[CANCER RESEARCH 50, 358-362, January 15, 1990]

Ultrastructural Differences in Junctional Intercellular Communication between Highly and Weakly Metastatic Clones Derived from Rat Mammary Carcinoma

Jin Ren,[1] Jun-ichi Hamada, Noritoshi Takeichi, Seizo Fujikawa, and Hiroshi Kobayashi

Laboratory of Pathology, Cancer Institute, Hokkaido University School of Medicine [J. R., J-i. H., N. T., H. K.], and Institute of Low Temperature Science, Hokkaido University [S. F.], Sapporo 060, Japan

ABSTRACT

We examined by electron microscopy the differences in junctional intercellular communications among highly metastatic clones, weakly metastatic clones, and the parent clone obtained from a spontaneously developed rat mammary carcinoma. We also investigated intercellular communications of the highly and weakly metastatic clone cells with normal fibroblasts. The results showed that ultrastructural changes of the highly metastatic clone cells, such as microvilli, microfilaments, and small organelles including endoplasmic reticulum, Golgi apparatus, and mucous particles, were more distinct than those of the weakly metastatic clone cells, and that the numbers of desmosome and gap junctions of weakly metastatic clone cells were significantly greater than those of highly metastatic clone cells. The formation of gap junctions and desmosomes was found only between weakly metastatic clone cells and normal fibroblasts. When both highly and weakly metastatic clone cells were cultured with normal fibroblasts, a tight junction was observed only in the culture of weakly metastatic clone cells and normal fibroblasts.

These results suggest that ultrastructural differences are related to the proliferation and detachment of tumor cells from the primary site in the initial stage of tumor metastasis.

INTRODUCTION

Tumor metastasis consists of serial steps such as release of tumor cells from the primary site, intravasation, circulation, lodging at a favorable site, extravasation, and colonial growth at the new sites. Interaction between tumor cells and the host's condition is naturally involved in all of these steps (1). Interactions of tumor cells with other tumor cells, normal cells, or extracellular matrices are also considered to be important factors, especially in the early stage of the metastatic process; for example, tissue homeostasis and stabilization are usually maintained by developing junctional intercellular communications between the cells, such as gap junctions, desmosomes, and tight junctions. These intercellular communications are inevitably considered to play an important role in tumor metastasis (2).

Although ultrastructural features of these junctional complexes in both human tissues (3) and experimental tumor tissues (4) have been described in many reports, there is no report on the characteristics and effects of such junctional complexes between tumor cells and between tumor cells and normal cells during the course of metastasis. As described in our previous report (2), we found by use of a dye-transfer method that the incidence of intercellular communication between weakly metastatic clone cells and fibroblasts was significantly higher than that between highly metastatic clone cells and fibroblasts. To further confirm the role of junctional intercellular communication in the tumor metastasis process, we examined by electron microscopy ultrastructural features which appear between the highly and weakly metastatic clone cells and between metastatic clone cells and normal cells *in vivo* and *in vitro*.

MATERIALS AND METHODS

Tumor Cell Lines. The highly metastatic cell clones 2 and 3, the weakly metastatic cell clones 4 and 4-2, and the parent clone, all of which were derived from c-SST-2 (a spontaneously developed mammary adenocarcinoma in a SHR[2] rat) cells, were used. Each clone cell suspension was inoculated s.c. into SHR rats. After 20 to 30 days, the tumor tissue was removed and examined by a light microscope and an electron microscope. Each clone cell was mixed with normal fibroblasts isolated from newborn skin tissues of normal SHR rats 24 h after the removal. Morphological studies were carried out after the mixture was cultured for 1 or 2 days in a CO_2 incubator.

Measurement of Intercellular Communication by Means of Fluorescent Dye Transfer. The dye transfer method was described in our previous reports (2, 5). A 10% (w/v) solution of Lucifer Yellow CH (Sigma Chemical Co., St. Louis, MO) in 0.33 M lithium chloride solution was injected through glass capillaries under a microinjectoscope (Olympus injectoscope). To examine the intercellular communication between c-SST-2 clone cells and fibroblasts, tumor cells (1×10^5) were seeded into fibroblast cultures when the fibroblasts were sparsely confluent in 60-mm culture dishes. One to 2 days after coculturing, dye was microinjected into either an isolated tumor cell or a fibroblast and tested to examine whether or not the dye had been transferred into an adjacent cell (fibroblast or tumor cell). The transfer of the dye into the surrounding cells was observed under a fluorescence microscope about 10 min after the injection.

Light and Electron Microscopic Observations. The tumor tissues and the cultured cells were fixed in 10% formalin for hemotoxylin staining. For electron microscopic study, they were fixed in 2.5% glutaraldehyde in cacodylate buffer at pH 7.4 overnight. The tissue blocks were then postfixed with 1% osmic acid for 2 h, followed by dehydration and embedding in Epon 812. Ultrathin sections were made by a microtome (Hitachi, Tokyo, Japan), stained with uranyl acetate and lead citrate, and examined by an electron microscope (Model H-800; Hitachi).

The Freeze-Fracture Method. In the method as described in the previous report (6), fresh tissue blocks were fixed with 2.5% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.2, at 4°C for 2 h, immersed in 30% glycerol overnight, and rapidly freeze fixed. Frozen replicates were made from the frozen blocks by Model JFD-7000 freeze-etching equipment (JEOL, Ltd., Tokyo, Japan) and examined by a Model JEM 100-C electron microscope.

RESULTS

Metastatic Ability of a Parental c-SST-2 Cell Line and Its Clones in SHR Rats. The SHR rats were inoculated s.c. with c-SST-2 clone cells (1×10^6 cells/rat) or given injections i.v. of the clone cells (1×10^4 cells/rat). Rats were sacrificed for autopsy 35 or 28 days after the inoculation. Metastasis incidences in the lung after s.c. inoculation of parent, clone 2, and clone 3 cells were 7 of 7, 5 of 5, and 5 of 5, respectively. The median numbers of lung colonies of parent, clone 2, and clone

Received 2/2/89; revised 7/11/89; accepted 10/9/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed.

[2] The abbreviation used is: SHR, spontaneous hypertensive rat.

3 cells were 99.0, 64.0, and 50.0, respectively. In contrast, incidences of lung metastasis of clone 4 and clone 4-2 cells were 3 of 6 and 2 of 6, respectively. The median numbers of pulmonary colonies of clone 4 and clone 4-2 were 3.5 and 0.0, respectively. In the cases of i.v. injection of the clone cells (1×10^4 cells/rat), however, differences of metastatic ability between highly (parent, clone 2, and clone 3) and weakly (clone 4 and clone 4-2) metastatic clones could not be detected in either the incidence of lung metastasis or the median numbers of pulmonary metastatic foci as shown in Table 1.

Communication Frequency of Dye Transfer between c-SST-2 Clones and Normal Fibroblasts. We used the dye transfer method to examine intercellular communication between highly or weakly metastatic clone cells and fibroblasts obtained from the subcutaneous tissues of normal SHR rats. Table 2 shows that the communication frequencies between highly metastatic clones and fibroblasts obtained from the subcutaneous tissues of normal SHR rats ranged from 2.3 to 2.4%, while those between weakly metastatic clones and the fibroblasts ranged from 25.0 to 26.2%. Between tumor cells and fibroblasts from normal subcutaneous tissues, the communication frequencies between weakly metastatic clones and the fibroblasts were also significantly higher than those between highly metastatic clones and the fibroblasts.

Morphological Differences between Highly and Weakly Metastatic Clones of c-SST-2. A light microscopic view of the c-SST-2 tumor presented a typical feature of a differentiated adenocarcinoma. After serial passages *in vitro* and *in vivo*, however, the histology of c-SST-2 cells began to present an undifferentiated sarcoma-like appearance.

The ultrastructural observation revealed that *in vivo* parental c-SST-2 cells and highly and weakly metastatic clone cells retained a general feature of adenocarcinoma. In the highly metastatic clone cells (clones 2 and 3), abundant irregular microvilli were seen on the cancer cell membrane, and small organelles including many mitochondria, endoplasmic reticula,

Table 1 *Metastatic ability of c-SST-2 clones in SHR rats*

c-SST-2 clones	Site of inoculation	Pulmonary metastases: Incidence	Median no. of colonies	Range
Parent	s.c.[a]	7/7	99.0	50–133
Clone 2	s.c.	5/5	64.0	32–107
Clone 3	s.c.	5/5	50.0	21–134
Clone 4	s.c.	3/6	3.5	0–19
Clone 4-2	s.c.	2/6	0.0	0–10
Parent	i.v.[b]	5/5	67.0	34–111
Clone 2	i.v.	5/5	53.0	30–110
Clone 3	i.v.	5/5	43.0	30–65
Clone 4	i.v.	5/5	52.0	46–70
Clone 4-2	i.v.	4/4	35.0	19–52

[a] Rats were inoculated s.c. with 1×10^6 c-SST-2 tumor cells, and pulmonary metastases were examined macroscopically 35 days after the inoculation.
[b] Rats were inoculated i.v. with 1×10^4 c-SST-2 tumor cells, and pulmonary metastases were examined macroscopically 28 days after the inoculation.

Table 2 *Frequency of dye transfer between c-SST-2 clones and normal fibroblasts*

Clones	Metastatic ability	No. of cells dye coupled/ no. of cells injected
Clone 2	High	1/43 (2.3)[a]
Clone 3	High	1/41 (2.4)
Clone 4	Weak	11/40 (25.0)
Clone 4-2	Weak	11/42 (26.2)

[a] Numbers in parentheses, percentage.

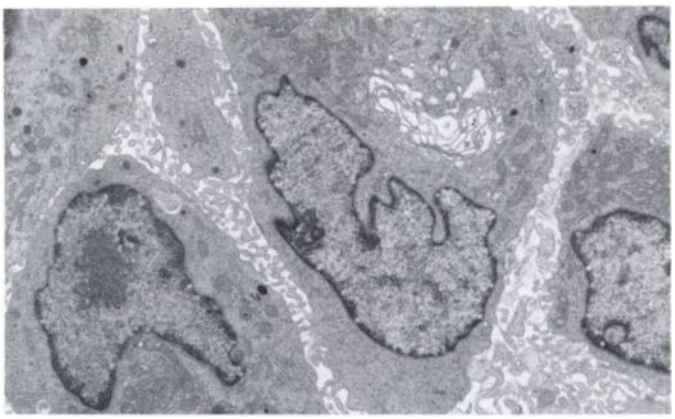

Fig. 1. Highly metastatic tumor cells (clone 3) with abundant microvilli on their cell surface and rich endoplasmic reticula and well-developed Golgi complexes in their cytoplasm. × 5,100.

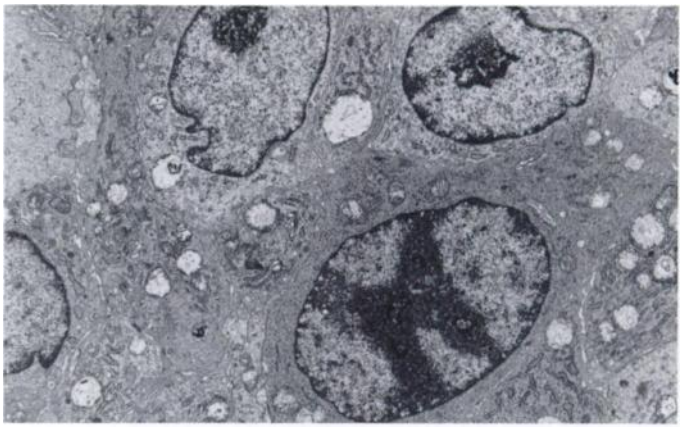

Fig. 2. Weakly metastatic tumor cells (clone 4-2) showing only a few organelles in their cytoplasm. × 3,850.

and secondary lysosomes were abundant in the cytoplasm. Furthermore, well-developed Golgi complexes and mucin granules were often found (Fig. 1). In particular, microfilaments were apparently present in these clone cells. In contrast, few small organelles were found in the weakly metastatic clone cells (clone 4 and clone 4-2) (Fig. 2; Table 3); instead, an irregularly enlarged nucleus which possessed more than one nucleolus was seen. The most obvious ultrastructural difference between the highly and weakly metastatic clone cells was intercellular junctions. The weakly metastatic clone cells (clone 4 and clone 4-2) were closely linked with each other by intercellular junctions, such as desmosomes (Fig. 3) and/or gap junctions (Fig. 4), and numbers of the desmosomes and the gap junctions were significantly higher than those in the highly metastatic clone cells (Table 4). The gap junctions and desmosomes were observed only between weakly metastatic clone cells and normal fibroblasts (Fig. 5). In contrast, the highly metastatic clone cells showed enlarged intercellular spaces, which were often connected with collagen fibers surrounding the tumor tissues. The number of intercellular junctions was very few between the highly metastatic clone cells.

The freeze-fracture method also revealed that a large number of gap junctions was often seen in the weakly metastatic clone cells compared with the highly metastatic clone cells (Table 4). The gap junctions were of different sizes in the weakly metastatic clone cells. The smallest gap junction was 0.8 cm in diameter, while the largest one was 4.7 cm in diameter when magnified ×90,000 (Fig. 6). In the highly metastatic clone cells,

Table 3 *Ultrastructural differentiation between highly and weakly metastatic clones of rat mammary carcinoma in vivo*

c-SST-2 clones	Metastatic ability	Frequency[a] of Microvilli	Desmosomes	Golgi complexes	Mitochondria	Rough endoplasmic reticula	Mucus	Microfilaments
Parent	High	+	−	+	+++	+++	+	++
Clone 2	High	++	−	++	+++	+++	++	+++
Clone 3	High	++	−	++	+++	+++	++	+++
Clone 4	Weak	+	−	−	+++	+++	−	+
Clone 4-2	Weak	−	++	−	+++	+++	−	+

[a] Frequency of ultrastructural findings per 100 cells: −, 0–5%; +, 5–10%; ++, 10–20%; +++, >20%.

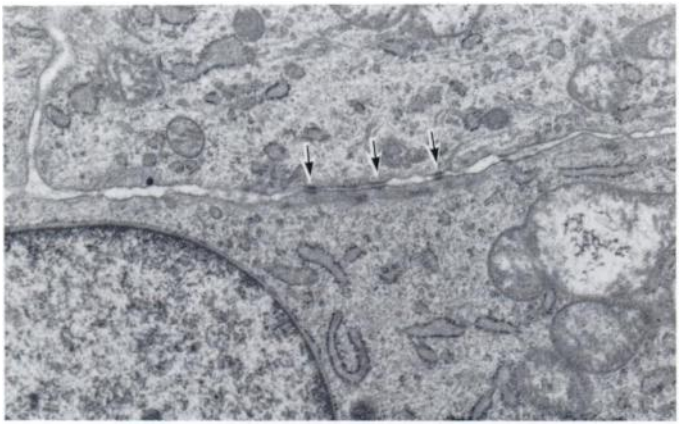

Fig. 3. Weakly metastatic tumor cells, showing numerous desmosomes (*arrows*). × 12,800.

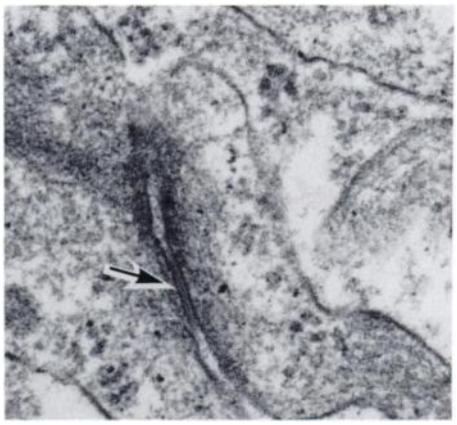

Fig. 4. Weakly metastatic tumor cells present typical gap junctions (*arrow*). × 66,400.

Table 4 *Comparison of numbers of desmosomes and gap junctions between highly and weakly metastatic clones of rat mammary carcinoma by transmission electron microscopy and the freeze-fracture method*

c-SST-2 clones	Metastatic	No. of desmosomes/ no. of cells observed[a]	No. of gap junctions/ no. of cells observed[a]	No. of gap junctions[b]/ area (μ^2)[c] of cells observed
Clone 2	High	5/342 (1.5)[d]	2/375 (0.5)	3/2091 (0.14)
Clone 4-2	Weak	75/389 (19.5)	26/339 (7.7)	45/2861 (1.57)

[a] Numbers of desmosomes or gap junctions counted during the observation by transmission electron microscopy.
[b] Numbers of gap junctions counted by the freeze-fracture method.
[c] Entire cell area measured by a Model KD4300 digitizer (Graphtec Co.) and a Model PC-286LE microcomputer (Epson Co., Tokyo, Japan).
[d] Numbers in parentheses, percentage.

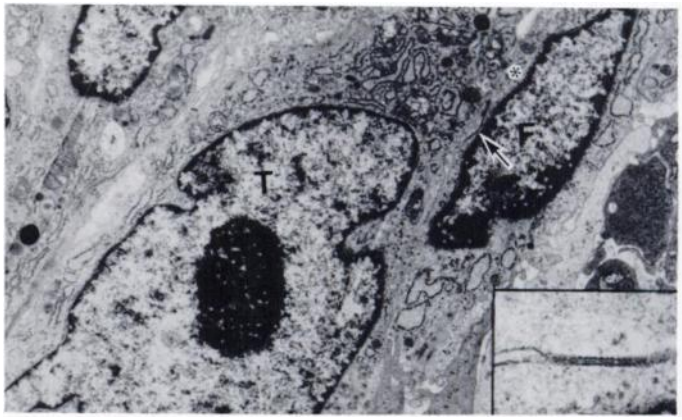

Fig. 5. Gap junction (*arrow*) and desmosomes (*) found between weakly metastatic tumor cells (*T*) and normal fibroblasts (*F*). × 3,300. *Inset* of higher magnification shows the gap junction. × 33,000.

three gap junctions were observed, in which the smallest gap junction was 0.4 cm in diameter, while the largest one was 1.5 cm in diameter when magnified ×90,000.

After cells of clones 2, 4, and 4-2 were cocultured with normal fibroblasts, ultrastructural differences emerged between the highly and weakly metastatic clone cells. Again, the most obvious difference was forming such a tight junction as observed only in the coculture of the weakly metastatic clone cells with normal fibroblasts (Fig. 7).

DISCUSSION

In our present study, we found that the ultrastructural changes of highly metastatic clone cells are more distinct than those of weakly metastatic clone cells; *i.e.*, the development of microvilli, microfilaments, and small organelles including endoplasmic reticulum, Golgi apparatus, and mucous particles is more apparent in the highly metastatic tumor cells. This observation indicates that the ultrastructural changes are possibly necessary for the active growth and metastasis of highly metastatic clone cells. It has been reported that morphological changes of the tumor cell membrane are closely correlated with aggressive proliferation (7). We also observed a number of microvilli on nearly the entire surface of the highly metastatic clone cells, in which antigen-antibody reactions are operative because of the receptors on microvilli of tumor cells as reported (8, 9). We suggest that the increase in small organelles of highly metastatic clone cells may be associated with some secretions, considering that a high amount of lactodehydrogenase, in the medium of metastatic hepatoblastoma cells can be an important parameter to distinguish the tumor growth speed (10).

The presence of intercellular junctional communication seems to have effects on the metastatic process. It has been

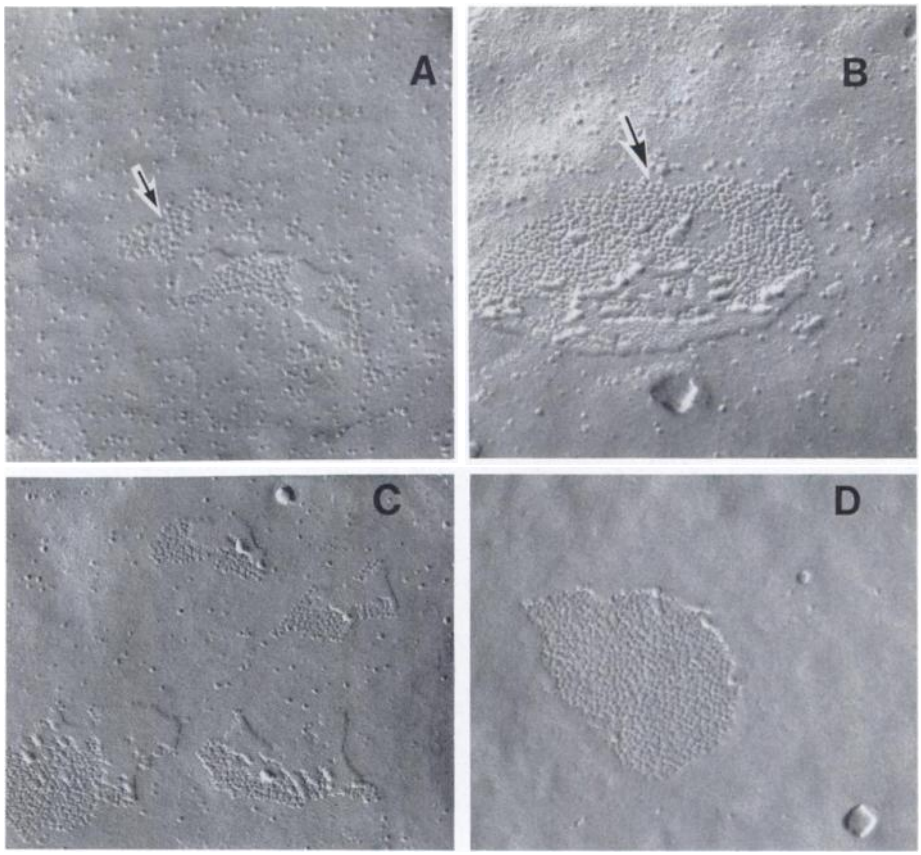

Fig. 6. Different sizes of gap junction revealed by the freeze-fracture method on the tumor cells of weakly metastatic clones (*arrows*). The *small arrow* shows the smallest gap junction, and the *large arrow* shows the largest gap junction. *a*, *b*, and *d*, × 90,000; *c*, × 72,000.

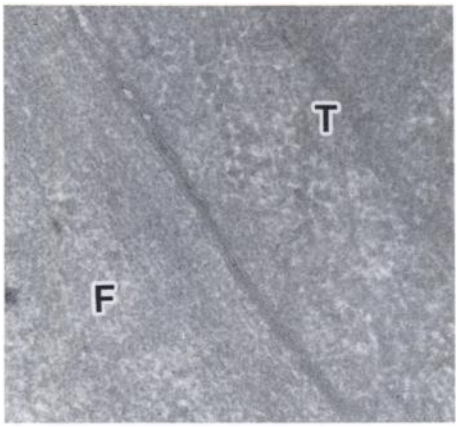

Fig. 7. A typical tight junction between a weakly metastatic tumor cell (*T*) and normal fibroblast (*F*). × 66,400.

reported that junctional complexes, such as desmosomes and intermediate junctions, are likely to increase intercellular adhesion (11), and that the initial phase of aggregation of tumor cells is followed by stabilization of the cells (12). Our present results provide further evidence that there are more distinct differences in the intercellular junctions between highly and weakly metastatic tumor cells, especially in the numbers of gap junctions and desmosomes. These results are coincident with the recent report that loss of intercellular junctional communication is closely correlated with highly metastatic potential in malignant mammary tumor cells (13). The gap junction is particularly considered to play an important role in maintaining tissue homeostasis (14). Therefore, we suggest that an absence or decrease of intercellular communication in the highly metastatic clone cells may be a promoting factor for tumor proliferation, detachment from the primary site, tumor invasion, and eventual formation of tumor metastasis.

A number of investigators have demonstrated that normal cells can control malignant transformation of tumor cells through gap junctions (15, 16). Hamada *et al.* found by using a dye-transfer method that the frequency of intercellular communication between weakly metastatic clone cells and fibroblasts was significantly higher than that between highly metastatic clone cells and fibroblasts, and they indicated that normal fibroblasts seem to regulate the metastatic ability of tumor cells by means of intercellular communication, especially by gap junction (5). Results of our present study by electron microscopic examination support their findings; *i.e.*, the formation of gap junctions is found only between weakly metastatic clone cells and normal fibroblasts. However, the biological and biochemical nature of the factors, produced by normal cells, which control the tumor metastasis through the gap junction is still under investigation.

REFERENCES

1. Fidler, I. J. Selection of successive tumor lines for metastasis. Nat. New Biol., *242:* 148–149, 1973.
2. Hamada, J., Takeichi, N., and Kobayashi, H. Inverse correlation between the metastatic capacity of cell clones derived from a rat mammary carcinoma and their intercellular communication with normal fibroblasts. Jpn. J. Cancer Res. (Gann), *78:* 1175–1178, 1987.
3. Bewtra, C., and Greer, K. P. Ultrastructural studies of cells in body cavity effusions. Acta Cytol., *29:* 226–238, 1985.
4. Jongen, W. M. F., Van der Leede, B. J. N., Chang, C. C., and Trosko, J. E. The transport of reactive intermediates in a co-cultivation system: the role of intercellular communication. Carcinogenesis (Lond.), *8:* 1239–1243, 1987.
5. Hamada, J., Takeichi, N., and Kobayashi, H. Metastatic capacity and intercellular communication between normal cells and metastatic cell clones derived from a rat mammary carcinoma. Cancer Res., *48:* 5129–5132, 1988.

6. Fujikawa, S. Intramembrane particle aggregation caused by membrane-to-membrane direct contact by freezing. J. Electron Microsc., *36:* 224–227, 1987.
7. Wallach, D. F. H. Cellular membranes and tumor behavior. Proc. Natl. Acad. Sci. USA, *61:* 868, 1968.
8. Haeffner, E. M., Paweletz, N., Hoffmann, C. J. K. Stohr, M., and Zimmerman, H-P. Translocation of microvilli and cell surface receptors of ascites tumor cells: alteration of the microfilament system by lipid composition in the culture medium. Cell. Mol. Biol., *32:* 359–368, 1986.
9. Kishimoto, T., and Tavassoli, M. Receptors for transferrin and transcobalamin II display segregated distribution on microvilli of leukemia L1210 cells. Biochem. Biophys. Res. Commun., *146:* 1102–1108, 1987.
10. Tsunoda, Y., Shibusawa, M., Okamatsu, T., Yatsuzuka, M., Gomi, A., Ishii, J., and Soejima, K. Characterization of human hepatoblastoma cell lines (second report). Growth rate of dissemination from hepatoblastoma. *In:* Proceedings of the 46th Annual Meeting of the Japanese Cancer Association, p. 205. 1987.
11. Staehelin, L. A. Structure and function of intercellular junctions. Int. Rev. Cytol., *39:* 191–283, 1974.
12. Wolfgang, M. K. Multicellular spheroids: a review on cellular aggregates in cancer research. J. Cancer Res. Clin. Oncol., *113:* 101–122, 1987.
13. Nicolson, G. L., Dulski, K. M., and Trosko, J. E. Loss of intercellular junctional communication correlates with metastatic potential in mammary adenocarcinoma cells. Proc. Natl. Acad. Sci. USA, *85:* 473, 1988.
14. Loewenstein, W. R. Junctional intercellular communication: the cell-to-cell membrane channel. Physiol. Rev., *61:* 829, 1981.
15. Mehta, P. P., Bertram, J. S., and Loewenstein, W. R. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. Cell, *44:* 187–196, 1986.
16. Enomoto, T., and Yamasaki, H. Lack of intercellular communication between chemically transformed and surrounding nontransformed BALB/c 3T3 cells. Cancer Res., *44:* 5200–5203, 1984.