VE-cadherin simultaneously stimulates and inhibits cell proliferation by altering cytoskeletal structure and tension

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Summary

Engagement of vascular endothelial (VE)-cadherin leads to the cessation of proliferation commonly known as 'contact inhibition'. We show that VE-cadherin inhibits growth by mediating changes in cell adhesion to the extracellular matrix. Increasing cell-cell contact decreased cell spreading and proliferation, which was reversed by blocking engagement of VE-cadherin. Using a new system to prevent the cadherin-induced changes in cell spreading, we revealed that VE-cadherin paradoxically increased proliferation. Treating cells with inhibitors of PKC and MEK abrogated the stimulatory signal at concentrations that disrupted the formation of actin fibers across the cell-cell contact. Directly disrupting actin fibers, blocking actin-myosin-generated tension, or inhibiting signaling through Rho specifically inhibited the cadherin-induced proliferative signal. By progressively altering the degree to which cell-cell contact inhibited cell spreading, we show that cell-cell contact ultimately increased or decreased the overall proliferation rate of the population by differentially shifting the balance between the two opposing proliferative cues. The existence of opposing growth signals induced by VE-cadherin that are both mediated through crosstalk with cytoskeletal structure highlights the complex interplay of mechanical and chemical signals with which cells navigate in their physical microenvironment.

Key words: Cell shape, Microfabrication, Intercellular adhesion, Adherens junctions

Introduction

The proliferation of endothelial cells is regulated by many factors in the surrounding microenvironment, including soluble cytokines, the underlying extracellular matrix (ECM), and neighboring cells. The complex interplay between these cues provides the signals that allow cells to form and maintain the architecture of the vascular tree. Growth factors and integrins cooperate to generate multiple mitogenic signals necessary for proliferation, including sustained ERK activity (Assoian and Schwartz, 2001; Danen and Yamada, 2001; Schwartz and Ginsberg, 2002). Although necessary, these signals fully support proliferation only in the presence of additional structural cues conveyed through the actin cytoskeleton (Chen et al., 1997; Huang et al., 1998; Huang and Ingber, 2002). Either restricting the ability of endothelial cells to spread and flatten against a substrate or disrupting the actin cytoskeleton arrests the cell cycle in mid-G1 despite the presence of growth factors, integrin ligation and ERK activation (Huang et al., 1998). This coordination between cell spreading and mitogenic signaling appears to be one critical mechanism that arrests proliferation once cells have crowded each other sufficiently to form an intact monolayer.

Although growth factors and integrins convey mitogenic signals leading to proliferation (Assoian and Schwartz, 2001; Danen and Yamada, 2001), adhesion between cells is thought to inhibit growth (Castilla et al., 1999; Caveda et al., 1996). As a result, cadherin engagement has been suggested to be another mechanism, distinct from decreased cell spreading, for the cessation of growth when cells reach confluence (Gumbiner, 1996). Transformed cells lacking a variety of components of the cell-cell adhesion machinery proliferate at higher rates than when they are transfected with neural (N)-cadherin (Levenberg et al., 1999) or epithelial (E)-cadherin (Stockinger et al., 2001). In endothelial cells, vascular endothelial (VE)-cadherin has been implicated as the major receptor responsible for this effect (Castilla et al., 1999; Caveda et al., 1996). Blocking the function of VE-cadherin increases proliferation, whereas exogenous expression of VE-cadherin in CHO cells decreases growth rates (Caveda et al., 1996). Despite these findings, little is known about the mechanism by which cadherins inhibit growth, or the relative contributions of changes in cell spreading versus cadherin engagement to growth arrest at confluence. Recent work has suggested that increasing cell-cell adhesion mechanically competes with and hence decreases cell-substrate adhesion (Lauffenburger and Griffith, 2001; Ryan et al., 2001). Thus, growth arrest by cell-cell contact and by changes in cell spreading may be linked; VE-cadherin could inhibit proliferation in part by altering cytoskeletal structure and decreasing cell spreading.

VE-cadherin can modulate the organization of the cytoskeleton through multiple mechanisms. The homotypic engagement of cadherins initiates both soluble signaling cascades as well as the recruitment of scaffolding proteins to form the adherens junction. Cadherins increase signaling

through ERK (Pece and Gutkind, 2000), PKC (Lewis et al., 1994) and Akt (Carmeliet et al., 1999), all of which have significant effects in reorganizing the actin cytoskeleton (Clark et al., 1998; Keenan and Kelleher, 1998; Klemke et al., 1997; Reif et al., 1996). Cadherins also modulate structural changes to microtubules (Chausovsky et al., 2000) and actin through proteins including the Rho family GTPases (Noren et al., 2001) and β-catenin (Barth et al., 1997). The formation of adherens junctions by VE-cadherin directly recruits and anchors the actin cytoskeleton to intercellular junctions (Breviario et al., 1995). Modulating cadherin-mediated intercellular adhesion also directly alters the expression of integrins (Zhu and Watt, 1996). Thus, VE-cadherin engages numerous pathways that can alter cytoskeletal structure and tension, cell shape and integrin signaling (Braga, 2000; Chen et al., 1997; Pawlak and Helfman, 2001; Schwartz and Assoian, 2001) – all potent regulators of proliferation.

The complex interplay between intercellular and extracellular matrix adhesion highlights the importance of developing an experimental system to independently manipulate these signals, and thereby decouple their respective effects on cell function. Using a new microengineering approach to control cell-cell contact and cell spreading independently, we recently reported that allowing physical contact between cells while preventing changes in cell spreading stimulated proliferation in multiple cell types, and that this stimulation was masked by an apparently distinct inhibitory signal when cell spreading was not controlled (Nelson and Chen, 2002). Now, using our new system to examine the role of cell spreading in VE-cadherin-mediated regulation of endothelial cell proliferation, we show that VE-cadherin exerts its inhibitory effects specifically by actively decreasing cell spreading. Preventing the cadherin-induced changes in cell morphology, we unexpectedly reveal that VE-cadherin also elicits a stimulatory signal for growth that depends on Rho-mediated tension in the actin cytoskeleton. Thus, VE-cadherin appears to regulate two distinct proliferative signals in endothelial cells, both through crosstalk with regulatory pathways traditionally associated with cell shape and cytoskeletal structure.

Materials and Methods

Cell culture and reagents

Bovine pulmonary artery endothelial cells (VEC Technologies, Rensselaer, NY) were cultured in 5% calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin in low glucose DMEM (all from Life Technologies). Bovine adrenal microvascular endothelial cells (VEC Technologies) were cultured in 10% fetal bovine serum (Hyclone), 100 units/mL penicillin, 100 μg/mL streptomycin in low glucose DMEM supplemented with 10 ng/mL EGF and 3 ng/mL bFGF. Human carcinoma A431D (null) and A431D-VE (VE^+) cells were a kind gift from K. Johnson (University of Nebraska), and were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin. Anti-VE-cadherin (clone 9H7) was a gift from R. Heimark (University of Arizona). The following antibodies were purchased from the given suppliers: β-catenin (Transduction Labs); connexin 43 (Chemicon); occludin (Zymed); PECAM-1 (Santa Cruz); VE-cadherin (BV9; Cell Sciences); polyclonal anti-VE-cadherin (Alexis); non-immune mouse and rabbit IgGs (Sigma). U0126, Ro-31-7549, H-7, cytochalasin D, 2,3-butanedione 2-monoxime (BDM), ML-7 and Y-27632 were all obtained from Calbiochem.

Fabrication of substrates with microwells

Stamps of poly(dimethylsiloxane) (PDMS, Sylgard 184, Essex Brownell, Fort Wayne, IN) containing a relief of the desired pattern (bowties raised from the surface) were fabricated as previously described (Nelson and Chen, 2002). Briefly, PDMS was cast on a silicon master with 20 μm-thick bowtie-shaped wells made by photolithography. To aid in release of the cured PDMS, the master was first silanized overnight with a vapor of (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Bristol, PA) under vacuum before casting the PDMS. A stamp oxidized under UV/ozone (UVO Cleaner, Jelight Company, Irvine, CA) was placed on a SuperFrost slide (Fisher Scientific) such that only the raised bowtie-shaped regions of the stamp sealed against the glass surface. A solution of 0.6% agarose (Life Technologies)/40% ethanol in water was heated (80°C), perfused through the channels formed between the sealed features, and allowed to cool for 12 minutes under vacuum. The stamps were peeled from the substrate, leaving behind bowtie-shaped wells with bases of glass and walls of agarose. The substrates were sterilized in ethanol, washed in PBS and treated with a 25 μg/mL solution of fibronectin (Collaborative Biomedical Products) in PBS, which coated the glass bases of the bowtie-shaped wells.

Measurement of proliferation

To obtain changes in cell number, cells were photographed with a Spot CCD camera (Diagnostic Instruments, Sterling Heights, NY). Cell proliferation was calculated by counting cells in microscopy images at indicated times, and normalized to initial cell number. Mean cell area was determined by outlining cells in phase contrast images with the Spot software. To determine entry into S phase, the percentage of cells incorporating 5-bromo-2′-deoxyuridine (BrdU) was quantified using a commercial assay (Amersham). Cells were G_0-synchronized by holding cultures at confluence for 2 days, then plated onto substrates in full culture media. BrdU was added to the media 2 hours after plating. Cells were fixed and stained according to the manufacturer's instructions at 24 hours. BrdU-positive fluorescent cells were visualized and scored using a Nikon epifluorescence microscope (Nikon). The DNA-binding dye Hoechst 33258 (Molecular Probes) was used as a counterstain (1 μg/ml). To distinguish between cell types in co-culture experiments, A431 cells were labeled with Cell Tracker Orange (Molecular Probes) prior to seeding with endothelial cells. For all proliferation conditions using random seeding, at least 200 cells were counted per condition across two independent experiments. For all proliferation conditions using patterned wells, at least 500 cells were counted per condition across three independent experiments.

Flow cytometry analysis

To determine position in the cell cycle, trypsinized cells were collected by centrifugation and resuspended in a 0.05 mg/mL solution of propidium iodide (PI) (Molecular Probes) in 1% sodium citrate, 0.1% Triton-X-100, and 7 units/mL DNAse-free ribonuclease A (Sigma). Analysis was performed on a FACScan flow cytometer (Becton Dickinson). All data were acquired and analyzed with the CellQuest software. For cell-cycle analysis, gating was set around cell populations based on fluorescence intensity and sideward scatter.

Immunofluorescence

Cells were fixed and stained at 24 hours after seeding. For the detection of cell-cell adhesion molecules, cells were fixed in 4% paraformaldehyde in PBS, permeabilized in 0.2% Triton-X-100 in PBS, washed in 33% goat serum in PBS, incubated in primary antibodies diluted in 33% goat serum in PBS, and visualized with Alexa 488- or 594-conjugated secondary antibodies (Molecular

Probes). For the detection of actin, fixed and permeabilized cells were stained with 0.1 μg/mL TRITC-conjugated phalloidin (Sigma) in PBS.

Construction of recombinant adenoviruses

Recombinant adenoviruses encoding RhoN19 and GFP were prepared using the AdEasy XL System (Stratagene) as per kit instructions. Briefly, the cDNA fragment encoding RhoN19 was mutagenized from pEGFP-WT-RhoA (gift from M. Philips, New York University). The fragment was PCR amplified and cloned into the shuttle vector pShuttle-IRES-hrGFP-1. After construction, the shuttle vector was linearized with *Pme* I and transformed into BJ5183-AD-1-competent cells pretransformed with the pAdEasy-1 adenoviral vector, to generate recombinant adenoviral plasmids, which were purified and transfected into HEK 293 cells. Adenoviral infection was monitored by GFP fluorescence, and adenoviral particles were obtained by cell extraction after 7-10 days. The virus was further amplified and purified by centrifugation on a CsCl gradient. Stocks of 10^9-10^{10} infectious particles/mL were retained and used in subsequent experiments. The virus was titrated by infecting HEK 293 cells with serially diluted stocks and counting GFP-expressing cells.

To infect endothelial cells, a solution of recombinant adenovirus was mixed with culture medium, and cells were exposed to the virus with a multiplicity of 10-100 viral particles/cell for 3 hours. Cells were then washed, trypsinized and plated onto substrates. Cells were analyzed 24 hours after plating; under these conditions, >95% of the cells were infected.

Results

VE-cadherin decreases cell spreading and proliferation

We first examined whether the engagement of VE-cadherin changes the degree to which endothelial cells spread. Cells

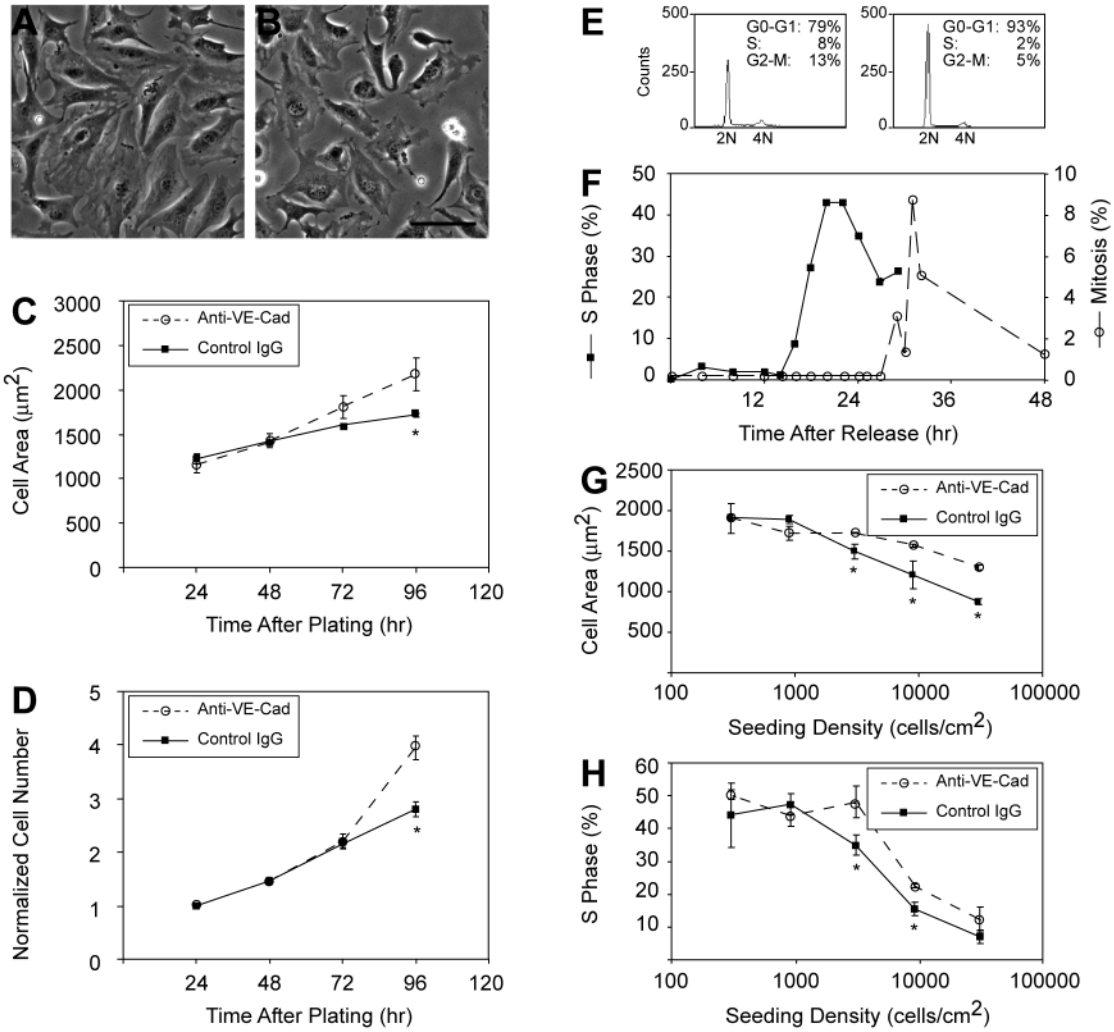

Fig. 1. Role of VE-cadherin in cell spreading and proliferation. (A,B) Phase contrast images of bovine pulmonary artery endothelial cells treated for 96 hours with anti-VE-cadherin (A) and control (B) antibodies. Scale bar: 50 μm. (C) Graph of mean projected area of endothelial cells with time after treatment with anti-VE-cadherin and control antibodies. (D) Graph of number of cells after treatment with anti-VE-cadherin and control antibodies, normalized to number of cells at 24 hours after plating. (E) Flow cytometry diagrams of cell-cycle distribution of unsynchronized cells (left) and synchronized cells (right). Quantification of cell-cycle distribution is shown for each condition. (F) Graph of percentage of cells in S phase (BrdU incorporation) and mitotic index with time after replating of synchronized cells. (G) Graph of mean projected area of endothelial cells as a function of seeding density after treatment with anti-VE-cadherin and control antibodies. (H) Graph of percentage of endothelial cells entering S phase (incorporating BrdU) as a function of seeding density after treatment with anti-VE-cadherin and control antibodies. Error bars represent the s.d., with (*) P<0.05 relative to controls as calculated by t-test.

were plated at 5000 cells/cm^2 and cultured with 50 μg/mL of either a function-blocking anti-VE-cadherin antibody or a non-reactive control at the time of seeding and subsequently every 24 hours for 4 days. Cell spreading was measured by outlining phase contrast images of cells at each time point, and calculating projected cell area from these outlines. Plating efficiency was not affected by antibody treatments. Initially, blocking VE-cadherin had no effect on cell spreading when the cell density was too sparse for cells to form cell-cell contacts. As the density of cells increased with time and cells began to form contacts with their neighbors, blocking VE-cadherin increased cell spreading relative to control (Fig. 1A-C). Consistent with previous reports (Caveda et al., 1996), treatment with anti-VE-cadherin antibody also increased the rate of proliferation (Fig. 1D). The time course of the increase in cell spreading closely followed that of the increase in cell number. However, the duration of these experiments made it difficult to interpret the roles of cell-cell contact and cell spreading in the regulation of proliferation.

To manipulate VE-cadherin engagement on shorter time scales and further explore its effects on cell spreading and cell-cycle progression, we altered cell-cell contact by seeding cells at different densities. Cells were synchronized by holding at confluence for 2 days, which arrests the cells in G0/G1 of the cell cycle (Davis et al., 2001), as confirmed by flow cytometry (Fig. 1E). Cells were released by plating onto fibronectin-coated substrates. Under these conditions, cells synchronously enter S phase at ~20 hours after plating, and subsequently enter mitosis 10 hours later at ~30 hours (Fig. 1F). Synchronized cells were plated at densities ranging from 300 to 30,000 cells/cm^2 (Fig. 1G,H), in the presence of either the function-blocking anti-VE-cadherin antibody or the non-reactive control. After 24 hours, cells were fixed and analyzed for cell spreading and cell proliferation. Increasing the density of cells simultaneously decreased cell spreading and entry into S phase. Blocking VE-cadherin increased cell spreading and proliferation rate relative to control at the highest plating densities. Thus, the engagement of VE-cadherin decreases endothelial cell spreading and proliferation. Because increased cell spreading itself is known to have a positive effect on the proliferation of endothelial cells (Chen et al., 1997), these findings raised the possibility that VE-cadherin may inhibit cell proliferation by causing a change in cell spreading.

Cell-cell contact increases proliferation when spreading is controlled

To investigate whether contact inhibition of proliferation requires the decrease in cell spreading, we used a method to prevent changes in cell spreading in cells cultured with or

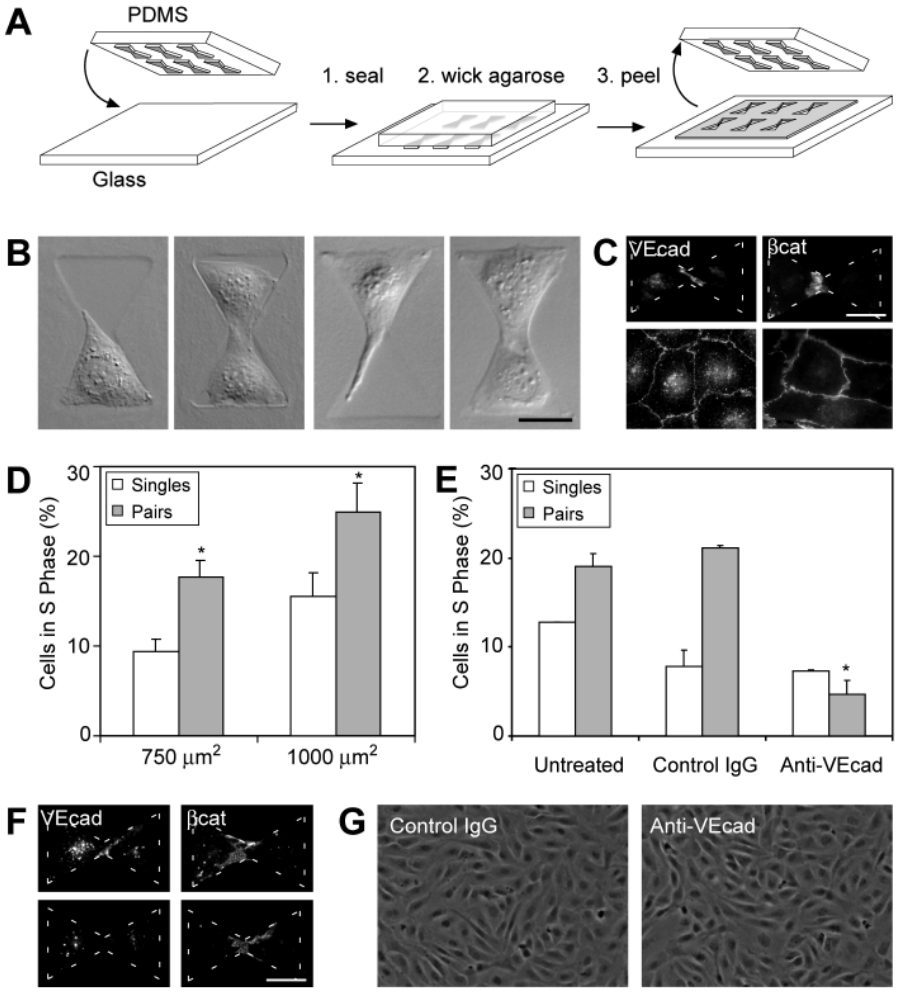

Fig. 2. Effect of cell-cell contact on proliferation when spreading is controlled. (A) Schematic outline of method used to pattern substrates to control cell spreading and cell-cell contact simultaneously. (B) Differential interference contrast images of single cells or pairs of cells in agarose wells of 750 μm^2/half (left two images) and 1000 μm^2/half (right two images). Bovine pulmonary artery and adrenal microvascular endothelial cells were G_0-synchronized and cultured on arrays of wells for 24 hours and fixed for analysis. Cells distributed randomly as single cells and pairs of cells in the wells. (C) Immunofluorescence images of pairs of cells in wells of 750 μm^2/half (top images) or monolayers (bottom images) stained for VE-cadherin (VEcad) or β-catenin (βcat). Both VE-cadherin and β-catenin specifically localized to the zone of contact. Broken lines (white) indicate the borders of the wells. (D) Graph of percentage of cells entering S phase (incorporating BrdU) for single cells and pairs of cells in both sizes of wells. (E) Graph of percentage of cells entering S phase for cells in wells of 750 μm^2/half treated with VE-cadherin antibody. Similar results were seen with both types of endothelial cells analyzed. Error bars represent the s.d., with (*) $P<0.05$ relative to single cells (D) or controls (E) as calculated by Student's t-test. (F) Immunofluorescence images of pairs of cells in wells treated with control (top) and anti-VE-cadherin (bottom) antibodies and stained for VE-cadherin (VEcad) or β-catenin (βcat). (G) Phase contrast images of endothelial cells treated with control (left) or anti-VE-cadherin (right) antibodies. Scale bars: 25 μm.

without cell-cell contact (Fig. 2A). Cells were plated on substrates that contained bowtie-shaped, micrometer-sized wells with bases made of fibronectin-coated glass and 20 μm-high walls made of agarose. Cells plated on these substrates attached only in the fibronectin-coated wells and not on the agarose. When two cells attached and spread in the well, each was constrained to spread triangularly to cover half of the area of the well (Fig. 2B). The cells contacted each other at the central constriction to form adherens junctions containing VE-cadherin and β-catenin identical to those of unpatterned cells (Fig. 2C). When a single cell attached in a well, it spread to fill half of the well, leaving the other half empty. Thus, pairs of cells only differed from single cells by the presence of a cell-cell contact; cell spreading was identical.

Using the patterned substrates, we first examined whether cell-cell contact would decrease proliferation in the absence of changes in cell spreading. Endothelial cells were synchronized at confluence, plated on two sizes of patterned substrates (750 μm^2 or 1000 μm^2 per half) such that single cells or pairs of cells populated the wells, and proliferation assessed by measuring the incorporation of BrdU. Consistent with previous studies (Chen et al., 1997), proliferation increased with cell spreading (1000 μm^2 as compared to 750 μm^2) in both single cells and pairs of cells (Fig. 2D). Interestingly, cells grown in pairs proliferated more than single cells of equal cell spreading. At both degrees of cell spreading, cell-cell contact increased proliferation by the same absolute magnitude, implying that the proliferative signals from spreading and intercellular contact are distinct and additive.

Engagement of VE-cadherin is required for cell-cell-induced proliferation

To determine whether VE-cadherin engagement was necessary for the contact-mediated stimulation of growth, we examined the proliferation of cells in the bowties with and without VE-cadherin function-blocking antibodies. Treatment with two different VE-cadherin function-blocking antibodies inhibited the contact-mediated increase in proliferation (Fig. 2E). At the concentration used to inhibit cell-cell-induced proliferation, treatment with function-blocking anti-VE-cadherin diminished the localization of VE-cadherin and β-catenin, but did not induce gap formation or retraction between pairs of cells or within monolayers (Fig. 2F,G), and did not affect the proliferation of cells without contacts. However, cadherin-blocking studies do not exclude the role of other junctional proteins, because inhibiting cadherin engagement has been shown to inhibit the formation of other types of contacts (Gottardi et al., 2001), including gap junctions, tight junctions and PECAM-1-containing contacts.

To determine whether VE-cadherin alone was responsible for the contact-mediated increase in proliferation, we co-cultured endothelial cells with A431 carcinoma cell lines that were either cadherin-null (null) or expressed recombinant human VE-cadherin (VE^+). In monolayers, endothelial cells formed VE-cadherin-containing contacts with VE^+ cells (Fig. 3A) but not with null cells (Fig. 3B). Neither null nor VE^+ cells formed gap junctions, tight junctions or PECAM-1 contacts with the endothelial cells as determined by immunofluorescence staining (Fig. 3A,B) and functional gap junction analysis (data not shown). Thus, the cell lines were used to present VE-cadherin to the synchronized endothelial cells. When endothelial cells were co-cultured with null cells in heterotypic pairs on the bowtie-shaped patterns, the proliferation rate of the endothelial cells was similar to that of single endothelial cells; co-culturing endothelial cells with VE^+ cells increased proliferation of the endothelial cells to that of pairs of endothelial cells (Fig. 3C). Thus, VE-cadherin alone reproduced the stimulatory signal for proliferation.

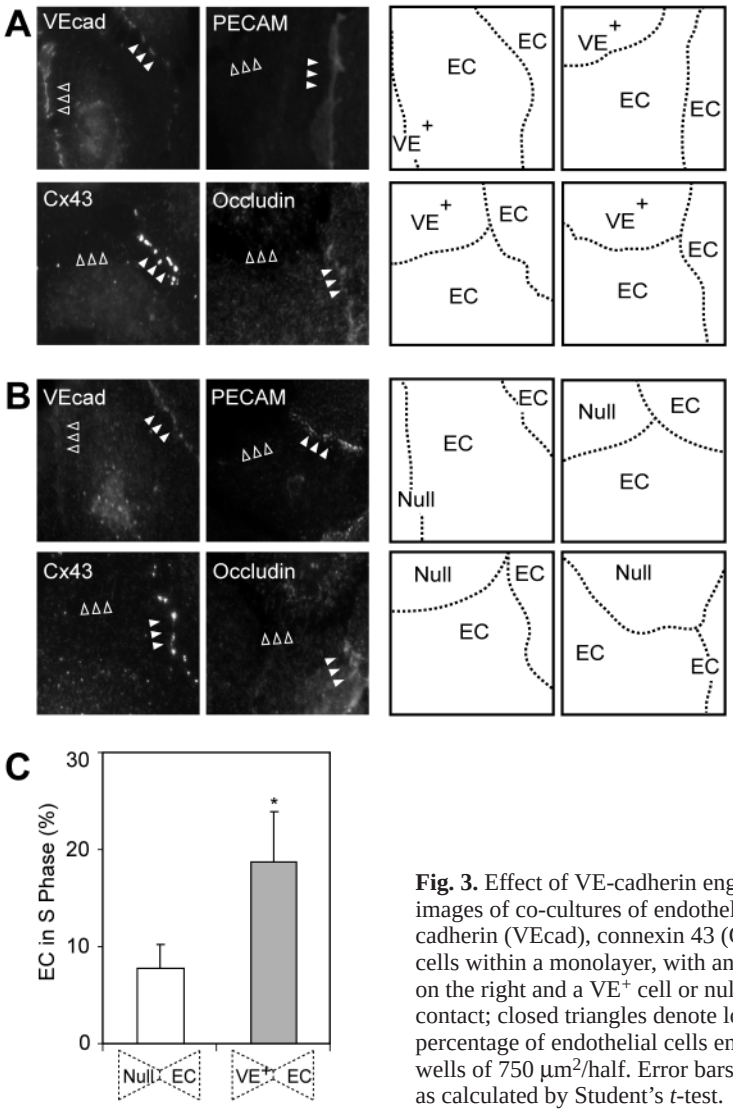

Fig. 3. Effect of VE-cadherin engagement on endothelial cell proliferation. Immunofluorescence images of co-cultures of endothelial cells and VE^+ cells (A) and null cells (B) stained for VE-cadherin (VEcad), connexin 43 (Cx43), PECAM-1 or occludin. Each image shows portions of three cells within a monolayer, with an endothelial cell in the center contacted by another endothelial cell on the right and a VE^+ cell or null cell on the left. Open triangles denote location of heterotypic contact; closed triangles denote location of homotypic endothelial cell contact. (C) Graph of percentage of endothelial cells entering S phase when co-cultured with null cells or VE^+ cells in wells of 750 μm^2/half. Error bars represent the s.d., with (*) $P<0.05$ relative to null cell co-cultures as calculated by Student's *t*-test.

VE-cadherin inhibits proliferation by decreasing cell spreading

Although these findings suggest that engagement of VE-cadherin can either inhibit (Fig. 1) or stimulate (Figs 2, 3) proliferation under different experimental conditions, it remained unclear what caused the switch in proliferative response. Because the inhibition of proliferation by VE-cadherin occurred in an experimental system that allowed cells to spread and form cell-cell contacts freely, whereas stimulation occurred when both cell spreading and cell-cell contact were constrained, we examined how constraining cell-cell contact but allowing cells to spread freely would affect the proliferative response. We controlled cell-cell contact but increased the ability of cells to spread by culturing endothelial cells in bowtie-shaped patterns with constant constriction size but increasing area. Cells plated on these substrates continued to form contacts, but as the sizes of the wells progressively increased, progressively fewer cells spread to fill the wells (Fig. 4A). The percentage of cells that fully spread to fill the wells was significantly lower in cells cultured in pairs than in single cells; this difference became more pronounced with the larger bowties. Cell-cell contact stimulated proliferation on the smaller bowties, but switched to an inhibitory effect on the largest bowties (Fig. 4B). Interestingly, when we divided the proliferation data for each condition into fully spread and partially spread sub-groupings, we revealed that the cadherin-mediated stimulus for proliferation was still present (Fig. 4C,D). The inhibition of proliferation observed for cells in pairs in the largest bowties was therefore a direct consequence of the high percentage of these cells that failed to spread fully. Co-culturing endothelial cells with null or VE^+ cells on the larger wells confirmed that VE-cadherin was responsible for the decrease in cell spreading (Fig. 4A). Taken together, these data suggest that cell-cell contact emits two opposing signals that regulate growth: one inhibits proliferation by decreasing cell spreading; the other promotes proliferation via a spreading-independent pathway. Both signals – the inhibition and stimulation of proliferation by intercellular contact – are mediated by VE-cadherin and are operating simultaneously.

Cell-cell-induced proliferation is blocked by inhibiting MEK or PKC

To explore further the contact-mediated stimulation of proliferation, we examined possible cellular pathways that might be involved. To determine whether specific signal transduction pathways associated with cadherin engagement were involved, we pharmacologically inhibited MEK and PKC. Pharmacological inhibitors were added to cells 2 hours after they were plated on bowtie-shaped patterns. The cell-cell

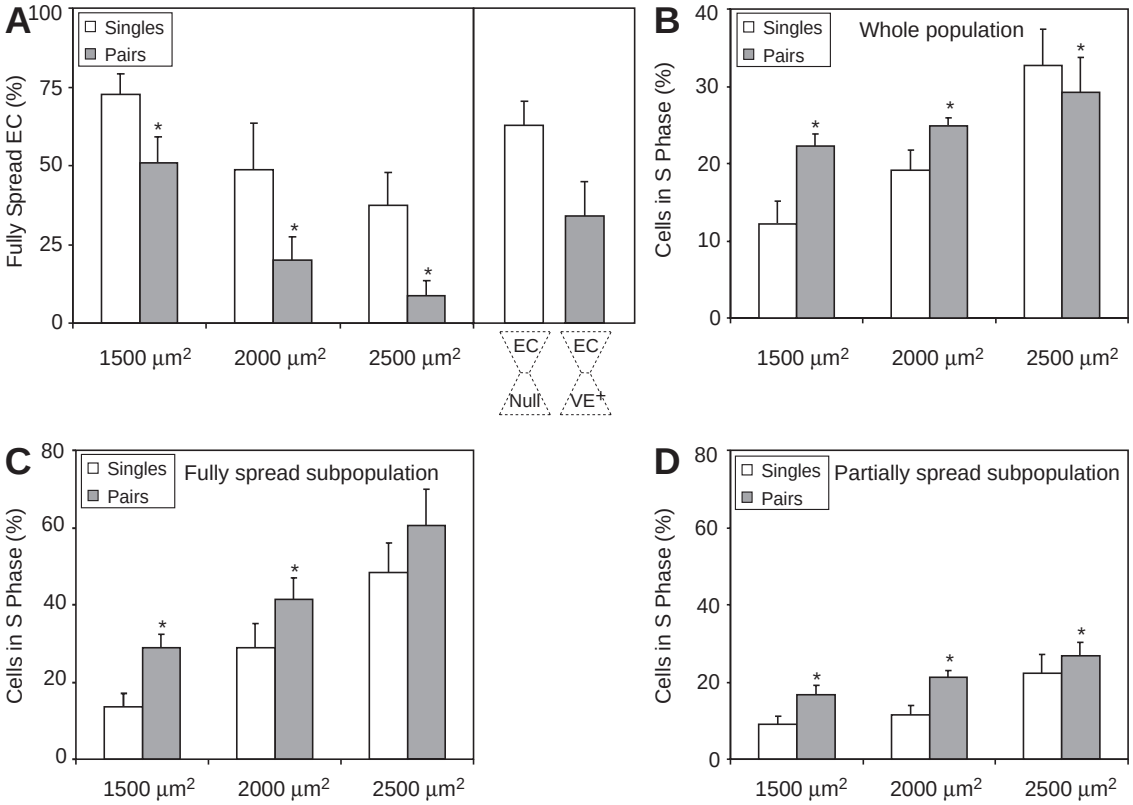

Fig. 4. Role of cell spreading in contact-mediated inhibition of proliferation. (A) Graph of percentage of fully spread cells for single cells and pairs of cells cultured in wells of 1500 μm^2/half, 2000 μm^2/half and 2500 μm^2/half (left); percentage of fully spread endothelial cells when co-cultured with null or VE^+ cells in wells of 1500 μm^2/half (right). (B) Graph of percentage of cells entering S phase for single cells and pairs of cells cultured in all three sizes of wells. (C,D) Graph of percentage of cells entering S phase for single cells and pairs of cells cultured in all three sizes of wells when cells were separated into (C) fully or (D) partially spread populations. Error bars indicate the s.d. of four experiments, with (*) $P<0.05$ relative to single cells, as calculated by the paired Student's *t*-test.

contact-induced increase in proliferation was selectively inhibited over single cell controls by the MEK inhibitor U0126 at concentrations greater than 60 nM (Fig. 5A). Similarly, inhibiting PKC with Ro-31-7549 (Fig. 5B) or H-7 (Fig. 5C) abrogated the increase in proliferation of cells grown in pairs. At higher concentrations, the drugs also affected cell spreading and equally inhibited proliferation in single cells and pairs of cells, indicating possible toxicity. Interestingly, treating cells with drugs at the concentrations that specifically inhibited cell-cell-induced proliferation also altered the actin cytoskeleton. Untreated cells cultured in pairs exhibited a characteristic, continuous band of actin fibers across the cell-cell contact, whereas treatment with U0126, Ro-31-7549 or H-7 dramatically altered or inhibited the formation of these fibers, without disrupting adherens junctions (Fig. 5D-G). Because adherens junctions are structurally analogous to focal adhesions, which regulate proliferation through both growth factor- and cytoskeleton-mediated pathways (Giancotti, 1997), and because both MAPK and PKC signaling dually affect proliferation and the cytoskeleton, our findings raised the possibility that the contact-mediated stimulatory signal for growth is mediated directly through the actin cytoskeleton.

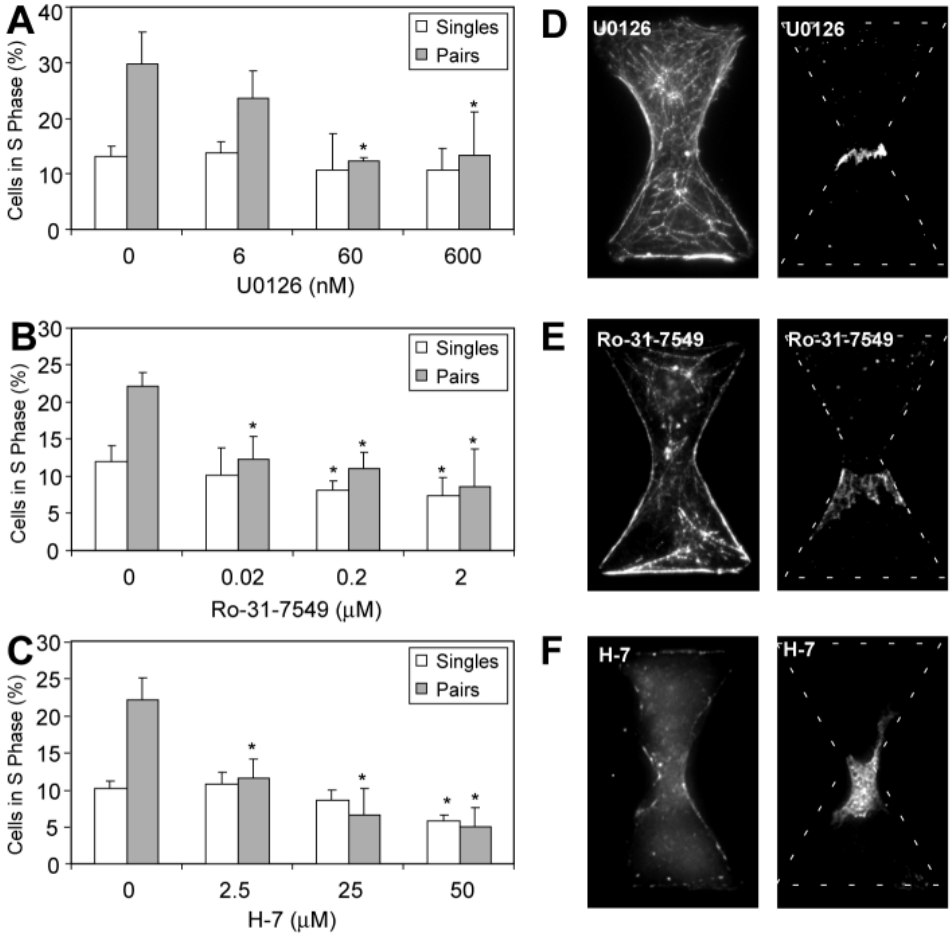

Fig. 5. Role of MEK and PKC pathways in cell-cell contact-mediated proliferation. Graphs of percentage of cells in S phase (A-C) and immunofluorescence images of actin (left) or β-catenin (right) staining (D-G) in cells on agarose patterns either treated with U0126 (A,D), Ro-31-7549 (B,E), or H-7 (C,F), or untreated (G). Scale bar: 25 μm. Actin and β-catenin images were acquired on different samples. Error bars indicate s.d. of three experiments, with (*) $P<0.05$ relative to untreated controls, as calculated by Student's *t*-test.

Cell-cell-induced proliferation requires actin cytoskeletal structure and tension

To examine directly whether the structural changes in actin we observed after drug treatment were involved in the specific inhibition of cell-cell-induced proliferation, we disrupted actin filament structure. Cells grown on the patterned substrates were treated with varying concentrations of cytochalasin D (Fig. 6A,B) or latrunculin B (Fig. 6C,D). At 0.01 μg/mL – which disrupted stress fibers but not cortical actin – cytochalasin D inhibited the contact-mediated increase in proliferation of pairs over single cells, which were unaffected. At 0.1 μg/mL – which disrupted both stress fibers and cortical actin – cytochalasin D dramatically reduced the proliferation of single cells. Higher concentrations of the drug interfered with cell spreading. Similar results were obtained with latrunculin B. These findings suggested that stress fibers alone were necessary and specific for the cell-cell-induced proliferative signal. To test directly whether it is the presence of the actin cytoskeleton or specifically the generation of cytoskeletal tension that is required for the increase in proliferation, we inhibited actin-myosin cycling with BDM (Fig. 6E,F). At low concentrations, BDM specifically reduced the cell-cell contact-mediated increase in proliferation without altering the growth of single cells. This result was confirmed with another myosin light chain kinase inhibitor, ML-7 (Fig. 6G,H). Staining for β-catenin and VE-cadherin in drug-treated cells verified that disrupting cytoskeletal structure and mechanics did not disrupt the formation of adherens junctions. These data suggest that cadherin-dependent proliferation depends on the integrity of the cytoskeleton and the generation of tension.

One of the major regulators affecting actomyosin tension generation is the Rho-ROCK pathway (Etienne-Manneville and Hall, 2002). To determine whether signaling through the Rho-ROCK pathway was required for the VE-cadherin-induced proliferation, we pharmacologically inhibited ROCK with Y-27632 (Fig. 7A,B).

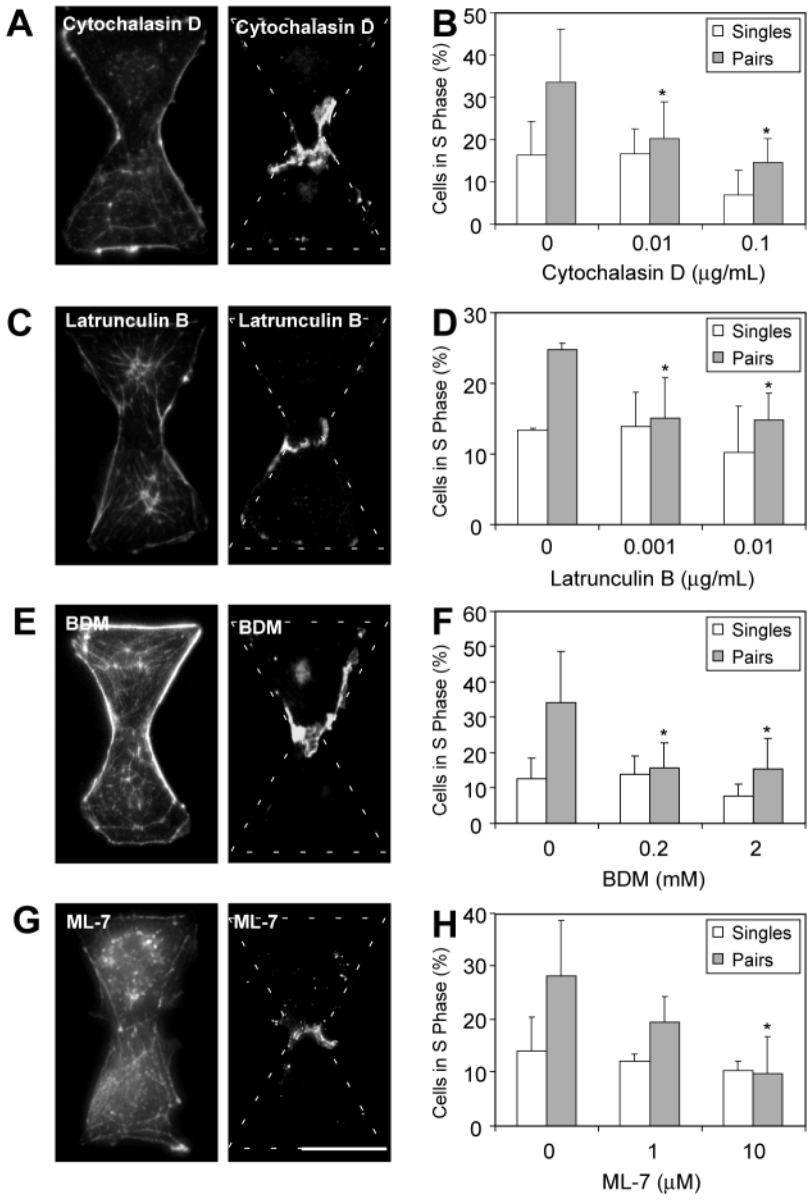

Fig. 6. Role of the cytoskeleton in cell-cell contact-mediated proliferation. Immunofluorescence images of actin (left) or β-catenin (right) staining and graphs of percentage of cells in S phase on agarose patterns treated with cytochalasin D (A-B) or latrunculin B (C-D) to disrupt the actin cytoskeleton and BDM (E-F) or ML-7 (G-H) to inhibit actomyosin dynamics. Broken lines (white) indicate the borders of the wells. Scale bar: 25 μm. Actin and β-catenin images were acquired on different samples. Error bars indicate s.d. of three experiments, with (*) $P<0.05$ relative to untreated controls, as calculated by Student's *t*-test.

Treatment with Y-27632 was sufficient to selectively inhibit cell-cell-induced proliferation. To confirm that Rho was required for the proliferative effect, we infected cells with an adenovirus expressing dominant-negative RhoA (Ad-RhoN19) bicistronic with GFP (Fig. 7C,D). Adenovirus expressing GFP alone was used as a control (Ad-GFP). Infection with Ad-RhoN19 specifically inhibited the cell-cell-induced proliferation in pairs of cells without altering the growth of single cells. Infection with Ad-GFP had no effect on proliferation or cell morphology. Inhibiting ROCK and Rho disrupted the actin cytoskeleton without disrupting the formation of adherens junctions, as verified by staining for actin and β-catenin. Collectively, these data suggest that the Rho-ROCK pathway is involved in the cytoskeletally dependent VE-cadherin-stimulated proliferation of endothelial cells.

Discussion

Previous studies have demonstrated the anti-proliferative effect of cell-cell contact and identified VE-cadherin as its principal mediator in endothelial cells (Caveda et al., 1996). Our findings suggest that the mechanism for this effect lies in the ability of VE-cadherin to actively decrease cell spreading (Fig. 8). The degree of cell spreading on extracellular matrix is a potent regulator of endothelial cell proliferation (Chen et al., 1997); inhibiting cell spreading increases levels of $p27^{kip1}$ and decreases cyclin D1, thereby arresting cell-cycle progression late in G1 (Huang et al., 1998). There are many mechanisms by which VE-cadherin could decrease cell spreading. Cell-cell contact formation and compaction through cadherins induce both local and global changes in the tension and structure of the actin cytoskeleton (Adams et al., 1998), each of which may change cell shape. Cadherin-based adhesions also decrease the expression of integrins (Zhu and Watt, 1996) as well as recruit vinculin to cell-cell contacts while reducing vinculin at focal adhesions (Levenberg et al., 1998). Thus, cell-cell contact could reduce the strength or stability of cell-ECM contacts essential for cell spreading. Although crosstalk between cadherin and integrin engagement is known to influence the balance of adhesive forces at cell-cell and cell-ECM boundaries (Dudek and Garcia, 2001), our work suggests that this crosstalk is also central to the cellular decision to proliferate. Our findings that VE-cadherin decreases proliferation by decreasing cell spreading are consistent with recent evidence demonstrating that the engagement of cadherins blocks proliferation by increasing $p27^{kip1}$ levels and inhibiting cell-cycle progression in S phase (St Croix et al., 1998). Thus, although it was previously thought that cadherin engagement and reduced cell spreading independently contributed to proliferation arrest at confluence, it now appears that these signals are linked through a direct causal mechanism.

Culturing pairs of cells on bowtie-shaped patterns revealed a distinct stimulatory signal for proliferation, also initiated by VE-cadherin. The patterned substrates that stimulated growth (Figs 2, 3) differed from the high-density cultures that inhibited growth (Fig. 1) in two important ways – first, the patterned cells could not change cell spreading upon formation of cell-cell adhesion, and second, they had less cell-cell contact. Holding the degree of cell-cell contact constant while freeing the cells

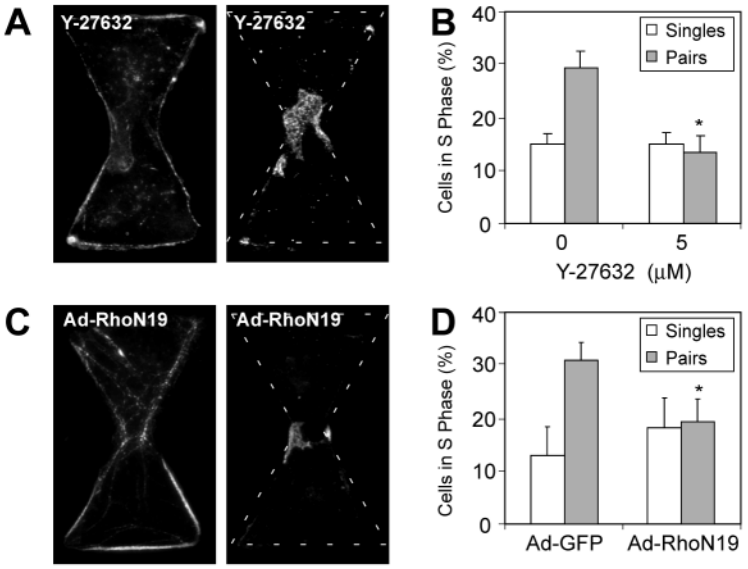

Fig. 7. Role of the cytoskeletal regulator Rho in cell-cell contact-mediated proliferation. Immunofluorescence images of actin (left) or β-catenin (right) staining and graphs of percentage of cells in S phase on agarose patterns treated with Y-27632 (A-B) to inhibit ROCK, or infected with Ad-GFP control or Ad-RhoN19 (C-D) to inhibit Rho. Broken lines (white) indicate the borders of the wells. Scale bar: 25 μm. Actin and β-catenin images were acquired on different samples. Error bars indicate s.d. of three experiments, with (*) $P<0.05$ relative to controls, as calculated by Student's *t*-test.

to change cell spreading, we found that 'small' contacts are sufficient to suppress proliferation (Fig. 4). Thus, the same amount of VE-cadherin engagement is sufficient for both the increases and decreases in proliferation. Although increasing the sizes or numbers of contacts may enhance either or both of these opposing signals, and may reveal additional layers of proliferative regulation, our data currently support a model in which VE-cadherin inhibits proliferation by decreasing cell spreading, and stimulates proliferation via a spreading-independent signal (Fig. 8).

Even when cell-cell contact inhibited the overall proliferation rate of bulk populations, examining subpopulations of cells of equal spreading demonstrated the existence of the stimulatory cue. Our data thus suggest the ubiquitous presence of both inhibitory and stimulatory signals by VE-cadherin engagement. The inhibitory signal can be attenuated by restricting how cells spread, whereas the stimulatory signal cannot. Which of these two signals then dominates the response of the population depends on how well the surrounding microenvironment supports cell spreading.

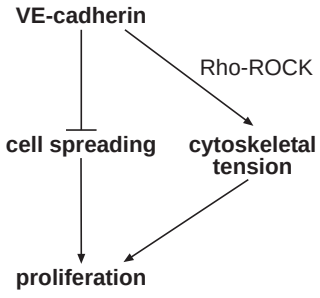

Fig. 8. Model proposing how VE-cadherin mediates simultaneous opposing signals for proliferation. VE-cadherin inhibits cell spreading, leading to proliferation arrest. When cells are already physically constrained, VE-cadherin engagement leads to an increase in proliferation by signaling through Rho-dependent changes in cytoskeletal tension.

These findings may explain why cell-cell contact appears to inhibit proliferation in vitro (when cells are freely spread and can therefore retract with cell-cell contact), but is capable of stimulating proliferation in vivo (when cells are physically restricted from changing cell shape). For example, it has been demonstrated in vivo that endothelial cells proliferate while in contact with their neighbors, such as during large vessel morphogenesis or capillary angiogenesis (Carmeliet and Jain, 2000; Hanahan and Folkman, 1996), and that these processes can be blocked by knocking out or pharmacologically inhibiting VE-cadherin (Carmeliet et al., 1999; Liao et al., 2000).

The newly identified VE-cadherin-dependent increase in proliferation appears to depend not only on signals previously known to be stimulated by cadherin engagement, including the MAPK and PKC pathways (Lewis et al., 1994; Pece and Gutkind, 2000), but also specifically on the cytoskeleton. The dual dependence on signaling and the cytoskeleton for VE-cadherin-dependent proliferation is analogous to the regulation of G1 progression by integrins and growth factors (Huang et al., 1998), suggesting a functional comparison between adherens junctions and focal adhesions. The linkages between extracellular environment, cytoskeleton and signaling machinery at these types of adhesions (Gumbiner, 1996; Yamada and Geiger, 1997) now appears to be a general mechanism for the integration of mechanical and chemical signaling. However, proliferative signals arising from cell-cell contacts are distinct, at least initially, from those from cell-ECM adhesions. The selective inhibition of cell-cell contact-induced proliferation over single cells with various inhibitors highlights these differences. Even the dependence of proliferative signaling on the actomyosin system appears to be selective: contact-mediated proliferation appears to require stress fibers, whereas integrin-mediated signals only require cortical actin (Zhu and Assoian, 1995). Although it is not yet clear how cytoskeletal tension modulates cadherin signaling, these findings suggest the possibility that the adherens junction may indeed act as a distinct mechanosensor.

The decreases in cell spreading and simultaneous tension-

dependent mitogenic signals generated from cadherin engagement each support the involvement of the Rho family of small GTPases. Inhibiting Rho and ROCK in our system both disrupted the actin cytoskeleton and blocked the VE-cadherin-mediated stimulation of proliferation. Members of the Rho family, including RhoA, Rac1 and Cdc42, are well-established regulatory molecules responsible for mediating specific changes in the actin cytoskeleton important to both cell spreading and cytoskeletal tension (Nobes and Hall, 1995). In addition to their direct effects on cytoskeletal structure, the GTPases also regulate mitogenic pathways. For example, Rho is required for sustained ERK activity and mid-G1 phase production of cyclin D1 for adhesion-dependent cell-cycle progression (Welsh et al., 2001). Our data are consistent with recent evidence from other groups suggesting that cadherins actively alter the actin cytoskeleton and focal adhesion components, possibly through Rho GTPases (Kovacs et al., 2002; Lampugnani et al., 2002). Thus, such crosstalk between cadherins and integrins may be responsible for the simultaneous cadherin-mediated changes in cell spreading and proliferation.

Previous linkages between the integrin and growth factor pathways have suggested a complex inter-relationship between chemical signaling, structural organization and mechanical cues. Our findings would suggest that cadherins and cell-cell contacts are also intricately linked to this web of mechanochemical signaling and highlight the presence of structural cues that coordinate neighboring cells in a multicellular environment. The fact that the same intercellular adhesion molecule, VE-cadherin, exerts both positive and negative growth signals simultaneously through cytoskeleton-dependent pathways emphasizes the organizational complexity of both the signaling and structural networks of the cell. The inextricable relationships between structure and function in the whole cell may point to a general paradigm for how cells are able to coordinate the many distinct cues from their environment, including juxtacrine, growth factor, extracellular matrix and mechanical signals, into a single network to produce functional responses such as proliferation. Such integration of mechanochemical signals may be fundamental to the development and maintenance of the spatial organization of multicellular life.

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