Cell adhesion via the α5β1 integrin receptor to fibronectin

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Motivation

Cell adhesion regulates a wide range of fundamental processes such as the development of individual organisms, cell proliferation and differentiation, wound healing, and immunological processes. Any aberration of mechanisms involved in cell adhesion and migration has a serious impact on the individual health. Therefore, one of the main interests of cell biology labs is to study molecular mechanisms that are involved in the regulation of cell adhesion to the extracellular matrix [1,2].

Integrins are the major adhesion receptors that mediate cell attachment to the extracellular matrix [3]. There are many functional assays available that allow crude measurement of cell attachment. However, most do not allow the precise evaluation of adhesion forces. The use of single cell force spectroscopy provides detailed information about forces and is thus an excellent tool to measure specific molecular contributions to cell attachment [4-6].

We used a CellHesion® 200 setup to analyze attachment of human foreskin fibroblasts (HFF) to the extracellular matrix protein fibronectin (FN). The rounded cells attached to the cantilever have a diameter of about 15 µm. When they attach to the cantilever which is decorated with extracellular matrix, these cells can be stretched to several times their original length before detaching. The CellHesion® 200 pulling range of 100 µm was sufficient to allow force measurements that exceeded the stretching capabilities of the cell under the chosen conditions.

CellHesion protocol

The first step of the assay required the attachment of a cell to a functionalized cantilever. After attachment to the cantilever the cell was lowered onto a fibronectin (FN) coated glass bottom dish for 5 s. The software recorded the cantilever approach and then retraction with the cell detachment forces.

1. *Functionalization of the cantilever*

For most experiments we coated the cantilever with commercially available fibronectin (20 µg/ml), to which HFF cells readily adhere. Coating of cantilevers was performed overnight at 4°C. We found adherence of cells to the cantilever improved by allowing FN to dry on the cantilever (2 min under sterile laminar flow) after rinsing it with PBS. Unused FN-coated cantilevers were kept for later use in sterile PBS at 4°C (up to 3 days).

Fig. 1: CellHesion 200 head and Life Science stage mounted on Zeiss Axiovert 200 with light transmission condenser for Phase contrast and DIC illumination.

2. Microscope setup

The force spectroscopy setup was installed on an Axiovert 200M (Zeiss, Jena, Germany, Figure 1) with a Roper CoolSnap HQ monochrome camera (Photometrics, Tuscon, Az, USA) attached. The camera was driven by IP Lab software (BD Biosciences, Rockville, MD, USA), and the force spectroscopy setup was driven by JPK's CellHesion 200 software. Images were captured with a 20X magnification lens (Zeiss).

3. Calibration

Tipless TL1 cantilevers from Nanoworld with a nominal spring constant of 0.03 N/m were used. The cantilever was mounted on the AFM and calibrated on uncoated glass bottom dishes filled with 2.5ml of PBS. To determine the spring constant of the cantilever the JPK-supported thermal noise method was used. Due to the lower noise the second resonance peak with the denoted correction factor of 0.251 was used (for more information see [7]).

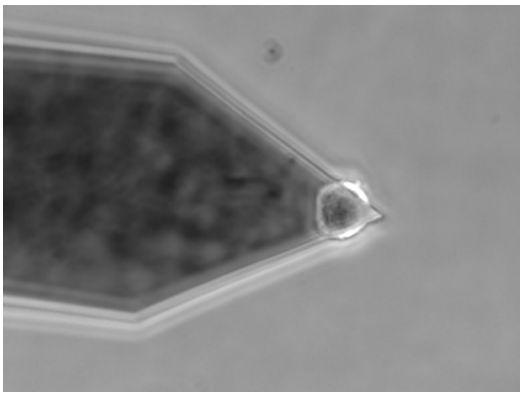

Fig. 2: Phase contrast image of a HFF cell attached to the tip of a FN-coated cantilever (TL-1).

4. Cell attachment to cantilever

Cell attachment was performed in a glass bottom dish (WPI, Herts, UK) where half of it was coated with 10µg/ml FN and the other half with 1% BSA (in PBS) at 4°C overnight. After rinsing twice with PBS, cells were plated sparsely in serum-free HEPES buffered (25mM) culture medium in dishes. Cells on BSA did not tightly adhere, which enabled easy capture by the FN coated cantilever. The glass bottom dish was placed in a heating device (JPK PetriDishHeater) that enabled performing experiments under physiological conditions (37°C). After attachment to the cantilever, cells were allowed to equilibrate for 30min. Figure 2 shows a phase contrast image of a HFF cell after attachment to a TL-1 cantilever.

5. Force measurements

Cell attachment curves were acquired for a cell contact time of 5 s to FN in constant height mode and an applied force of 1nN. Approach and retract speed were set to 5µm/s. Cells were allowed to contact the area of attachment 5 times in minute intervals before a new contact area was chosen. After force measurements to 4-6 different areas, drugs or antibodies were added to the glass-bottom dish. Antibodies diluted in a volume of 300 µl were added with a 1 ml syringe attached with a connection tube to the fluidic port of the holder of the glass bottom dish. After 30 min incubation, force measurements were continued under modified conditions.

Cell adhesion to fibronectin

The main adhesion receptor of a fibroblast is the α5β1 integrin, which mediates cell attachment to FN. After having attached the cell to the cantilever, force measurements were performed with contact times of 5 s and an applied force of 1 nN in constant height mode. The short contact time of 5s was chosen to avoid integrin-mediated cytoskeletal reorganization and resulting contributions of actomyosin-induced contractile tension to the force spectrum (see also [5]).

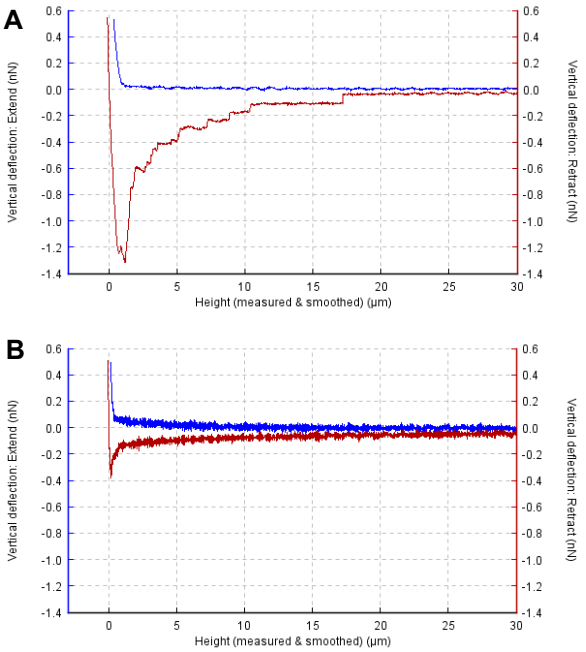

Fig. 3: Measurement of HFF attachment to FN (A). Attachment is inhibited using a β1-integrin adhesion-blocking antibody (B). Blue and red lines represent the approach and retraction curve, respectively. The force curves have been processed to set the baseline and contact points.

After several cycles of force measurements, specific integrin function blocking antibodies were added to the medium and after further incubation for 30min force measurements were continued. Typical force curves before and after addition of β1 integrin-blocking antibodies are presented in Figures 3 and 4.

There are several different parameters that can be extracted from such force curves in order to characterize the adhesion. One critical parameter is the maximum adhesion force reached during the retract cycle. It is also important to have a measure of the separation distance required to break all the cell contacts. In the example curve from Figure 3A, it can be seen that the cell must be moved around 17 µm from the FN-coated surface before the last connection is broken. The force measurements in Figure 3B on the antibody-blocked surface have a much lower peak adhesion force (around 0.3 nN compared with 1.3 nN) and adhesion range.

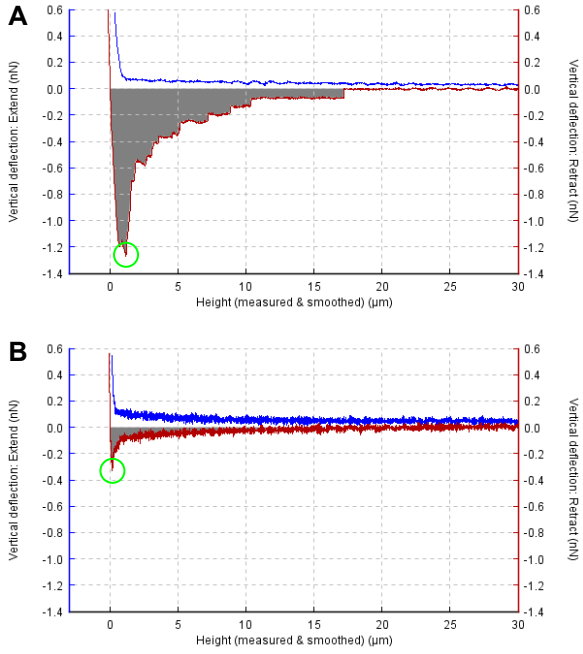

Fig. 4: Processed data from Figure 3 of HFF cells attached to FN (A). Attachment is inhibited using a β1-integrin adhesion blocking antibody (B). The lowest point of the retraction curve represents the maximal unbinding force, and is marked with a green circle. The area enclosed by the retraction curve (colored gray) describes the work required for cell detachment.

We analyzed the force-distance-curves using the JPK data analysis software with (i) baseline and tilt correction for the retract curve, (ii) measurement of the maximum (adhesion) force peak, and (iii) calculation of the area under the curve (Figure 4). The baselines for approach and retract curves can be vertically shifted, because of hydrodynamic drag. The tilt indicates cantilever drift (for more details see [8]). The area under the curve gives a measure of the work (energy) required to remove the cell from the surface, and can be an equivalent to the adhesion energy [5].

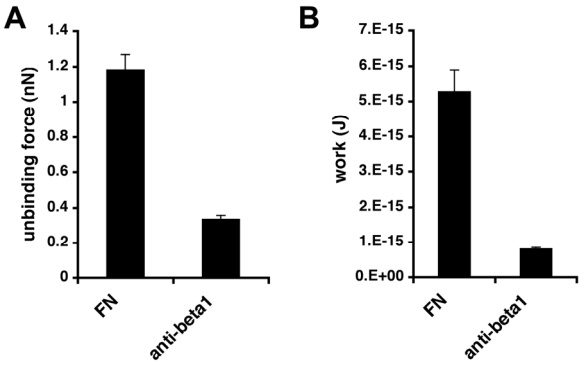

Fig. 5: Quantification of unbinding (A) and work of removal (B) of non-treated cells and cells treated with a blocking anti-β1 integrin antibody (contact time 5s; A/B – average and s.d. of 61/65 curves respectively).

While maximal unbinding forces of HFF cells to FN reached levels of more than 1nN (Figure 5), with many tethers due to small unbinding events during detachment (Figure 4A), addition of blocking antibodies inhibited adhesion to background levels (Figures 4B and 5); background levels show no tethers and the measured residual binding was due to non-specific interactions.

Conclusion

The CellHesion® 200 AFM has enabled us to accumulate precise and controlled measurements of cell attachment forces to a given substrate. Such measurements will allow us to proceed with more complex experiments to identify the molecular basis of cell attachment regulation. The variability of the CellHesion system with its large pulling range together with the highly developed software for the analysis of multiple parameters will facilitate this research.

The integration of this setup on a fluorescence microscope and the use of GFP-fusion constructs will further allow the recording of molecular dynamics under force.

Literature

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