Association of Decreased Intercellular Communication with the Immortal but not the Tumorigenic Phenotype in Human Mammary Epithelial Cells

Sandra R. Eldridge, Thomas W. Martens, Carol A. Sattler, and Michael N. Gould

University of Wisconsin-Madison, Department of Human Oncology, University of Wisconsin Clinical Cancer Center [S. R. E., T. W. M., M. N. G.], McArdle Laboratory for Cancer Research [C. A. S.], and Environmental Toxicology Center [S. R. E., M. N. G.], Madison Wisconsin 53792

ABSTRACT

Intercellular communication was compared in early passage cultures of human mammary epithelial cells (HMEC) from normal and malignant breast tissues and immortalized nonmalignant human breast cell lines (184A1 and 184BS). A clonogenic assay for the cell-mediated transfer of toxic metabolites of 6-thioguanine (TG) between cells was used as a measure of intercellular communication. We examined the effects of wild-type TG-sensitive (TG+) HMEC density on the survival of mutant TG-resistant (TG-) immortalized HMEC in TG-containing medium. Survival rates of TG+ HMEC cocultured with TG- normal HMEC, malignant HMEC, or immortalized nonmalignant HMEC were dependent on the density of TG+ cells. For example, the percentage of recovery of TG+ cells cocultured with 10^5 TG-immortalized, normal, or carcinoma HMEC was 88 ± 4, 41 ± 10, or 2.0 ± 1.7, respectively. Gap junction-mediated intercellular communication between homologous HMEC types was also studied, as quantitated on the basis of Lucifer yellow dye transfer between cells in culture. Results from the dye transfer studies supported those from the metabolic cooperation studies. These results using nonimmortalized tumor cells differ from previous reports in which immortal tumor cells have been found to communicate less than their normal counterparts. Previous reports suggesting that tumor cell lines communicate less than normal cells may have resulted from the confounding influence of the immortal phenotype on the tumor phenotype.

INTRODUCTION

The potential role of gap junction-mediated intercellular communication in carcinogenesis has been reviewed extensively (1, 2). In general, malignant cell lines have been reported to be communication deficient. Since intercellular communication is important for normal growth and differentiation, the loss of the ability to communicate has been suggested to be important in neoplastic progression by allowing potential tumor cells to escape local growth control (3, 4). Additional support for the role of cell communication in neoplastic transformation comes from observations that tumor promoters, such as 12-O-tetradecanoylphorbol 13-acetate, inhibit cell communication (5, 6). Evidence for the role of junctional communication in neoplastic progression comes from the recent observation that the loss of intercellular communication correlates with metastatic potential in a murine mammary tumor cell line (7).

Many methods have been used to study intercellular communication including both nucleotide and dye transfer. The transfer of the toxic nucleotide formed in TG+ cells to TG- cells through gap junctions provides a specific method to quantitate intercellular communication (8). HGPRT+ cells cannot proliferate in the presence of TG because HGPRT converts the purine analogue to its toxic metabolite. HGPRT- cells, on the other hand, survive in TG-containing medium, since they lack the ability to metabolize TG. However, because of the cell-mediated transfer of the toxic nucleotide from HGPRT+ to HGPRT- mutant cells, the survival of HGPRT- cells in TG-containing medium is reduced when cocultured with HGPRT+ cells. The loss of recovery of HGPRT+ cells, in the presence of a fixed number of wild-type cells, is a measure of intercellular communication. This method, however, requires the use of a TG+ cell of the type under study. This latter requirement cannot always be met, especially when studying primary cells. Fortunately, an alternative method of dye transfer can be used in these cases. In this method, Lucifer yellow dye is introduced into cells by "scrape-loading" (9). This dye passes between cells via gap junctions. This transfer can be quantitated and is a measure of intercellular communication.

Most reported in vitro studies on intercellular communication use fibroblast cell strains and immortalized cell lines to compare communication patterns in normal and transformed cells. It is thus useful to extend communication studies to nonimmortalized human epithelial cells. In this paper we report differential intercellular communication in early passage cultures of HMEC from normal and carcinomatous tissue and from two immortalized nonmalignant human breast cell lines.

MATERIALS AND METHODS

Cells. Normal HMEC were derived from residual surgical material from reduction mammoplasties of five healthy women. Carcinomatous HMEC were derived from five different samples of malignant human breast tissue.

Procedures for the isolation of HMEC have been previously described (10), and identical techniques were used for both normal and carcinomatous HMEC. Briefly, tissue was grossly dissected, and skin and apparent fat were discarded. The remaining tissue was minced and then enzymatically digested overnight in minimal essential medium (Gibco, Grand Island, NY) containing 2 mg/ml of collagenase (type III; Worthington, Freehold, NJ), 0.2 mg/ml of hyaluronidase (Sigma Chemical Co., St. Louis, MO), 10% fetal bovine serum, 5 μg/ml of insulin (Collaborative Research, Waltham, MA), glutamine (Sigma), and gentamycin (United States Biochemical Corp., Cleveland, OH) while shaking at 37°C. Dnase (0.5 ng/ml; Sigma) was added to samples during the final 10 min of incubation. The samples were centrifuged to remove fat, and the cell pellet was resuspended and filtered through a 53-μm pore size nylon mesh filter (Tetko, Elmsford, NY). The filter was inverted and washed to collect the ductal fragments. The epithelial cells in the form of ductal fragments (>10 cells/fragment) were counted. Primary cultures were started from either fresh or cryopreserved cells and were plated at an approximate density of 10^6 cells/100-mm plastic tissue culture dish (Lux; Miles Scientific, Naperville, IL). Epithelial cells grew out from the attached ductal fragments. These cultures were maintained for 7 days with regular feedings with MCDB 170 medium (described below), trypsinized with 0.25% trypsin (Gibco) plus 0.2 ng/ml of EDTA (Sigma), and replated as monodispersed cells into second-
ary cultures which were maintained for 7 days before metabolic cooperation experiments were started.

Early passage cultures of normal and carcinomatous HMEC have been shown to express epithelial-cell-specific keratin (11, 12). In addition, HMEC from similar cell cultures express mammary cell-specific human milk fat antigen (13). Cultures of both normal and carcinomatous HMEC prepared by these methods have normal diploid or near-diploid karyotypes, although some cells taken from carcinomas show distinct clonal karyotypic abnormalities such as deletions and translocations (14). HMEC from malignant tumors grow in agar, whereas normal HMEC do not (15). Finally, using similar methodology, Smith et al. (16) have shown that carcinoma-derived cells but not normal HMEC demonstrate invasive properties.

Immortalized HMEC (lines 184A1 and 184BS) were derived independently from normal HMEC taken from one donor (Case 184) by Dr. Martha Stamper (17). Karyotypic analyses show the cells to be near diploid. Neither cell line grows in agar, nor do they produce tumors in nude mice. Cells from both cell lines express keratin and human milk fat antigen (17). Late passage, nonimmortalized cells from the same cell line (Case 184, passage 9) as the immortalized cells were also obtained from Dr. Stamper. Late passage HMEC strain (Case B482, passage 14) was established in our laboratory from normal HMEC. TG' cells were selected from 184BS cells exposed to 10 J/m² of UV radiation and retained the characteristics of immortalized cells. Sufficient numbers of TG' cells could not be propagated from normal or carcinomatous HMEC due to their finite life spans in culture.

The growth medium requirements for all three cell types (normal, carcinomatous, and immortalized HMEC) are identical. Growth kinetic values of all three cell types, as shown by propidium iodide staining and flow cytometric analyses, are also very similar. The doubling time of all three cell types is approximately 24 h during exponential growth. Ultimately growth is inhibited at confluence.

Metabolic Cooperation Assay. Experiments to quantify intercellular communication were performed by plating 100 TG' mutant immortalized cells with various numbers of TG' HMEC in 100-mm dishes containing 30 μM TG. Control cultures consisted of the same numbers of TG' cells in TG-containing medium without the TG' mutant cells, in order to correct for spontaneous mutants arising from TG' cells. Five dishes were used for each determination and were incubated at 37°C in 5% CO₂ for 21 days with two changes of medium. The cells were examined.

RESULTS

Early passage HMEC from both normal and carcinomatous breast tissue were shown to communicate with immortalized TG' HMEC. This was demonstrated by increasing loss of TG' mutants as the number of cocultured wild-type cells was increased (Fig. 1). Fig. 1 also shows a difference in survival of TG' cells cocultured with either normal or carcinomatous HMEC from five separate donors; HMEC derived from malignant breast tissue communicated to a greater extent than normal HMEC. For example, when 10⁵ normal or carcinomatous HMEC were cocultured with 100 TG' immortalized HMEC, survival of TG' HMEC was 41 ± 9.9% and 2.0 ± 1.7%, respectively. Results were independent of the TG' clone used, since similar results were obtained using two independently derived TG' clones in replicate experiments (data not shown). In addition, interindividual variation was comparable to experimental variation.

Cell communication was measured in two immortalized HMEC lines. Immortalized HMEC communicated to a lesser...
extent than normal and carcinomatous HMEC, as illustrated in Fig. 2. However, late passage, nonimmortalized HMEC from the same individual (184) and from another woman (B482) exhibited levels of communication similar to early passage normal HMEC (Fig. 2).

The above data for both normal and carcinomatous cells were obtained using a mixed cell system. In all measurements, the tester cell (TG') was an immortalized cell. Since it was not possible to obtain sufficient numbers of TG' HMEC from primary cultures of malignant and normal tissues, two alternative methods were used in which the ability to extend these results to a homologous HMEC system could be evaluated.

Fig. 3 shows the results of the first of these assays. Uncorrected and corrected background mutation frequencies at two different cell densities of carcinomatous HMEC during selection of spontaneous mutants are presented. Observed background mutation frequencies at the HPRT locus in carcinoma-derived HMEC plated at 5 \times 10^4 cells per dish during selection of TG' mutants were less than that at 10^5 cells per dish. However, if the number of observed mutants was corrected for loss of TG' mutants, using correction factors taken from carcinoma-immortal cell communication data presented in Fig. 1, then the mutation frequencies at both cell densities were approximately the same.

In order to further examine intercellular communication in homologous HMEC, we used a dye transfer assay. Gap junction-mediated transfer of fluorescent Lucifer yellow dye between homologous human mammary cell types is shown in Figs. 4 and 5 and quantitated in Table 1. The marker dye rhodamine dextran was confined to the scrape-loaded cells in all three cell types. An example of this is shown in Fig. 4B. Limited dye coupling was observed between immortalized HMEC (Fig. 4C). Dye transfer was more extensive in cultures of carcinoma HMEC than normal HMEC (Fig. 5).

Finally, we examined our various HMEC cell cultures for the presence of gap junctions by electron microscopy. After extensive investigation, gap junctions were not observed in immortalized HMEC, although the plasma membranes were well developed between cells. In contrast, gap junctions were readily found in early passage normal and carcinomatous HMEC and late passage normal HMEC. An example of a gap junction in carcinomatous HMEC is shown in Fig. 6.

**DISCUSSION**

Our results suggest that carcinoma-derived early passage HMEC communicate to a greater extent than early passage HMEC derived from normal tissue. These results are qualitatively different from many previous reports suggesting that cancer cells communicate less efficiently than their normal counterparts (2, 5, 21–25).

Junctional communication, as measured by electrical conductance, was shown to be extensive in primary normal liver epithelial cells, whereas cancerous liver cells from transplanted tumor cell lines did not communicate (22). Using the metabolic cooperation method, efficient cell communication was observed in normal human fibroblast strains, whereas their transformed counterparts showed decreased communication (23). These transformed cells displayed multiple phenotypic differences from their normal counterparts. These included immortality, growth in agar, and tumorigenicity. Chang et al. recently reported defect junctional intercellular communication in many human carcinoma cell lines, using dye transfer as an indicator of intercellular communication (24). SV40-immortalized human keratinocytes did not communicate when microinjection of fluorescein dye was used as the measure of cell communication; normal keratinocytes were fully coupled to each other (25). Nonimmortalized cells derived from tumors of human colon were used to demonstrate communication in premalignant epithelial cells in contrast to malignant cell strains, which were uncoupled as shown by dye transfer (26). Using the transfer of radioactive nucleotide (27) from prelabeled breast epithelial donor cells to non-breast epithelial recipient cells, Fentiman and coworkers (28–30) found most human breast cancer epithelial cell lines to be noncommunicators. These investigators also reported that primary breast tumor cells were noncommunicators when cocultured with non-breast tester cells. However, normal primary breast cells communicated with homologous cells (29, 30).

Most of the tumor cells used in previous studies were cell lines, possessing both the tumorigenic and immortal phenotypes, whereas the tumor cells used in our studies were derived from nonimmortal early passage cultures of tumor-derived cells. Thus, in order to separate these two phenotypes with respect to intercellular communication, we examined cell communication in two human nontumorigenic breast cell lines derived from normal HMEC. We found that these immortalized HMEC did not communicate to the same extent as did early or late passage normal HMEC. The two cell lines were derived independently from normal HMEC from one individual. The decrease in communication observed in the two cell lines was not a characteristic of the individual from which the immortal
INTERCELLULAR COMMUNICATION IN HUMAN BREAST CELLS

Fig. 4. Gap-junctional intercellular communication in immortalized HMEC. Confluent monolayer cultures of immortalized HMEC were scrape loaded with Lucifer yellow and rhodamine dextran as described in "Materials and Methods." The scrape line is located to the left of the fluorescent row of cells. A, immortalized HMEC; phase contrast. B, immortalized HMEC; fluorescence, rhodamine dextran. C, immortalized HMEC; fluorescence, Lucifer yellow. Photomicrographs represent the same scraped area.

cells were derived, since nonimmortalized HMEC from this donor communicated to a similar extent as the other normal HMEC. This suggests that the decrease in intercellular communication observed in the two cell lines is a function of the immortalization process. Previous observations using a variety of techniques to show decreased communication in tumor cells may therefore have resulted from the confounding influence of the immortal phenotype on the tumor phenotype.

In the metabolic cooperation studies reported here, intercellular communication is defined by the cell-mediated transfer of the toxic ribonucleoside monophosphate metabolite of TG from TG' cells to TG' immortalized cells. Presumably this transfer occurs via gap junctions, since the ribonucleotide cannot penetrate cell membranes (31). The significant differences in survival rates of TG' immortalized cells could not be explained by differences in HGPRT activity or sensitivity to TG, since these parameters were similar in the normal, carcinomatous, and immortal cell types. It is important to point out that, in these studies, cell communication was examined between TG' immortalized HMEC and one of three cell types (normal, carcinomatous, or immortalized HMEC). This was necessitated by the inability to expand mutant clones of normal or carcinomatous HMEC with finite life spans. It is important to ask whether similar results are obtained if cell communication is determined between homologous cell types. Indirect evidence suggesting that carcinoma-carcinoma cell communication is equivalent to carcinoma-immortal cell communication comes from specific locus mutagenesis studies using primary HMEC from carcino-
Fig. 5. Gap-junctional intercellular communication in normal and carcinomatous HMEC. Identical methods were used as described in Fig. 4. The scrape line is located above the fluorescent Lucifer yellow-containing cells. A, normal HMEC; B, carcinomatous HMEC. All photomicrographs in Figs. 4 and 5 are at the same magnification.

Table 1 Intercellular communication between homologous human mammary cell types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of independent experiments</th>
<th>No. of cells coupled from scrape edge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immortalized</td>
<td>16</td>
<td>0.69 ± 0.1d</td>
</tr>
<tr>
<td>Late passage normal</td>
<td>18</td>
<td>2.8 ± 0.3f</td>
</tr>
<tr>
<td>Early passage normal</td>
<td>10</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>Early passage carcinoma</td>
<td>9</td>
<td>6.0 ± 0.4d</td>
</tr>
</tbody>
</table>

* Two immortalized human mammary epithelial cell lines (184A1 and 184B5) independently derived from normal breast tissue taken from one individual (Case 184). Late passage, nonimmortalized HMEC from the same cell lineage as the immortalized cells (Case 184, passage 9) and late passage HMEC derived from another individual (Case B482, passage 14). Second or third passage cultures of normal and carcinomatous HMEC from two individuals were tested.

* At least two independent experiments per individual or cell line.

d Mean ± SE.

f Significantly different from all other cell types tested ($P < 0.0001$).

* No statistical difference from early passage normal HMEC ($P > 0.05$); pooled $t$ test.

mas. When the number of observed TG' mutants was corrected for loss of mutants resulting from intercellular communication, using values obtained from the metabolic cooperation assays, the same mutation frequencies at both cell densities were obtained. These findings suggest that HMEC from carcinomas communicate among themselves to a similar extent that they communicate with immortalized HMEC.

We extended these investigations of homologous cell communication by using the scrape-loading/dye transfer assay described by El-Fouly et al. (9) to investigate gap junction-mediated communication. Results from these studies are qualitatively consistent with the results from the metabolic cooperation assays. Immortalized HMEC communicated to a lesser extent than both normal and carcinoma-derived HMEC, while carcinoma cells were more efficient at dye transfer than normal HMEC. These results were further supported by electron microscopic studies in which gap junctions were found in cultures of normal and carcinomatous HMEC, but not in immortalized HMEC.

At this time we can only speculate as to why tumor cells might communicate to a greater extent than their normal counterpart. There are many examples of tumors displaying gene expression patterns of embryonic cells. Since it is clearly documented that "the presence of extensive junctional communication is a general feature of embryonic development" (1), it is not surprising to find a high level of communication in neoplastic cells. Increased junctional coupling in tumor cells in vivo may help support extensive neoplastic growth. Tumors generally grow beyond the ability of their vasculature to provide normal levels of oxygen and nutrients. Intercellular junctions may provide an efficient means for passage of nutrients and their metabolites from cells located near a capillary to those at distant locations. In addition, specific growth stimulation molecules or their "second messengers" may more efficiently pass through these specialized junctions. Thus, as rare tumor cells

Fig. 6. Electron micrograph of carcinomatous HMEC in culture. Confluent monolayer culture was en bloc stained; section was stained with uranylacetate and lead citrate. A gap junction (GJ) and a desmosome (D) are present on the plasma membrane between two cells. ×60,000.
develop a growth advantage by increasing or modifying their receptors or producing growth-stimulatory peptides, they may share this advantage with surrounding tumor cells. These possibilities require further investigation.

In summary, we have presented data which demonstrate that decreased intercellular communication in HMEC is associated with the immortal but not the tumorigenic phenotype. Our data support the importance of using nonimmortalized cells in studies comparing normal and tumorigenic cells (32). Our data suggest that caution be applied in extrapolating cell communication data from cell lines having both tumorigenic and immortal phenotypes to primary tumor cells in vitro and in situ which are tumorigenic but not necessarily immortal.

ACKNOWLEDGMENTS

We thank Dr. Martha Stampfer for the generous gift of 184, 184A1, and 184B5 cells; Steven Howard for providing primary carcinoma cell cultures; and Dr. Cynthia J. Moore for critical review of the manuscript. Pituitary extract was obtained from Susan Hammond, University of Colorado, Boulder, CO; present address: Hammond Cell Technologies, Palo Alto, CA.

REFERENCES

Association of Decreased Intercellular Communication with the Immortal but not the Tumorigenic Phenotype in Human Mammary Epithelial Cells

Sandra R. Eldridge, Thomas W. Martens, Carol A. Sattler, et al.

Cancer Res 1989;49:4326-4331.

Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/15/4326

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.