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Loss of intercellular junctional communication correlates with metastatic potential in mammary adenocarcinoma cells

(tumor cell interactions/malignancy/cellular diversification/tumor progression)

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ABSTRACT **A series of rat 13762NF mammary adenocarcinoma cell sublines and clones of various spontaneous pulmonary metastatic potentials from the mammary fat pads of syngeneic rats were examined for their intercellular junctional communication. Using the scrape-loading dye-transfer technique to introduce Lucifer yellow (M_r 457) into cells, we measured the abilities of 13762NF cells to transfer dye to adjacent cells. There was an excellent correlation between loss of Lucifer yellow dye transfer and spontaneous metastatic potential (average total volume of lung metastases inversely correlated to % cells coupled, $r = 0.93$; average total number of lung metastases inversely correlated to % cells coupled, $r = 0.91$). The data suggest that high metastatic potentials are closely correlated with loss of intercellular junctional communication in these malignant mammary tumor cells.**

Intercellular communication through gap junctions between adjacent mammalian cells allows the exchange of nutrients, ions, and regulatory molecules with a relative molecular mass up to ≈1500 (1). This type of communication is considered important in regulating normal cell proliferation, embryogenesis, and development (2–5). Inhibition of gap-junctional communication by various chemicals, including tumor promoters, is thought to be a factor in carcinogenesis (6–8), teratogenesis (9–11), and other disorders (12). Indeed, neoplastic transformation mediated by oncogenes such as v-*ras*, v-*src*, and the gene coding for polyoma virus middle-sized tumor (middle T) antigen can decrease intercellular junctional communication in certain cell systems (13–16), although not all cells show the same degree of junctional uncoupling as transformed epithelial cells (17, 18).

Less is known concerning the possible role that junctional communication plays in the neoplastic progression of tumor cells to the highly malignant phenotype. Evidence suggests that intercellular junctional communication exists in benign epithelial tumors, and there is some evidence that invasive epithelial tumor cells show reduced abilities to communicate via intercellular junctions (19, 20). To examine the relationship of cell communication and malignancy in metastasizing epithelial tumor cells, we used a series of cloned rat 13762NF mammary adenocarcinoma cells with various potentials for spontaneous metastasis to lymph node and lung (21, 22). Intercellular junctional communication occurred in cultures of nonmetastatic or barely metastatic cells but not in cultures of highly metastatic cells.

MATERIALS AND METHODS

Cells. An early transplant of the 13762NF tumor was used to establish a parental cell line (MTPa), which was injected subcutaneously in the mammary fat pads of F344 rats (21). Tumor cells were grown in alpha minimal essential medium (GIBCO) containing 10% fetal bovine serum (Flow General, McLean, VA) and no antibiotics. Cell clones were established from the locally growing tumor (MTC, MTF7) or from spontaneous lymph node (MTLY) or lung (MTLn3) metastases by colony growth. When tested for their spontaneous metastatic potentials after subcutaneous injection of 10^6 viable cells into the mammary fat pad, the various cell lines or clones were quite different in their abilities to produce spontaneous lung metastases (Table 1).

Intercellular Communication. We measured intercellular junctional communication in the 13762NF cells *in vitro* by the scrape-loading dye-transfer technique (24). This technique introduces macromolecules into cells by a transient perturbation of the cell membrane that does not affect cell viability or colony-forming ability (23, 24). Lucifer yellow (M_r 457) does not diffuse through intact cell membranes, but its relative molecular mass permits diffusion through patent gap junctions (25). Rhodamine dextran ($M_r \approx 10{,}000$) is used as a control dye, because it cannot diffuse through intact cell membranes or gap junctions (26). When added simultaneously, the two dyes can be used to verify that dye transfer occurs through intercellular junctions (24). Several studies have demonstrated that such dye transfer is related to the morphological presence of gap junctions, radioactive metabolite transfer, and electrical coupling between cells (24, 27–29).

RESULTS

Examination of 13762NF cell clones revealed that cells of nonmetastatic or low metastatic potential (MTC.4 and MTPa, respectively) were completely coupled; after confluent cultures of the cells were scrape-loaded, they all transferred Lucifer yellow to adjacent cells (Fig. 1*a*). Tumor cells of intermediate metastatic potential (MTF7) showed less coupling than cells of low metastatic potential (Fig. 1*c*), and highly metastatic cells (MTLn3) were completely uncoupled (Fig. 1*e*). As in previous experiments (23, 28), rhodamine dextran was not transferred in any of the cultures (data not shown), indicating that the transfer of Lucifer yellow was not nonspecific. Table 1 summarizes the results of six independent experiments (at least 100–200 cells of each cell line or clone were examined in each individual experiment) using Lucifer yellow dye transfer to assess intercellular communication between 13762NF cells. There was an excellent correlation between metastatic potential and loss of intercellular junctional communication (average total volume of metastases inversely correlated to % cells coupled, $r = 0.93$; average number of metastases inversely correlated to % cells coupled, $r = 0.91$). In addition, when grown subcutaneously as tumors in the mammary fat pads of F344 rats, nonmetastatic MTC.4 but not highly metastatic

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Abbreviation: PMA, phorbol 12-myristate 13-acetate.

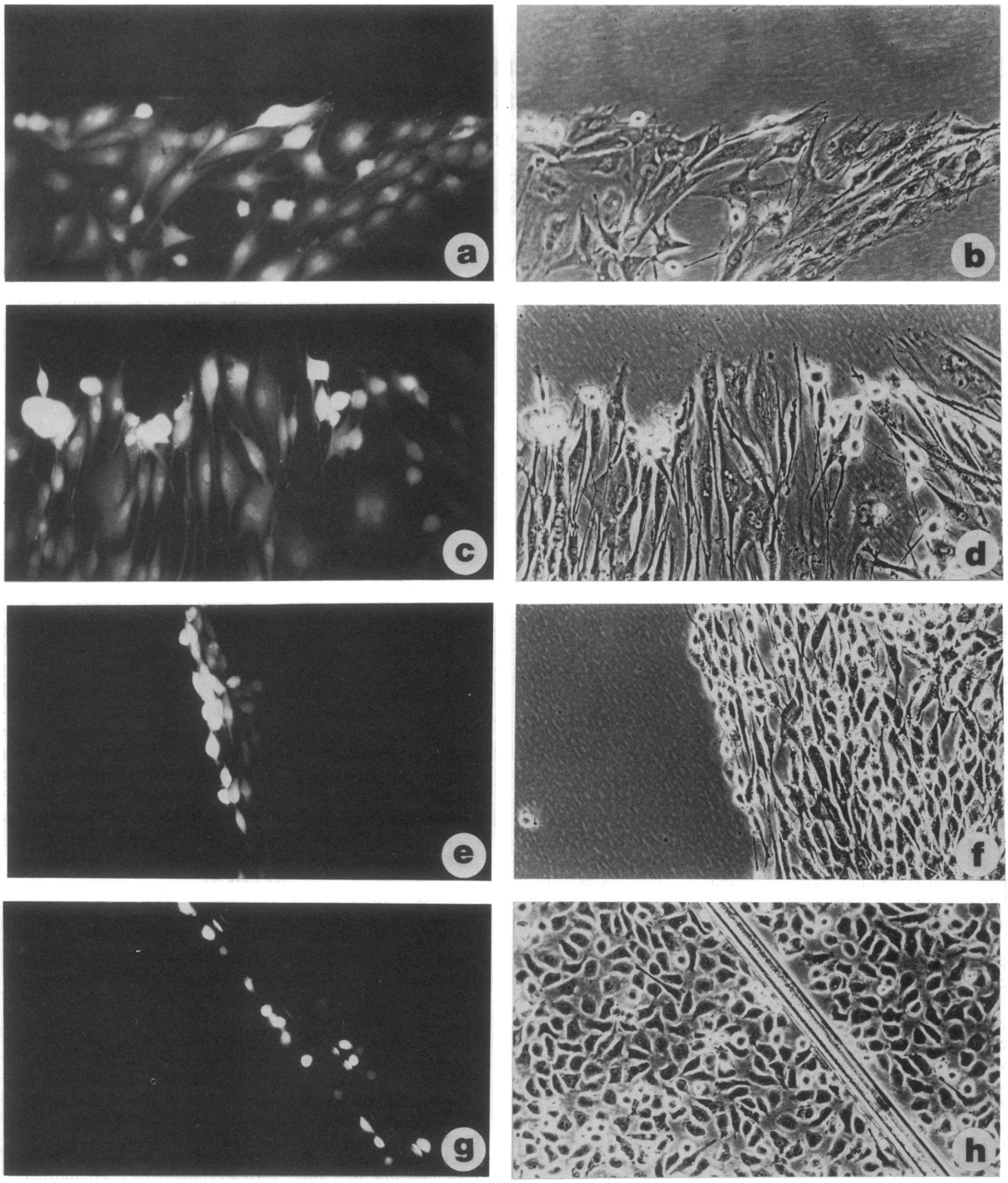

FIG. 1. Intercellular gap-junctional communication in rat 13762NF mammary adenocarcinoma cell cultures. Cell communication was assessed by scrape-loading confluent cell monolayers with Lucifer yellow according to the legend to Table 1. (*a*) MTC.4 (fluorescence). (*b*) MTC.4 (phase-contrast). (*c*) MTPa (fluorescence). (*d*) MTPa (phase-contrast). (*e*) MTF7 (fluorescence). (*f*) MTF7 (phase-contrast). (*g*) MTLn3 (fluorescence). (*h*) MTLn3 (phase-contrast). (×150.)

MTLn3 cells contained gap junctions as assessed by electron microscopy (G.L.N. and B. Meyers, unpublished data).

DISCUSSION

A variety of cellular interactions are important in controlling cell growth and development. Among these are cellular interactions with tissue stroma and extracellular matrix (30) and interactions with similar and different cells (31). After neoplastic transformation, such cell–cell communication is often altered (13–19, 32). For example, impaired intercellular communication is observed in rat kidney cells (33) and human keratinocytes (34) after transformation by avian

Table 1. Intercellular junctional communication in rat 13762NF mammary adenocarcinoma cells of various spontaneous metastatic potentials

Cells	Passage number	Spontaneous lung metastases: Average number (95% C.I.)*	Spontaneous lung metastases: Average total volume (95% C.I.),* mm^3	% cells coupled (mean ± SD)†	No. of cells coupled from scrape edge (mean ± SD)†
MTC.4	14–17	0	0	100 ± 0‡	5.15 ± 1.1‡
MTPa	14–16	0.4 (0–12)	0.2 (0–1.9)	88.6 ± 11.4‡	3.40 ± 0.8‡
MTF7	14–17	28.5 (22.7–40.1)	18.1 (12.8–24.9)	37.6 ± 24‡	1.23 ± 0.4‡
MTLY	16–19	45.3 (34.1–51)	26.4 (22.2–30.8)	12.4 ± 6.9‡	0.45 ± 0.3‡
MTLn3	14–17	78.6 (51.6–100.8)	43.1 (35.1–51.6)	0 ± 0‡	0 ± 0‡

Cells were grown in alpha minimal essential medium containing 10% fetal bovine serum. Spontaneous metastatic potential was assessed according to Welch *et al.* (22) by subcutaneous injection of 10^6 viable tumor cells in the mammary fat pads of each of fifteen F344 rats. Metastases to the lymph nodes and lung after 43 days were assessed by counting the number and estimating the total tumor volume (22). Intercellular junction-mediated cell communication was performed by scrape-loading confluent cell cultures, previously rinsed with phosphate-buffered saline (PBS, containing Mg^{2+} and Ca^{2+}), with 0.05% Lucifer yellow or a mixture of 0.05% Lucifer yellow and 0.05% rhodamine dextran (Molecular Probes, Junction City, OR) in PBS at room temperature, using a sharp steel blade or Pasteur pipet tip. The cell cultures were incubated for 5 min in the dye solution, rinsed in PBS, and examined in PBS in a Nikon phase-contrast microscope equipped for epifluorescence (23). Percentage of cells coupled was determined in each experiment by dividing the number of cells capable of Lucifer yellow dye transfer to adjacent cells by the total number of cells initially loaded with the dye at a scrape edge. Number of cells coupled was determined by estimating the number of cells containing Lucifer yellow in the cell monolayer perpendicular to Lucifer yellow scrape-loaded cells at the scrape edge.

*Ninety-five percent confidence interval, computed using propagation of error.

†Mean ± standard deviation for six independent experiments; at least 100–200 cells were examined in each experiment.

‡$P < 0.0001$; statistical significance between test and all other lines, calculated by one-way analysis of variance.

sarcoma virus and simian virus 40, respectively. Using the dye-transfer technique, Enomoto and Yamasaki (35) found that chemically transformed BALB/c 3T3 cells could no longer communicate with surrounding untransformed cells, and Chang *et al.* (15) showed that cultures of v-*src*-transformed NIH 3T3 cells lost their abilities to communicate and transfer [^{3}H]uridine nucleotide concomitant with an increase in protein kinase $pp60^{src}$. Using the 13762NF tumor system, we have shown recently that protoplast transfection of the T24 EJ c-Ha-*ras* oncogene into MTC.4 cells results in an increase in spontaneous metastatic potential. In addition, transfected MTC.4 subclones with increased metastatic potential and expression of EJ c-Ha-*ras* show loss of intercellular communication by the Lucifer yellow scrape-loading technique (G.L.N. and K.M.D., unpublished data).

In studies by Chang *et al.* (15), treatment with phorbol 12-myristate 13-acetate (PMA) reduced intercellular communication [results that are similar to those seen in many other cell systems (6–8)] and increased the cellular activities of protein kinase C. Diacylglycerol, the endogenous activator of protein kinase C, has also been shown to inhibit junctional communication (36–39). These observations suggest that in certain cells the *src* gene product and PMA can directly or indirectly interfere with intercellular communication through a cellular protein kinase (15), although studies by Saez *et al.* (40) imply that there are several mechanisms by which intercellular junctional communication can be inhibited. The recent demonstration that protein kinase C, an enzyme activated by tumor promoters such as PMA, can phosphorylate liver gap-junction proteins provides further suggestive evidence for the role of intercellular junctions in carcinogenesis (41).

Tumor promoters can also have profound effects on cellular diversification. For example, Chow (42) found that PMA accelerated the rate of cellular diversification. Thus, disruption of normal cell interactions and spatial arrangements necessary for the maintenance of normal tissue architecture may stimulate greater cellular heterogeneity (43, 44). This is often seen when polyclonal cell cultures are dispersed into single cells and allowed to proliferate as separate cell clones (43–47). Such results have also been obtained with the 13762NF system by cloning on plastic (48) or in semisolid agarose (49).

Cell–cell communication by means of intercellular junctions may be one of the processes involved in stabilizing benign epithelial cells and limiting their rates of cellular diversification, and this may also be one of the cellular characteristics circumvented during tumor progression to more malignant phenotypes (44). The rapid phenotypic shifts seen in tumor cells as they progress to invasive and metastatic phenotypes, as well as the increased diversity of cell populations treated with chemicals that modify cell–cell communication, could be explained, in part, by the loss of regulatory signals that are passed between adjacent epithelial cells through intercellular junctions.

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