Fixed endothelial cells exhibit physiologically relevant nanomechanics of the cortical actin web

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Abstract
It has been unknown whether cells retain their mechanical properties after fixation. Therefore, this study was designed to compare the stiffness properties of the cell cortex (the 50–100 nm thick zone below the plasma membrane) before and after fixation. Atomic force microscopy was used to acquire force indentation curves from which the nanomechanical cell properties were derived. Cells were pretreated with different concentrations of actin destabilizing agent cytochalasin D, which results in a gradual softening of the cell cortex. Then cells were studied ‘alive’ or ‘fixed’. We show that the cortical stiffness of fixed endothelial cells still reports functional properties of the actin web qualitatively comparable to those of living cells. Myosin motor protein activity, tested by blebbistatin inhibition, can only be detected, in terms of cortical mechanics, in living but not in fixed cells. We conclude that fixation interferes with motor proteins while maintaining a functional cortical actin web. Thus, fixation of cells opens up the prospect of differentially studying the actions of cellular myosin and actin.

Keywords: cell mechanics, atomic force microscopy, cytoskeleton, fixation, cell cortex

1. Introduction
Fixation is a common means of preserving cell samples. It allows for much less difficult handling of the specimen because in contrast to live cells, fixed cells can withstand a wide range of temperature, electrolyte concentration and pH values and do not require complex culture media. Furthermore, unlike living specimens, fixed samples are suitable for long-distance transportation allowing in-depth analysis in special competence centres. However, until now it has been questionable whether the mechanical properties of fixed cells reflect those of living cells.

The mechanical properties of the cell are associated with cell function in many ways. For example, cell stiffness changes during the cell cycle [1], is affected by the aging of cells [2], controls the function of blood cells [3, 4] and is crucial for the spreading of cancer cells [5]. In the present study we focused on the stiffness of the cell cortex (i.e. a 50–100 nm thick layer beneath the plasma membrane [6, 7]) that is regarded as a functional cell compartment of its own [8]. In endothelial cells the mechanical properties of the cell cortex control the synthesis of nitric oxide (NO), the prominent regulator of vascular smooth muscle tone [9–11]. The elasticity of the endothelial cell cortex (K_cortex) therefore plays a crucial...
role in blood pressure regulation, in general, and in the pathogenesis of arterial hypertension, in particular. In a variety of other cell types the cortex plays a role during mitosis and cell migration [12, 13].

The cellular cortex is, on the one hand, made up of actin which forms a web of ultra-fine filaments. While polymerization of actin (i.e. the chain formation, from globular actin to filamentous actin) requires energy, once constructed actin filaments can be sustained virtually without energy consumption. On the other hand, the mechanical properties of the cortex are dependent on the activity of non-muscle myosin II which links actin filaments and can contract isometrically in order to create the basal cell tone. In return, myosin II activity consumes energy. Both actin and myosin are known to be of paramount importance for the mechanical properties of the cell cortex [14–17].

In order to assess the viability of fixed cells for the analysis of cell mechanics we performed stiffness measurements on both living and fixed endothelial cells with the help of atomic force microscopy (AFM). AFM has been shown to be a suitable tool for examining the mechanical properties of endothelial cells [18–21]. The obtained force-indentation curves allow the differential analysis of different cell layers [7, 8, 22, 23] and the measurement of \( K_{\text{cortex}} \).

In this study we treated endothelial cells with cytochalasin D (CD) which changes the G-/F-actin ratio by inhibiting actin polymerization [24]. In another series, we applied blebbistatin which inhibits myosin motor protein activity [16]. We used different concentrations of CD demonstrating that cortical myosin activity can be detected as a \( K_{\text{cortex}} \) decrease only in live cells. Fixed cells do not report any changes in cortical myosin activity.

2. Methods

2.1. Cell culture

Bovine aortic endothelial cells GM-7373 (DSMZ GmbH, Braunschweig, Germany) [25] were cultured in minimal essential medium (Life Technologies, Carlsbad, CA, USA) supplemented with 20% fetal calf serum (PAA Laboratories/GE Healthcare Bio-Sciences, Piscataway, NJ, USA), 1% non-essential amino acids (Life Technologies), 1% MEM vitamins (Life Technologies) and 1% penicillin and streptomycin (Biochrom, Berlin, Germany) at 37 °C and 5% \( \text{CO}_2 \).

2.2. Cortical stiffness measurements

Cells were seeded on glass bottomed dishes (Ø 40 mm, WillCo Wells, Amsterdam, Netherlands) and cultured until the cell layers were confluent. Monolayers were incubated with various concentrations of CD (10 to 100 nM; Sigma-Aldrich, St Louis, MO, USA) or 5 μM blebbistatin (Sigma-Aldrich) in HEPES buffer (concentrations in mM: 140 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 5 glucose, 10 HEPES, 1L-arginine, pH 7.4) at 37 °C for 30 min.

For experiments on fixed cells the cells were subsequently fixed with 0.1% glutaraldehyde (GA) or 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS; PAA Laboratories) for 15 min at room temperature. Subsequent to fixation some of the GA fixed cells were treated with 1% triton x-100 (Carl Roth, Karlsruhe, Germany) in PBS for 10 min at room temperature in order to disperse the plasma membrane.

All measurements were performed with the BioScope Catalyst (Bruker, Karlsruhe, Germany). We used cantilevers with spherical polystyrene tips with a diameter of 10 μm (nominal spring constant of 0.01 N m\(^{-1}\); Novascan Technologies, Ames, IA, USA). Cantilevers with large spherical tips allow indentation measurements with low loading forces (range: 200 pN) that do not disturb cell function. According to Hooke’s law the applied force (\( F \)) can be calculated from the registered deflection (\( D \)) after deflection sensitivity (\( S \)) and spring constant (\( C \)) were determined:

\[
F (\text{pN}) = S \left( \frac{\text{nm}}{V} \right) \times D (V) \times C \left( \frac{\text{pN}}{\text{nm}} \right).
\]

Furthermore, the indentation (\( I \)) of the sample can be calculated on the basis of the piezo displacement (\( P \)):

\[
I (\text{nm}) = P (\text{nm}) - S \left( \frac{\text{nm}}{V} \right) \times D (V).
\]

By plotting the force value (in Newton) against the indentation depth (in metre) one generates force-indentation curves from which \( K_{\text{cortex}} \) (pN nm\(^{-1}\)) can be derived:

\[
K \left( \frac{\text{pN}}{\text{nm}} \right) = \frac{F (\text{pN})}{I (\text{nm})}.
\]

Figure 1 displays typical force-indentation curves of living and fixed endothelial cells. The curves were evaluated...
using Protein Unfolding and Nano-Indentation Software (PUNIAS, http://site.voila.fr/punias).

Experiments with living cells were performed at 37 °C while experiments with fixed cells were performed at room temperature. K_{cortex} was derived from force-indentation curves that were recorded at 1 μm s^{-1} indentation velocity because the curves are less reproducible at higher velocities [26, 27]. In all experiments the cell cortex was indented right above the nucleus because cells are known to exhibit different stiffness properties at different cell sites [28].

2.3. Confocal imaging

Cells were seeded on glass cover slips (Ø 15 mm), cultured as described above and utilized in a subconfluent state. The treatment with CD and blebbistatin, and cell fixation were performed in a similar way as described for the AFM experiments. Then, cells were permeabilized with 0.1% triton X-100 in PBS for one minute at room temperature and washed three times with PBS. 10% goat serum (Sigma-Aldrich) was used as a blocking agent and applied for 30 min. The sample was then stained with Alexa Fluor 546 phalloidin (Life Technologies) and DAPI (Life Technologies) for 45 min, washed three times and stored in fluorescent mounting medium (Dako North America, Carpinteria, CA, USA).

Cell imaging was performed by means of a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). DAPI was used for staining the cell nucleus and Alexa Fluor 546 phalloidin for staining the actin filaments. DAPI was excited at a wavelength of 405 nm (emitted wavelength: 420 to 520 nm). Alexa Fluor 546 phalloidin was excited at a wavelength of 561 nm (emitted wavelength: 570 to 700 nm).

In order to analyse the fluorescence originating mainly from the cell cortex we chose a focal plane right above the cell nucleus. This is the first focus plane, optically sectioning the cell from top to bottom. In endothelial cells this first optical section represents mostly the fluorescent structures of the cortical compartment. Figure 2 sketches the chosen focal plane.

2.4. Statistics

Statistical dispersion is given as the standard error of the mean. The obtained data were analysed using t-tests if parametrically distributed. For all experiments with more than two groups we used Kruskal–Wallis–ANOVA because the data of some groups were not distributed parametrically. Data in all experiments were visualized as box plots with the borders of the boxes representing the 25% and 75% percentiles and the whiskers displaying outliers within 1.5 interquartile range of the lower and upper quartile, respectively. Results of all experiments were considered statistically significant if P<0.05 (indicated by *). For correlation analysis relative changes were calculated and R^2 was used as correlation coefficient. The number of experiments and the number of cells probed are indicated as N and n, respectively.

3. Results

3.1. Staining shows dose-dependent depolymerization of cortical actin in response to CD

The confocal images show cortical actin filaments and nuclei of endothelial cells. The cortical actin network of an untreated cell is mostly organized in the shape of fibres as can be observed in both the overview (figure 3(a)) as well as in the detailed image of the focal plane above the cell nucleus (figure 3(b)). Cells treated, prior to fixation, with a very low CD concentration of 10 nM are indistinguishable from control condition cells. However, after treatment with 50 and 100 nM CD the actin filaments (indicated by arrows) show an increasing disassembly (indicated by arrow heads) and the remaining filaments aggregate in spot-shaped clusters instead of fibres (indicated by asterisks).

3.2. CD-induced softening of the actin web can still be detected after fixation

The main objective of this work is to determine whether living and fixed cells exhibit comparable mechanical properties. The parameter chosen for answering this question is K_{cortex}. In order to explore the influence of different fixatives we performed stiffness measurements on living cells as well as on cells that were fixed with either GA or PFA.

With increasing concentrations of CD, living cells show a gradual decrease in K_{cortex} as expected (figure 4(a), N ≥ 8, n ≥ 32). Live control (not treated with CD) cells exhibit a stiffness of 1.76 ± 0.02 pN nm^{-1} while cells treated with 50 and 100 nM CD exhibit stiffness values of 1.26 ± 0.03 pN nm^{-1} and 1.2 ± 0.03 pN nm^{-1}, respectively. This reflects statistically significant decreases in K_{cortex} of 28% and 32%, respectively.

Control cells fixed with GA exhibit a K_{cortex} of 3.78 ± 0.03 pN nm^{-1} which indicates that their cortices are about twice as stiff as the cortices of living cells (figure 4(b), N ≥ 8, n ≥ 172). When cells were treated with 50 and 100 nM CD prior to fixation K_{cortex} decreases to 3.55 ± 0.04 pN nm^{-1} and 3.46 ± 0.03 pN nm^{-1}, respectively. The changes correspond to decreases in K_{cortex} of 6.0% and 8.6%, respectively. The correlation of the CD-induced changes in K_{cortex} detected in live cells and those fixed after CD treatment is highly significant (R^2 of 0.91, P<0.001).
Figure 3. Alexa Fluor 546 phalloidin (actin, red) and DAPI (nucleus, blue) staining of endothelial GM7373 cells on glass. The cells were pretreated with different concentrations of CD. (a) Overview images. (b) Close-up views. Arrows indicate the submembraneous actin web that disappears with increasing CD concentrations (arrow heads and asterisks).

Figure 4. (a) Cortical stiffness of live endothelial cells in response to CD. (b) Cortical stiffness of GA fixed cells in response to CD. (c) Cortical stiffness of PFA fixed cells in response to CD. (d) Correlation in $K_{\text{cortex}}$ between live and fixed endothelial cells.
Cells fixed with PFA respond similarly. $K_{cortex}$ is $4.09 \pm 0.04 \text{ pN nm}^{-1}$ in control cells and $3.82 \pm 0.06 \text{ pN nm}^{-1}$ for cells treated with 50 nM CD prior to fixation (figure 4(c), $N \geq 7$, $n \geq 144$). When all stiffness values were placed in one graph, a high correlation in mechanical stiffness between living and fixed cells is obvious (figure 4(d)).

In another set of experiments we removed the lipid bilayer of the plasma membrane of fixed cells with triton x-100. For this series we used cells treated with 100 nM CD and subsequently fixed with GA. By applying triton x-100 we aimed at exploring whether the integrity of the plasma membrane had an influence on the $K_{cortex}$ of fixed cells. As shown in figure 5 the presence or absence of an intact lipid bilayer has no impact on the mechanical properties of a fixed cell indicating that the submembraneous actin web determines cortical stiffness ($N \geq 2$, $n \geq 42$).

### 3.3. Myosin II contributes to cortical stiffness only in living cells

After we found that the mechanical properties of the actin web of living and fixed cells correlate highly with each other, we aimed at finding out whether this is also true for motor protein myosin II. While actin retains a filamentous structure virtually without energy consumption, myosin motor proteins crosslink different actin filaments in an energy consuming process. Myosin filaments thereby generate the basal tone of the cell. The activity of myosin can be inhibited with blebbistatin. As shown in figure 6, living cells show a significant decrease in stiffness when exposed to blebbistatin: the mean $K_{cortex}$ decreases from $2.07 \pm 0.05 \text{ pN nm}^{-1}$ to $1.53 \pm 0.04 \text{ pN nm}^{-1}$ which corresponds to a 26% decrease in $K_{cortex}$ (live cells: $N=2$, $n=18$, fixed cells: $N \geq 4$, $n \geq 43$). However, when the cells get fixed with GA after application of blebbistatin a decrease in $K_{cortex}$ cannot be observed anymore.

### 4. Discussion

Stiffness of cells is associated with cell function in a number of ways. It plays a role in cell migration, adhesion [12] and mitosis [13] and has been linked to pathological processes. When cells age [29, 30] and in diseases such as arthritis [31], asthma [32] and sickle cell anaemia [33], cells exhibit more rigid cytoskeletal properties. On the other hand, malignant transformation causes cells to become much softer [34].

Recently, the cortex of endothelial cells received attention because it is a crucial cellular zone that determines endothelial function [7]. The main structural component of the cell cortex is actin which stiffens the cell when globular monomeric actin (G-actin) assembles into actin filaments (F-actin). The G-/F-actin ratio and the stiffness of the cortex are regulated, among others, by the concentrations of ambient sodium [35] and potassium [8], and possibly the magnitude of the plasma membrane electrical potential difference [36]. A high extracellular concentration of sodium, slightly above the physiological range, stiffens the cortical actin web while, in contrast, high potassium causes the cell cortex to fluidize.

$K_{cortex}$ of endothelial cells is highly relevant for the function of blood vessels, because it regulates NO release [9]. NO is synthesized in the endothelial cell cortex and quickly reaches smooth muscle cells that form the outer layer of the vessel. There, NO causes the muscle to relax followed by vasodilation.

Another component besides actin that influences the submembraneous cell mechanics is non-muscle myosin II. The filamentous myosin crosslinks actin filaments and—in an energy dependent process—pulls them together and thereby creates the basal cell tone [16, 37].

In view of the fact that elasticity of the cell cortex is most likely a crucial parameter for the physiology of the vascular
system, we addressed the question of whether the mechanical properties of the cell can be analysed not only in living but also in fixed cells. The reason for the comparison ‘live versus fixed cortex’ was the potential advantage of fixed over live cells in terms of experimental handling. If it turned out that some important mechanical properties of the cell cortex are still retained despite fixation, then such a preparation could be most useful for medical diagnosis in specialized centres such as AFM laboratories.

With confocal imaging and AFM we show that $K_{cortex}$ is highly dependent on the amount of filamentous actin present in the cell cortex. Treatment of living endothelial cells with rising concentrations of CD changes the G/F-actin ratio, consequently causing softening of the endothelial cell cortex. Most importantly, this CD effect can still be observed if the cells get fixed after CD treatment. It turned out that the $K_{cortex}$ of living and fixed cells are highly correlated. The experiments using triton x-100 imply that an intact plasma membrane is not a prerequisite for such a correlation and thus can be neglected.

Cortical softening of the living cell can also be observed when myosin activity is inhibited with blebbistatin. Fixation eliminates this mechanical response. An explanation could be the energy dependent nature of the myosin II motor activity.

The fact that fixation interferes with motor protein activity but still allows one to study the function of the actin web opens up new experimental perspectives. Derived from the finding that a fixed cell has ‘dead’ myosin motors but still has functional actin we conclude that by comparing the mechanical measurements of ‘live versus fixed’ cells the contribution of the two cytoskeletal proteins—actin and myosin—could be differentially studied. For example, cancer cells harvested by surgery from patients could be fixed and sent to an AFM laboratory. There, without using any interim cell culture procedures, the mechanical properties such as cortical elasticity could be quantified and compared to those of a ‘reference’ cell. Fixation excludes many, often unknown, variables that could occur in live cells. Moreover, the handling of fixed cells is easy and safe, and the time between ‘harvesting’ and ‘diagnosis’ can be varied over a wide range.

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