Cortical stiffness of keratinocytes measured by lateral indentation with optical tweezers

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Abstract

Keratin intermediate filaments are the principal structural element of epithelial cells. Their importance in providing bulk cellular stiffness is well recognized, but their role in the mechanics of cell cortex is less understood. In this study, we therefore compared the cortical stiffness of three keratinocyte lines: primary wild type cells (NHEK2), immortalized wild type cells (NEB1) and immortalized mutant cells (KEB7). The cortical stiffness was measured by lateral indentation of cells with AOD-steered optical tweezers without employing any moving mechanical elements. The method was validated on fixed cells and Cytochalasin-D treated cells to ensure that the observed variations in stiffness within a single cell line were not a consequence of low measurement precision. The measurements of the cortical stiffness showed that primary wild type cells were significantly stiffer than immortalized wild type...
cells, which was also detected in previous studies of bulk elasticity. In addition, a small difference between the mutant and the wild type cells was detected, showing that mutation of keratin impacts also the cell cortex. Thus, our results indicate that the role of keratins in cortical stiffness is not negligible and call for further investigation of the mechanical interactions between keratins and elements of the cell cortex.

**Introduction**

Mechanical stiffness of animal cells is provided by the cytoskeleton composed of three main structural elements: actin filaments (AF), microtubules (MT) and intermediate filaments (IF) [1]. MT and IF predominantly reside in the cell interior, whereas AF compose the submembrane acto-myosin cortex that provides the cortical tension on the cell surface [2]. In a functional cytoskeleton, all three filament systems are not only intertwined but also have a large number of associated proteins that interact or bind to them (e.g., motor proteins, focal adhesion proteins, desmosome and hemidesmosome proteins, tight junction proteins, etc.) [3, 4]. In such a complex structure, a disruption of one type of filaments can cause rearrangement of the others [5], and *in vivo* this can also lead to diseases.

In epithelial cells, the incidence of IF can be much higher than that of AF and MT, especially in the perinuclear region [6]. The important role of IF can be witnessed in keratinocytes – epithelial cells that form the skin’s outer shell – where a mutation of a single IF protein (either keratin 5 or keratin 14) causes a genetic disease epidermolysis bullosa simplex, which manifests in the inability of skin keratinocytes to resist physical stress and induces severe skin lesions that may even lead to death. A typical characteristic of those keratin mutants is the presence of highly dynamic keratin particles in the cell periphery [7-10].

It has been shown that vimentin IF contribute significantly to intracellular mechanics but little to the stiffness of the sub-membrane cortex [11]. Atomic force microscopy (AFM) on
keratinocytes have demonstrated that a mutation or knock-out of specific keratin IF also significantly impacts cell stiffness against deformations that penetrate deep into the cell [12, 13], but the role of keratin in cell mechanics under small deformations has not been investigated. As keratins interact biochemically with actin filaments and contribute to focal adhesions at the cell surface [14], one can hypothesize that the distinctive accumulation of keratin particles at the cell periphery in mutant cells might be related to altered mechanical properties at the level of cell cortex and plasma membrane.

To examine, if keratin IF contribute to the mechanical properties of the cell cortex, we set-up a method based on optical tweezers (OT) for indenting the cells gently from the lateral side with deformations in the range of a few 100 nm. The cell indentation was carried out by steering an optically trapped microbead with acousto-optic deflectors (AOD) without employing any moving mechanical part. The measurements were performed on three cell lines: NEB1 (an immortalized wild type cell line), KEB7 (an immortalized K14 R125P mutant cell line) and NHEK2 (primary wild type cells). It was found that the cortical stiffness of primary cells was significantly higher than one of immortalized wild type cells, which is in line with previous studies of bulk stiffness. Interestingly, a small difference between the mutant and the wild type immortalized cells was also detected.

**Materials and methods**

**Cell lines and sample preparation** Optical tweezers experiments were performed on two previously extensively characterized immortalized keratinocyte cell lines [15, 16], which were derived from skin punch biopsies of a healthy individual (NEB1 cell line) and an epidermolysis bullosa simplex patient with a severe phenotype (KEB7 cell line, expressing the K14 R125P mutation), and primary cells (NHEK2) obtained from a control/healthy individual.
All cells were grown in serum-free EpiLife medium supplemented with EpiLife defined growth supplement and gentamicin/amphotericin (Cascade Biologies, Thermo Fischer Scientific, Waltham, MA, USA), at 37°C and 5% CO2. Mammary gland/breast epithelial cells derived from the metastatic site (MDA-MB-231) were obtained from ATCC (USA). MDA-MB-231 cells were grown in RPMI 1640 medium (Genaxxon bioscience, Germany) supplemented with 4.5 g/l of glucose, 2 mM L-glutamine, 1 mM pyruvate and 10% fetal bovine serum (FBS; Sigma-Aldrich). They were maintained at 37°C in a humidified atmosphere with 5% (v/v) CO2. To validate the method, the experiments were performed as well on MDA-MB-231 cells first treated with Cytochalasin D, and on NEB-1 cells fixed with a 4% paraformaldehyde for 20 min at 37°C.

Optical tweezers. Cell deformation experiments were performed on an Eclipse Ti inverted microscope (Nikon, Tokyo, Japan) equipped with laser tweezers (Tweez 250si, Aresis, Ljubljana, Slovenia). The optical tweezers setup used an infrared laser beam (λ=1064 nm), which was set on a constant optimal power so that there was no evidence of cell damage or bleb formation. The laser beam was focused through a water immersion objective (60x, NA 1.00, Nikon) into a sample chamber, which was maintained at a constant temperature (37°C). The laser trap position and velocity were computer-controlled with acousto-optic deflectors (AOD) with 100 kHz rate. The laser power was calibrated according to the manufacturer’s protocol to be uniform across the central part of the field of view, where the force measurements were performed. The experiments were recorded at approximately 100 frames/s with a CMOS camera (PLB-741, PixeLink, Montreal, Canada) and the bead position was tracked by TweezPal software [17]. Before each set of experiments, the stiffness of the laser trap (α) was determined by the equipartition theorem from at least 30000 images of the
Brownian motion of a bead in a stationary trap [18]. In a typical experiment, the trap spring constant was $\alpha = 140$ pN/µm.

**Indentation experiments.** Streptavidin-coated silica microbeads with a radius of a 5.06 µm (CS01N, Bangs Labs, Fishers, IN) were used for indentation experiments. The beads were functionalized by an anti-integrin beta 1 antibody (Abcam, UK) to increase the adhesion of the bead to the cell membrane. A custom-made sample chamber containing adherent keratinocytes was mounted on the microscope and the beads were added to the sample. A bead was trapped by the optical tweezers approximately 2 µm above the bottom of the chamber and positioned near the trailing edge of a cell, i.e., on the side away from the cell leading edge. The bead was then pushed laterally into the cell by moving the position of the optical trap at a constant velocity, and then retracted at the same constant velocity (Fig 1A and B, Supporting Movie S1). The force-deformation curve was determined from the known positions of the trap and the bead, where the force on the bead was calculated from the displacement of the bead from the trap center ($\Delta x$) as $F = \alpha \Delta x$ (Fig 1C). The stiffness of the cell was quantified as the slope of the force-deformation curve [19] in the linear regime at the indentation of 200 nm. If the bead adhered to the membrane, the maximal force during the retraction, $F_{\text{max}}$, was measured. In a subset of adhesion incidents, a thin membrane tether was pulled from the cell membrane during retraction.

**Fig 1.** Experimental protocol. (A) A schematic representation of the experiment (side view). A silica microbead is trapped at the cell’s trailing edge by optical tweezers and pushed into the cell at a constant velocity by moving the optical trap with acousto-optic deflectors (top). The force exerted on the cell by the bead is determined from the displacement of the bead from the center of the optical trap ($\Delta x$; bottom). The bead is then retracted from the cell at the same constant velocity. (B) A brightfield microscope image of the cell and the bead during
the indentation experiment. The bead diameter is 5.06 μm. The whole experimental sequence is shown in Movie S1 in the Supporting information. (C) A typical force-deformation curve. The cell stiffness \(k\) is determined as the slope of the linear part of the curve during the pushing phase. The adhesion of the bead onto the membrane is revealed as a maximal positive force \(F_{\text{max}}\) during retraction. In some cases, the force during the retraction never decreased to zero, which indicated that a membrane tether was extracted from the cell.

To test for a possible viscoelastic response, i.e., the dependence of the apparent cell stiffness on the rate of deformation, the measurements were performed at bead velocities of 0.1 and 1 μm/s. These velocities corresponded to the slowest and the fastest velocity attainable by the experimental set up (the high velocity is limited by the camera recording speed and the low velocity by the typical duration of the experiment for which the optical trap does not attract debris present in the sample). For trap velocities of 1 μm/s, the measurement was repeated twice at the same position.

**Statistical analysis.** The statistical analysis was performed by Mathematica (Wolfram Research). Because the cell stiffness data did not exhibit a normal distribution, the Mann-Whitney-Wilcoxon test was used to calculate the p-values for comparison of the median cell stiffness between two data sets, and \(p<0.05\) was considered a significant difference (note that, if the student t-test was used to calculate the p-values, they were \(<0.05\) for almost all comparisons). The differences in variances were tested by the Variance Equivalence Test. The nominal data for adhesion events was tested by the Fisher’s exact test.
Results

Experimental protocol validation
Probing cell stiffness with optical tweezers allows repeated measurements on the same spot of the same cell and under the same experimental conditions. During the setting up of the experimental protocol on live keratinocytes, we not only observed considerable variations between individual cells but also between two consecutive measurements on the same spot of the same cell (Fig 2A). To verify that the observed variations were a consequence of the continuous remodeling of the cytoskeleton rather than of inaccurate measurements, we validated the experimental protocol also on fixed NEB1 cells and found good repeatability of the force-deformation curves on the same spot of one cell (Fig 2B). The variations in stiffness between two consecutive measurements on the same cell were significantly higher for live than for fixed cells (p<0.005, Fig 2A), indicating that the experimental approach is susceptible to continuous remodeling of live cells, which was evident during indentation experiments (Movie S1). In the case of KEB7, NHEK2, and fixed NEB1 cells, the median difference between two consecutive measurements was less than 6%, while in the case of NEB1 the median difference was 38%, meaning that cell stiffness of NEB1 cells decreased from the first to the second measurement.

Fig 2. Repeatability of the cell stiffness measurements. (A) The relative difference between two consecutive measurements of the cell stiffness on the same spot of individual cells ($\frac{k_1 - k_2}{(k_2 + k_1)/2}$). Measurements on living cells show a substantial variation, most likely due to active remodeling of living cells. On the other hand, the stiffness measurements performed on paraformaldehyde fixed cells indicate exceptional experimental reproducibility. The deformation rate in these experiments was set to 1 µm/s. The median value and the quartiles are denoted for each cell type. (B) A typical example of two force-deformation curves.
obtained on the same spot of a fixed cell. For clarity, the curves are shifted horizontally with
respect to each other.

To further verify our experimental protocol against a positive control on living cells, we also
measured the stiffness of Cytochalasin D treated cells (Cytochalasin D disrupts actin
filaments making the cells softer). However, as keratinocytes have been shown to exhibit only
a weak response to Cytochalasin D at small deformations [13], we measured the stiffness of
MDA-MB-231 breast cancer cells before and after Cytochalasin D treatment. We chose this
cell line because it is one of the standard lines in our lab and because cancer cells are known
to respond to Cytochalasin D treatment [20]. The obtained results (Fig S1 in the Supporting
Material) are in accordance with previous reports, i.e., the observed softening of MDA-MB-
231 cells due to Cytochalasin D treatment was approximately 30% [13, 20].

Keratinocyte cortical stiffness
We compared the stiffness of an immortalized wild type cell line (NEB1) with an
immortalized keratin mutant cell line (KEB7), as well as with the stiffness of wild type
primary cells (NHEK2) (Fig 3 and Table S1 in the Supporting information). To test for a
possible viscoelastic behavior, we performed the indentation experiments at deformation rates
of 0.1 µm/s and 1 µm/s. Only measurements that exhibited a distinctive force-deformation
curve (Fig 1C) were taken into account. We found that the stiffness of severe keratin mutant
KEB7 cells was higher than that of immortalized wild type cells NEB1 when the lower bead
velocity was used (p<0.005 at 0.1 µm/s) but not significantly different at the higher bead
velocity (p=0.21 at 1 µm/s). The stiffness of primary wild type keratinocytes NHEK2 was
significantly higher than that of the immortalized wild type cell line NEB1 at both
deformation rates (p<0.005), which is in line with the previously published data on AFM experiments [21]. As expected, the stiffness of fixed cells is significantly higher than the one of any of the live cells (p<0.005).

**Fig 3. Cell stiffness of three keratinocyte types.** Cell stiffness of NEB1, KEB7 and NHEK2 cells was measured at two deformation rates 0.1 µm/s (orange) and 1 µm/s (blue). Stiffness of fixed NEB1 cells was measured at 1 µm/s. The median value and quartiles are indicated for each cell type, the numbers of measured cells are above the data points, and the p-values of relevant pairs are specified at the top.

In general, cells do not respond to mechanical stress as pure elastic bodies but exhibit a passive flow or an active remodeling of the cytoskeleton with timescales from several tenths of a second to several minutes [2]. With the deformation rate of 0.1 µm/s, the duration of the cell indentation is in the order of magnitude of seconds, which may cause cortex remodeling and apparent cell softening, as typically observed in experiments on cellular mechanics [20, 22]. However, the measured effect of the deformation rate on cell stiffness was small when compared to variations within a given rate (Fig 3). The only statistically significant cell softening at a slow deformation rate was observed for NEB1 cells (78 pN/µm at 0.1 µm/s vs. 105 pN/µm at 1 µm/s).

**Bead adhesion and membrane tether formation**
Keratins have been shown to participate in focal adhesions at the surface of the cells [23, 24]. To verify, if the mutations affect also the adhesion between the plasma membrane and the bead, coated with anti-integrin beta 1 antibodies, we quantified the adhesion events in the
indentation experiments (this adhesion can be detected as a positive force during bead retraction, Fig 1C). The incidence of adhesion in indentation experiments on different cell types is presented in Fig 4A. As expected, in all cases, a stronger adhesion was detected at the lower deformation rate due to a longer time of contact between the bead and the membrane. Wild type cells (NEB1) exhibited a larger proportion of adhesion events than the mutant cells (KEB7) (p=0.002 at 0.1 µm/s and p=0.17 at 1 µm/s). In all cell lines, the tether formation occurred in approximately 50% of adhesion events. In these experiments the relative magnitudes of the maximal force $F_{\text{max}}$ (Fig 4B) during bond rupture correlated with the adhesion strength and were larger for lower deformation rates. In the case of fixed cells, some adhesion between the bead and the cell also occurred, indicating the presence of non-specific adhesion, but no membrane tethers were formed (data not shown).

**Fig 4. Bead-membrane adhesion.** (A) The percentage of experiments with manifested bead adhesion on the cell membrane and subsequent membrane tether formation. A stronger adhesion was detected at the lower deformation rate due to a longer time of contact between the bead and the membrane. The vertical lines represent 95% confidence intervals. (B) The maximal force $F_{\text{max}}$ during bead retraction for experiments with manifested bead adhesion. $F_{\text{max}}$ during bond rupture correlated with the adhesion strength and was larger for lower deformation rates. The median value and the quartiles are denoted for each measurement.

**Discussion**

Data for vimentin IFs indicated that its disruption influences primarily the stiffness of the cell interior but not the stiffness of the cell cortex [11], and that the measured cell stiffness
depends on the indentation depth [25]. AFM studies on keratinocytes also showed that keratin disruption has a significant impact on cell stiffness under large deformations [12, 13, 21], but the role of keratin at small deformations that penetrate only a few hundreds of nanometers into the cell remained unexplored. The goal of our study was to compare the cortical stiffness of three keratinocyte cell lines, and in particular if the stiffness is influenced by the incidence of keratin particles in the periphery of mutant cells [7-10].

In established methods for assessing cell mechanics, the cells are typically indented from the top by moving an AFM cantilever or a piezo-stage with fixed OT. In the approach presented in this paper, the cells were indented from the lateral side by AOD-steered optical tweezers without employing moving mechanical components (Fig 1), which can be an advantage in many closed experimental systems, e.g., in microfluidic devices. The use of lateral indentation and a relatively large 5 µm microbead as an indenter allowed for straightforward microbead tracking and accurate measurement of the force-deformation curve. On the downside, the indentation could only be performed on the trailing edge side of the cell, where the cell geometry was relatively upright. In AFM experiments, the cell stiffness is often modeled by a Hertz model [26], which is sensitive with respect to inaccuracies in resolving the point of contact between the indenter and the cell. However, in our experiments the point of contact was hard to determine accurately, possibly due to the presence of a glycocalyx, which surrounds the cells and obscures their boundary. We therefore quantified the cell stiffness as the slope of the force-deformation curve in the linear part at a 200 nm indentation (Fig 1C). The value of this stiffness cannot be directly compared to the value of the Young modulus measured with AFM, but it is nevertheless a robust way to compare the cortical stiffness of different cell lines and to compare relative effects of cell treatments [2].
Large variations in the measured stiffness among cells within the same cell line were observed, possibly resulting from physiological and geometrical differences, which are always present in a population of living cells. In addition, there were variations between two consecutive measurements on the same spot of one cell (Fig 2), probably due to active remodeling of cells, which was apparent during experiments (Supporting Movie S1). To validate the protocol, we therefore measured the stiffness of paraformaldehyde (PFA) fixed cells and found that it was indeed significantly higher than the one of living cells as well as repeatable (Fig 2). Cell stiffening upon PFA fixation had been shown also in AFM measurements [27]. The protocol was further validated by measuring the stiffness of cells treated with Cytochalasin D, and the results (Fig S1) were in line with published data [13, 20]. In general, the variability among mutant KEB7 and primary NHEK2 cells was larger than the one among wild type NEB1 cells. Consequently, the latter was also the only cell type exhibiting a statistically significant dependence of the cell stiffness on the deformation rate.

The results of indentation experiments (Fig 3) revealed that the cortex in mutant keratinocytes (KEB7) was slightly stiffer than in the wild type cells (NEB1), but this stiffening was not statistically significant at the higher deformation rate and much less pronounced than the softening of the cell interior found in the studies by AFM [12, 13, 21]. Thus, these results strengthen the view that the main mechanical role of IF is in providing the bulk cellular stiffness. However, it is clear that keratins are also involved in cortical mechanics and it remains to explore if the observed cortex stiffening is related directly to the emergence of keratin particles in the periphery of mutant cells or indirectly to remodeling of acto-myosin cortex caused by impaired keratin filaments. Notably, the experiments also revealed that keratin mutations caused a reduction in adhesion between the membrane and the bead coated with anti-integrin beta 1 antibodies (Fig 4). This result is not unexpected because
it has been shown that keratin is closely related to structures at the cell surface, e.g., to focal
adhesions [14, 23]. Still, further studies are needed to scrutinize whether the reduced adhesion
in mutant cells is a consequence of an altered surface expression of the integrin or of less
specific pathways, such as a reduced interaction between the membrane and the underlying
actin cortex.

Finally, a notable finding in our study was that the cortex of the primary cell line (NHEK2)
was significantly stiffer than the one of the immortalized WT cell line (NEB1), which agrees
with the findings obtained with AFM for cell interior [21]. That indicates that the
immortalization process in vitro causes changes that induce cell mechanical softening, which
is an important feature given that many cancerous mutations undergo similar changes [28,
29]. Again, a mechanistic description of this process remains obscure.

In summary, our study showed that the cellular cortical stiffness can be examined by
indenting the cells laterally with AOD-steered optical tweezers without employing any
moving mechanical component, such as a cantilever or a piezo microscope stage. The method
was applied to compare three keratinocyte lines. The results showed the cortical stiffness of
primary keratinocytes was significantly higher than that of immortalized cells. The mutant
cells were also found to be slightly stiffer than wild type cells, but this difference was less
prominent that the softening reported in previous AFM experiments that probed the bulk
cellular stiffness. While the study largely supports the current understanding of the role of
intermediate filaments in cortical mechanics, it also indicates the need for further studies of
the interactions between keratin and the cellular cortex.
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Supporting information

S1 Fig. Validation of the experimental protocol with Cytochalasin D. The relative stiffness of breast cancer MDA cells before and after the treatment with Cytochalasin D, which disrupts the actin skeleton and softens the cells. After the treatment, the median stiffness decreased for 25% from 156 pN/µm to 117 pN/µm (p=0.02). The stiffness was measured at the deformation rate of 1 µm/s. The median value and the quartiles are indicated and the number of measured cells is denoted above the data points.

S1 Table. A summary of the results presented in Fig. 3.

| Cell Type | NEB1 | KEB7 | NHEK2 | fixed NEB1 |
|-----------|------|------|-------|-----------|
| deformation rate [µm/s] | 0.1  | 0.1  | 0.1   | 0.1       |
| N         | 29   | 71   | 42    | 21        |
| median value [pN/µm] | 78   | 132  | 218   | 366       |
| first quartile [pN/µm] | 70   | 73   | 130   | 261       |
| third quartile [pN/µm] | 90   | 207  | 336   | 566       |

S1 Movie. Brightfield microscopy recording of an indentation experiment. A microbead is trapped in an optical trap, pushed into the cell with a constant velocity and then retracted. The movie spans over 2 minutes. Continuous cell remodeling during the indentation experiment is clearly visible. A thin membrane tether, extracted from the cell upon retraction, is barely noticeable.
Figure 1
Figure 2
Figure 3
Figure 4