Junctional Communication Is Induced in Migrating Capillary Endothelial Cells

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Abstract. Using an in vitro model in which a confluent monolayer of capillary endothelial cells is mechanically wounded, gap junction–mediated intercellular communication has been studied by loading the cells with the fluorescent dye, Lucifer Yellow. Approximately 40–50% of the cells in a nonwounded confluent monolayer were coupled in groups of four to five cells (basal level). Basal levels of communication were also observed in sparse and preconfluent cultures, but were reduced in postconfluent monolayers. 30 min after wounding, coupling was markedly reduced between cells lining the wound. Communication at the wound was partially reestablished by 2 h, exceeded basal levels after 6 h and reached a maximum after 24 h, at which stage ∿90% of the cells were coupled in groups of six to seven cells. When the wound had closed (after 8 d), the increase in communication was no longer observed. Induction of wound-associated communication was unaffected by exposure of the cells to the DNA synthesis inhibitor mitomycin C, but was prevented by the protein synthesis inhibitor, cycloheximide. The induction of wound-associated communication was also inhibited when migration was prevented by placing the cells immediately after wounding at 22°C or after exposure to cytochalasin D, suggesting that the increase in communication is dependent on cells migrating into the wound area. In contrast, migration was not prevented when coupling was blocked by exposure of the cells to retinoic acid, although this agent did disrupt the characteristic sheet-like pattern of migration typically seen during endothelial repair. These results suggest that junctional communication may play an important role in wound repair, possibly by coordinating capillary endothelial cell migration.

The vascular endothelium consists of a highly ordered quiescent monolayer of cells that can be induced to migrate in response to a variety of stimuli. Migration thus occurs during the process of new blood vessel formation (Ausprunk and Folkman, 1977), and during repair of the endothelial lining of large vessels after injury (Fishman et al., 1975; Buck and Malczak, 1977; Schwartz et al., 1978). In contrast to other vessel wall cells such as fibroblasts and smooth muscle cells, which usually migrate as individual cells (Abercrombie et al., 1970; Thorgeirsson et al., 1979; Gotlieb and Spector, 1981), capillary endothelial cells migrate as well-organized, tube-like sprouts (Ausprunk and Folkman, 1977; Montesano and Orci, 1985), whereas large vessel endothelial cells migrate as a coherent sheet with a well-defined leading front (Schwartz et al., 1978). Intercellular contact between migrating endothelial cells is essential for the maintenance and regeneration of structurally and functionally competent blood vessels endowed with a continuous nonthrombogenic luminal surface, and has been suggested to provide a mechanism for coordinating the migration process (Gotlieb et al., 1987; Larson and Haudenschild, 1988).

In many systems, coordination of cellular functions is achieved by intercellular communication mediated by gap junctions. Gap junctions are clusters of transmembranous hydrophobic channels, which allow the direct exchange of ions and small molecules (up to 900 D) between adjacent cells (Gilula et al., 1972; Simpson et al., 1977). Related but nonidentical gap junction proteins (connexins) appear to have a tissue specific distribution (reviewed in Beyer et al., 1987; 1988). In excitable tissues such as the myocardium and some electrotonically coupled neurons, gap junctions provide the means for rapid propagation of electrical signals, thereby synchronizing the cells (reviewed in Bennett and Spray, 1985). Gap junction–mediated cell-to-cell communication has also been demonstrated in most nonexcitable tissues, where it is believed to play a role in the control of various cellular functions such as proliferation (Loewenstein, 1968; 1979) and secretion (Meda et al., 1986; 1987; Chanson et al., 1989).

Intercellular communication, as revealed by the diffusion of microinjected Lucifer Yellow, has been demonstrated in both micro- and macrovascular endothelium (Larson and Sheridan, 1982; Larson and Haudenschild, 1988; Larson et al., 1987), but its role in endothelial cell function remains hypothetical. It has recently been reported that intercellular

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communication between aortic endothelial cells migrating from the edges of an experimental wound was decreased relative to cells outside the wound (Larson and Haudenschild, 1988). In this study, we addressed the following questions: (*a*) do capillary endothelial cells communicate, and if so, does wounding affect the extent of communication? (*b*) which repair-associated cell functions might be correlated with intercellular communication? (*c*) does intercellular communication play a role in the repair process? To investigate these issues, we have wounded a confluent monolayer of capillary endothelial cells and assessed the extent of communication by evaluating the intercellular diffusion of Lucifer Yellow using either microinjection or scrape-loading techniques.

Materials and Methods

Cell Culture and Wounding

Cloned microvascular endothelial cells from bovine adrenal cortex (BME[1] cells) (Furie et al., 1984), were routinely subcultured in gelatin-coated tissue culture flasks (Falcon Labware; Becton-Dickinson & Co., Oxnard, CA) in complete medium consisting of MEM, alpha modification (Gibco AG; Basel, Switzerland), supplemented with 15% heat-inactivated donor calf serum (Flow Laboratories, Baar, Switzerland), 500 U/ml penicillin and 100 μg/ml streptomycin. Cells were used between passages 11 and 20. The cells were seeded at 1–2 × 10^5 cells/35-mm gelatin-coated culture dish (Falcon Labware), and grown to confluence in complete medium (2–3 d). At confluence, the monolayers were wounded with either a 5-mm-wide rubber policeman or with a blade to mark the original wound edge (Bürk, 1973), and fresh complete medium was replaced. Unless otherwise stated, wounded monolayers were incubated thereafter at 37°C in an air/CO_2 (97%:3%) atmosphere for varying lengths of time.

Determination of Junctional Communication

Cell-to-cell coupling was determined by two complementary approaches. In the first, individual endothelial cells were injected with the gap junction–permeant fluorescent tracer Lucifer Yellow (Stewart, 1978) (Sigma Chemical Co., St. Louis, MO), and in the second, monolayers were scrape-loaded with a mixture of Lucifer Yellow and dextran rhodamine (Molecular Probes, Eugene, OR), using a modification of the technique of McNeil et al. (1984), as described by El-Fouly et al. (1987).

For microinjection, medium was removed, Krebs-Ringer-bicarbonate medium containing 12.5 mM Hepes and 0.1% BSA was added, and the dishes were transferred to the heated stage (37°C) of an inverted microscope (ICM405; Carl Zeiss, Inc., Oberkochen, FRG). Individual endothelial cells were injected for 3 min as reported in Meda et al. (1986). After removal of the electrode, the injected field was photographed either under fluorescence or using combined fluorescence and phase-contrast illumination. Microinjections were performed both at the edge of the endothelial wound and at a distance from it. The first region, called "wound," was arbitrarily defined as filling one microscopic field along the wound as seen under a 25× objective in an inverted photomicroscope (ICM405; Carl Zeiss, Inc.), and comprised ~10 cell rows from the wound edge. The second region, called "outside the wound," was separated from the first by at least two microscopic fields, and comprised an entire field as seen under the 25× objective. Photographs taken at the end of each injection in these two regions were used to score the number of Lucifer Yellow–labeled cells. The incidence of coupling was expressed as the percentage of injections showing cell-to-cell dye transfer. The extent of dye transfer was represented by the number of labeled cells receiving dye from the injected cell (i.e., excluding the injected cell), and is expressed as the mean ± SEM of the number of injections demonstrating coupling.

For scrape-loading, monolayers were rinsed with PBS, and incubated in 1 ml PBS containing 20 mg/ml Lucifer Yellow and a few crystals of 10,000-mol wt dextran-tetramethylrhodamine to obtain a Bordeaux wine-colored solution. The monolayers were then scraped perpendicular to the original endothelial wound using a mini-glass cutter, and incubated in the dark for 2 min at room temperature. The Lucifer Yellow-dextran mixture was then removed, the cultures were washed several times in PBS, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and stored in the dark at 4°C. Scrape-loaded cultures were photographed under both phase-contrast and fluorescence illumination. The presence or absence of coupling was evaluated in the "wound" and "outside the wound" regions in two ways. In the first, cell layers labeled by Lucifer Yellow on both sides of the scrape line were evaluated under a fluorescence microscope for the presence or absence of coupling by scoring − or +. Score − referred to the absence of coupling, as indicated by labeling of only the first layer of cells lining the scrape (these cells were also labeled with dextran-rhodamine indicating that they had been permeabilized during the scrape-loading procedure). Score + referred to the presence of coupling as indicated by the labeling of at least the first two rows of cells along the scrape, the second and subsequent rows being labeled exclusively with Lucifer Yellow. This approach allowed for the rapid and simultaneous evaluation of different regions of the monolayer within individual culture dishes. In the second method of evaluation, the levels of Lucifer Yellow fluorescence were evaluated by scanning the scrape-loaded dishes perpendicular to the scrape line (i.e., parallel to the original wound), using a microspectrophotometer (MPMOlK; Carl Zeiss, Inc.) equipped with the UV illumination provided by a mercury HB0100 lamp and a highly stabilized power supply, filters for fluorescein detection, a water immersion 40× objective and a rectangular measuring diaphragm representing approximately the surface area of a single endothelial nuclear profile. The intensities of fluorescence were measured relative to a constant fluorescence source provided by a uranyl glass standard (GG17038; Carl Zeiss, Inc.) and recorded as a function of the scan position on a two-channel chart recorder (2210; LKB, Bromma, Sweden). Each monolayer was randomly sampled in the "wound" and "outside the wound" regions (two or three scans for each region). The tracings obtained were used to score the number of fluorescent peaks on either side of the nonfluorescent scrape line, one peak on each side indicating the absence of coupling, and more than one peak on at least one side of the scrape line indicating the presence of coupling. The incidence of coupling was expressed as the percentage of measurements with more than one peak.

Identification of Gap Junctions

Gap junctions were studied in the "wound" and "outside the wound" regions by freeze-fracture, 24 h after wounding. For this purpose, wounded monolayers on plastic tissue culture coverslips (Thermanox TM; Miles Laboratories, Naperville, IL) were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer pH 7.4 for 20 min, infiltrated with 30% phosphate-buffered glycerol and frozen in Freon 22 that had been cooled in liquid nitrogen. Fracture and shadowing were carried out in a Balzers BAF 301 apparatus (Balzers High Vacuum Corp., Balzers, Liechtenstein). The replicas were washed in a sodium hypochlorite solution, rinsed in distilled water, mounted on Formvar- and carbon-coated grids and examined in an electron microscope (EM 301; Philips Electronic Instruments, Eindhoven, The Netherlands).

Cell Migration and Wound-Edge Coupling at 22°C

To determine the effect of temperature on the coupling and migration of BME cells, cultures were wounded and immediately incubated at 22°C in complete medium containing 50 mM Hepes, pH 7.4. Communication and migration were assessed after a 24-h incubation in ambient air. In all experiments, the pH of the medium remained unchanged at 7.4 during this period. To determine whether the effects of low temperature were reversible, wounded cultures that had been at 22°C for 24 h were transferred to 37°C without changing the medium, and migration and communication were assessed after a further 24-h incubation at 37°C. To determine whether established junctional communication was affected at 22°C, wounded monolayers that had been incubated at 37°C for 24 h were transferred to 22°C and coupling was determined as described above.

Results

24 h after wounding, extensive transfer of Lucifer Yellow was observed between cells situated in the first 10–15 rows behind the leading front, both by microinjection and scrape-loading techniques (Figs. 1, *a* and *b*; 2, *a–c*; and 3 *a*). This was in marked contrast to the minimal communication seen

1. *Abbreviation used in this paper*: BME, bovine microvascular endothelial cells.

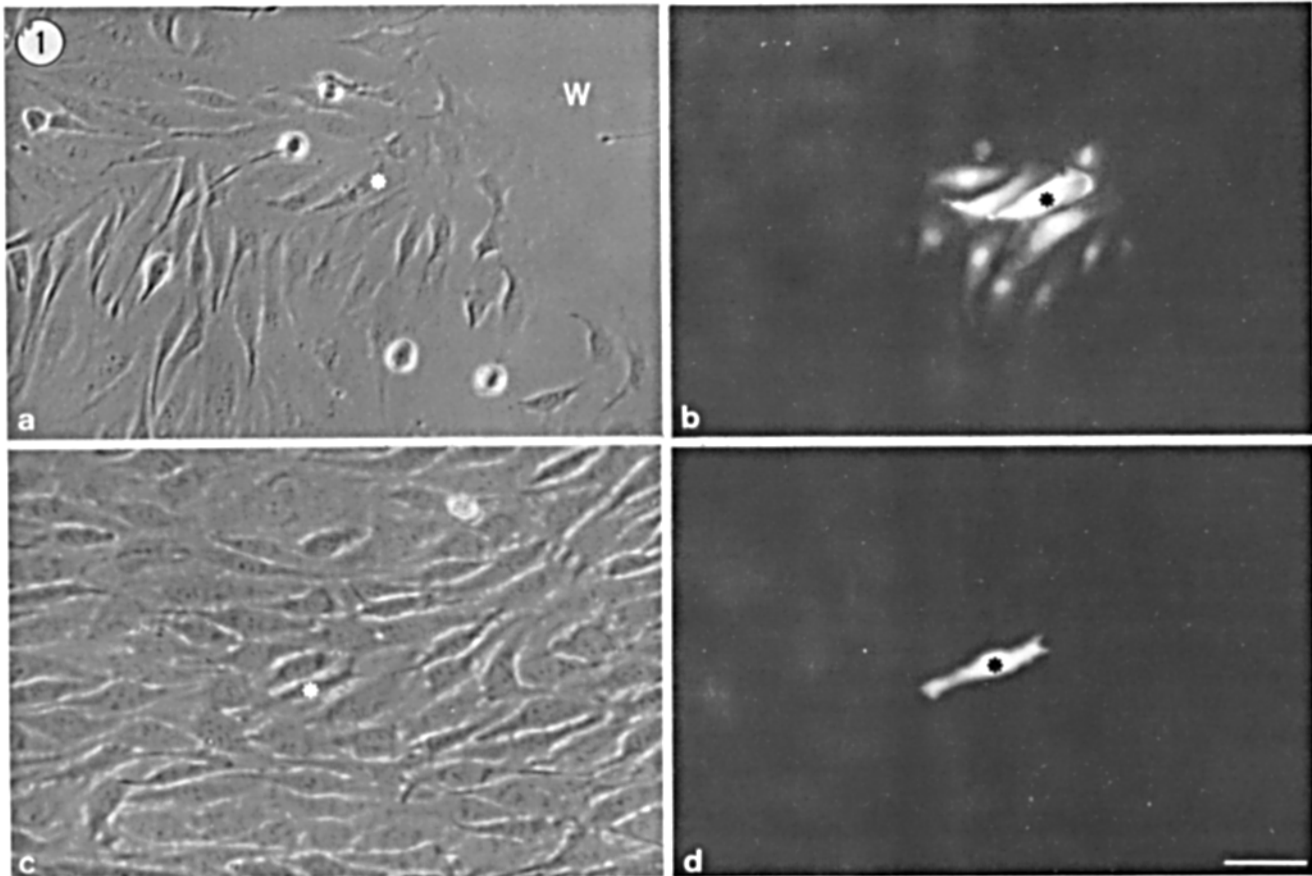


Figure 1. Junctional communication of BME cells as evaluated by microinjection, 24 h after wounding. Phase-contrast views of (*a*) the "wound" and (*c*) "outside the wound" regions of the monolayer; (*b* and *d*) corresponding fluorescence views after microinjection of Lucifer Yellow into individual endothelial cells (*asterisk*). The fluorescent tracer was transferred to adjacent cells in *b*, indicating the occurrence of cell-to-cell coupling at the wound edge. In contrast, tracer was retained within the injected cell in *d*, indicating the absence of coupling at a distance from the wound. *W*, original wound area denuded of cells. Bar, 50 μm.

between cells in the regions of the same monolayers situated outside the wound (Figs. 1, *c* and *d*; 2, *a* and *b*; and 3 *b*). A quantitative analysis revealed that in confluent nonwounded BME monolayers ~40–50% of the cells were coupled in groups of four to five cells (Table I and Fig. 5, *a* and *b*). Communication was next assessed at various times after wounding. Dye transfer between cells in the wound region was unaffected up to 30 min after wounding (Table I and Fig. 5, *a* and *b*). From 30 min to 2 h after wounding, coupling was markedly reduced between cells lining the wound edge (Table I; Figs. 4 and 5, *a* and *b*). Communication at the wound edge was partially reestablished 2 h after wounding, exceeded basal levels after 6 h, and reached a maximum after 24 h, at which stage ~90% of the cells were coupled in groups of six to seven cells (Table I; Figs. 4, *c–f*; and 5, *a* and *b*). In contrast, communication decreased with time between cells outside the wound, and after 24 h was restricted to ~15–35% of the cells in groups of two to three cells (Table I and Fig. 5). After 8 d, when the wound had closed, increased levels of junctional communication were no longer observed (Fig. 4, *g* and *h*). As shown in Table I and Fig. 5, *a* and *b*, similar data were obtained whether communication was quantitated by scrape-loading or microinjection techniques. It should also be noted that in the scrape-loading assay, the cells are of necessity wounded. However, communication between cells in the wound region is unaffected up to 30 min after wounding with a rubber policeman or a blade (Table I and Fig. 5, *a* and *b*), indicating that dye transfer is unlikely to be affected during the two minute period between scrape-loading (in which the cells are wounded with a glass cutter) and cell fixation (see Materials and Methods).

We assessed whether the coupling between cells at the wound edge could be correlated with the presence of morphologically identifiable gap junctions in this region. Freeze fracture of wounded monolayers of BME cells revealed the presence of aggregates of particles (on the P fracture face) (Fig. 6) and pits (on the E fracture face), which are typical of gap junctions. The size of these aggregates varied widely from one cell to another as well as on different membrane regions of the same cell, where aggregates of only a few particles were found nearby much larger aggregates consisting of several hundred particles. Typical gap junctions were seen in cells at the wound edge, but were rarely seen in regions of the BME monolayer outside the wound. However, even at the wound edge, the majority of exposed fracture faces of BME plasma membranes appeared devoid of morphologically identifiable gap junctions.

We next examined whether the increase in cell-to-cell communication at the wound edge was related to cell division by treating the BME monolayers with mitomycin C (10

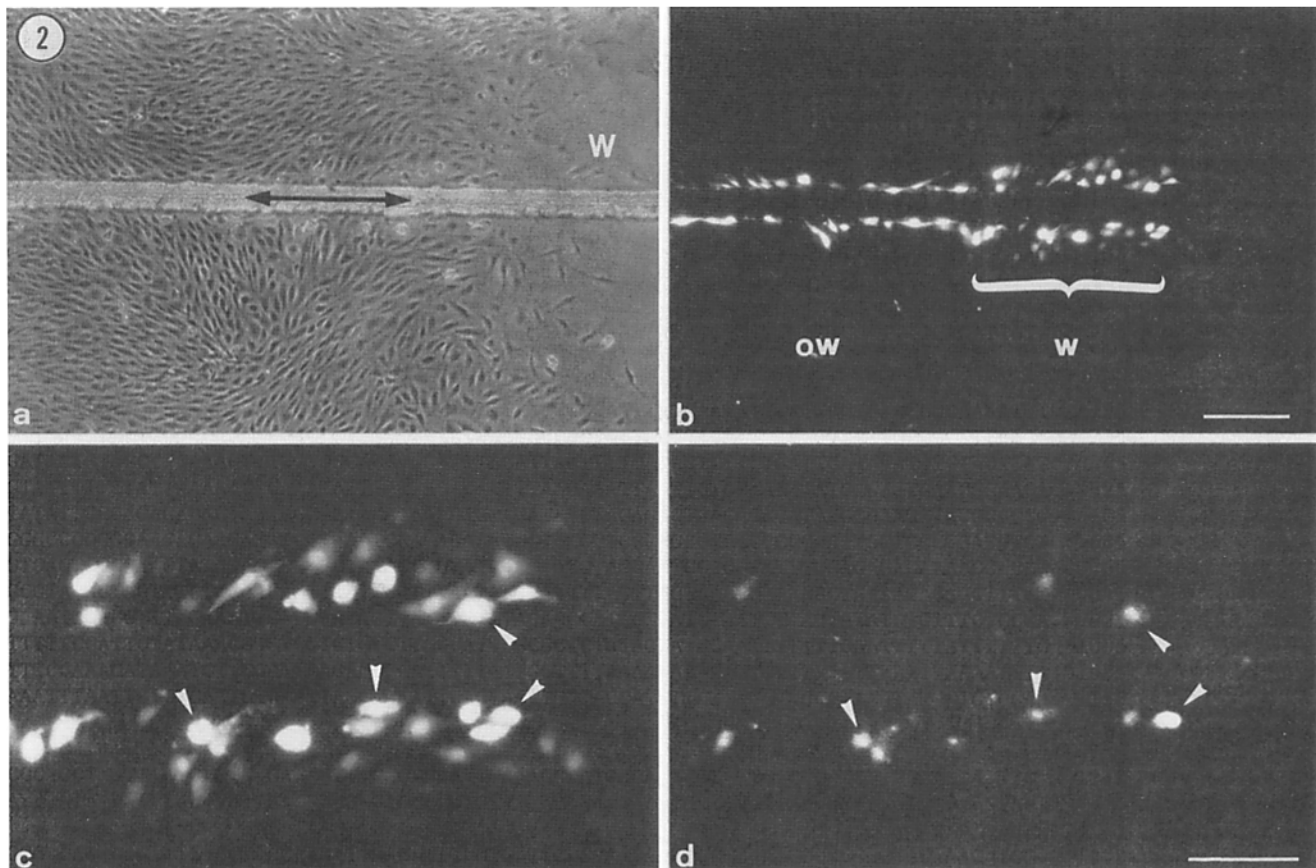


Figure 2. Junctional communication of BME cells as evaluated by scrape loading, 24 h after wounding. (*a* and *b*) Low power phase-contrast (*a*) and fluorescence (*b*) views showing the positions of wound (*w*) and outside the wound (*ow*) regions of the monolayer, as well as the original wound area (*W*) not yet invaded by the leading edge of migrating cells. The former region (*w*) comprises 10–15 rows of cells from the leading front, whereas the latter region (*ow*) comprises the undisturbed confluent portion of the monolayer. *Doubleheaded arrow* indicates the scrape line perpendicular to the original wound, used to load the cells with both Lucifer Yellow (a gap junction permeant tracer) and dextran-rhodamine (a molecule too large to cross the junctional channels); (*b*) the distribution of Lucifer Yellow–labeled cells along the scrape line. In the wound region (*w*), the fluorescent tracer has labeled several rows of BME cells on either side of the scrape line, indicating the occurrence of cell-to-cell coupling. In contrast, outside the wound (*ow*), Lucifer Yellow has labeled only the row of cells immediately adjacent to the scrape line, indicating the absence of cell-to-cell coupling; (*c* and *d*) higher magnification views of the wound region (*w*) shown in *a* and *b* and seen with filters for fluorescein (*c*) and rhodamine (*d*). Comparison of the two figures shows that whereas only cells lining the scrape (*arrowheads*) had incorporated the large molecular weight dextran as a result of transient permeabilization during scrape-loading (*d*), Lucifer Yellow is transferred to four or five adjacent cell rows in *c*. Thus, transfer of the gap junction–permeant tracer had occurred from the row of permeabilized cells lining the scrape to adjacent coupled cells. Bar, 200 μm in *a* and *b* and 100 μm in *c* and *d*.

μg/ml) for 4 h before wounding, a procedure which has previously been shown to induce a complete and sustained block of mitosis of BME cells at the wound edge (Pepper et al., 1987). We observed that mitomycin C treatment did not alter the increase in cell-to-cell communication that occurs between migrating BME cells, 24 h after wounding (Table II). In contrast, the increase in coupling seen at the wound edge was reversibly inhibited by incubating wounded monolayers in the presence of the protein synthesis inhibitor, cycloheximide (0.1 μg/ml), for 24 h after wounding (Table II and Fig. 7, *a* and *b*). Treatment with cycloheximide, which markedly reduced the uptake of [^{3}H]leucine in wounded monolayers (compare Fig. 7, *c* and *d*), also inhibited cell migration (compare Fig. 7, *a–d*).

To determine whether the increase in communication was due to the regional decrease in cell density at the wound, we compared cell-to-cell communication in sparse, preconfluent/confluent, and postconfluent BME cultures. We observed that BME cells were coupled in 50–60% of cases in groups of four cells in sparse and preconfluent/confluent monolayers (Table III). This value is significantly lower than that observed in the wound region 24 h after wounding (Table I and Fig. 5, *a* and *b*). In postconfluent monolayers, the incidence of coupling was reduced to ~20% (Table III).

To determine whether increased coupling might be related to cell migration, BME monolayers were wounded and immediately incubated in the presence of 0.5 μg/ml cytochalasin D in complete medium for 24 h. Cytochalasin D prevented both migration and the establishment of communication at the wound edge (Table IV; Fig. 8 *c*). The effects of this drug were reversible: removal of cytochalasin D, washing, and a further 24 h of culture in the absence of the drug, restored cell-to-cell communication and migration in parallel (Table IV; Fig. 8 *f*). In addition, migration and cell-to-cell

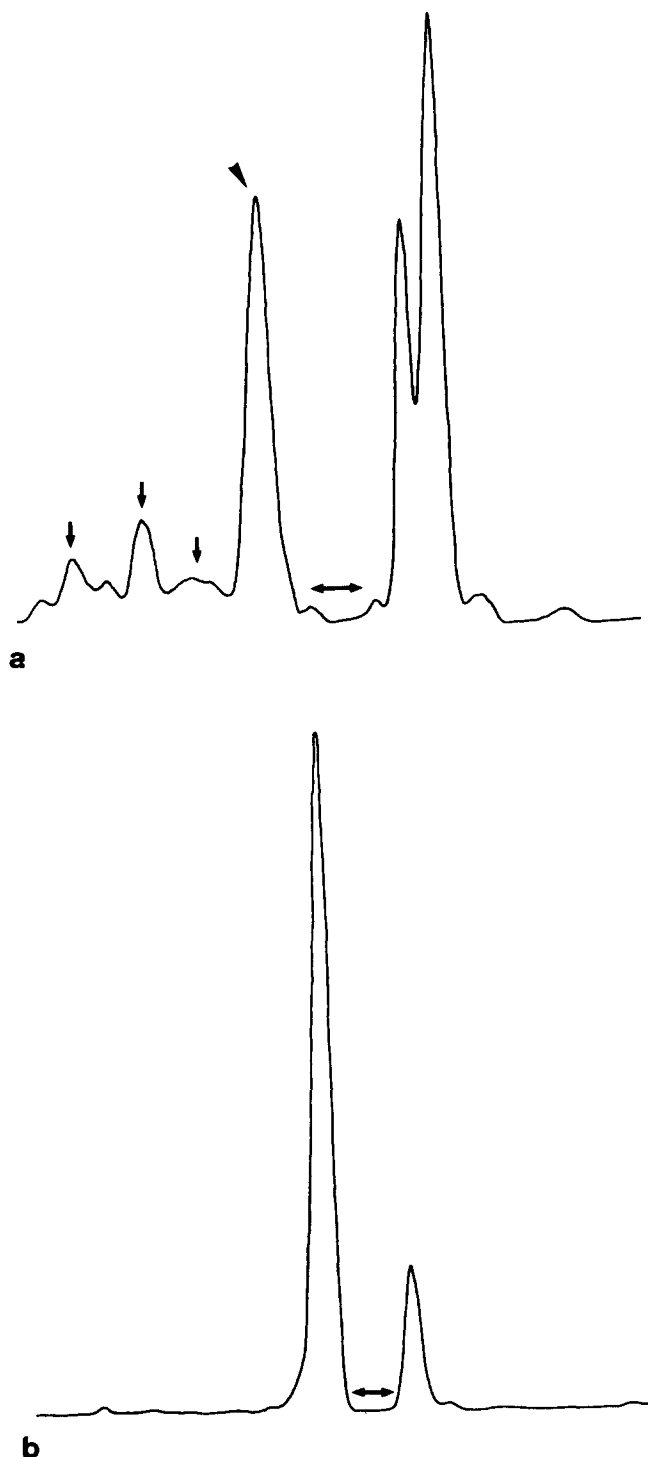


Figure 3. Spectrophotometric tracings of Lucifer Yellow fluorescence in scrape loaded monolayers, 24 h after wounding. Monolayers were scanned perpendicular to the scrape line (i.e., parallel to the original wound). (*a*) In the wound region, the presence of multiple fluorescence peaks on either side of the scrape (*double-headed arrow*) indicates the transfer of Lucifer Yellow from the layer of cells lining the wound (*arrowhead*) to several adjacent layers of coupled cells (*arrows*); (*b*) outside the wound, the presence of a single peak of fluorescence indicates the lack of transfer of Lucifer Yellow to adjacent cells, demonstrating absence of coupling.

Table I. Incidence of Cell-to-Cell Communication in Nonmigrating and Migrating Endothelial Cells as Determined by Scrape-Loading

		MI	SP
	h		
Nonwounded		47.0 (n = 17)	48.6 (n = 35)
Wounded			
Wound	t = 0	32.3 (n = 34)	43.5 (n = 23)
	t = <0.5	56.2 (n = 16)	50.0 (n = 4)
	t = 1	3.6 (n = 28)	16.7 (n = 12)
	t = 2	38.9 (n = 18)	25.0 (n = 4)
	t = 2–6	62.5 (n = 40)	64.3 (n = 14)
	t = 6–24	83.5 (n = 176)	94.8 (n = 77)
	t = 24–48	88.7 (n = 71)	94.4 (n = 36)
Outside the wound	t = 0	50.0 (n = 12)	54.5 (n = 11)
	t = 24	28.6 (n = 112)	35.5 (n = 76)

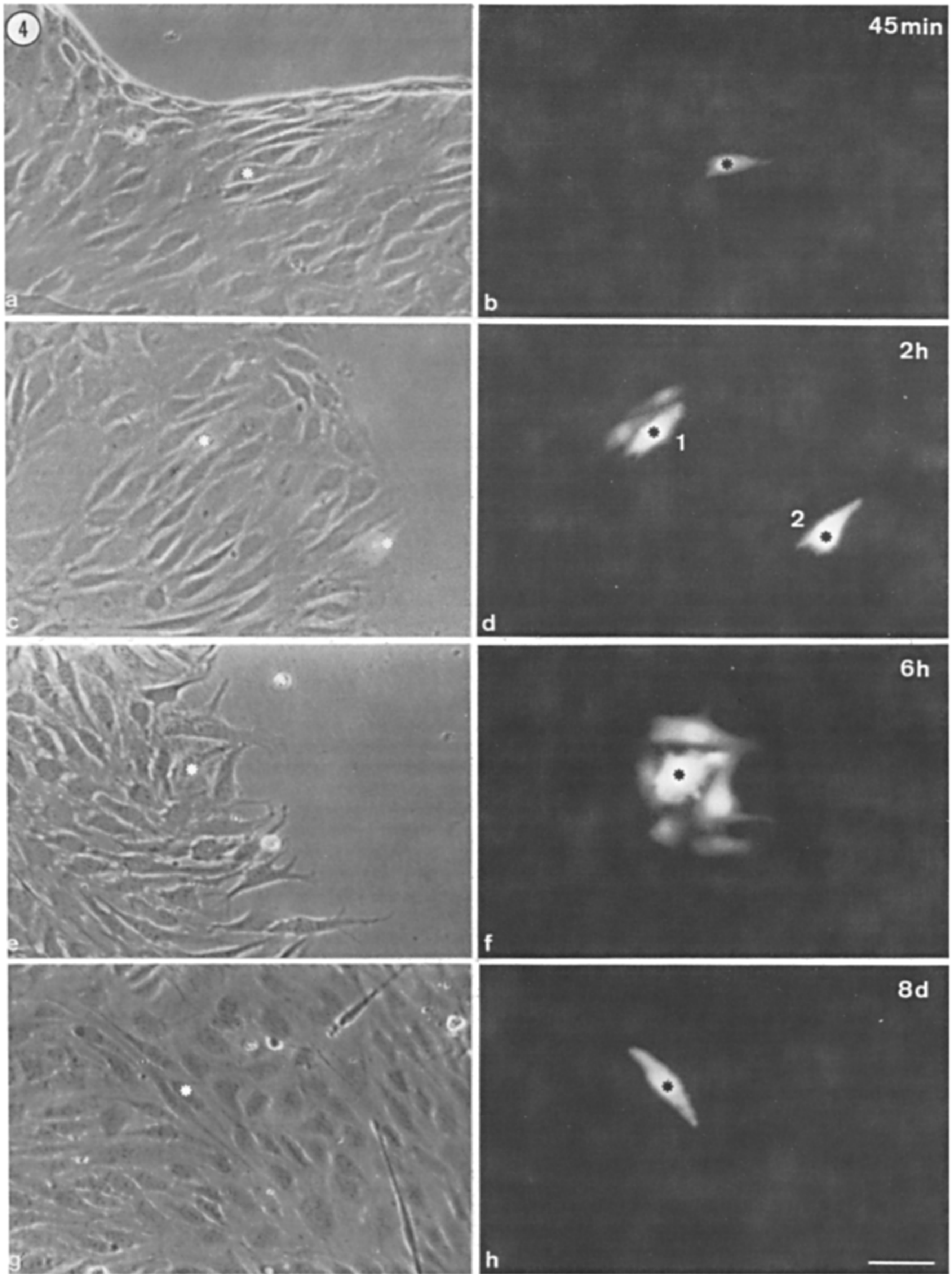
Values are percent of scrape-loadings that revealed coupling in nonwounded (confluent) and wounded monolayers. n, number of measurements per time point. MI, microscopic quantitation; SP, spectrophotometric measurement. t, time after wounding.

communication at the wound edge were completely prevented by placing BME cultures immediately after wounding at 22°C for 24 h (Table IV; Fig. 8 *b*). The inhibition of both junctional communication and migration was reversed when the monolayers were transferred back to 37°C for a further 24-h period; under these conditions, the incidence of coupling was reestablished to control values, in parallel with restoration of migration (Table IV; Fig. 8 *e*). Increased coupling between migrating cells in wounded monolayers which had been cultured at 37°C for 24 h was not affected by subsequently transferring the cells to 22°C for a further 24 h (not shown).

To further explore the apparent relationship between cell coupling and migration, BME cells were exposed to retinoic acid, an agent that has been reported to block junctional communication in other systems (Walder and Lutzelschwab, 1984; Pitts et al., 1986). Retinoic acid (10^{-4} M) inhibited the increase in communication typically seen at the wound edge (Table V; Fig. 9 *a*). Migration was not inhibited under these conditions (i.e., in the absence of coupling), as judged by the ability of retinoic acid–treated cells to migrate as far into the wound as cells in control cultures (Fig. 9, *b* and *d*). However, in the presence of retinoic acid a significantly greater number of cells ($p < 0.005$) showed reduced contact with the migrating sheet ("separate cells") when compared with controls (Table V and Fig. 9, *c* and *d*).

Discussion

Previous studies on microvascular endothelium have demonstrated gap junctions between endothelial cells and the presence of junctional communication in vitro (Larson et al.,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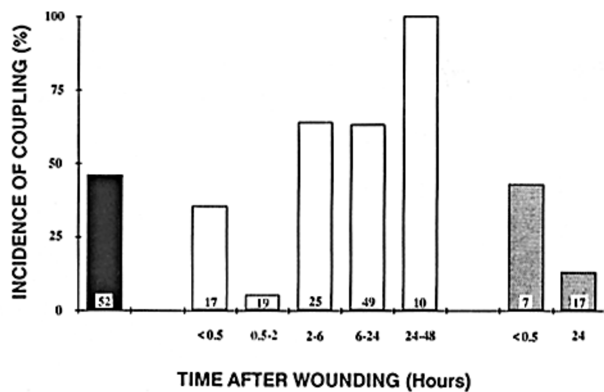


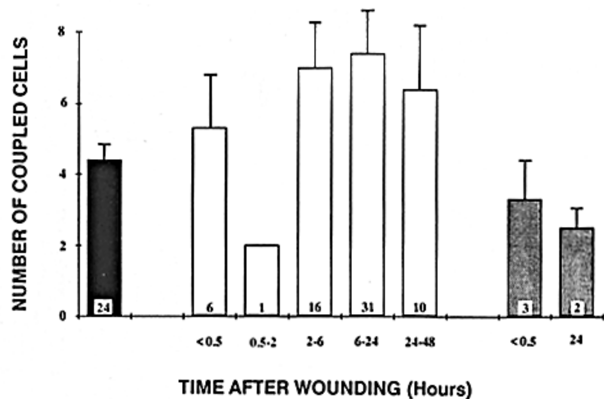


Figure 5. Quantitative data on BME cell coupling, as evaluated by microinjection of Lucifer Yellow in nonwounded confluent monolayers (*solid bars*) and at various times after wounding in either the wound (*open bars*) or "outside the wound" regions (*shaded bars*). (*a*) Incidence of coupling expressed as the percentage of microinjections that resulted in the intercellular transfer of Lucifer Yellow from the injected cell into at least one adjacent cell. (Numbers indicate the number of microinjections for each condition or time point.) (*b*) Extent of coupling expressed as the mean ± SEM of the number of cells that received Lucifer Yellow from the injected cell, per injection. (Numbers indicate the number of microinjections for each condition or time point which demonstrated coupling.) As shown, coupling was markedly reduced between 30 min and 2 h after wounding, but increased markedly in both incidence (*a*) and extent (*b*) for the next 48 h (*open bars*). During this time period, coupling decreased progressively in the nonwounded regions of the same culture dishes (*shaded bars*).

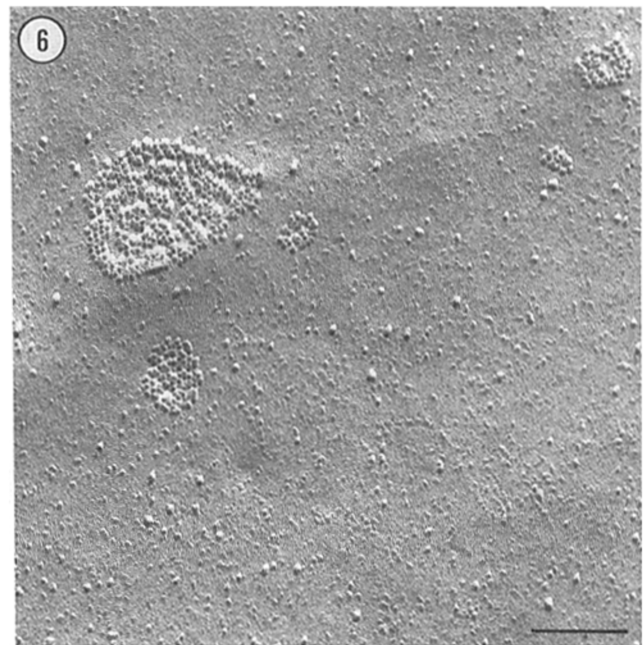


Figure 6. Gap junctions of BME cells in the wound region, 24 h after wounding. A freeze-fracture replica of this region reveals aggregates of uniformly large particles characteristic of gap junctions on the P-fracture face of BME membranes. As shown, the number of aggregated particles varies widely from one gap junction to another. Bar, 150 nm.

1987). In this study, we have investigated whether junctional communication is altered after experimental wounding of a confluent monolayer of microvascular endothelial cells and, if so, whether this alteration can be related to specific wound-induced changes in cellular function. We have found that the frequency and extent of junctional communication between BME cells is initially unaffected, but subsequently decreases and then markedly increases along the wound edge, but not in regions of the same cultures where cells remained as a nondisturbed confluent monolayer. This correlated with the presence of morphologically identifiable gap junctions between BME cells lining the wound edge, 24 h after wounding. We also found that the increased communication of BME cells returned to the lower values observed in the nonwounded regions of the monolayer when the wound had closed. An analogous sequence of injury-related uncoupling and recoupling has been observed in regenerating liver, although with different kinetics, and this has been correlated with alterations in connexin expression (Meyer et al., 1981; Dermietzel et al., 1981; Traub et al., 1989).

Experimental wounding induces a time-dependent sequence of changes in junctional communication that occur concomitantly with changes in other endothelial cell functions that are differentially controlled between cells outside the wound and at the wound edge. It was therefore important to determine whether a change in one or several of these

Figure 4. Time course of junctional communication in the wound region, as evaluated by microinjection of Lucifer Yellow into individual cells (*asterisks*). (*a* and *b*) 45 min after wounding: retention of the tracer within the injected cell indicates the absence of junctional communication; (*c* and *d*) 2 h after wounding, some injections (e.g., no. 2) revealed the absence of coupling, whereas others (e.g., no. 1) revealed the intercellular exchange of Lucifer Yellow, seen here between three coupled cells; (*e* and *f*) 6 h after wounding, cells had begun to migrate into the denuded area, as indicated by the extension of thin cytoplasmic processes into the wound (*e*). At this time, junctional communication was consistently observed in the wound region (*f*). In the case illustrated, the communication territory comprised six adjacent cells; (*g* and *h*) after 8 d, the cells had completely closed the original wound (*g*) and microinjection revealed the absence of junctional communication in this region. Bar, 50 μm.

Table II. Effect of Inhibition of Protein Synthesis and Cell Division on the Incidence of Coupling at the Wound Edge

	Injection	Scrape-loading	
		MI	SP
Control (*24 h*)	100 (n = 5)	87.5 (n = 24)	80.0 (n = 10)
Mitomycin C*	66.7 (n = 3)	87.5 (n = 24)	91.7 (n = 12)
Cycloheximide‡	ND	20.0 (n = 15)	12.5 (n = 8)
Reversibility			
Control (*48 h*)	ND	87.5 (n = 32)	100 (n = 8)
Cycloheximide§	ND	87.5 (n = 16)	87.5 (n = 8)

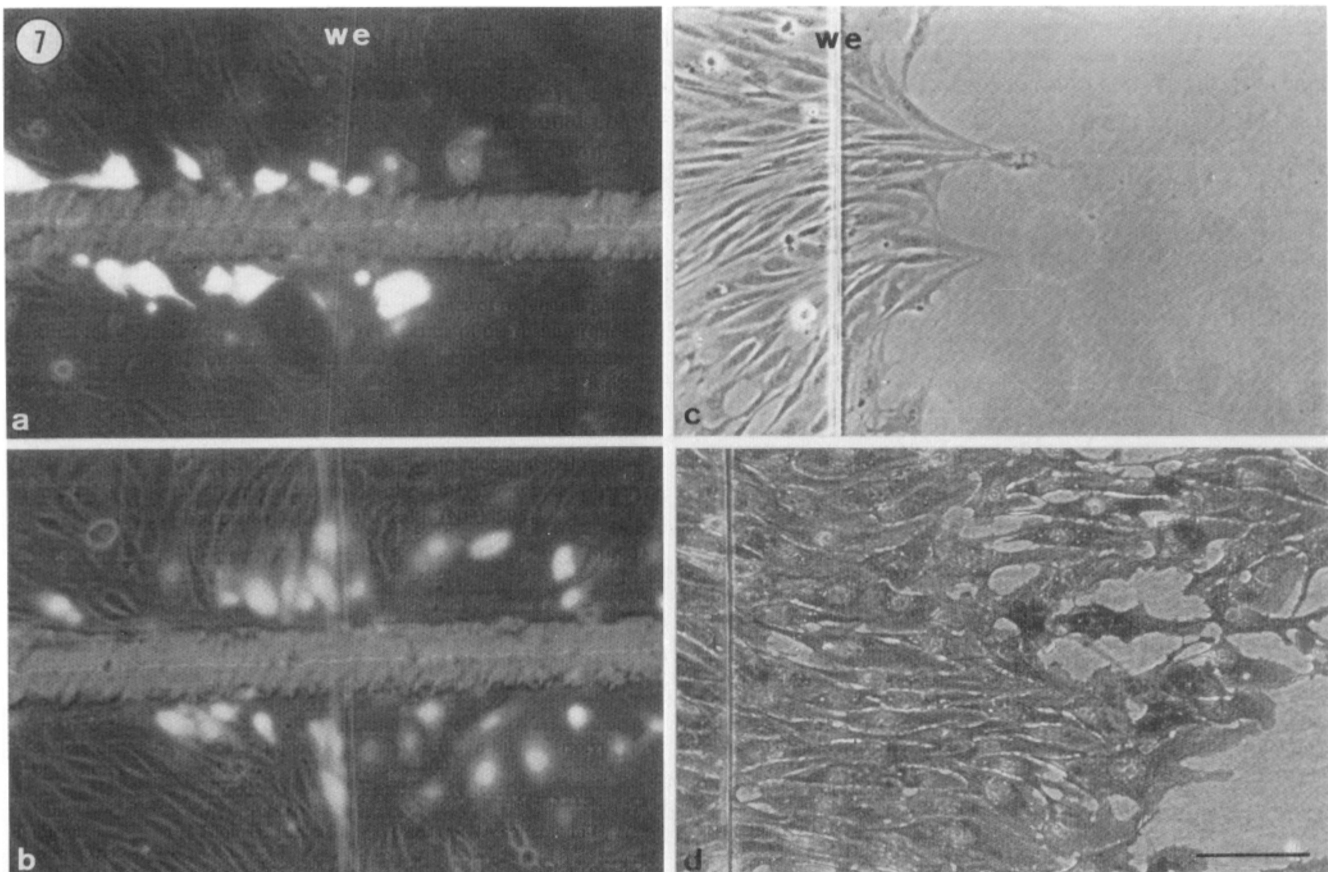
Values are percent of microinjections and scrape-loadings that revealed coupling. n, number of injections or measurements per condition; MI, microscopic quantitation; SP, spectrophotometric measurement.
* Confluent monolayers of BME cells were treated with mitomycin C (10 μg/ml) for 4 h before wounding; fresh complete medium without mitomycin C was added after wounding, and the incidence of cell-to-cell coupling determined 24 h later.
‡ Confluent monolayers of BME cells were wounded, and fresh complete medium containing cycloheximide (Sigma Chemical Co.) (0.1 μg/ml) added; the incidence of cell-to-cell coupling was determined 24 h later.
§ Confluent monolayers of BME cells were wounded, and fresh complete medium containing cycloheximide (0.1 μg/ml) added; 24 h later the monolayers were washed with PBS and fresh complete medium added; the incidence of cell-to-cell coupling was determined after a further 24-h culture.

Table III. Effect of Cell Density on the Incidence and Extent of Cell-to-Cell Dye Transfer

	Incidence			
		Scrape-loading		
	Injection	MI	SP	Extent
Sparse*	68.2 (n = 22)	50.0 (n = 24)	ND	3.9 ± 0.3 (n = 15)
Preconfluent and confluent‡	52.6 (n = 38)	56.8 (n = 44)	60.0 (n = 5)	4.0 ± 4.0 (n = 20)
Postconfluent§	21.4 (n = 14)	16.1 (n = 56)	17.9 (n = 28)	3.0 ± 1.5 (n = 3)

Values are percent of microinjections and scrape-loadings that revealed coupling ("incidence") and number of coupled cells (mean ± SEM) that received Lucifer Yellow per injection ("extent").
BME cells were seeded at different densities and the incidence and extent of cell-to-cell dye transfer determined by microinjection or scrape loading. n, number of injections or measurements per condition. MI, microscopic quantitation; SP, spectrophotometric measurement.
* Cells seeded at 5 × 10^4 cells/35-mm culture dish, and allowed to attach and spread overnight.
‡ Cells seeded at 1 × 10^5 cells/35-mm culture dish and allowed to attach and spread overnight (preconfluent) or grown to confluence (2–3 d).
§ Cells seeded at 1 × 10^5 cells/35-mm culture dish and grown to postconfluence (4–5 d).

functions could account for the regional difference in coupling. Firstly, although cell density is relatively homogeneous throughout a nonwounded culture, endothelial cells that migrate into a wound are moving from a high- to a low-density state, where cell-to-cell contact is continuously maintained (Schwartz et al., 1978; Thorgeirsson et al., 1979; Gotlieb and Spector, 1981; Ryan et al., 1982). Thus, cell density is significantly reduced in several rows of cells at the wound edge when compared with those located outside the wound.



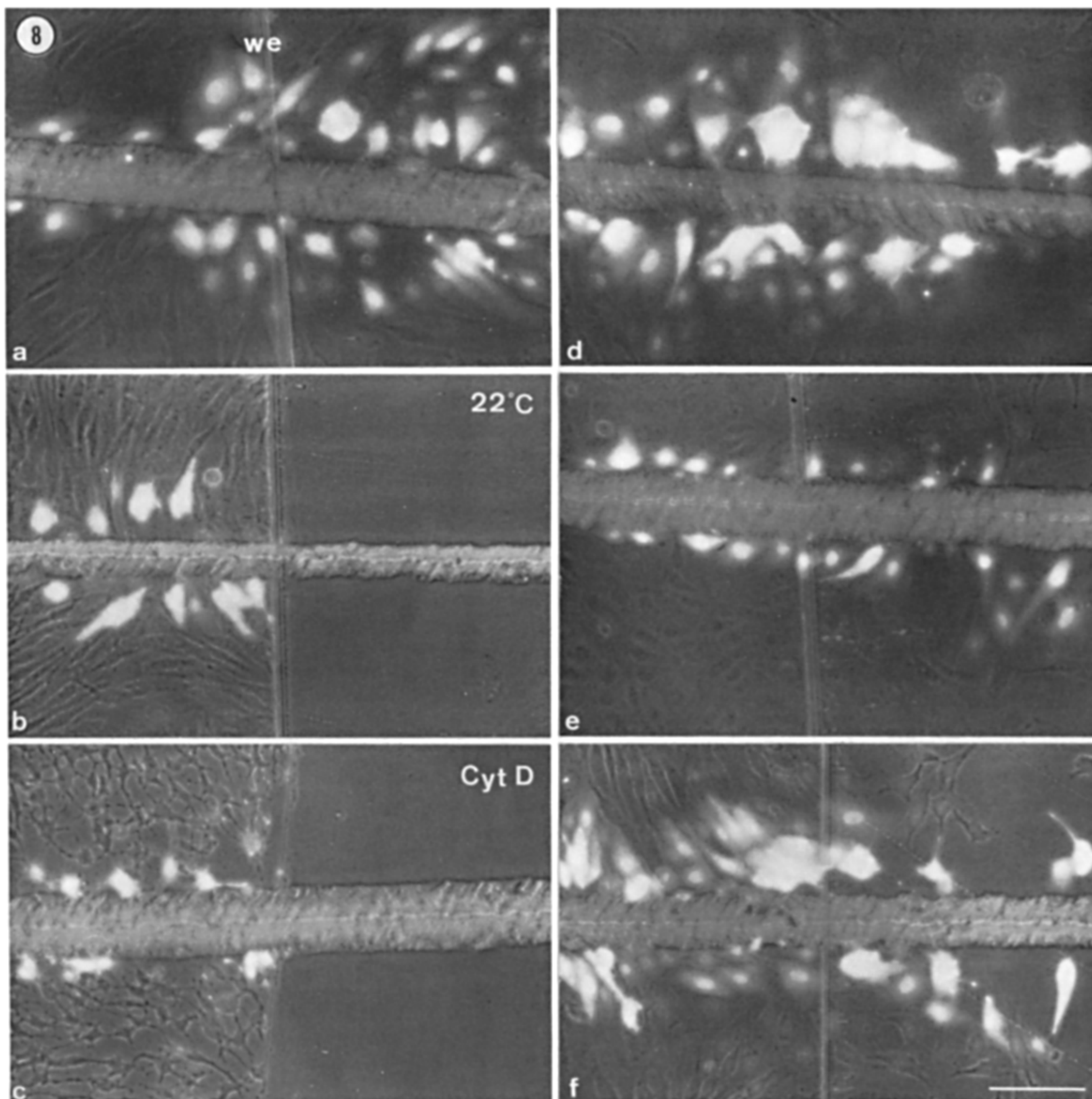


Figure 8. Effect of inhibition of cell migration on the coupling of BME cells, as evaluated by scrape-loading. In control cultures incubated at 37°C for 24 h after wounding (*a*), coupling was increased between the BME cells that had migrated across the original wound edge (*we*). In contrast, coupling and cell migration were not observed in the wound region of cultures that were placed at 22°C immediately after wounding (*b*) or that were incubated at 37°C in the presence of cytochalasin D (*c*). The inhibition of migration and coupling observed under the two latter conditions were reversible after returning of the cultures to 37°C (*e*) or removal of cytochalasin D (*f*), respectively. Thus, after a further 24 h (i.e., 48 h after wounding), cells that had previously been exposed to either 22°C (*e*) or to cytochalasin D (*f*) had migrated from the original wound edge and had established communication, as observed in control cultures processed in parallel (*d*). Bar, 100 μm.

Figure 7. Effect of cycloheximide on communication and cell migration in wounded BME monolayers. (*a*) Combined phase contrast and fluorescent micrographs show that addition of 0.1 μg/ml cycloheximide to BME monolayers for 24 h after wounding inhibited the increase in communication typically seen in the wound area of control cultures (*b*). Cell migration was also inhibited after cycloheximide treatment (*a* and *c*), under which condition the uptake of [^{3}H]leucine by BME cells was markedly inhibited (*c*) when compared with controls (*d*). (*c* and *d*) are phase-contrast views of monolayers processed for autoradiography after a 90-min incubation with [^{3}H]leucine (0.4 mCi/ml; 69 Ci/mmol; Amersham, Buckinghamshire, UK) commencing 22 h after wounding. Cells which have incorporated [^{3}H]leucine into newly synthesized proteins are labeled by dark autoradiographic grains. *we* is the original wound edge. Bar, 100 μm in *a* and *b*, and 50 μm in *c* and *d*.

Table IV. Effect of Inhibition of Cell Migration on the Incidence of Coupling at the Wound Edge

	Injection	Scrape loading	
		MI	SP
Control (*24 h*)	75.0 (n = 4)	86.6 (n = 67)	78.9 (n = 38)
Cytochalasin D*	0 (n = 2)	24.3 (n = 37)	14.3 (n = 7)
22°C (*24 h*)‡	0 (n = 5)	8.0 (n = 25)	18.7 (n = 16)
Reversibility			
Control (*48 h*)	ND	65.9 (n = 44)	68.7 (n = 16)
Cytochalasin D§	ND	82.5 (n = 63)	87.5 (n = 24)
22°C (*24 h*) + 37°C (*24 h*)‖	ND	80.0 (n = 20)	50.0 (n = 4)

Values are percent of microinjections and scrape-loadings that reveal coupling. n, number of injections or measurements per condition; MI, microscopic quantitation; SP, spectrophotometric measurement.
* Confluent monolayers of BME cells were wounded, and fresh complete medium containing cytochalasin D (Sigma Chemical Co.) (0.5–1.0 μg/ml) added; the incidence of cell-to-cell coupling was determined 24 h later.
‡ Confluent monolayers of BME cells were wounded, and fresh complete medium containing 50 mM Hepes (pH 7.4) was added, and the cells placed at 22°C; the incidence of cell-to-cell coupling was determined 24 h later.
§ Confluent monolayers of BME cells were wounded, and fresh complete medium containing cytochalasin D (0.5–1.0 μg/ml) added; 24 h later the monolayers were washed and fresh complete medium added; the incidence of cell-to-cell coupling was determined after a further 24 hour culture.
‖ Confluent monolayers of BME cells were wounded, fresh complete medium containing 50 mM Hepes pH 7.4 was added, and the cells placed at 22°C; 24 h later the cells were transferred to 37°C, and the incidence of cell-to-cell coupling determined after a further 24 h.

Since junctional communication is cell density dependent in some systems (Flagg-Newton and Loewenstein, 1981), it is conceivable that a change in cell density could account for regional differences in communication within a wounded monolayer of BME cells. To address this issue, we tested the effects of cell density on intercellular communication in our cells. In sparse and preconfluent cultures that mimicked the cell density found at the wound edge, the incidence and extent of coupling were not increased above the control values of confluent monolayers. In addition, these values were lower than the incidence and extent of coupling observed at the wound edge. Our findings suggest therefore that the increase in junctional communication observed at the wound edge may not be accounted for solely by the decrease in cell density that occurs in this region. It is possible that geometric factors such as cell thickness, which may differ between low-density cultures and cells at the wound edge, could contribute to the density- versus wound-related differences in communication that we have observed. Differences in cell density might explain the marked reduction in coupling observed in postconfluent cultures, as well as the decrease in coupling seen with time, outside the wound.

Secondly, wounding has been shown to induce cell division of endothelial cells lining the wound edge (Sholley et al., 1977; Ryan et al., 1982). We therefore considered the possibility that enhanced junctional communication may be related to increased proliferation. However, the observation that a complete and sustained block of BME mitosis by mitomycin C (Pepper et al., 1987) did not prevent the increase in junctional communication at the wound edge suggests that junctional communication and BME cell proliferation are controlled independently.

The most striking difference between cells located at the wound edge and those located at distance from it is that whereas the former are induced to migrate the latter are not. Endothelial cell migration occurs in a way that continuously preserves cell-to-cell contacts, even though these contacts may be continuously remodeled between the actively moving cells. Therefore, we addressed the question of whether the increased cell-to-cell communication in the wound region may be related to, or perhaps dependent on, BME migration. We found that either incubation at 22°C or treatment with cytochalasin D, two conditions which have been previously demonstrated to inhibit large vessel endothelial cell migration (Selden et al., 1981), inhibited BME migration after wounding, and prevented the increase in junctional communication normally observed at the wound edge. These observations suggest that wounding alone is insufficient to induce an increase in communication, and that this increase is therefore dependent on cells migrating into the wound.

Retinoic acid (10^{-4} M) is known to block junctional communication in some systems (Walder and Lutzelschwab, 1984; Pitts et al., 1986). This drug was therefore used to explore whether cell migration might be affected by blocking junctional communication. We observed that a retinoic acid–induced block in communication did not inhibit BME cell migration. However, a significantly greater percentage of cells among those that did migrate across the wound edge, demonstrated reduced contact with the migrating sheet when compared to controls. These results therefore indicate that inhibition of communication does not inhibit cell migration per se. It remains to be established whether inhibition of communication and disruption of the coordinated sheet-like pattern of migration are causally related events.

Table V. Effects of Retinoic Acid on Migration and Cell-to-Cell Dye Transfer at the Wound Edge

	Dye coupling		Migration	
	MI	SP	Cell no.	Separate cells
				%
Control (*24 h*)	87.1 (n = 31)	69.2 (n = 13)	287.2 ± 34.0 (n = 12)	11.6 ± 1.19 (n = 12)
Retinoic acid	4.8 (n = 21)	18.7 (n = 16)	152.7 ± 12.1 (n = 12)	27.3 ± 4.80 (n = 12)
Retinoic acid reversibility	93.7 (n = 16)	42.8 (n = 7)	436.6 ± 11.9 (n = 12)	14.7 ± 1.20 (n = 12)

Values are percent of scrape loadings that revealed coupling ("dye coupling") and the number of migrating cells (mean ± SEM) per field ("migration"). n = number of injections/measurements (for coupling) or photographs analysed (for migration). MI = microscopic quantitation; SP, spectrophotometric measurement.

Confluent monolayers were wounded, the medium was removed, and fresh complete medium containing all-trans retinoic acid (10^{-4} M) was added. After 24 h, the medium was removed, the monolayers were washed, fresh complete medium added and the cells incubated for a further 24 h (= reversibility).

The incidence of cell-to-cell dye transfer was assessed after 24 and 48 h by scrape loading. In each experiment (n = 2), migration was assessed after 24 and 48 h in 6 randomly selected 1.8 × 1.2 mm photographic fields in which the wound edge was perpendicular to the long axis of the field.

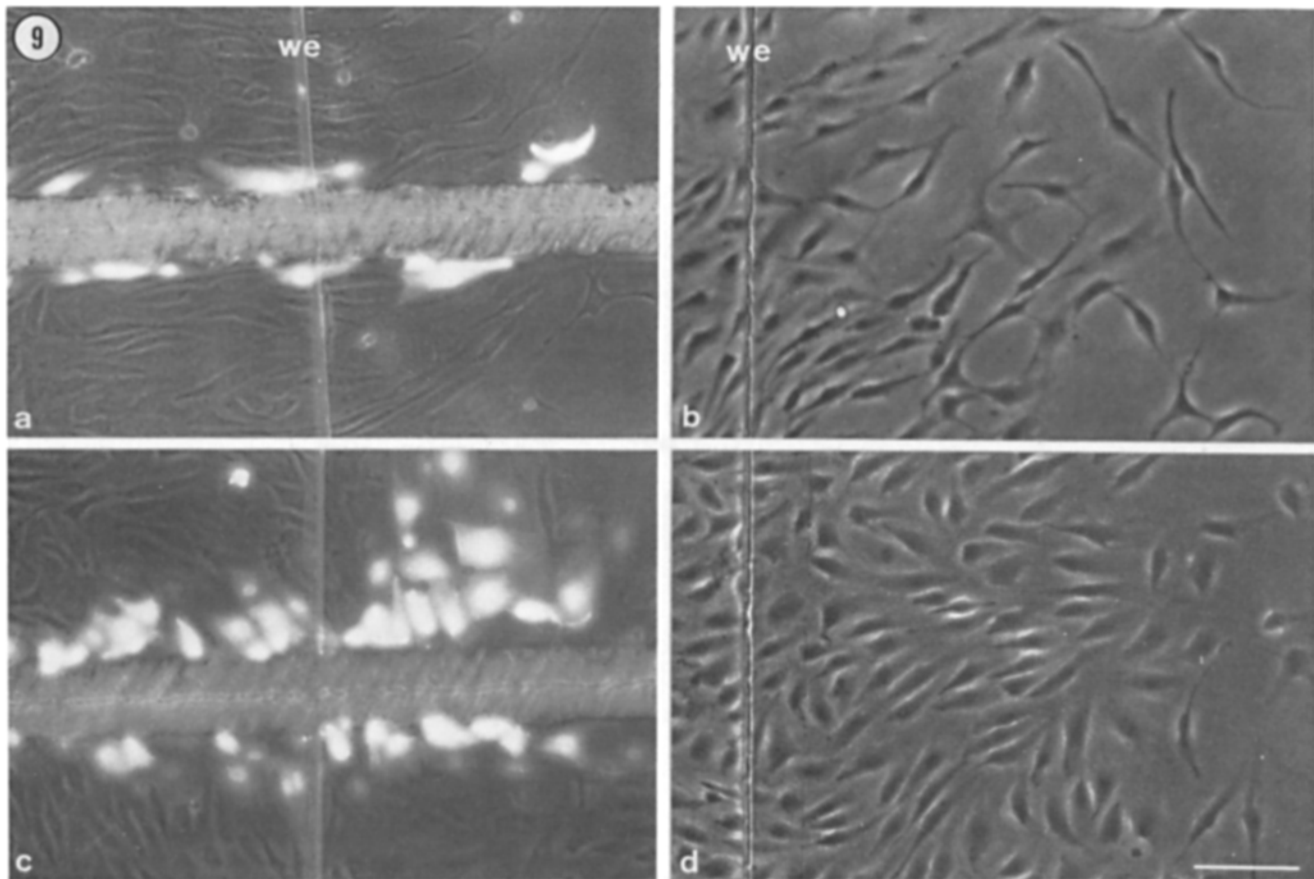


Figure 9. Effects of retinoic acid on the migration and communication of BME cells. (*a*) Addition of 10^{-4} M all-*trans* retinoic acid for 24 h after wounding blocked the dye transfer that was typically seen between cells in the wound region of control cultures (*c*). The sheet-like pattern of cell migration typically seen during endothelial repair (*d*), was also disrupted in cultures treated with retinoic acid for 24 h after wounding (*b*). *we* is the original wound edge. Bar, 100 μm.

Finally, our findings with capillary endothelial cells are at variance with observations on large vessel endothelial cells after wounding. Larson and Haudenschild (1988) have recently reported a small but lasting reduction in junctional communication between bovine aortic endothelial (BAE) cells at the wound edge. In agreement with these findings, we have recently observed in BAE cultures scrape loaded 24 h after wounding, that coupling between cells at the wound edge is reduced when compared with coupling between cells in the remaining quiescent monolayer (unpublished). The reasons for the major difference in the regulation of coupling between capillary and aortic endothelial cells are at present unclear, but may reflect inherent functional differences between the two cell types studied, i.e., cloned adrenal capillary (our work) versus early passage aortic endothelial cells (Larson and Haudenschild, 1988). It also remains to be determined whether these differences are significant in vivo.

In summary, a number of experimental observations point to coordinated migration as the endothelial cell function induced by wounding that, in our system, correlates best with the concomitant regional increase in junctional communication. Whether the process of migration is the only factor responsible for this increase in communication is at present unclear. Because the region of increased communication clearly extends over several cell rows at the wound edge, junctional-mediated transfer of signals may well represent a mechanism whereby endothelial cells along the leading front could communicate both with adjacent cells as well as with cells situated further behind. Our observations are therefore compatible with the previous suggestion that communication may be required for the coordinated movement of endothelial cells (Gotlieb et al., 1987; Larson and Haudenschild, 1988), which is necessary for reendothelialization of a denuded wound area, and for capillary sprouting during angiogenesis.

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References

Abercrombie, M., J. E. M. Heaysman, and S. M. Pegrum. 1970. The locomotion of fibroblasts in culture: I. Movements of the leading edge. *Exp. Cell. Res.* 59:393–398.

Ausprunk, D. H., and J. Folkman. 1977. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor

angiogenesis. *Microvasc. Res.* 14:53–65.

Bennett, M. L. V., and D. C. Spray. 1985. Gap Junctions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Beyer, E. C., D. A. Goodenough, and D. L. Paul. 1988. The connexins, a family of related gap junction proteins. *Mod. Cell. Biol.* 7:167–175.

Beyer, E. C., D. L. Paul, and D. A. Goodenough. 1987. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* 105:2621–2629.

Buck, R. C., and H. T. Malczak. 1977. Regeneration of endothelium in rat aorta after local freezing. *Am. J. Pathol.* 86:133–148.

Bürk, R. R. 1973. A factor from a transformed cell line that affects cell migration. *Proc. Natl. Acad. Sci. USA.* 70:369–372.

Chanson, M., R. Bruzzone, D. Bosco, and P. Meda. 1989. Secretion and junctional coupling of pancreatic acini: parallel changes revealed by N-alcohols. *J. Cell. Physiol.* 139:147–156.

Dermietzel, R., S. B. Yancey, O. Traub, K. Willecke, and J.-P. Revel. 1981. Major loss of the 28-kD protein of gap junction in proliferating hepatocytes. *J. Cell Biol.* 105:1925–1934.

El-Fouly, M. H., J. E. Trosko, and C.-C. Chang. 1987. Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell Res.* 168:422–430.

Fishman, J. A., G. B. Ryan, and M. J. Karnovsky. 1975. Endothelial regeneration in the rat carotid artery and the significance of endothelial denudation in the pathogenesis of myointimal thickening. *Lab. Invest.* 32:339–351.

Flagg-Newton, J. L., and W. R. Loewenstein. 1981. Cell junction and cyclic AMP: II. Modulations of junctional membrane permeability, dependent on serum and cell density. *J. Membr. Biol.* 63:123–131.

Furie, M. B., E. B. Cramer, B. L. Naprstek, and S. C. Silverstein. 1984. Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electrical current. *J. Cell Biol.* 98:1033–1041.

Gilula, N. B., O. R. Reeves, and A. Steinbach. 1972. Metabolic coupling, ionic coupling and cell contacts. *Nature (Lond.).* 235:262–265.

Gotlieb, A. I., and W. Spector. 1981. Migration into an in vitro experimental wound. A comparison of porcine aortic endothelial and smooth muscle cells and the effect of culture irradiation. *Am. J. Pathol.* 103:271–282.

Gotlieb, A. I., M. K. K. Wong, P. Boden, and A. C. Fone. 1987. The role of the cytoskeleton in endothelial repair. *Scanning Electron Microsc.* 1:1715–1726.

Larson, D. M., M. P. Carson, and C. C. Haudenschild. 1987. Junctional transfer of small molecules in cultured bovine brain microvascular endothelial cells and pericytes. *Microvasc. Res.* 34:184–199.

Larson, D. M., and C. C. Haudenschild. 1988. Junctional transfer in wounded cultures of bovine aortic endothelial cells. *Lab. Invest.* 59:373–379.

Larson, D. M., and J. D. Sheridan. 1982. Intercellular junctions and transfer of small molecules in primary vascular endothelial cells. *J. Cell Biol.* 92:183–191.

Loewenstein, W. R. 1968. Communication through cell junctions. Implications in growth control and differentiation. *Dev. Biol.* 190(Suppl. 2):151–183.

Loewenstein, W. R. 1979. Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta.* 560:1–65.

McNeil, P. L., R. F. Murray, F. Lanni, and D. L. Taylor. 1984. A method for incorporating macromolecules into adherent cells. *J. Cell Biol.* 98:1556–1564.

Meda, P., R. Bruzzone, M. Chanson, D. Bosco, and L. Orci. 1987. Gap junctional coupling mediates secretion of exocrine pancreas. *Proc. Natl. Acad. Sci. USA.* 84:4901–4904.

Meda, P., R. Bruzzone, S. Knodel, and L. Orci. 1986. Blockage of cell-to-cell communication within pancreatic acini is associated with increased basal release of amylase. *J. Cell Biol.* 103:475–484.

Meyer, D. J., S. B. Yancey, and J.-P. Revel. 1981. Intercellular communication in normal and regenerating liver: a quantitative analysis. *J. Cell Biol.* 91:505–523.

Montesano, R., and L. Orci. 1985. Tumor-promoting phorbol esters induce angiogenesis in vitro. *Cell.* 42:469–477.

Pepper, M. S., J.-D. Vassalli, R. Montesano, and L. Orci. 1987. Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. *J. Cell Biol.* 105:2535–2541.

Pitts, J. D., A. E. Hamilton, E. Kam, R. R. Burk, and J. P. Murphy. 1986. Retinoic acid inhibits junctional communication between animal cells. *Carcinogenesis.* 7:1003–1010.

Ryan, U. S., M. Absher, B. M. Olazabal, L. M. Brown, and J. W. Ryan. 1982. Proliferation of pulmonary endothelial cells: time-lapse cinematography of growth to confluence and restitution of monolayer after wounding. *Tissue Cell.* 14:637–649.

Schwartz, S. M., C. C. Haudenschild, and E. M. Eddy. 1978. Endothelial regeneration. I. Quantitative analysis of initial stages of endothelial regeneration in rat aortic intima. *Lab. Invest.* 38:568–580.

Seldon, S. C., P. S. Rabinovitch, and S. M. Schwartz. 1981. Effects of cytoskeletal disrupting agents on replication of bovine endothelium. *J. Cell. Physiol.* 108:195–211.

Seldon, S. C., and S. M. Schwartz. 1979. Cytochalasin B inhibition of endothelial proliferation at wound edges in vitro. *J. Cell Biol.* 81:348–354.

Sholley, M. M., M. A. Gimbrone, and R. S. Cotran. 1977. Cellular migration and replication in endothelial regeneration. A study using irradiated endothelial cultures. *Lab. Invest.* 36:18–25.

Simpson, I., B. Rose, and W. R. Loewenstein. 1977. Size limit of molecules permeating the junctional membrane channels. *Science (Wash. DC).* 195: 294–296.

Stewart, W. W. 1978. Functional connections between cells as revealed by dye coupling with a highly fluorescent naphthalimide tracer. *Cell.* 14:741–759.

Thorgeirsson, G., A. L. Robertson, and D. H. Cowan. 1979. Migration of human vascular endothelial and smooth muscle cells. *Lab. Invest.* 41:51–62.

Traub, O., J. Look, R. Dermietzel, F. Brümmer, D. Hülser, and K. Willecke. 1989. Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. *J. Cell Biol.* 108:1039–1058.

Walder, L., and R. Lutzelschwab. 1984. Effects of 12-O-tetradecanoylphorbol-13-acetate (TPA), retinoic acid and diazepam on intercellular communication in a monolayer of rat liver epithelial cells. *Exp. Cell Res.* 152:66–76.