation of hydrated sample for environmental scanning electron microscopy

RCJ-P cells were seeded on glass cover slips and incubated for 1 h, 3 h or 24 h at 37 °C, then fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, 5 mM $CaCl_2$, pH 7.2, for 30 min. Following rinsing with 0.1 M cacodylate buffer and with water, the cells were incubated for 45 min with 2% uranyl acetate in water at pH 3.5, then washed with water. Suspended cells were fixed and treated in the same way, then seeded on serum-coated glass cover slips at 4°C for 16 h. The samples were examined in the environmental scanning electron microscope, XL 30 ESEM FEG (Philips; FEI, Netherlands) at 10 kV, operated in the wet mode at 5°C, 6.4 Torr (867 Pascal), at 7.8 mm working distance. 0.4 μm silica beads (kindly provided by Prof. S. Margel, Bar Ilan University, Ramat Gan, Israel) were added to the sample just before introducing it into the ESEM.

Flow experiment

Suspended RCJ-P or A6 cells were seeded on serum-coated glass cover-slips (Marienfeld, Bad Mergentheim, Germany), and incubated for 25 min at 37 °C (RCJ-P) or 5 min at 27 °C (A6). The cells were then placed in a parallel-plate flow chamber (GlycoTech, MD, USA) and subjected to flow, exerting a shear stress of 6.5 dyne/cm^2, applied for three minutes by a peristaltic pump (Gilson minipuls3). Time-lapse movies were taken with a DeltaVision digital microscope (DeltaVision, Applied Precision, Inc., Issaqua, WA, USA) at 2 second intervals.

Results and Discussion

The environmental scanning electron microscope (ESEM) technique enabled us to visualize a fully hydrated hyaluronan pericellular coat enclosing RCJ-P chondrocytes and A6 epithelial cells (1). Cells were incubated at pH 3.5 with uranyl acetate; the heavy and positively charged uranyl ion binds to the negatively charged hyaluronan and simultaneously operates as an efficient stimulator of secondary electron emission, enabling visualization of the pericellular coat (Figure 1a). This layer is otherwise transparent to the electron beam. We were able to visualize a 4.4±0.7 μm-wide coat around chondrocytes (Figure 1a), and a 2.2±0.4 μm-wide coat around A6 cells (1). The model of pericellular hyaluronan as a mediator of the initial stage of cell-matrix adhesion both in chondrocytes and in A6 cells was thus substantiated by actual detection of the layer in suspended cells (1,8). Upon attachment, the hyaluronan coat becomes less dense and more difficult to visualize (Figure 1b). After cells spread the coat thickness around chondrocytes reduces to 2 μm, and becomes less dense, judging by the fact that it dehydrates rapidly upon scanning (Figure 1c). By twenty-four hours after plating, a thick hyaluronan matrix is developed, covering the cells. The thickness of the gel causes it to appear dark in the ESEM mode (Figure 1d).

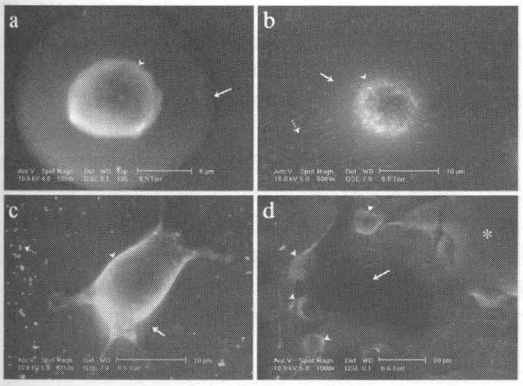

Figure 1: **Visualization of hydrated pericellular coats using the Environmental Scanning Electron Microscope (ESEM).** Chondrocytes (RCJ-P) in suspension (a), or 1 h, 3 h, 24 h after plating on glass (b, c, d, respectively) were fixed and labeled with uranyl acetate at pH 3.5; the uranyl ions bind to hyaluronan, resulting in visualization of a 4.4±0.7 μm-thick halo around suspended cells (a). (b,c) In order to distinguish between hyaluronan gel and water, 0.4 μm silica beads (dashed arrows) were added to the sample just before introducing it into the ESEM. These beads vibrated in water when scanned with the electron beam, but were essentially immobile when attached to or embedded in the gel. (b): the coat associated with cells 1 h after plating (arrow) is less dense and harder to visualize. (c): The hyaluronan coat in spreading cells is about 2 μm wide (arrow), and dehydrates rapidly upon scanning. (d): Fully-spread cells are covered with a hyaluronan gel that appears dark in the ESEM. Arrows indicate the gel, arrowheads point to the cell membrane, and asterisk indicates water.

In order to check the properties of hyaluronan-mediated adhesion, chondrocytes and A6 cells were plated on glass cover slips, allowed to adhere to the glass, then placed in a flow chamber and subjected to constant flow exerting a sheer force of 6.5 dyne/cm^2 (1). Continuous microscopic monitoring of these cells indicated that chondrocytes drift in the direction of the flow, leaving hyaluronan tracks behind (Figure 2, compare a to a'). The hyaluronan tracks where visualized by labeling the cell culture with biotinylated hyaluronan-binding protein (bHABP) and fluorescent streptavidin (1). In contrast, A6 cells adhered firmly to the surface, did not drift but occasionally "vibrated" around their attachment sites (Figure 2, compare b to b'). Hyaluronidase treated cells did not attach to the surface and were washed away with the flow (1).

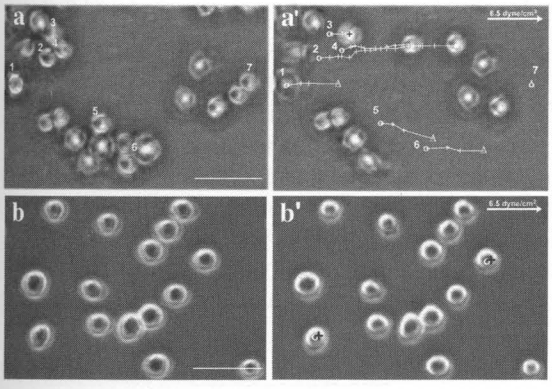

Figure 2: **The resistance of hyaluronan-mediated adhesion to shear stress.** RCJ-P (a, a') and A6 cells (b, b') were allowed to adhere to serum-coated glass for 25 min and 5 min, respectively. They were then washed with a continuous flow of medium which exerted a shear force of 6.5 dyne/cm^2. Cell movement was recorded by time-lapse phase microscopy. The arrows indicate the flow direction. (a, b): 2 seconds within flow. (a', b') 60 sec later. Circles mark the original position of selected cells; the crosses mark the cell position at 4 sec intervals; the triangles mark the detachment position; the lines mark the cells path. Scale bar 50 μm.

The above-mentioned observations imply that the thick pericellular coat associated with chondrocytes mediates a "soft adhesion" that is susceptible to shear force. This is consistent with the possibility that hyaluronan molecules are entangled in a multiple-layer coat. In contrast, adhesion, mediated by a thinner coat, such as the one associated with A6 cells, essentially immobilizes the cells or limits them to small amplitude vibrations, which is consistent with hyaluronan molecules being attached to the membrane in a "brush" configuration, where each molecule is directly attached to a receptor (1). In both cases, however, the cells are anchored to the matrix indirectly, via the hyaluronan coat, and need to proceed to integrin-mediated adhesion. Thus in chondrocytes, an integrin molecule embedded in the cell membrane, needs to overcome a 4.4±0.7 μm-coat of hyaluronan in order to interact, via their integrin receptors with the matrix. We are currently studying the translocation of pericellular hyaluronan during the transition from hyaluronan-mediated adhesion to integrin-mediated adhesion, by wet electron microscopy (QX^{TM} capsules, 9) and total internal reflection microscopy (TIRFM).

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