Loss of Elasticity of Ageing Epithelial Cells, and Its Possible Reversal

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ABSTRACT

We investigated human epithelial cells of different age in-vitro by means of the atomic force microscopy. We found that older cells are considerably (~2-4 times) more rigid that younger cells. This helps to explain why skin often looks and feels more leathery as we age. Despite the loss of elasticity of ageing skin has been know, previously researchers believed the problem was only the biochemical "glue" that holds epithelial tissue together (dermis layer) rather than the cells themselves (epidermis layer).

Further research has been focused on understanding the cause of the elasticity loss. We developed a novel method of studying cellular cytoskeleton by means of the atomic force microscopy, which allowed us to find that the elasticity change is associated with the increase of fiber density in the cytoskeleton. Based on this finding we found a biochemical way to reverse the loss of elasticity of due to aging. A new treatment causes the old cells to decrease in rigidity to the level of young cells.

The discovered loss of elasticity has been implicated in the pathogenesis of many progressive diseases/problems of aging including hardening of the arteries, joint stiffness, cataracts, Alzheimer's and dementia. We hope that our finding can inspire the search for new treatments.

Keywords: epithelial cells, ageing, mechanics of cells, scanning probe microscopy

1 INTRODUCTION

The loss of elasticity of human epithelial tissues with aging has been known [1] for a long time. It has been implicated in the pathogenesis of many progressive diseases of aging including vascular diseases, kidney disease, stiffness of joints, cataracts, Alzheimer's Dementia [2,3] , impaired wound healing, complications of diabetes, and cardiomyopathies [4]. It is believed that the loss of tissue elasticity results from rigidification of the extra cellular matrix due mostly to an increase of polymerization of collagen and elastin [1]. Many treatments [4,5] for age-related diseases are based on this belief. Here we report that individual epithelial cells also become considerable more rigid while ageing *in-vitro*.

Previous works on the cytoskeleton of human peripheral blood lymphocytes showed that the concentration of F-actin fibres increases in older cells [6]. Recent works on measurement of rigidity of rat liver macrophages [7] and transformed mouse fibroblasts [8,9] using atomic force microscopy (AFM) demonstrated that actin fibres have a great influence on cell rigidity. Recently, we have shown that the older cells of human epithelial cells indeed become up to an order of magnitude more rigid [10]. Further investigation on the cause of such increase resulted in the conclusion that it is connected with overdeveloped cellular cytoskeleton [11]. Developing this idea, here we show that using cytotoxic drugs [12], we can decrease the amount of cytoskeleton, and consequently, reverse the elasticity loss back to the young level.

This observation may have relevance for the treatment of age-related diseases that are caused by loss of elasticity of epithelial tissues.

2 MATERIALS AND METHODS

2.1 Atomic force microscopy

A Nanoscope™ Dimension 3100 (Digital Instruments/Veeco, Inc., Santa Barbara, CA) atomic force microscope (AFM) was used in the present study. A standard cantilever holder cell for operation in liquids was employed. To measure the Young modulus, the force-volume mode of operation was utilized. The AFM software version used was 5.12, release 4. To image the cells, either contact or tapping mode of scanning was used. All scanning and measurements related to the rigidity measurements were performed on cells maintained in Hank's balanced salt solution (HBSS). Scanning of the cytoskeleton was done in both air and the HBSS solution.

2.2 AFM probe preparations

A V-shaped standard narrow 200 µm AFM cantilever (Digital Instruments/Veeco, Santa Barbara, CA) was used throughout the study. A 5 µm diameter silica ball was glued to the cantilever by 10min epoxy resin using the AFM built-in micromanipulator. Such a modified probe was an essential part in this study due to the following three reasons: (1) this probe has a considerably larger contact area with the cell in comparison with the typical commercial probe (the radius of curvature ~ 5-50nm) broadly used for such measurements. Because of the averaging of the force curves due to the large probe-cell contact, the variation of the Young's modulus was a few times lower than if measured with the regular sharp probe.

This means that we needed fewer measurements to gather good statistics. This was rather important because of limited time (ca. two hours) of the measurements when the cells were alive in the HBSS solution. (2) Because of low rigidity of the edge areas of the cell, it would be very hard, if not impossible, to measure it with a sharp AFM tip. A sharp tip simply penetrates the thin areas of the cell and shows even higher rigidity due to toughing the rigid substrate. (3) Using the 5 μm probes, we caused much less damage to the cell during the scanning (see the Results and Discussion section).

After each series of measurements, the probe was changed. In some cases it was possible to clean it in acid/base solutions. The radius of the probe and its cleanness were tested by scanning the reversed grid (TGT01, Micromash, Inc., Estonia), and sometimes with SEM. The cantilever spring constant was measured using the built-in option of the Nanoscope software. In each experiment, the AFM cantilever sensitivity was calibrated against a small piece of silicon wafer immersed into the HBSS solution directly in the Petri dish.

2.3 Cell Culture

Primary cultures of human foreskin epithelial cells were prepared by a two-stage enzymatic digestion as described [13] and cells were maintained in keratinocyte serum free medium (Invitrogen, Carlsbad, CA). Young cultures typically consisted of cells that had been maintained for less that 3 passages *in vitro* (less than 25 population doublings). Old, *in vitro* aged cell cultures were maintained between 8 and 12 passages (over 50 population doublings). The 60mm cell culture dishes were mounted on the chuck of the AFM with a double sticky tape.

2.4 Cell Treatment

To inhibit actin polymerization, we used 1 μg/μL physiological solution of cytochalasin B, a cell permeable fungal toxin which binds to the barbed end of actin filaments inhibiting both the association and dissociation of its subunits. Treatment in such a solution was carried over night (12 hours).

2.5 Young's Modulus Calculations

To find the Young's modulus, we used the Hertz-Sneddon model [14,15,16] because of relatively low adhesion between the AFM probe, a clean silica sphere, and the cell. The Hertz-Sneddon model has been developed for a spherical probe of radius R over a plane surface. In this model, the Young's modulus E is given by

$$E = \frac{3}{4}\frac{1-\nu^2}{\sqrt{R}}\frac{dF}{d\left(p^{3/2}\right)}, \tag{1}$$

where F is the load force, R is the radius of the ball, p is the probe penetration into the cell. The Poisson ratio ν of the cells was chosen to be 0.5.

Because the model is developed for a sphere over a plane, we calculated the Young's modulus only over relatively flat areas of the cells. It was possible to find by scanning in the Force-Volume mode, which provides information about both the surface topography and the force curves at the same time. The force curves were collected over areas of 20 x20 μm^2 with the height change within 7 μm, the limit of the scanner. The global position of the AFM scan was controlled by the built-in video system, which allows observation of areas from 150x110 to 675x510 μm^2 with 1.5μm resolution. More precise positioning of the probe over the cell was found from the AFM topological image (a part of Force-volume image). A home-written routine was utilized to calculate the Young modulus of the studied cells. It is important to note that the cell is not a homogenous medium. It was expected and observed that the Young's modulus changed when the probe penetrated too deep into the cell. In this work, the values of Young's modulae were collected as calculated for small penetrations, *ca*. 100nm, while being relatively constant. This was done in each point on the cell surface.

2.6 Imaging of the Cytoskeleton

We used a novel technique for imaging the cytoskeleton with the AFM. The cells attached to the Petri dish were washed twice with HBSS solution. Then, the cells were treated overnight at 4°C with a solution of 0.5% Triton X-100 detergent (Sigma, Ronkonkoma, NY) mixed with buffer (10 mM M Tris–HCl, pH 7.6, 0.14 M NaCl, 5 mM $MgCl_2$, 4% polyethylene glycol 6000). After the treatment, the cells were washed twice for 2 min in the buffer and then fixed in the buffer with 1% formalin for 10 min. The treating solution was removed and the cells were washed twice with HBSS solution. The HBSS solution was added to scan the cell cytoskeleton in liquid. For scanning in air, the cells were washed with MilliQ ultrapure water and dried under ambient conditions. The details of this technique are described elsewhere [11].

3 RESULTS AND DISCUSSION

Despite intrinsic heterogeneity of cell rigidity [8,17,18], we identified at least three different regions of the cell in which the variations of the Young's modulus were relatively small. Each area has a characteristic thickness, or height above the substrate. The highest area is the nucleus, the cytoplasmic area is lower, and the lowest is the cell edge.

The Young's modulus was measured by means of the atomic force microscopy (AFM) for 12 old and 20 young

cells. The old cells become 2.4, 3.0, and 3.9 times more rigid in the nucleus, cytoplasm, and edge regions, respectively.

A typical cytoskeleton of an old and young cell ease shown in Figure 1.

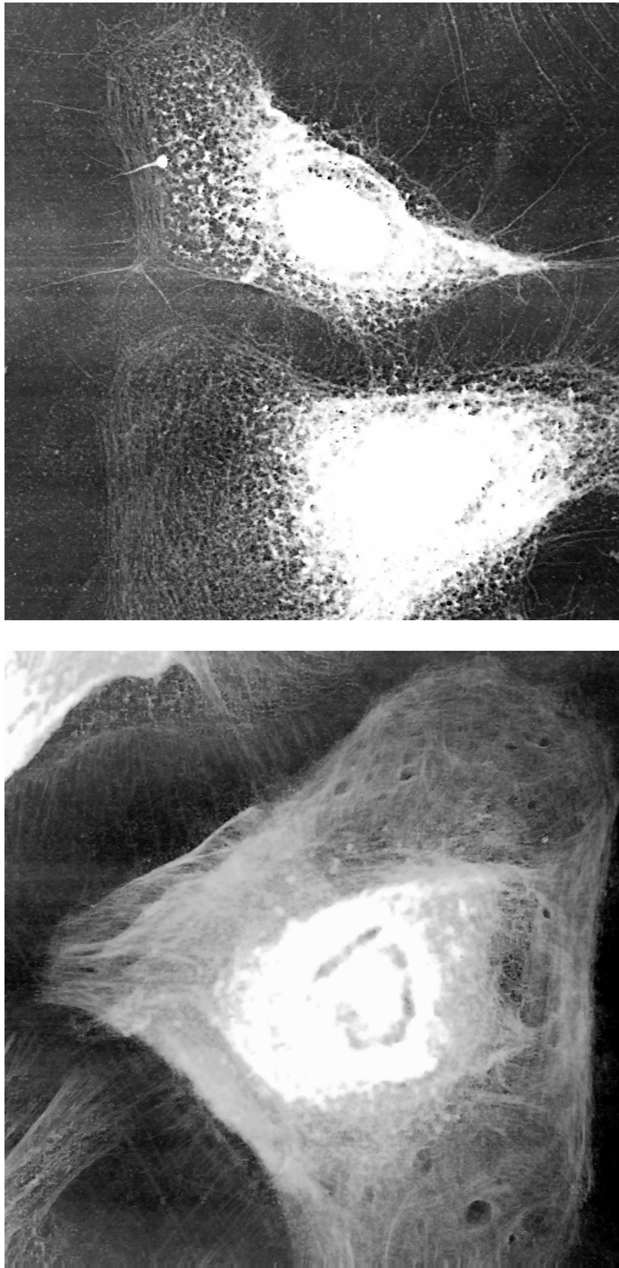

Figure 1: typical cytoskeleton of two young cells (the top image), and an old cell (the bottom image).

To quantify this visual difference, we have used a novel method involving the AFM directly visualize the cytoskeleton. We showed that the increased rigidity of ageing cells was correlated with a higher density of cytoskeletal fibres. Fig.2 shows the index of the fiber density distribution measured for both young and old cells. The surface fiber density index is defined as $SFDI = \frac{surface\ area - projected\ area}{projected\ area} 100\%$, where the surface area is as measured by the AFM.

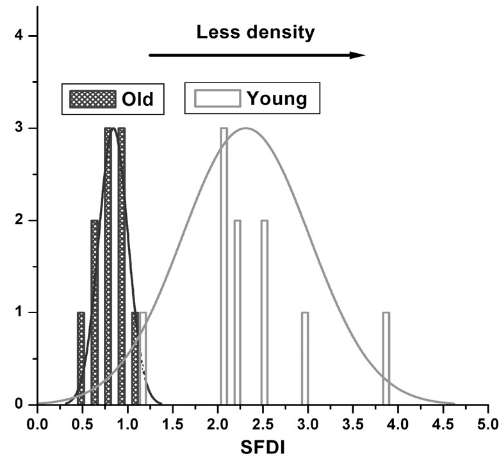

Figure 2: Statistics of the cytoskeleton, a histogram, number of cytoskeletons vs. an SFDI.

One can see obvious correlation between the increase of rigidity and the density of the cytoskeleton. Based on this observation, the proceed with our treatment to decrease the amount of cytoskeleton. In general, the used drug, cytochalasin B is poisonous that kills the cells. However, we use smaller than usual concentration of the drug [12]. Consequent measurements of the Young's modulus show that the cells indeed decreased their rigidity, Figure 3.

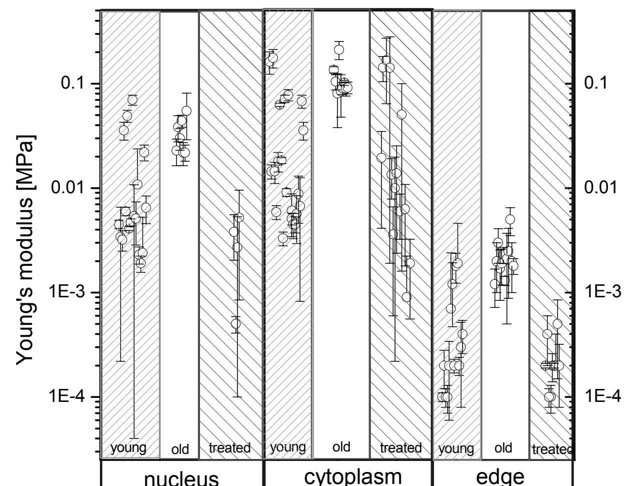

Figure 3: Results of the rigidity measurements grouped for the nucleus, cytoplasm, and edge areas of the young, old, and treated old cells

The most important, however, is the fact that the different parts of the cells decreased its rigidity right back to the young level. This is quite remarkable result because the amount of cytoskeleton was quite different in those of three areas of interest, and therefore, it was not obvious to expect the decrease of rigidity to the right level **all** three regions simultaneously.

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