Gap Junctional Intercellular Communication in Cells Isolated from Urethane-Induced Tumors in A/J Mice

ADINA VULTUR,¹ EVANGELIA TOMAI,¹ KATHERINE PEEBLES,³ ALVIN M. MALKINSON,³
NICHOLAS GRAMMATIKAKIS,¹ POH-GEK FORKERT,² and LEDA RAPTIS¹

ABSTRACT

Studies using normal or neoplastically transformed established mouse lung epithelial cell lines revealed a reduction in gap junctional, intercellular communication (GJIC) with transformation. To determine the stage in tumor development at which GJIC is interrupted, we used the well-established model of lung tumors induced in strain A/J mice by urethane. In this system, tumor development follows a well-characterized pattern; hyperplasias, adenomas, and carcinomas are manifested at approximately 8, 16, and 40 weeks after urethane treatment, respectively. GJIC levels were examined using a novel technique where cells are grown on a glass slide, half of which is coated with electrically conductive, optically transparent, indium–tin oxide. An electric pulse that opens transient pores on the plasma membrane is applied in the presence of the fluorescent dye, Lucifer yellow, causing dye penetration into cells growing on the conductive part of the slide. Migration of the dye through gap junctions to nonelectroporated cells growing on the nonconductive area is then microscopically observed under fluorescence illumination. Unexpectedly, primary cells cultured from urethane-induced tumors, even late stage carcinomas, possessed extensive GJIC immediately upon isolation. Upon passage for several months however, these cells lost GJIC. These results suggest that the molecular changes that lead to the formation of the tumor *in vivo* are not sufficient to interrupt gap junctions. Propagation of tumor cells in culture induces additional alterations that can lead to gap junction closure.

INTRODUCTION

GAP JUNCTIONS ARE CLUSTERS of plasma membrane channels that serve as conduits for the passage of small molecules and ions between the interiors of cells (Goodenough *et al.*, 1996). Gap junction channels open and close in a regulated manner. This “gating” is achieved through phosphorylation of connexins, the major gap junction proteins, and changes in intercellular pH, calcium concentration, and redox status depending upon the particular connexin(s) (Bruzzone *et al.*, 1996). Changes in gap junctional intercellular communication (GJIC) modulate proliferation and differentiation of normal cells. It has been postulated that a GJIC reduction facilitates abnormal growth, given that a number of activated oncogenes such as Src and Ras or tumor promoters interrupt gap junctions (Azarnia *et al.*, 1988; De Feijter *et al.*, 1992; Raptis *et al.*, 1994). At the same time, GJIC reduction also accompanies the cessation of

proliferation and differentiation of murine preadipocytes (Azarnia and Russell, 1985; Brownell *et al.*, 1996). In the case of human lung carcinomas, examination of gap junctions in fresh tumor tissue by electron microscopy suggested that a tumor’s ability to spread and metastasize is inversely related to its number of intercellular junctions visible by electron microscopy (McDonagh *et al.*, 1991), but no functional gap junctions were found in cells cultured from a number of human lung cancer specimens (Tomai *et al.*, 1988, 1999).

Primary lung tumors in mice have been used extensively to model human lung adenocarcinomas and provide insights into molecular events during carcinogenesis, due to their morphologic, histogenic, and molecular similarities (Malkinson, 2001). Studies using normal versus neoplastically transformed established mouse lung epithelial cell lines revealed a reduction in GJIC upon transformation (Cesen-Cummings *et al.*, 1998). However, since the immortalization process itself may have al-

¹Departments of Microbiology and Immunology and Pathology, and ²Anatomy and Cell Biology, Queen’s University, Kingston, Ontario, Canada.

³Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, Colorado.

tered the cells, a correlation between GJIC and the characteristics of the cells when they were in their natural environment as part of normal tissue or a naturally occurring tumor remains to be established; primary cultures may be more representative of the *in vivo* situation. Moreover, to determine when GJIC is interrupted, it is necessary to examine GJIC in cells at different stages of neoplastic development. To address these questions, we used the well-established model of lung tumors induced in strain A/J mice by urethane. This strain displays enhanced susceptibility to chemical carcinogens, perhaps due to overexpression of a *Ki-ras* allele (Chen *et al.*, 1994), and offers a highly reproducible model of tumor progression (Malkinson, 1992). Tumor development follows a well-characterized pattern; hyperplasias, adenomas, and carcinomas are manifested at approximately 8, 16, and 40 weeks after urethane treatment, respectively (Forkert *et al.*, 1996). Lung adenomas and carcinomas grow in solid or papillary patterns. Solid tumors are derived from alveolar type II cells (Thaete *et al.*, 1987); the cell of origin of papillary tumors has not been established, but it has been proposed that they may originate from either the alveolar type II or the nonciliated bronchiolar Clara cells (Forkert *et al.*, 1996). To examine the effects of tumor progression upon GJIC, we measured Lucifer yellow dye transfer and connexin 43 expression in cells cultured from tumors at different stages after urethane administration, and compared with primary cultures derived from their cells of origin.

Assays to detect GJIC in cultured cells include the introduction of nontoxic, fluorescent dyes such as Lucifer yellow, into a cell usually through microinjection or scrape-loading (el-Fouly *et al.*, 1987), followed by observation of dye migration into neighboring cells. These methods are expensive and time-consuming, and introduce the complication of cellular damage (McKarns and Doolittle, 1992). In this communication we used a novel technique for measurement of dye transfer, termed *in situ* electroporation on a partly conductive slide (Raptis *et al.*, 1994). This method takes advantage of the ability of short electric pulses to create transient "pores" in the cell membrane through which Lucifer yellow can enter simultaneously and into large numbers of cells with minimal disturbance to cellular physiology (Raptis *et al.*, 1994; Brownell *et al.*, 1996; Raptis and Vultur, 2001; Tomai *et al.*, 1998). Cells are grown on a glass slide, half of which is coated with electrically conductive, optically transparent, indium-tin oxide. An electric pulse is applied in the presence of Lucifer yellow causing its penetration into cells growing on the conductive half of the slide. The migration of the dye through gap junctions into nonelectroporated cells growing on the nonconductive area is then microscopically observed under fluorescence illumination. The results demonstrate that primary Clara cells have low GJIC, which, however, increases within a few days of growth in culture. Unexpectedly, primary cells cultured from urethane-induced tumors, even at late stages of carcinoma development, also possessed extensive GJIC. However, further passage of these cells in culture led to a loss of junctional communication. These results suggest that, while the urethane-induced, molecular changes that are sufficient for tumor growth *in vivo* do not preclude junctional communication in cultured cells immediately after isolation, subsequent propagation in culture induces additional alterations that lead to gap junction closure.

MATERIALS AND METHODS

Cell lines and culture techniques

E10 is a mouse lung type II epithelial cell line that is non-tumorigenic and exhibits contact-dependent inhibition of growth (Malkinson *et al.*, 1997); E9 is a spontaneous transformant of E10 cells, selected for resistance to growth inhibition by dexamethasone. E9 cells are not contact inhibited, and upon subcutaneous injection into immunocompetent, syngeneic mice, form tumors (Smith and Lykke, 1985; Malkinson *et al.*, 1997). E10 and E9 cells were grown in CMRL 1066 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS). LM1, a cell line derived from a urethane-induced, solid lung tumor, and LM2, derived from a urethane-induced, papillary tumor, were grown in alpha-MEM supplemented with 10% fetal calf serum. PCC4 and 82-132, lines derived from mouse lung papillary and solid tumors, respectively, were grown in McCoy's and DMEM, respectively, supplemented with 10% FCS (Cesen-Cummings *et al.*, 1998). All cells were grown as monolayers in plastic Petri dishes in a 5% CO₂ incubator and passaged by trypsinization.

Isolation of bronchiolar Clara cells

The technique described by Malkinson *et al.* (1993) was used with minor modifications. Briefly, two sets of five mice were anesthetized by intraperitoneal injection of 0.1 ml pentobarbital/heparin. The chest was opened and a #20 gauge Luer stub adaptor cannula inserted into the trachea and secured with suture silk. The abdominal aorta was severed and blood absorbed with sterile gauze. A perfusion needle, attached to a tuberculin syringe containing buffer A (0.8% NaCl, 5.5 mM KCl, 2.5 mM sodium phosphate buffer pH 7.4, 10.5 mM HEPES pH 7.4, 1 mg/ml glucose, 0.1% gentamicin), was inserted into the right ventricle of the heart, until it reached the pulmonary artery, and lungs were perfused with buffer A. At the same time, air was introduced into the lungs through the cannula, to inflate the lungs during perfusion. Lungs were subsequently removed from the thoracic cavity along with the cannulated trachea, placed in a tissue culture Petri dish containing buffer A supplemented with 2 mM EGTA, and lavaged eight times with buffer A/EGTA through the tracheal cannula. Lungs were slowly filled with a total of 8 ml of elastase solution (4.3 units/ml, Worthington Biochemical Corp., Freehold, NJ, in buffer B, 0.8% NaCl, 5.5 mM KCl, 2.5 mM sodium phosphate buffer pH 7.4, 10.5 mM HEPES pH 7.4, 2 mM CaCl₂, 1.5 mM MgSO₄, 1 mg/ml glucose, 0.1% gentamicin) through a syringe attached to the tracheal cannula. Lungs were subsequently cut away from the trachea and minced into approximately 1 mm³ pieces, which were placed into a sterile 250-ml Erlenmeyer flask containing 10 ml of calf serum. The flask was gently shaken at 37°C for 20 min, the suspension filtered through a nylon mesh, and centrifuged through a layer of calf serum (2000×g, 10 min) in a 15-ml conical tube. Macrophages were removed by adsorption onto mouse IgG-coated plastic Petri dishes for 1 h at 37°C. Clara cells were collected from the supernatant by centrifugation and plated on partly conductive slides or regular Petri dishes for further analysis. An aliquot was fixed by air drying and stained with nitroblue tetrazolium (NBT) (0.1% NBT, 0.1% NADPH in HEPES-buffered saline, for 20 min). More than 90%

of the cells stained strongly the day after isolation (Malkinson *et al.*, 1993).

Induction of tumors in A/J mice and growth of tumor-derived cells in culture

An intraperitoneal injection of urethane was administered to 20 1-month-old male A/J mice (1 mg/g body weight, dissolved in 0.9% NaCl; Forkert *et al.*, 1996). At 8, 16, and 40 weeks thereafter, six, seven, and seven mice, respectively, were sacrificed and the tumors sterilely dissected using a low-magnification, dissecting microscope. Several tumors from each mouse were minced into $\sim 1 \text{ mm}^3$ pieces and cells separated by trypsinization. Trypsin was removed by centrifugation in DMEM containing 10% FCS and the cells plated directly onto partly conductive slides for GJIC determination, or in Petri dishes in DMEM with 10% FCS. Because cell-cell contact is a prerequisite for the formation of gap junctions, the cells were plated at a relatively high-initial density (50–80% of confluence). Upon reaching confluence, approximately 2–6 days later, GJIC was determined by electroporation in the presence of Lucifer yellow. Cytokeratin immunostaining using a polyclonal rabbit antikeratin antiserum (Dako, Z0622) indicated that the cells were of epithelial origin.

Western blotting for connexin 43 quantitation

This assay was performed as previously described (Dupont *et al.*, 1988). Briefly, washed cells were lysed in 50 mM Tris,

pH 8.0, 30 mg/ml SDS, 0.1 mM leupeptin, 10 mg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and DNA was degraded by sonication. Protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL), and 30 μg of protein resolved through polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA), the membrane probed for Cx43 using a monoclonal antibody (Chemicon, Temecula, CA), a horseradish-peroxidase-coupled, antimouse secondary antibody and ECL reagents according to the manufacturer's instructions.

Examination of intercellular, gap junctional communication

The technique described by Raptis *et al.* (1994, 2000) was used with minor modifications. Briefly, cells were grown on a glass slide, half of which was coated with electrically conductive, optically transparent indium-tin oxide, which permits growth of adherent cells (Fig. 1). A solution of the nonpermeant, fluorescent dye, Lucifer yellow (5 mg/ml in Ca^{++} -free DMEM) was added to the cells. An electrode set was placed on top of the slide resting on the frame, linked to a circuit, and six pulses of 30–40 V were delivered from a 0.1 μF capacitor. Cells were washed with Ca^{++} -free DMEM, supplemented with 10% dialysed calf serum, and observed under phase contrast and fluorescence illumination using an Olympus IX70 inverted

FIG. 1. GJIC in type II cell lines. (A, B) E10 cells established from mouse lung epithelial type II cells were plated on partly conductive slides and at confluence were electroporated in the presence of 5 mg/ml Lucifer yellow. After washing away any unincorporated dye, cells from the same field were photographed under fluorescence (A) or phase contrast (B) illumination (Raptis *et al.*, 1994). Note the gradient of fluorescence, indicating dye transfer through gap junctions. To quantitate intercellular communication, the number of cells into which the dye transferred through gap junctions per electroporated border cell was calculated by dividing the total number of fluorescing cells on the nonconductive side (white circles) by the number of cells growing at the border with the conductive coating (black stars) (Raptis *et al.*, 1994). (C, D) E9 cells, a spontaneous transformant of the E10 line, were plated on partly conductive slides, electroporated, washed and photographed as above. (C) fluorescence, (D) phase contrast illumination photograph of the same field. Note the absence of dye transfer through gap junctions. To better illustrate these differences, dense cultures of both types of cells are shown. In all photographs, the left side is conductive. Arrows on the conductive side point to the interphase between conductive and non-conductive areas. Magnification: 140 $\times$.

phase contrast microscope equipped with epifluorescence. Lucifer yellow tracer movement was evaluated 5 min after the electrical pulse by overlapping the phase contrast and fluorescence images of the cells. Cells growing on the conductive side of the slide are electroporated, while those on the neighboring, nonconductive area do not receive any current, therefore are not permeated (Raptis *et al.*, 1994). The dye can only enter these cells from electroporated adjacent cells through gap junctions, if any are present. To maximize the likelihood that cells will attach close to the border between conductive and nonconductive areas, this transition line ran laterally across the slide, as described before (Raptis *et al.*, 2000). The equipment is available from Ask Science Products, Kingston, Ontario, Canada.

To quantitate intercellular communication, the number of cells into which the dye migrated through gap junctions per electroporated border cell was calculated by dividing the total

number of fluorescing cells on the nonconductive side by the number of cells growing at the border with the conductive coating (Fig. 1A). For each experiment, transfer from a minimum of 200 contiguous border cells was calculated. Results from at least eight separate slides are presented as means $\pm$ SEM. This number is referred to as GJIC.

RESULTS

GJIC in established lung epithelial cell lines

E10 is an established, type II mouse alveolar epithelial cell line. Previous work, using microinjection of the fluorescent dye, Lucifer yellow, indicated that a large proportion of these cells possess gap junctions (Cesen-Cummings *et al.*, 1998). To pre-

TABLE 1. GJIC^a IN NORMAL AND TRANSFORMED MOUSE LUNG TYPE II CELL LINES AND CELLS DERIVED FROM URETHANE-INDUCED TUMORS

Cell designation		GJIC ^a
Established lines		
E10	Type II, normal, immortalised	2.7 $\pm$ 0.2
E9	Spontaneous transformant of E10	0.2 $\pm$ 0.1
LM1	Solid tumor-derived, slow growing	1.0 $\pm$ 0.2
LM2	Papillary tumor-derived, fast growing	0.2 $\pm$ 0.2
PCC4	Papillary tumor-derived, fast growing	0.3 $\pm$ 0.2
82-132	Solid tumor-derived, fast growing	0.3 $\pm$ 0.2
Primary cells		
I. Normal cells		
Clara cells	2 days after isolation	0.3 $\pm$ 0.2
Clara cells	10 days after isolation	2.1 $\pm$ 0.2
II. Tumor cells ^b		
Lung hyperplasia	8 weeks after urethane injection	2.7 $\pm$ 0.2
Lung adenoma	16 weeks after urethane injection	2.5 $\pm$ 0.2
Lung carcinoma	40 weeks after urethane injection	2.5 $\pm$ 0.2
Carcinoma cells, grown in culture ^c		
M1.p5	3 weeks in culture	2.5 $\pm$ 0.2
M1.p70	6 months in culture	0.6 $\pm$ 0.2
M3.3b	6 months in culture	0.2 $\pm$ 0.1
M5.1a	8 months in culture	0.3 $\pm$ 0.2
M11.1c	8 months in culture	0.2 $\pm$ 0.1
M1.4b	4 months in culture	0.7 $\pm$ 0.2
M1.p12	4 weeks in culture	1.5 $\pm$ 0.2
M1.e.p14	5.5 months in culture	0.5 $\pm$ 0.2
M3.u.p12	6 months in culture	1.0 $\pm$ 0.2
M2.e.p13	6 months in culture	1.1 $\pm$ 0.2
M1-mix	5 months in culture (mixed clones)	0.7 $\pm$ 0.2
M11-mix	6 months in culture (mixed clones)	0.5 $\pm$ 0.2

^aGJIC was calculated as described in Fig. 1A, by dividing the total number of fluorescing cells on the nonconductive side, where the dye transferred through gap junctions (white dots, Fig. 1A and Materials and Methods), by the number of cells growing at the border with the conductive coating, which were loaded with the dye by electroporation (black stars). Numbers refer to means $\pm$ SEM.

^bFollowing urethane administration, tumors were explanted at the indicated times and cells placed in culture. Results from one representative tumor each, 3 days after plating are shown.

^cCloned carcinoma cells were grown in culture for the indicated times and their GJIC determined as above.

cisely quantitate GJIC, cells were plated on partly conductive slides and electroporated in the presence of Lucifer yellow, as described in Materials and Methods. This technique offers the possibility of observing intercellular communication among large numbers of cells simultaneously, which permits accurate assessment of the degree of intercellular communication; fol-

lowing pulse delivery, the number of cells into which the dye transferred, per cell loaded with the dye by electroporation, was calculated by dividing the total number of fluorescing cells on the nonconductive area (Fig. 1A and B, marked with a dot) by the number of cells growing on the conductive side, at the border with the nonconductive area (Fig. 1A and B, marked with

FIG. 2. GJIC in primary Clara cells and cells from urethane-induced tumors. (A, B) Primary Clara cells 2 days following isolation were plated on partly conductive slides and electroporated in the presence of 5 mg/ml Lucifer yellow, as described in Fig. 1. Note the absence of dye transfer through gap junctions. (C, D) Primary Clara cells 10 days following isolation and growth in culture were plated on partly conductive slides and electroporated in the presence of 5 mg/ml Lucifer yellow as described in Fig. 1. Note the extensive dye transfer through gap junctions. (E, F) Cells from urethane-induced lung carcinomas were plated on partly conductive slides and 3 days after plating, dense cultures electroporated in the presence of 5 mg/ml Lucifer yellow as described in Fig. 1. Note the extensive dye transfer through gap junctions. (G, H) Cells from urethane-induced lung carcinomas were plated on partly conductive slides and electroporated at lower densities than in E and F, 12 h after plating, in the presence of 5 mg/ml Lucifer yellow as described in Fig. 1. Note the less extensive dye transfer through gap junctions. Magnification: 140 $\times$.

a star). As previously shown (Raptis and Vultur, 2001), a wide range of voltages (25–45 V) permitted efficient introduction of Lucifer yellow into all lines tested with no detectable cellular damage and no effect upon GJIC (Raptis *et al.*, 1994, 1998; Raptis and Vultur, 2001). In addition, results obtained using this method closely correlate with results obtained using more conventional techniques, such as scrape-loading (Raptis *et al.*, 1994). As shown in Figure 1, transfer into several rows of E10 cells was evident (GJIC of 2.7 ± 0.2), as revealed by the distinct gradient of fluorescence from the cells at the edge of the conductive border, to the nonconductive area. This degree of junctional communication is similar to that previously seen in the rat fibroblast line, F111 (Raptis *et al.*, 1994). In contrast, GJIC in line E9, the spontaneous transformant of E10 (Smith *et al.*, 1984), was very low (0.2 ± 0.1 , Fig. 1 and Table 1), indicating that spontaneous transformation of E10 cells dramatically decreases GJIC.

Gap junctional communication in primary Clara cell isolates and cells derived from urethane-induced tumors

Expression of connexin 43, a prevalent connexin in pulmonary epithelial cells, is elevated in lung adenomas induced in mouse lung upon urethane injection, compared to uninvolved lung tissue or normal lung, then reduced in carcinomas (Ruch *et al.*, 2001). Given that connexins may possess other functions in the cell, in addition to the formation of gap junction channels (Ruch, 2000), direct GJIC examination would be a better measure of cell to cell transfer than connexin content alone. Besides, because the uninvolved tissue and normal lung possess additional cell types to those that proliferate to give rise to the tumors, to follow the effect of tumor progression upon gap junctions, it is important to compare GJIC in the progenitor Clara and type II cells, and hyperplasias, adenomas, and carcinomas. Examination of GJIC in primary Clara cells from untreated mice immediately after isolation revealed very low levels of junctional communication (Fig. 2A and B and Table 1). No transfer was detected between Clara cells and any of the small number of fibroblasts present (not shown). However, as previously shown for rat type II cells (Lee *et al.*, 1997), Clara cells progressively acquired a more extended morphology upon culture from their initial more rounded appearance, and by day 10 had extensive GJIC (2.1 ± 0.2 , Fig. 2C and D). As previously documented (Malkinson *et al.*, 1993), nitroblue tetrazolium reduction to the insoluble purple formazan (Devereux and Fouts, 1980) was very strong in Clara cells during the first day in culture but gradually waned, although it remained present. Cells continued to stain more strongly than mesenchymal cell lines (not shown), indicating that the main cell type present in long-term cultures is Clara cells. As expected, Clara cell cultures stained strongly for cytokeratins.

Tumors produced at different times after urethane injection were excised and the cells plated on partly conductive slides. As shown in Table 1, cells from hyperplasias and adenomas appearing at 8 and 16 weeks, respectively, had extensive GJIC (2.7 ± 0.2 and 2.5 ± 0.2 , respectively). Unexpectedly, as also shown in Fig. 2 (E and F, G and H), cells grown from carcinomas appearing at 40 weeks or even later after urethane administration also had extensive junctional communication. This

observation is consistent with the high levels of connexin 43 previously noted in urethane-induced carcinomas in A/J mice and may imply that the molecular changes that lead to the formation of a tumor *in vivo*, are not able to interrupt gap junctions in cells cultured from these tumors. Consistent with earlier observations (Chaudhuri *et al.*, 1993, Lee *et al.*, 1997), GJIC was lower in cells within a few hours after passage onto the conductive slides (Fig. 2G and H) but increased dramatically upon subsequent growth to confluence, permitting extensive cell–cell contact (Fig. 2E and F). Similar results were obtained with cells from all mice bearing hyperplasias, adenomas, or carcinomas (not shown).

Explanted carcinoma cells were propagated in culture for several months and their junctional communication examined before and after isolation of individual clones (Table 1). Contrary to primary tumor cells, eight clones that were derived from cells cultured from carcinomas that had been dissected from five individual mice had dramatically reduced GJIC levels after 5–6 months in culture (approximately 0.2 to 1.1, Table 1), while seven clones derived from three different mice had levels of 1 to 1.5 ± 0.3 (not shown). A substantial reduction in GJIC was also seen in mixed populations of tumor cells, prior to cloning (Table 1). As another estimate of junctional permeability, we examined connexin-43 content in a number of lines. As shown in Figure 3, there was an excellent correlation between connexin-43 levels and Lucifer yellow dye transfer, using our assay. These results are in agreement with data from the tumor-derived, established cell lines LM1, LM2, PCC4, and 82-132 (Table 1). The extensive communication of primary carcinoma cells is in sharp contrast to the absence of GJIC in the majority of the established tumor lines (e.g., E9, Fig. 1C and D), and indicates that propagation in culture appears to be a

FIG. 3. Connexin-43 quantitation. Total protein extracts from the lines indicated at the top (Table 1) were resolved by polyacrylamide gel electrophoresis and proteins transferred to a Nitrocellulose membrane, which was probed with an antibody against connexin-43 (see Materials and Methods). GJIC: numbers correspond to Lucifer yellow transfer assessed by *in situ* electroporation (Table 1). Arrows point to the positions of connexin-43 (Cx43) or its phosphorylated form (Cx43-P). Horizontal bars point to the positions of molecular weight markers.

necessary step for gap junction closure of urethane-induced lung tumor cells.

DISCUSSION

Extensive evidence from a number of cell culture systems has demonstrated a correlation between low GJIC levels and neoplasia. Oncogene proteins, such as Src, the middle tumor antigen of polyoma virus (mT) (Azarnia and Loewenstein, 1987; Raptis *et al.*, 1994) and Ras (De Feijter *et al.*, 1992; Brownell *et al.*, 1996), as well as tumor promoting agents (Oh *et al.*, 1993) interrupt junctional permeability in a variety of cultured cells derived from mouse or rat. Moreover, using systems of inducible mT or v-Ras expression, it was demonstrated that the levels of these oncogenes, which are sufficient to block gap junctions, are lower than those required for full neoplastic conversion and tumorigenicity (Raptis *et al.*, 1994; Brownell *et al.*, 1997). In addition, overexpression of pp60^{c-src}, which does not lead to full neoplastic transformation, is able to interrupt GJIC (Azarnia *et al.*, 1988). These results imply that a decrease in GJIC may be an important *early* event preceding full neoplastic conversion at least in these *in vitro* systems.

We attempted to establish the stage in tumor development *in vivo* when gap junctional communication is interrupted, using the well-studied model of urethane-induced tumors in A/J mice. Previous results indicated that a mutationally activated Ki-Ras oncogene, and inactivated or deleted forms of the p53 tumor suppressor gene occur in urethane-induced tumors with a high frequency; Ki-Ras mutations are already present in most hyperplasias and adenomas, while p53 mutations appear at later stages (Horio *et al.*, 1996). Our results revealed that although junctional communication is eliminated in type II cells upon spontaneous transformation in culture (e.g., line E9) (Cesen-Cummings *et al.*, 1998), primary cultures from urethane-induced lung tumors, even at late stages of tumor development, possess extensive junctional permeability. These data are in agreement with findings indicating that connexin-43 is expressed in both benign and malignant lung tumors (Ruch *et al.*, 2001). However, repeated passage of the tumor cells in culture reduced GJIC and connexin-43 levels. Therefore, it appears that the mutations present in urethane-induced carcinomas are not sufficient to interrupt gap junctions in cells isolated from these tumors; additional changes brought about through passage in culture, possibly enhanced Ras expression (Finney and Bishop, 1993; Fisher *et al.*, 2001), are required for this effect.

A rapid, precise, and efficient method of GJIC measurement is especially important for GJIC quantitation in primary cells, because a considerable degree of variation might be expected. The technique described here can assess GJIC in a large number of cells simultaneously without cellular injury (Brownell *et al.*, 1998), and it does not involve any mechanical manipulation of the cells that could disturb cell-to-cell contact areas and interrupt gap junctions. Previous results (Cesen-Cummings *et al.*, 1998) using microinjection of Lucifer yellow found that approximately 90% of E10 and 20% of E9 cells had gap junctions; our corresponding numbers using *in situ* electroporation are 2.7 ± 0.2 and 0.2 ± 0.1 (Table 1). This apparent discrepancy in absolute values is probably related to differences in experimental approach. When Lucifer yellow is microinjected into

cells growing as a monolayer, transfer from a single cell is assessed; cells score as positive if any transfer is observed. In the present study, on the other hand, transfer from cells lined up along the edge of the conductive coating is measured, so that quantitation of the average dye transfer from a large number of cells in the population can be precisely achieved. In addition, contrary to electroporation, which opens a large number of small pores in the cell membrane that immediately reclose, microinjection can cause damage to the cell that could affect junctional communication. In any event, our results indicate that mutations caused by urethane administration that lead to neoplastic growth *in vivo*, do not cause a concomitant interruption of GJIC in cells grown from these tumors; other molecular changes occurring during passage in culture are required for GJIC loss. The nature of these alterations will be the subject of future investigations.

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Address reprint requests to:

Leda Raptis, Ph.D.

Department of Microbiology and Immunology

Queen's University

Botterell Hall, Rm. 715

Kingston, Ontario, Canada K7L3N6

E-mail: raptis1@post.queensu.ca

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