Chapter 9

INTERCELLULAR COMMUNICATION IN THREE-DIMENSIONAL CULTURE

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I. INTRODUCTION

The coordination of cell growth and differentiation as well as of tissue homeostasis and synchronization of tissue functions requires the transfer of information between cells. This may be achieved by an unidirectional signal flow, as is the case with secreted molecules (hormones, neurotransmitters) and/or with membrane-bound receptors (sperm-egg binding, immune system). It can also follow a "nonrectifying" mode, as is observed for direct intercellular communication via gap junctions that are formed in regions of close cell-to-cell contact. These membrane channels are not only bidirectionally permeable and facilitate the free exchange of charged and neutral molecules, but also connect adjacent cells mechanically. They must be clearly discriminated from desmosomes, which anchor cells together to form structural or functional units, as well as from tight junctions that seal membranes of epithelial cells to each other so that the paracellular path becomes impermeable to molecules, and a polarity of apical and basolateral surface is maintained.

Gap junctions are found throughout the animal kingdom; only a few terminally differentiated cell types no longer express these channel proteins. A typical gap-junction plaque contains several hundred channels and is best visualized on freeze-fracture replicas of plasma membranes, as is shown in Figure 1. Each contacting cell contributes a hemichannel, the connexon, so that a communicating gap-junction channel is formed. The connexon is made of six intramembranous proteins, the so-called connexins, as is schematically illustrated in Figure 2. Several monographs have been published since 1988 in which the functional and structural aspects of gap junctions are discussed.[1–4]

The size of the proteins isolated from gap-junction plaques ranges from 16 kD[5] over the first-described 26 kD (now connexin cx32)[6] and the protein found in heart tissue (cx43)[7] to a 70 kD[8,9] protein. The topological models of these proteins show four α-helical transmembrane regions that may be part of the hydrophilic channels. Thus, a connexin has two extracellular loops and another loop at the intracellular side where also the amino- and the carboxy-terminus of the protein is located.

The gap-junction permeability is influenced by two endogenous signal pathways, using as second messengers cAMP or diacylglycerol.[10,11] cAMP up-regulates the gap junctional permeability, whereas the diacylglycerol effects its down-regulation. Besides a possible regulation by phosphorylation, the fine tuning may well vary in different cells, since many other substances with regulatory effects have been described.[12] However, the closing of gap-junction channels is not only caused by physiological regulations with pH, Ca^{2+}, and retinoic acid, but also by nonphysiological reactions induced by heptanol, benzhydrol, or glutardialdehyde.

The permeability limit for molecules that can pass gap junctions in vertebrate cells is about M_r 800 to M_r 1000.[13,14] With molecules of this size, a metabolic cooperation is possible that allows cells with certain enzymatic

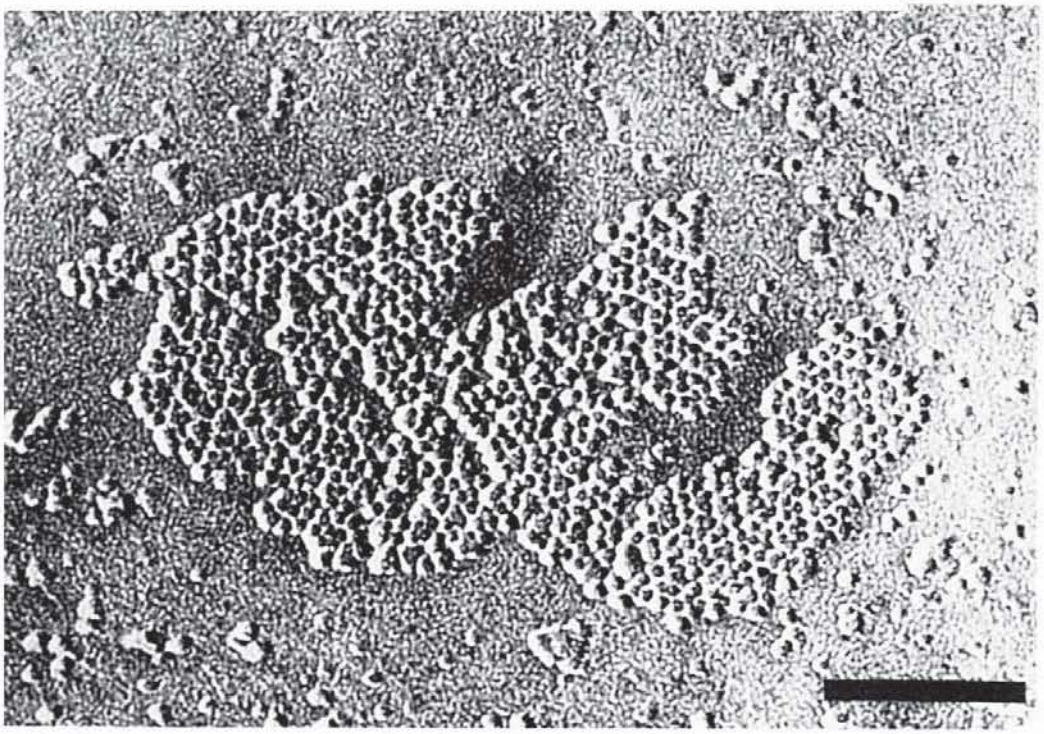

FIGURE 1. Freeze-fracture replica of gap-junction plaque in a 2-day-old multicell spheroid of BICR/M1R$_k$ cells. (Horizontal bar, 100 nm.)

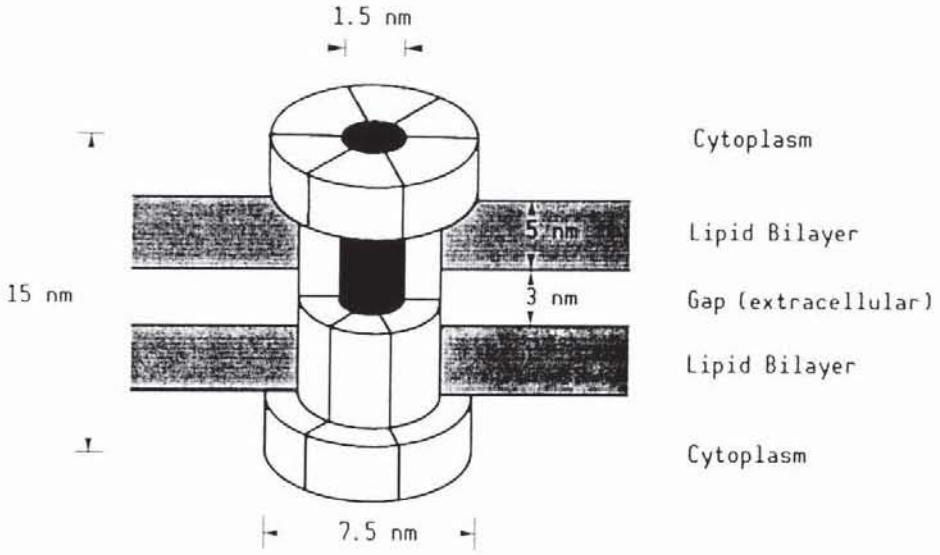

FIGURE 2. Schematic model of a gap-junction channel. Six connexins are assembled to one hemichannel (= connexon). Two connexons are linked together forming a gap-junction channel that bridges the extracellular gap between two contacting cells.

defects to proliferate without a substitution for the missing enzyme or its product as long as they are coupled to wild-type cells by gap junctions.[15] Since in most cases the intercellular signal that is exchanged between the cells is not known, artificial signals, e.g., the fluorescent dye Lucifer yellow, are used for

the demonstration of open gap junctions. However, if Lucifer yellow is retained in the injected cell, this does not necessarily indicate a complete closure of gap-junction channels, since ionic coupling may still be present.[16,17]

Gap-junction communication has also been detected in mixed cultures of vertebrate cells derived from different tissues and different organisms.[18–21] One may therefore ask the question whether tumor cells that are coupled to normal cells have a reduced proliferative capacity[22,23] or whether they have an advantage for invading normal tissue.[24,25] Loewenstein and Kanno[26] found noncoupled tumor cells and correlated the absence of gap-junction communication to tumorigenicity. This hypothesis has stimulated the research of junction communication, but the role of gap junctions in tumor development and tumor invasion is still under controversial discussion. Since numerous well-coupled tumor cells have also been described,[16,21,27–30] it is obvious that gap junctions play a more complex role in growth control than can be explained by two-channel configurations (open vs. closed). Interestingly, coupled cells might be less sensitive to changes of the cells microenvironment or stress factors than cells without gap junctions, as has been shown for irradiated multicellular tumor spheroids.[31,32]

Cells that are interconnected by gap junctions, when grown as a two-dimensional monolayer, are also coupled when cultivated as a three-dimensional multicell spheroid. In the latter case, however, the amount of coupling and/or the permeability of gap junctions may be somewhat reduced,[16,33] but information transfer is still effective; this can be evaluated through synchronously beating reaggregated heart cells.

This contribution summarizes some results on gap-junction communication in both two- and three-dimensionally grown cultures and reveals some methodological difficulties that need to be solved for a quantification of the gap-junction permeability in the three-dimensional case. Furthermore, it provides some evidence for the hypothesis that the invasive behavior of tumor cells depends on their capacity to participate in the host cells metabolism. For this purpose an *in vitro* invasion assay developed by Mareel and coworkers[34] was used, and precultured fragments of embryonic chick heart were confronted with multicell spheroids of both coupled and noncoupled tumor cells. Comparative investigations with this three-dimensional culture model and tumor-cell implantation into syngeneic animals demonstrated a strict correlation between invasion *in vitro* and formation of invasive tumors *in vivo*.[35]

II. MATERIALS AND METHODS

A. Cell Cultures

1. Permanently Growing Cells

Fibroblastioid BICR/M1R$_k$ cells were derived from the BICR/M1R transplantable mammary tumor of the Marshall rat.[36] The fibroblastioid EMT6/Ro cells were selected from a mammary tumor cell line of a Balb/c mouse.[37]

Fibroblastioid C6 cells were derived from N-nitrosomethylurea-induced glial tumors of the rat.[38] Epithelioid HeLa cells were established from a human cervix carcinoma.[39,40] Fibroblastioid L cells originated from a 20-methylcholanthrene-treated primary strain of C3H mouse fibroblasts.[41] These cells were cultivated as monolayers or as multicell tumor spheroids.

Spheroids were initiated by seeding single-cell suspensions (1 to 2×10^6 cells per dish) in 94-mm-diameter plastic Petri dishes (Greiner, Nürtingen, FRG) with nonadherent surfaces. Within 3 to 7 d, the aggregated cells were transferred into spinner flasks (Bellco Glass, Vineland, NJ) filled with 60 ml medium and cultured at 120 rpm.

2. *Primary Embryonic Chick Heart Cells*

Single-cell suspensions of 9-day-old embryonic chick hearts were prepared as described by Freshney.[42] Chick hearts were explanted from 9-day-old embryos and placed overnight in 1 ml of ice-cold trypsin. After trypsin removal, the organ was incubated in the residual trypsin at 37°C for 10 to 15 min and dispersed by gentle pipetting in 2 ml of culture medium. Embryonic chick heart fragments were obtained by dissecting organs of 9-day-old embryos under stereomicroscopical control. Freshly cut fragments were cultured in spinner flasks.

3. *Culture Conditions*

All cell lines were grown at pH 7.4 and 37°C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (Biochrom KG, Berlin, FRG) supplemented with 3.7 g/l $NaHCO_3$, 100 mg/l streptomycine sulfate, 150 mg/l penicillin G, and 5% calf serum. Cells were passaged in tissue culture flasks (Nunc, Roskilde, Denmark) by treatment with 0.25% trypsin in phosphate-buffered saline (PBS) without calcium and magnesium. For electrophysiological experiments, the cells were cultured in Petri dishes (Falcon 3002 F, Falcon, Becton Dickinson, Mountainview, CA). The bicarbonate/CO_2-buffered culture medium was replaced for Hepes-buffered medium (25 m*M*) only for embryonic heart cells during the measurements.

Heterologous cultures of embryonic chick heart cells with permanently growing mammalian tumor cells were obtained by seeding tumor cells to subconfluent heart monolayer cultures. For better identification, tumor cells had been stained with vital red (0.8 mg/ml of culture medium; Chroma, Köngen, FRG) for 2 to 3 d prior to trypsinization and subsequent confrontation.

4. *Confrontation Cultures*

After 4 d, precultured heart fragments of 400-µm diameter were collected under stereomicroscopical control and individually brought in contact with multicell tumor spheroids of 200-µm diameter on a semisolid agar medium (0.2 g of agar in 10 ml of ringer solution and 20 ml of culture medium) in a Petri dish, as described by Mareel and co-workers.[34] After an incubation period of

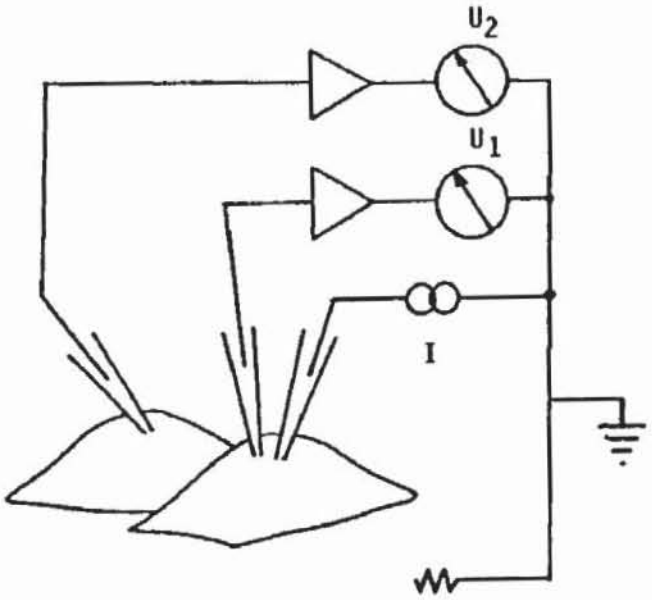

FIGURE 3. Schematic setup for the determination of intercellular communication with three intracellularly inserted glass microelectrodes.

1 to 2 h, confronting pairs were transferred into medium-filled multiwell dishes (Costar 3548), basecoated with semisolid agar medium, and individually cultured.

B. Electrophysiology

Measurements of membrane potentials and intracellular injections of electrical current or dye were performed under light microscopical observation. Glass microelectrodes were freshly pulled from capillary glass (outer diameter 1 mm, with inner filament; Hilgenberg Glas, Malsfeld, FRG) and back-filled with 3 *M* KCl or with a 4% solution of the fluorescent dye Lucifer yellow CH (M_r 457, Sigma, St. Louis, MO) in 1 *M* LiCl. Membrane potentials were measured with two microelectrodes by a capacity-compensated electrometer (750, WP Instruments, New Haven, CT). Hyperpolarizing current pulses were injected into a cell by a third microelectrode. The resulting hyperpolarizing pulses were registered in the same and, in case of coupling, in adjacent cells, as is schematically shown in Figure 3. Dye spreading was monitored 2 min after a 30 s injection with a negative current step of up to 20 nA under a Zeiss Standard microscope with epifluorescence illumination (exciter filter BP 450-490, dichroic mirror 510, barrier filter LP 520). All electrophysiological experiments were performed with electrically driven micromanipulators (Gebr. März häuser, Wetzlar, FRG).

C. Histology

1. Light Microscopy

Spheroids, heart fragments, and confrontation cultures were washed in PBS and fixed in Bouin solution for 2 h at room temperature. Cell cultures were then

dehydrated in 70% ethanol for 2 to 3 h, 80 and 96% isopropanol for 30 min at room temperature, and in 100% isopropanol (replaced for three times) for 90 min at 45°C. After impregnation overnight at 57°C in a mixture of equal parts isopropanol and the embedding medium Paraplast Plus (Polyscience, Warrington, PA), cell cultures were infiltrated for 2 × 7 h at 57°C with pure Paraplast Plus and finally embedded in Paraplast Plus. For routine histology all specimens were cut to 5-µm-thick sections and stained with hematoxylin/erythrosine (Chroma, Köngen, FRG).

Immunohistochemical staining of embryonic chick heart cells in 5-µm-thick sections was performed with rabbit anti-chick-heart serum, kindly provided by Mareel and co-workers,[43] using the unlabeled antibody-enzyme method.[44-46] Sections were counterstained with Papanicolaou's Hemalaun (Merck, Darmstadt, FRG).

2. *Electron Microscopy*

For thin sections, monolayer cell cultures were washed in PBS twice and fixed with 2.5% glutardialdehyde (Merck, Darmstadt, FRG) in 0.1 *M* PBS at pH 7.1 for 30 to 60 min at room temperature. After 5 rinses in 0.1 *M* PBS, the cells were fixed with 1% osmium tetroxide (Merck) in 0.1 *M* PBS for 30 to 60 min, rinsed in PBS, and dehydrated in increasing concentrations of ethanol. The cells were impregnated stepwise 10 to 15 h with mixtures of 2:1, 1:1, and 1:2 parts ethanol and epoxy resin Glycidether 100 (formerly Epon 812, C. Roth, Karlsruhe, FRG), as well as twice with pure embedding medium. After complete polymerization (8 h at 40°C, and 12 h at 70°C), the embedded material was removed from the Petri dishes, thin-sectioned with a diamond knife on a Reichert OM U 3 ultramicrotome (Reichert-Jung, Nußloch, FRG), placed on pioloform-coated (Wacker, München, FRG) slit grids, and poststained with uranyl acetate and lead citrate.[25]

For freeze fracture, cell cultures were washed twice in PBS and fixed in 2.5% glutardialdehyde in 0.1 *M* PBS at pH 7.1 for 30 to 90 min at room temperature. After five rinses in 0.1 *M* PBS, the cells were treated with increasing concentrations of glycerol (20 min in 5, 10, and 30% glycerol in 0.1 *M* PBS). Monolayer cells were removed from the Petri dishes with a rubber policeman. Fixed and unfixed cells were rapidly frozen in liquid Freon 22, freeze-fractured, and replicated under high vacuum (≤260 µPa) at 123 K in a BAF 301 instrument (Balzers AG, Liechtenstein). All replicas and thin sections were examined with a Zeiss EM 10 A electron microscope.

For scanning electron microscopy, multicellular spheroids have been washed and fixed as described for the freeze-fracture procedure, transferred on coverslips that were coated with cell Tak (Biopolymers, Farmington, USA). The cells were dehydrated in ethanol and dried in a Balzers CPD 020 apparatus (Balzers, Liechtenstein). Before examination in a low-voltage scanning electron microscope (Hitachi S-900), the material was sputtered in an Ion Tech EM Microsputter (Teddington, U.K.).

FIGURE 4. Determination of intercellular communication with three intracellularly inserted glass microelectrodes. (Left) Coupled BICR/M1R$_k$ monolayer cells. (Right) Noncoupled HeLa monolayer cells. (Horizontal bar, 10 ms.) (Vertical bar, 10 mV [upper traces] or 10 nA [lower trace].)

III. RESULTS

A. Homologous Coupling

In monolayer cultures, both mammary tumor cell lines BICR/M1R$_k$ and EMT6/Ro as well as the glial tumor cell line C6 were ionically coupled (Figure 4a). On the other hand, HeLa cells as well as L cells were not ionically coupled when tested with intracellularly inserted microelectrodes, as is shown in Figure 4b. Embryonic chick heart cells in a monolayer culture generate action potentials that were used as intrinsic voltage signals to measure the gap-junction coupling in these cultures. Figure 5 depicts two traces of membrane potentials in heart fibroblasts with synchronously superimposed action potentials of a junctionally coupled heart muscle cell.

Iontophoretical injection of the fluorescent dye Lucifer yellow into these monolayer cultures revealed different degrees of dye coupling. After dye injection into a BICR/M1R$_k$ cell, dye spreading into all contacting cells was observed (Figure 6). Lucifer yellow injected into EMT6/Ro cells spread only into some of the attached cells. In C6 glioma cells, dye coupling was apparent in only one or two directly connected cells. Dye coupling could not be registered in L or HeLa cells; the injected cells retained the dye, no matter how many cells were in direct contact with the injected cell. In embryonic chick heart cells, dye transfer was restricted to one or two contacting cells.

In three-dimensionally grown cultures, the intracellular coupling was similar to that seen in monolayer cultures, as long as small spheroids were investigated; in larger spheroids the gap-junction communication was down-regu-

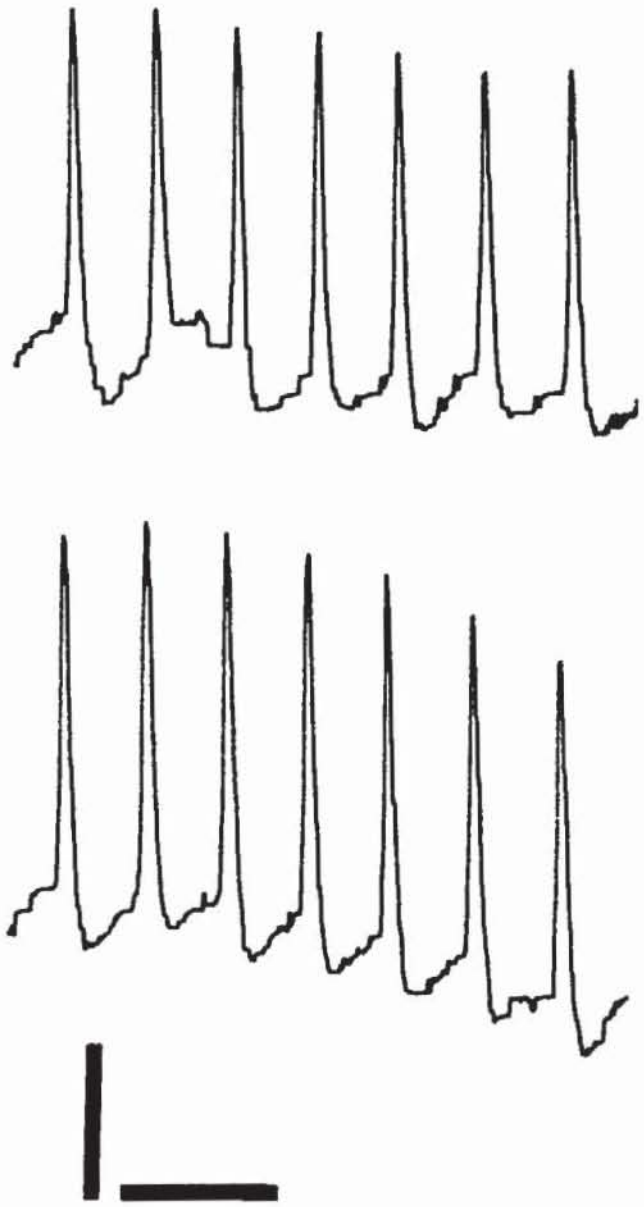

FIGURE 5. Intercellular communication in embryonic chick heart cells grown as monolayer culture. The action potential of a muscle cell is synchronously recorded in attached fibroblasts. (Horizontal bar, 10 s. Vertical bar, 10 mV.)

lated. This phenomenon is demonstrated in Figure 7: in small spheroids of BICR/M1R$_k$ cells, Lucifer yellow spread into all cells of the spheroid (Figure 7a, b), whereas in larger spheroids the dye diffused only into some of the neighboring cells (Figure 7c, d). Under these conditions the electrical coupling in multicell spheroids was also reduced.[16] HeLa and L cells were always noncoupled, regardless of the cultivation condition, as is demonstrated with HeLa spheroids of different sizes (Figure 7e–g).

Under the electron microscope, gap junctions have always been observed for both monolayer and spheroid cultures of coupled cells. With BICR/M1R$_k$ cells, many gap junctions were found in freeze-fracture replicas (Figure 2).

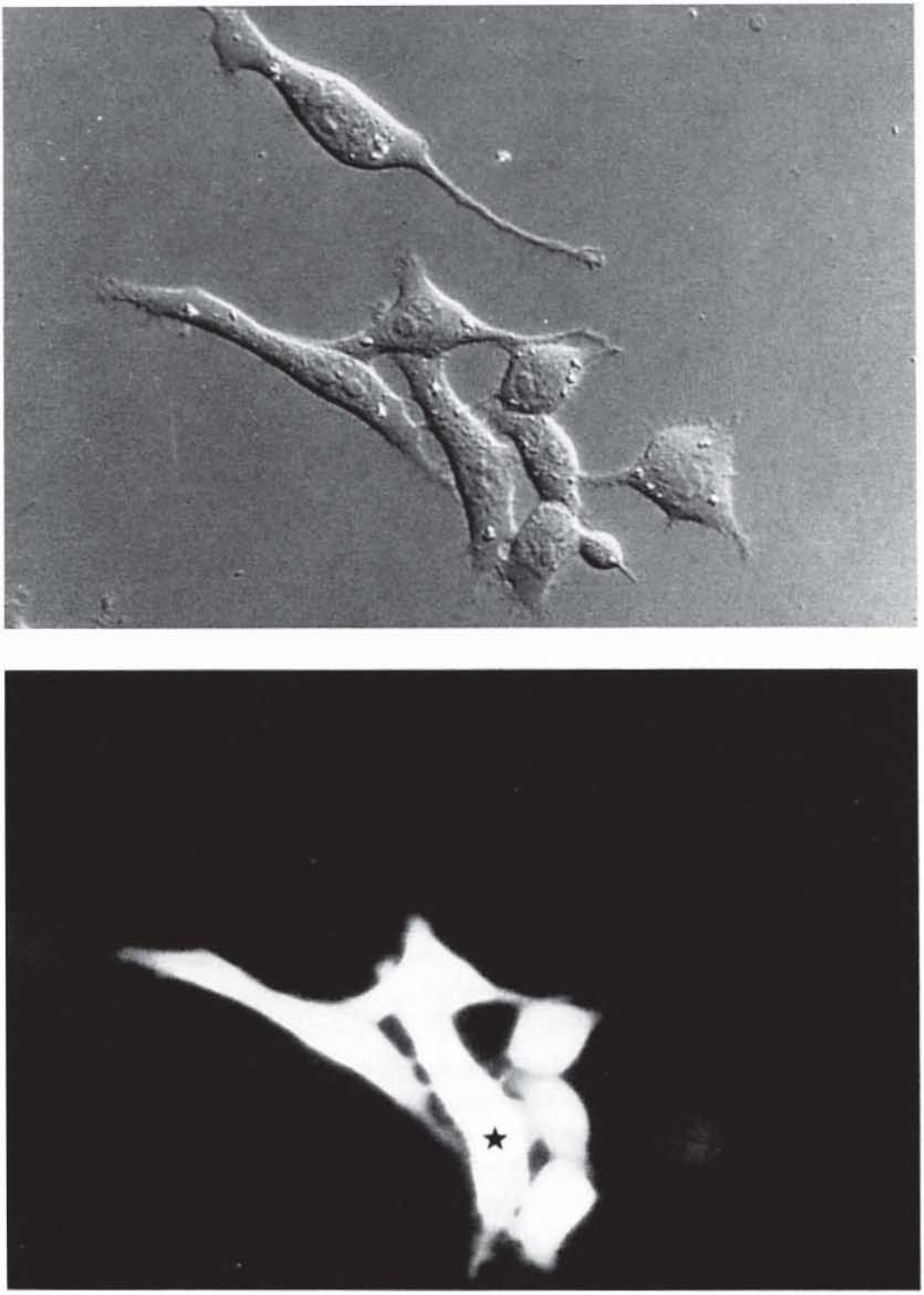

FIGURE 6. Dye coupling demonstrated with Lucifer yellow in BICR/M1R$_k$ cells. (Top) Differential interference contrast; (bottom) epifluorescent illumination of the same area. * cell where Lucifer yellow had been injected. Note: dye did not spread into the isolated cell.

Their spheroids were composed of large cells that were loosely packed, best demonstrated with the scanning electron microscope (Figure 8). In the thin sections for the electron microscope, this wide intercellular space was also apparent, but at sites of intercellular contacts, gap junctions and desmosomes

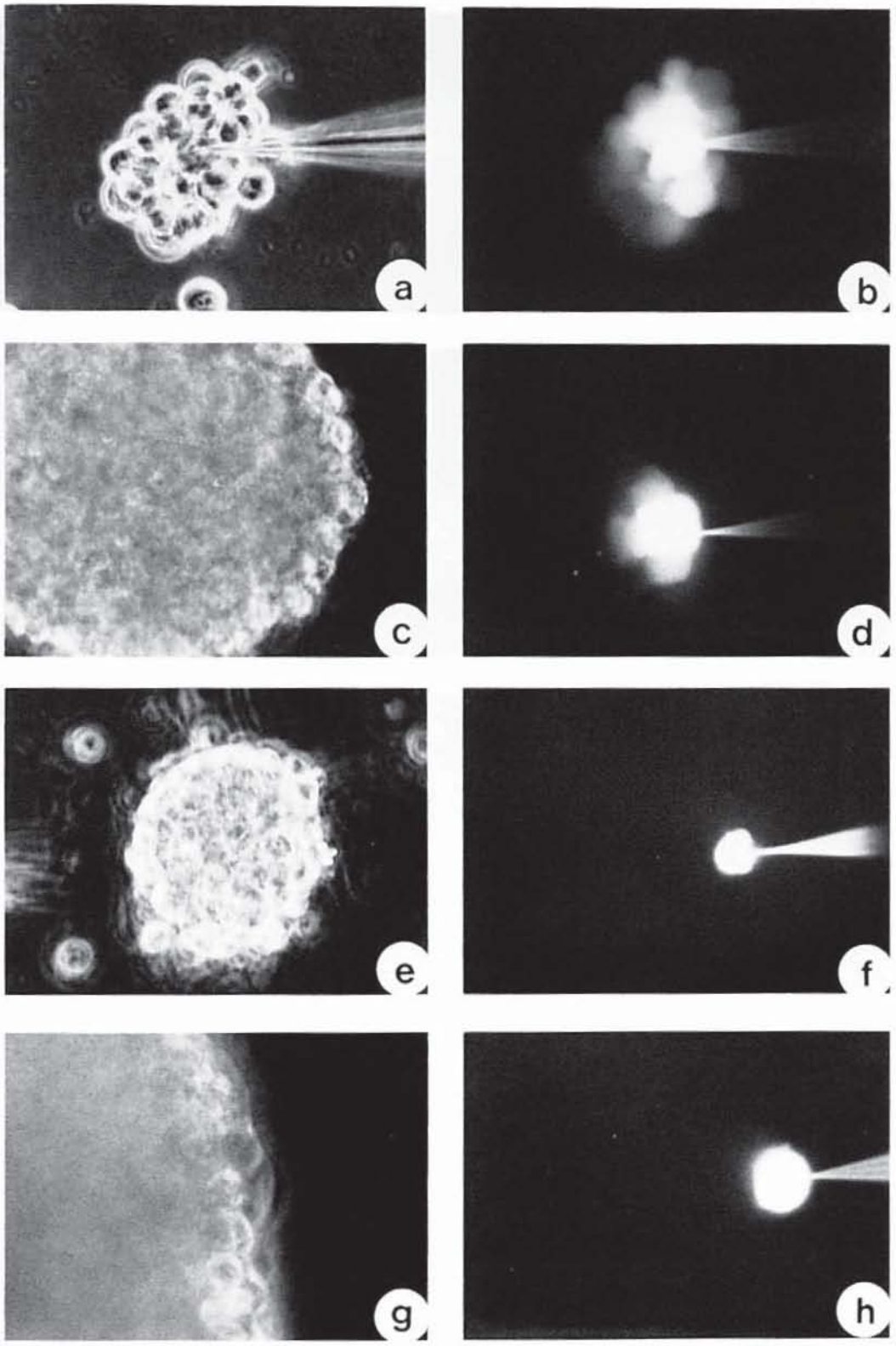

FIGURE 7. Lucifer yellow spreading in small (a, b) and big (c, d) multicell spheroids of BICR/M1R$_k$ cells. HeLa cells always retained the injected dye, regardless of the spheroid size (e–h). Phase contrast (a, c, e, g); epifluorescent illumination (b, d, f, h).

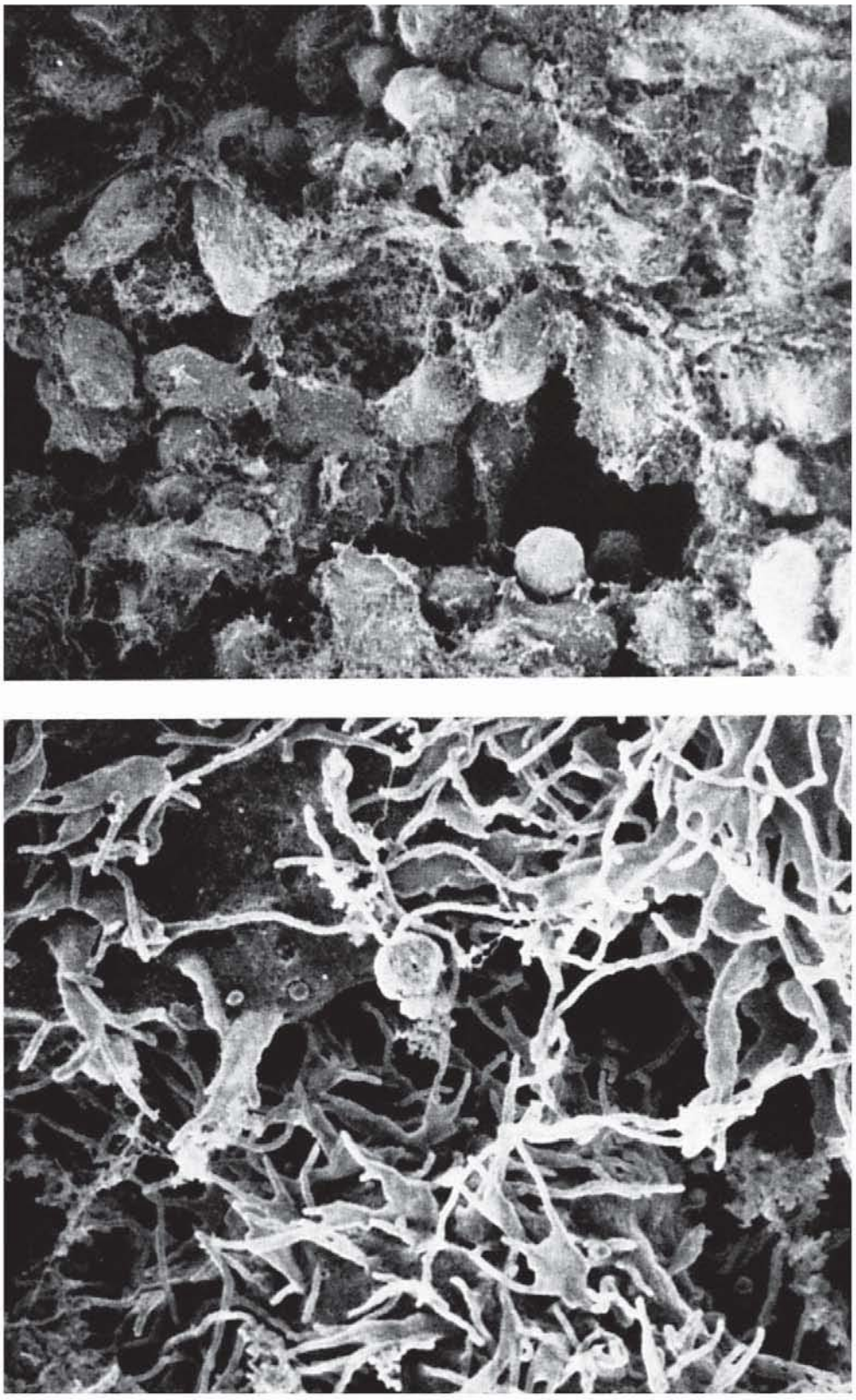

FIGURE 8. Extracellular space in a BICR/M1R$_k$ spheroid as seen with scanning electron microscopy. (Original magnification: (top) × 840; (bottom) × 10,600.)

were frequently found. Even in completely down-regulated multicell spheroids of BICR/M1R$_k$ cells, numerous gap junctions could be detected.[16,33]

A less-frequent appearance of gap junctions was observed with EMT6/Ro monolayer cells when freeze-fracture replicas were screened. In spheroids of EMT6/Ro cells, a high packing density of small fibroblastioid cells could be observed. Ultrathin sections exhibited a large number of fine cellular processes and numerous gap junctions. In ultrathin sections of C6 monolayer cells, gap junctions were very rarely detected. In spheroids these small fibroblastioid cells were densely arranged with large areas of intercellular contacts, but only a few gap junctions were found.

In freeze-fracture replicas of embryonic chick heart monolayer cells, gap junctions appeared as small-sized, irregularly shaped plaques. Rotation culture of freshly prepared heart fragments resulted in the loss of damaged cells from the surface of the fragments and a rounding off to spheroidal cell aggregates. A few layers of fibroblastioid cells surrounded a core of muscle cells, forming the so-called precultured heart fragment. In thin sections, both linear and annular gap junctions were found.

In thin sections and freeze-fracture replicas of the fibroblastioid L cells and the epithelioid HeLa cells, which did not exhibit ionic or dye coupling, gap junctions were not detected in monolayer or spheroid cultures. L cells exhibited only desmosomes as specialized cell junctions, even in spheroids where they were tightly packed and made numerous contacts by fine cellular processes. HeLa cells revealed numerous tight junctions in freeze-fracture replicas in both monolayer and spheroid cultures. As spheroids they were tightly packed, forming a dense external cell layer of epithelial morphology.

B. Heterologous Coupling

Monolayer confrontation cultures of embryonic chick heart cells with each of the five investigated mammalian cell lines were tested for heterologous gap-junction communication. The spontaneous heart action potentials were used as endogenous signals that could be detected in neighboring tumor cells when they were coupled by gap junctions to the heart cells. All cell lines that were ionically coupled in homologous monolayer cultures also coupled to embryonic heart cells. Figure 9 demonstrates the recording of action potentials of a heart cell superimposed to the membrane potential of a BICR/M1R$_k$ cell. The amplitude of the chick heart action potentials detected in coupled mammalian cells depended on cell density and the ratio of tumor to heart cells. With the noncoupled cell lines L and HeLa, heterologous coupling was never recorded. Because of the limited dye spreading in cultured heart cells, no measurements with Lucifer yellow injection were carried out in confrontation cultures.

C. Confrontation in Three-Dimensional Culture

For confrontation with multicell tumor spheroids, only precultured heart fragments with a diameter of about 400 μm were selected as host tissue. Spheroids of all five tumor cell lines readily attached to the heart tissue and

FIGURE 9. Heterologous coupling between heart muscle and BICR/M1R$_k$ cells. The action potential of an embryonic chick heart cell led to synchronous depolarizations of the coupled tumor cell membrane potential. (Horizontal bar, 10 s. Vertical bar, 10 mV.)

formed stable contacts within 2 h of incubation. Cells of spheroids migrated to their attached host tissue, covering it totally within about 40 h. This cellular arrangement did not allow measurements of heterologous coupling with microelectrodes. Histological sections, however, revealed significant differences in the interactions, which might be attributed to the junction-coupling capacity. As can be seen from Figure 10, fine extensions of tumor cells are present within the heart tissue already 15 min after the formation of stable contacts. After about 60 to 70 h, invasion of the coupled tumor cells into the heart fragments could be detected at many sites. A serious destruction of the host tissue by invading tumor cells was apparent in confrontation cultures after 120 h. With increasing incubation periods, further replacement of the heart tissue by invading coupled tumor cells was observed. A large number of peripheral tumor cells had accumulated immunoreactive material.

The noncoupled tumor cell lines HeLa and L did not exhibit protrusions of cytoplasmic tumor cell extensions between adjacent fibroblastioid heart cells. Over long cultivation periods, the host tissue was still clearly delineated (Figure 11). Within 90 h, HeLa cells did not exhibit invasive growth, although

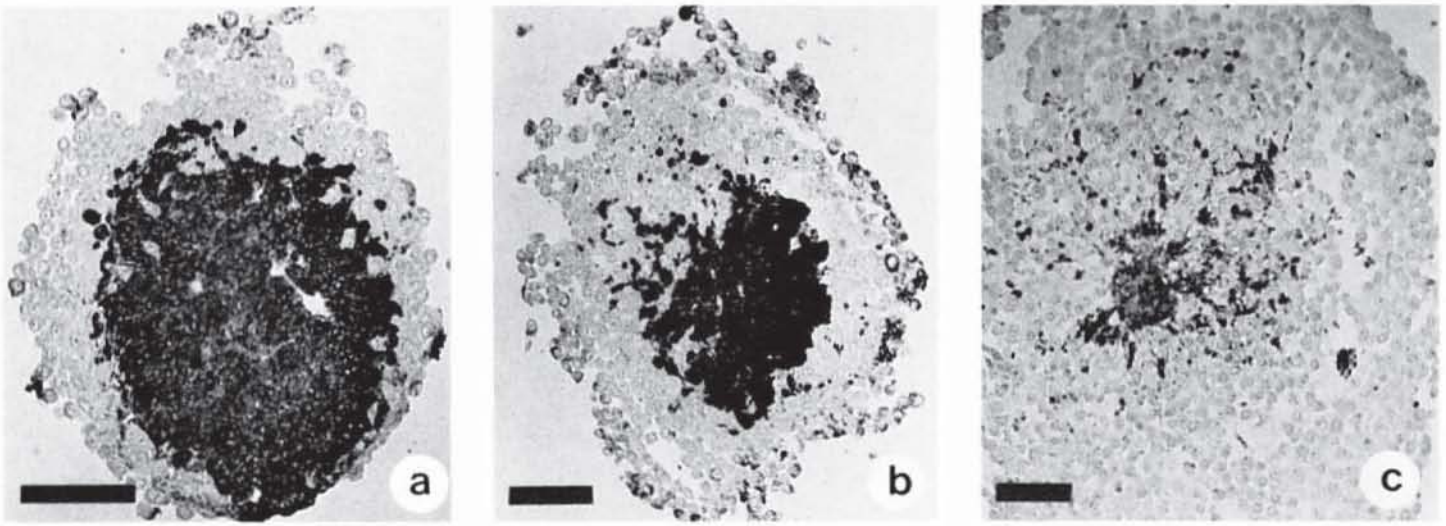

FIGURE 10. Light and electron microscopical investigations of confrontation cultures of precultured heart fragments with BICR/M1R$_k$ spheroids (a–c), EMT6/Ro spheroids (d–f), and C6 spheroids (g–i). (a) After 65 h; (b) after 122 h; (c) after 163 h; (d) after 15 min; (e) after 44 h; (f) after 136 h; (g) after 15 min; (h) after 56 h; (i) after 84 h. T, tumor cell; H, heart cell. (Staining with uranyl acetate and lead citrate in d and g and with an antiserum against embryonic chick heart in remaining figures. Scale bar, 2 µm in d and g; 100 µm in remaining figures.) (From Bräuner, T. and Hülser, D. F., Tumor cell invasion and gap junctional communication. II. Normal and malignant cells confronted in multicell spheroids, *Invasion Metastasis*, 10, 16, 1990. With permission of S. Karger AG, Basel.)

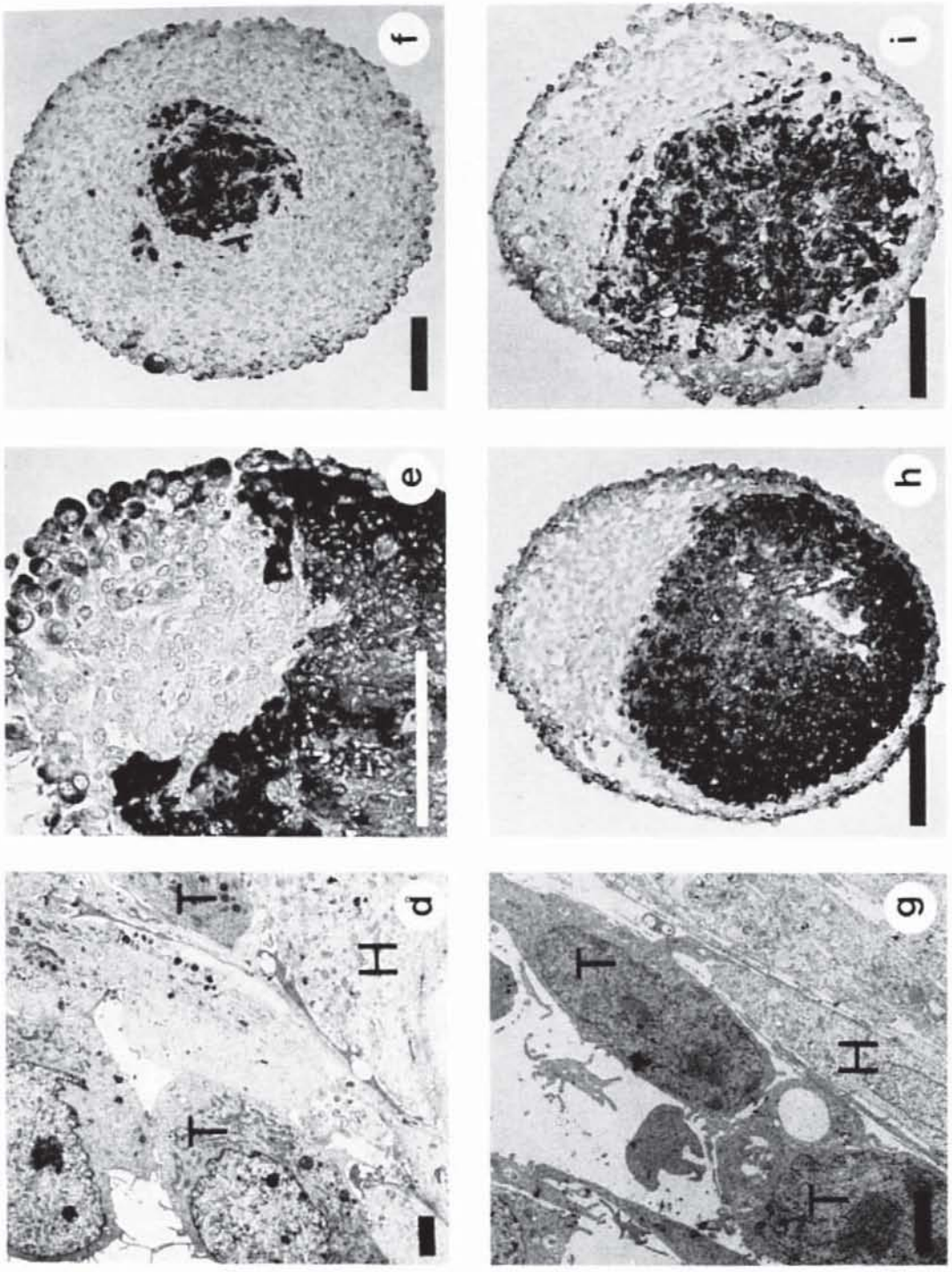

FIGURE 10 (continued).

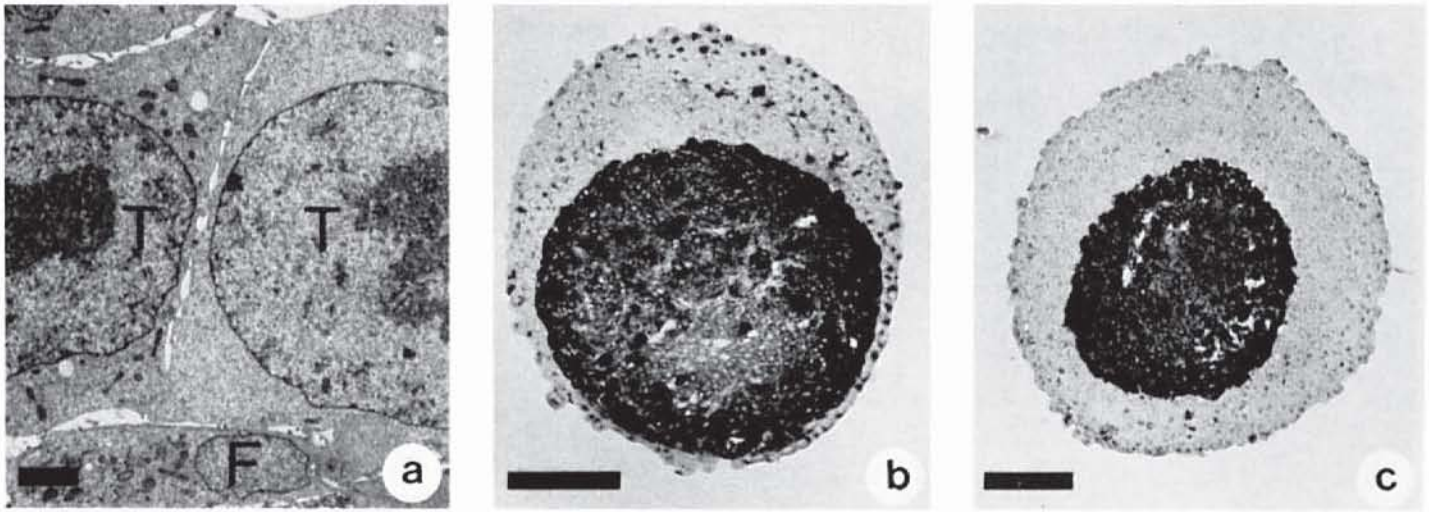

FIGURE 11. Light and electron microscopical investigations of confrontation cultures of precultured heart fragments with HeLa spheroids (a–f) and L spheroids (g–i). (a) After 19 h; (b) after 56 h; (c) after 84 h; (d) after 90 h (1 µm section of an epon-embedded culture, stained with azur II and methylene blue); (e) after 90 h (inset: tight junction; scale bar, 0.25 µm); (f) after 125 h; (g) after 75 min; (h) after 65 h; (i) after 160 h. T, tumor cell; F, fibroblastoid heart cell; M, heart muscle cell. (Staining with uranyl acetate and lead citrate in a, e, and g, and with an antiserum against embryonic chick heart in remaining figures. Scale bar, 2 µm in a, e, g; 100 µm in remaining figures.) (From Bräuner, T. and Hülser, D. F., Tumor cell invasion and gap junctional communication. II. Normal and malignant cells confronted in multicell spheroids, *Invasion Metastasis*, 10, 16, 1990. With permission of S. Karger AG, Basel.)

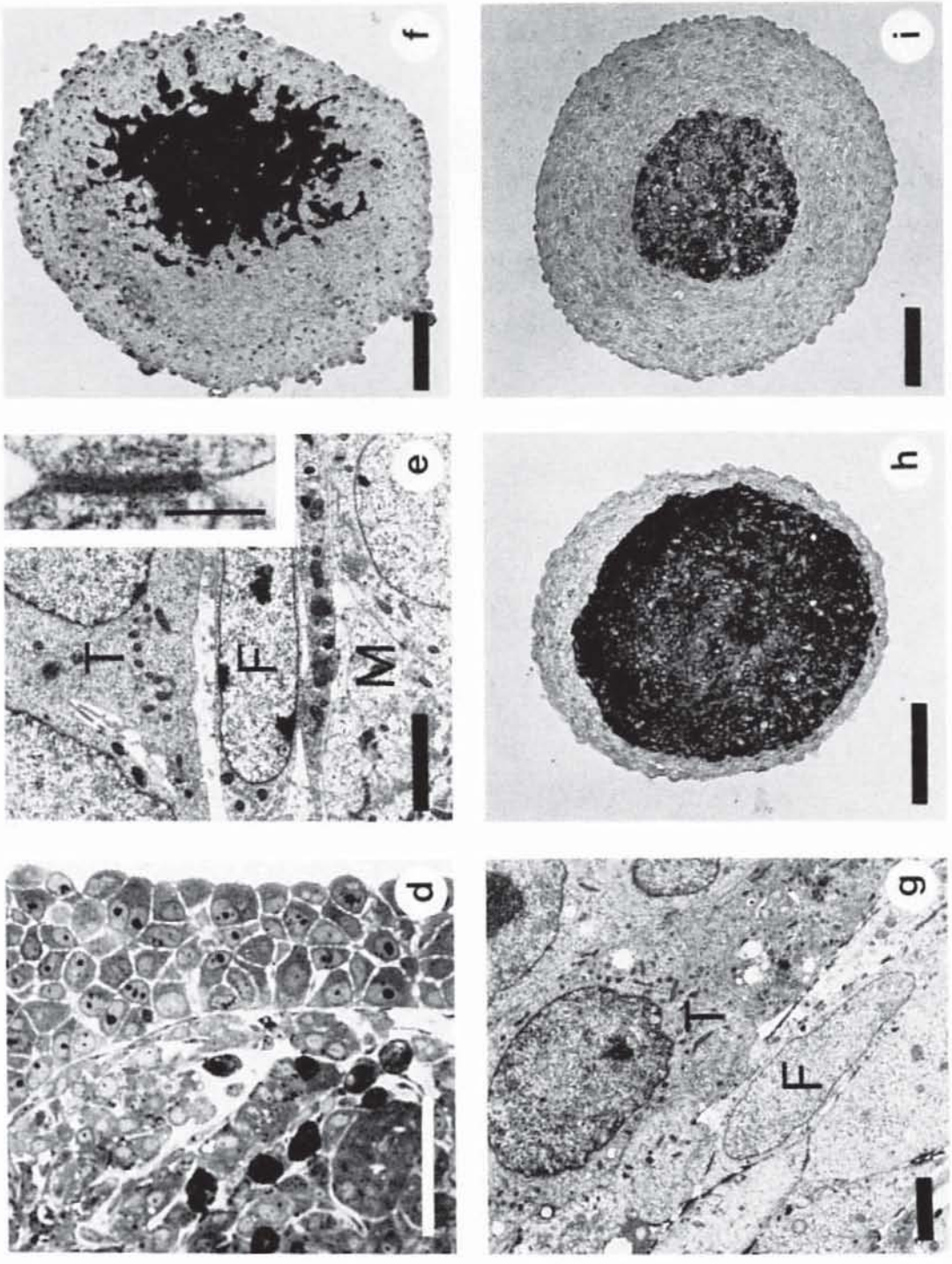

FIGURE 11 (continued).

they accumulated immunoreactive material. A dramatic change was observed after 125 h; HeLa cells infiltrated into the heart tissue from all directions, completely destroying its peripheral region. The cellular organization of central parts of the host tissue, however, was conserved much longer compared with the coupled tumor cell lines. For L cells, even after 160 h, no destruction of the heart tissue was observed, although it was surrounded by a thick L-cell capsule. Immunohistochemical staining revealed no immunoreactive material in L cells.

IV. DISCUSSION

Gap junctions provide a path for intercellular transport of ions and metabolites and form a syncytium-like situation in tissue and organs. In spite of this coupling, the cells preserve their individuality and control the opening or closing of gap-junction channels, which is necessary for development and cell differentiation. On the other hand, coordinated cellular functions, such as synchronous beating of heart muscle cells or contraction of the smooth muscle cells in the intestine, may be modulated by additional superior mechanisms. Furthermore, gap-junction communication may not only affect the transformation of normal cells to malignant cells, but may also determine some prospective reactions and the fate of tumor cells within the body.

Most investigations on intercellular communication have been performed with cells in monolayer cultures. It seems, therefore, essential to question whether these results obtained for the two-dimensional case are also valid in three-dimensional growth — the natural situation in animals. Many coupling-competent cells remain coupled over a wide range of culture conditions (e.g., cell density, pH, or temperature of medium, serum, etc.) when they grow as monolayer cultures. This coupling is best visualized by the injection of Lucifer yellow and its diffusion into neighboring cells. However, when dye spreading is not observed, this does not necessarily indicate an absence of intercellular communication.[16] The complete closure of gap-junction channels can only be demonstrated with high-resolution electrophysiology, using the double whole-cell patch-clamp technique.[47] This method can only be performed with a sufficient accuracy at isolated cell pairs; in multicell spheroids, complex junction paths obscure these measurements. A total absence of gap junctions in a cell line must be ascertained with molecular techniques by demonstrating the lack of gap-junction RNA or protein.

For the tumor cells presented here, the degree of coupling in monolayer cultures was highest for BICR/M1R_k cells and lowest for C6 cells, which compared to the coupling in embryonic chicken heart cells. Since for cocultures the amount of coupling is given by cells with the lower coupling capacity, heart cells should be the limiting factor for metabolic coupling in the *in vitro* invasion assay. It is unclear, however, if this is also true for the three-dimensional multicell spheroids. Under this more complex growth condition,

regulatory mechanisms are activated that are not effective in monolayer cultures. Figure 7a–d illustrated this situation for BICR/M1R$_k$ cells, where the Lucifer yellow spreading decreased with increased spheroid size. Even at complete inhibition of dye transfer between these cells, electrical coupling was measured, and numerous gap junctions were still found at their contact sites when ionic coupling was highly reduced.

A closer view between the cells (Figure 8) reveals many filopodia that provide a close contact even between cells that are loosely packed in a three-dimensional architecture. In addition, a specific extracellular matrix is found that is not as evident between monolayer cells.[48] Intracellular regulation processes may well depend on feedback mechanisms that are triggered by this extracellular matrix. Indeed, cells grown as multicell spheroids are subject to a limited endogenous regulation, as has been shown for the radiosensitivity of tumor cells. This resistance against radiation had been termed contact effect;[49,50] its magnitude was correlated with the degree of coupling.[32] Cell lines without gap-junction coupling did not exhibit this contact effect. It is noteworthy that in these cases, the electrical coupling was measured in monolayer cells, whereas the contact effect was determined for cells grown as multicell spheroids, where the amount of coupling was already reduced.

These few examples shed some light on the complex situation with three-dimensional cultures and reveal the dilemma that one faces in the quantification of intercellular communication under these growth conditions. On the one hand, exact measurements of intercellular communication are possible only in monolayer cultures or in cell pairs, while on the other hand, sufficient evidence has been accumulated to strengthen the idea of physiological regulations that take place only in three-dimensional growth and that clearly depend on the coupling capacity of the cells, as determined in a monolayer culture. As a consequence of activated regulation processes, the gap-junction conductance may well be reduced in spheroids. Other techniques besides the electrophysiological measurements must therefore be used to determine the coupling status of cells in a three-dimensional culture.

When gap-junction permeability is reduced, the concentration of intracellular messengers that interfere with the junction coupling ought to be varied. A key molecule for the regulation of gap-junction conductance is cAMP (for review, see De Mello[51]). This signal molecule was always reduced in multicell spheroids of coupled cells when compared with the same cells in monolayer culture; noncoupled cells had the same (low) cAMP concentrations under both growth conditions.[32,52] Thus, a reduced cAMP concentration may well indicate a decreased junction coupling but gives no clues to the actual junction conductance.

Another possibility for the detection of the coupling status of three-dimensionally grown cells may be obtained from electron microscopical techniques. However, conventional electron microscopical pictures give no information about the activity of the replicated channels. Since the addition of fixatives

(e.g., glutardialdehyde) to cell material causes a rapid and irreversible uncoupling of gap-junction communication, only degenerated gap junctions are seen under the electron microscope. This effect is amplified by cryoprotectants (e.g., glycerol) that enhance an aggregation of gap-junction proteins. Thus, a picture of tightly packed channels within a plaque normally represents closed gap junctions. Only with rapid freezing methods may different gap-junction plaques be found: the tightly packed hexagonal arrays rather indicating nonactive (permanently closed) channels, whereas the active channels are represented by the gap-junction plaques with a wider center-to-center space.[53] Detailed analyzes of the ratio of active to nonactive gap-junction plaques in multicell spheroids are still lacking and may well fail because of the size of the spheroids, which makes rapid freezing difficult.

In spite of all these difficulties in evaluating the actual coupling status in a multicell spheroid, some interesting consequences of junction coupling for the behavior of tumor cells can be demonstrated. Comparing electrophysiological measurements, light microscopical histology, and ultrastructural data, an invasion of gap-junction-coupled tumor cells into precultured heart fragments was observed, whereas noncoupled cells were unable to protrude into the normal tissue (see Figures 10 and 11).[24,25] Since the onset of gap-junction coupling could be demonstrated in electrophysiological measurements with high resolution only 1 min after the formation of intercellular mechanical contacts,[54] heterologous intercellular communication might well influence the process of tumor cell invasion from the moment of initial contact between host and tumor cells.

A possible participation of intercellular communication in tumor invasion may be discussed under two aspects: (1) advantages resulting from homologous gap-junction coupling between tumor cells, and (2) advantages resulting from heterologous gap-junction coupling between host and tumor cells. In the first case, tumor cells that leave their bulk aggregate and start invasion soon face the alien metabolic environment of the normal tissue. Under these conditions tumor cell survival is an essential prerequisite for the process of invasion, which might be supported by homologous intercellular spreading of metabolites[15] and regulatory molecules[55] into tumor cells at the invasion front. Heterologous gap-junction coupling might result in a parasitic participation of invasive tumor cells in the metabolism of the normal host cell. Numerous mitotic tumor cells were detected in close contact with the normal tissue,[24] demonstrating a high metabolic and proliferative activity, which might partly be supported by a transjunctional flow of metabolites or signal molecules from host to tumor cells. Mehta et al.[56] observed growth inhibition of various chemically and virally transformed monolayer cells when cocultured with normal cells, and postulated a transmittance of growth-regulating signals from cell to cell via gap junctions. Since this growth inhibition correlated with the frequency of heterologous communication, the authors postulated a flow of growth-inhibiting signals via gap-junction channels from the growth-arrested

normal cell population into the transformed cells. These contradictory results may have their roots in the different test systems: monolayer 10 T1/2 cells as a host for transformed cells, compared to embryonic heart fragments, which clearly did not affect tumor cell proliferation. Furthermore, for tumor cell invasion the growth rate of tumor cells is of minor importance: growth-inhibited mouse fibrosarcoma cells were able to invade embryonic chick heart fragments.[57]

An interesting phenomenon could be observed with the noncoupled epithelioid HeLa cells. In three-dimensional culture these cells exhibited a high packing density, with numerous tight junctions sealing the plasma membranes of neighboring cells together. In confrontation cultures there was a 4-day phase of coexistence between host and HeLa cells during which neither degradation of host tissue structures nor any other invasive activities of the tumor cells could be detected. Later, the growth of tightly packed HeLa cells around the host tissue caused its progressive destabilization, resulting in destruction and infiltration.

In our confrontation cultures with the noncoupled fibroblastioid L cells, no invasive activities have been observed over a cultivation period of 7 d, although L cells established a very thick cellular wall around the host tissue. Due to their fibroblastioid morphology with numerous protruding cellular processes, the proportion of free intercellular space was much higher as compared with HeLa cells. In addition, L cells do not form tight junctions; thus, the diffusion of nutrients to the host tissue in the center of confrontation cultures may not be blocked. This could explain why L cells do not destroy host tissue by the passive mechanism proposed for HeLa cells.

Active tumor cell invasion coincided with the ability of tumor cells to communicate with the host cells via gap junctions. The lack of gap-junction communication is associated either with a failure to invade or with a destruction of the host tissue according to a totally different, more passive mechanism. This conclusion is supported by comparing electrophysiological data of several tumor cell lines and histological results obtained with the same *in vitro* invasion assay. Invasive activities were detected with nine tumor cell lines[35] described as electrically coupled.[30] Embryonic rat cells that spontaneously transformed to permanent growth, did not exhibit ionic coupling[30] and were noninvasive in the *in vitro* assay.[35]

V. CONCLUSIONS

Cells that are interconnected by gap junctions in a monolayer culture change their degree of coupling when cultured as multicell spheroids. This reduction of junction permeability seems to be correlated with some physiological cell changes. The trigger that leads to this selective permeability in spheroids is not yet known. Nevertheless, these findings underline the advantage of multicell spheroids over monolayer cultures for the simulation of *in vivo* situations. A

down-regulation of the gap-junction channels may well be an expression of a more selective permeability or of a reduction of the exclusion size for permeating molecules. When heterologous coupling is established, this regulation may be controlled by each of the contacting cells or dominated by one cell only. Additional information about these interactions will contribute to a better understanding of the invasion and/or survival of tumor cells in normal tissue.

ACKNOWLEDGMENTS

I thank Mrs. Beate Rehkopf for her skillful technical and secretarial assistance. I am grateful to Dr. Gerald Schatten and his colleagues in Madison, WI, for their help with the scanning electron microscope. Work from my laboratory reported in this review was supported by grants from the Deutsche Forschungsgemeinschaft and the Bundesministerium für Forschung und Technologie.

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