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Research Article

Engineering amount of cell–cell contact demonstrates biphasic proliferative regulation through RhoA and the actin cytoskeleton

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ABSTRACT

Endothelial cell–cell contact via VE-cadherin plays an important role in regulating numerous cell functions, including proliferation. However, using different experimental approaches to manipulate cell–cell contact, investigators have observed both inhibition and stimulation of proliferation depending on the adhesive context. In this study, we used micropatterned wells combined with active positioning of cells by dielectrophoresis in order to investigate whether the number of contacting neighbors affected the proliferative response. Varying cell–cell contact resulted in a biphasic effect on proliferation; one contacting neighbor increased proliferation, while two or more neighboring cells partially inhibited this increase. We also observed that cell–cell contact increased the formation of actin stress fibers, and that expression of dominant negative RhoA (RhoN19) blocked the contact-mediated increase in stress fibers and proliferation. Furthermore, examination of heterotypic pairs of untreated cells in contact with RhoN19-expressing cells revealed that intracellular, but not intercellular, tension is required for the contact-mediated stimulation of proliferation. Moreover, engagement of VE-cadherin with cadherin-coated beads was sufficient to stimulate proliferation in the absence of actual cell–cell contact. In all, these results demonstrate that cell–cell contact signals through VE-cadherin, RhoA, and intracellular tension in the actin cytoskeleton to regulate proliferation.

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Introduction

Spatial regulation of proliferation, coordinated by numerous factors in the local microenvironment, is necessary at every stage of multicellular life from embryogenesis through adulthood. Regulators of proliferation include soluble factors [1], cell–ECM interactions [2], cell shape [3,4], mechanical forces [5,6], and cell–cell adhesions [7,8]. In endothelium, cells at wound edges proliferate at greater rates than those in the interior of the monolayer [9]. Similarly, cells comprising the tips of sprouts during angiogenesis proliferate while their neighbors remain quiescent [10]. In these spatially regulated cases the degree of cell–cell contact correlates with, and may be a direct regulator of, changes in proliferation. While previous studies have demonstrated a role for cell–cell contact in regulating proliferation, the mechanisms of such control appear to be complex, and have not been fully elucidated [11,12].

Most previous studies of cell–cell adhesions in endothelial cells have concluded that their formation inhibits proliferation [7,11,13–16]. The classical method which lead to the widespread belief that cell–cell contact decreases proliferation was to compare proliferation rates in sparse cells, having few or no cell–cell contacts, to

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proliferation rates in cells contacting multiple neighbors within densely crowded monolayers [1]. This phenomenon, known as contact-dependent inhibition of cell proliferation, has been shown to require VE-cadherin, since cadherin-null cells fail to fully arrest proliferation at confluence [7,11,13–15]. However, in these early studies, a high degree of cell–cell contact inhibited proliferation under conditions where cell adhesion and spreading on the underlying extracellular matrix was restricted by virtue of cell crowding. Since cell spreading itself is known to affect proliferation [4,17], our group studied the proliferative effect of VE-cadherin using a new strategy, based on culturing cells in microfabricated wells, in order to separate the independent roles of cell–cell and cell–matrix adhesion [8,18]. Using this system, contact with a single neighboring cell unexpectedly increased proliferation under conditions of constant spreading, and this effect required VE-cadherin. While our previous studies suggested that cell–cell contact arrested proliferation in monolayers because contact decreased cell spreading on ECM, another possibility is that the small amount of cell–cell contact in our two-cell patterns stimulates proliferation while the large amount of contact in monolayers arrests proliferation.

Here, we used a novel micropatterning approach to investigate whether the number of contacting neighbors can differentially regulate endothelial proliferation. While the microfabricated wells used previously facilitated the formation of pairs of cells, it was difficult to form groups of three or more cells with specified arrangements of cell–cell contacts. To overcome this limitation we developed a method which uses dielectrophoretic traps to actively and simultaneously position the cells onto a substrate [19]. Several studies have demonstrated that, under appropriate conditions, dielectrophoresis (DEP) can in fact be used to harmlessly manipulate endothelial cells and a variety of other cell types [19–23]. This active positioning technique enables the patterning of cells in configurations that are otherwise unobtainable by passive micropatterning techniques in which the pattern fidelity is determined randomly.

In the current study, we set out to discern the effect of cell–cell contact on proliferation in a relatively complex yet well-controlled environment. Modulation of cell–cell contact from zero to three uniformly spread neighbors demonstrated a biphasic relationship between cell–cell contact and proliferation. While one neighbor increased proliferation, two or three neighbors diminished this increase. We then investigated the hypothesis that the pathway responsible for these proliferative effects involves a VE-cadherin-derived signal mediated by the actin cytoskeleton. This study demonstrates that quantitative changes in cell–cell contact modulate proliferation through RhoA signaling and intracellular tension, and highlights a novel control mechanism by which cells autoregulate their responses as a function of subtle changes in multicellular organization.

Materials and methods

Cell culture and reagents

Bovine pulmonary arterial endothelial cells (BPAECs, VEC Technologies, Rensselaer, NY) were cultured in a standard growth media containing Dulbecco's Modified Eagle Medium supplemented with 10% calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Prior to experiments using dielectrophoresis, cells were detached using 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid in PBS, rinsed with 22 μg/mL Soybean trypsin inhibitor (Invitrogen) in growth media, pelleted by centrifugation at 240 *g* for 4 min, resuspended in 3 mL of 300 mOsm sucrose with 1% calf serum (sucrose media), vacuum degassed, and pulled into syringes already containing 1 mL of 10% CO_2/air. After dielectrophoresis, sucrose media was replaced with growth media. Cells plated on passive substrates were resuspended in growth media immediately after trypsinization.

Patterning cells onto substrates

Groups of one to two cells were patterned without the assistance of dielectrophoresis as described previously [8,18]. Briefly, agarose was perfused under a polydimethylsiloxane (PDMS) mold containing raised regions of various geometries, and sealed over a glass surface. Where the raised regions sealed against the glass, fluid was prevented from flowing between the PDMS and the glass, and thus these areas remained free of agarose. Upon agarose curing, and peeling the mold off the glass substrate, the agarose remained adhered to the glass. Substrates were then immersed in 10 μg/mL human fibronectin (Collaborative Biomedical Products), which adsorbed only to the agarose-free areas. Cells were then seeded onto the substrates, attaching only in the regions coated with fibronectin. For maximum patterning efficiency, cells were seeded ~10^4 cells/cm^2 in growth media, and rinsed with fresh growth media at 2 h after seeding.

Substrates used to pattern groups of one to four cells via dielectrophoresis were embedded with arrays of 3 μm electrodes, designed to trap one cell per electrode, as previously described [19]. To increase adhesion between the substrates and the agarose, substrates were coated with an amino functionality using 3-(Aminopropyl)trimethoxysilane (APTES, Aldrich Chemical). Briefly, after treatment with an air plasma (Plasma Prep II, SPI supplies, West Chester, PA) for ~1 min, substrates were placed in a desiccator also containing a drop of APTES on a microscope slide. The desiccator was evacuated and placed at 60 °C overnight. To align the agarose layer with the electrodes, a PDMS mold (see above) was adhered to a glass backing to prevent feature distortion and to facilitate alignment using a mask aligner (Karl Suss, Munich, Germany). Agarose was then wicked under the aligned mold as described above.

To pattern cells using dielectrophoresis (DEP), a parallel plate flow chamber (Fig. S1, S2), similar to that described previously [19,24], was used to introduce cells to the substrates, remove extra cells, and provide a constant supply of fresh media. The floor of the chamber consists of the substrate itself, and a silicone gasket forms the walls of the 160 μm high, 15 mm wide chamber. The electrical signal (5 V, 2 MHz) used to operate the traps was applied to substrates and the counter electrode using a battery operated dual signal generator. The chamber was sterilized with ethanol and dried before each use. As previously described [19], sucrose media containing ~10^6 cells/mL was introduced into the system from 3 mL syringes via rubber tubing. After cells began to flow over the substrate, as monitored by a microscope, the electrodes were energized and began to trap cells. A flowrate of ~50 μL/min for 5 min allowed single and multiple cells to be trapped at the electrodes. A 4-way valve was then used to switch between cells and cell-free media without introducing bubbles into the system.

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