Cell-cell adhesion in human fibroblasts requires calcium signaling

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SUMMARY

In connective tissues, intercellular adhesion is essential for tissue morphogenesis, development and wound healing. However, the signaling mechanisms initiated by cell-cell adhesion in fibroblasts and that regulate it are not known. In this study we tested the hypothesis that intracellular calcium signaling is required to mediate intercellular adhesion between fibroblasts. Fura-2 or fluo-3 labeled human fibroblasts were used to investigate calcium homeostasis during intercellular adhesion. After contact with suspended fibroblasts there was a rise in cytosolic free calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) and multiple calcium oscillations in substrate-attached cells. Antibodies against the extracellular but not the cytoplasmic domain of cadherin induced a similar calcium response, indicating that these responses were initiated by cadherin binding. As shown by the near-plasma membrane Ca\textsuperscript{2+} indicator (Fura-C18) and by confocal microscopy of fluo-3-loaded cells, [Ca\textsuperscript{2+}]\textsubscript{i} transients probably originated at sites of cell-cell contact. Cell-cell adhesion was dependent on both calcium influx through membrane channels and release of Ca\textsuperscript{2+} from internal calcium stores, because the calcium channel inhibitor LaCl\textsubscript{3} or pretreatment of cells with thapsigargin significantly inhibited (>35%) cell-cell attachment. The [Ca\textsuperscript{2+}]\textsubscript{i} changes induced by cell-cell adhesion were temporally correlated with increased recruitment of intercellular junctional proteins into the cytoskeleton and movement of GFP-actin to sites of cell-cell contact. [Ca\textsuperscript{2+}]\textsubscript{i} responses induced by intercellular adhesion were essential for both junctional protein recruitment and the establishment of strong cell-cell contacts, as loading cells with BAPTA/AM significantly inhibited cell-cell adhesion and recruitment of cadherins and β-catenin to the actin cytoskeleton. Actin depolymerization by cytochalasin D dramatically reduced cell-cell adhesion and recruitment of cadherins and catenin to the actin cytoskeleton. These results demonstrate that cadherin-cadherin interaction induces [Ca\textsuperscript{2+}]\textsubscript{i} transients during cell-cell adhesion in fibroblasts, and these calcium signals regulate cell-cell adhesion through remodeling of cortical actin and recruitment of cadherins and β-catenin into intercellular junctions.

Key words: Cell-cell adhesion, Calcium, Fibroblast, Cadherin, Catenin

INTRODUCTION

Intercellular adhesion is important in tissue differentiation and remodeling. For example, the intercellular adherens junctions mediated by cadherins are essential for tissue morphogenesis (Gumbiner, 1996) and are crucial for the maintenance of solid tissues as well as cell recognition and cell sorting during development (Takeichi, 1991). Homotypic cell-cell adhesion is largely mediated through cadherins, which are calcium-dependent cell-cell adhesion molecules (Gumbiner, 1996; Takeichi, 1991). Cadherins cluster to cell-cell contacts, then complex with catenins, which crosslink to actin filaments and then contribute to the formation of adherens junctions. The interaction of the cadherin cytoplasmic tail with the actin cytoskeleton is required for cell-cell adhesive activity (Knudsen et al., 1998).

The requirement for extracellular calcium in the formation of intercellular adherens junctions has been shown and extensively studied (Volberg et al., 1986; Green et al., 1987; Chitaev and Troyanovsky, 1998), but how calcium signaling regulates cell-cell adhesion is poorly understood. Intracellular calcium signaling is important in mediating actin rearrangement (Janmey, 1994), a required process in the formation of adherens junctions; however, there has not been any study on the possible role of intracellular calcium signaling in the development and subsequent strengthening of fibroblastic intercellular contacts. Intercellular contact-induced intracellular Ca\textsuperscript{2+} signaling has only been studied in highly specialized heterotypic cell-cell interactions such as those between lymphocyte and cytokine-treated endothelial cells (Pfau et al., 1995) as well as between T cell and antigen-presenting cell in T cell activation (Liu and Golan, 1999; Negulescu et al., 1996). However, intracellular Ca\textsuperscript{2+} signaling and its regulation of actin organization has not yet been characterized in the formation of adherens junctions.

In human fibroblasts, cell-cell junctions are thought to be essential structures for connective tissue remodeling and wound healing in vivo (Gabbiani and Rungger-Brandle, 1981). However, while cell-cell junctions have been extensively studied in epithelial cells, there are few studies on the formation of adherens junctions in human fibroblasts. Fibroblasts from periodontal connective tissues provide a good
model for the study of intercellular adhesion and its importance in tissue remodeling since fibroblasts from these tissues form extensive adherens junctions in vivo (Beertsen and Everts, 1980; Shore et al., 1981) and these cells mediate rapid turnover of the extracellular matrix (Sodek and Ferrier, 1988).

To study the formation of cell-cell adhesion, previous models have used: (1) switching cells from Ca^{2+}-free medium to high-Ca^{2+} medium (Volberg et al., 1986; Green et al., 1987; Chitaev and Troyanovsky, 1998); (2) waiting for the filopodia from different cells to collide (Glushankova et al., 1998); and (3) decompaction and recompaction studies of embryos (e.g. Pey et al., 1998). Some reports have used retrospective immunohistochemistry to examine the dynamic nature of cell-cell contact formation (McNeill et al., 1993), but it is not possible to perform real-time mechanistic studies with this approach.

Here we investigated Ca^{2+} signaling during homotypic cell-cell adhesion in periodontal fibroblasts by studying the attachment of suspended cells (S cells) onto an attached monolayer of cells (A cells). This model allows the synchronous formation of cadherin-mediated cell-cell adherens junctions in a cohort of cells (Ko et al., 2000). We examined the underlying mechanisms by which intracellular calcium signaling regulates the formation of cell-cell adherens junctions. We found that intracellular calcium signaling is initiated upon cadherin-mediated cell-cell contact, through Ca^{2+} influx and Ca^{2+} release from thapsigargin-sensitive stores. Intercellular adhesivity is reduced by perturbation of intracellular calcium homeostasis. Furthermore, our results show that intercellular adhesion-induced intracellular calcium signaling mediates the recruitment of cadherins and β-catenin to the cytoskeleton, a process that is required for strengthening of cell-cell adhesive contacts.

**MATERIALS AND METHODS**

**Reagents**

Primary antibodies against human antigens including mouse monoclonal anti-connexin43 (Clone 2) and anti-β-catenin (Clone 14) were purchased from Transduction Laboratories (Lexington, NY, USA). Mouse monoclonal antibodies for β1 integrin (clone 4B4-RD1) were purchased from Coulter (Burlington, ON, Canada). Rat monoclonal (clone DECMA-1) anti-ε-cadherin, monoclonal mouse anti-pan-cadherin (Clone CH-19) and FITC-goat anti-mouse antibodies, FITC-phalloidin, Cytochalasin D and Thapsigargin were purchased from Sigma Chemical Co. (St Louis, MO, USA). BAPTA/AM, Calcein/AM, DiI-CM (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate-chloromethylbenzamido), Fluo-3/AM, Fura-2/AM, Fura-C18, saponin and Texas Red-dextran (10 kDa) were purchased from Molecular Probes (Eugene, OR, USA). The RGD peptides GRGDSP and GRGESP were purchased from Life Technologies Inc. (Grand Island, NY, USA). The E-cadherin mimetic peptide (amino acid sequence LFSHA VSSNG) and E-cadherin scrambled peptide (amino acid sequence LHSNVSFGSA) were synthesized by the Alberta Peptide Institute (Edmonton, Alberta, Canada).

**Cell culture**

Human gingival fibroblasts (HGF) were derived from primary explant cultures as described (Pender and McCulloch, 1991). Cells from passages 6-15 were grown as monolayers in T-75 flasks. Full growth medium consisted of α-minimal essential medium (α-MEM), antibiotics (0.017% penicillin G; Ayerst Lab., Montreal, PQ, Canada), 0.01% gentamycin sulphate (Life Technologies, Grand Island, NY, USA) in α-MEM, and 10% (v/v) heat-inactivated fetal bovine serum (FBS; ICN Biomedicals, Costa Mesa, CA, USA). 2 days before each experiment cells were harvested with 0.01% trypsin and approximately 100,000 cells were plated onto 35 mm diameter culture dishes (Becton Dickinson, Mississauga, ON, Canada). The cells were grown to confluence prior to all experiments except when sparse cultures were used, as indicated.

Rat-2 cells (ATCC CRL1764; American Type Culture Collection, Rockville, MD, USA) were incubated in Dulbecco’s modified Eagle’s medium (DMEM; high glucose) containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO2 and 95% air. According to the ATCC, Rat-2 cells are diploid, and are easily transfectable by exogenous DNA, one of the principal rationales for the choice of these cells in the studies reported herein. Rat-2 cells also exhibit several common phenotypic traits as periodontal fibroblasts including rapid collagen synthesis, osteopontin synthesis, collagen degradation by phagocytosis and fibroelastic morphology (Hui et al., 1997).

**Intracellular calcium**

For measurement of whole cell intracellular calcium ion concentration ([Ca^{2+}]_{i}), cells on coverslips were loaded at 37°C with 3 μM Fura-2/AM (a Ca^{2+}-sensitive fluorescent dye) for 20 minutes. For measurement of near-plasma membrane Ca^{2+} concentration, cells attached on coverslips were briefly permeabilized with saponin and incubated with 20 μM Fura-C18, pentapotassium salt (Etter et al., 1994) at 25°C for 10 minutes, followed by three washes with PBS. The calcium-free buffer (CF buffer) consisted of a bicarbonate-free medium containing 150 mM NaCl, 5 mM KCl, 10 mM D-glucose, 1 mM MgSO4, 1 mM NaHPO4 and 20 mM Heps, pH 7.4, with an osmolality of 291 mOSm. For experiments requiring external calcium, 1 mM CaCl2 was added to the buffer. The attached cells were washed twice and transferred to a tissue culture chamber (Corning, Corning, NY, USA). After incubation with Fura-2/AM, inspection of cells by fluorescence microscopy demonstrated no vesicular compartmentalization of Fura2, suggesting that the dye loading method allows measurement of cytosolic [Ca^{2+}]; and not that of calcium located in membrane-bound compartments such as endosomes. Visual inspection of Fura-C18 loaded cells showed fluorescent labeling of the plasma membrane.

Whole cell [Ca^{2+}]_{i}, measurements and plasma membrane [Ca^{2+}]_{i} were obtained with a Nikon Diaphot II inverted microscope optically interfaced to a Deltascan 4000, dual beam, epifluorescence spectrophotometer and analysis system (Photon Technology Int., London, ON, Canada) operating on a 386 SX personal computer. The dual excitation fluorochromes Fura-2 or Fura-C18 were excited at alternating (approximately 100 Hz) wavelengths of 346 and 380 nm from dual monochromators with slit widths set at 2 nm. Emitted fluorescence was collected with a 40× quartz I.32 NA oil immersion Nikon Fluor objective and passed through a 530/20 nm barrier filter (Omega Optical, Brattleboro, VT, USA). A variable aperture, intrabeam mask was used to restrict measurements to single cells or a small group of cells. Signals from the photomultiplier tube (D104, PTI) were recorded at five points per second. Estimates of [Ca^{2+}]_{i}, independent of the precise intracellular concentration of Fura-2 were calculated from dual excitation emitted fluorescence according to the equation of Grynkiewicz et al. (Grynkiewicz et al., 1985) and as described earlier (Ko et al., 1998). Oscillations in [Ca^{2+}]_{i} during cell-cell contact formation (McNeill et al., 1993), but it is not possible to perform real-time mechanistic studies with this approach.
cytoplasmic actin cDNAs (Kaech et al., 1997). Specifically, the GFP-β-actin construct was in a pcDNA3 vector driven by an actin promoter. The construct was cut from the plasmid with EcoR1 and HindIII and was propagated in XL-Blue bacteria. The GFP coding region of pEGFP-N1 (Clonetech, Palo Alto, CA, USA) was fused in-frame to the actin coding region either directly at the N terminus or at the C terminus after an 11-amino-acid linker from vesicular stomatitis coat virus protein (Kaech et al., 1997). For transient transfections, the GFP-β-actin expressing plasmids (3 µg) were coated with cationic lipid, (6 µL; Lipofectamine, Gibco BRL, ON, Canada) to form liposomes. The liposomes were incubated with Rat-2 cells plated on 2-well chamber coverglass (Labtek) in DMEM without serum for 6-12 hours and then with DMEM containing 20% serum for another 24 hours to facilitate integration of the cDNA into these cells. The transfected cells were observed by confocal microscopy.

**Intercellular adhesion assay**

To measure cell-cell adhesion, HGF were grown to a confluent monolayer in full growth medium. Another set of suspended HGF (S cells) were fluorescence-labeled with Calcein-AM (5 µg/mL, 1 hour incubation at 37°C, followed by three washes with α-MEM) and plated onto the established monolayer of attached cells (A cells). Attachment and spreading of the S cells were monitored and recorded at specific time points (0-180 minutes) with a fluorescence microscope coupled to a CCD camera (Princeton Instruments, NJ, USA). Quantification of relative adhesivity under different experimental conditions was done by counting the number of S cells per high power microscope field after 90 minutes of cell-cell contact and that remained attached following three washes with PBS (Ko et al., 2000). The A cell monolayer was grown at least overnight to ensure that the acceptor cells were highly adherent to the tissue culture plate and were not detached during jet-washing. For immunoblotting experiments, the A cell layer was grown to approx. 60-70% confluency and at specific time points (0-180 minutes) with a fluorescence microscope coupled to a CCD camera (Princeton Instruments, NJ, USA). Staining for both Fura-2 and Texas Red. Minimal dye-transfer between Fura-2-loaded and Texas Red-dextran-loaded cells. Evidence of dye-coupling (a measure of gap junctional communication) was detected using anti-pan-cadherin monoclonal antibody (Clone CH-19) and anti-β-catenin monoclonal antibody (Clone 14), respectively. Blots were blocked for 1 hour with 5% skimmed milk in PBS and incubated with the indicated antibody in 0.1% Tween-TBS for 30 minutes. For visualization of actin filaments, cells were fixed with 1% paraformaldehyde in PBS and incubated with the indicated antibody for 30 minutes. To identify and localize specific molecules involved in cell-to-cell adhesion, immunocytochemistry was performed for cadherins (using pan-cadherin antibody) and β-catenin. Cells grown on coverslips were fixed and permeabilized with methanol at −20°C for 10 minutes, blocked with 1:1000 mouse serum in PBS for 10 minutes and incubated with primary antibody (1:100 dilution) for 1 hour at room temperature, washed three times with PBS containing 0.2% BSA, and incubated with FITC-conjugated goat anti-mouse (1:100). Non-specific control staining was performed on a separate coverslip using irrelevant isotype control antibody and secondary antibody. Coverslips were washed with PBS and mounted with an anti-fade mounting medium (ICN). For visualization of actin filaments, cells were stained with TRITC-phalloidin and examined using a ×40, 1.3 NA oil immersion objective under epifluorescence optics and confocal imaging (Leica CLSM, Heidelberg, Germany).

**Confocal microscopy**

Laser scanning confocal microscopy was used to locate and identify adhesive and gap junctional proteins at the intercellular interface between donor and acceptor cells. For localization of Ca2+ signals, attached fibroblasts on coverslips were incubated with a Ca2+ indicator fluo-3/AM (3 µM) for 20 minutes at 37°C. For FITC-labeled probes or for fluo-3, excitation was set at 488 nm and emission was collected with a 530/20 nm barrier filter. For TRITC, excitation was set at 530 nm and emission was collected at 620/40 nm. Cells were imaged with a ×63 oil immersion lens (numerical aperture = 1.4) and transverse optical sections were obtained from the level of cell attachment at the substratum of the A cell to the dorsal surface of the S cell (as verified by phase-contrast microscopy). The cell-to-cell interface was estimated to be located at about the middle optical section between the cells and further verified by visual assessment of the position of the nuclei of the top and bottom cells (DAPI staining).

**Calcium signaling during cell-cell adhesion**

Cells were washed once with PBS and lysed directly with 2% SDS Laemmli sample buffer for production of whole cell lysates or with 1% Triton X-100 in PBS for production of cytoskeletal fractions. For both methods, the buffer contained 5 mM EDTA, 50 µM VO42−, 10 mM NaF, and protease inhibitors (2 mM PMSF, 10 µg/mL aprotinin and 1 µg/mL leupeptin). The Triton X-100 insoluble fraction (i.e. cytoskeletal pellet) was solubilized with 2% SDS-sample buffer. Proteins were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membranes. Cadherins and β-catenin were detected using anti-pan-cadherin monoclonal antibody (Clone CH-19) and anti-β-catenin monoclonal antibody (Clone 14), respectively. Blots were blocked for 1 hour with 5% skimmed milk in PBS and incubated with the indicated antibody in 0.1% Tween-TBS. Blots were washed with 0.5% Tween-TBS for 30 minutes. Primary antibody was detected using affinity-purified, peroxidase-conjugated goat antibody (Chemicon International, Temecula, CA, USA) for 1 hour at room temperature, washed 5× in Tween-TBS and developed by chemiluminescence (ECL, Amersham, Oakville, ON, Canada).

**Flow cytometry and quantification of dye-coupling**

For measurement of the amount of Fura-2 dye-transfer between S and A cells (Ko et al., 2000), S cells were loaded with Fura-2/AM as described above. Cocultures of S and A cells were incubated in α-MEM for 120 minutes to allow formation of cell-to-cell adhesions. Single cell suspensions were prepared from the coculture monolayer after one wash by trypsinization (0.01% w/v trypsin in PBS for 10 minutes). Three samples for each experimental group were analysed with a FACStar Plus flow cytometer (Becton Dickinson FACS Systems, Mountain View, CA, USA) as described (Ko et al., 2000). For detection of Fura-2 fluorescence, a UV laser was used. Emitted fluorescence was divided between two detectors by beam splitters and band pass filters for green fluorescence (515-545 nm; for Fura-2) and red fluorescence (606-644 nm; for Texas Red). Photomultiplier tube voltage settings were determined for each experiment on the basis of thresholds established from unlabelled cell samples and from a mixture of Fura-2-loaded and Texas Red-dextran-loaded cells. Evidence of dye-coupling (a measure of gap junctional communication) was indicated by the appearance of cells that exhibited supra-threshold staining for both Fura-2 and Texas Red. Minimal dye-transfer between S and A cells was observed, indicating that for the time intervals used here, there was negligible gap junctional communication. This confirmed that the results observed were not due to the intercellular transfer of fluorescent dye or gap junctional transfer of signaling molecules.

**Electron microscopy**

Microspheres (2 µm, Polysciences; Warrington, PA, USA), which were phagocytosed by fibroblasts after overnight incubation, were used to discriminate donor cells from acceptor cells. Permeabilization of cells was obtained with 10% PHEM (0.6 M Pipes, 0.25 M Heps, 0.1 M EGTA, 20 mM MgCl2, 0.75% Triton X-100). Fixation was done with 1% glutaraldehyde. After 30 minutes, samples were embedded in Lowicryl-K4M. Thin sections were cut and placed on nickel grids. The grids were stained with uranyl acetate and lead citrate and observed under an electron microscope (Hitachi-60).

**Cell viability**

Propidium iodide staining was used to determine if thapsigargin, BAPTA/AM, cytochalasin D and E-cad HAV peptides were toxic to cells during the duration of the experiments. Flow cytometry analyses were performed as described (Lee et al., 1996). Briefly, following treatment with each of the above reagents for 45 minutes, cells were
trypsinized and suspended in PBS, pelleted, resuspended in PBS to a cell concentration of 1x10^6/ml and stained with propidium iodide (10 μg/ml; Calbiochem, CA, USA). After 5 minutes incubation, cells were analysed by flow cytometry. Only cytochalasin D was found to slightly increase the percentage of propidium iodide stained cells (9.2±0.2%) compared to control cells (4.9±1.5%). Cells treated with thapsigargin (4.2±0.4%), BAPTA/AM (4.4±0.04) and E-cad HA V peptides (4.6±0.3%) were not statistically different from control cells in propidium iodide staining, indicating that treatment with the above reagents does not significantly affect cell viability.

**Statistical analysis**
For continuous variable data, means and standard errors of the mean were computed and, when appropriate, comparisons between two groups were made with unpaired Student’s t-tests with statistical significance set at P<0.05. Analyses of multiple groups were made by ANOVA followed by a post-comparison Scheffe test with statistical significance set at P<0.05.

**RESULTS**
**Intracellular calcium signals are initiated after cadherin-mediated cell-cell contact**
Using a simple cell-cell adhesion model (Fig. 1A), we previously showed that adherens junctions form rapidly (in <15 minutes of cell-cell contact; Fig. 1B) in human fibroblasts and that these junctions are cadherin-mediated (Ko et al., 2000). In this model, vital, Calcein-labeled suspended cells (S cells) are added onto an attached cell monolayer (A cells) that is used as substrate for attachment. The S cells attached to (within 15 minutes of cell-cell contact) and spread on (within 60 minutes of cell-cell contact) the A cell layer (Fig. 1C), indicating formation of adhesive intercellular junctions. To characterize the adhesion molecules involved in fibroblast cell-cell adhesion, we measured cell-cell adhesion in the presence of antibodies against cadherins and integrins as well as mimeric peptides (Fig. 1D). Since human fibroblasts express abundant α5β1 and α2β1 integrins on their surface (Lee et al., 1996), we tested whether integrins are involved in cell-cell adhesion by using an RGD peptide (amino acid sequence GRGDSP; 500 μM), which inhibits α5β1 integrin-mediated adhesion through competitive binding to integrin binding sites (Knowles et al., 1991). A 4B4-anti-β1-integrin antibody, which inhibits binding to β1 integrin (Shimizu et al., 1990), was also used to assess if any β1-integrins were involved. We found that both inhibitory (GRGDSP; 500 μM) and control (GRGESP; 500 μM) peptides had no effect on cell-cell adhesion, while the 4B4-anti-β1-integrin antibody (1:50 dilution) inhibited cell-cell adhesion by less than 30% (Fig. 1D). Notably, part of the inhibitory effect of the 4B4-anti-β1-integrin antibody in the cell-cell adhesion assay was caused by rounding of the substrate-attached A cell monolayer and not because of a direct effect mediated by β1 integrin inhibition. Cell rounding in this assay reduces the likelihood of A cell-S cell interactions because the surface area of the A cells is substantially reduced. On the other hand, while HGFs express low levels of the E-cadherin (immunoblotting data; K. S. Ko, unpublished), antibodies against the extracellular domain of the E-cadherin (anti-E-cad; 1:50) or against the cytoplasmic domain of cadherins (anti-pan-cad; 1:50) had no effect on cell-cell adhesion. An E-cadherin mimetic peptide (amino acid sequence LFSHAVSSNG), which includes the common cadherin-cadherin binding HAV domain (Noe et al., 1999), significantly reduced cell-cell adhesion by more than tenfold (Fig. 1D; P<0.001), while the E-cadherin scrambled peptide (amino acid sequence LHSNSVGFSAA) had no effect. These results indicated that cell-cell adhesion in fibroblasts is mediated primarily through cadherins. To better understand intercellular adhesion-mediated signaling in fibroblasts, we used the A cell-S cell model to investigate the relationship between intercellular adhesion and changes in [Ca2+].

In many cell types, intracellular Ca2+ signaling involves both a rise in basal [Ca2+], and oscillatory changes in [Ca2+]; that are graded in response and spatially organized (Berridge, 1993; Clapham, 1995; Thomas et al., 1996; Berridge et al., 1999). In this study we examined the immediate and early events of cell-cell adhesion by measuring the spatial and temporal organization of [Ca2+]i following cell-cell attachment in fibroblasts. We assessed [Ca2+]i changes in Fura-2-loaded S cells and A cells during formation of cell-cell contact by ratio spectrofluorimetry. After a delay of a few minutes following initial S cell-A cell contact, [Ca2+]i transients were triggered in 100% of A cells (Fig. 2A), having formed adhesive contacts with S cells (attachment between S cells and A cells was confirmed by jet-washing at the end of [Ca2+]i measurements). The observed calcium responses in A cells were characterized by a rise in basal [Ca2+], (Fig. 2A,B) and more frequent [Ca2+], oscillations (Fig. 2C). During the first 60 minutes of cell-cell contact, fourfold more frequent [Ca2+], oscillations (compared to resting cells) were observed in A cells (Fig. 2A), leading to a significant and sustained rise in basal [Ca2+]; by >40% after 90 minutes (Fig. 2B). After contact with the A cells, S cells maintained a stable baseline [Ca2+]; with only a twofold increase in the frequency of [Ca2+], oscillations (Fig. 2A,C). In resting attached cells that were observed for 90 minutes without addition of S cells, there was no increase in basal [Ca2+]; with only a few oscillations (Fig. 2A,C). Notably, the more frequent [Ca2+], oscillations induced by cell-cell contact were not due to mechanical perturbation as [Ca2+]i transients induced by attachment of fibronectin-coated latex beads (20 μm diameter) to the A cells exhibited very different characteristics. We observed a >300% increase in [Ca2+]; but with no [Ca2+], oscillations (Fig. 2A,C). The longer delay in Ca2+ signaling of cell-substrate attachment compared to cell-cell attachment is consistent with previous findings showing that cell-cell adhesion occurs more rapidly than cell-substrate adhesion in fibroblasts (Ko et al., 2000).

Since the HAV peptide significantly inhibited cell-cell adhesion, we tested whether cadherin-cadherin interactions initiate the Ca2+ signal. We measured [Ca2+], after addition of an antibody to the extracellular domain of E-cadherin (anti-E-cad; 1:50 dilution), a cadherin that is expressed but is evidently not involved in cell-cell adhesion (Fig. 1D). We found that the mere binding to cadherins by the antibody induced calcium transients similar to those observed in intercellular adhesion-induced Ca2+ responses (Fig. 2A). Control experiments using an antibody against the cytoplasmic domain of cadherin (anti-pan-cad) showed no change in [Ca2+].

We also found that both the rise in basal [Ca2+], and [Ca2+], oscillations induced by cell-cell adhesion were abolished by pretreatment of S and A cells with BAPTA/AM (5 μM), which buffers intracellular Ca2+ (<5). Similar inhibition of [Ca2+],
responses induced by cell-cell contact can be achieved by pretreatment of the S and A cells with thapsigargin (1 μM; Fig. 2A; N=5). Since thapsigargin blocks the ATPase required for Ca\(^{2+}\) reuptake into intracellular pools and they leak until they are empty (Berridge, 1990), these results indicate that cell-cell contact requires release of Ca\(^{2+}\) from internal Ca\(^{2+}\) stores.

**Rises in [Ca\(^{2+}\)]\(\text{i}\) are initiated at cell-cell contact sites**

As localized signaling events have been shown to cause global changes in whole cell [Ca\(^{2+}\)]\(\text{c}\) (e.g. Xia and Ferrier, 1996), we examined the spatial distribution of Ca\(^{2+}\) during cell-cell adhesion. Fluo-3 loaded A cells and Dil-labeled S cells were imaged with a laser scanning confocal microscope. There was a transient increase in Fluo-3 fluorescence intensity at sites of cell-cell contact (Fig. 3A), indicating that localized [Ca\(^{2+}\)]\(\text{i}\) transients occur during early stages of contact formation (approx. 10-15 minutes of cell-cell contact).

To characterize further the temporal sequence of this localized [Ca\(^{2+}\)]\(\text{c}\) change at the cell-cell contact area, we used a lipophilic membrane-associated Ca\(^{2+}\) indicator, Fura-C18, which monitors rapid changes in free [Ca\(^{2+}\)] immediately adjacent to the plasma membrane (Etter et al., 1994). We found that there was a significant single rise in Fura-C18 fluorescence intensity within 5-10 minutes of cell-cell attachment (Fig. 3B), which preceded the increase in whole-cell basal [Ca\(^{2+}\)]\(\text{c}\) and calcium oscillations measured by Fura-2 loading.

**Cell-cell contact and the cytoskeleton**

We studied the temporal relationship between cell-cell contact induced [Ca\(^{2+}\)]\(\text{c}\) changes and the assembly of adherens junctions. Previous studies have shown that Triton-insoluble cadherins and catenins are preferentially found at cell-cell contacts (Adams et al., 1996) and we have shown previously clustering of cadherins and β-catenin at sites of cell-cell

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**Fig. 1.** Model system for studying the formation of intercellular adhesion. (A) Schematic diagram showing the model system used for studying intercellular adhesion. Calcein-loaded (green) S cells are added onto Texas Red-dextran-labeled (red) A cell layer as substrate for attachment. (B) Electron micrograph showing formation of intercellular adherens junction (arrow) after 15 minutes of contact between the S cell and A cell. S cells, identified by intracellular microspheres as a marker (not shown), were added to an A cell monolayer and cells were fixed after one wash. This representative image is typical of five separate analyses. (C) Phase-contrast (top row) and epifluorescence (bottom row) micrographs of S and A cells at the indicated times of cell-to-cell contact. Calcein-labeled S cells were added at t=0 minutes to the A cell monolayer and incubated at 37°C. Note that the S cell was rounded at t=0 minutes, started to form membrane ruffles and filopodia within 15 minutes, indicating formation of cell-cell adhesive contacts, spread well within 60 minutes, and was very well spread by 180 minutes. (D) Histogram showing quantification of cell-cell adhesion after treatment with antibodies against β1-integrins and cadherins as well as RGD and HAV peptides. Cell-cell adhesion was measured by counting the number of S cells per high power microscope field after 90 minutes of cell-cell contact. Cells counted were those that remained attached following three washes with PBS (Ko et al., 2000). Cell counts were normalized against cell counts in normal (1 mM) Ca\(^{2+}\) medium (Control). An asterisk indicates a statistically significant difference from the control (P<0.05). RGD peptides had no effect while 4B4-anti-β1-integrin antibody inhibited cell-cell adhesion by <30%. Antibodies against the E-cadherin extracellular domain (anti-E-cad) and against a common cytoplasmic domain (anti-pan-cad) did not affect cell-cell adhesion. Cadherin-binding-site mimetic (HAV) peptide (LFSHAVSSNG) inhibited cell-cell adhesion >fivefold (P<0.001) while the scrambled (LHSNVGFSVA) peptide had no effect. These results indicate that cell-cell adhesion in fibroblasts is primarily cadherin-mediated. Data are from N=4 replicate samples.
Fig. 2

A

Resting cell

S cell

A cell

FN-coated bead

Thapsigargin

BAPTA

Anti-cadherin Ab
(extracellular domain)

Anti-cadherin Ab
(cytoplasmic domain)

B

C

Basal [Ca^{2+}]_i (nM)

Time (min.)

Resting cell  A cell  S cell  FN-A cell

# of [Ca^{2+}] oscillations cell/hour
Fig. 2. Intercellular adhesion-induced calcium signals. (A) Typical tracings showing the [Ca\textsuperscript{2+}]; response of fibroblasts induced by cell-cell and cell-substrate attachment. Ratio fluorometry of fura2-loaded fibroblasts was used to measure [Ca\textsuperscript{2+}]. Attached resting cells (no S cells or beads added) exhibited a stable baseline ([Ca\textsuperscript{2+}]) fluctuated by <5 nM during a 40 minute recording. S cells attached to A cells at approx. 1 minute after start of recordings. A rise in baseline [Ca\textsuperscript{2+}], and a dramatic increase in frequency of [Ca\textsuperscript{2+}] oscillations were observed in the attached A cell within 5 minutes of S cell addition and formation of intercellular adhesive contacts between the S and A cells. S cells showed a slight increase in frequency of [Ca\textsuperscript{2+}] oscillations while basal [Ca\textsuperscript{2+}] was stable. Attachment of fibronectin-coated beads induced a significant rise in basal [Ca\textsuperscript{2+}] of attached A cells without any increase in frequency of [Ca\textsuperscript{2+}] oscillations. Depletion of sarcoplasmic reticulum stores of calcium by pretreatment of both S and A cells with thapsigargin or buffering of [Ca\textsuperscript{2+}], by pretreatment with BAPTA/AM eliminated [Ca\textsuperscript{2+}] transients and oscillations induced by cell-cell contact in the A cell. Note that intracellular BAPTA significantly reduced basal [Ca\textsuperscript{2+}] in low calcium buffer. Antibody against the extracellular domain of E-cadherin (anti-E-cad, DECMa-1; 1:50 dilution; N=4) induced a robust calcium transient, as shown in this representative trace of N=4 experiments. Control experiments using antibody against a common cytoplasmic domain of cadherin (anti-pan-cad, clone CH-19; 1:50 dilution; N=4) showed no calcium response, indicating that specific binding to the extracellular domain of cadherin is sufficient to induce an intracellular calcium signal. All experiments were repeated at least three times and showed similar results. (B) Basal [Ca\textsuperscript{2+}] during formation of intercellular adhesive contacts between S and A cells (mean ± s.e.m.; N=6). Note the significant rise in basal [Ca\textsuperscript{2+}] after 90 minutes of cell-cell contact (P<0.05). (C) Histogram showing cell-cell contact-induced [Ca\textsuperscript{2+}], oscillations in fibroblasts. Each bar represents the mean (± s.e.m.) number of [Ca\textsuperscript{2+}], oscillations per cell over 60 minutes, as measured by ratio spectrofluorimetry in calcium-containing buffer (N=6). [Ca\textsuperscript{2+}] oscillations are defined here as transient increases of [Ca\textsuperscript{2+}] >10 nM. Note the significant increase in [Ca\textsuperscript{2+}], oscillations during the initial 60 minutes of cell-cell contact formation, especially in A cells (P<0.05), and the absence of [Ca\textsuperscript{2+}], oscillations during cell-substrate contact formation (cell-fibronectin-coated beads), indicating that the increase in [Ca\textsuperscript{2+}], oscillations is not due to mechanical disturbance but is specific to cell-cell contact formation.

Calcium signaling during cell-cell adhesion

Intercellular adhesion. Cell-cell adhesion was inhibited approx. fivefold in the presence of 1 μM cytochalasin D (19.73±0.05% of control, P<0.05), indicating that an intact actin cytoskeleton is required to establish strong cell-cell adhesive contacts. This is consistent with previous studies showing that the adhesiveness of the apical (free) plasma membrane of uterine epithelial cells to trophoblasts requires an intact actin cytoskeleton during human embryo implantation (Thie et al., 1997).

Cell-cell adhesion requires calcium signaling

To assess the requirement for an intact calcium signaling system during adhesions junction assembly, we measured cell-cell adhesion (Ko et al., 2000) in the presence of various inhibitors of calcium signaling. We found that in medium containing a normal Ca\textsuperscript{2+} concentration, agents that inhibit intracellular calcium signaling such as the calcium channel blocker LaCl\textsubscript{3} (250 μM) significantly reduced cell-cell adhesion (by approx. 60%, P<0.05; Fig. 6A). Thapsigargin pretreatment (1 μM) also reduced cell-cell adhesion by approx. 40% (P<0.05; Fig. 6A); this inhibition required the presence of thapsigargin during cell-cell adhesion and could be reversed by washing the pretreated cells. Complete removal of extracellular Ca\textsuperscript{2+} in the medium with 2 mM EGTA abolished any cell-cell adhesion (Fig. 6A), indicating that it is a Ca\textsuperscript{2+}- dependent process in fibroblasts. However, under our experimental conditions, we found that cell-cell adhesion in

contact between S and A fibroblastic cells (Ko et al., 2000). Furthermore, the cadherin-catenin complex is recruited into a Triton-insoluble pool and associates with actin microfilaments during maturation of adherens junction assembly (McNeill et al., 1993; Adams et al., 1996; Glushankova et al., 1998). Therefore to study the time course of recruitment of cadherins and β-catenin into the cell-cell contacts and cytoskeleton, we used immunoblots of Triton-insoluble samples prepared after various times of cell-cell adhesion between S and A cells (Fig. 4). The design of the experimental model allows the formation of a cohort of cell-cell couples that are approximately at the same stage of assembly of cell-cell junctions. Sparse A cell monolayers (minimal cell-cell contact) were used as substrates for attachment. There was a minimal amount of cytoskeletal-associated cadherins and β-catenin at t=0 minutes since contacts had not formed between the S and A cells and there were minimal intercellular contacts between the A cells. Over time, as S cells attached to A cells, we observed an increased amount of these cell-cell junctional proteins in the Triton-insoluble cytoskeletal fraction. Consistent with previous studies using retrospective immunohistochemistry (McNeill et al., 1993), there was a dramatic increase in the amount of Triton-insoluble cadherins and β-catenin within 15 minutes of cell-cell contact, which then plateaued at approx. 60 minutes. This was temporally correlated with the time when the most frequent [Ca\textsuperscript{2+}] oscillations were observed (Fig. 2A). The amount of whole-cell cadherins and β-catenin remained constant during this time, indicating that the increase was not because of altered levels of total cell protein but was due to recruitment of these proteins to the cytoskeletal fraction.

Accumulation of actin filaments at intercellular contacts

In epithelial cells, induction of cell-cell adhesion by switching cells from low to high extracellular calcium concentration causes a rapid (<10 minutes) recruitment of Cy3-actin to intercellular junctions (Braga et al., 1997), where it colocalizes with E-cadherins. We have shown (Ko et al., 2000) that distinct cell-cell adherens junctional plaques are able to form within 15 minutes of cell-cell contact in fibroblasts. We therefore examined actin rearrangement during cell-cell contact formation in fibroblasts that is concomitant with changes in [Ca\textsuperscript{2+}]. To test how actin reorganizes during cell-cell contact formation using our cell-cell adhesion model (Ko et al., 2000), Rat-2 fibroblasts were transfected with an EGFP-β-actin construct. The transfected cells were grown as a monolayer and used as substrate for attachment of S cells (Fig. 5). While substrate-attached single Rat-2 cells showed prominent actin stress fibres (Fig. 5A), bundles of actin filaments reorganized within 60 minutes of cell-cell attachment and accumulated at sites of cell-cell contact (Fig. 5B). This rearrangement of actin filaments was a short-lived event as overnight culture and examination of adjacent cells did not show such localized actin accumulation (Fig. 5C).

We then asked if actin remodeling is required for intercellular adhesion. Cell-cell adhesion was inhibited approx. fivefold in the presence of 1 μM cytochalasin D (19.73±0.05% of control, P<0.05), indicating that an intact actin cytoskeleton is required to establish strong cell-cell adhesive contacts. This is consistent with previous studies showing that the adhesiveness of the apical (free) plasma membrane of uterine epithelial cells to trophoblasts requires an intact actin cytoskeleton during human embryo implantation (Thie et al., 1997).
fibroblasts was only slightly reduced (by <15% of normal Ca\(^{2+}\) control) in low Ca\(^{2+}\) conditions (nominally Ca\(^{2+}\)-free buffer; Fig. 6A), where the extracellular Ca\(^{2+}\) concentration was estimated to be in the range 10-40 \(\mu M\) (Nohmi et al., 2000).

To investigate the requirement for intracellular Ca\(^{2+}\) signaling in cell-cell adhesion, we used BAPTA-loaded S and A cells. Our preliminary experiments showed that low extracellular Ca\(^{2+}\) concentration is required to maintain the buffering capacity of intracellular BAPTA. We therefore use low Ca\(^{2+}\) conditions (which allowed cell-cell adhesion of >85% of normal Ca\(^{2+}\) control) to facilitate our experiments using BAPTA-loaded cells. Pretreatment of both S cells and A cells with the intracellular calcium buffer BAPTA/AM (5 \(\mu M\)) significantly reduced cell-cell adhesion by approx. 40% \((P<0.05)\) as compared to the low calcium control group (Fig. 6B). This inhibition by BAPTA/AM was reversible as normal cell-cell adhesion could be restored in BAPTA/AM pretreated cells in medium with 1 mM Ca\(^{2+}\). As shown in Fig. 2A, the reduction of cell-cell adhesion by BAPTA/AM or thapsigargin pretreatment of both S and A cells was associated with the elimination of cell-cell contact-induced [Ca\(^{2+}\)] responses in A cells as measured by Fura-2. Together, these results indicate that the observed increase of blot density was not due to increased cell number in the sample or increased expression. Note also that significant amounts of cadherins and \(\beta\)-catenin were recruited to the cytoskeleton within 15 minutes of cell-cell contact formation. Similar results were obtained in three independent experiments. The same amount of proteins (40 \(\mu g\); determined by Bradford assay, Ko et al., 2000) was loaded in each lane. Quantification by densitometry of immunoblots are shown as histograms of mean density ± s.e.m. \((N=3\) experiments).

Calcium and recruitment of cadherins and \(\beta\)-catenin to cell-cell contacts

Since we demonstrated that intracellular calcium signaling and actin assembly are required for the formation of adhesive cell-cell contacts, we investigated whether Ca\(^{2+}\) signaling mediates cell-cell attachment via its regulation of actin remodeling and recruitment of cell-cell junctional proteins. S cells were
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allowed to attach to A cells for 90 minutes, a time period at which there was maximal cadherin and β-catenin recruitment to the Triton-insoluble fraction. Immunoblots of the cytoskeletal fractions were compared as described in Fig. 4. We found that there was a significant reduction in the amount of cadherin (approx. 30% reduction by densitometry; Fig. 7) and β-catenin (>2.5-fold reduction, as determined by densitometry; Fig. 7) in the S cell-A cell couples that were pretreated with BAPTA/AM and cocultured in low calcium medium compared to the group in low calcium medium alone. As mentioned above, the low calcium condition used here was sufficient to allow significant cell-cell adhesions and was required to maintain the buffering capacity of intracellular BAPTA. Consistent with our cell-cell adhesion data, in low calcium medium, the amounts of Triton-insoluble cadherins and β-catenin were only slightly reduced when compared to the group in the medium with normal (1 mM) calcium concentration (Fig. 7). The amounts of whole-cell cadherins and β-catenin were not affected by low calcium or BAPTA/AM treatment, indicating that buffering intracellular calcium

Fig. 5. Actin rearrangement. Confocal fluorescence images of GFP-actin transfected Rat-2 fibroblasts showing actin reorganization during the formation of cell-cell contacts. In a substrate-attached single GFP-actin transfected Rat-2 A cell, prominent actin fibres were evident (A). (B) After approx. 90 minutes incubation with an S cell (labeled with DiI; top cell), there was formation of cell-cell contacts and rearrangement of actin in the A cell (bottom cell). Note the accumulation of actin along the cell-cell contact between the S and A cell (white arrowheads). Actin accumulation at cell-cell junctions was transient as overnight culture of confluent cells restored actin stress fibres in cells in close contact (C). These images are representative of three independent trials.

Fig. 6. Cell-cell adhesion after treatment with agents that perturb intracellular Ca²⁺ homeostasis. Cell-cell adhesion was measured by counting the number of S cells per high power microscope field after 90 minutes of cell-cell contact that remained attached following three washes with PBS (Ko et al., 2000). The cell counts were normalized against cell counts in normal (1 mM) Ca²⁺ medium (control in A; norm. Ca²⁺) or against cell counts in low Ca²⁺ (nominally Ca²⁺-free) medium (control in B; low Ca²⁺). An asterisk indicates a statistically significant difference from the control (P<0.05). (A) S cells were allowed to attach to A cells in normal Ca²⁺ medium except for the low Ca²⁺ group. Low extracellular Ca²⁺ concentration slightly reduces (by approx. 15%) cell-cell adhesion. Complete removal of extracellular calcium with 2 mM EGTA abolished cell-cell adhesion. Thapsigargin (1 μM) was used to deplete internal Ca²⁺stores and inhibit cell-cell adhesion by approx. 40% (P<0.05; N=3 in three independent experiments). LaCl₃ (250 μM) was used to block Ca²⁺ channels and caused a >50% reduction in cell-cell adhesion (P<0.05; N=5 in three independent experiments) (B) S cells were allowed to attach to A cells in low Ca²⁺ medium. BAPTA/AM (5 μM) pretreatment of both S and A cells was used to buffer cytosolic [Ca²⁺] and reduced cell-cell adhesion by approx. 35% (P<0.05; N=5 in three independent experiments). Note that there is minimal added effect of inhibition by a combination of BAPTA/AM and LaCl₃ treatment.
inhibited the recruitment of cell-cell junctional proteins to the cytoskeleton pool but had no effect on protein content.

To investigate the effect of buffering intracellular calcium on accumulation of cadherins and catenins at cell-cell contacts, we used optical sectioning by confocal microscopy to study the distribution of cadherins and β-catenin at the S cell-A cell interface (Fig. 8; Ko et al., 2000). Clustering of cadherins, β-catenin and actin filaments was evident at cell-cell junctions in medium with normal calcium (1 mM) and in low calcium. In the BAPTA/AM pre-treated group, there was a significant reduction in staining for cadherins, β-catenin and actin at the S cell-A cell interface. Notably, buffering of intracellular calcium inhibited the reorganization of actin and the recruitment of cadherins and β-catenin to sites of cell-cell contact.

**DISCUSSION**

[Ca^{2+}]] signaling is thought to be involved in a variety of heterotypic cell-cell interactions. In this study, we provide experimental evidence for a role of [Ca^{2+}]] in homotypic fibroblastic cell-cell adhesion, a process essential for cell differentiation (Takeichi, 1991) and wound healing (Matsuyoshi and Imamura, 1997). We studied [Ca^{2+}]] during formation of cell-cell adhesive contacts by: (1) measuring intracellular free calcium concentration; (2) a functional study of cell-cell adhesion in the presence of various inhibitors of calcium signaling; and (3) confocal microscopy and biochemical analysis of cell-cell junctional and cytoskeletal proteins in a cohort of cell-cell couples. The major finding is that cell-cell adhesion induces [Ca^{2+}]] transients in fibroblasts and these signals are required for actin remodeling, an important process that is involved in the recruitment of junctional proteins (e.g. cadherins and β-catenin) to cell-cell contacts.

Our cell-cell adhesion model creates conditions in which a large number of intercellular contacts form in a relatively short time period, thereby enabling studies of synchronized intercellular adhesive events. We have previously shown that cell-cell adherens junctions form shortly (in <15 minutes) after the S cells and A cells are in contact (Ko et al., 2000). In this study, we used Ca^{2+}]] indicator-loaded A cells to study [Ca^{2+}]], signaling initiated by contact with S cells. Using the same model, we quantified the amount of cadherin and β-catenin recruitment into cell-cell contacts by immunoblotting of S cell-
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A cell couples after Triton-extraction. We observed a dramatic increase of both Triton-insoluble cadherins and β-catenin within 60 minutes of cell-cell contact. This is consistent with retrospective immunocytochemistry analyses of epithelial cells showing that the amounts of Triton-insoluble E-cadherin and β-catenin increase two- to fourfold at contact sites within 45 minutes of cell-cell contact (Adams et al., 1996). Previous studies have shown that cadherins are at or near the top of a molecular cascade of events that mediates homotypic cell-cell adhesion and initiates the structural and functional reorganization of cells leading to tissue differentiation (Vlemingckx and Kemler, 1999). However, the specific role of intracellular calcium signaling in cell-cell adhesion has not been determined. We focused on the temporal and spatial regulation of cell-cell adhesive complex formation during the initiation and strengthening of cell-cell adhesion. The data show that localized Ca²⁺ signaling events occur within minutes of cell-cell contact and that the global [Ca²⁺]ᵢ response is initiated at intercellular contact sites. The localized nature of [Ca²⁺]ᵢ changes may be related to the observation that early (<45 minutes) cell-cell contacts exhibit discrete, punctate cadherin and catenin staining, indicating that the initiation of cell-cell adhesion is a localized molecular event (Adams et al., 1996). We observed more frequent oscillations and sustained increases in global [Ca²⁺]ᵢ of A cells in the initial 60 minutes of cell-cell contact. This is the first report to demonstrate early stage intracellular signal transduction events in the formation of intercellular adherens junctions. [Ca²⁺]ᵢ oscillations have been observed in T cell-APC interactions (Liu and Golan, 1999; Negulescu et al., 1996) and lymphocyte-endothelial cell interactions (Pfau et al., 1995), but the type of junctions in these studies were not characterized. We have also examined the functional relevance of these calcium oscillations in the context of intercellular adhesion. Intracellular calcium signaling was required for the formation of stable cell-cell contacts, since buffering intracellular calcium by pretreatment of cells with BAPTA/AM (Glogauer et al., 1998) blocked cell-cell contact-induced rises in [Ca²⁺]ᵢ and significantly reduced cell-cell adhesion. BAPTA/AM was used to block intracellular Ca²⁺ signaling because it can effectively chelate Ca²⁺ fast enough to suppress the biological response to mobilized Ca²⁺. To maintain the buffering capacity of intracellular BAPTA, low calcium conditions were used to prevent re-uptake of Ca²⁺ through Ca²⁺ ion channels and endocytosis (Gerasimenko et al., 1998). The low calcium condition used here contains sufficient Ca²⁺ to allow robust cell-cell adhesion, although

**Fig. 8.** Ca²⁺ regulation of recruitment of cadherins, β-catenin and actin to cell-cell contacts. Clustering of cadherins, β-catenin and actin at the cell-cell interface was shown by confocal optical sections in the Z-axis from the bottom of the A cell to the top of the S cell (see diagram at top). Only sections at the cell-cell interface are shown. Fibroblasts were stained with anti-pan-cadherin antibody or with anti-β-catenin antibody followed by FITC-conjugated goat anti-mouse antibody; actin filaments were labeled with FITC-phalloidin. S cells in (A) were allowed to attach onto A cell monolayers in medium containing 1 mM Ca²⁺. S cells in (B) and (C) were allowed to attach in low Ca²⁺ medium for 90 minutes. Note that cells in C were pretreated with 5 μM BAPTA/AM before the S and A cells were allowed to attach; significantly fewer β-catenin and cadherins were detected at the cell-cell junction (C). Formation of actin stress fibres was also significantly reduced adjacent to the cell-cell junction (C); S cell spreading was also inhibited. These images are typical of three independent experiments.
complete removal of Ca\textsuperscript{2+} by 2 mM EGTA prevented any measurable cell-cell adhesion. Finally, the inhibitory effect of intracellular BAPTA on cell-cell adhesion is unlikely due to the low extracellular [Ca\textsuperscript{2+}], but is rather due to its elimination of [Ca\textsuperscript{2+}]; signaling, since thapsigargin-treated cells also showed reduced adhesion and abolished cell-cell contact-induced [Ca\textsuperscript{2+}] response in the presence of normal extracellular [Ca\textsuperscript{2+}]. Since thapsigargin depletes the sarcoplasmic reticulum stores of Ca\textsuperscript{2+}, we also showed that the calcium responses induced by intercellular adhesion depended on Ca\textsuperscript{2+} release from the sarcoplasmic reticulum stores.

The temporal sequence of our data on cell-cell contact-induced [Ca\textsuperscript{2+}]; transients is in agreement with Angres et al., who showed that strengthening of adhesion in E-cadherin transfected fibroblasts occurs over a 30-40 minute period after initiation of cell-cell contacts (Angres et al., 1996). In certain specialized cell-cell interactions such as T cell activation, intracellular Ca\textsuperscript{2+} signaling in T cells is known to be required for stabilization of the T cell-APC contact (Liu and Golan, 1999; Negulescu et al., 1996). We show here that cell-cell contact-induced intracellular calcium transients mediate and strengthen homotypic intercellular adhesion in human fibroblasts.

Our electron microscopic and confocal studies indicated that the intercellular adhesive contacts which we observed in fibroblasts resemble cadherin-mediated adhesions junctions. Since cadherin-catenin complex binding to actin filaments is required for cell-cell adhesivity (Angres et al., 1996), we considered that calcium signals initiated by cell-cell contact might trigger actin-mediated recruitment of cadherins and catenins to the cell-cell contact. The interaction of catenin with the actin cytoskeleton may also provide an important potential pathway of regulating cell-cell adhesion. Catenin interacts with a number of actin-binding proteins, including α-actinin, vinculin, ZO-1, and actin itself (Knudsen et al., 1995; Imanura et al., 1999). Using GFP-β-actin transfected A cells, we found that cell-cell adhesion induces an early and short-lived accumulation of actin filaments at the cell-cell interface. The recruitment of actin to the cell-cell contact sites shown here is similar to the focal recruitment of cortical GFP-β-actin to adhesive contacts mediated by laminin at the cell surface (Heidemann et al., 1999). We also demonstrated that actin rearrangement during cell-cell adhesion in fibroblasts is consistent with previous reports on epithelial cells showing actin polymerization at cell-cell contact sites (Vasioukhin et al., 2000).

Although actin reorganization during the formation of cell-cell adherens junctions has been described in epithelial cells (e.g. Vasioukhin et al., 2000), the signaling mechanisms initiated by cell-cell attachment leading to cytoskeletal reorganization is not well understood. We demonstrated that actin rearrangement induced by cell-cell contacts requires intracellular calcium signaling. Using optical sectioning by confocal microscopy (Ko et al., 2000) and immunoblotting of Triton-insoluble fractions of S cell-A cell couples, we showed clustering of cadherins, β-catenin and β-actin at intercellular contacts. In comparison with cells in low calcium, this recruitment of cell-cell junctional proteins and actin was significantly inhibited by buffering intracellular calcium. Since clustering of cadherins significantly increases intercellular adhesive strength (Yap et al., 1997), we examined the importance of an intact [Ca\textsuperscript{2+}]; signaling system in the formation of stable and secure cell-cell contacts. The observed calcium-dependent nature of actin filament accumulation at the cell-cell interface may be due in part to the important role of calcium in regulating the activity of a variety of actin-binding proteins (Janmey, 1994).

Collectively, our results indicate that the formation of intercellular contacts in human fibroblasts induces intracellular calcium transients that mediate reorganization of cortical actin and the recruitment of cadherins and β-catenins to cell-cell contacts. Spatial and temporal changes in cytoplasmic Ca\textsuperscript{2+} signals are a potential pathway through which activation of cell surface receptors is translated into intracellular signals (Thomas et al., 1996) such as those that trigger actin reorganization. We suggest that the [Ca\textsuperscript{2+}]; transients induced by cell-cell adhesion are important for regulation of cell-cell adherence through modulation of actin remodeling.

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