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Growth Inhibition in G_1 and Altered Expression of Cyclin D1 and $p27^{kip-1}$ After Forced Connexin Expression in Lung and Liver Carcinoma Cells

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Abstract Gap junctional intercellular communication (GJIC) and connexin expression are frequently decreased in neoplasia and may contribute to defective growth control and loss of differentiated functions. GJIC, in E9 mouse lung carcinoma cells and WB-aB1 neoplastic rat liver epithelial cells, was elevated by forced expression of the gap junction proteins, connexin43 (Cx43) and connexin32 (Cx32), respectively. Transfection of Cx43 into E9 cells increased fluorescent dye-coupling in the transfected clones, E9-2 and E9-3, to levels comparable to the nontransformed sibling cell line, E10, from which E9 cells originated. Transduction of Cx32 into WB-aB1 cells also increased dye-coupling in the clone, WB-a/32-10, to a level that was comparable to the nontransformed sibling cell line, WB-F344. The cell cycle distribution was also affected as a result of forced connexin expression. The percentage of cells in G_1-phase increased and the percentage in S-phase decreased in E9-2 and WB-a/32-10 cells as compared to E9 and WB-aB1 cells. Concomitantly, these cells exhibited changes in G_1-phase cell cycle regulators. E9-2 and WB-a/32-10 cells expressed significantly less cyclin D1 and more $p27^{kip-1}$ protein than E9 and WB-aB1 cells. Other growth-related properties (expression of platelet-derived growth factor receptor-β, epidermal growth factor receptor, protein kinase C-α, protein kinase A regulatory subunit-Iα, and production of nitric oxide in response to a cocktail of pro-inflammatory cytokines) were minimally altered or unaffected. Thus, enhancement of connexin expression and GJIC in neoplastic mouse lung and rat liver epithelial cells restored G_1 growth control. This was associated with decreased expression of cyclin D1 and increased expression of $p27^{kip-1}$, but not with changes in other growth-related functions. J. Cell. Biochem. 79:347–354, 2000.

Key words: gap junctions; connexin; cell cycle; growth control; cyclins; cyclin-dependent kinase inhibitors

INTRODUCTION

GJIC regulates cellular growth by unknown mechanisms and is usually reduced in neoplastic cells [Ruch, 2000]. Most neoplastic cells have fewer gap junctions, decreased gap junction protein (connexin) expression, and less GJIC than normal cells [Cesen-Cummings et al., 1998; Mehta et al., 1996; Wilgenbus et al., 1992]. Metastatic potential was also greater in mammary adenocarcinoma cells with low GJIC [Nicolson et al., 1988]. Growth in vitro and tumorigenicity were reduced when poorly communicating, neoplastic cells were transfected with connexin genes [Chen et al., 1995; Eghbali et al., 1991; Hirschi et al., 1996; Huang et al., 1998; Mehta et al., 1991; Mesnil et al., 1995; Rae et al., 1998; Zhu et al., 1991]. Conversely, reduction of GJIC by treating cells with connexin antisense DNA, transfection of dominant-negative connexin genes, or connexin gene deletion ("gene knockout") increased cell growth and neoplastic transformation [Goldberg et al., 1994; Omori and Yamasaki, 1998; Oyoyo et al., 1997; Ruch et al., 1995; Temme et al., 1997].

GJIC may affect cell growth and the neoplastic phenotype by modulating the cell cycle and its regulatory factors [Ruch, 2000]. Decreased

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GJIC, altered connexin phosphorylation, and decreased expression of connexins occur at specific stages in the cell cycle. For example, in regenerating rat liver following partial hepatectomy, Cx32 expression, gap junction density, and GJIC were markedly reduced in hepatocytes at S-phase [Kren et al., 1993; Meyer et al., 1981; Traub et al., 1989]. GJIC was also reduced in late G_1-phase in rat liver epithelial cells coincident with the protein kinase C (PKC)-dependent phosphorylation of Cx43 [Koo et al., 1997]. During mitosis in rat fibroblasts and human endothelial cells, GJIC was markedly reduced and this was associated with $p34^{cdc2}$-dependent phosphorylation of Cx43 [Kanemitsu et al., 1998; Lampe et al., 1998]. Transfection of Cx43 into neoplastic dog kidney epithelial cells restored GJIC, lengthened the duration of the G_1- and S-phases of the cell cycle, and decreased the contents of cyclins A, D1, D2, and cyclin-dependent kinases (CDK) 5 and 6. Cyclin E, CDK2, and CDK4 protein levels were not affected [Chen et al., 1995]. Thus, downregulated GJIC is involved in cell cycle regulation and progression, but the specific cell cycle stage(s) that are modulated appear to vary among cell types.

We investigated how the Cx43 transfection of E9 mouse lung carcinoma cells and the Cx32 transduction of neoplastic WB-aB1 rat liver epithelial cells affected cell proliferation and cell cycle transit and whether or not this was due to alterations in the concentrations of cell cycle regulatory proteins. We found that Cx43-transfected E9 cells and Cx32-transduced WB-aB1 cells proliferated more slowly than nontransfected cells, had an increased percentage of cells in G_1-phase, and had greater $p27^{kip-1}$ and lower cyclin D1 contents.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

E10 cells are a mouse lung epithelial cell line derived from a clone of Type II cells. They are non-tumorigenic and exhibit contact-dependent inhibition of growth [Smith et al., 1984]. E9 cells, which arose from E10 cells after spontaneous transformation in vitro, are not contact-inhibited, and are tumorigenic [Smith and Lykke, 1985]. The origin of E10 and E9 cells, and the genetic and phenotypic differences between the two, were recently reviewed [Malkinson et al., 1997]. E9 cells were transfected with a Cx43 cDNA expression plasmid and several stable, Cx43-expressing clones were recently described [Ruch et al., 1998]. In this study, we utilized clones E9-2 and E9-3. Clones of E9 cells that had been transfected with the expression plasmid minus the Cx43 cDNA (E9-41 and E9-42 cells) [Ruch et al., 1998] were also used as controls. All lung cell lines were cultured as described [Ruch et al., 1998].

WB-F344 rat liver epithelial cells are diploid, contact-inhibited, non-tumorigenic, have properties of oval cells, and exhibit a high level of GJIC [Rae et al., 1998; Tsao et al., 1984]. WB-aB1 cells are a poorly communicating, highly tumorigenic derivative of WB-F344 cells [Rae et al., 1998]. WB-a/32-10 cells were derived from WB-aB1 cells by transduction with a Cx32 retroviral expression vector, communicate well, and are weakly tumorigenic [Rae et al., 1998]. WB cells were cultured as described [Rae et al., 1998].

Dye-Coupling Assay of GJIC

GJIC was assessed by fluorescent dye microinjection as we described [Cesen-Cummings et al., 1998]. Ten cells per 35 mM dish were microinjected with Lucifer Yellow CH dye (Sigma). Those cells directly adjacent to microinjected cells were evaluated for dye accumulation (dye-coupling) under epifluorescence illumination five min after dye injection. Dye-coupled and noncoupled neighboring cells were counted and the dye-coupling percentage determined for each dish. Three to four dishes were sampled per cell line.

Cell Cycle Analysis

The cell cycle distribution of the lung and liver cell lines was determined by flow cytometry after staining cellular DNA with propidium iodide. Cells were cultured in 10 cm dishes and sampled either at 50% confluency or two days postconfluence. The cells were suspended with trypsin and incubated in staining solution (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/ml of propidium iodide, and 10 μg/ml of RNase A) for 30 min at room temperature. Stained cells were analyzed using a Coulter Epics Elite flow cytometer in the Flow Cytometry Laboratory at the Medical College of Ohio. At least 20,000 cells per sample were analyzed. The cell cycle stage distribution was determined using computer algorithms available in the laboratory.

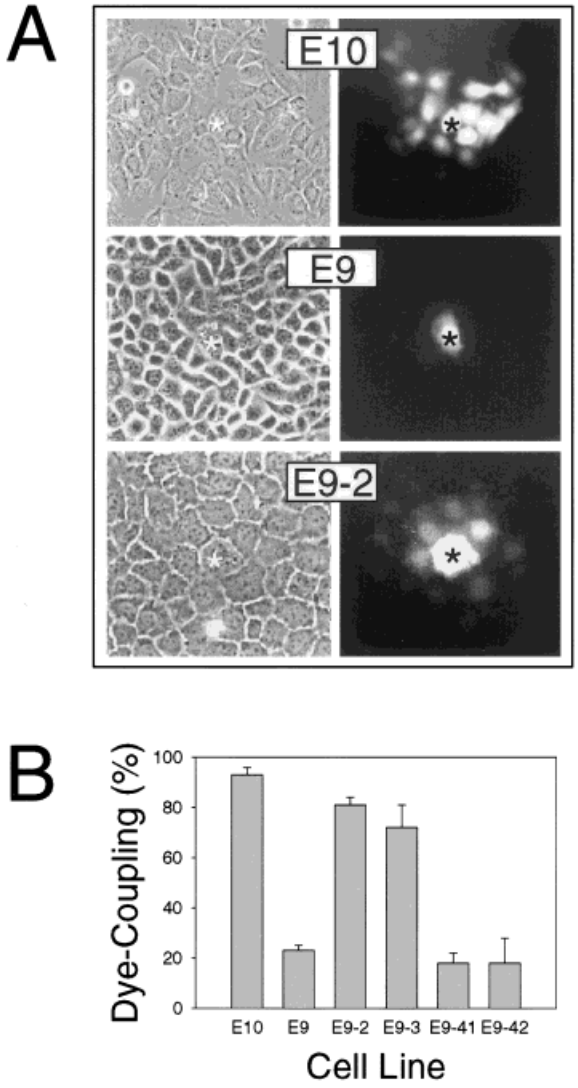

Fig. 1. Dye-coupling in lung cells. **A**: A single cell (*) was microinjected with Lucifer Yellow CH dye and the field of cells photographed five min later (left side - phase contrast; right side - fluorescence; 20X magnification). **B**: Dye-coupling percentages of lung cell lines (mean ± S.D.; n=6–8 dishes).

Western Blot and Protein Kinase Assays

The content of cyclin D1, $p27^{kip-1}$, EGFR, PDGFR-β, PKCα, and PKA-RIα were determined by Western blotting. Cell extracts were prepared from two days postconfluence cultures by harvesting the cells in cold phosphate-buffered saline (PBS), pelleting the cells by centrifugation (3,000x*g* for five min), and lysing the cells in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride]. Cell debris was sedimented by centrifugation (14,000x*g* for 30 min), and aliquots (40 μg protein) of the supernatant fractions were electrophoresed on 10% polyacrylamide SDS-PAGE gels. The separated proteins were transferred by electrophoresis to Immobilon-P membrane (Millipore Corp., Bedford, MA) and proteins were detected using mouse monoclonal antibodies (anti-cyclin D1 and anti-$p27^{kip-1}$, Santa Cruz Biotechnologies, Inc., South San Francisco, CA; anti-PKCα, Upstate Biotechnology, Inc., Lake Placid, NY) or rabbit polyclonal antibodies (anti-EGFR, anti-PDGFR-β, and anti-PKA-RIα, Santa Cruz Biotechnologies, Inc.). Band densities were analyzed by densitometry. The activities of PKA and PKCα were determined as described [Dwyer et al., 1994; Lange-Carter et al., 1993].

Effects of Cytokines on Nitric Oxide Production

A mixture of pro-inflammatory cytokines (interleukin-1β, tumor necrosis factor-α, and interferon-γ), collectively referred to as Cytomix, stimulates lung epithelial cells to generate NO. Interleukin-1β (murine, 10 U/ng) was obtained from Genzyme; tumor necrosis factor-α (human, 10 U/ng) and interferon-γ (murine, 15 U/ng) were purchased from Sigma. E10 cells are more responsive than E9 cells to this mixture [Thompson et al., 1998]. We examined NO production in E10, E9, and E9-2 cells following stimulation with Cytomix to determine whether Cx43 transfection altered the response of E9 cells. After the cells were serum-starved for 24 h, Cytomix was added to give final concentrations of 10 ng/ml for each cytokine. Forty-eight hours after the addition of Cytomix, aliquots (0.1 ml) of the culture media were removed and stored at -80° C. The production of NO was determined by measuring nitrite levels by flow injection analysis as described [Thompson et al., 1998].

RESULTS

Relationship between GJIC and Growth

We first investigated whether forced Cx43 expression affected GJIC and the growth of E9 mouse lung carcinoma cells. E10 cells exhibited extensive GJIC; nearly all cells adjacent to microinjected, dye-loaded cells demonstrated dye-coupling (Fig. 1A). E9 cells, in contrast, exhibited poor GJIC with only approximately one in five neighbors dye-coupled with microinjected

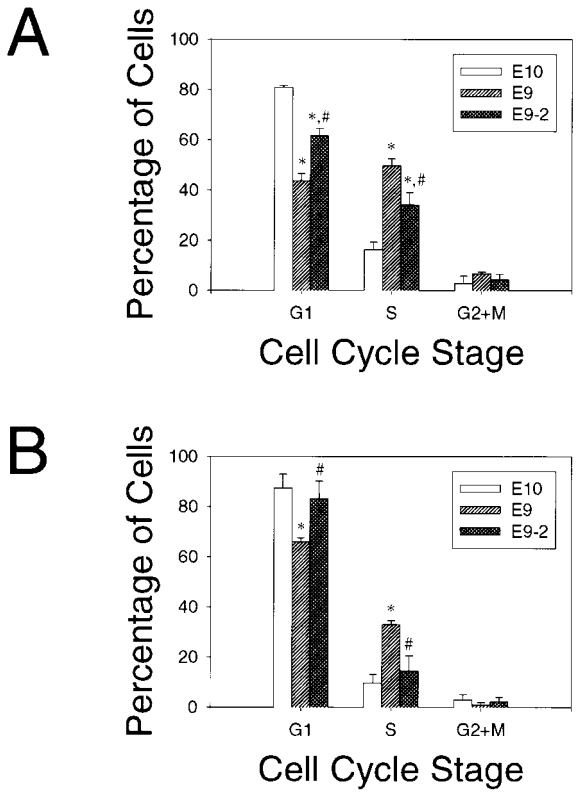

Fig. 2. Cell cycle distribution of mouse lung cells when approximately (**A**) 50% confluent and (**B**) two days postconfluence (mean± S.D.; n=3–4 cultures). Significantly different ($P<0.05$) vs. E10 cells (*) or vs. E9 cells (#).

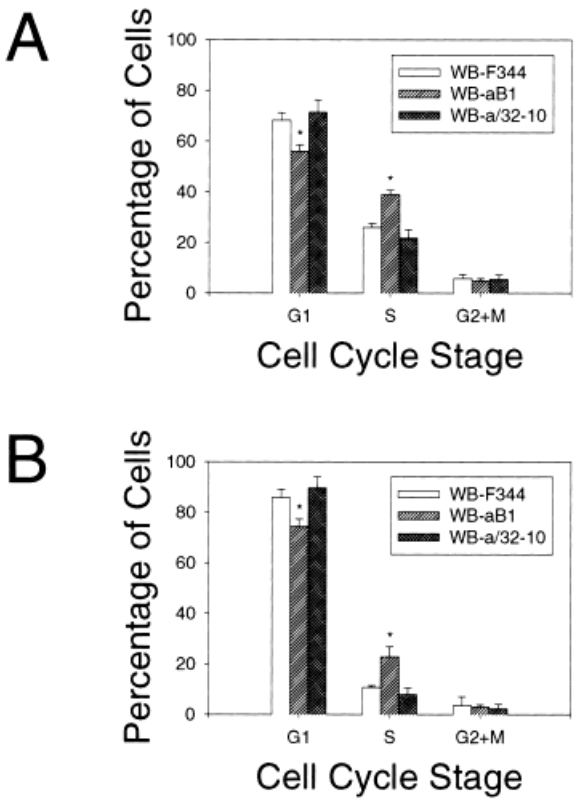

Fig. 3. Cell cycle distribution of rat liver cells when approximately (**A**) 50% confluent and (**B**) two days postconfluence (mean± S.D.; n=3–4 cultures). Significantly different ($P<0.05$) vs. WB-F344 cells (*).

cells. Cx43-transfected E9-2 and E9-3 cells exhibited high dye-coupling levels that were comparable to E10 cells and approximately three- to fourfold higher than E9 cells or the E9-41 and E9-42 plasmid controls (Fig. 1B).

We previously reported that the population doubling time (PDT) and saturation density (SD) of Cx43-transfected E9-2 and E9-3 cells were intermediate between E10 and E9 cells, and that the PDT and SD of transfection control E9-41 and E9-42 cells were similar to E9 cells [Ruch et al., 1998]. In the present study, we quantified the cell cycle distribution of subconfluent and postconfluent cultures of E9, E9-2, and E10 cells. In 50% confluent cultures, the percentage of G_1-phase cells was approximately twofold greater in E10 cells than in E9 cells, whereas the opposite was true for S-phase cells; no differences were evident for G_2+M cells (Fig. 2A). In postconfluent cultures, these patterns were similar except that the percentages of G_1-phase cells increased and S-phase cells decreased in E9 cells (Fig. 2B). Cx43-transfected E9-2 cells exhibited distributions of G_1 and S-phase cells that were intermediate between E9 and E10 cells.

We had also previously reported that Cx32 transduction increased GJIC and decreased the growth of neoplastic rat liver epithelial cells [Rae et al., 1998]. WB-F344 cells had a dye-coupling level of 97%, a PDT of 20.1 h, and a SD of $2.2x10^5$ cells/cm^2. In neoplastic WB-aB1 cells, the corresponding parameters were 3%, 13.7 h, and $4.0x10^5$ cells/cm^2. In Cx32-transduced WB-a/32-10 cells, dye-coupling was elevated to 91%, the PDT was 19.4 h, and the SD was $2.1x10^5$ cells/cm^2 [Rae et al., 1998]. Analyses of cell cycle distribution in these lines revealed that WB-aB1 cells had lower G_1-phase, higher S-phase, and similar G_2+M percentages to WB-F344 cells, and that these percentages in WB-a/32-10 cells were similar to WB-F344 cells (Fig. 3).

Expression of Cyclin D1 and p27^{kip-1}

Because GJIC increased the percentage of G_1-phase cells, we investigated whether the

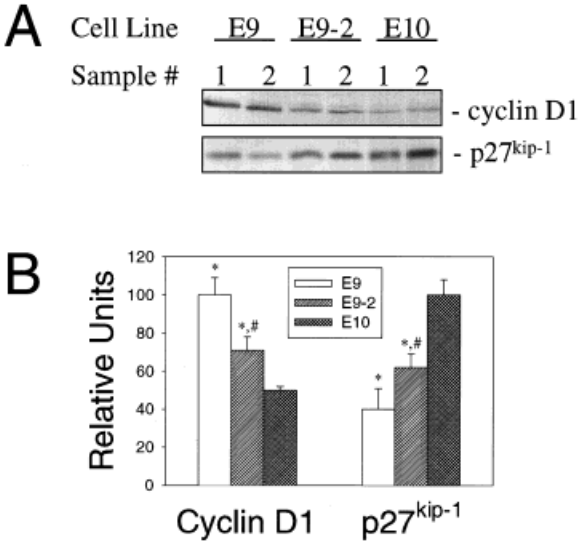

Fig. 4. **A**: Western blotting of cyclin D1 and $p27^{kip-1}$ in mouse lung cells. **B**: Densitometric analyses of mouse lung cell cyclin D1 and $p27^{kip-1}$ western blots (mean ± S.D.; n=4 samples from separate cultures; two of these samples are shown). Significantly different ($P<0.05$) vs. E10 cells (*) or vs. E9 cells (#).

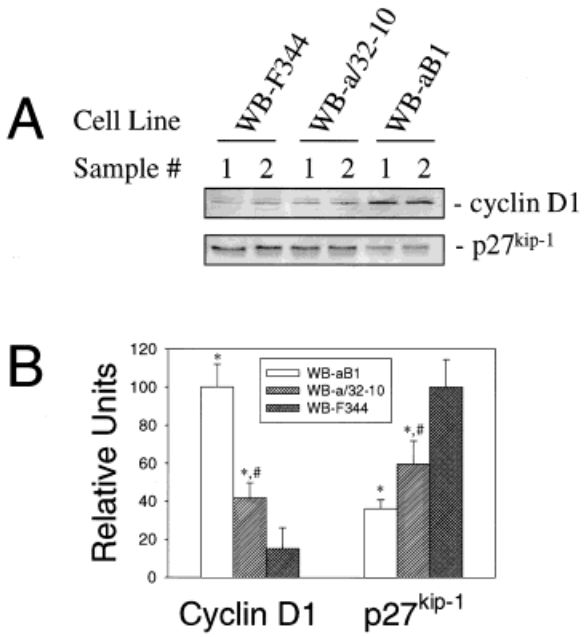

Fig. 5. **A**: Western blotting of cyclin D1 and $p27^{kip-1}$ in rat liver cells. **B**: Densitometric analyses of rat liver cell cyclin D1 and $p27^{kip-1}$ western blots (mean ± S.D.; n=6 samples from separate cultures; two of these samples are shown). Significantly different ($P<0.05$) vs. WB-F344 cells (*) or vs. WB-aB1 cells (#).

expression of the G_1-phase cell cycle regulatory proteins, cyclin D1 and $p27^{kip-1}$, differed among the cell lines. Western blot analyses revealed that cyclin D1 content was approximately twofold greater, and $p27^{kip-1}$ content approximately twofold lower in postconfluent cultures of E9 cells compared to E10 cells (Fig. 4). In agreement with the changes in cell growth (Fig. 2), the levels of both proteins were intermediate in Cx43-transfected E9-2 cells. In the WB cell series, cyclin D1 content was greater and $p27^{kip-1}$ content lower in WB-aB1 cells than in WB-F344 cells; the levels of these proteins were intermediate in WB-a/32-10 cells (Fig. 5). The levels of both proteins in empty vector-transfected control cells were similar to nontransfected cells (data not shown).

Expression of Growth Factor Receptors and Protein Kinases

We examined additional growth-related proteins that had different expressions in E10 and E9 cells [Malkinson et al., 1997], and that might be affected by upregulated GJIC. E10 cells expressed EGFR and PDGFR-β, but E9 cells did not (Fig. 6A,B). E9-2, E9-3, and E9-42 cells also did not express these receptor proteins. E9 cells expressed much less PKCα and PKA-RIα than E10 cells, and this was not significantly affected by Cx43 transfection in E9-2 cells (Fig. 6C-E). The activities of these kinases also did not change as a consequence of Cx43 transfection (data not shown).

NO Production in Response to Cytokines

We next determined whether Cx43 transfection of E9 cells affected cytokine-induced NO production. Cytomix strongly stimulates normal lung epithelial cells to generate NO, but has little effect on their neoplastic counterparts, although they stimulate proliferation [Thompson et al., 1998]. In response to these cytokines, E10 cells generated much more NO than E9 cells (Fig. 7). E9-2 cells gave a reproducible, intermediate response.

DISCUSSION

GJIC is an important mechanism of growth regulation. Defective GJIC contributes to neoplastic transformation, but the mechanisms are unknown [Ruch, 2000]. Mammalian gap junction channels permit the direct cell-to-cell spread of molecules less than approximately one kDa and thus facilitate cellular homeostasis. Many second messengers and other signal

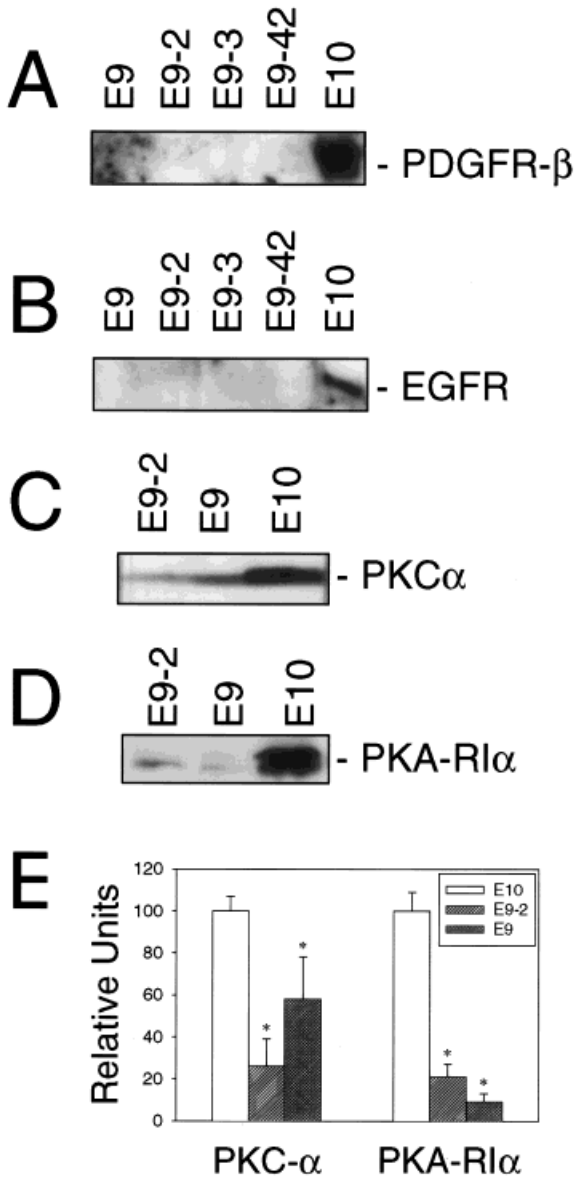

Fig. 6. Western blotting of (**A**) platelet-derived growth factor receptor-β (PDGFR-β), (**B**) epidermal growth factor receptor (EGFR), (**C**) protein kinase C-α (PKCα), and (**D**) protein kinase A regulatory subunit-Iα (PKA-RIα) in mouse lung cells. **E**: Densitometric analyses of PKCα and PKA-RIα (mean ± S.D.; n=4; *, $P<0.05$ vs. E10 cells).

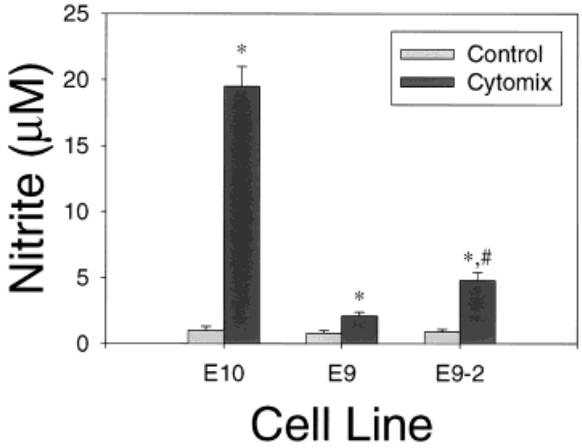

Fig. 7. Nitrite production in mouse lung cells stimulated with Cytomix, a mixture of pro-inflammatory cytokines [interleukin-1β (100 U/ml), tumor necrosis factor-α (100 U/ml), and interferon-γ (150U/ml)]. Cells were serum-starved for 24 h then refed with serum-free medium containing the cytokines. Nitrite levels in the medium were determined 48 h later (mean ±S.D.; n=6–8; *, $P<0.05$ vs. control group; #, $P<0.05$ vs. E9 cells).

molecules that are involved in cell growth control and differentiation pass through gap junction channels, including cAMP, calcium ion, ATP, and inositol-triphosphate [Goldberg et al., 1998; Kam et al., 1998; Lawrence et al., 1978; Leybaert et al., 1998; Saez et al., 1989]. Changes in the intracellular concentrations of second messengers, and other small signal molecules, are critical for cell cycle progression and signal transduction [Pastan et al., 1975; Whitfield et al., 1979]. In growth-stimulated cells, these signal fluctuations might be buffered by neighboring cells through GJIC and thus prevent cellular replication. To progress through the cell cycle, cells would need to uncouple their gap junctions from neighboring cells at critical points in the cell cycle, such as the restriction point (R) in G_1-phase, when cells commit to DNA synthesis and completion of the cell cycle. One potential G_1-phase regulatory signal molecule that might be buffered by GJIC is cAMP. In most cells, cAMP levels must fall in late G_1-phase before S-phase can begin; if cAMP levels are maintained by treating growth-stimulated cells with cell-permeant cAMP analogues, the cells do not progress past G_1-phase [Pastan et al., 1975]. This G_1-phase block is not fully understood, but it might be due to the elevation of $p27^{kip-1}$ and decreased cyclin D1 caused by cAMP [Kato et al., 1994; L'Allemain et al., 1997]. GJIC might also decrease other signals generated in neoplastic cells as a consequence of oncogene activation. Thus, the reduction of GJIC observed in neoplastic cells and cells treated with tumor promoters [Ruch, 2000] may enhance cellular replication by preventing this buffering of growth-related signals.

The data we have presented support the hypothesis that GJIC regulates cell growth and

that this control is mediated at least in part in G_1-phase. Enhancement of GJIC in E9 cells by Cx43 transfection and in WB-aB1 cells by Cx32 transduction reduced their growth and increased the percentage of cells in G_1-phase and decreased the percentage in S-phase. In agreement with this, E9-2 and WB-a/32-10 cells expressed approximately 50% less cyclin D1 and about twice as much $p27^{kip-1}$ than E9 and WB-aB1 cells, respectively. These changes are consistent with other studies in which cyclin D1 and $p27^{kip-1}$ levels were quantified. Mann et al. [1997] reported that cyclin D1 content increased fourfold after stimulation of quiescent Swiss 3T3 cells with insulin. Coats et al. [1996] found that low serum-induced G_1-phase arrest in Balb/c 3T3 fibroblasts correlated with a six- to eightfold increase in $p27^{kip-1}$ content. Consistent with the hypothesis that GJIC is involved in G_1-phase control, GJIC was markedly decreased in rat liver epithelial cells in late G_1-phase [Koo et al., 1997], and Cx43 transfection of neoplastic dog kidney epithelial cells increased the duration of G_1-phase (and S-phase) and decreased the expression of cyclins A, D1, D2 and CDK 5 and 6 [Chen et al., 1995]. The expression of cyclin E, CDK2, and CDK4 was unaffected and CDK inhibitors such as $p27^{kip-1}$ were not examined in those studies. The results of past studies, and our data, therefore support the hypothesis that GJIC regulates transit through G_1-phase. It is still unclear, however, whether these changes in the expression of G_1-phase cell cycle regulators were directly due to enhanced GJIC, or secondary to another effect of GJIC on cell growth.

We also examined other growth-related phenotypic properties that differ between E10 and E9 cells [Malkinson et al., 1997], including the contents of EGFR, PDGFR-β, PKCα, and PKA-RIα, and the production of NO in response to Cytomix. With the exception of a slight increase in NO production in E9-2 cells, we saw no correlation with GJIC. Cytomix increases the proliferation of E10 and E9 cells, but only strongly affects NO production in E10 cells [Thompson et al., 1998]. These results demonstrate that GJIC does not affect all growth-related, phenotypic traits.

In summary, the growth in vitro of neoplastic mouse lung and rat liver epithelial cells was decreased by forced connexin expression. This was associated with reduced exit from G_1-phase, decreased cyclin D1 expression, and increased $p27^{kip-1}$ expression. Other growth-related properties were minimally affected. The mechanisms for these changes remain to be determined.

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