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Flow induced adherens junction remodeling driven by cytoskeletal forces

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ABSTRACT

Adherens junctions (AJs) are a key structural component for tissue organization and function. Under fluid shear stress, AJs exhibit dynamic assembly/disassembly, but how shear stress couples to AJs is unclear. In MDCK cells we measured simultaneously the forces in cytoskeletal α-actinin and the density and length of AJs using a genetically coded optical force sensor, actinin-sstFRET, and fluorescently labeled E-cadherin (E-cad). We found that shear stress of 0.74 dyn/cm^2 for 3 h significantly enhanced E-cad expression at cell-cell contacts and this phenomenon has two phases. The initial formation of segregated AJ plaques coincided with a *decrease* in cytoskeletal tension, but an *increase* in tension was necessary for expansion of the plaques and the formation of continuous AJs in the later phase. The changes in cytoskeletal tension and reorganization appear to be an upstream process in response to flow since it occurred in both wild type and dominant negative E-cad cells. Disruption of F-actin with a Rho-ROCK inhibitor eliminated AJ growth under flow. These results delineate the shear stress transduction paths in cultured cells, which helps to understand pathology of a range of diseases that involve dysfunction of E-cadherin.

1. Introduction

Adherens junctions (AJs) are key structural components for tissue organization [1–3]. In response to mechanical forces, AJs interact with F-actin and adhesion proteins through its core structural protein E-cadherin (E-cad) [4–7]. This force transduction and adaptation is important for the maintenance of tissue integrity. Loss of E-cad expression is correlated with disease states including chronic kidney disease (CKD) [8] and cancer [9,10].

AJs are highly dynamic and continuously undergo remodeling even in differentiated epithelia [1,11]. This dynamic process involves changes in actomyosin contractility, remodeling of cell-cell adhesions, and actin polymerization [12–15]. Previous studies have focused on the regulatory role of internal contractile forces in the junction formation and stability. It was found that this dynamic process is regulated by actomyosin contractility via interaction between E-cadherin complexes and the underlying actin dynamics [13–17]. An increase in myosin-II dependent force stimulates growth of AJs, whereas disrupting the cytoskeleton disassembles AJs [18–21].

Recent studies showed that external forces have a direct effect on E-cad expression and junction growth [22–24]. Exposure of renal tubule cells to chronic flow shear stress results in a dramatic increase in E-cad expression at cell-cell junctions and a significant cytoskeletal reorganization [25,26], which was accompanied by a reduction in cytoskeletal stress [27]. Similarly, cells grown on soft substrates exhibit less cytoskeletal tension compared to cells grown on hard substrates, and soft substrates promote junction formation at the cell-cell contacts [28,29]. These data suggest that reduced cytoskeletal tension is favorable to junction formation; actin cytoskeleton may apply inwardly directed forces at cell edges, and a high cytoskeletal tension reduces stability of cell-cell junctions. Conversely, the application of external force by stretching a flexible substrate promoted an increase of contact length in a force-dependent fashion [23,24]. The cell's response to oscillatory forces applied via the E-cad coated magnetic beads on the cell surface decayed with time [22], indicating the actin bundles linked to the attached AJs became stiffer [22]. This evidence suggests mechanical stimuli may activate cell defense functions that enhance the adherens junctions to resist the external forces. One of the first responses of cells to mechanical stimuli like shear stress is cytoskeletal tension variation. The role of cytoskeletal tension in shear stress transduction in cells regulating AJ assembly remains unclear.

Two signaling mechanisms may participate in AJ formation and

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retraction under flow. The E-cad based junctions could sense the shear stress and activate actin-myosin-II contraction, leading to actin reorganization. Alternatively, the shear stress could change the cytoskeletal tension and organization that modulates the junction dynamics under flow. Here we propose that kinetic analysis may help resolve the causal relation between AJ formation and actin reorganization in response to mechanical force.

In this study, we simultaneously measured AJ dynamics and cytoskeletal stress in epithelial cells using fluorescently labeled E-cadherin (E-cad-RFP) and a force sensor, actinin-sstFRET, in cells under flow. We analyzed time dependent force redistribution and E-cad dynamics in cells. The results show that changes in cytoskeletal tension and reorganization triggers AJ remodeling in epithelial cells under flow, demonstrating a causal role of mechanical force in AJ dynamics.

2. Materials and methods

2.1. Flow experiments

The microfluidic chip used to perfuse the cells consisted of a PDMS channel between two parallel glass coverslips and was fabricated using soft lithography [30]. The channel was 1000 µm wide, 100 µm high and 15 mm long. Holes through the PDMS formed the inlet and outlet. During flow experiments, the chamber was placed in a stage-top incubator (INUB-ZILCSD-F1-LU, Tokai Hit Co., Ltd, Japan) maintained at 37 °C and 5% CO_2. Isotonic solution was perfused through the channel using a syringe pump (PHD2000, Harvard Apparatus). The fluid shear stress (τ) was calculated using $\tau = 6\mu Q/wh^2$, where $\mu = 1.0 \times 10^{-3}$ Pa s is the dynamic viscosity of the solution, Q is the volume flow rate, and w and h are the width and the height of the channel, respectively. A typical physiological urine flow rate of 7.4 µl/min corresponding to 0.74 dyn/cm^2 shear stress was used for all experiments [31]. Kidney epithelial cells undergoes significant reorganization at this shear stress [25–27], although partial reorganization could occur at lower flow rate [27].

2.2. Cell culture and transfection

Madin-Darby Canine Kidney (MDCK) cells (ATCC) were grown to confluence in Dulbecco's Modified Eagle Medium (DMEM) having 10% fetal bovine serum and 1% penicillin and streptomycin. The suspension of cells was perfused into a microfluidic channel coated with fibronectin. Cells were grown in the chamber and the culture media was changed every 24 h. Cells were co-transfected with actinin-sstFRET and Ecad-RFP after 48 h of culture, at ~60% confluency, using Effectene (0.2 µg DNA each; 1:50, DNA to Effectene ratio) and incubated in solution for next 16 h. The transfection efficiency was ~20%. The cells were washed with media and experiments were done at 80–90% confluency. MDCK cells engineered to express E-cadherin lacking extracellular domain, which compete with endogenous E-cadherin in a dominant negative fashion (abbreviated here as DN-E-cad cells), were cultured and transfected in similar fashion and were incubated in doxycycline for another 24 h before experiments. Isotonic solution was used during imaging to avoid background fluorescence from media.

2.3. FRET probes and force measurements

The force-sensitive FRET probe consisted of Cerulean (donor) and Venus (acceptor), linked together with a spectrin repeat domain; originally termed sstFRET for spectrin-repeat-stretch-sensitive-FRET sensor [32]. The probe sequence is incorporated into the DNA for α-actinin at position 300. Mechanical forces stretch the protein thus change the FRET ratio. The details of the probe construction and properties have been described previously [32]. The force sensitivity of the probe was calibrated using DNA springs following a previously described method [33]. The calibration shows that the probes are sensitive to forces on the scale of 0–10 pN [32]. The chimeric constructs behave physiologically as indistinguishable from endogenous proteins [32,34,35]. E-cad-RFP was a gift from Dr. W. James Nelson's laboratory at Stanford University.

2.4. Fluorescence imaging and FRET analysis

Fluorescence images were obtained using three channel recording using an inverted microscope (Axiovert 200M, Zeiss) with 63x oil immersion objective and an EM-CCD camera (ImagEM C9100-13, Hamamatsu, Japan). Three filter sets (Ex: 436/20, Em: 480/40), (Ex: 500/20, Em: 535/30), and (Ex: 550/25, Em: 605/70) were used for CFP, YFP and RFP channels, respectively. Micromanager was used to record time-lapse images of the three channels.

FRET energy transfer efficiency was calculated as the acceptor to donor ratio (FRET A/D ratio), where the donor (CFP channel) and acceptor (YFP channel) fluorescence was measured with donor and acceptor excitations respectively [32]. The A/D ratio represents quenching of the donor fluorescence due to energy transfer and is monotonically inverse to the tension. CFP and YFP images were processed using Image-J (NIH) following previously published methods [32]. The results were displayed as 16-color maps where warm colors (red) indicated higher FRET ratios equivalent to lower tension and cool colors (blue) indicated a lower FRET ratio (higher tension).

For quantitative analysis, we developed an automated image processing workflow utilizing two programs, CellProfiler (an open source cell image analysis software) and ImageJ. RFP images were first processed using ImageJ to subtract the background and background cytosolic fluorescence. Images were then analyzed using a custom designed analysis procedure in Cell Profiler. E-cad plaques were identified at the cell periphery as primary objects at each time point based on an intensity threshold, allowing tracking of the AJs over time. A mean E-cad intensity was obtained by averaging integrated intensities for selected regions of interest (ROI)/detected AJs, and this was normalized to the intensity at time zero. The average length of AJ plaques was normalized by the average length before shear. To obtain regional force distribution (FRET ratio at AJs along cell edges), FRET ratio images were generated using ImageJ as previously described [34,36]. The average FRET ratio was then calculated for the identified objects (AJs) using CellProfiler. For DN-E-cad cells, E-cad intensity and FRET ratio was calculated along the cell periphery that was manually identified using actinin/YFP images. The normalized data for each type of measurement was averaged across multiple experiments. Each measurement used a fresh cell culture in a fresh flow chamber. The data is shown as means ± standard error of the means (s.e.m.). Statistical analysis was done using paired sample t-test. Value of $p < 0.05$ was considered statistically significant.

2.5. Construction of dominant negative E-cadherin cell line

A doxycycline regulatable vector containing dominant negative E-cadherin (DN-E-cad) expression cassette was created as described previously [37]. This vector expressed a truncated form of E-cad lacking the extracellular domain that is required for adherens junction formation upon doxycycline treatment. In addition, DN-E-cad vector also contained a puromycin selection cassette for selection of transfected cells. Second generation DN-E-cad lentivirus was produced as described previously [38]. Briefly, 293T/17 cells (ATCC, Manassas,VA) were co-transfected with DN-E-cad plasmid, envelope glycoprotein VSV-G encoding plasmid pMD2.G (Addgene plasmid 12259) and virus packaging plasmid psPAX2 (Addgene plasmid 12260) using the calcium phosphate method. To this end all three plasmids were mixed in equimolar ratio and upon calcium phosphate precipitation, plasmid mixture was added to the 293T/17 cells (~60% confluent). After 8 h post-transfection, medium (DMEM plus 10% fetal bovine serum) was

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