Influence of microfluidic shear on keratin networks in living cells

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\textbf{Abstract.} Intermediate filaments play a key role in cell mechanics, providing cells with compliance to small deformations and reinforcing them when large forces are applied. Here, we present a study of networks of keratin intermediate filaments in living cells under the influence of external forces. We expose the cells to controlled shear forces applied by microflow and investigate the response of the keratin network \textit{in situ}. Our results show that bundle dynamics are reduced upon the application of shear flow. It is likely that cytoskeletal cross-talk is involved in this shear stress response via actin–keratin coupling.

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1. Introduction

Intermediate filaments (IFs) are part of the cytoskeleton along with microtubules, actin filaments and associated (binding) proteins and molecular motors. One function of these fibrous proteins is to withstand potentially harmful mechanical influences and to guarantee the integrity of the cell [1–3]. An example of the importance of mechanical properties for cells is found in cancer research: it has been shown that cancer cells show higher deformability than healthy cells [4].

In contrast to microtubules and actin filaments, which are highly conserved in different cells and different organisms, IF proteins are grouped into five categories according to their amino acid sequence. Types I and II of the IF family consist of keratin proteins, which assemble into keratin filaments. Keratins are further grouped into subcategories, an example of which is epithelial keratin [5]. In epithelial cells, keratin intermediate filaments (KIFs) form networks of bundles. This bundle formation greatly enhances the mechanical resilience of the KIF network [6–9], and the ability to withstand mechanical stresses is one of the major functions of KIFs [10–12]. This circumstance becomes most evident in diseases that are associated with keratin malfunctions. For example, keratin mutations may cause mechanical fragility of epithelial cells associated with diseases like skin blistering [13, 14].

Although one of the main functions of KIFs is to guarantee the mechanical stability of the cell, the intracellular KIF structure is not static but by contrast highly dynamic. These dynamics become evident on different length scales. On the scale of the entire cell, a keratin cycle takes place in which keratin disassembles near the nucleus and reassembles at the cell periphery [15, 16]. The KIF bundles themselves are dynamic displaying oscillations and undulations [17–19]. Additionally, on a molecular scale, there is a continuous exchange of keratin subunits between polymeric and soluble cytosolic keratin as revealed by fluorescence recovery after photobleaching (FRAP) experiments with epithelial cells containing K8/K18 keratin [18–20].

All these dynamic processes are influenced when the cells are exposed to shear stress. For very high shear stresses (3 Pa) and long time periods (24 h) the KIF network deteriorates [21].
When cells are sheared for shorter time periods, however, a reorganization of the KIF network takes place that effectively reinforces the network and helps to protect the cell by making it more resistant to shear stress. In particular, within 4 h at 1.5 Pa shear stress, Sivaramakrishnan et al observed that the KIF network mesh size becomes smaller near the cell periphery leading to a more uniform distribution of mesh sizes across the entire cell. This reorganization results in a 40% increase of the mean storage modulus [22]. For even shorter time periods of cell shearing (1 h at 0.7 or 3 Pa), thick and wavy tonofibrils are built, a process that is promoted by increased phosphorylation of the keratin [20, 23]. The network reorganization is furthermore accompanied by changes in the exchange rate of keratin subunits. Sivaramakrishnan et al found that the exchange rate between keratin subunits is about doubled when shear stress is present (FRAP recovery time: $t_{1/2} = 55 \pm 16$ min versus $t_{1/2} = 120 \pm 22$ min without shear stress) [20]. An immediate response to shear stress is observed as well: within 2–4 min at 0.15 Pa shear stress the majority of the KIF bundles are visibly dislocated in the direction of the flow as shown by Yoon et al [18].

All these studies support the idea that the keratin network plays a major role in serving the mechanical integrity of cells. The network structure flexibly adapts to externally applied forces. So far, however, it is unknown whether the bundle motion itself is affected by shear stress and by which mechanisms the observed KIF bundle dynamics are regulated. Here, we analyze the KIF bundle motion under shear stress (0.14 Pa) on a time scale shorter than necessary for network reorganization but after the direct response in the form of network displacement takes place. To this end, live-cell image sequences are analyzed. To generate controlled shear conditions, we use microfluidic methods. We find that the bundle dynamics are reduced when cells are exposed to shear forces and interpret this finding as cytoskeletal cross-talk between keratin and actin networks.

2. Materials and methods

2.1. Cell culture

The SK8/18-2 cell line was kindly provided by Rudolf Leube (RWTH Aachen, Germany). The cell line was derived from human adrenal cortex carcinoma SW-13 cells (ATCC CCL-105) [24], which were then stably transfected with DNA encoding for fluorescent human keratin hybrids (HK8-CFP, HK18-YPF) [19, 25, 26]. We seeded the cells on culture dishes in high glucose (4.5 g l$^{-1}$) Dulbecco’s modified Eagle medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal calf serum (Invitrogen GmbH, Darmstadt, Germany), 100 U ml$^{-1}$ penicillin and 0.1 mg ml$^{-1}$ streptomycin (Sigma-Aldrich, Munich, Germany). The cells were kept at 37 °C in a water saturated atmosphere with 5% CO$_2$.

2.2. Microfluidic devices

Microfluidic devices were fabricated by soft lithography [27] using a negative photo resist SU-8 3025 (MicroChem, Newton, USA) to obtain a structure width of 150 $\mu$m and a structure height of 40 $\mu$m. From the structured wafer, we then produced polydimethylsiloxane (PDMS) casts. One side of the channels was cut to later be able to insert cells into the microchannels, while at the other side holes were punched for the attachment of tubing. The PDMS casts were then sealed with a glass cover slip (see figure 1(a)).
Figure 1. (a) Sketch of a microfluidic device used for shear stress experiments on cells. The cells are inserted into the PDMS device by applying a cell suspension to the cut edge of the microfluidic channel. Shear flow is established via tubing which is connected to the device via holes in the PDMS. (b) Simulation of shear stresses in a microfluidic device with respect to the channel height (channel width: 150 µm; total channel height: 40 µm). The wall shear stress is about 0.14 Pa when there is no obstacle in the microfluidic channel. The maximum shear stress at the top of the cell is about 0.27 Pa.

For insertion of the cells into the microfluidic device, the cells were detached from the culture dish with trypsin and rinsed with cell culture medium. Several drops of this suspension (1 Mio cells ml⁻¹) were placed in front of the cut edge of the microfluidic device and sucked through the channel using a syringe from the opposite side of the channel. After inserting the cells, the device was covered completely with cell culture medium to prevent the medium in the channel from evaporating. The device was kept in a cell culture incubator overnight to allow the cells to adhere to the glass bottom of the channel.

Finite element method (FEM) simulations of the flow conditions in the microchannels containing single cells were performed using COMSOL Multiphysics 4.2 (COMSOL Multiphysics GmbH, Göttingen, Germany) based on the Navier–Stokes equation and under the constraint that velocities are zero at the channel walls (no slip). The typical dimensions of a cell were extracted from confocal microscopy z-stacks of several cells (Leica Confocal-TCS SP5/LAS AF setup, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). To this end, 18 cells were surveyed to extract their three-dimensional (3D) shape. Since the cells were randomly orientated in the channel we obtained on average a circular footprint of the cells. Based on these
confocal images, we approximated the cell to be a spherical cap with a total height of $9\,\mu\text{m}$ and a footprint radius of $17\,\mu\text{m}$. This information was used to model an average cell in the simulation. As output of the simulation, we obtained the flow velocity $u$ as well as the shear stress $\sigma$ inside the channel and thus also the shear stress distribution over the cell surface:

$$\sigma(x, y, z) = \mu \frac{\partial u(x, y, z)}{\partial z},$$

where $\mu$ is the dynamic viscosity and $u$ is the component of the flow velocity in the $x$-direction.

2.3. Live-cell imaging

The microfluidic devices were connected with polyethylene tubing to gas tight syringes (Hamilton, Bonaduz, Switzerland), which were actuated by precise pumps (neMESYS, Cetoni GmbH, Korbüfen, Germany). For image acquisition, we used an inverted epifluorescence microscope (IX 81, Olympus, Hamburg, Germany) with a water-cooled CCD camera (Hamamatsu Orca-R2; 1344 × 1024, Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany). To establish physiological conditions ($37^\circ\text{C}$ in a water saturated atmosphere with $5\%\text{ CO}_2$) during the whole experiment, the microfluidic device was placed in a top-stage incubation chamber (INU2E-ONICS, Tokai Hit, Shizuoka-ken, Japan). Images were recorded every 2 s with an exposure time of 35 ms using a 100×, 1.4 NA oil immersion objective. During the first 15 min, we observed the cells in the channel without flow for control. Afterwards, a flow of cell culture medium with beads (diameter 500 nm; 2% solid, diluted 1 : 50 000 in cell culture medium) was initiated where the flow rate was kept constant at $25\mu\text{L}\,\text{h}^{-1}$ leading to a wall shear stress of 0.14 Pa. The beads serve as passive markers for visual control of the flow. The bead concentration was low enough that the fluid flow was not affected. Furthermore, no direct interaction between the beads and the cells was observed. After the onset of flow and an additional 5 min waiting time, images were taken again for 15 min. We focused on a plane located under the cell nucleus, where the network shows reduced out-of-focus motion and network reorganization is minimal.

2.4. Data analysis

For recording live-cell images and no-neighbor filtering, xcellence software (Olympus, Hamburg, Germany) was used. We used MATLAB functions (version 7.9.0.529 (R2009b) with Image Processing Toolbox; The MathWorks, Natick, MA, USA) for further filtering (median, Wiener, Gaussian) to reduce noise in the images. The processed images were then analyzed with respect to network dynamics. As a global approach to data analysis, image-to-image cross-correlations with subpixel accuracy were performed using a Matlab program by Guizar-Sicairos et al [28]. In brief, the normalized cross-correlation function $G(\tau)$ was calculated:

$$G(\tau) = \left\langle \frac{F(t, t + \tau)}{\sqrt{F(t, t)F(t + \tau, t + \tau)}} \right\rangle, \quad \text{with}$$

$$F(t, t + \tau) := \max_{x', y'} \left\langle I(x, y, t)I(x + x', y + y', t + \tau) \right\rangle,$$

where $I(x, y, t)$ is the recorded fluorescence intensity at time $t$ and pixel coordinates $x, y$. Here, $F(t, t + \tau)$ was determined by Guizar-Sicairos’ program, the result of which was used to obtain $G(\tau)$ using a self-written Matlab program. For improved computational efficiency $F(t, t + \tau)$
was calculated in Fourier space. Furthermore, the script by Guizar-Sicairos carried out drift detection and correction in the images.

In addition to this analysis of a complete image stack, we analyzed local responses of the keratin network to shear forces by direct tracking of individual nodes in the network. The dynamics of network nodes induce changes in node morphology in addition to 3D motion. This leads to slightly out-of-focus regions within an image, making automatic node tracking very challenging. Hence, we opted for a manual approach to node tracking. In this procedure, the cell images were upsampled by a factor of 5 through bicubic extrapolation. The center of the node was then marked for every image using an interactive Matlab program.

Having determined the node positions, we calculated the radius of gyration $R_g$, a parameter that describes the spreading of an ensemble of $N$ points with their position vectors $\vec{r}_k$. In our case, $R_g$ characterizes the spreading of the trajectory of a keratin network node.

$$ R_g^2 = \frac{1}{N} \sum_{k=1}^{N} (\vec{r}_k - \langle \vec{r} \rangle)^2, $$

where $\langle \vec{r} \rangle$ is the position of the centroid of the trajectory.

Furthermore, we investigated the time-averaged two-dimensional (2D) mean square displacement of the node positions:

$$ \langle (\Delta r)^2 \rangle = 4D_{\text{eff}} \tau^\beta, $$

where $\tau$ corresponds to the time shift between two images and the exponent $\beta$ characterizes the type of motion ($\beta < 1$ subdiffusive, $\beta > 1$ superdiffusive). This equation was used in analogy to studies of 2D Brownian motion, where $\beta = 1$. In analogy to passive Brownian motion, we use this relation as a measure for the mobility of the network nodes, although, in our case, the motion is based on active processes. Accordingly, the diffusion constant becomes an effective diffusion constant $D_{\text{eff}}$. We assume a 2D motion of the nodes corresponding to the fact that we observe a projection of the node motion to the focal plane of the microscopy setup. Nevertheless, the approximation of 2D motion is a good representation of the real node motion because in the observed region of the cell the nucleus and the channel surface restrict the node fluctuations mainly to a plane.

2.5. Blebbistatin experiments

Cells were grown on culture dishes overnight. Before treating the cells with blebbistatin (Sigma-Aldrich, Munich, Germany) a time sequence of the fluorescent KIF network in the cells was recorded over 2 min with time intervals of 2 s. Blebbistatin treatment was carried out by removing cell culture medium and replacing it with 1 ml of 100 $\mu$m blebbistatin in cell culture medium. After a time period of 4 h following blebbistatin addition, we recorded a second time series of the KIF network dynamics over 2 min with 2 s intervals between consecutive time frames.

3. Results

3.1. Shear stress distribution in the microchannels

To characterize the applied shear stress, we perform simulations of the flow properties inside our microfluidic chamber. The result of an FEM simulation of the shear stress distribution inside
Figure 2. Example of an image-to-image cross-correlation. Exponential decay fits to the cross-correlations are shown as dashed/solid lines. We find that for small time shifts $\tau$ the curve decays faster when the flow is absent (blue data points). Here, the characteristic decay time $t$ for no-shear conditions is $69 \pm 1$ s which increases to a value of $184 \pm 4$ s when shear flow is present (decay time errors are fit errors).

the microfluidic channel is shown as a function of the channel height in figure 1(b). We obtain a maximum shear stress of 0.14 Pa at the top or bottom surface of the channel in our layout (solid line, channel height: 40 $\mu$m; width: 150 $\mu$m). We have a nearly linear shear stress distribution inside the channel when there are no obstacles present. When a cell with typical dimensions is placed inside the channel, the reduced height leads to higher flow velocities resulting in higher shear forces of up to 0.27 Pa (dashed line).

3.2. Global approach: two-dimensional cross-correlation

Knowing the shear stress distribution in the channel, we characterize the influence of shear stress on the bundle dynamics of the keratin network. To this end, we separately analyze typical time scales for bundle oscillations before and during shear stress application by cross-correlating fluorescence microscopy time sequence images of the keratin network under the cell nucleus for each condition. For the shear stress condition, the first 5 min of recording are excluded from the cross-correlation to allow the cell to adjust to the new condition. The time period of 5 min is chosen since Yoon et al have shown that within 2–4 min after the application of flow most of the bundles are singularly and collectively displaced in the direction of flow [18]. Thus, the results of the cross-correlation solely reflect changes in the bundle oscillations without being influenced by the immediate, flow-induced dislocation of the KIF network. By calculating the cross-correlation, we obtain a value which describes the similarity between two images. This value is averaged for all image-to-image correlations of one image stack, pairwise with a certain time shift $\tau$ (see an example for a typical cell in figure 2). By fitting an exponential function to the data, we obtain characteristic decay times that reflect the typical time scales for bundle dynamics. Here, we find larger average decay times when shear flow is present ($210 \pm 15$ s).
3.3. Local effects of shear flow on the keratin network

The previously described cross-correlation approach characterizes the bundle motion in the KIF network in its entirety in the region of interest (ROI) under the nucleus. To complement this global approach, we also analyze the local bundle dynamics under shear and no-shear conditions by tracking individual nodes of the keratin network in the same ROI. An example of a typical node trajectory is shown in figure 3(a). To characterize node dynamics, we calculate the radius of gyration according to equation (3). When exposing the cells to shear forces the radius of gyration decreases from an average of 0.18–0.13 µm (38 nodes in 10 cells). An estimate of the error, containing node tracking inaccuracies as well as the standard error of mean (SEM) of the averaging, leads to about 0.01 µm. The spreading of the node trajectories is reduced when shear flow is present as compared to no-shear conditions.

To further describe the dynamics of the keratin network nodes, we calculate the mean square displacement of each node and extract an effective diffusion constant. According to equation (4), we obtain this constant by plotting the mean square displacement on a logarithmic scale and by fitting the data of each node linearly (example shown in figure 4). In our case, node dynamics are not characterized by thermal Brownian motion. Instead, the parameter $D_{\text{eff}}$ represents an effective diffusion constant. We find that the average value of $D_{\text{eff}}$ decreases on average from $(1.57 \pm 0.01) \times 10^{-16} \text{m}^2 \text{s}^{-1}$, when there are no shear forces, to a value of $(0.83 \pm 0.01) \times 10^{-16} \text{m}^2 \text{s}^{-1}$ under shear conditions. This result confirms our previous observation that the nodes of the keratin network move less when shear stress is applied than in the absence of shear.

Two possible explanations may account for the reduced KIF bundle motion: the passive material properties of the KIF bundles may be changed or active processes may influence the

Figure 3. (a) Typical trajectory of a node in the keratin network. After extracting the positions of the node for each time point, the trajectory is corrected by the cell centroid motion. (b) The radius of gyration of the trajectory decreases when shear flow is present. Data are based on the evaluation of 38 nodes of the keratin network in 10 cells (error bars are SEM).
Figure 4. (a) Evaluation of the mean square displacement of the network nodes gives access to the effective diffusion constant. (b) The effective diffusion constant decreases when shear flow is present (error bars are SEM).

KIF motion indirectly by affecting the KIF surrounding (see the discussion). Direct influence of motor proteins on keratin can be excluded, since there are no such IF associated motor proteins known. The most prominent active components in the cell that regulate pre-stress of the cytoskeleton are myosin motors acting in conjunction with the cytoskeletal actin network. To address the question of whether myosin motors are involved in the observed KIF network dynamics, we inhibit myosin II motor activity with the drug blebbistatin [29] in an experimental setup without shear stress. When comparing the position of the KIF network in a cell at two points in time ($\Delta t = 30$ s) of an untreated cell, a displacement of the KIF bundles is clearly visible (see figures 5(a) and (c)). After an incubation of 4 h with blebbistatin in cell culture medium the KIF bundle motion is clearly reduced (figures 5(b) and (d)).

4. Discussion

We have investigated the influence of micro-flow-induced shear stress on the keratin bundle dynamics in a region below the nucleus of SK8/18-2 cells. The motivation to study the response of the keratin bundle motion to mechanical stress is that keratins are assumed to help withstand external mechanical forces on a cellular level.

In our analysis, we have followed both a global approach to characterize bundle motion by calculating cross-correlations of time series of microscopy images of cells containing a KIF network, and a local approach by tracking single nodes in the keratin network. The results from both approaches show that the motion of the keratin network is reduced within minutes when the cells get sheared by the applied flow. There are two possible explanations for the observed reduction in KIF bundle motion. Firstly, the passive mechanical properties of the keratin network itself could be changed under shear stress leading to a stiffer KIF network. Secondly, the cells may respond actively to shear stress by altering motor protein activity which enhances the effective rigidity of the cell.

In line with the first possibility mentioned above, rheology measurements by Ma et al have revealed strain stiffening of in vitro keratin gels upon increased shear [30], which is caused by pulling out entropic fluctuations in the bundles [31]. However, in living cells this
Figure 5. (a), (b) Fluorescence images (ROIs) of SK8/18-2 cells at two different time points, color coded ($\Delta t = 30$ s). As shown in the intensity profile (c), taken along the blue line in (a), the KIF bundles in cells without any external influence show a distinct displacement due to active motion. When cells are treated with the drug blebbistatin, which inhibits myosin II motor activity, for 4 h the bundle displacement is reduced (d). The scale bar corresponds to 5 µm.

strain hardening effect should be less important because the keratin is not directly exposed to externally applied shear stress. Nevertheless, network stiffening has been observed in KIF networks of living cells after exposure to shear stress on time scales of hours. Here, the underlying process leading to network stiffening is an active reorganization of both the KIF network structure and the KIF bundle itself. Sivaramakrishnan et al [20] showed that after 1 h at 3 Pa the keratin network in cells is reinforced and thicker bundles are formed. Additionally, the mesh sizes of the KIF network reduce after shear (1.5 Pa after 4 h) near the periphery of the cell, resulting in a more homogeneous network across the whole cell and an increase in the average cell elasticity [22]. This behavior is related to an enhanced phosphorylation of keratin that leads to a faster keratin subunit exchange when shear stress is present [23].

We have focused our analysis on the network below the cell nucleus, where network reorganization is not as pronounced as elsewhere in the cell. Moreover, we have looked at time scales that were small enough to exclude reorganization and the consequent network stiffening
as a possible cause for the reduced KIF bundle motion. Hence, we can exclude strain stiffening as the major cause for the observed reduced bundle activity. By contrast, an indirect mechanism caused by altered motor protein activity could play a role. Evidence supporting this idea has been provided in a previous study by Woo et al [32], who have pointed out that intracellular calcium concentration is increased as soon as epithelial cells get mechanically sheared by at least 0.009 Pa. Calcium, in turn, is known to regulate myosin activity. The actin-associated myosin motors then contract the actin network, which may in turn influence keratin bundle motion. Since the actin network spans the cell in a cable-like structure, as revealed by fluorescence micrographs (data not shown), we postulate that the enhanced myosin activity increases the effective actin network rigidity which is transmitted to the KIF network. This could be achieved either by direct coupling of the actin and the keratin networks through coupling proteins, or by the confinement imposed by the entire elastic cytoskeleton surrounding the keratin bundles [33]. In fact, mechanical coupling proteins between actin and keratin, such as members of the plakin family, are known [34–36]. Additional experimental evidence confirming myosin II motor protein involvement in KIF bundle motion has been provided in our experiments in which myosin II activity was inhibited by the drug blebbistatin in the absence of shear stress. Here, we see that the observed KIF bundle dynamics are reduced 4 h after drug addition (figure 5). This demonstrates that the observed bundle motion is not Brownian and indicates that myosin II is involved in driving the KIF bundle dynamics. If, in turn, myosin II activity changes upon shear stress application, so will in effect the KIF bundle dynamics itself. Hence, we suggest that the enhanced myosin activity upon shear stress application increases the rigidity of the actin network and pre-stresses the network, which in turn restricts the KIF bundle motion leading to the observed reduction in KIF bundle dynamics.

A simple estimate supports the idea that the observed bundle motion is not Brownian and instead dependent on motor protein activity. From the mean square displacement analysis, we know that the maximum squared distance that a KIF network node travels is of the order of $10^{-13}$ m$^2$. Multiplying this squared distance with a typical mesh size of the KIF network ($\sim 1 \mu m$) we obtain a volume scale of $10^{-19}$ m$^3$. If we assumed the motion of the keratin network to be Brownian and purely thermal we would estimate the elastic modulus of the network by dividing the thermal energy $k_B T \approx 4 \times 10^{-21}$ J by the previously calculated volume scale. We thus arrive at a modulus of 0.04 Pa which is extremely small. Walter et al [37] showed that the elastic modulus of an isolated, epithelial keratin network is about 10 Pa. This estimate clearly shows that the observed bundle motion is not Brownian but arises from active processes.

In conclusion, shear stress does not only affect the keratin network organization which leads to a reinforced network within a few hours, as has been shown before, but there is also a fast response to shear within a few minutes observed in the bundle motion. We would like to suggest the following idea of the processes taking place in the sheared epithelial cells. External shear flow may initiate an increase in the intracellular calcium concentration, possibly via mechanosensitive ion channels. This increase enhances myosin motor activity, leading to a higher effective actin network rigidity that is transmitted directly or indirectly to the KIF network. As a result, the KIF bundle motion is reduced, leading to more persistent network dynamics. This hypothesis needs to be confirmed by further experiments involving, for example, labeling of the actin network and members of the plakin family to study the actin–keratin coupling or by studying dynamics in composite, in vitro keratin-acto-myosin gels.
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