

## Tumor Cell Invasion and Gap Junctional Communication

### II. Normal and Malignant Cells Confronted in Multicell Spheroids<sup>1</sup>

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**Key Words.** Invasion · Gap junction · Intercellular communication · Multicell spheroids · Heterologous coupling

**Abstract.** The invasive behavior of five tumor cell lines was investigated with an in vitro invasion assay developed by Mareel et al. Spheroids of all 5 cell lines readily attached to precultured heart fragments (PHF), resulting in confronting pairs. Tumor cells (BICR/MIR<sub>4</sub>, C6, and EMT6/Ro) which communicated with the host tissue via gap junctions, rapidly invaded the PHF within 3 days. Population doubling time or migratory activities had no influence on the invasion process. The noncoupled HeLa and L cells formed a cellular capsule around the PHF and showed no invasive activities. HeLa cells, however, started to destroy the PHF after 4 days. We suggest a mechanism different from that of coupled tumor cells. Epithelioid HeLa cells are linked by numerous tight junctions and may, therefore, cut off the nutrition supply for the inner-laying PHF, resulting in a disintegration of the heart tissue.

### Introduction

Invasion of malignant cells into normal host tissue is a common process during the metastatic cascade. Therefore, much attention has been focused on model systems which simulate tumor-host cell interactions. In general, in vitro systems allow a separate analysis of specific factors potentially involved in tumor cell invasion. They provide the advantage of reproducible experimental conditions in a defined and controlled culture environment and offer an easy access for technical manipulations. A large

<sup>1</sup> This work was supported by a grant from the Bundesministerium für Forschung und Technologie (03-8549-0).

variety of *in vitro* models have been introduced into cancer research, in most cases employing primary cultures of normal cells as a host for cultured tumor cells [11, for review].

Two-dimensionally growing monolayer cultures offer the possibility to observe under the microscope tumor-host interactions during the cultivation period, thereby allowing measurements of cellular properties in individual cells, as described in part 1. The behavior of tumor cells in these monolayer cultures, however, is mainly influenced by their contact with the artificial plastic substrate of the culture dish [1, 2, 13], thus limiting the relevance of results from such experiments for the *in vivo* situation. Considerable progress has been made by using three-dimensionally growing cell cultures introduced by Holtfreter [7] and Moscona [17, 18] for the cultivation of reaggregated embryonic cells. With this technique, cell aggregates are either cultivated on a nonadhesive agar surface or kept in constant motion on a gyratory shaker, thus avoiding adhesion to glass or plastic surfaces and regaining the three-dimensional histotypical organization of the tissue. Multicell spheroids, cultured from tumor cells of various origins, exhibited structures analogous to those observed in the original tumor [3, 21]. Modification of this technique and systematical investigations on multicell tumor spheroids by Sutherland et al. [26, 27] triggered numerous studies employing these cell aggregates in many fields of experimental cancer research [19, for review]. For studies on tumor cell invasion, Mareel et al. [13] developed an *in vitro* invasion assay using precultured fragments of embryonic chick heart confronted with multicell tumor spheroids. Comparative investigations with this three-dimensional culture model and tumor cell implantation into syngeneic animals demonstrated a direct correlation between invasion *in vitro* and formation of invasive tumors *in vivo* [4]. Using this assay in a slightly modified version, we investigated whether gap junctional communication between normal and malignant cells – i.e. the ability of tumor cells to participate in the host cells' metabolism – has an influence on their invasive behavior.

## Material and Methods

### *Cells*

Origin and maintenance of the tumor cell lines BICR/M1R<sub>k</sub>, EMT6/Ro, C6, L and HeLa is described in the first paper of this series.

Multicell tumor spheroids (MTS) were initiated by seeding single cell suspensions ( $1-2 \times 10^6$  cells per dish) in 94-mm diameter plastic Petri dishes (Greiner, Nürtingen,



FRG) with a nonadherent surface. These cell suspensions were obtained after treatment of monolayer cultures grown in tissue culture flasks (Nunc, Roskilde, Denmark) with 0.25% trypsin [in phosphate-buffered saline (PBS) without calcium and magnesium]. Within 3–7 days, cells aggregated and were transferred into spinner flasks (Bellco Glass, Vineland, N.J.) filled with 60 ml culture medium at pH 7.4 and 37 °C in a humidified incubator with 5% CO<sub>2</sub> atmosphere. Cell aggregates were cultured on magnetic stirrers (type EOA-W, IKA-Werk, Staufen, FRG) at 120 rpm regulated with a control unit (type ES 5, IKA-Werk). 2/3 of the culture medium were renewed daily and MTS were screened microscopically for growth and morphology.

The directional migration of each cell line was measured with 22–24 multicell spheroids which were selected for size (300 µm diameter) under stereomicroscopical control and seeded individually in each of the 48 wells of a plastic multiwell dish (Costar 3548, Cambridge, Mass.) filled with culture medium and incubated in 5% CO<sub>2</sub> atmosphere at 37 °C. The area, covered by cells that migrated radially from the spheroids, was determined during a 5-day period. The daily increase of the mean distance was plotted and the time points for 2 mm were taken from these curves.

For the determination of population doubling times, monolayer cultures were trypsinized, suspended in culture medium and seeded in 60-mm diameter plastic Petri dishes (Falcon, 3002 F, Becton Dickinson, Mountainview, Calif.) at cell densities between  $2.75 \times 10^5$  and  $6.30 \times 10^5$  cells per Petri dish. Over 4 days, the cell number per Petri dish was determined in a hemocytometer (2 Petri dishes per time point, 2 time points per day) after trypsinization of monolayer cultures. Cell counts (mean values) were plotted logarithmically versus time and population doubling times were taken from the exponential growth phase.

Embryonic chick heart fragments were obtained by dissecting organs of 9-day-old embryos under stereomicroscopical control. Freshly cut fragments were transferred into spinner flasks filled with 60 ml culture medium and stirred at 120 rpm for 4 days at 37 °C in a humidified incubator with 5% CO<sub>2</sub>.

#### *Confrontation Cultures*

After 4 days, precultured heart fragments (PHF) of 400 µm diameter were collected under stereomicroscopical control and individually brought in contact with an MTS of 200 µm diameter on semisolid agar medium (0.2 g agar in 10 ml Ringer solution and 20 ml culture medium) in a Petri dish, as described by Mareel et al. [13]. After an incubation period of 1–2 h, confronting pairs were transferred into medium-filled multiwell dishes (Costar 3548) base coated with semisolid agar medium and individually cultured at 37 °C in a humidified incubator with 5% CO<sub>2</sub>.

#### *Light Microscopical Histology*

MTS, PHF and confrontation cultures were washed in PBS and fixed in Bouin-solution for 2 h at room temperature. They were dehydrated in ethanol and in isopropanol and embedded in Paraplast Plus (Polyscience, Warrington, Pa.). 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 coworkers [14, for details], using the unlabeled antibody enzyme method [24] with details pub-

lished by Fritz et al. [5] and Mulhaupt et al. [20]. Briefly, the primary antiserum was diluted 1:250 in a mixture of unspecific pig serum and Tris-saline and applied to the tissue sections overnight in a refrigerator. After washing procedures, tissue sections were incubated for 2 h at room temperature with pig antirabbit immunoglobulin G serum (Dakopatts, Denmark), diluted 1:20 in a mixture of unspecific pig serum and Tris-saline. Sections were again washed and incubated for 1 h at room temperature with a rabbit peroxidase/antiperoxidase complex (Dakopatts) diluted 1:100 in a mixture of unspecific pig serum and Tris-saline. After washing, sections were incubated for 30 min at room temperature in the substrate and 3-amino-9-ethyl-carbazole as the electron donor. The enzyme reaction was stopped by washing the slides in distilled water and sections were counter-stained for 15 s in Papanicolaou's hemalaun solution (Merck, Darmstadt, FRG), rinsed with water and mounted in glycerol-gelatin (Merck).

#### *Electron Microscopical Histology*

Cell cultures were washed twice in PBS, fixed in 2.5% glutardialdehyde (Merck) and postfixed in 1% osmium tetroxide (Merck) at room temperature. Cells were embedded, stained and sectioned as described in the first paper of this series.

## Results

### *Multicell Tumor Spheroids*

BICR/M1R<sub>k</sub>-MTS were composed of large cells, which were loosely packed (fig. 1a). In thin sections, a wide intercellular space was apparent (fig. 1b). At sites of intercellular contacts, gap junctions and desmosomes could be frequently detected (fig. 1c). A high packing density of small fibroblastoid cells could be observed in EMT6/Ro-MTS (fig. 1d). Ultrathin sections exhibited a large number of fine cellular processes (fig. 1e) and numerous gap junctions (fig. 1f). In MTS of C6 rat glioma cells, small fibroblastoid cells were densely arranged (fig. 1g, h), with large areas of intercellular contacts. Specialized cell junctions (fig. 1i), however, could only rarely be detected. L-MTS exhibited a high packing density (fig. 1j), with many contacts between the fibroblastoid cells (fig. 1k). However, only few desmosomes or focal contacts have been found (fig. 1l). Thin sections revealed a large number of fine cellular processes (fig. 1k). In HeLa-MTS, the epithelioid cells are tightly packed (fig. 1m), forming a dense external cell layer of epithelial morphology (fig. 1n) with numerous tight junctions (fig. 1o).

### *Directional Migration and Population Doubling Time*

MTS of all cell lines adhered within 1 or 2 h to the plastic surface of the multiwell dish. After 24 h, cells that migrated radially from the spher-

**Table 1.** Population doubling times and directional migration of the investigated tumor cell lines

Cell line	Population doubling time <sup>1</sup> , h	Directional migration <sup>2</sup> , h
BICR/M1R <sub>k</sub>	10.5	92.75
C6	14.75	80.0
EMT6/Ro	15.75	70.0
L	19.0	93.5
HeLa	18.5	126.5

<sup>1</sup> Population doubling times were derived from the exponential growth phase of monolayer cell cultures.

<sup>2</sup> Directional migration was quantified by measuring the time  $t_{2\text{mm}}$  at which cells migrating radially from MTS with a diameter of 300  $\mu\text{m}$  covered a circular area with a diameter of 2 mm.

roids already covered a circular area with diameters reaching from 0.66 mm for BICR/M1R<sub>k</sub> cells to 0.89 mm for EMT6/Ro cells. For all cell lines we determined the time  $t_{2\text{mm}}$ , at which maximally migrating cells covered a circular area with a diameter of 2 mm. These data are listed in table 1.

The population doubling time of the investigated cell lines was determined from growth curves which were obtained with monolayer cultures. It ranged from 10 h 30 min for BICR/M1R<sub>k</sub> cells to 19 h for L cells (table 1).

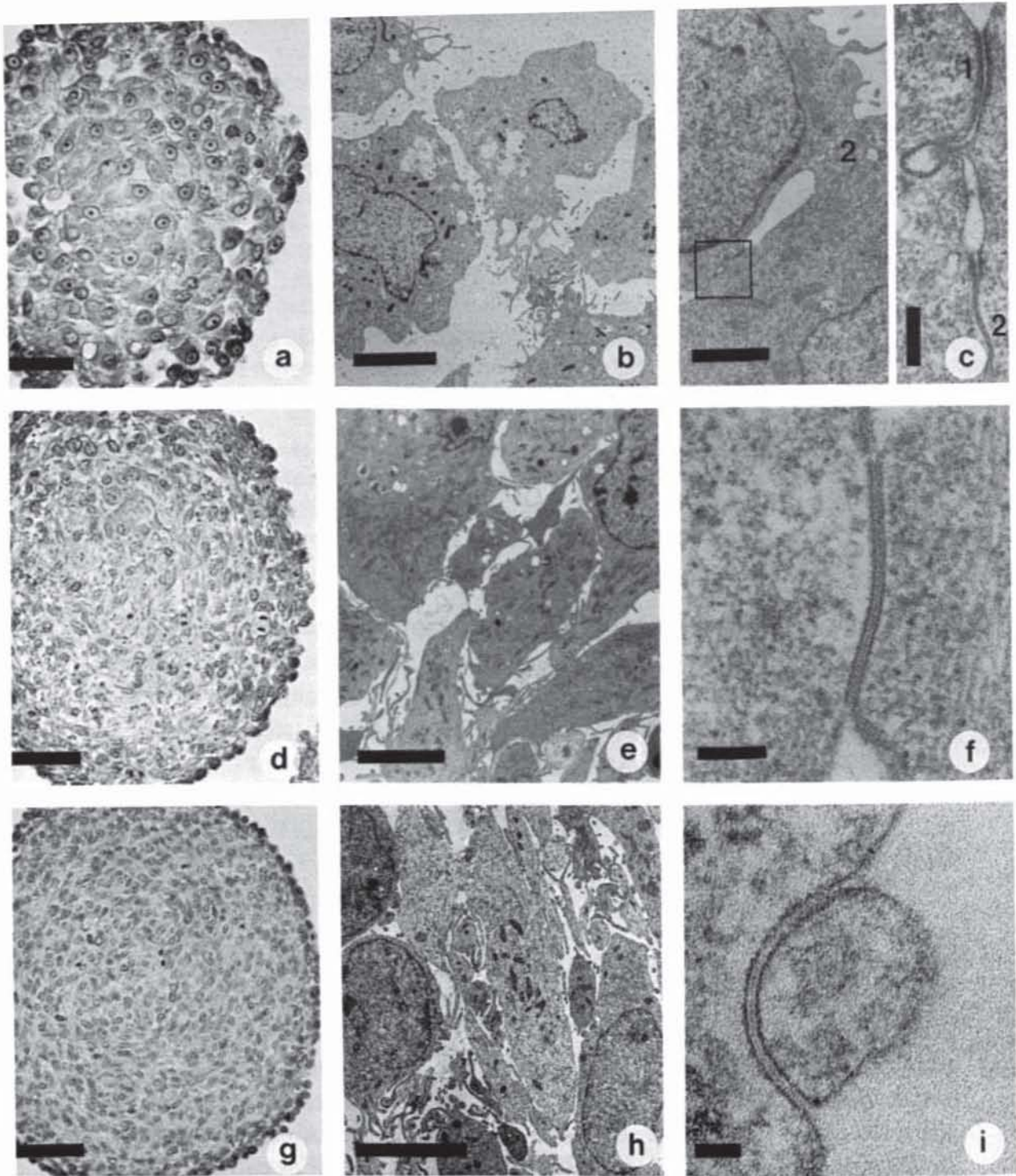
#### *Heart Fragments*

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 fibroblastoid cells surrounded a core of muscle cells (fig. 1p, q), forming the PHF. In thin sections, both linear and annular gap junctions were found (fig. 1r). These PHF remained almost constant in diameter over the experimental periods, and exhibited rhythmical spontaneous contractions over several days. For confrontation with MTS, only PHF with a diameter of about 400  $\mu\text{m}$  were selected.

#### *Immunohistochemical Staining*

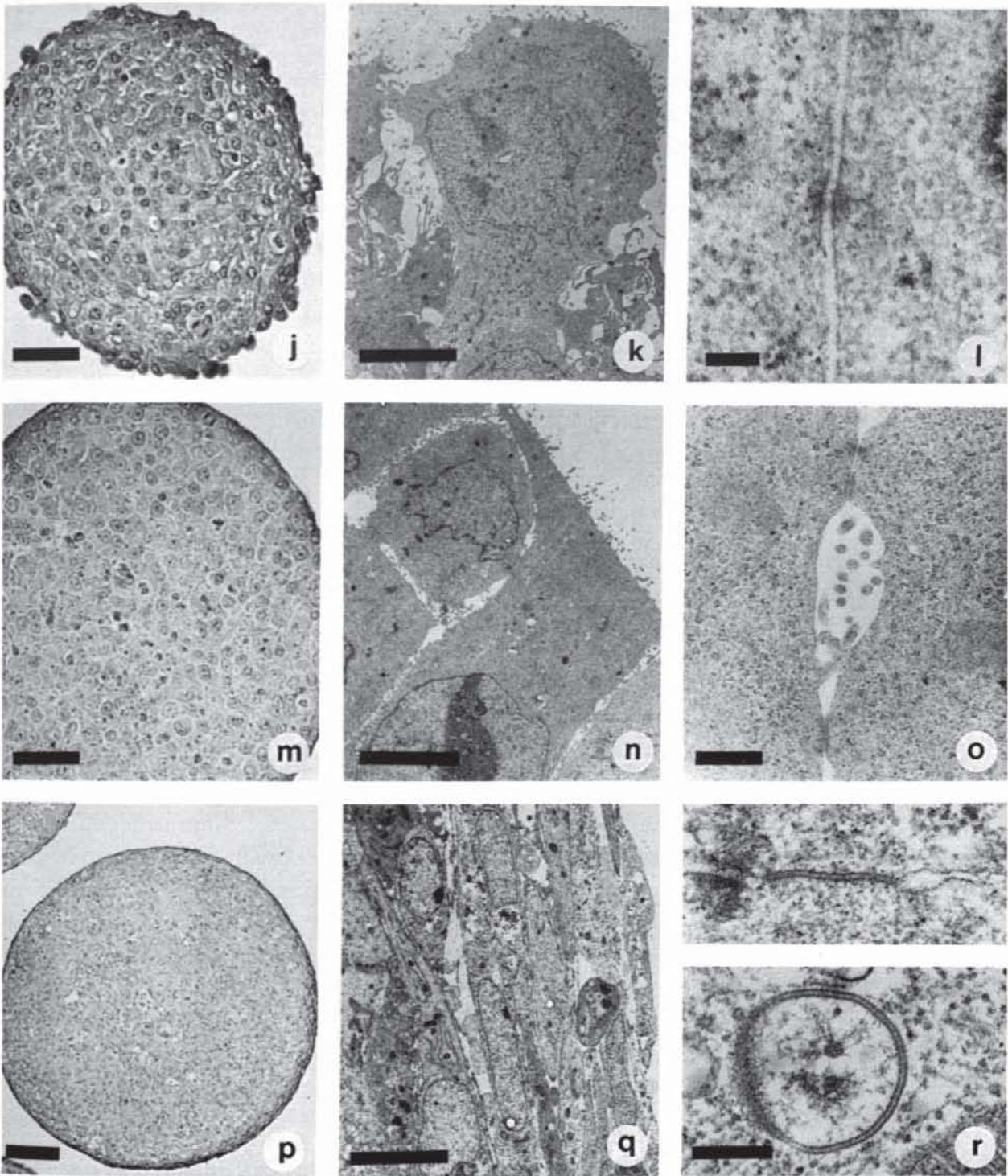
The staining pattern of PHF with the antiserum against total embryonic chick heart corresponded with the results described by Mareel et al. [14]. Briefly, myoblasts were heavily stained, whereas the peripheral





**Fig. 1.** Light and electron microscopical investigations of MTS and PHF. **a, b** BICR/M1R<sub>k</sub>-MTS after 6 days; bar: 50  $\mu$ m (**a**), 5  $\mu$ m (**b**). **c** Regions of contact between BICR/M1R<sub>k</sub> cells; bar: 2  $\mu$ m. Detail: 1 desmosome, 2 gap junction; bar: 0.25  $\mu$ m. **d, e** EMT6/Ro-MTS after 6 days (**d**) and 4 days (**e**); bar: 50  $\mu$ m (**d**), 5  $\mu$ m (**e**). **f** Gap junction between EMT6/Ro cells; bar: 0.1  $\mu$ m. **g, h** C6-MTS after 6 days (**g**) and 5 days (**h**); bar: 50  $\mu$ m (**g**), 5  $\mu$ m (**h**). **i** Gap junction between C6 cells; bar: 0.05  $\mu$ m (see also fig. 1i of part 1 in





this volume). **j, k** L-MTS after 8 days (**j**) and 6 days (**k**); bar: 50  $\mu\text{m}$  (**j**), 5  $\mu\text{m}$  (**k**). **l** Focal contact between L cells; bar: 0.1  $\mu\text{m}$ . **m, n** HeLa-MTS after 6 days; bar: 50  $\mu\text{m}$  (**m**), 5  $\mu\text{m}$  (**n**). **o** Tight junctions and focal contact between HeLa cells; bar: 0.5  $\mu\text{m}$ . **p, q** PHF after 4 days; bar: 100  $\mu\text{m}$  (**p**); 5  $\mu\text{m}$  (**q**). **r** Linear (top) and annular (bottom) gap junctions in a PHF; bar: 0.2  $\mu\text{m}$ . Light microscopical sections were stained with hematoxylin-erythrosin, thin sections with uranyl acetate and lead citrate.

fibroblast layers exhibited only faint staining. The antiserum was tested for cross-reactions with the cell lines used in confrontation cultures with PHF and immunostaining was absent in homogeneous MTS of all cell lines.

#### *Confrontation Culture*

MTS of all 5 cell lines readily attached to the PHF and formed stable contacts within 2 h of incubation. This stage marks the onset for the chronology of all confrontation cultures. During the first 20 h, cells of MTS migrated to their attached PHF, totally covering it within another 20 h. Histological sections, however, revealed significant differences in the interactions of the respective tumor cells with the heart tissue. The results for confrontation cultures of PHF with MTS of each cell line are therefore presented separately.

#### *PHF + BICR/M1R<sub>k</sub>-MTS*

After 65 h, invasion of BICR/M1R<sub>k</sub> cells into the PHF could be detected at many sites. A large number of peripheral tumor cells contained immunoreactive material (fig. 2a). Very frequently, mitotic tumor cells could be observed adjacent to the normal tissue. A pronounced destruction of the PHF by invading tumor cells was apparent in confrontation cultures of 120 h (fig. 2b), after 160 h only a small central part of the PHF remained (fig. 2c).

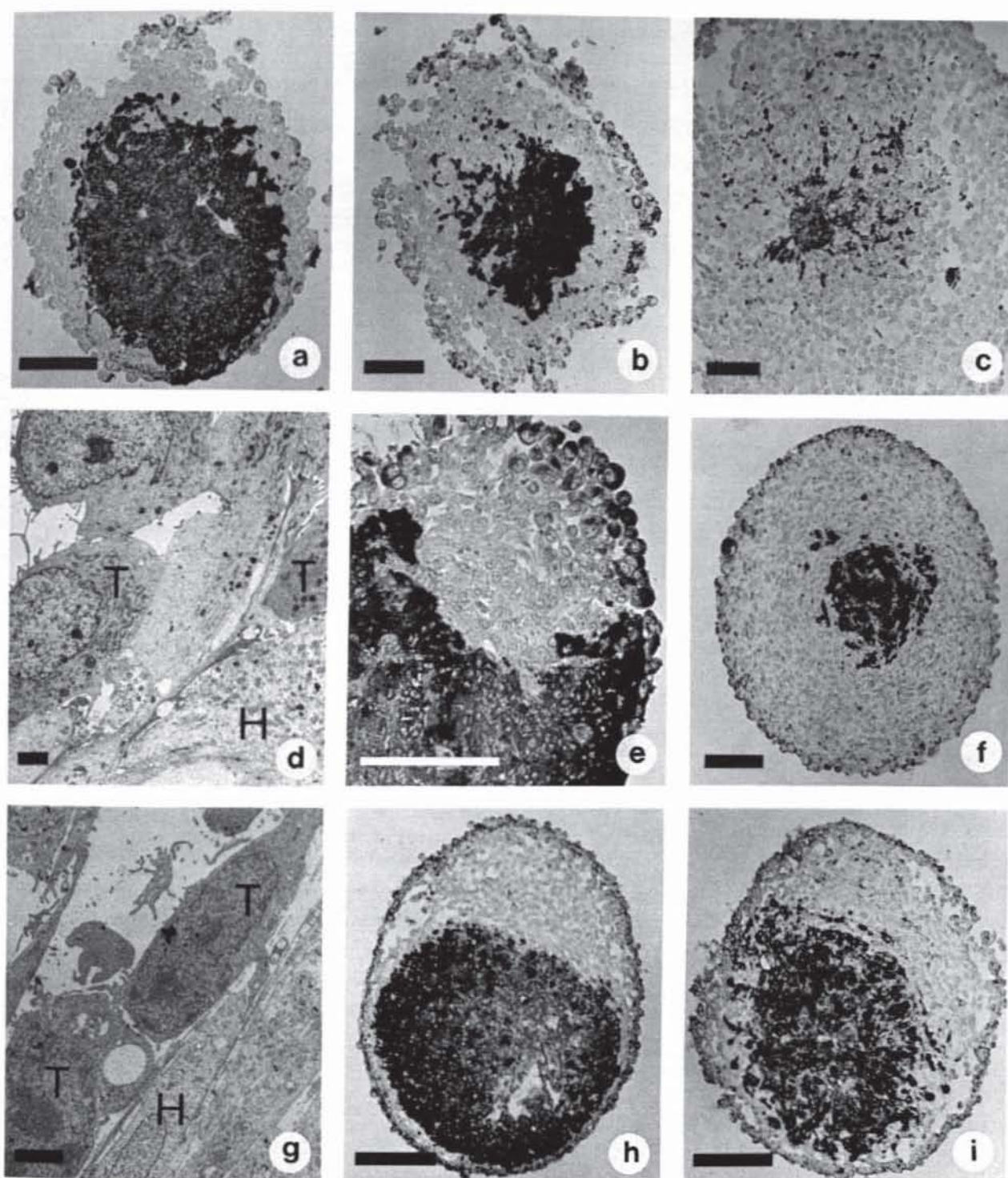
#### *PHF + EMT6/Ro-MTS*

Thin sections revealed extension of fine tumor cell processes between fibroblastoid heart cells already after 15 min (fig. 2d). With these tumor cells, destruction of the heart tissue could be clearly detected after 44 h. In these cultures peripheral EMT6/Ro cells also accumulated immunoreactive material (fig. 2e). With increasing incubation periods, further replacement of the heart tissue by invading tumor cells was observed (fig. 2f).

#### *PHF + C6-MTS*

With C6 glioma cells cytoplasmic extensions have been detected in the intercellular space between fibroblastoid heart cells 15 min after formation of stable cell contacts (fig. 2g). After 56 h, C6 cells had invaded the outer regions of the PHF (fig. 2h). At this time, tumor cells at the periphery of the MTS exhibited a high amount of immunoreactive material. At 84 h, C6 cells from all directions had already penetrated deeply into the PHF (fig. 2i), completely destroying the architecture of the heart tissue.





**Fig. 2.** Light and electron microscopical investigations of confrontation cultures of PHF with BICR/M1R<sub>k</sub>-MTS (a-c), EMT6/Ro-MTS (d-f,) and C6-MTS (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 [14] in remaining figures; bar: 2 μm in **d** and **g**, 100 μm in remaining figures.

### *PHF + HeLa-MTS*

With the noncoupled epithelioid cell line HeLa, protrusions of cytoplasmic tumor cell extensions between adjacent fibroblastoid heart cells could not be observed even after 19 h (fig. 3a). Over cultivation periods of up to 90 h (fig. 3b–d), the PHF was still clearly delineated, faced by epithelially arranged HeLa cells. Within this time HeLa cells did not exhibit invasive activities, although they accumulated immunoreactive material (fig. 3b). In the electron microscope a large number of coated pits in the HeLa cell membrane opposite to heart cells (fig. 3e) and numerous tight junctions between HeLa cells (fig. 3e, inset) were detected. A dramatic change was observed after 125 h: from all directions, HeLa cells were infiltrating into the PHF, completely destroying its peripheral region (fig. 3f). The cellular organization of central parts of the PHF, however, was much longer conserved, compared with the coupled cell lines BICR/M1R<sub>k</sub>, EMT6/Ro and C6.

### *PHF + L-MTS*

The noncoupled fibroblastoid L cells attached to the PHF without penetrating between fibroblastoid heart cells (fig. 3g). After 65 h, the PHF was completely encapsulated by a few layers of L cells, which did not exhibit any invasive growth (fig. 3h). Even after 160 h, no destruction of the heart tissue could be observed, although the PHF was surrounded by a thick L cell capsule (fig. 3i). Immunohistochemical staining revealed no immunoreactive material in L cells.

### *Controls*

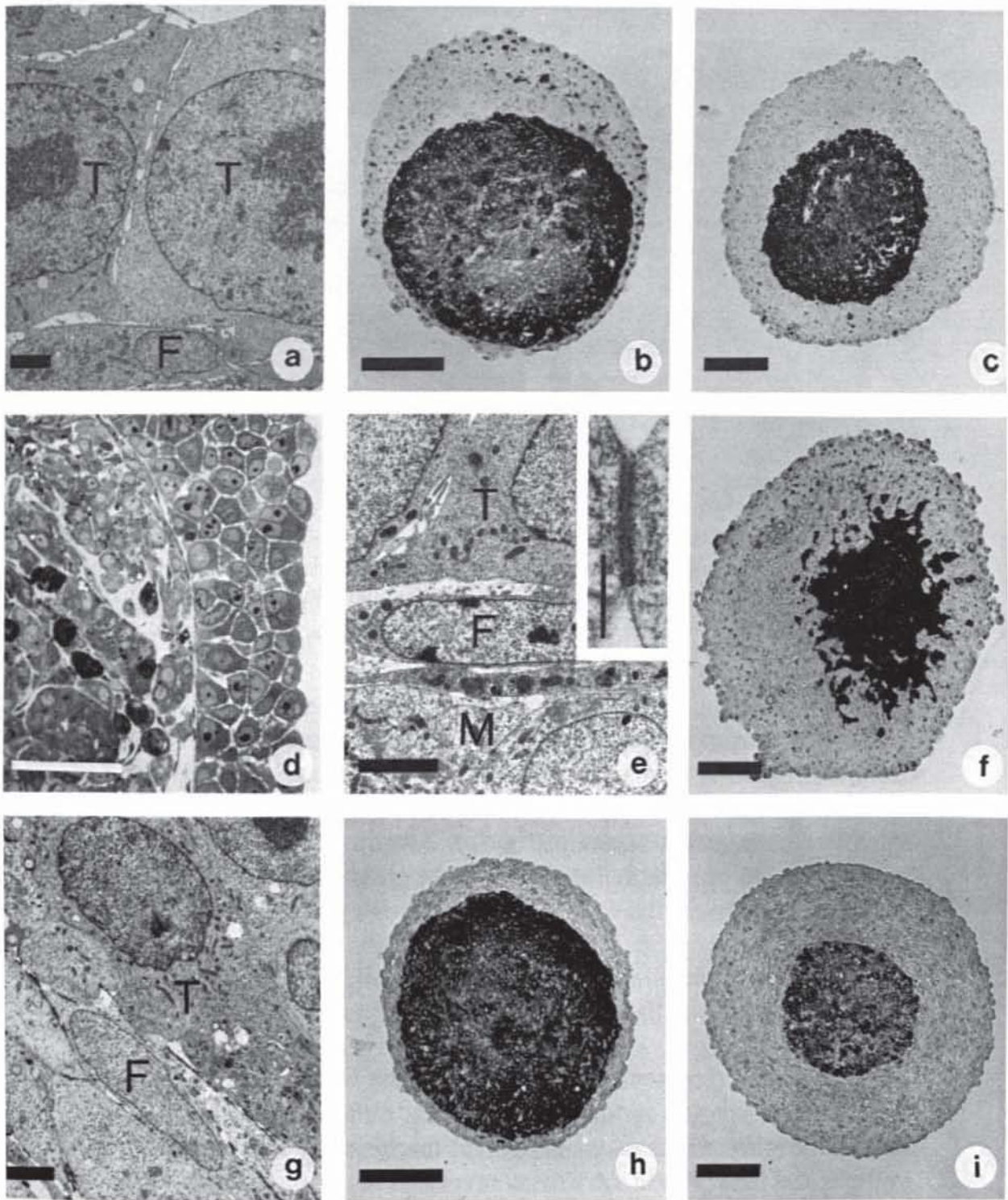
PHF were individually cultured on semisolid agar medium in absence of any MTS over 5 days. In histological sections, no degenerative alterations could be observed (fig. 4a), except for a slight reduction in the thickness of the fibroblastoid cell capsule.

In addition, cellular interactions between PHF and normal cells were investigated by confronting PHF with reaggregated cerebral cells of 9-day-old embryonic chicks. These spheroidal reagggregates also firmly attached

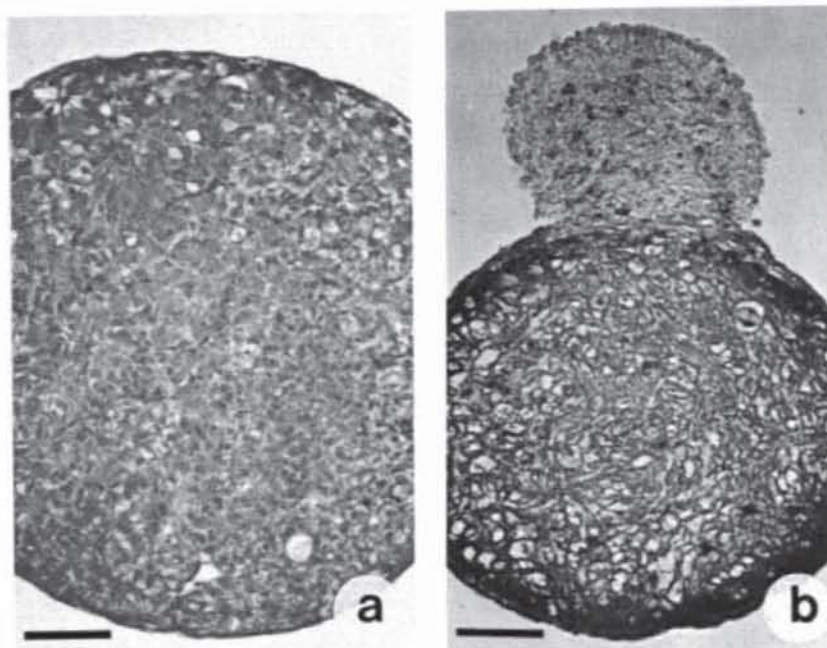
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**Fig. 3.** Light- and electron microscopical investigations of confrontation cultures of PHF with HeLa-MTS (a–f) and L-MTS (g–i). a after 19 h; b after 56 h; c after 84 h; d after 90 h (1- $\mu$ m section of an epon-embedded culture, stained with azur II and methylene blue); e after 90 h (inset: tight junction; bar: 0.25  $\mu$ m); 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, g and with an antiserum against embryonic chick heart [14] in remaining figures. Bar: 2  $\mu\text{m}$  in a, e, g; 100  $\mu\text{m}$  in remaining figures.



**Fig. 4.** Controls. **a** PHF after 125 h, stained with hematoxylin-erythrosin. **b** Confrontation of PHF with reaggregated embryonic brain cells after 136 h, stained with an anti-serum against embryonic chick heart [14]. Bar: 50  $\mu$ m.

to the PHF during incubation for 2 h. Histological sections of these confrontation cultures after a cultivation period of 136 h, however, revealed neither migration of embryonic cerebral cells onto the PHF, nor proliferation of the reaggregate culture (fig. 4b). Any invasive activities of embryonic chicken cerebral cells could never be observed in the confrontation cultures.

### Discussion

Our studies represent the first investigation on the effect of gap junctional communication on tumor cell invasion, obtained by a comparison of electrophysiological measurements, light microscopical histology, and ultrastructural data. The *in vitro* assay revealed the invasion of gap junctionally coupled tumor cells into PHF only minutes after the initial 2-hour incubation period which is necessary for the formation of stable cell contacts. BICR/M1R<sub>k</sub>, C6, and EMT6/Ro cells which communicate with embryonic chick heart cells via gap junctions (see part 1 in this volume)



occupied the intercellular space of the outer fibroblastoid heart cell layers via fine cellular protrusions, whereas the noncoupled HeLa and L cells were unable to protrude into the PHF. With all coupled tumor cell lines, invasion of PHF was evident within rather short culture periods of 2–3 days. Referring to this early occurrence of invasive behavior it is important to note that the onset of gap junctional coupling could be demonstrated in electrophysiological measurements with high resolution only 1 min after formation of intercellular mechanical contacts [8]. Heterologous intercellular communication might therefore influence the process of tumor cell invasion from the moment of initial contact formation 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 junctional coupling between tumor cells, and (2) advantages resulting from heterologous gap junctional coupling between host and tumor cells. In the first case, tumor cells that leave their bulk aggregate and start invasion are soon facing the metabolic environment of the normal tissue. Since tumor cell survival under these conditions is an essential prerequisite for the process of invasion, it is worth asking for mechanisms that might support tumor cells at the invasion front. In this regard, gap junctional coupling could play a major role, since gap junctions do not only allow equilibration of different intracellular ion concentrations, but also mediate the intercellular spreading of metabolites [25] and regulatory molecules [10].

Miller et al. [16] suggested that junctional communication might affect drug sensitivity of a tumor, especially when the inhibitory activity of the drug is related to its ability to be metabolically activated. In case of coupling the sensitivity of a cell can be modified, depending not only on its own metabolism, but also on that of its communicating neighbor cell. These authors mentioned that metabolites of small anticancer drug molecules, e.g. thioguanine or 5-fluorouracil may pass through gap junctional channels.

Referring to the second, above-mentioned aspects, gap junctional coupling between host and tumor cells 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, 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. [15] observed growth

inhibition of various chemically and virally transformed cells when they were cultured in contact with normal cells in Petri dishes and postulated a transmittance of growth-regulating signals from cell to cell via gap junctions. This growth inhibition correlated with the frequency of heterologous communication, implying – according to the authors – 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 partly be explained by the different test systems, i.e. Mehta et al. [15] used confluent monolayers of normally growing mouse 10 T1/2 cells as a host for transformed cells, whereas in our in vitro invasion assay, embryonic PHF served as host tissue. With this three-dimensional assay we could not measure tumor cell growth. However, the remarkable increase in the volume of our confrontation cultures with both coupled and noncoupled tumor cells during the incubation period and the presence of numerous mitotic tumor cells adjacent to the normal host tissue indicate that tumor cell proliferation was not affected by contacting normal cells. Considering tumor cell invasion, the growth rate of tumor cells was shown to be of minor importance: mouse fibrosarcoma cells which were growth inhibited by various anticancer agents, were able to invade embryonic chick PHF [12]. In addition, the three-dimensional tissue architecture of our confrontation cultures resembles much more the in vivo conditions than monolayer cultures. For example, our previous experiments with monolayer cultures of rat embryonic brain cells and various tumor cell lines demonstrated that most tumor cells did not adhere to normal cells, but readily settled on cell-free areas of the plastic Petri dish, piling up to cell aggregates in midst of normal brain cells – regardless of their homologous and heterologous coupling capacities [2].

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.

In confrontation cultures of PHF with HeLa cells we observed 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. During these 4 days, however, immunoreactive granules could be detected in HeLa cells. Since this immunoreactive material could not be correlated with any heart cell degradation at sites of host-to-tumor contacts in ultrastructural investigations, these granules may result from normal phagocytosis, which is not a specific property of



tumor cells, but a continuous process in all eucaryotic cells. Immunoreactive material in tumor cells could also be found in confrontation cultures with BICR/MIR<sub>k</sub>, C6 and EMT6/Ro cells, and have also been described by Mareel et al. [14] in 4 different tumor cell lines confronted with heart tissue.

The destruction and infiltration of PHF by HeLa cells after 5 days seems to be primarily a result of a progressive host tissue destabilization, caused by the growth of tightly packed HeLa cells around the PHF. HeLa cells encapsulating the PHF may cause a massive restriction of the nutrition supply for the inner-laying PHF due to numerous tight junctions acting as permeability barriers. Gabbert [6] postulated from observations made with an *in vivo* invasion model that the isolation of host tissue cells from nutritive blood vessels by surrounding tumor cells increased the diffusion distance for oxygen and other nutrients, thereby causing a shortage of these substrates. This effect can be intensified by the competition of tumor cells and neighboring host cells for essential substrates [6].

This more passive mechanism proposed for the noncoupled HeLa cells is clearly differing from that of coupled tumor cells which exhibited an early-starting active invasion. This view is supported by results obtained with two different experimental systems [22]. In the first case pre-cultured multicell spheroids of normal human fibroblasts were confronted with a HeLa single cell suspension in shaker culture. In this assay HeLa cells were not able to intrude into the host tissue but encapsulated the normal fibroblast spheroid due to their high proliferation rate. In a second system this author confronted reaggregates of normal adult human endometrium with HeLa cells. Intrusion of HeLa cells could only be observed at sites where either a wound – even of the size of one missing cell – or a lumen existed. In confrontation cultures with PHF and HeLa cells Mareel et al. [14] described a longer conservation of the tissue-specific organization of the PHF during invasion as compared with the fibroblastoid MO<sub>4</sub> or B16 cells.

In our confrontation cultures of PHF with the noncoupled fibroblastoid L cells no invasive activities have been observed over a cultivation period of 7 days, although L cells established a very thick cellular wall around the PHF. As demonstrated in homogeneous spheroids L cells are also densely packed. Due to their fibroblastoid morphology with numerous protruding cellular processes, the proportion of free intercellular space, however, was much higher as compared with HeLa cells. In addition, L cells do not form tight junctions, so that the diffusion of nutrients to the

PHF 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.

With the communicating tumor cell lines a major influence of tumor cell migration and proliferation on the process of invasion could not be detected. For example, migrating BICR/M1R<sub>k</sub> cells reached a 2-mm distance from their bulk spheroid more than 22 h later than EMT6/Ro cells, however, the rate of invasion detected in histological sections was similar. Comparison of electron microscopical sections with the results of the directional migration assay indicated that migratory capacity is influenced as well by the number of fine cellular processes as by the number and type of specialized cell contacts. The epithelial HeLa cells which exhibited only few cellular processes and were sealed together by numerous tight junctions revealed the slowest migration value. On the other hand, the highest migration rate was detected with EMT6/Ro cells, which were characterized by a very large number of cellular processes. Proliferation of tumor cells has obviously no effect on the invasion, as BICR/M1R<sub>k</sub> cells with a population doubling time of 10½ h and EMT6/Ro cells with a population doubling time of 15 h 45 min invaded PHF with a similar rate.

Summarizing our results we conclude that active tumor cell invasion of PHF coincided with the ability of tumor cells to communicate with the host cells via gap junctions. On the other hand, the lack of gap junctional communication is associated either with a failure to invade PHF 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 [9] and histological results [4] obtained with the same in vitro invasion assay as used in our studies. Invasive activities were detected with 9 tumor cell lines [4] described as electrically coupled [9]. Embryonic rat cells which spontaneously transformed to permanent growth did not exhibit ionic coupling [9] and were noninvasive in the in vitro assay [4]. The three-dimensional in vitro invasion assay enabled us to investigate the influence of intercellular communication, cell proliferation, and directional migration on an important step of the metastatic cascade, i.e. local tumor cell invasion, without interference of cellular and humoral immunity, stromal reactions, innervation, and blood supply. The uniform cellular organization of PHF and their regular spheroidal shape in connection with a standardized selected size offered the advantage of reproducible and comparable results and clearly demonstrated the suitability of this in vitro invasion model.



## Acknowledgments

The technical assistance of Mrs. Ulrike Reber and Mrs. Beate Rehkopf is greatly appreciated. We are especially grateful to Prof. Dr. M. Mareel and his staff (Laboratory of Experimental Cancerology, University Hospital, Ghent, Belgium) for helpful technical demonstrations and the donation of rabbit anti chick heart serum [14]. We thank Prof. Dr. R.M. Sutherland, Rochester, N.Y., USA for the donation of EMT6/Ro cells and Dr. H. Mulhaupt, Stuttgart, FRG for advice in immunohistochemistry.

## References

- 1 Benke, R.; Paweletz, N.: Scanning electron microscopic observations on cells grown in vitro. VII. HeLa cells can penetrate WI 38 fibroblasts. *Eur. J. Cell Biol.* 33: 52-54 (1984).
- 2 Bräuner, T.: Interzelluläre Kommunikation und invasives Wachstum maligner Zellen; thesis Stuttgart (Verlag Stöffler & Schütz 1987).
- 3 Dabrowska-Piaskowska, K.: Observations on the histoformative capacities of tumor cells dissociated by digestion with trypsin. *Exp. Cell Res.* 16: 315-323 (1959).
- 4 de Ridder, L.J.; Laerum, O.D.: Invasion of rat neurogenic cell lines in embryonic chick heart fragments in vitro. *J. natn. Cancer Inst.* 66: 723-728 (1981).
- 5 Fritz, P.; Müller, H.; Wegner, G.; Braun, U.; Grau, A.; Tuzcek, H.-V.; Moessner, E.; Schenk, R.: Immunohistochemie: theoretische Möglichkeiten, praktische Anwendung. *Zentbl. allg. Path. path. Anat.* 130: 187-203 (1985).
- 6 Gabbert, H.: Mechanisms of tumor invasion: evidence from in vivo observations. *Cancer Metastasis Rev.* 4: 293-309 (1985).
- 7 Holtfreter, J.: A study of the mechanics of gastrulation. *J. exp. Zool.* 95: 171-212 (1944).
- 8 Hülser, D.F.: Electrical characterization of gap junction formation. *Studia biophys.* 90: 197-198 (1982).
- 9 Laerum, O.D.; Hülser, D.F.; Rajewsky, M.F.: Electrophysiological properties of ethylnitrosourea-induced, neoplastic neurogenic rat cell lines cultured in vitro and in vivo. *Cancer Res.* 36: 2153-2161 (1976).
- 10 Lawrence, T.S.; Beers, W.H.; Gilula, N.B.: Transmission of hormonal stimulation by cell-to-cell communication. *Nature, Lond.* 272: 501-506 (1978).
- 11 Mareel, M.M.: Recent aspects of tumor invasiveness. *Int. Rev. exp. Pathol.* 22: 65-129 (1980).
- 12 Mareel, M.; De Brabander, M.: Effect of microtubule inhibitors on malignant invasion in vitro. *J. natn. Cancer Inst.* 61: 787-792 (1978).
- 13 Mareel, M.; Kint, J.; Meyvisch, C.: Methods of study of the invasion of malignant C3H-mouse fibroblasts into embryonic chick heart in vitro. *Virchows Arch. Abt. B Zellpath.* 30: 95-111 (1979).
- 14 Mareel, M.M.; De Bruyne, G.K.; Vandesande, C.; Dragonetti, C.: Immunohistochemical study of embryonic chick heart invaded by malignant cells in three-dimensional culture. *Invasion Metastasis* 1: 195-204 (1981).

- 15 Mehta, P.P.; Bertram, J.S.; Loewenstein, W.R.: Growth inhibition of transformed cells correlated with their junctional communication with normal cells. *Cell* 44: 187–196 (1986).
- 16 Miller, B.E.; Roi, L.D.; Howard, L.M.; Miller, F.R.: Quantitative selectivity of contact-mediated intercellular communication in a metastatic mouse mammary tumor line. *Cancer Res.* 43: 4102–4107 (1983).
- 17 Moscona, A.: Cell suspensions from organ rudiments of chick embryos. *Exp. Cell Res.* 3: 535–539 (1952).
- 18 Moscona, A.: Rotation-mediated histogenetic aggregation of dissociated cells. A quantifiable approach to cell interactions in vitro. *Exp. Cell Res.* 22: 455–475 (1961).
- 19 Müller-Klieser, W.: Multicellular spheroids. A review on cellular aggregates in cancer research. *J. Cancer Res. Clin. Oncol.* 113: 101–122 (1987).
- 20 Multhaupt, H.; Fritz, P.; Schumacher, K.: Immunohistochemical localisation of arginase in human liver using monoclonal antibodies against human liver arginase. *Histochemistry* 87: 465–470 (1987).
- 21 Schleich, A.: Studies on aggregation of human ascites tumor cells. *Eur. J. Cancer* 3: 243–246 (1967).
- 22 Schleich, A.: The confrontation of normal and malignant cells in vitro. An experimental system in tumor invasion studies; in Garattini, Franchi, *Chemotherapy of cancer dissemination and metastasis*; pp. 51–58 (Raven Press, New York 1973).
- 23 Spray, D.C.; Bennett, M.V.L.: Physiology and pharmacology of gap junctions. *Ann. Rev. Physiol.* 47: 281–303 (1985).
- 24 Sternberger, L.A.; Hardy, P.H.; Cuculis, J.J.; Meyer, H.G.: The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of Spirochetes. *J. Histochem. Cytochem.* 18: 315–333 (1970).
- 25 Subak-Sharpe, J.H.; Bürk, R.R.; Pitts, J.D.: Metabolic cooperation between biochemically marked cells in tissue culture. *J. Cell Sci.* 4: 353–367 (1969).
- 26 Sutherland, R.M.; McCredie, J.A., Inch, W.R.: Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *J. natn. Cancer Inst.* 46: 113–120 (1971).
- 27 Sutherland, R.M.; Carlsson, J., Durand, R.; Yuhas, J.: Meeting report: spheroids in cancer research. *Cancer Res.* 41: 2980–2984 (1981).

Received: November 15, 1988; revised version accepted: May 30, 1989

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