A new perspective on cell-cell adhesion

Introduction

In multicellular organisms, cell adhesion is fundamental to cellular organisation (i.e. tissue formation, development), cell cycle control, cancerous transformation, angiogenesis and metastasis, cell migration through tissues and cell layers and multiple events in the interactions of cells of the immune system. In short, cells are constantly in contact with other cells and with the surrounding extracellular matrix, and these contacts modulate cell activity.

The study of cell adhesion is complicated by a number of factors, including the heterogeneity of molecules involved in the adhesion of any given cell. To effectively study cell adhesion, the contribution of various molecules to a given adhesion event need to be distinguished. Force-spectroscopy using an atomic force microscope generates data containing information on non-specific and specific contributions to adhesion and in-situ experiments can be conducted to distinguish which molecules are involved. While some interesting studies on cell adhesion have been conducted using force spectroscopy [1,2] there has not been a commercially available instrument that can be used to effectively study cell-cell adhesion. Force-spectroscopy experiments on the adhesion of two cells can often require an effective pulling range of up to 80µm, due to the extrusion of membrane tethers during cell separation. With its extended pulling range the CellHesion can be used to study the binding of cells to, not only coated surfaces, but also other cells.

Overview of CellHesion protocol

There are a number of important steps in acquiring force spectroscopy data with an atomic force microscope. The central principle is based on bringing a cantilever-bound cell into contact with a second cell or a coated surface and monitoring cantilever deflection during separation. The basic steps involved are:

1. *Functionalize the cantilever*

The cantilever must be functionalized with a molecule to which a cell will bind with a stronger interaction than the cell with form with the surface to be tested.

2. *Calibrate the cantilever*

By calibrating the cantilever using inbuilt routines in the JPK SPM software, cantilever deflection will be presented in Newtons.

3. *Introduce cells to be bound to the cantilever in the sample chamber.*

By using the Biocell™ from JPK Instruments, the temperature of the sample can be controlled. As the CellHesion module is integrated into an inverted light microscope, phase contrast or fluorescence microscopy can then be used to select an appropriate cell to be bound to the cantilever.

4. *Attach cell to cantilever*

By using the light microscope the functionalized cantilever can be placed over the top of a specific cell and gently lowered onto the top of the cell. After a few seconds contact time the cantilever is retract and the cell should be attached. The cell is then allowed some time to form strong contacts with the cantilever

5. *Select a region to aquire force curves*

The light microscope can be used to select an area to collect force curves. In such a manner different regions of a target cell or monolayer can be specifically probed

6. *Acquire force curves*

After setting the desired parameters, the cantilever-bound cell is then brought into contact with the surface and cantilever deflection and separation distance monitored. By plotting the applied force (calculated automatically from cantilever deflection) against separation distance, the force required to break the component bonds involved in cell adhesion can be measured.

7. *Vary conditions and collect a second data set with the same cell.*

By using the JPK Biocell™ sample holder, after the acquisition of the first set of curves the conditions can easily be changed and the same cell used to collect a second data set. For a temperature sensitive mutant the temperature control of the Biocell™ could be used to take a set of force curves at permissive temperature, before increasing the temperature and collecting a second set. Alternatively, the Biocell™ is designed such that it is easy to introduce a blocking agent. In such a manner, the contribution of a particular ligand-receptor interaction to a particular cell adhesion interaction could be determined.

For a more detailed overview of the technique, see our technical report “Using the CellHesion module- a practical guide” or for a precise protocol please see reference [3]. The number of experimental questions that could be addressed with the CellHesion is extensive. Here we will describe two examples, one from developmental biology and the other from the field of oncolocy.

Zebrafish binding to fibronectin

During zebrafish gastrulation (a model for tissue morphogenesis during vertebrate gastrulation) cell adhesion is involved in germ layer formation with progenitor cells of different germ layers display varying adhesive properties. In such a complex system it can be difficult to distinguish the role of various proteins in adhesion and signalling. Recently, the question of whether a particular protein, Wnt11, that is known to be involved in the wnt signalling pathway during gastrulation, is also involved in adhesion was addressed using force spectroscopy [4].

Previous available data could not distinguish whether the Wnt signalling pathway mediated control of gastrulation movements was due to an effect on actual adhesion or on intracellular signalling mediated by adhesion molecules. To investigate this question the authors compared the binding of wild type and silverblick (containing a wnt11 mutation) cells to fibronectin coated surfaces.

The authors isolated primary cells from zebrafish embryos. A single zebrafish cell was selected using phase-contrast microscopy and attached to a cantilever. Multiple force-distance curves were obtained on fibronectin coated glass. Integrin binding was then blocked using an RGD peptide and a second series of force curves obtained. It was observed that the wild type cells exhibited a larger maximal unbinding force and more work required for total dissociation. In addition, the small force jumps corresponding to single unbinding events were mostly absent from the force-distance curves collected with the silberblick mutant, whereas there were multiple events such as these for the wild type, increasing with increased contact time. However, when the integrin binding of the wild type cells was blocked using the RGD peptide, the force jumps corresponding to individual unbinding events were significantly reduced in number.

From these data the authors conclude that Wnt11 is involved in the binding of speficic cells to fibronectin during gastrulation. As experiments conducted using force spectroscopy directly measure the force of cell interactions, the effect of mutations on adhesion vs a signalling response to adhesion can be distinguished. Additionally, the authors show that, in this case, the binding to fibronectin in the wild type cells can be blocked using RGD peptide, indicating that the effect of Wnt11 on cell adhesion to fibronectin is mediated by an effect on integrin binding. In further experiments it was shown that Wnt11 also has a role in E-Cadherin mediated binding of mesendodermal cells [5].

Melanoma binding to endothelial cells

The progression of melanoma is linked with changes in cell surface markers. The untransformed melanocytes in the basement membrane have a low proliferative capacity as their cell cycle is tightly regulated by interaction with surrounding keratinocytes. On transformation, melanoma cells switch their cadherin profile and lose E-cadherin mediated contact with keratinocytes, displaying instead N-Cadherin which promotes cell-cell interactions with fibroblasts and endothelial cells (For a review see [6]). In addition, the distribution of integrins at the surface changes such that integrins become associated with actin-based

ridge-like structures (Figure 1) that protrude from the surface [7]. As such, the interaction of the transformed melanoma cells with surrounding tissue changes with disease progression. As melanoma progresses the cells invade the dermis and then metastasize, at which point patient prognosis becomes significantly worse.

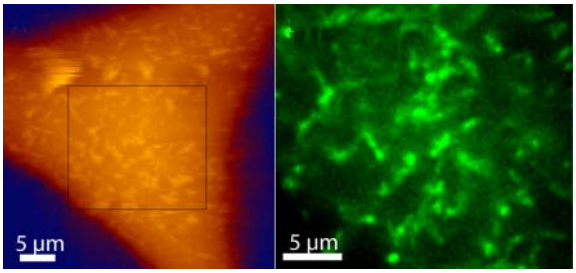

Fig 1. Atomic force (left) and corresponding fluorescence (right) images of melanoma cells with FITC-phalloidin labelled actin

The changing surface molecular expression linked with melanoma transformation reflects changes in which the cells interact with the extracellular matix (for example via beta1 integrin) and with other cells (for example cadherin mediated interactions with endothelial cells). To demonstrate that the CellHesion and force spectroscopy can be a powerful tool for investigating adhesion of cancer cells to surfaces involved in disease progression, force-distance curves of the binding of WM115 melanoma cells were acquired on fibronectin coated surfaces and on endothelial cells [8].

WM115 cells were released from culture dishes by washing with trypsin/EDTA, followed by incubation of 20 min in PBS without Ca^{2+}/Mg^{2+}. After harvesting by centrifugation, cells were resuspended in media without serum, but supplemented with HEPES. After half an hour the WM115 cells in suspension were injected into the Biocell™, which contained a coverslip coated with fibronectin. After allowing the cells to settle, a WM115 cell was attached to the ConA-coated cantilever. A series of force-distance curves were acquired at 37°C, in constant height mode, with an applied contact force of around 500 pN and a contact time of 10 seconds. The cell was brought into contact with the fibronectin-coated surface not more than 10 times on any single position, to avoid artefacts that may arise from passivation of the surface. After the acquisition of a set of control curves, an RGD peptide was added to block the any integrin-mediated binding. A second set of curves was then acquired.

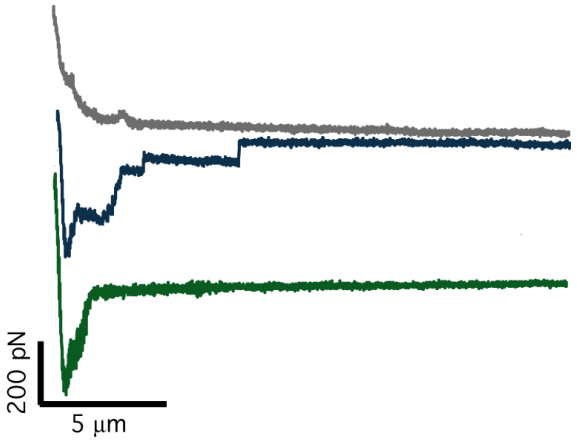

Fig 2. Force-distance curves of WM115 interaction with a fibronectin coated surface. The grey curve is during approach, the blue curve corresponds to a typical retract curve before blocking and the green after blocking with RGD peptide.

Presented in Figure 2 is a representative force-distance curve from before (blue) and after (green) blocking with RGD peptide. The grey curve represents the approach curve. It can be seen that before blocking with RGD peptide, there were a number of discrete unbinding events, downstream of the initial rupture of bonds. After blocking integrin binding, these discrete unbinding events disappear and only the initial peak remains. These data indicate that the initial unbinding event is predominantly non-specific interactions between the cell and the fibronectin-coated glass surface. The downstream events, however, correspond to the unbinding of specific integrin-fibronectin interactions.

Traditionally, cell binding assays looking at the adhesion of melanoma cells have used human umbilical vein endothelial cells (HUVEC) to study melanoma cell binding. The CellHesion has been used to determine whether such studies can be reproduced in terms of force-distance

curves, as opposed to cellular binding over the course of 24 hours.

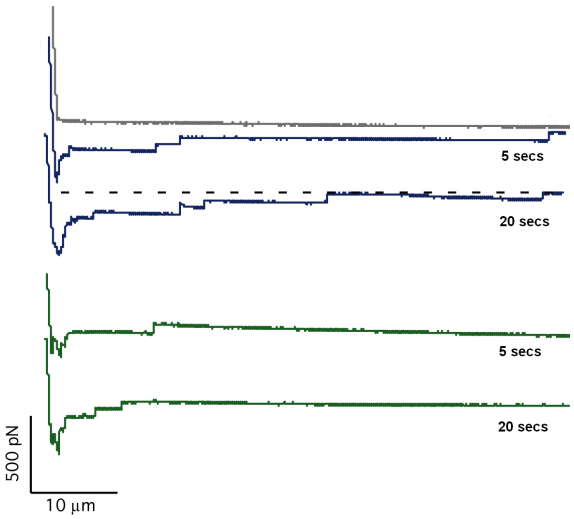

Fig. 3 Force-distance curves of WM115 cell binding to HUVEC monolayers. The blue curves correspond to typical retract curves before the addition of EGTA. The green curves are typical retraction curves after the addition of EGTA.

WM115 melanoma cells were released from tissue culture dishes as described above. After the 30 minute recovery period these WM115 cells were introduced into a Biocell™ containing a monolayer of HUVEC cells. After being allowed to settle, a single WM115 cell was attached to the cantilever. Force-distance curves were acquired between the cantilever-bound WM115 cell and the surface associated HUVEC monolayer. At a pulling distance of 60 µm, control curves were obtained with contact times of 5 and 20 seconds. After the acquisition of a set of curves EGTA was introduced into the Biocell™, in order to chelate the divalent metal ions in the media. Treatment with EGTA blocks Ca^{2+} dependent cadherin-mediated binding.

Representative force curves are presented in Figure 3. Force curves obtained before the addition of EGTA are displayed in blue, those after in green with the approach curve presented in grey. It can be seen that again there is an initial unbinding event that corresponds to both specific and non-specific interactions. Before blocking with the EGTA, there are more specific interactions, and more membrane tethers form during separation. The treatment with EGTA does not block all of the discrete unbinding events- suggesting that cadherin is not the only specific receptor on the melanoma cell surface that interacts with the HUVEC cells.

Additional examples- cell attachment

The early stages of cell adhesion to various components of the extracellular matrix, or to other cells, has also been studied using single cell force spectroscopy experiments. Taubenberger and colleagues investigated the binding of $\alpha 2\beta 1$ integrin to defined collagen substrates using the CHO-A2 cell line, which lacks endogenous collagen binding integrins, but stably expressing the $\alpha 2$ integrin subunit [9]. They used single cell force spectroscopy to show that the $\alpha 2\beta 1$ integrin specifically binds collagen in a manner that is dependent on Mg ions, with adhesion of the cells to the collagen increasing over time for the cells expressing $\alpha 2\beta 1$ integrin. Additionally, they were able to show that a population of activated cells arises, where the individual bonds between cell and surface are broken at higher applied force, with contact times over 120 seconds. As such, it is possible to use this technique to study the changes in cell binding properties that occur after the initial binding of the cell to the surface.

Recently single cell force spectroscopy, in combination with RNA interference technology, has been used to study the contributions of galectins to the binding of MDCK cells to different components of the extracellular matrix [10]. The authors found that short term interactions of MDCK cells with laminin was not integrin dependent, but mediated by galectin-3 and -9 and carbohydrate interactions. In the case of the initial phase of MDCK binding to collagen I the opposite was found, with integrin mediated binding playing a role.

Such studies highlight the flexibility of such a technique for assessing the relative contributions of cell surface components to the process of cell adhesion.

Conclusion

Cell-cell adhesion is a complex process that is involved in not only the tethering of cells, but also in cell-cell

communication, tissue formation, cell migration and the development and metastasis of tumors. The complexity of cell adhesion and subsequent signalling, and the heterogeneity of the cell surface make it difficult to identify the contribution to cell adhesion by individual elements. Force spectroscopy, using an atomic force microscope, can allow one to distinguish between interactions in the adhesion of a cell to a second cell or a coated substrate. In situ blocking experiments can help determine which surface molecules are involved in cell adhesion to a given surface. Such experiments will prove useful in distinguishing between effects of mutations on cell adhesion vs adhesion mediated signalling, as shown for the effect of the silverblick mutation in zebrafish gastrulation [4]. This system could also be used to investigate the contribution of various surface proteins in the progression of various tumors. Above and beyond the applications alluded to here, the CellHesion could be used for the investigation of a number of other systems, from interactions between cells of the immune system to the interactions involved in the invasion of cells by intracellular pathogens. The extended pulling range of the CellHesion and its integration into an inverted, optical microscope now enables force-spectroscopy to be conducted at the level of cell-cell adhesion, opening the door to a new field of investigation.

References:

[1] Benoit M., Gaub H.E. "Measuring cell adhesion forces with the atomic force microscope at the molecular level". Cells Tissues Organs. 172:174-89. (2002)
[2] Zhang X., Chen A., De Leon D., Li H., Noiri E., Moy V.T., Goligorsky M.S. "Atomic force microscopy measurement of leukocyte-endothelial interaction". Am. J. Physiol. Heart Circ. Physiol. 286:H359-67. (2004)
[3] Franz C., Taubenberger A., Puech P.H., Müller D.J. „Studying integrin-mediated cell adhesion at the single-molecule level using AFM force spectroscopy." Sci. STKE (406), pl5. (2007)
[4] Puech P.H., Taubenberger A., Ulrich F., Krieg M., Muller D.J., Heisenberg C.P. "Measuring cell adhesion forces of primary gastrulating cells from zebrafish using atomic force microscopy". J. Cell Science, 118:4199-206. (2005)
[5] Ulrich F., Krieg M., Schötz E.M., Link V., Castanon I., Schnabel V., Taubenberger A., Mueller D., Puech P.H., Heisenberg C.P. "Wnt11 functions in gastrulation by controlling cell cohesion through Rab5c and E-Cadherin". Developmental Cell, 9:555-64. (2005)
[6] Haass N.K., Smalley K.S., Li L., Herlyn M. "Adhesion, migration and communication in melanocytes and melanoma". Pigment Cell Res. 18:150-9. (2005)
[7] Poole K., Muller D. "Flexible, actin-based ridges colocalise with the beta1 integrin on the surface of melanoma cells". Br. J. Cancer. 92:1499-505. (2005)
[8] Puech P.H., Poole K., Knebel D., Mueller D.J. "A new technical approach to quantify cell-cell adhesion forces by AFM". Ultramicroscopy. 106: 637–644 (2006).
[9] Taubenberger A., Cisneros D.A., Friedrichs J., Puech P.H., Muller D.J., Franz C.M. „Revealing early steps of $\alpha 2\beta 1$ integrin-mediated adhesion to collagen type I by using single-cell force spectroscopy" Mol. Biol. Cell. 18:1634-1644. (2007)
[10] Friedrichs J., Torkko J.M., Helenius J., Teräväinen T.P., Füllekrug J., Muller D.J., Simons K., Manninen A. "Contributions of galectin-3 and -9 to epithelial cell adhesion analysed by single cell force spectroscopy. J. Biol. Chem. 282:29375-29383 (2007).