Biochemical and functional characterization of intercellular adhesion and gap junctions in fibroblasts

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Ko, Kevin, Pamela Arora, Wilson Lee, and Christopher McCulloch. Biochemical and functional characterization of intercellular adhesion and gap junctions in fibroblasts. Am J Physiol Cell Physiol 279: C147–C157, 2000.—Despite their significance in wound healing, little is known about the molecular determinants of cell-to-cell adhesion and gap junctional communication in fibroblasts. We characterized intercellular adherens junctions and gap junctions in human gingival fibroblasts (HGFs) using a novel model. Calcein-labeled donor cells in suspension were added onto an established, Texas red dextran (10 kDa)-labeled acceptor cell monolayer. Cell-to-cell adhesion required Ca$^{2+}$ and was $>$30-fold stronger than cell-to-fibronectin adhesion at 15 min. Electron micrographs showed rapid formation of adherens junction-like structures at $\sim$15 min that matured by $\sim$2–3 h; distinct gap junctional complexes were evident by $\sim$3 h. Immunoblotting showed that HGF expressed $\beta$-catenin and that cadherins and connexin43 were recruited to the Triton-insoluble cytoskeletal fraction in confluent cultures. Confocal microscopy localized the same molecules to intercellular contacts of acceptor and donor cells. There was extensive calcein dye transfer in a cohort of Texas red dextran-labeled cells, but this was almost completely abolished by the gap junction inhibitor $\beta$-glycyrrhetinic acid and the connexin43 mimetic peptide GAP 27. This donor-acceptor cell model allows large numbers ($\sim$$10^3$) of cells to form synchronous cell-to-cell contacts, thereby enabling the simultaneous functional and molecular studies of adherens junctions and gap junctions. Interactions. Because a large number of synchronized intercellular contacts were established with this model, biochemical studies of intercellular adhesive and gap junctional proteins and their regulation are possible. Our results indicate that the double-label synchronized cohort model provides a simple and effective approach to studying functional interactions in intercellular adhesion and communication.

Both adherens junctions and gap junctions are thought to be important in cellular signaling in connective tissue cells. For example, cadherin-mediated intercellular adhesion is required for human osteoblast differentiation (6), and gap junctional communication can modulate gene expression in osteoblastic cells (22). Despite their significance in tissue remodeling and wound healing, there are few studies on adherens junctions and gap junctions in human fibroblasts. Periodontal connective tissues provide a good model for study of intercellular adhesion and communication in vivo because the fibroblasts from these tissues form extensive adherens junctions and gap junctions (3, 32). However, neither the molecular components of adherens junctions (e.g., cadherins, catenins, and actin) (17) nor the kinetics of formation have been well characterized in fibroblasts.

Previous studies of intercellular contacts in fibroblasts involved observation of colliding lamella of two adjacent cells in the x-y plane (13, 29, 30). Studies based on this and similar model systems present several limitations in that only a few cells can be examined at any time; because cell processes are moving over a substrate, observation of intercellular contacts is affected by cell-to-substrate interactions. Cognizant of these limitations, we developed and characterized a simple intercellular adhesion model that allows the study of key events in the early stages of intercellular adhesion that is independent of cell-to-substrate interactions. Because a large number of synchronized intercellular contacts were established with this model, biochemical studies of intercellular adhesive and gap junctional proteins and their regulation are possible.

MATERIALS AND METHODS

Reagents. Primary antibodies against human antigens including mouse monoclonal anti-connexin 43 (Clone 2) and anti-$\beta$-catenin (Clone 14) were purchased from Transduction Laboratories (Lexington, NY); mouse monoclonal anti-con...
nexin26 antibody (Clone CX-12H10) and anti-connexin32 antibody (Clone CX-2C2) were purchased from Zymed Laboratories (San Francisco, CA). Pan-cadherin (Clone CH-19) and FITC-goat anti-mouse antibodies, tetramethylrhodamine isothiocyanate (TRITC)-phallolidin, and β-glycyrrhetinic acid (BGA) were purchased from Sigma Chemical (St. Louis, MO). Calcein-AM, Texas red dextran (10 kDa), and FITC-dextran (70 kDa) were purchased from Molecular Probes (Eugene, OR). Conexin43 mimetic peptides including GAP 20 (amino acid sequence EIKFKFYGC), GAP 27 (amino acid sequence SRPTKETIF), and modified GAP 27 (amino acid sequence SRPTKETIF) were synthesized by the Alberta Peptide Institute (Edmonton, Alberta, Canada).

Cell culture. Human gingival fibroblasts (HGFs) were derived from primary explant cultures as described (27). Cells from passages 6–15 were grown as monolayers in T-75 flasks. Full growth medium consisted of α-MEM, antibiotics [0.017% penicillin G (Ayerst Lab, Montreal, PQ), 0.01% gentamycin sulfate (Life Technologies, Grand Island, NY), and 1% (vol/vol) heat-inactivated fetal bovine serum (FBS; ICN Biomedicals, Costa Mesa, CA)]. Two days before each experiment, cells were harvested with 0.01% trypsin, and ~100,000 cells were plated into 35-mm-diameter culture dishes (Falcon, Becton Dickinson, Mississauga, ON). The cells were grown to confluence before all experiments except when sparse cultures were used as indicated.

Intercellular adhesion assay. To characterize the functional properties and the intercellular adhesion molecules in our simplified model, HGF were grown overnight to a confluent monolayer in α-MEM (supplemented with 10% FBS and antibiotics). Another set of HGF (donor cells) were fluorescence-labeled with calcein-AM (5 μg/ml) and Dil-CM (10 μg/ml) for 1 h at 37°C, followed by three washes with α-MEM. These cells were then plated onto the established cell monolayer (acceptor cells). Attachment and spreading of the plated cells were monitored and recorded at specific time points (0–180 min) with a fluorescence microscope coupled with a charge-coupled device (CCD) camera (Princeton Instruments, NJ). Quantification of relative adhesion under different experimental conditions was done by counting the number of donor cells per high-power microscope field that remained attached after three washes with PBS. The acceptor cell monolayer was grown overnight to ensure that the acceptor cells were highly adherent to the tissue culture plate and were not detached during jet washing.

Immunocytochemistry. To identify and localize specific molecules involved in cell-to-cell adhesion, immunocytochemistry was performed for cadherins (using pan-cadherin antibody), β-catenin, and the gap junction protein connexin43. Cells grown on coverslips were fixed with methanol at −20°C for 10 min, blocked with 1:1,000 mouse serum in PBS for 10 min, incubated with primary antibody (1:100 dilution) for 1 h at room temperature, washed 3 times with PBS containing 0.2% BSA, and incubated with FITC-conjugated goat anti-mouse (1:100). Nonspecific control staining was performed on a separate coverslip using secondary antibody only. Coverslips were washed with PBS and mounted with an antifade mounting medium (ICN Biomedicals). For visualization of actin filaments, cells were stained with TRITC-phalloidin and examined using a ×40, 1.3 numerical aperture (NA) oil-immersion objective under epifluorescence optics and confocal imaging (Leica Confocal Laser-Scanning Microscopy, 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 FITC-labeled probes, 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 (NA = 1.4), and transverse optical sections were obtained from the level of cell attachment at the substratum of the acceptor cell to the dorsal surface of the donor 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 (4’,6-diamidino-2-phenylindole staining).

Immunoblotting. 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. The cytoskeletal buffer also contained 5 mM EDTA, 50 μM VO₄, 10 mM NaF, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 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, β-catenin, connexin43, conexitin26, and connexin32 were detected using anti-pan-cadherin monoclonal antibody (Clone CH-19), anti-β-catenin monoclonal antibody (Clone 14), and anti-connexin43 monoclonal antibody (Clone 2), anti-connexin26 monoclonal antibody (Clone CX-12H10), and anti-connexin32 antibody (Clone CX-2C2). Blots were blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) and incubated with the indicated antibody in 0.1% Tween-TBS. Blots were washed with 0.5% Tween-TBS for 30 min. Primary antibody was detected using affinity-purified, peroxidase-conjugated goat antibody (Chemicon International, Temecula, CA) for 1 h at room temperature, washed 5 times in TBS-Tween, and developed by chemiluminescence (Amersham, Oakville, ON).

Flow cytometry and quantification of dye coupling. HGF monolayers (acceptor cell population) were grown on 35-mm dishes in the presence of Texas red dextran (10 kDa; 1 mg/ml) overnight for intracellular loading through endocytosis (31). Texas red dextran in acceptor cells was not able to pass through the gap junctions because of its size. Single cell suspensions of donor HGF were prepared, labeled with 0.05 μg/ml calcein-AM, incubated at 37°C for 45 min, and followed by a 2× PBS wash. Cell counts were obtained with an electronic cell counter (Coulter Electronics, Hialeah, FL) and included separate estimates of acceptor cells (number of cells per 35-mm dish) and donor cells (number of cells per milliliter of growth medium). An aliquot (1 ml) of donor cells was added to each 35-mm dish of acceptor cells so that the ratio of donor to acceptor cells was 1:4. Cocultures were incubated in α-MEM with 10% FBS at 37°C for various time periods (30, 60, 120, 240, and 360 min) to allow formation of cell-to-cell adhesions and gap junctions. At the indicated time points, cocultures were gently washed once with PBS to remove unbound cells.

For qualitative observation of dye coupling, cocultures were monitored and images were recorded at specific time points (0, 15, 30, 60, and 180 min) with a fluorescence microscope coupled to a CCD camera. Quantification of dye coupling between the donor and acceptor cells was assessed by dual-excitation flow cytometry. Single cell suspensions were prepared from the coculture monolayer by trypsinization (0.01% vol/vol trypsin in PBS for 10 min). The suspension was neutralized with growth medium, pelleted, and resuspended in PBS for flow cytometry analysis. Three samples were analyzed for each experimental group, and >10⁶ cells were analyzed in each sample. Samples were analyzed by dual-excitation flow cytometry. Single cell suspensions were prepared from the coculture monolayer by trypsinization (0.01% vol/vol trypsin in PBS for 10 min). The suspension was neutralized with growth medium, pelleted, and resuspended in PBS for flow cytometry analysis. Three samples were analyzed for each experimental group, and >10⁶ cells were analyzed in each sample. Samples were analyzed by dual-excitation flow cytometry.
with a FACStar Plus flow cytometer (Becton Dickinson FACS Systems, Mountain View, CA) at a sheath pressure of 11 lb/in.² with an Innova 70 argon laser (Coherent Laser Products, Palo Alto, CA) at a light regulation mode setting of 250 mW and a wavelength of 488 nm. Emitted fluorescence was divided between two detectors by beam splitters and bandpass filters for green fluorescence (515–545 nm) and red fluorescence (606–644 nm). Photomultiplier tube voltage settings were determined for each experiment on the basis of thresholds established from unlabeled cell samples and from a mixture of calcein-loaded and Texas red dextran-loaded cells. Evidence of dye coupling was indicated by the appearance of cells that exhibited suprathreshold staining for both calcein and Texas red. Negative controls included 1) incubation of FITC-dextran (70 kDa)-labeled donor cells with Texas red dextran (10 kDa)-labeled acceptor monolayers, and 2) use of a porous filter (Falcon membrane, 0.4 μm) to separate donor and acceptor cells. In both cases, no cells with both red and green fluorescence were detected for up to 6 h of incubation.

To further characterize the model system, two different inhibitors of gap junction communication were used to assess whether these agents inhibit dye coupling in the above assay. The inhibitors included BGA (20–100 μM) (8, 33) and GAP 27 peptide [synthetic connexin-mimetic peptide; GAP 20 and truncated GAP 27 peptides were used as controls (5, 8); 500 μM]. Cells were pretreated with the above inhibitors for 3 h before experiments.

Electron microscopy. Microspheres (2 μm; Polysciences, Warrington, PA) that 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 HEPES, 0.1 M EGTA, 20 mM MgCl₂, and 0.75% Triton X-100). Fixation was done with 1% glutaraldehyde. After 30 min, samples were embedded in Lowicryl-K4M, and thin sections were placed on nickel grids. The grids were stained with uranyl acetate and lead citrate and observed under an electron microscope (Hitachi-60).

Statistical analysis. For continuous variable data, means and SE 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.

RESULTS

Intercellular adhesion. We studied cell-to-cell adhesion and gap junctional communication in fibroblasts with a model in which two-color fluorescence labeling of donor and acceptor cell populations were used for

Fig. 1. A: schematic diagram showing the model system used in the study of intercellular adhesion and gap junctional communication in human gingival fibroblasts (HGFs). Fluorescence dyes are used to distinguish the two cell populations. Calcein-labeled (green) donor cells are added onto Texas red dextran-labeled (red) acceptor cell monolayers as substrates for attachment. At the early stage (<15 min) of cell-to-cell contact, the donor cell is rounded and adherens junctions are starting to form between the donor and acceptor cells. At later stages (60–180 min), the donor cell spreads on the acceptor cell and forms membrane ruffles. More adherens junctions and gap junctions form as indicated by transfer of calcein from donor to acceptor cells. B: phase-contrast (top) and epifluorescence micrographs (bottom) of donor and acceptor cells at indicated times of cell-to-cell contact. Calcein-labeled donor cells were added at time 0 to the acceptor cell monolayer and incubated at 37°C. Note that donor cells were rounded at time 0, started to form membrane ruffles and filopodia within 15 min, spread well within 60 min, and were very well spread and formed dye couples to acceptor cells underneath by 180 min.
discrimination and tracking (Fig. 1A). Calcein-labeled donor cells were added onto the Texas red dextran-labeled acceptor cell monolayer as a substrate for attachment. Cell morphology and calcein dye transfer from donor to acceptor cells were studied at various time points after the coincubation was started. The donor cells rapidly formed attachments with the acceptor cells; membrane ruffles on the acceptor cell dorsal surface were observed within 15 min after coincubation at 37°C (Fig. 1B). With increased time, the donor cells continued to spread on the acceptor cells, and by 60 min, there was evidence of calcein dye transfer from the donor to the acceptor cells, indicating the formation of functional gap junctions. Within 180 min, the donor cells became well spread, and by phase-contrast microscopy, blended in with the acceptor cells. More extensive dye coupling was also evident by 180 min. These data indicated that fibroblasts can form adhesive cell-to-cell junctions (15 min) and gap junctions (60 min) shortly after they are in contact with each other.

Morphology of intercellular contacts formation. We initially characterized cell-to-cell junctions in HGF monolayers (i.e., no donor cells) by transmission electron microscopy. At sites of contacting cell processes, HGFs formed structures with the morphology of gap junctions and adherens junctions (Fig. 2, A and B). Unlike epithelial cells that form abundant and well-defined intercellular junctions (2), we found that cell-to-cell contacts in fibroblasts often involved overlap-
ping cell processes that complicate the study of cell-to-cell contacts in the x-y plane. Therefore, to investigate the ultrastructure of cell-to-cell junctions during their formation, donor cells labeled with phagocytosed microparticles were used to distinguish donor from acceptor cells. Cells were prepared for electron microscopy at various times after the addition of donor cells. Adherens junction-like structures were formed within 15 min of cell-to-cell contact (Fig. 2, C and D) that were coincident with membrane ruffling seen by phase-contrast microscopy (Fig. 1B). Adherens junctions were frequently adjacent to vesicles in the donor cells (Fig. 2E). Abundant close contacts between donor and acceptor cells were formed within 60 min of coincubation (Fig. 2F). Close contacts continued to develop over ~2 h, and well-defined adherens junctions were evident after ~3 h of attachment (Fig. 2G). Distinct structures that resembled gap junctions were evident also after ~3 h (Fig. 2H). The structure of the adherens and gap junctions in HGFs appeared to “mature” over the course of 3 h after the initial cell-to-cell contact. Thus, within 3 h, close contacts between the plasma membranes of donor and acceptor cells developed into well-defined structures resembling that of adherens junctions and gap junctions in 3-day-old HGF monolayer cultures (Fig. 2, A and B).

Cadherin, β-catenin, and connexin43 expression and localization. Because electron micrography showed that adjacent human fibroblasts were linked by structures that resembled adherens junctions and gap junctions, we used immunoblotting to study the expression of proteins responsible for intercellular adhesion. High levels of cadherins (using pan-cadherin antibody) and β-catenin (Fig. 3A) indicated that these two proteins may play a role in intercellular adhesion in fibroblasts. A relatively low level of P-cadherin and cadherin-5 was also detected in HGF (data not shown). HGFs strongly expressed both the phosphorylated (46 kDa) and the unphosphorylated isoforms (43 kDa) of connexin43 but not connexin26 or connexin32 (Fig. 3A).

Because cadherins, β-catenin, and connexins are normally concentrated at the cell-to-cell contacts (17) but not in cell-to-substrate contacts (e.g., focal adhesion complexes), we investigated the distribution of these proteins and their relationship with the cytoskeleton in monolayer cultures. Triton-insoluble cell lysates were prepared from sparse (no cell-to-cell contacts) and confluent cell cultures (abundant cell-to-cell contacts). Western blot analysis showed that in sparse cultures, cadherins, although present in whole cell lysates (Fig. 3B, lane 3), were not recruited to the Triton-insoluble cytoskeletal fraction (Fig. 3B, lane 1). In contrast, β-catenin was associated with the cytoskeletal fraction in the absence or presence of intercellular contacts (Fig. 3B, lanes 1 and 2). Connexin43 was minimal in the cytoskeletal fraction of sparse cultures (Fig. 3B, lane 1) but was abundant in confluent cultures (Fig. 3B, lane 2). Because Triton extraction was enriched for the slower migrating, presumably phosphorylated species of connexin43 (46 kDa) that are incorporated into junctional plaques (26, 35), the abundance of phosphorylated connexin43 in confluent HGF monolayers (Fig. 3B, lane 2) suggests that they may be able to form gap junctions with cytoskeletal associations under these conditions.

Immunofluorescence microscopy was used to determine the localization of intercellular attachment proteins at cell-to-cell contacts in monolayer cell cultures. In confluent monolayers, cadherins, β-catenin, and connexin43 were concentrated at cell-to-cell contact points (Fig. 4A) where filopodia meet and overlap. Both
Cadherins and β-catenin were concentrated in discrete linear structures perpendicular to the peripheral margin of the cell. The cell monolayer that we studied (Fig. 4A) exhibited two types of contacts: 1) cell-to-substrate contacts with the tissue culture plate (e.g., focal adhesion complexes), and 2) intercellular contacts between adjacent cells (i.e., adherens junctions and gap junctions). The appearance and distribution of cadherins and β-catenin were very different from those found in typical focal adhesion complexes, consistent with the fact that these cadherins and β-catenin are intercellular adherens junctional proteins. Although cytoplasmic staining for connexin43 was evident, very strong connexin43 staining appeared as punctate junctional plaques at intercellular contacts.

Immunocytochemistry and confocal microscopy were used to localize connexin43, β-catenin, and cadherins at intercellular contacts in z-axis scans of donor-acceptor cell preparations. Optical sections (2 μm apart; perpendicular to the z-axis of the culture dish; Fig. 4, top diagram) midway between the nuclei of the donor (top) cell and the acceptor cell (bottom) showed clustering of cadherins, β-catenin, and connexin43 at the level of cell-to-cell contact in both donor and acceptor cells (Fig. 4B). Staining for cadherins, β-catenin, and connexin43 was not observed at acceptor cell-substrate sites. Notably, during early cell-to-cell contact formation, cadherins and β-catenin were concentrated more toward the periphery of donor cells, a finding that is consistent with electron microscopy showing cell-to-cell contacts at the tip of the filopodia of donor cells (Fig. 2C). Unlike adherens junctions, staining for gap junctions with connexin43 exhibited a more dispersed distribution at the cell-to-cell interface.
Kinetics of intercellular adhesive contacts. We compared the rate of formation of cell-to-cell and cell-to-substrate adhesive contacts. Donor cells were added to highly adherent acceptor cell monolayer and allowed to attach for various lengths of time at 37°C (15, 30, 60, and 180 min) before vigorous washing with PBS. We optimized previously plating conditions by overnight attachment so that the acceptor cells were not detached by jet washing. The DiI-chloromethylbenzamido (CM)-labeled donor cells remaining attached to the HGF acceptor monolayer (cell-to-cell) or those that were freshly plated on the fibronectin-coated surface (cell-to-substrate) were counted in a fluorescence microscope and used to estimate the rate of adhesive contact formation. Based on counts of DiI-CM-labeled cells, cell-to-cell adhesive contacts formed at a faster rate than cell-to-substrate contacts (Fig. 5). The difference in adhesion rate was most significant during the time of early contact formation (>30-fold at 15 min; \( P < 0.001 \)), suggesting that cell-to-cell adhesive contacts may be stronger than cell-to-substrate contacts at their early stages of formation. Cell-to-cell adhesion was Ca\(^{2+}\)-dependent because when the same adhesion assay was repeated in Ca\(^{2+}\)-free medium containing 2 mM EGTA, no donor cells remained attached on the acceptor cell monolayer after washing.

Quantification of gap junctional communication. We used flow cytometry to measure the amount of dye transfer between donor-acceptor couples in a 3-h time course. Gap junctional communication was quantified by measuring the mean calcein fluorescence of the acceptor population (Gate R2; Figs. 6 and 7). The consistency in calcein fluorescence among samples at each time point (Fig. 8) and the general trend of calcein increase in the acceptor cells over time indicate that our model generates a synchronous cohort of dye-coupled cells, findings that are consistent with previous studies of single cells forming junctions in the x-y plane (9). When dye coupling was measured over time, there was a dramatic increase after \(~120\) min of cell-to-cell contact at 37°C (Fig. 8). This finding was consistent with the electron microscopy data that showed progressive formation of gap junctions between the donor and the acceptor cells over time and in which distinct gap junctional structures appeared after \(~120\–180\) min of cell-to-cell contact. The increase in cell-to-cell adhesion (Fig. 5) and the increase in gap junctional communication (Fig. 8) exhibited different kinetics. The rate of increase in cell-to-cell adhesion was highest in the first 60 min, whereas the rate of increase of calcein dye transfer was dramatically increased only after \(~120\) min. This apparent delay in gap junctional communication is consistent with the generally accepted theory that gap junctions are formed after cell-to-cell adherens junctions are formed (10).

Inhibition of gap junction communication. We investigated whether the formation of functional gap junctions is temperature dependent by conducting cell-to-cell adhesion assays at 4°C for 3 h. Whereas cell-to-cell adhesion was only slightly reduced at 4°C compared with control cells when incubated at 37°C (\(<20\%\) reduction), dye transfer was significantly inhibited to \(<5\%\) of control \((P < 0.001;\) Fig. 6), indicating that the formation of functional gap junctions is indeed temperature dependent.

We perturbed donor-acceptor cell couples with different inhibitors of gap junctional communication, including BGA (8, 33) and several different connexin43 mimetic peptides (5, 9). BGA, a saponin that causes gap junction disassembly and connexin43 phosphorylation (16), significantly reduced dye transfer in a dose-dependent manner (to \(<10\%\) of control \(P < 0.001;\) Figs. 6 and 7) without affecting cell-to-cell adhesion (105% of control at 20 \(\mu\)M, \(P > 0.2\)). Specific blockade of gap junctional communication was achieved by incubation with the connexin43 mimetic peptide GAP 27 (amino acid sequence SRPTEKTIF) that has the same amino acid sequence as a part of the extracellular loop of connexin43. This peptide likely acts by perturbing connexin-connexin interactions that probably maintain channel integrity (5, 9). Accordingly, significant inhibition by GAP 27 of dye transfer was observed at 500 \(\mu\)M \((<10\%\) of control, \(P < 0.001;\) Figs. 6 and 7). On the other hand, a truncated version of GAP 27 (amino acid sequence SRPTEKTFI) that lacks the two isoleucine residues required for membrane insertion did not inhibit dye transfer (Figs. 6 and 7). Further, a connexin-mimetic peptide that resembles part of the intracellular loop of connexin43 (GAP 20; amino acid sequence EIKKFKYG) (5, 9) was included as a negative control and also did not inhibit dye transfer (Figs. 6 and 7).
Fig. 6. Flow cytographs showing the effects of various inhibitors of gap junctional communication. Dye transfer was indicated by increased calcein fluorescence of the acceptor cell population (upper left quadrant). Significant dye transfer occurred within 180 min of incubation at 37°C in the presence (ii) or absence (iii) of serum when compared with time 0 (i). Note the absence of calcein-labeled donor cells in (i) but their presence in the bottom right quadrant at later times (ii–ix, t = 180 min). Low temperature significantly inhibited dye transfer (iv). The gap junctional communication blocker β-glycyrrhetinic acid (BGA) significantly reduced calcein dye transfer at 20 μM (v), and further reduction was evident at 100 μM (vi). The connexin43 mimetic peptide GAP 27 significantly reduced the extent of dye transfer (viii), whereas its truncated version GAP 27-II (viii) and GAP 20 (ix) had relatively no effect. These results are representative of 3 parallel samples (n = 3) in 2 independent experiments.
DISCUSSION

*Double-label synchronized cohort model.* Studies of epithelial cells (29) and developing tissues (1) have provided much of our current understanding of intercellular adhesive structures and gap junctional communication. However, there has been only limited characterization and less understanding of intercellular adhesion and gap junctions in fibroblasts despite the apparent importance of these structures in wound healing (14, 21) and remodeling of connective tissues.

To facilitate the study of intercellular adhesion and gap junctional communications, we have developed and characterized a simple model using human fibroblasts derived from the periodontium. In these tissues, gap junctions and adherens junctions are prominent structures of adjacent fibroblasts in vivo (3, 32).

Most previous studies of intercellular adhesions and gap junctions in cultured fibroblasts have been limited to morphological examinations in the x-y plane (13, 30, 35) of only a few cells attached on a substrate. We considered that the ability to study the formation of intercellular contacts without undue interference from cell-to-substrate interactions is important for resolving the dynamics of cell-to-cell interactions. In addition, many studies of cell-to-cell contacts use transformed cells (28, 30) or epidermal cells (12), and there are few reports on normal human fibroblasts. The new model presents several useful features. First, it 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. With this approach, we have characterized their morphology at discrete stages of their development by transmission electron microscopy, confocal microscopy, and optical sectioning. These data showed that the donor-acceptor model not only features types of intercellular junctions similar to those that formed in monolayer cultures in the x-y plane but that they are much more prominent and more easily recognized. Because large numbers of cells can be analyzed, the model also enables biochemical analysis of intercellular junctions that are associated with the cytoskeleton during the formation of intercellular adhesions (e.g., immunoblotting). Furthermore, functional assays of intercellular adhesion and gap junctional communication by dye transfer measurements with flow cytometry can be performed.

A second useful feature relates to the design of the model. Because donor cells are added onto a confluent acceptor cell monolayer, the attachment and subsequent spreading of donor cells depend largely on cell-to-cell interactions between the donor and acceptor cells and only minimally on cell-to-substrate interactions between acceptor cells and their substratum. Third, because the donor and the acceptor cells are separately labeled with different fluorescence dyes, any donor, acceptor, or dye-coupled acceptor populations can be preferentially selected by fluorescence-activated cell sorting for later analysis, including biochemical analysis. The model could therefore be used to test the permeability of gap junctions to specific molecules between donor and acceptor cells. Molecules to be tested could be loaded into the cytoplasm of donor cells by electroporation (11), scrape loading, or endocytosis. For example, we have shown that endocytosed FITC or Texas red dextran (>10 kDa) does not transfer between cells after a 6-h coincubation.

In general, gap junctions are formed by connexin subunits that are a family of proteins including connexins43, 26, 32, and 45 (15). Another advantage of using human gingival fibroblasts is that of simplifica-
tion: only connexin43 was detected by immunoblotting. Notably, the degree of dye transfer inhibition in HGFs was significantly more than previous reports in which the GAP 27 peptide was used to inhibit gap junctional communication in other cell types (5, 9). With the availability of inhibitory peptides, our model provides a sensitive, specific, and simplified assay for study of gap junctions.

**Intercellular adhesion.** We found that intercellular adhesive contacts formed rapidly (<15 min) between donor and acceptor fibroblasts and were typical of the appearance of adherens junctions (3, 32). The intercellular adhesion was Ca$^{2+}$ dependent. Our immunofluorescence and confocal images showed clustering of cadherins, β-catenin, and connexin43 at intercellular contacts. We also found an enrichment of these proteins in the Triton-insoluble cytoskeletal fraction in confluent cell cultures. These observations are consistent with the notion that intercellular adherens junctions are cadherin-mediated complexes comprised of cytoplasmic plaque proteins such as catenins and are connected to the actin cytoskeleton (17).

For functional analysis, we employed a jet-washing assay to study cell-to-cell adhesive strength. This method has been used previously to estimate relative adhesive strength of collagen-coated beads to fibroblasts (7). The increased number of Dil-CM-labeled donor cells remaining attached to acceptor cells after jet washing provides an estimate of the increase in intercellular adhesive strength over time. Presumably, the progressive maturation of the adherens junctions strengthens intercellular adhesion by expansion of cell-to-cell contact sites into larger junctional plaques as shown by our electronmicrographic data and by forming more connections to the actin cytoskeleton. The adherens junctions that formed evidently engaged the cytoskeleton because cadherins, β-catenin, and connexin43 were recruited into the cytoskeletal network when there were abundant cell-to-cell contacts.

**Interdependence between intercellular adhesion and gap junctions.** Previous reports have shown a dependence of gap junction formation on cadherin-mediated intercellular adhesion in epidermal cells (19) as well as an interdependence between these two types of junctions (24). The pattern of spatial association between gap junctions and cell adhesion junctions is likely an important factor in maturation of mammalian cardiac tissues (1). In thyroid cells, neoplastic alterations of the complex cellular network established by adherens receptors and gap junctions can lead to an imbalance of cell-to-cell communication: this imbalance allows transformed cells to escape from the tissue to generate metastases (28). Therefore, the ability to study simultaneously both adherens and gap junctions is of considerable biological importance and can be realized with this model system. We have shown that structures resembling adherens junctions appeared well before gap junctional plaques. Kinetic studies showed that adhesive cell-to-cell contacts increased most rapidly during the initial 60 min of coincubation that was followed by a dramatic increase in dye transfer starting at ~120 min. This temporal relationship suggests that intercellular adhesion is a prerequisite for gap junction formation. Presumably, the close apposition of adjacent cell membranes mediated by adherens junctions facilitates cell-to-cell interactions. These interactions include connexon-to-connexon couples that lead in turn to the formation of gap junctions.

The ability to measure intercellular adhesion and gap junctional communication simultaneously by flow cytometry enhances the analytical power of our model. We showed that although the gap junctional inhibitor BGA had no effect on cell-to-cell adhesion, GAP 27 peptide significantly reduced adherence of donor cells by >90%. Because BGA dephosphorylates connexins (16, 18) while GAP 27 probably inhibits the formation of gap junctions by perturbing connexin-connexin interactions (5, 9), we interpret these data as indicating that connexon assembly has a more important effect on intercellular adhesion than does connexin phosphorylation.

We conclude that the synchronized cell cohort model reported here provides information on the structure and kinetics of the formation of intercellular adherens and gap junctions in human fibroblasts. The data suggest that it is a sensitive, specific, and quantitative model for investigating the dynamics of intercellular adherens junctions and gap junctions. The model should facilitate studies of intercellular signaling in fibroblasts and other stromal cells that are involved in wound healing and tissue remodeling.

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**REFERENCES**


