Intercellular Junctions and Transfer of Small Molecules in Primary Vascular Endothelial Cultures

DAVID M. LARSON and JUDSON D. SHERIDAN

Zoology Program and Department of Anatomy, University of Minnesota, Minneapolis, Minnesota 55455. Dr. Larson's present address is the Vascular Pathophysiology Laboratory, Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115

ABSTRACT The ultrastructure of gap and tight junctions and the cell-to-cell transfer of small molecules were studied in primary cultures and freshly isolated sheets of endothelial cells from calf aortae and umbilical veins. In thin sections and in freeze-fracture replicas, the gap and tight junctions in the freshly isolated cells from both sources appeared similar to those found in the intimal endothelium. Most of the interfaces in replicas had complex arrays of multiple gap junctions either intercalated within tight junction networks or interconnected by linear particle strands. The particle density in the center of most gap junctions was noticeably reduced. In confluent monolayers, after 3–5 days in culture, gap and tight junctions were present, although reduced in complexity and apparent extent. Despite the relative simplicity of the junctions, the cell-to-cell transfer of potential changes, dye (Lucifer Yellow CH), and nucleotides was readily detectable in cultures of both endothelial cell types. The extent and rapidity of dye transfer in culture was only slightly less than that in sheets of freshly isolated cells, perhaps reflecting a reduced gap junctional area combined with an increase in cell size in vitro.

Endothelial cell cultures have become a popular model for investigations into the physiology and pathophysiology of the vascular intima (9, 24). Many of the in vitro characteristics of endothelial cells, e.g. contact inhibition, monolayer growth, Factor VIII antigen, micropinocytosis, and hormone receptors are retained in short- or long-term primary culture and, in some cases, even after multiple passages (5, 6, 7, 9, 11, 16, 24, 39). Rather little attention, however, has been given to the ability of these cells to maintain and form gap and tight junctions in vitro, even though these junctions are often especially prominent in vivo and occur in quite characteristic patterns in various regions of the vascular tree (14, 15, 38, 44, 45, 50).

Although variations in tight junction structure or distribution may indicate differences in the control of vascular permeability in vivo, such variations are unlikely to be important for most studies on endothelial functions in vitro (unless endothelial tight junctions contribute to junctional coupling [43]). Because gap junctions, however, presumably allow the intercellular transfer of small molecules (2, 8, 18, 25), differences in gap junctional size and distribution might affect any process dependent on small molecular transfer, either in vivo or in vitro. For example, it has been suggested that junctional transfer plays some role in the regulation of cell proliferation (22). If so, the suitability of endothelial cultures for studying cell proliferation would rely on maintaining structurally and functionally intact gap junctions in vitro.

We have investigated the ultrastructure and distribution of endothelial gap and tight junctions, by thin-section and freeze-fracture electron microscopy, in intact calf aortae and umbilical veins, in sheets of freshly isolated cells and in confluent primary cultures. In addition, we have tested for the functional correlates of gap junctions, the movement of ions and small molecules from cell to cell, by electrophysiological, dye transfer, and nucleotide transfer methods in confluent primary endothelial cultures. For comparison we have investigated dye and nucleotide transfer in freshly isolated sheets of cells. A brief report of this work has appeared elsewhere (20).

MATERIALS AND METHODS

Isolation and Culture of Cells

Fetal bovine umbilical cord veins (near term) and young calf aortae (thoracic to upper abdominal) were collected within 15 min of death and transported to the laboratory in cold, sterile, phosphate-buffered saline (PBS: NaCl, 136.8 mM; KCl, 2.7 mM; $CaCl_2/2H_2O$, 0.7 mM; $MgCl_2/6H_2O$, 0.5 mM; KH_2PO_4, 1.5 mM; $Na_2HPO_4/7H_2O$, 7.9 mM; pH 7.4). Sheets and clumps of endothelial cells

(effluent cells) were released from the intima by collagenase treatment (0.5 mg/ml, Sigma Chemical Co. [St. Louis, Mo.], Type IV, 30 min at 37°C in PBS) and agitation according to methods modified from Macarak et al. (23) and Booyse et al. (4). The endothelial cells were grown in 60-mm culture dishes containing Medium 199 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N.Y.) with 30% heat-inactivated calf serum (Gibco Laboratories), 4 mM L-glutamine, 4 U/ml penicillin - 5 μg/ml streptomycin (Gibco Laboratories), 2 ng/ml amphotericin B (Fungizone/Squibb and Sons, Inc., Princeton, N. J.), and 50 μg/ml gentamycin (Schering Corp., Kenilworth, N. J.) at 37°C in a humidified atmosphere of 95% air/5% CO_2. Identification of the endothelial cells was based primarily on morphological characteristics. In addition, some of the cultures were grown on glass cover slips and the identity of the endothelial cells was confirmed using indirect immunofluorescence staining (data not shown) for Factor-VIII-associated protein (Calbiochem-Behring Corp., La Jolla, Calif.; 16).

Vascular smooth muscle cells (SMC) from bovine umbilical vein were isolated by explant outgrowth methods modified from Ross (36) including a 30-min pretreatment of the explants with 1 mg/ml collagenase in PBS at 37°C. The culture conditions of SMC were the same as above, except that 10% heat-inactivated fetal calf serum (Gibco Laboratories) was used instead of the calf serum. SMC grew in typical "hill-and-valley" patterns (10).

Electron Microscopy

Ultrastructural observations were made on intact vessel segments, freshly isolated sheets, and confluent primary cultures (3–5 d after plating). For thin-section microscopy, small pieces of freshly obtained vessels, pellets of effluent cells, and dishes of cultured cells were fixed for 30 min in 2.5% glutaraldehyde in PBS, postfixed in osmium tetroxide, stained en bloc with tannic acid and uranyl acetate (19, 44), dehydrated in ethanol and embedded in Epon-Araldite. Sections were stained with uranyl acetate and lead citrate and examined in a Phillips 201 electron microscope operated at 60 kV.

For freeze-fracture, intact vessel pieces, effluent cells, and cultures were fixed in 2.5% glutaraldehyde in PBS as described above and then stored at 4°C for 1–6 d (the length of time in storage had no effect on junctional structure). After fixation, the pellets and cultures were washed with cold PBS and treated with 30% glycerol in PBS at 4°C for 2 h. Some of the cultures were gently scraped from the dishes with a rubber policeman and pelleted. The small pieces of intact vessel and the pellets of effluent and cultured cells were frozen in liquid Freon, fractured, and replicated by standard techniques in either a Balzers 301 (with a knife) or Balzers 360M (with a double-replica device). Other cultures were loosened from the dishes by less vigorous scraping with the rubber policeman. The resulting large sheets of cells, along with a small amount of 30% glycerol, were placed between two flat-top specimen holders, frozen in liquid Freon, and fractured using the double-replica device. This technique for fracturing the cultured cells gave substantially larger membrane faces and requred less initial material. In all cases, fracturing was carried out at −105°C with no etching. The replicas were examined in either an AEI 801 microscope or Philips 201 microscope operated at 60 kV.

Junctional Transfer

ELECTROTONIC TRANSFER: Three- to five-day-old confluent primary cultures of endothelial cells were tested electrophysiologically (18, 46) for their ability to transfer small ions. Experiments were carried out at room temperature (20°C) and atmosphere in standard growth medium and were visually monitored by inverted phase microscopy. Current-passing and voltage-recording electrodes were glass micropipets (Omega Dot/F. Haer) filled with 3 M KCl/0.1 M K-acetate and connected to the amplifier circuit (M4A; W-P Instruments, New Haven, Conn.) by Ag/AgCl wires. The microelectrodes had tip resistances of 5–8 × 10^7 ohms, in medium, against the Ag/AgCl bath electrode. Current was injected in 1–5 × 10^{-9} A rectangular hyperpolarizing pulses of 100–300 ms duration. Oscilloscope tracings were recorded on linagraph film (Kodak RAR 2495), and phase micrographs were taken of each penetration.

DYE TRANSFER: Freshly isolated cells and 3–5-day-old confluent primary endothelial cultures were tested (2, 41) for their ability to transfer the fluorescent dye, Lucifer Yellow CH (mol wt 457.3; generously supplied by W. Stewart; 47). Cultures and effluent cells were washed twice with PBS to reduce the background fluorescence of the medium. Experiments were performed in PBS at room temperature. Omega Dot glass microelectrodes were filled from tip to shoulder with Lucifer Yellow (4% in distilled water) and in the barrel with 1 M LiCl by the fiber fill method (48). Iontophoretic injection of the dye was carried out by rectangular, hyperpolarizing current pulses (5–10 × 10^{-9} A, 200 ms duration, 1/s) for 1–5 min. The injection and spread of dye were monitored by darkfield fluorescence microscopy (Leitz Labolux II; BG12/K510). Photographs were taken on Tri-X film (10 s to 1 min exposure) for permanent records.

NUCLEOTIDE TRANSFER: Uridine-nucleotide transfer experiments were conducted using the general techniques of Pitts and Simms (29). For "effluent recipient cell" experiments, freshly isolated sheets were plated out at a low density (10^2–10^3 cells/well) on 4-chamber Lab-Tek slides (Lab-Tek Products, Naperville, Ill.), incubated overnight in standard growth medium, and then prelabeled for 4–6 h with 10–15 μCi/ml of [^{3}H]-5-uridine (New England Nuclear, Boston, Mass.) in medium. After thorough washing with PBS to remove extracellular label, recipient effluent cells were added in a 10- to 100-fold excess. After 12–18 h of incubation, the cocultures were washed with PBS, fixed with cold 5% trichloroacetic acid, and processed for autoradiography with NTB-2 nuclear track emulsion (Kodak). After ~1 wk at 4°C, the slides were developed in D-19 (Kodak), fixed, stained with Harris' hematoxylin, dehydrated, and cover slipped with Permount (Fisher Scientific Co., Pittsburgh, Pa.).

In addition, uridine-nucleotide transfer experiments were conducted using confluent monolayers of primary endothelial cells (3–5 d in culture) as recipients and subcultured vascular smooth muscle cells as donors ("trypsinized-donor" technique). The endothelial recipients were cultured for 3–5 d in 35-mm plastic culture dishes (Falcon Labware, Oxnard, Calif.). SMC were loaded with 15 μCi/ml of [^{3}H]-5-Uridine for 4 h. These cells were then thoroughly washed with PBS, gently dispersed with trypsin EDTA (0.05% trypsin - Gibco Laboratories, 1:250; 0.02% EDTA-Fisher Scientific Co.), counted, and plated on the endothelial recipient monolayers at ratios of 1:1000 to 1:5000 (donors:recipients). After 4–6 h of incubation in endothelial growth medium, the cocultures were washed thoroughly with PBS, fixed, and processed for autoradiography as above. After development, the cultures were stained with Safranin O and cover slipped using glycerin jelly.

In control experiments, mouse L-929 fibroblasts were used as recipient or donor cells. These cells do not usually form gap junctions (8) and are a standard control for nonjunctional transfer (28). In addition, BHK-C13 fibroblasts, known to form gap junctions and participate in junctional transfer (19, 28), were used as positive controls.

RESULTS

Morphology

The freshly isolated cells consisted mainly of sheets of up to several hundred rounded cells (Fig. 1 *a*). After 2 h in culture,

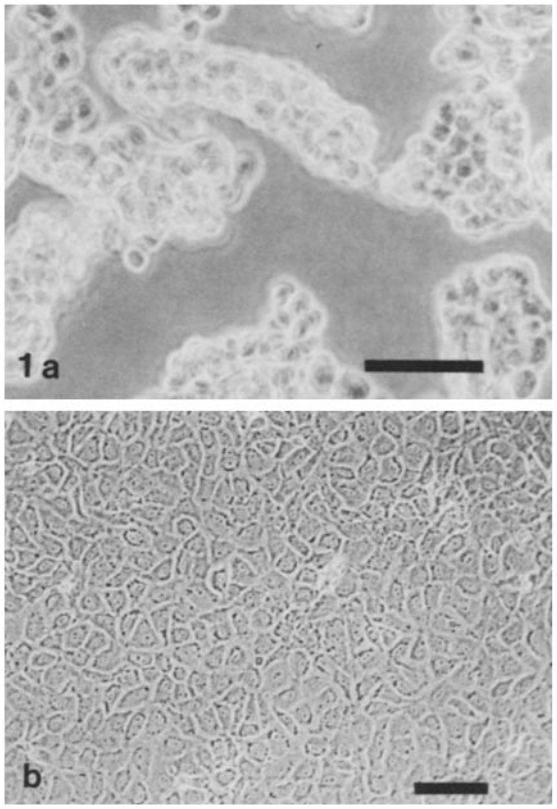

FIGURE 1 Cell identification and general morphology. (*a*) Phase-contrast micrograph of umbilical vein effluent cells. (*b*) Phase-contrast micrograph of confluent aortic endothelial primary culture. Bars, 50 μm.

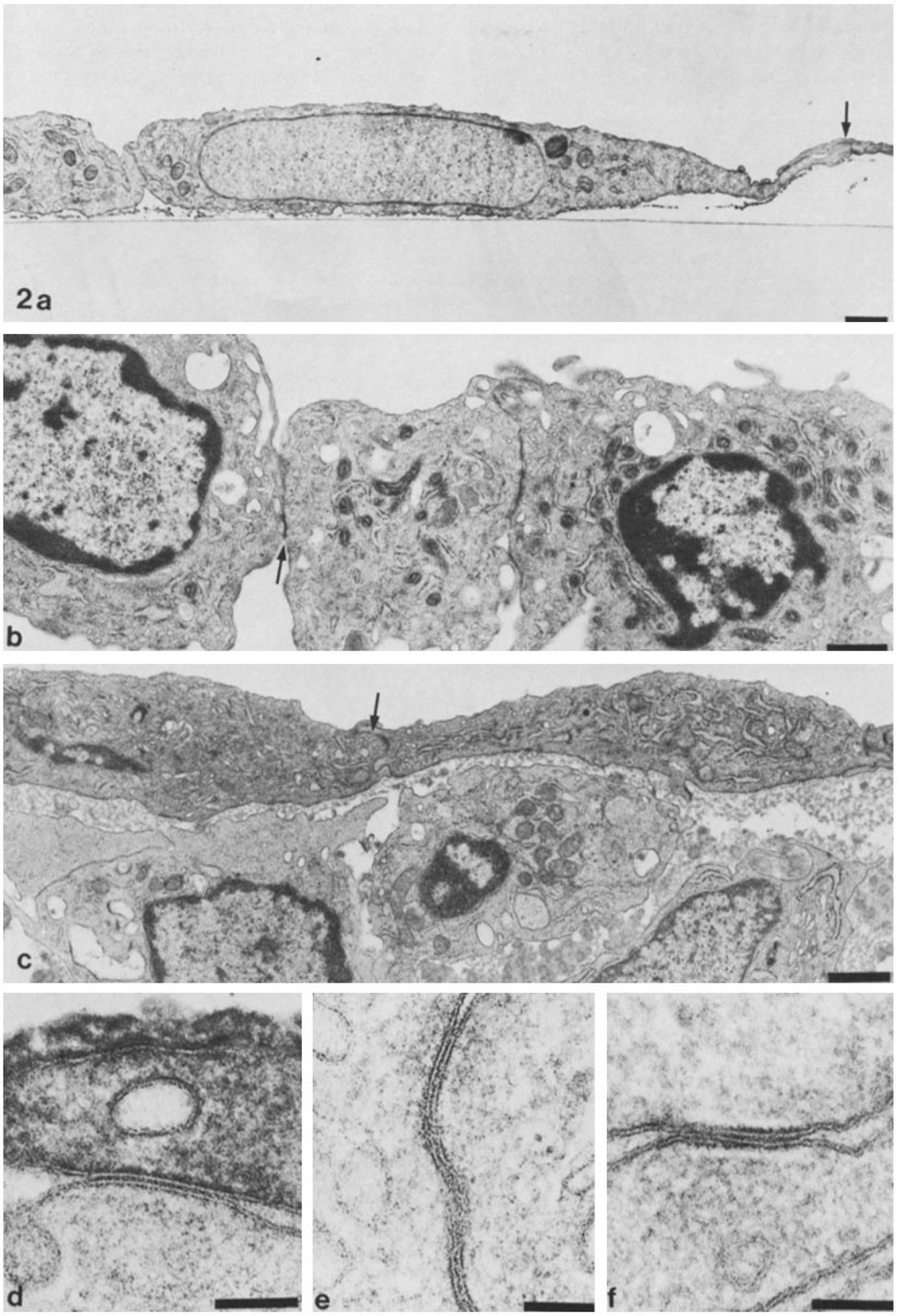

FIGURE 2 Thin section of aortic endothelial cells. (*a*) Cultured cells. Note that the cells are thin, especially away from the nucleus. Nucleus has uniform chromatin. Interfaces with occasional junctions often involve overlapping of thin processes (arrow). Bar, 1 μm. (*b*) Effluent cells showing peripheral clumping of nuclear chromatin, swollen cristae, and dense matrix in mitochondria, numerous micropinocytotic vesicles, and groups of intermediate filaments. Each interface (arrow) typically has a junctional complex. Bar, 1 μm. (*c*) Intact aortic segments. The upper endothelial layer is separated from underlying smooth muscle cells by a thin basal lamina and diffuse extracellular material. Arrow indicates interface between adjacent cells. Each interface typically has a junctional complex. Bar, 1 μm. (*d–f*) Gap junctions between intimal, freshly isolated, and cultured endothelial cells, respectively. Bars, 0.1 μm.

most of these sheets had settled and attached to the plastic substrate. Over the next 3- to 5-day culture period, the sheets expanded by migration and cell division to form a confluent monolayer with the "cobblestone" pattern (Fig. 1*b*) characteristic of other endothelial culture systems (4, 11, 13, 37, 39).

In thin section, the cultured aortic (Fig. 2*a*) and venous (not shown) cells resembled other endothelial cells in vitro (13, 37) and, except for shape variations, they were similar to the corresponding cells in freshly released sheets (Fig. 2*b*) or in the intact endothelium (Fig. 2*c*). Gap junctions were quite

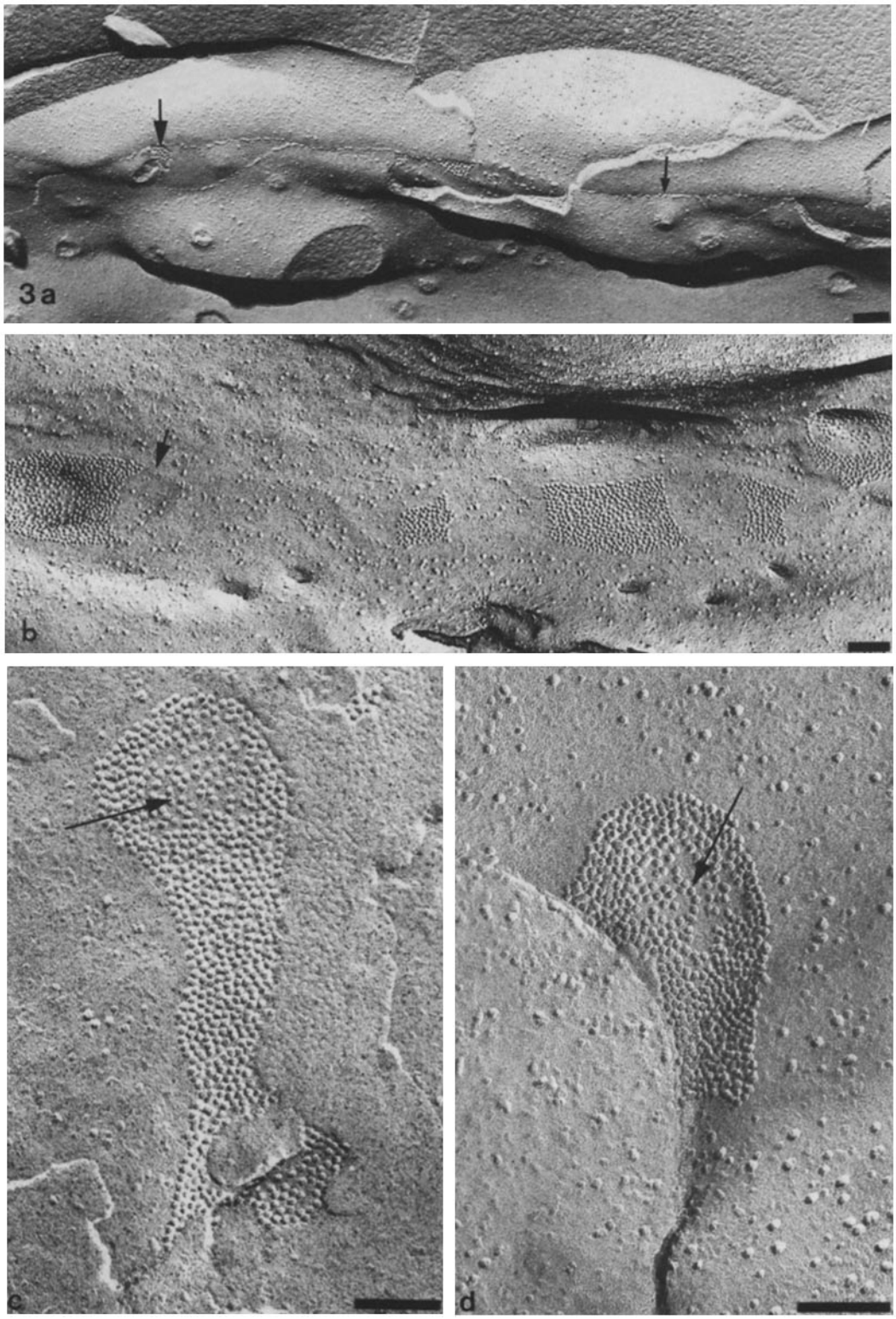

FIGURE 3 Freeze-fracture of aortic endothelial cells. (*a*) Cultured cells. Gap junctions (large arrow) interconnected by tight junctions having typical E-face grooves with particles (small arrow). (*b*) Freshly isolated cells. Note P-face tight junction ridge (arrow) that is part of network interconnecting gap junctions. (*c* and *d*) Effluent and cultured cells, respectively. Isolated gap junctions have central decrease in particle density (arrows). Bars, 0.1 μm.

common in thin sections of intact vessels (Fig. 2*d*) and freshly isolated cells (Fig. 2*e*), and apparently less common in cultures (Fig. 2*f*). Areas resembling tight junctions were also seen (not shown), but the orientation of the sections was rarely suitable for clear identification.

In freeze-fracture, gap junctions between cultured cells (Fig. 3*a*, *d* and Fig. 4*a*) and between effluent cells (Fig. 3*b*, *c* and Fig. 4*b*) were similar to those between endothelial cells in situ (Fig. 4*c*; also 14, 15, 44, 45, 50). In contrast to previous reports, there was a striking decrease in particle density in the centers of most gap junctions in effluent cells (Fig. 3*a*, *c*), in culture (Fig. 3*d*), and in intact endothelium (not shown).

Tight junctions were often seen with the gap junctions and showed the characteristic fracture pattern previously reported for other endothelia (44, 45, 50). The E-face grooves were filled with numerous particles, spaced irregularly, while the P-face

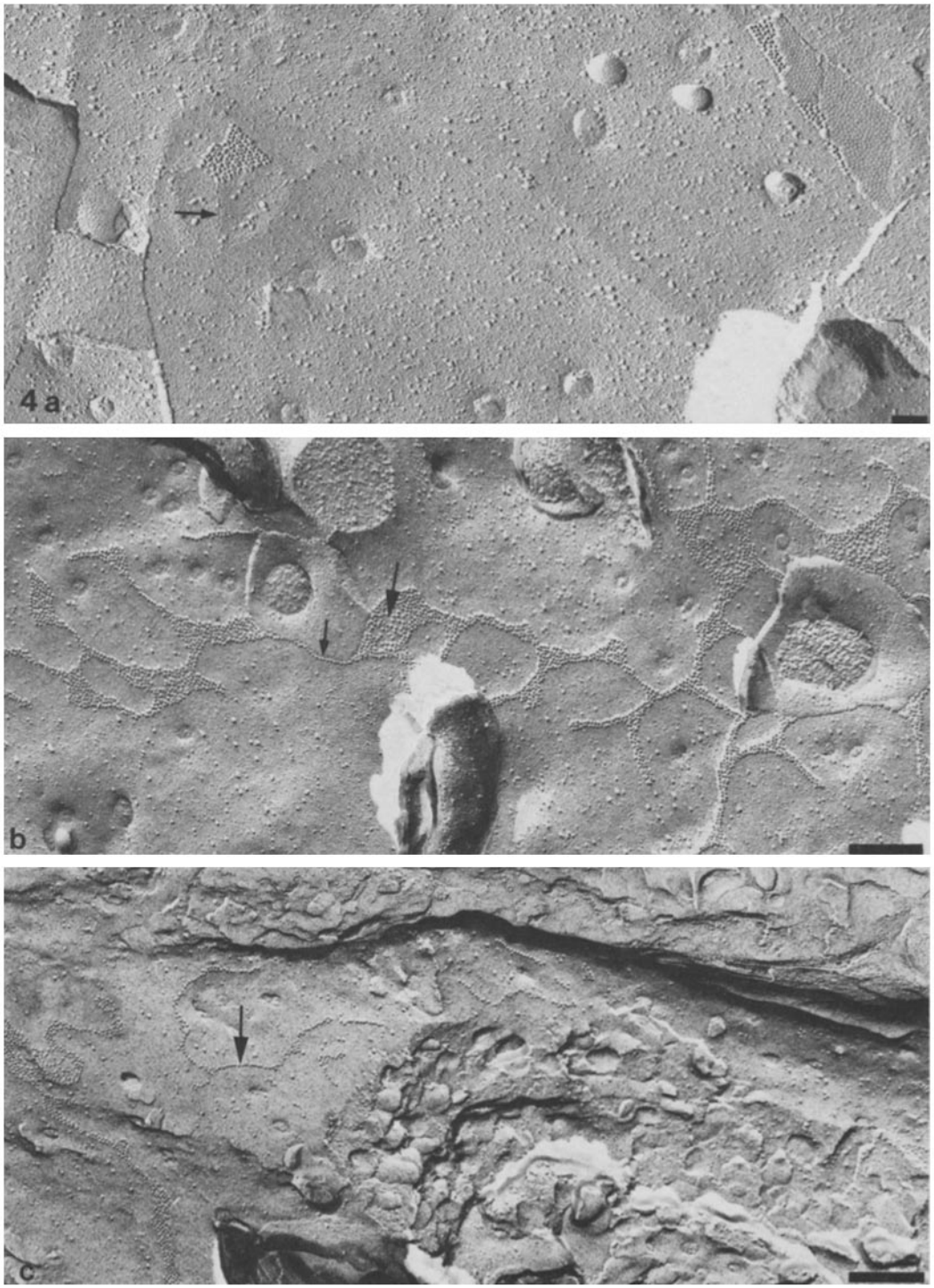

FIGURE 4 Freeze-fracture of umbilical vein endothelial cells. (*a*) Cultured cells showing less complex arrangement of tight junctions and gap junctions. The arrow indicates a fine groove alone a P-face ridge. The gap junctions here show a more crystalline array of particles than usual. Bar, 0.1 μm. (*b*) Freshly isolated cells showing extensive network of particle strands (small arrow) and interconnected gap junctions. Large arrow indicates region of (arrow) associated with gap junction particle aggregates. Bar, 0.2 μm. (*c*) Intact vessel segment showing P-face particle strands (arrow) and gap junction particle aggregates. Bar, 0.2 μm.

ridges often had fine grooves with few attached particles (Fig. 3 *a*, *b*). In many of the preparations of effluent cells and intact vessel pieces, instead of tight junctions, there were single, double, or triple strands of particles linking macular gap junctions (Fig. 4 *b*, *c*).

In cultures, the gap junctions were generally smaller and less complex. A rare example of a gap-tight array is shown in Fig. 3 *a*. More often the gap junctions occurred without associated tight junctions (Fig. 3 *d*) or where the tight junction was quite simple (Fig. 4 *a*). Tight junctions were also seen without associated gap junctions (not shown). The relative frequencies with which the various junctional arrangements occurred in freshly isolated sheets and cultures are tabulated in Table I. The similarity of the aortic and venous cells and the dissimilarity of effluent cells and cultures are especially striking.

Junctional Transfer

ELECTROTONIC TRANSFER: Experiments to test for electrotonic transfer were carried out using two electrodes placed in the monolayer. In a typical experiment, the current electrode was left in one cell and the voltage recording electrode was

TABLE I

Junctional Complexity in Freeze-fracture Replicas from Effluent and Cultured Endothelial Cells

	Aortic				Venous			
	Effluent		Cultured		Effluent		Cultured	
	Faces*	% Total	Faces	% Total	Faces	% Total	Faces	% Total
Gap only‡	20	45	39	70	30	51	25	60
Simple	10	23	38	68	10	18	22	53
Complex	10	23	1	2	20	33	3	7
Tight only§	5	11	12	21	1	2	8	19
Simple	4	9	9	16	1	2	3	7
Complex	1	2	3	5	0	0	5	12
Combination‖	19	43	5	9	28	50	9	21
Complex gap	9	20	0	0	14	25	0	0
Complex tight	9	20	1	2	15	27	1	2

* The faces were classified by the most complex junction seen.

‡ Faces having only gap junctions were termed either "simple" if the particle aggregates were isolated from each other and few in number or "complex" if there were multiple aggregates interconnected by linear particle strands.

§ Faces having only tight junctions were termed either "simple," if there were two or fewer parallel tight junction strands, or "complex," if there were three or more strands.

‖ "Combination" refers to faces with both gap and tight junctions. The gap and tight junctions on each face were classified separately. "Complex" tight junctions were defined as described above according to the number of strands. "Complex" gap junctions, however, were defined somewhat differently than above and consisted of multiple particle aggregates intercalated within a tight junction network. In each case, faces with "simple" junctions can be easily derived using the total number of "combination" faces. For simplicity, the faces with particular combinations of "simple" and/or "complex" junctions are not listed; generally faces having complex tight junctions also had complex gap junctions.

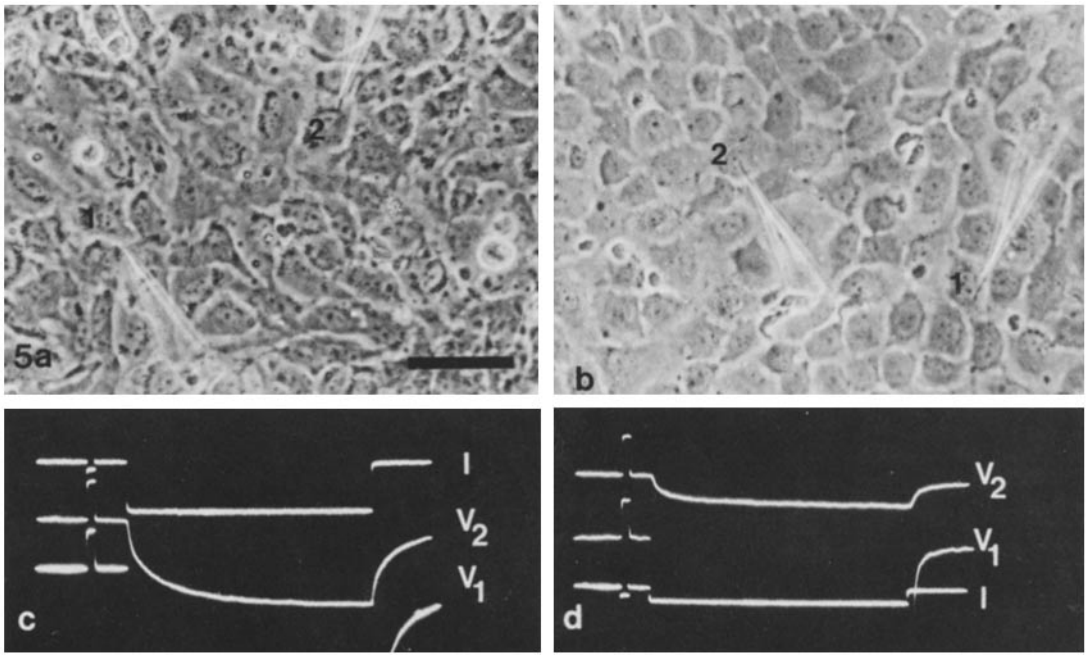

FIGURE 5 Electrotonic transfer. (*a* and *b*) Phase-contrast micrographs of aortic and umbilical vein cultured cells (4 d) with current injecting (*1*) and voltage-recording (*2*) electrodes. Bar, 50 μm. (*c* and *d*) Corresponding oscilloscope tracings; *I*, current injected into cell 1 (calibration pulse corresponds to a current of 1.01×10^{-9} A × 10 ms); V_1, voltage trace from cell *1* (bridge unbalanced) and V_2, voltage trace from cell *2* (calibration pulse, 10^{-2} V × 10 ms).

moved from cell to cell within the monolayer (Fig. 5). In more than 20 experiments, in which more than 300 cell pairs were tested, all successful electrode penetrations at interelectrode distances of 15 to over 300 μm (1–10 cell diameters) resulted in demonstrable electrotonic transfer. There was no obvious qualitative difference in the capabilities of the aortic and umbilical vein endothelial cells to engage in electrotonic transfer.

DYE TRANSFER: Iontophoretic injection of the fluorescent dye, Lucifer Yellow CH, into a cell in a confluent monolayer of primary aortic or venous endothelium resulted in the rapid appearance of fluorescence in contiguous cells (Fig. 6*a*). In general, dye was usually detectable in adjacent cells within 30 s of the onset of injection. Transfer was commonly detected out to 3–10 cell diameters with 1–5 min of dye injection.

In comparison, dye injected into cells in unattached sheets of effluent cells (Fig. 6*b*) was detected more rapidly in adjacent cells. Transfer was usually seen within 5–10 s and the extent of detectable transfer, in terms of cell diameters, was typically greater than that seen in the cultured cells (cf. Fig. 6*a* and *b*). Although there was a quantitative difference in the transfer between PEC and cultured cells (see Discussion), there was no obvious difference between aortic and venous endothelial cells.

NUCLEOTIDE TRANSFER: Further indication that the aortic and venous cells were capable of junctional transfer was tested by prelabeling cells with [^{3}H]uridine and coculturing these donor cells with nonprelabeled cells (29). Once again, we tested both effluent and cultured cells for their capabilities for transfer. When freshly isolated cells were used as recipients, most of the sheets and small clumps acquired significant label during the 12- to 18-h coculture (shorter times gave similar results). Typically, each recipient clump showed relatively uniform incorporation, although some clumps were negative, presumably reflecting a lack of junction formation with donors during the coculture period (see Fig. 7*a*). When primary cultures 3- to 5-d-old were used as recipients for trypsinized donors, transfer again was detected in most cases (see Fig. 7*b*). Vascular smooth muscle cells, endothelial cells, and BHK-C13 fibroblasts were competent donors. However, L-929 fibroblasts rarely engaged in transfer either as donors or as recipients. (In a few isolated cases, apparent transfer was seen with these cells. It is not clear whether these cases were artifactual or due to rare instances of junction formation.)

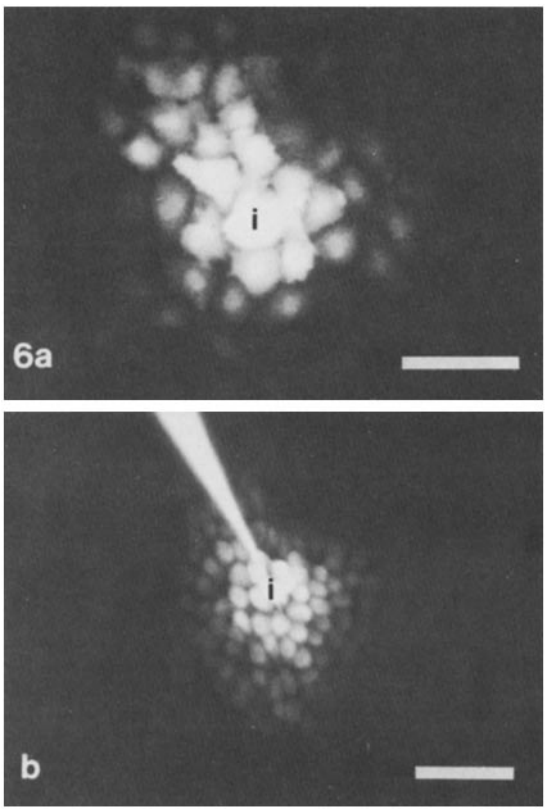

FIGURE 6 Dye transfer. Dark-field fluorescence micrographs. (*a*) Aortic endothelial primary culture; dye injected into cell *i*. (*b*) Aortic effluent cell sheet; dye injected into cell *i*. Bars, 50 μm.

DISCUSSION

Our results demonstrate for the first time that primary cultures of endothelial cells from bovine aortae and umbilical veins have both gap and tight junctions and have the capacity to exchange small ions, tracer dyes, and nucleotides. The qualitative similarity in the structure and transfer capability of the

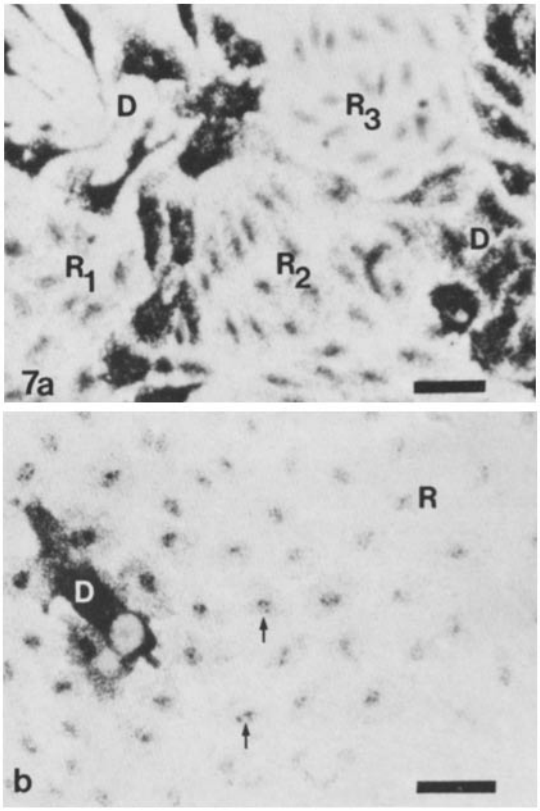

FIGURE 7 Nucleotide transfer autoradiographs. Bright-field micrographs. (*a*) "Effluent recipient cell" technique. *D*, donor endothelial cells; R_{1-3}, recipient endothelial islands. The density of autoradiographic grains over cells in R_1 and R_2 is fairly heavy, indicating that transfer occurred. The uniformity of the grain density shows that any nucleotides transferred into the recipient islands rapidly equilibrated throughout all the cells, suggesting that the formation of junctions between donors and recipients was the rate-limiting step in transfer. The grain density over cells in R_3 does not exceed background levels for this experiment, indicating no transfer and arguing against exchange via extracellular space. (*b*) "Trypsinized donor" technique. *D*, donor SMC; *R*, recipient endothelial monolayer; arrows, nucleolar labeling in endothelial recipients. Bars, 50 μm.

gap junctions[1] in vitro and in freshly isolated sheets of cells further supports the use of such cultures as models for studying the biology of the vascular endothelium.

Although the general structure and distribution of junctions in our preparations were similar to those reported for endothelial cells in vivo (14, 15, 38, 44, 45, 50), two peculiarities were noted. The first was the reduction in particle density in the center of most of the gap junctions seen in freeze-fracture replicas. This morphology has not been reported for endothelial cells fixed in vivo. Because the state of particle aggregation may influence junctional permeability (27), it will be important to determine whether the normal state of these particles is more aggregated or dispersed, using, for example, rapid freezing techniques (31).

The second peculiarity was seen primarily in freeze-fracture replicas of effluent cells and of intact endothelium where the macular gap junctions were often linked by networks of particle bands, 1–3 particles wide. The rarity of tight junctions in these assemblages distinguishes them from the special endothelial junctions described by Simionescu et al. (45). They may be complex gap junctions, like those seen in differentiating lens fibers (1), or they may represent formation or degradation of tight junctions encircling and linking the gap junctions. Similar networks of particles are seen during tight junction development in the liver (26) and in hepatoma cultures (30).

The extent and frequency of gap junctions and the complexity of tight junctions is reportedly different in aortae and certain veins (45). The differences in gap junctions could be reflected in the capabilities of the two endothelial populations to carry out junctional communication. In our studies, however, there was little difference in the structure of aortic and umbilical vein junctions, either in freshly isolated sheets or in short-term primary culture. Furthermore, both cell types were well coupled electrically in culture (see also reference 49) and were capable of dye and nucleotide transfer in cultures and in effluent preparations. Thus, we observed neither a structural nor physiological basis for a difference in junctional communication in aortic and umbilical vein endothelium.

We did find some differences between the junctions in effluent cells and in the corresponding cultured cells of either type. The most obvious differences were structural. The extent and complexity of the gap and tight junctions in the cultures were notably reduced in comparison with the freshly isolated cells (Table I). Two factors are likely to have contributed to this reduction. Once the effluent cells adhered, they flattened and spread. Preexisting junctions might well have been reduced in size and complexity during cell spreading. In addition, new junctions were formed as the spreading cells from one clump made contact with those of the next clump and, probably, as the cells divided. Conceivably, the newly formed junctions were simpler than those in the effluent sheets. It may be that the few complex and extensive junctions detected in cultures were retained from the effluent stage, while the remaining junctions were newly constructed. We have not examined subcultured cells, but, according to one report (40), endothelial cells from the rabbit superior vena cava have small gap junctions and no tight junctions after multiple subculturing.

Whatever the source, the decrease in junctional complexity may have contributed to the apparent reduction in dye transfer in culture. However, the decrease in transfer was small and probably was influenced by the decreased size and increased thickness of the effluent cells. (A more quantitative analysis of transfer by densitometry of photographic negatives corrected for cell and nuclear thickness suggests a small but definite junction effect; in preparation). Whether a small reduction in transfer could have any physiological effect remains unclear.

Because the transfer of dye and labeled metabolites is readily detectable between effluent cells, it is likely that cells in the intact intima are capable of similar or even more extensive transfer. (This possibility has been confirmed in dye experiments on vessels in bovine chorion and rat omentum; 43). The physiological roles for such transfer are not known, but the numerous possibilities include the homeostatic regulation of concentrations of ions, intermediary metabolites, and regulatory molecules throughout the tissue. Junctional transfer could also mediate cooperative responses of the intimal cells to normal or pathological external stimuli and contribute to growth regulation and the maintenance of a monolayer topology (21, 22, 42). The concept of the intima as a functional syncytium rather than a collection of individual cells could be important in understanding the physiology and pathology of this tissue.

In addition, our experimental observations of nucleotide transfer between cultured endothelial and vascular smooth muscle cells and the descriptions of endothelial-smooth muscle junctions in situ (12, 17, 32–35) are consistent with Rhodin's suggestion (32) that physiologically significant signals might be transferred between these two cell types in vivo. As an example, endothelial cells, in vivo and in vitro, have been shown to respond to a variety of vasoactive substances, via membrane receptors, with changes in the intracellular concentrations of ions and small molecules (5, 6, 7). Such responses could be transferred to smooth muscle cells and thereby affect vessel tonus. This possibility is further supported by the work of Bevan and Duckles (3) who demonstrated contraction of aortic strips with the application of norepinephrine-derivatized glass beads to the intimal surface. The particularly intriguing possibility of direct endothelial-smooth muscle transfer is being investigated further.

The authors are grateful to Ms. Patricia Anaya and Ms. Susan Anderson for their excellent technical assistance on this project, and to Dr. Michael A. Gimbrone, Jr., for helpful discussion of the revised manuscript.

This work was supported by National Institutes of Health grants HL06314 and HL21166.

Received for publication 8 June 1981, and in revised form 20 August 1981.

REFERENCES

1. Benedetti, E. L., I. Dunia, and H. Bloemendal. 1974. Development of junctions during differentiation of lens fibers. *Proc. Natl. Acad. Sci. U. S. A.* 71:5073–5077.
2. Bennett, M. V. L. 1973. Permeability and structure of electrotonic junctions and intercellular movements of tracers. *In:* Intracellular Staining in Neurobiology. S. B. Kater and C. Nicholson, editors. Springer Verlag, N. Y. 115–134.
3. Bevan, J. A. and S. P. Duckles. 1975. Evidence for α-adrenergic receptors on intimal endothelium. *Blood Vessels.* 12:307–310.
4. Booyse, F. M., B. J. Sedlak, and M. E. Rafelson, Jr. 1975. Culture of arterial endothelial cells. *Thromb. Diath. Haemorrh.* 34:825–839.
5. Buonassisi, V. and P. Colburn. 1980. Hormone and surface receptors in vascular endothelium. *In:* Advances in Microcirculation, Vol. 9, B. M. Altura, editor. S. Karger, Basel. 76–94.
6. Buonassisi, V. and J. C. Venter. 1976. Hormone and neurotransmitter receptors in an established vascular endothelial cell line. *Proc. Natl. Acad. Sci. U. S. A.* 73:1612–1616.
7. D'Amore, P. and D. Shepro. 1977. Stimulation of growth and calcium influx in cultured, bovine, aortic endothelial cells by platelets and vasoactive substances. *J. Cell. Physiol.* 92: 177–183.
8. Gilula, N. B., O. R. Reeves, and A. Steinbach. 1972. Metabolic coupling, ionic coupling,

[1] The gap junctions are the most likely sites of transfer although some contribution by endothelial tight junctions (43) or cytoplasmic bridges cannot be ruled out.

and cell contacts. *Nature (Lond.)*. 235:262–265.
9. Gimbrone, M. A., Jr. 1976. Culture of vascular endothelium. *In:* Progress in Hemostasis and Thrombosis, Vol. III. T. H. Spaet, editor. Grune and Stratton, N. Y. 1–28.
10. Gimbrone, M. A., Jr. and R. S. Cotran. 1975. Human vascular smooth muscle in culture: growth and ultrastructure. *Lab. Invest.* 33:16–27.
11. Gimbrone, M. A., Jr., R. S. Cotran and J. Folkman. 1974. Human vascular endothelial cells in culture. Growth and DNA synthesis. *J. Cell Biol.* 60:673–684.
12. Haudenschild, C. C. 1980. Growth control of endothelial cells in atherogenesis and tumor angiogenesis. *In:* Advances in Microcirculation, Vol. 9 B. M. Altura, editor S. Karger, Basel. 226–251.
13. Haudenschild, C. C., R. S. Cotran, M. A. Gimbrone, Jr., and J. Folkman. 1975. Fine structure of vascular endothelium in culture. *J. Ultrastruct. Res.* 50:22–32.
14. Hüttner, I., M. Boutet, and R. H. More. 1973. Gap junctions in arterial endothelium. *J. Cell. Biol.* 57:247–252.
15. Hüttner, I. and H. Peters. 1978. Heterogeneity of cell junctions in rat aortic endothelium: a freeze-fracture study. *J. Ultrastruct. Res.* 64:303–308.
16. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* 52:2757–2764.
17. Jasiński, A. 1977. Endothelial-to-perivascular cell junctions. *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* 25:455–457.
18. Johnson, R. G. and J. D. Sheridan. 1971. Junctions between cancer cells in culture: ultrastructure and permeability. *Science (Wash. D. C.)*. 174:717–719.
19. Kalderon, N., M. L. Epstein, and N. B. Gilula. 1977. Cell-to-cell communication and myogenesis. *J. Cell Biol.* 75:788–806.
20. Larson, D. M. and J. D. Sheridan. 1979. Structure of gap and tight junctions and transfer of small molecules from cell to cell in cultured bovine endothelial cells. *Anat. Rec.* 193:599 (Abstr.).
21. Lawrence, T. S., W. H. Beers, and N. B. Gilula. 1978. Transmission of hormonal stimulation by cell-to-cell communication. *Nature (Lond.)* 272:501–506.
22. Loewenstein, W. R. 1979. Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta.* 560:1–65.
23. Macarak, E. J., B. V. Howard, and N. A. Kefalides. 1977. Properties of calf endothelial cells in culture. *Lab. Invest.* 36:62–67.
24. Mason, R. G., S. F. Mohammad, H. I. Saba, H. Y. K. Chuang, E. L. Lee and J. U. Balis. 1979. Functions of endothelium. *In:* Pathobiology Annual editor. Vol. 9. H. L. Ioachim, editor. Raven, N. Y. 1–48.
25. McNutt, S. and R. Weinstein. 1973. Membrane ultrastructure at mammalian intercellular junctions. *Prog. Biophys. Molec. Biol.* 26:45–101.
26. Montesano, R., D. S. Friend, A. Perrelet, and L. Orci. 1975. In vivo assembly of tight junctions in fetal rat liver. *J. Cell Biol.* 67:310–319.
27. Peracchia, C. and A. F. Dulhunty. 1976. Low resistance junctions in crayfish: structural changes with functional uncoupling. *J. Cell Biol.* 70:419–439.
28. Pitts, J. D. 1972. Direct interaction between animal cells. *In:* Cell Interactions. L. G. Silvestri, editor. North Holland, Amsterdam. 227–285.
29. Pitts, J. D. and J. W. Simms. 1977. Permeability of junctions between animal cells: intercellular transfer of nucleotides but not macromolecules. *Exp. Cell Res.* 104:153–163.
30. Porvaznik, M., R. G. Johnson, and J. D. Sheridan. 1976. Intercellular junctions and other cell surface differentiations of H4IIE hepatoma cells *in vitro*. *J. Ultrastruct. Res.* 55:343–359.
31. Raviola, E., D. A. Goodenough, and G. Raviola. 1978. The native structure of gap junctions rapidly frozen at 4°K. *J. Cell Biol.* 79(2, Pt. 2):229*a* (Abstr.).
32. Rhodin, J. A. G. 1967. The ultrastructure of mammalian arterioles and precapillary sphincters. *J. Ultrastruct. Res.* 18:181–223.
33. Rhodin, J. A. G. 1968. Ultrastructure of mammalian venous capillaries, venules and small collecting veins. *J. Ultrastruct. Res.* 25:452–500.
34. Roggendorf, W. and J. Cervós-Navarro. 1977. Ultrastructure of arterioles in the cat brain. *Cell Tissue Res.* 178:495–515.
35. Roggendorf, W., J. Cervós-Navarro, and M. D. Lazaro-Lacalle. 1978. Ultrastructure of venules in the cat brain. *Cell Tissue Res.* 192:461–474.
36. Ross, R. 1971. The smooth muscle cell: II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* 50:172–186.
37. Ryan, U. S., E. Clements, D. Habliston, and J. W. Ryan. 1978. Isolation and culture of pulmonary artery endothelial cells. *Tissue Cell.* 10:535–554.
38. Schneeberger, E. E. and M. J. Karnovsky. 1976. Substructure of intercellular junctions in freeze-fractured alveolar-capillary membranes of mouse lung. *Circ. Res.* 38:404–411.
39. Schwartz, S. M. 1978. Selection and characterization of bovine aortic endothelial cells. *In Vitro.* 14:966–980.
40. Shaklai, M., D. Loskutoff, and M. Tavassoli. 1978. Membrane characteristics of cultured endothelial cells: identification of gap junction. *Isr. J. Med. Sci.* 14:306–313.
41. Sheridan, J. D. 1971. Dye movement and low-resistance junctions between reaggregated embryonic cells. *Dev. Biol.* 26:627–636.
42. Sheridan, J. D. 1976. Cell coupling and cell communication during embryogenesis. *In:* Cell Surface in Animal Embryogenesis and Development. G. Poste and G. L. Nicholson, editors. Elsevier North-Holland, Amsterdam. 409–447.
43. Sheridan, J. D. 1980. Dye transfer in small vessels from the rat omentum: homologous and heterologous junctions. *J. Cell Biol.* 87(2, Pt. 2):61*a* (Abstr.).
44. Simionescu, M., N. Simionescu, and G. E. Palade. 1975. Segmental differentiations of cell junctions in the vascular endothelium: the microvasculature. *J. Cell Biol.* 67:863–885.
45. Simionescu, M., N. Simionescu, and G. E. Palade. 1976. Segmental differentiations of cell junctions in the vascular endothelium: arteries and veins. *J. Cell Biol.* 68:705–723.
46. Socolar, S. J. and W. R. Loewenstein. 1979. Methods for studying transmission through permeable cell-to-cell junctions. *In:* Methods in Membrane Biology, Vol. 10. E. D. Korn, editor. Plenum Press, N. Y. 123–179.
47. Stewart, W. W. 1978. Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. *Cell.* 14:741–759.
48. Tasaki, K., Y. Tsukahara, S. Ito, M. J. Wayner, and W. Y. Yu. 1968. A simple and direct and rapid method for filling microelectrodes. *Physiol. Behav.* 3:1009-1010.
49. Venter, J. C., V. Buonassisi, S. Bevan, S. Heinemann, and J. A. Bevan. 1975. Hormone and neurotransmitter receptors on the intimal endothelium. *Blood Vessels.* 12:381–382.
50. Yee, A. G. and J. P. Revel. 1975. Endothelial cell junctions. *J. Cell Biol.* 66:200–204.