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BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
12 (2016) 1013–1021



nanomedjournal.com

Original Article

Quantifying cellular mechanics and adhesion in renal tubular injury using single cell force spectroscopy

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Received 12 July 2015; accepted 2 December 2015

Abstract

Tubulointerstitial fibrosis represents the major underlying pathology of diabetic nephropathy where loss of cell-to-cell adhesion is a critical step. To date, research has predominantly focussed on the loss of cell surface molecular binding events that include altered protein ligation. In the current study, atomic force microscopy single cell force spectroscopy (AFM-SCFS) was used to quantify changes in cellular stiffness and cell adhesion in TGF- β 1 treated kidney cells of the human proximal tubule (HK2). AFM indentation of TGF- β 1 treated HK2 cells showed a significant increase (42%) in the elastic modulus (stiffness) compared to control. Fluorescence microscopy confirmed that increased cell stiffness is accompanied by reorganization of the cytoskeleton. The corresponding changes in stiffness, due to F-actin rearrangement, affected the work of detachment by changing the separation distance between two adherent cells. Overall, our novel data quantitatively demonstrate a correlation between cellular elasticity, adhesion and early morphologic/phenotypic changes associated with tubular injury.

From the Clinical Editor: Diabetes affects many patients worldwide. One of the long term problems is diabetic nephropathy. Here, the authors utilized atomic force microscopy single cell force spectroscopy (AFM- SCFS) to study cellular stiffness and cell adhesion after TGF1 treatment in human proximal tubule kidney cells. The findings would help further understand the overall disease mechanism in diabetic patients.

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Key words: Atomic force microscopy; TGF- β 1; Fibrosis; Nanomechanics; Cell elasticity

A multifaceted condition, diabetic kidney disease encompasses a number of structural and function disturbances, including increased glomerular filtration rate, albuminuria and mesangial expansion. Of these changes, tubulointerstitial fibrosis (TIF) represents the key underlying pathology of diabetic nephropathy, with overwhelming evidence implicating isoform 1 of the pro-fibrotic cytokine transforming growth factor beta (TGF- β 1) as the principle mediator in early tubular injury.^{1,2} Characterised by loss of cell integrity and decreased expression of proteins critical to intercellular junction formation, tubular injury is linked to altered cell morphology, re-organisation of the cell cytoskeleton and *de-novo* expression of fibroblastic

markers.³ A pivotal step in progression of these events is the early loss of the epithelial cell adhesion protein, E-cadherin.^{4,5} Cadherins are important in forming the multi-protein adherens junction (AJ) that links the cell exterior to the actin cytoskeleton.⁶ The extracellular domain of E-cadherin mediates ligation with partner proteins on adjacent cells, whilst the cytoplasmic domain binds to β -catenin, linking cadherin to the actin cytoskeleton. Interaction of cadherin with F-actin not only increases the adhesive strength of the junction, but also acts as a signalling ‘hub’ for proteins that influence adhesiveness and initiate intracellular signalling.

In addition to the many biochemical processes altered in tubular injury and renal fibrosis, changes in cell architecture ultimately contribute to impaired tubular function. Investigation of single cell mechanics is essential for the fundamental understanding of disease progression and the development of potential therapies.⁷ AFM based force spectroscopy can be utilised for the study of single cell mechanics in various pathophysiological conditions. Recent studies by Buckley et al⁸ investigated a role for TGF- β 1 in the mechanical properties of

AFM-SCFS was made possible by the support of an equipment grant from Diabetes UK (BDA: 12/0004546). This work was also supported by a Leverhulme Trust (PRG-2012-738) and an EFSD/Janssen Kidney grant.

The authors declare no conflict of interest.

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<http://dx.doi.org/10.1016/j.nano.2015.12.362>

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alveolar epithelial cells (A549) undergoing epithelial to mesenchymal transition, a process in which cells are converted from an epithelial to mesenchymal phenotype and thus contribute to, and exacerbate the state of pulmonary fibrosis. Their data showed a strong correlation between cellular elasticity and cytoskeletal arrangement and suggested that the mechanical dynamics of transitioning alveolar epithelial cells are greatly controlled by their cyto-architecture. Elasticity is defined as the ability of a cell to resist an applied force or stress and return to its original configuration when the stress is removed. If the cell is assumed as linear elastic material, the elasticity can be expressed quantitatively by modulus, such as Young's modulus, which is a measure of the intrinsic mechanical properties of a material as a resistance to deformation under the applied stress. Deformation is the degree of a cell transforming from an original shape to a deformed shape. In the current study we have applied AFM-single cell force spectroscopy (SCFS) to investigate the effects of early TGF- β 1 induced tubular injury on single cell mechanics and cell-to-cell adhesion. The AFM-SCFS system incorporates an improved positioner that allows displacement measurements up to 100 μ m for completely separating two adherent cells, at a high force resolution (\sim pN) over a large dynamic range (\sim 5 pN to \sim 100 nN). Fitted with a spherical bead-attached cantilever the system permits indentation of single cells and enables cell elasticity to be calculated from the measured force-displacement curves using the Hertzian contact mechanics model.

Using the well established human proximal tubule kidney (HK2) cell line, the main aim of our study was to determine the effect of the pro-fibrotic cytokine TGF- β 1 on cytoskeletal re-arrangement and E-cadherin mediated cell-adhesion. These early events are linked to tubular injury, renal fibrosis and ultimately end stage renal disease (ESRD). We provide novel evidence that elevated levels of TGF- β 1 modify the mechanical properties of single cells, such as elastic (*E*) modulus, due to reorganization of the cytoskeleton. In addition, systematic investigation of the separation process of two adherent cells suggests that the corresponding changes in the elastic properties of cells, contribute to the loss of adhesion characteristics, apart from the surface molecular ligation. To our knowledge, this is the first study that correlates single cell mechanics and loss of cell adhesion in early proximal tubular injury.

Methods

Materials

HK2 cells were purchased from the American Type Culture Collection (ATCC; Gaithersburg, MD 20878, USA). Recombinant human TGF- β 1, fibronectin and TRITC-conjugated phalloidin was obtained from Sigma (Poole, UK). Tissue culture media and plastic ware were purchased from Invitrogen Life Technologies (Paisley, UK). Immobilon P membranes (Millipore, Watford, UK), ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK) and anti-fade Citifluor (glycerol/PBS solution: Agar Scientific, Essex, UK) were also obtained. For preparation of compartmental protein a Qproteome kit was

obtained from Qiagen (Sussex, UK). E-cadherin antibody was obtained from R&D systems.

Maintenance of HK2 cells

HK2 cells (passages 18–30) were maintained in DMEM/Hams F12 (DMEM/F12) medium, supplemented with 10% foetal calf serum (FCS), glutamine (2 mM), and EGF (5 ng/mL). Cells were seeded onto 40 mm Petri-dishes and cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air. Prior to treatment, cells were cultured in DMEM/F12 low glucose (5 mM) for 48 h. Basal (5 mM) glucose culture media were generated as described previously.⁹ For all experiments, cells were cultured in low glucose containing un-supplemented DMEM/F12 medium for 48 h. Cells then treated with TGF- β 1 (2–10 ng/mL) for 48 h. In all experiments, cells were serum starved overnight before agonist stimulation.

Immunoblotting

Cytosolic proteins were prepared and separated by gel electrophoresis and electro-blotting onto Immobilon P membranes as described previously. Membranes were probed overnight with a specific polyclonal anti-E-cadherin antibody (1:1000).

Immunocytochemistry

Cells at 80% confluence were fixed with 4% paraformaldehyde (PFA). Following blocking, the nuclear stain 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; 1 mM) was added for 3 min. Cells were then incubated with TRITC-conjugated phalloidin (Sigma) diluted at 1:100 in PBS-Triton for 1 h at 25 °C. Fluorescence was visualised using an Axiovert 200 fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK).

AFM-SCFS

Experiments were performed using the CellHesion®200 module (JPK Instruments, Berlin, Germany) that was installed on an Eclipse TE 300 inverted microscope (Nikon, USA). During each experiment, cells were maintained at a physiological temperature (37 °C) by combining the BioCell™ temperature controller (JPK, Berlin, Germany) with the AFM stage. Phase microscopy images were acquired using a CCD camera (DFK 31AF01 Firewire, The Imaging Source, Germany) connected on the side port of the microscope. The whole AFM-FS set-up with the CCD camera was driven by JPK's CellHesion200 software. Images were captured using a 20 \times magnification lens. The entire optical microscope and AFM headset-up was supported on an anti-vibration table (TMC 63-530, USA). Arrow sensors (TL1, Nanoworld AG, Switzerland), which are tipless cantilevers with a force constant of 0.03 N/m, were used for performing SCFS measurements.

Cell-cell adhesion experiments

The tip-less cantilevers were chemically functionalized for a single suspended cell to be attached. Initially the cantilevers were sterilised by UV treatment (15 min), before being incubated in poly-L-lysine (25 μ g/mL in PBS, 30 min, RT) and then

fibronectin (20 µg/mL in PBS, 2 h, 37 °C).¹⁰ After functionalization cantilevers were stored in PBS solution at 4 °C and used within 3 days. To record a force curve for calibration, the cantilever was configured to approach the base of a cell-free Petri-dish once, to minimise the loss of coating (set-point <2 V). Suspended cells were dispensed into the Petri dish using a pipette. Free cells stick on the substrate within 2–3 min, hence the cell-cantilever attachment procedure was performed rapidly (<1 min), with set-point force and contact time at 0.8–1.0 nN and 8–10 s respectively. During the contact period, the instrument was set in a constant force mode, in which force is kept constant by adjustments of the piezo-actuator height. Once a single cell was attached to the cantilever, it was left to recover for at least 5 min to form strong binding with the functionalized surface.¹¹

The cantilever-attached cell was brought in contact with another cell adhering on the substrate, until a preset contact force of 1 nN was reached. The cells remained in contact for 10 s, in which surface bonding was formed. Next, the cantilever was retracted and force versus displacement measured until the two cells were completely separated. The procedure was repeated three times for each cell tested, with 30 s intervals between each measurement. The attached cell was used to perform measurements on approximately 5 cells for each dish, using multiple dishes from at least 3 separate samples of cells in each experiment ($n = 3$).

Cell indentation experiments

Using a small amount of two-part fast setting epoxy glue (5 min), colloidal probes were prepared by gluing an 11 µm polystyrene microsphere (Polybeads®, Polysciences, USA) on a tipless TL-1 cantilever. The attachment procedure was performed on the stage of AFM with the aid of the inverted optical microscope. The microsphere was attached immediately by performing an approach curve directly above the sphere. The spring constant of the bead-attached cantilever beam was measured before each set of experiments using the thermal noise method. Each substrate cell was indented 5 times with an interval pause of 60 s, whilst force-displacement ($F-d$) curves were recorded simultaneously. For consistency, all cells were indented immediately at the position above the nucleus. Approach and retraction speed was kept constant at 5 µm/s to minimize hydrodynamic forces acting on the cantilever.¹² Prior to indentation of each cell, its height was determined¹³ in order to minimise the effects of the hard substrate in the calculation of E modulus. Since the displacement positions of the substrate and the plasma membrane were determined for each single cell prior testing, the depth of indentation was tailored for each cell under investigation. Eventually the E modulus was calculated from the acquired loading $F-d$ curves using the Hertz model. The criteria and assumption for applying this model to biological material are described elsewhere.^{14–17} However, it has been well recognised that the Hertz model often overestimates the elasticity of cells due to substrate effects. Alternatively, the classic Hertz model which was modified by Chen to take account of the influence of the substrate to the measured elasticity may be applied for modelling the cell indentation tests.¹⁸ Generally the Chen model

is valid for well attached cells which are joined closely with the substrate. However, it is worth pointing out that the cells in this study, were always indented at their central regions away from the cell boundary. In addition, the first 10% of the loading $F-d$ curves (i.e. cell under very small deformation) was used to calculate the cell elasticity, in order that the substrate effect could be reasonably ignored.

Data analysis

To process all force-displacement curves the JPK Data analysis software was used. To signify statistical differences data were evaluated using a paired t -test. Data are expressed as mean in the text and as median and interquartile range at the graphs. Number of experiments are represented with 'n'. $P < 0.05$ was taken to indicate statistical significance.

Results

TGF-β1 evokes classic changes of early tubular injury in human proximal tubule cells

The transition of tubular epithelial cells from a typical, cobblestone morphology to a fibrotic phenotype is commonly associated with reorganization of cell architecture and alterations in the expression of epithelial proteins involved in adherens and tight junction formation. HK2 cells were cultured in 5 mM glucose containing media for 48 h prior to overnight serum starvation. Cells were stimulated for 48 h with TGF-β1 (2–10 ng/mL) under serum-free conditions and morphological and phenotypic changes assessed. Under control non-stimulated conditions, control HK2 cells exhibited typical cobblestone morphology consistent with tubular epithelia. At 48 h, phase contrast microscopy confirmed that TGF-β1 induced a dose-dependent change in cell morphology towards an elongated fibroblast-like phenotype (Figure 1, A). Staining of TGF-β1 treated HK2 cells with TRITC conjugated phalloidin confirmed that these morphological changes were accompanied by reorganization of the actin cytoskeleton from a diffuse transcellular network of F-actin filaments that spanned the cytosol, into more dense peripheral stress fibres (Figure 1, B). The pro-fibrotic cytokine TGF-β1 (48 h, 10 ng/mL) altered the architecture of the cells and produced an elongated, fibroblast-like phenotype, characteristic of early TGF-β1 induced tubular damage.

Architectural reorganization of HK2 cells in response to TGF-β1 indicates that the cytokine can alter the stability of the adherens junction complex, an event that has serious repercussions for the integrity of the epithelial sheet. To resolve these changes, we examined the effect of TGF-β1 on expression of the epithelial cell adhesion protein E-cadherin. TGF-β1 induced a concentration-dependent decrease in whole-cell expression of E-cadherin to $19.3 \pm 5.7\%$ ($P < 0.05$), $11.3 \pm 0.6\%$ ($P < 0.001$) and $10 \pm 1.5\%$ ($P < 0.001$) as compared to control at 2, 4 and 10 ng/mL respectively ($n = 3$, Figure 1, C).

TGF-β1 evokes a variable effect on adhesion parameters

In order to relate changes in surface expression of E-cadherin to functional cell-to-cell adhesion, SCFS was used to quantitatively characterise the detachment process of control and

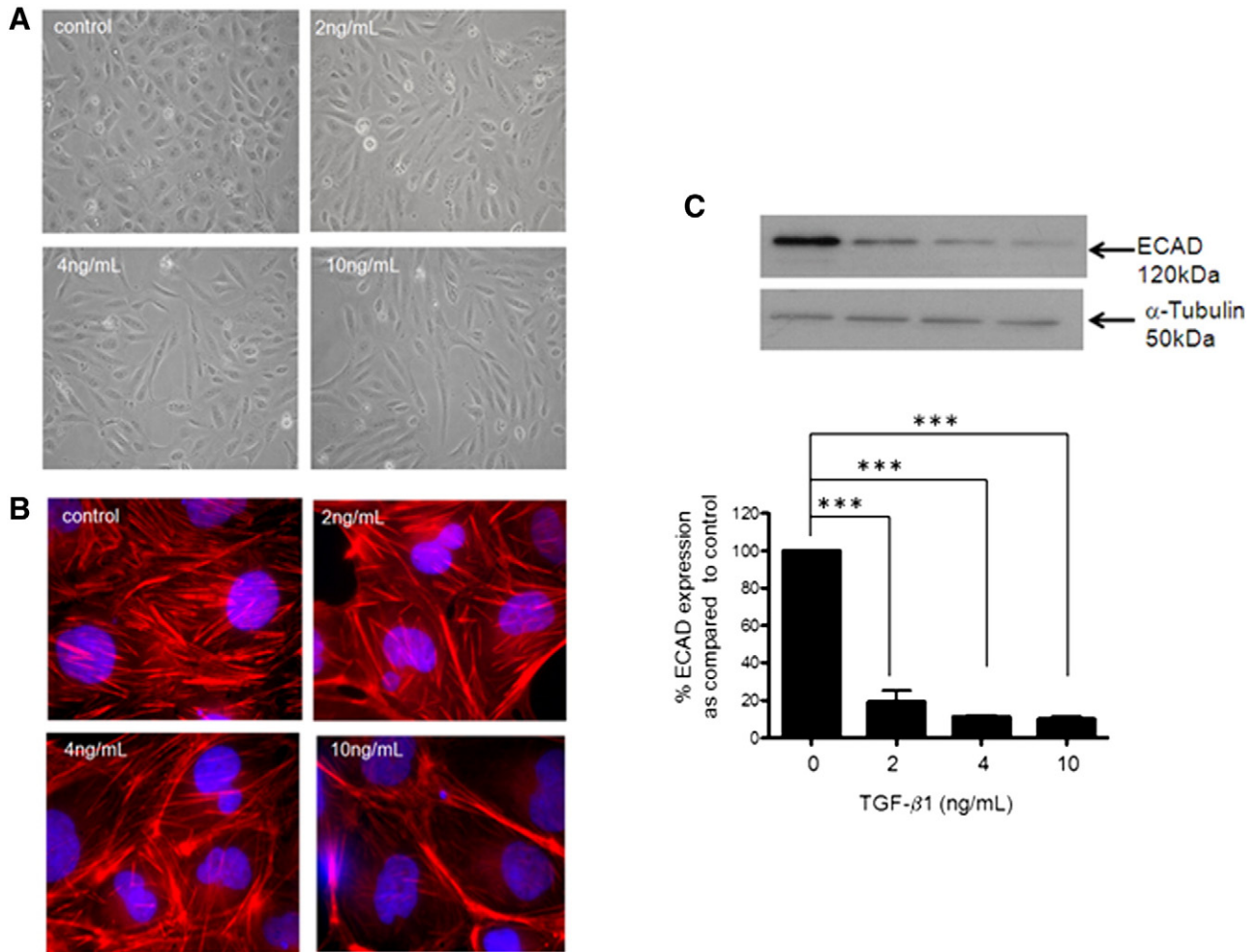


Figure 1. TGF- β 1 evokes classic morphological changes of early tubular injury in HK2 cells. To assess the effect of TGF- β 1 on cell morphology and cytoskeletal reorganization, HK2 cells were cultured in 5 mM glucose containing media for 48 h prior to overnight serum starvation. Cells were stimulated for 48 h with TGF- β 1 (2–10 ng/mL) under serum-free conditions and morphological and phenotypic changes assessed. Phase contrast microscopy (**A**) and TRITC conjugated phalloidin (**B**) confirmed the dose dependent effects of TGF- β 1 on cell morphology and cytoskeletal reorganization respectively. (**C**) A pivotal step in early tubular injury is the loss in expression of epithelial E-cadherin. Incubation of HK2 cells with increasing concentrations of TGF- β 1 (2–10 ng/mL) evoked a significant dose dependent decrease in E-cadherin at 48 h as confirmed by Western blot analysis. Upper panels show representative blots for each protein and re-probed for α -tubulin as a loading control. Lower panels show mean (\pm SEM) densitometry data, normalised against the non-stimulated low glucose control (100%), from 3 or more separate experiments. Each lane in the representative blot corresponds to the associated bar in the graph. Key significances are shown, $*P < 0.05$, $*P < 0.01$, $***P < 0.001$.

TGF- β 1 (48 h, 10 ng/mL) treated HK2 cells. The elongated shape of the treated cells was used as a visual marker of the transformation occurred after treatment (Figure 1, A). By using identical SCFS experimental specifications more than 40 cells from 5 separate experiments, were characterised in order to investigate the function of the adhesion molecule.

Retraction F - d curves provide important information regarding the adhesion parameters between the cells under investigation (the cantilever-attached cell and the cell adhering on the substrate). Figure 2 shows the various adhesion parameters that can be extracted from SCFS experiments. The retraction curve in Figure 2, A, illustrates the detection of the maximum unbinding force (F_{max}), the energy or work of complete detachment (W_d) and the maximum distance of complete separation (d_s). Complete detachment of

control cells was achieved by using an effective retraction range of at least 80 μ m, for 5 μ m/s speed. Figure 2, B, illustrates the detection of the tethering rupture events (TREs) during detachment between the two cells. TREs were identified by detecting sharp steps of force that occur in the x-axis, which correspond to surface bond ruptures.¹⁹ Sharp steps of force that are preceded by a ramp in force (j' events) occurred early in the pulling process. As the pulling distance was increased rupture events were preceded by a displacement plateau (t' events). As shown in Figure 2, B, a window was observed in which both j' and t' events were present, indicating the initiation of extended displacement plateaus. The subsequent range of the retraction curve was characterised by a large number of extended displacement plateaus, which are related to the ability of soft biological cells to deform.

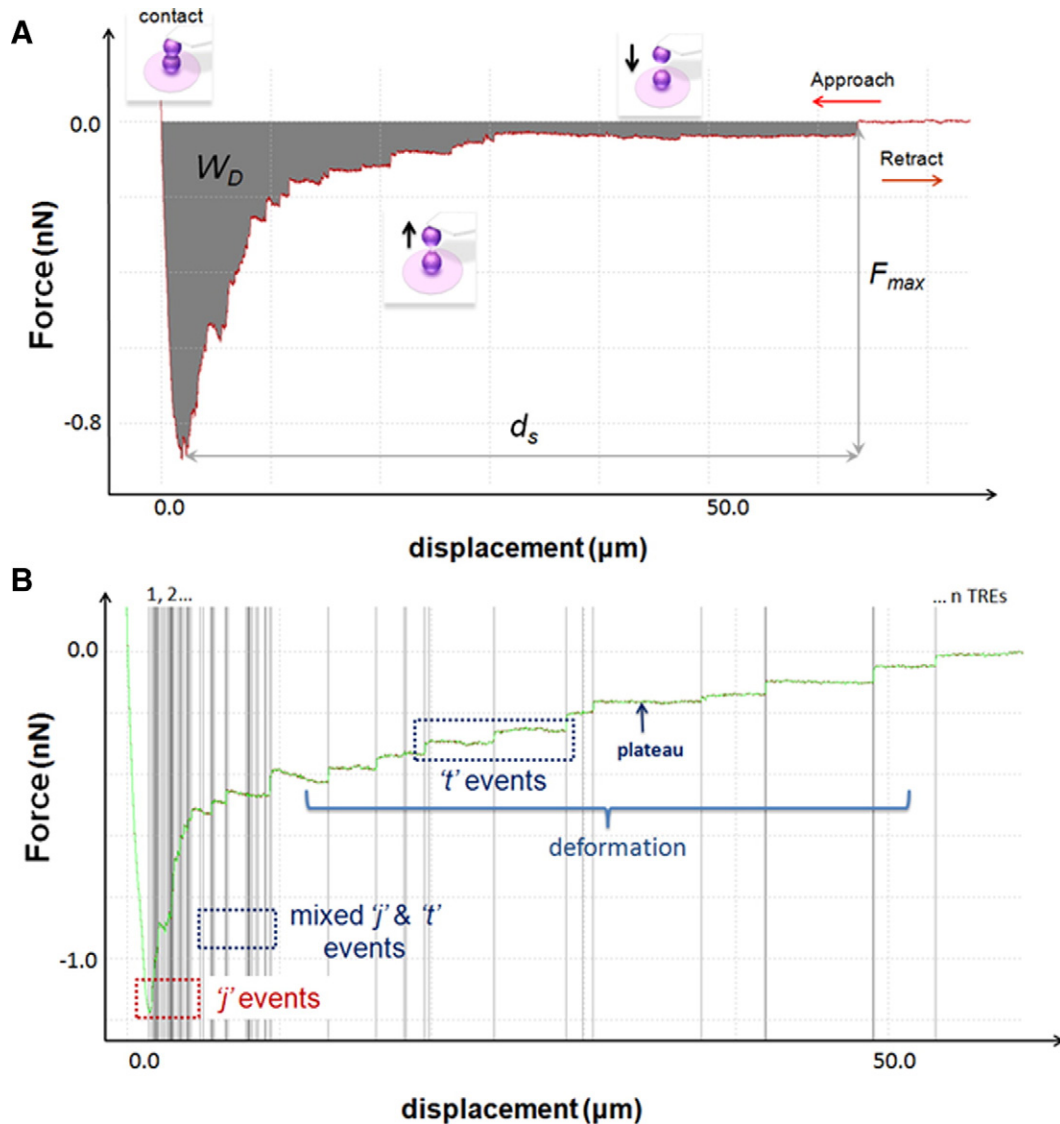


Figure 2. Retraction $F-d$ curves illustrating the various adhesion parameters that can be extracted from SCFS measurements. **(A)** Maximum unbinding force, F_{max} , is the difference between the maximum force value and the point of complete detachment. Work of detachment, W_D , is the integral of the continuous area under the baseline of complete separation. Distance of separation, d_s , is the difference between F_{max} and the point of complete separation. **(B)** The number of tethering rupture events, TREs, was calculated by detecting the j' events (force ramp preceded) and t' events (displacement plateau preceded).

More than 100 curves from 5 separate experiments were analysed and the data points are shown in Figure 3. The results indicate that TGF- β 1 decreased the number of tether rupture events by 35%, resulting in a decrease of the maximum unbinding force by 19%. However, the work of detachment (or adhesion energy) was decreased more significantly by 53%, consistent with a reduction of 46% in distance of separation (>30 cells, $n = 5$, $P < 0.001$) (Figure 3, A-D).

TGF- β 1 significantly increases single cell stiffness

In order to investigate the role of the cytoskeleton in the E-cadherin mediated functional adhesion measurements, single cell indentation was used to characterise cell elasticity. Extension

$F-d$ curves provide important information regarding the elastic properties of a cell by commonly fitting the Hertz model for spherical beads. Figure 4, A, shows an extension curve of a treated cell, illustrating the calculated values of E modulus with incremental fitting of the model. The contact point with the plasma membrane was found to be between 400 and 500 nm. The corresponding force range was 60–100 pN, rather than the initial point when the cantilever was deflected in the positive force value. In consideration of tip-cell adhesion, we have applied the Johnson–Kendall–Roberts (JKR) adhesion theory to recalculate the cell elasticity based on the loading curve of cell indentation testing under a spherical probe.²⁰ The elasticity difference determined by JKR and Hertz is between 2% and 10%; the detailed calculations are described elsewhere.²¹ Such a

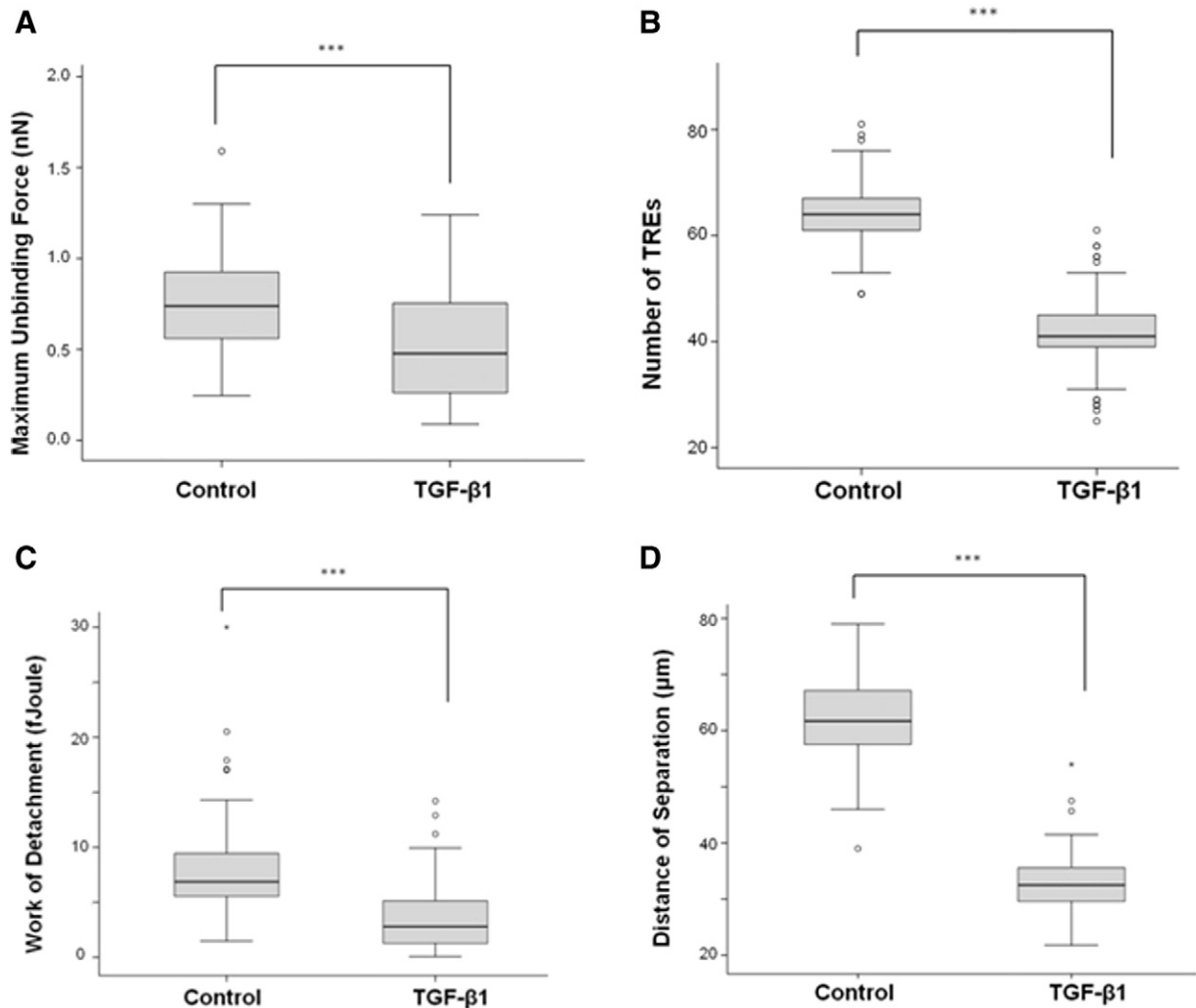


Figure 3. The effects of the cytokine TGF- β 1 (48 h, 10 ng/mL) on adhesion parameters. The values inside the box represent the first (lower value) and third quartile, the line within the box represents the median, the (–) shows the minimum and maximum observations and the (○) symbol indicates outliers. **(A)** The maximum unbinding force (decreased by 19%), **(B)** the number of tethering rupture events (decreased by 35%), **(C)** the work of detachment (decreased by 53%) and **(D)** the distance to complete separation (decreased by 46%) are shown. Key significances of more than 40 cells from 5 separate experiments are shown, *** $P < 0.001$.

minor variation shows the tip-cell adhesion may be negligible in the current testing. In fact such a low tip-cell adhesion was expected, because the spherical tips were not functionalized and the indentation experiments were conducted in liquid medium, which resulted in low magnitudes in both specific and non-specific adhesions. Figure 4, B, shows the changes in elasticity between the two groups of treatments, and is the culmination of processing more than 150 curves for each treatment obtained from 5 separate AFM-SCFS indentation experiments. The results were analysed using paired samples *t*-test and the data are expressed as mean \pm SEM. The data indicate that the TGF- β 1 (48 h, 10 ng/mL) increased the elastic modulus by 71% (>30 cells, $n = 5$, $P < 0.001$) (Figure 4, B), clearly showing that cells treated with the cytokine became considerably more rigid. The pro-fibrotic cytokine altered the architecture of normal HK2 cells and produced an elongated, fibroblast-like phenotype, characteristic of TGF- β 1 induced

tubular damage. These morphological changes were associated with reorganization of the cytoskeleton into peripheral stress fibres, which ultimately resulted in the alteration of the mechanical properties of the treated cells.

Discussion

It is well established that the pro-fibrotic cytokine TGF- β 1 is instrumental in mediating the morphological and phenotypic events of early tubular injury in diabetic nephropathy, where the loss in epithelial characteristics is associated with disassembly of the adherens junction. E-cadherin is the most characterised adhesion protein that mediates epithelial cell-to-cell adhesion.⁵ In the current study the effects of TGF- β 1 on E-cadherin mediated cell-to-cell adhesion in renal proximal tubule HK2 cells were systematically investigated using AFM-SCFS. The HK2

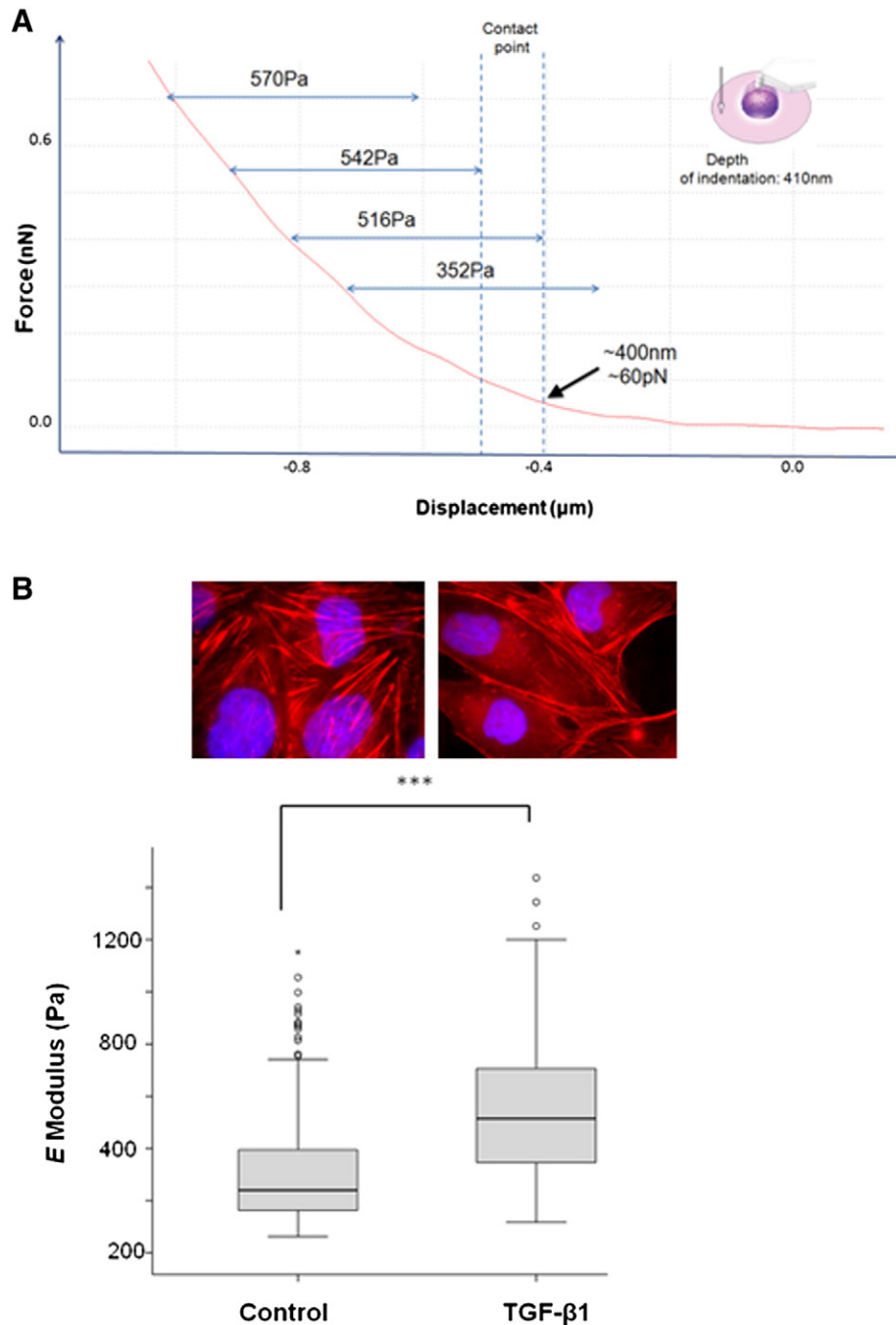


Figure 4. The effects of the cytokine TGF- $\beta 1$ (48 h, 10 ng/mL) on the E modulus of HK2 cells. The values inside the box represent the first (lower value) and third quartile, the line within the box represents the median, the (–) shows the minimum and maximum observations and the (O) symbol indicates outliers. **(A)** Fitting of Hertz model in incremental parts of displacement helped to determine the contact point for each cell separately. The depth of indentation was customised to 10% of the cell's height. **(B)** Treated cells showed an increase of 71% compared to the untreated group. Key significances of more than 30 cells from 3 separate experiments are shown, *** $P < 0.001$. Cell morphology was affected by cytoskeletal reorganisation (red), resulting in such a significant increase of E modulus.

cell line maintains functional characteristics of the proximal tubular epithelium and thus is an ideal model for the study of events, which precede the early loss of epithelial characteristics in tubular injury. Altering the expression of the adhesion protein does not absolutely indicate that the function of the cell-to-cell adhesion will correspondingly change.¹⁶ The involvement of

binding partners linked to the cellular architecture adds to the complexity of surface molecular interactions and hence cell adhesion is affected by changes in binding affinity, bond number and mechanical deformation. In the current study, the TGF- $\beta 1$ evoked reduction in E-cadherin expression was matched by a decrease in functional tethering between cells, as shown by the

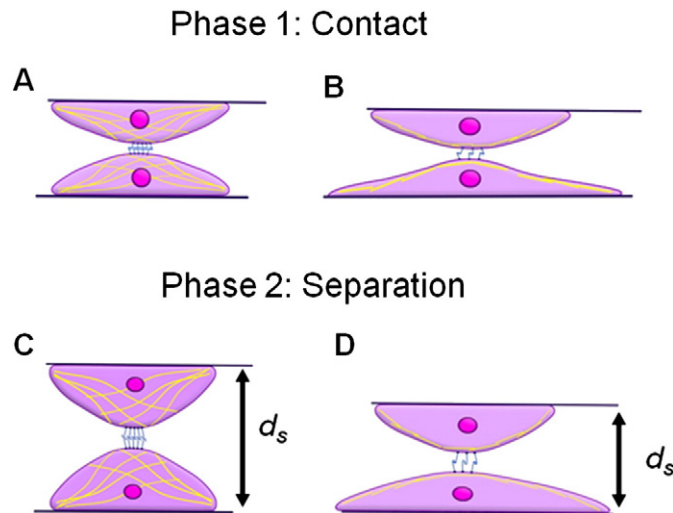


Figure 5. Schematic concept of E-cadherin mediated cell-to-cell adhesion process of control (A, C) and TGF- β 1 (10 ng/mL) treated (B, D) cells. In phase 1 (A) and (B) the cells are in contact and bonding is formed. In phase 2 (C) and (D) the separation process is shown, in which the interplay between cytomechanics and surface molecular binding is manifested.

reduction in the number of TREs after TGF- β 1 treatment. By quantifying the unbinding of the ligation complexes ruptures we suggest that the loss of adhesion is directly associated with a reduction in bond number, which in turn is associated with the decrease in unbinding forces between cells.

Cell adhesion is a complex process regulated by the involvement of the cytoskeleton as well as a number of surface proteins. In order to assess the effects of TGF- β 1 cytoskeletal reorganisation, single cell indentation experiments were performed to depths up to 10% of cell height. At this moderate degree of indentation changes in elasticity are largely dictated by the changes in cytoskeletal rearrangement.^{22,23} Morphological changes in the TGF- β 1 treated cells were associated with the remodelling of the cytoskeleton from F-actin filaments to dense stress fibres towards the periphery of the cell. Li et al²⁴ investigated the changes in E modulus of breast cancer cells through AFM indentation, suggesting that changes in cell morphology reflect rearrangement of the cytoskeleton and changes in elasticity. This is in agreement with current findings regarding the effects of the TGF- β 1 on single cell elasticity. The average calculated value of E modulus for the control cells was 320 Pa whilst for the treated cells was 549 Pa, indicating a raise of 71% that signifies a dramatic increase in cell stiffness upon treatment. This result combined with phase and fluorescence imaging of the cells demonstrates that changes in E modulus could be compared with changes in cell architecture, resulting from cytoskeletal rearrangement (Figure 4, B). Therefore, a potential structure-mechanics-disease correlation is suggested during TGF- β 1 induced early injury in diabetic nephropathy.

In addition to morphological changes, rearrangement of the cytoskeleton influenced cell-to-cell adhesion characteristics of HK2 cells. Retraction F - d curves confirmed that treatment of HK2 cells with TGF- β 1 reduced the number of tethering rupture events by 35% resulting in the reduction of maximum unbinding force by 19% but causing a notable decrease in the work of detachment by 53% (Figure 3). The greater reduction in the work

of detachment was accompanied by a decrease in distance of separation (46%) and could be partly explained by the increase in cell stiffness as demonstrated by the large increase in E modulus. Weder et al²⁵ reported that the increased E modulus of mitotic cells is related to a reorganization of the cytoskeleton into peripheral stress fibres and suggested that in SCFS adhesion measurements, cytoskeletal components are inevitably deformed during the separation process. Diz-Munoz et al²⁶ applied AFM-SCFS to show that cytomechanics have a profound influence on tether forming during the pulling or separation process of an AFM-tip binding with cell membrane. Our results suggest that the altered stiffness of the treated cells affects significantly the progression of the disease, since the dramatic decrease in adhesive characteristics is contributed by the higher loss of elastic deformation.

The importance of characterising both elasticity and adhesion strength using AFM based SCFS was recently highlighted by Andolfi et al²⁷ in cancer migration. In the current study, we clearly demonstrate that TGF- β 1 produced a distinguished effect in the cytoskeleton and the surface molecular ligation. We provide novel evidence, however, highlighting the fact that whilst TGF- β 1 evokes a decrease in E-cadherin ligation complexes, this is not the sole factor determining changes in cell-to-cell adhesion. The considerable influence of the stiffness of the cells in the detachment process suggests an intricate interplay between adhesion and cytomechanics, which may be pivotal for the disease progression (or potential regression) and is worthy of attention.⁷ Based on our data, it would be reasonable to suggest that biochemical changes altered biomechanical behaviour, which in turn affected too, the separation process between HK2 cells. Overall, the cohesive results between cell elasticity and the various parameters of adhesion facilitate better understanding of the role of the cytoskeleton and surface ligation in the changes of cell morphology and the development of the disease. The net effect of these changes can be conceptually illustrated in Figure 5. In phase 1, A, a schematic of control cells

is shown whilst in *B*, a schematic of cells treated with TGF- β 1 is shown. During this phase the cells are in contact with each other and E-cadherin ligation is formed. In phase 2 the separation process between the two cells is illustrated. In *C*, increased distance of separation, due to cell deformation, corresponded to higher work of detachment for normal cells whilst in *D* cells became more rigid due to cytoskeletal reorganisation into the periphery after TGF- β 1 resulting in a decreased distance of separation inevitably leading to a dramatic reduction of work of detachment. Therefore, the changes in the elasticity of single cells had a significant effect on work of detachment corresponding to the increase of the distance of complete separation. Overall, the reduction in surface binding affinity was partially responsible for the subsequent loss of cell adhesion.

In the current study, changes in E-cadherin mediated cell-to-cell adhesion upon treatment with the pro-fibrotic cytokine TGF- β 1 (48 h, 10 ng/mL) in the renal proximal tubule HK2 cell line have been investigated. TGF- β 1 affected the extracellular domain by decreasing the expression of E-cadherin causing a reduction in the number of tethering rupture events, whilst in the intracellular domain TGF- β 1 altered cytoskeletal architecture, resulting in the increase in elasticity of individuals cells. These changes in cell stiffness affected in turn the separation process between two adherent cells. As a consequence, TGF- β 1 induced renal tubular injury instigates a reduction of the adhesive properties between cells by decreasing the number of molecular ligation and increasing cell stiffness.

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