Modulation of pulmonary alveolar type II cell phenotype and communication by extracellular matrix and KGF

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The alveolar epithelium consists of two cell types, alveolar type I (AT1) and alveolar type II (AT2) cells. We have recently shown that 7-day-old cultures of AT2 cells grown on a type I collagen/fibronectin matrix develop phenotypic characteristics of AT1 cells, display a distinct connexin profile, and coordinate mechanically induced intercellular Ca2+ changes via gap junctions (25). In this study, we cultured AT2 cells for 7 days on matrix supplemented with laminin-5 and/or in the presence of keratinocyte growth factor. Under these conditions, cultured AT2 cells display AT2 type morphology, express the AT2-specific marker surfactant protein C, and do not express AT1-specific cell marker aquaporin 5, all consistent with maintenance of AT2 phenotype. These AT2-like cells also coordinate mechanically induced intercellular Ca2+ signaling, but, unlike AT1-like cells, do so by using extracellular nucleotide triphosphate release. Additionally, cultured cells that retain AT2 cell-specific markers express connexin profiles different from cultured cells with AT1 characteristics. The parallel changes in intercellular Ca2+ signaling with cell differentiation suggest that cell signaling mechanisms are an intrinsic component of lung alveolar cell phenotype. Because lung epithelial injury is accompanied by extracellular matrix and growth factor changes, followed by extensive cell division, differentiation, and migration of AT2 progenitor cells, we suggest that similar changes may be vital to the lung recovery and repair process in vivo.

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ALVEOLAR TYPE I (AT1) and alveolar type II (AT2) cells form the mammalian lung alveolar epithelium. Although both cells are found in approximately equal numbers, AT2 cells are typically found in the corners of the alveoli, whereas AT1 cells are flattened and cover >90% of the alveolar surface area. Traditionally, AT2 cells have been viewed as the important cell phenotype for much of the physiology of the lung epithelium (e.g., surfactant secretion, salt movement, and progenitors for new AT2 and AT1 cells). AT1 cells facilitate gas exchange and fluid barrier function; recent reports suggest that they also may help to facilitate AT2 function (2). As in any tissue, it is most likely that both cells contribute a variety of functions in lung alveolar epithelial homeostasis. A problem in attributing physiological functions to individual alveolar cell types has been the difficulty in maintaining stable phenotypes in cell cultures.

The lung extracellular matrix, which supports the alveolar epithelium, is a dynamic set of structural molecules permeated by soluble molecules that together can regulate alveolar cell growth and differentiation (15, 34, 43). Structural studies on lung extracellular matrix have shown that healthy lungs can withstand transient or permanent changes in extracellular matrix and growth factors after lung damage. Such changes can help guide inflammatory responses and differentiation of AT2 to AT1 cell phenotype in vivo, which can contribute to the reformation of the functional lung epithelium (29, 35, 39). Although it is suggested that alveolar epithelial repair is initiated by cell division and differentiation of AT2 cells to restore the alveolar epithelial integrity (reviewed in Ref. 43), mechanisms for coordinated cell function and communication among and between AT2 and AT1 cells are not well defined.

One pathway observed by mammalian cells to help coordinate cellular function is the communication of small molecules to neighboring cells using gap junctions (reviewed in Ref. 40). Gap junctions consist of complementary connexons, one from each cell, that combine to form an intercellular aqueous channel. Each connexon is a hexamer of connexin proteins. Currently, there are >20 known cell- and tissue-specific mammalian connexins (reviewed in Ref. 8). Connexin expression patterns in isolated and cultured AT2 cells suggest a complex regulation during development in response to growth factors or in response to changes in the extracellular matrix (1, 9, 20, 25, 27). While it is likely that such changes in connexin expression alter...
cell communication, communication of physiologically significant molecules among and between AT2 and AT1 cells is not understood.

An increasingly common method for showing cell communication is the coordination of changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (reviewed in Ref. 36). Mechanicall stimulation provides a technique to initiate intercellular Ca\(^{2+}\) signals and study their propagation (38). In tracheal epithelial cells, such changes can be coordinated by gap junctional intercellular communication (e.g., Ref. 4) or extracellular release of nucleotides (e.g., Ref. 23). Two recent reports have shown that cultured alveolar epithelial cells that display AT1 phenotypic characteristics display a connexin expression pattern that differs from that previously seen in 7-day-old cultures of cells with AT2 phenotypic characteristics (25). We propose that a similar change in signaling mechanism may be important in vivo for lung epithelium homeostasis.

MATERIALS AND METHODS

Materials. All growth media, buffers, fetal bovine serum (FBS), and trypsin inhibitor were purchased from Gibco BRL. Antibodies to connexin proteins connexin 40 (Cx40) and Cx46 were purchased from Alpha Diagnostic International. Antibodies to Cx26, Cx32, Cx43, and the gap junctional-inhibiting peptide with amino acid sequence SRPTERTIFII (gap27) were kindly provided by Dr. W. Howard Evans (University of Wales). Antibodies to surfactant protein C (SP-C), aquaporin 5 (AQP5), and all secondary antibodies were purchased from Santa Cruz Biochemical. Fura 2-acetoxymethyl ester (fura 2-AM) and fura 2 were purchased from Calbiochem. Laminin-5 was from 804G cell culture supernatants (3); the cell line was kindly provided by Dr. J. C. R. Jones (Northwestern University). ATP (A-2383), UTP (U-6875), fibronectin, KGF, DNase, IgG, Lucifer yellow, and apyrase were from Sigma Chemical. Elastase was purchased from Worthington Biochemical. All other chemicals were purchased through Fisher or VWR and were of the highest biochemical grade.

Cell culture. Primary AT2 cells were harvested by modified methods described in Ref. 12. Briefly, male Sprague-Dawley rats weighing 250–300 g were injected peritoneally with pentobarbital sodium. The lungs were initially washed with solution 1 (136 mM NaCl, 2.2 mM Na\(_2\)HPO\(_4\), 5.3 mM KCl, 5.6 mM glucose, and 10 mM HEPES, pH 7.4), removed from the animal, and lavaged with solution 2 (136 mM NaCl, 2.2 mM Na\(_2\)HPO\(_4\), 5.3 mM KCl, 5.6 mM glucose, 1.9 mM CaCl\(_2\), 1.3 mM MgSO\(_4\), and 10 mM HEPES, pH 7.4) supplemented with ~4 U/ml elastase. The lungs were minced in a tryptic inhibitor solution (10 ml of solution 1 supplemented with 100 mg of BSA, 10 mg of trypsin inhibitor, 10 mg of DNase, and 400 \(\mu\)M EDTA per rat) and panned on IgG plates for 1 h at 37°C. Cells were recovered and plated on matrix-coated coverslips (see below) at a density of 1.8 \(\times\) 10\(^6\) cells/ml. Cells were grown in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% FBS, penicillin, streptomycin, and amphotericin with or without 100 ng/ml KGF at 37°C in a 5% CO\(_2\) atmosphere for 7 days. Culture media were changed at day 1 and every other day thereafter. Cell viability was determined using 0.5% trypan blue. Cells were considered completely (>90%).

Coverlip preparation. Collagen-coated coverslips were prepared as described in Ref. 11. Coverslips that contained fibronectin were additionally incubated with 300 \(\mu\)g/ml of 50 \(\mu\)g/ml fibronectin at 3°C for 1 h and then warmed to room temperature before use. For culture on a matrix that contained laminin-5, coverslips were incubated with 300 \(\mu\)l of supernatants obtained from the 804G cell line (hereafter referred to as Ln-5) at 37°C and 5% CO\(_2\) for 1 h. Coverslips that contained both fibronectin and Ln-5 were treated similarly, with the fibronectin laid down first. In all cases, coverslips were used immediately after preparation.

Morphology staining. Cultured AT2 cells were stained using a modified 1.0% tannic acid stain as described (30). Briefly, cells were washed in phosphate-buffered saline (PBS; 8 mM Na\(_2\)HPO\(_4\), 128 mM NaCl, 2 mM KCl, and 10 mM HEPES, pH 7.4) and fixed with 1.5% glutaraldehyde (in PBS) for 15 min. Cells were washed twice with PBS and then fixed with 2.0% OsO\(_4\) for 1.5 h. Cells were again washed twice with PBS and then incubated in 1.0% tannic acid (in PBS, pH 6.8) overnight. Cells were then washed twice with PBS and twice with distilled H\(_2\)O, and images were obtained on an Olympus IX70 inverted microscope by using a \(\times\)40 oil-immersion 1.35 NA objective under differential interference contrast optics.

Immunocytochemistry. Cells were washed with cold Hanks’ balanced salt solution (HBSS; 1.3 mM CaCl\(_2\), 5.0 mM KCl, 0.3 mM KH\(_2\)PO\(_4\), 0.5 mM MgCl\(_2\), 0.4 mM MgSO\(_4\), 137.9 mM NaCl, 0.3 mM Na\(_2\)PO\(_4\), and 1% glucose additionally buffered with 25 mM HEPES, pH 7.4) and fixed with cold 50:50 acetone:methanol for 3 min. Cells were washed with PBS, incubated for 20 min with antibody dilution solution (0.25% gelatin, 3.0% BSA, 0.05% Tween 20, and 0.2% NaN\(_3\) in Tris-buffered saline (150 mM NaCl and 15 mM Tris, pH 7.4), and then incubated with the appropriate primary antibodies for 1 h at 37°C. The cells were washed with PBS and antibody dilution solution and incubated in appropriate FITC-linked secondary antibodies for 1 h at 37°C. The coverslips were thoroughly washed with PBS and deionized water, mounted, and viewed on a Leica TSD-4D confocal laser scanning microscope. When scoring cells for connexin protein expression, cells were first examined under phase-contrast microscopy. If cells were surrounded on all sides by neighboring cells, they were determined to be “adjacent” cells (~80% of the culture). If cells had no cell-cell contact, they were determined to be “solitary” cells (~5% of the culture). Cells that did not fall into one of these categories were not counted. Only cells that displayed clear punctate stains were
considered positive for connexin expression. At least 100 cells from three separate isolations were counted for each connexin isoform and each cell type.

Dye coupling. Seven-day-old AT2 coverslip cultures were placed in 100-cm petri dishes containing HBSS. Microinjection into the cytoplasm of individual cells was conducted using an Eppendorf Micromanipulator 5171 and Transjector 5426. Eppendorf femtotips were backfilled with 5 mg/ml Lucifer yellow. Injections were monitored on a Nikon Eclipse TE300 inverted microscope with a ×20 objective by phase contrast for injections and epifluorescence for dye coupling. Images were captured immediately after injection and at 5 min postinjection with a DAGE 300T-RC ICCD video camera using QED imaging software and a Macintosh G3 computer. All injections were conducted at room temperature.

$\text{Ca}^{2+}$ imaging. Seven-day-old AT2 cell cultures were loaded with fura 2 by a 75-min incubation in 5 μM fura 2-AM in HBSS. Cells were washed for 20 min in HBSS, mounted on an inverted Olympus IX70 microscope, and observed using a ×40 1.35 NA oil-immersion objective. Fura 2 fluorescence was observed after alternating excitation at 340 and 380 nm by a 150-W xenon lamp linked to a Delta Ram Illuminator (Photon Technologies, Lawrenceville, NJ). Images of emitted fluorescence >505 nm were recorded by an ICCD camera (Photon Technologies) and simultaneously displayed on a 21" Vivitron color monitor. The imaging system was under software control (ImageMaster, Photon Technologies). Calculations of $\text{[Ca}^{2+}]_i$ were by published equations (19). ATP and UTP were supplemented to HBSS at 10 μM concentrations. Mechanical stimulation was administered through a glass micropipette (tip diameter ~1 μm) under piezoelectric control, positioned using a hydraulic micromanipulator, and deflected downward for 150 ms to deform an individual cell. Only experiments where the stimulated cell membrane was left intact (as determined by phase-contrast observation and fura 2 dye retention measured at 340-nm excitation) were included in the analysis. This precluded nucleotide triphosphate loss from the wounded cell that could contribute to $\text{[Ca}^{2+}]_i$ changes in adjacent cells (e.g., Ref. 25). Changes in $\text{[Ca}^{2+}]_i$ of 150 nM, a two- to threefold change over resting values, were considered a positive $\text{[Ca}^{2+}]_i$ change. In mechanical stimulation experiments, all cells exhibiting a positive change in $\text{[Ca}^{2+}]_i$ were counted as communicating cells. Thus a count of one cell represents a change in $\text{[Ca}^{2+}]_i$, restricted to the stimulated cell.

Inhibition of gap junctional signaling. One-hundred thirty micromolar gap27 peptide (SRPTEKTPIID) in HBSS was used as gap junction inhibitor (5, 10, 25). Cells were exposed to the inhibitor for at least 50 min before microinjection or mechanical stimulation experiments. Apyrase (50 U/ml in HBSS) was used as a nucleotide triphosphate inhibitor and was applied 1 min before ATP or UTP application.

Statistics. Two-way ANOVA tests were used in all data comparisons unless otherwise noted. Significance was assumed when $P < 0.05$. Graphs show averages ± SD.

RESULTS

Identification of cultured cell phenotype. Isolated AT2 cells were cultured for 7 days on different matrices and/or in the presence of KGF. Cell phenotype at day 7 was initially characterized by morphometric staining with tannic acid (30). Tannic acid stains of isolated AT2 cells grown on a fibronectin/collagen matrix displayed typical AT1-like features; the cells were flattened and contained few lamellar bodies (Fig. 1). In contrast, isolated AT2 cells cultured on matrices supplemented with laminin-5 (Ln-5) or in media supplemented with KGF were more compact in shape and displayed distinct lamellar bodies, typical of freshly isolated AT2 cells. Immunocytochemistry with specific antibodies generated to the AT2 phenotypic marker surfactant protein C (SP-C) and the alveolar type I (AT1) phenotypic marker aquaporin 5 (AQP5). Each row represents typical differential interference contrast micrographs (tannic acid stain) or confocal micrographs (SP-C, AQP5, and controls using secondary antibody alone) under the following growth conditions: type I collagen/fibronectin matrix (Fibro+, KGF --, Ln-5 ---); type I collagen/fibronectin matrix in medium supplemented with keratinocyte growth factor (KGF; Fibro+, KGF+, Ln-5 --); type I collagen/fibronectin/laminin-5 matrix (Fibro+, KGF --, Ln-5+); type I collagen/laminin-5 matrix supplemented with KGF (Fibro+, KGF+, Ln-5 --); or type I collagen/laminin-5 matrix (Fibro+, KGF --, Ln-5+). Bars, 5 µm. On the type I collagen/fibronectin matrix, the morphological and immunocytochemical stains were indicative of an AT1 phenotypic type. On all other matrices tested, the smaller cells with distinct lamellar bodies were indicative of an AT2 cell phenotype. Distinct punctate staining for SP-C predominated in cells grown on matrix supplemented with Ln-5 or in the presence of culture medium supplemented with KGF, also indicating an AT2 cell phenotype. Ln-5 and KGF supplements can maintain AT2 phenotypic staining.

<table>
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<tr>
<th>Tannic Acid</th>
<th>SP-C</th>
<th>AQP-5</th>
<th>Control</th>
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<tr>
<td>Fibro + KGF -- Ln-5 --</td>
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Fig. 1. Phenotypic characterization of 7-day-old primary cultured alveolar type II (AT2) cells. Cultured AT2 cells were stained with tannic acid and immunostained for the AT2 phenotypic marker surfactant protein C (SP-C) and the alveolar type I (AT1) phenotypic marker aquaporin 5 (AQP5). Each row represents typical differential interference contrast micrographs (tannic acid stain) or confocal micrographs (SP-C, AQP5, and controls using secondary antibody alone) under the following growth conditions: type I collagen/fibronectin matrix (Fibro+, KGF --, Ln-5 ---); type I collagen/fibronectin/laminin-5 matrix (Fibro+, KGF --, Ln-5+); type I collagen/laminin-5 matrix supplemented with KGF (Fibro+, KGF+, Ln-5 --); or type I collagen/laminin-5 matrix (Fibro+, KGF --, Ln-5+). Bars, 5 µm.
collagen matrix resulted in >80% of the cells staining positive for SP-C (Fig. 1). However, ~10% of these cells displayed some staining for AQP5 (Fig. 1). The loss of AT2-specific markers in 7-day-old cultures of AT2 cells can be prevented by the addition of Ln-5 to the type I collagen matrix or KGF to the growth medium.

Connexin expression in cultured AT2 cells. Previous reports on connexin expression in cultured AT2 cells have demonstrated at least six known isoforms (1, 20, 25, 27); however, the connexin expression patterns have not been consistent among reports. Immunocytochemistry and specific antibodies generated to individual connexin isoforms were used to determine whether extracellular matrix components or growth medium supplemented with KGF could alter connexin expression in 7-day-old cultures of AT2 cells. Immunocytochemistry with antibodies generated to Cx26 and Cx46 showed diffuse punctate staining in cells from each of the matrix/KGF conditions that maintained AT2 phenotypic markers, suggesting a constitutive expression among cultured AT2 cells (Fig. 2). However, Cx26 was not expressed in cells grown on a type I collagen/fibronectin matrix, whereas Cx46 was expressed in these cells (Table 1) (25). Thus Cx46 was independent of cell phenotype and constitutively expressed, whereas Cx26 was determinative for an AT2 phenotype. Alternatively, Cx43 was determinative for an AT1 phenotype; it was not expressed in cells grown under any of the conditions in which AT2 phenotypic markers were expressed (Fig. 2) but was expressed in cells with AT1 phenotypic markers (Fig. 1, Table 1). Two of the connexins tested were variably expressed in AT2 cell phenotypes: Cx32 showed limited punctate staining in the presence of KGF (Fig. 2); and Cx40 showed punctate staining when KGF was absent (Fig. 2, Table 1). Together, these results suggest that changing the extracellular matrix or adding KGF will alter phenotype and/or connexin isoform expression in AT2 cells (Table 1).

Although most of the cultured AT2 cells were in contact with at least one neighboring cell, there was always a fraction (~5%) of the cultured cell population

### Table 1. Connexin isoform expression in 7-day-old cultured AT2 cells under varying matrix and/or growth factor conditions

<table>
<thead>
<tr>
<th>Phenotypic Dependent</th>
<th>AT2</th>
<th>AT1</th>
<th>Variable</th>
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<td>Cx26</td>
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Extracellular matrix and/or keratinocyte growth factor (KGF) supplement are indicated in the extreme left column (all matrices include type I collagen). Each subsequent column is representative of connexin (Cx) isoform. +, Positive stain, –, negative stain, and +/–, positive stain with reduced punctate staining. Table is descriptive of whether individual isoforms are associated with alveolar type II (AT2) phenotypic characteristics or alveolar type I (AT1) phenotypic characteristics. Accordingly, Cx26 and Cx43 were phenotypic dependent; Cx32 and Cx40 were variable; and Cx46 was constitutive.

*Data summarized from Ref. 25.

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Fig. 2. Connexin protein expression in primary cultured AT2 cells. Cultured AT2 cells that retained AT2 morphology and markers in culture were immunostained for connexin isoforms and viewed with confocal microscopy. Bar, 5 μm. Connexin (Cx) 26 expression was evident by distinct punctate staining in each condition tested, and none of the culture conditions displayed Cx43 expression. These connexins were labeled “determinative” for cell phenotype. Cx32 was evident in cultures grown on KGF, and Cx40 was evident in cultures grown without KGF. These connexins were labeled “variable” due to their differential expression patterns despite the consistent AT2 phenotypic markers. Similar to Cx26, Cx46 expression was evident in all culture conditions. However, unlike Cx26, Cx46 was also expressed in AT2 cells grown on a type I collagen/fibronectin matrix that results in positive staining for AT1 phenotypic markers (Fig. 1) (25). For comparison, control stains using secondary antibody alone are shown for each condition tested.
that remained solitary. To determine whether cell-cell contact influenced connexin expression, the percentage of cells expressing positive punctate staining for connexin isoforms was independently plotted for adjacent cells and solitary cells (Fig. 3). Expression of connexin in adjacent cells is consistent with confocal images from Fig. 2 (Fig. 3A). Significant punctate staining of connexins in solitary cultured AT2 cells is seen only in Cx26, Cx32, and Cx46, and only under conditions that combine an Ln-5 matrix component with KGF supplement (Fig. 3B). Thus cell-cell contact can contribute to the observed connexin expression patterns.

Dye coupling in cultured AT2 cells. The ability of cells to transfer the 437-Da negatively charged fluorescent tracer Lucifer yellow after microinjection into a single cell was used to assess gap junctional coupling in the cultured AT2 cells (1, 20). Seven-day-old cultures expressing AT2 or AT1 phenotypic characteristics displayed functional dye coupling (Fig. 4). Additionally, in both cell phenotypes, dye coupling was restricted by preinubcation with the gap junctional inhibitor gap27 (Fig. 4). Thus the change in connexin profiles between AT2 and AT1 phenotypes does not preclude functional dye coupling nor inhibitor sensitivity in the 7-day-old cultured cells.

Cultured AT2 cells respond to ATP and UTP by changing [Ca\(^{2+}\)]. Two nucleotide triphosphates, ATP and UTP, have been used as effector molecules to raise [Ca\(^{2+}\)], in lung epithelium (13, 22, 24). These nucleotide triphosphates additionally have been shown to coordinate mechanically induced intercellular Ca\(^{2+}\) signals in upper airway epithelial cells (23) and wound-induced intercellular Ca\(^{2+}\) signals in cultured cells with AT1 phenotypic markers and morphology (25). [Ca\(^{2+}\)]\(_i\) was monitored in 7-day-old cultured cells with AT2 phenotype after bath application of 10 \(\mu\)M ATP (Fig. 5, A–D) or 10 \(\mu\)M UTP (Fig. 5, E–H) to determine whether these cells displayed similar purinergic responses. Application of either nucleotide triphosphate resulted in an increase of [Ca\(^{2+}\)]\(_i\) in all cells in the field of view within 25 s. [Ca\(^{2+}\)]\(_i\) returned to near baseline levels within 60 s despite the continued presence of ATP or UTP. Apyrase effectively eliminated the ATP- (Fig. 5, I–L) or UTP-induced (not shown) [Ca\(^{2+}\)]\(_i\) responses in 7-day-old cultured AT2 cells with AT2 phenotypic characteristics. These responses are consistent with P2Y2 receptor activation (31).

Mechanically induced intercellular Ca\(^{2+}\) signaling in cultured AT2 cells. [Ca\(^{2+}\)]\(_i\) was monitored in a field of 7-day-old cultured cells with AT2 phenotype to determine whether these cells could propagate intercellular Ca\(^{2+}\) signals following mechanical stimulation of a single cell. In all matrix/KGF combinations that maintained AT2 cell markers and morphology, mechanical stimulation of a single cell resulted in [Ca\(^{2+}\)]\(_i\) changes in six to seven cells (Fig. 6, A–D, and Fig. 7). Similar to the ATP/UTP-induced [Ca\(^{2+}\)]\(_i\) changes, [Ca\(^{2+}\)]\(_i\) recovered within 60 s following mechanical stimulation. Cultured cells were exposed to the nucleotide phosphatase apyrase or the gap junction inhibitor gap27 to determine the mechanism of intercellular Ca\(^{2+}\) signaling. After application of apyrase, mechanical stimulation resulted in an increase of [Ca\(^{2+}\)]\(_i\) that was restricted mostly to the stimulated cell (Fig. 6, A–D, and Fig. 7), significantly lower than mechanical signals (Fig. 7). A 5-min washout of apyrase allowed for a return to normal cell participation in mechanically induced Ca\(^{2+}\) signals (Fig. 7). Similar experiments with gap27 did not significantly reduce intercellularly propagated [Ca\(^{2+}\)]\(_i\) changes in response to mechanical stimulation (Fig. 6, I–L, and Fig. 7). There were no significant differences in response to mechanical stimulation in cultured AT2 cells grown in any of the matrix/KGF conditions that maintained AT2 phenotypic markers (Fig. 7).
In the lung alveolar epithelium, much of the physiological maintenance is attributed to AT2 cells. These cells are typically found in the corners of the alveoli, surrounded by AT1 cells, which are derived from AT2 cells. Maintenance and differentiation of alveolar cells can be profoundly altered by both the soluble and insoluble macromolecules that compose the extracellular matrix (7, 28, 29, 34). The developmental fate of AT2 cells can be altered by differing matrix or growth factor components (6, 29, 34, 44). KGF has been indicated as a mitogen for AT2 cells (44) and has been shown to maintain AT2 cell phenotype in cultured AT2 cells (1, 6). Because the extracellular matrix and growth factors change in response to injury (i.e., when cell growth and differentiation are necessary), they have been suggested as triggers for cell division, differentiation, and migration that underlie reepithelialization in vivo (35, 45).

In this study, the effects of KGF on maintenance of the AT2 cell phenotype in primary culture are confirmed and extended to 7-day-old cultures. Additionally, a new set of matrix conditions (type I collagen enriched with Ln-5) is shown to also be capable of maintaining the AT2 cell phenotype in culture for at least 7 days. The effects on cell phenotype for both these conditions are demonstrated using standard morpho-

**DISCUSSION**

In the lung alveolar epithelium, much of the physiological maintenance is attributed to AT2 cells. These cells are typically found in the corners of the alveoli, surrounded by AT1 cells, which are derived from AT2 cells. Maintenance and differentiation of alveolar cells can be profoundly altered by both the soluble and insoluble macromolecules that compose the extracellular matrix (7, 28, 29, 34). The developmental fate of AT2 cells can be altered by differing matrix or growth factor components (6, 29, 34, 44). KGF has been indicated as a mitogen for AT2 cells (44) and has been shown to maintain AT2 cell phenotype in cultured AT2 cells (1, 6). Because the extracellular matrix and growth factors change in response to injury (i.e., when cell growth and differentiation are necessary), they have been suggested as triggers for cell division, differentiation, and migration that underlie reepithelialization in vivo (35, 45).

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HBSS supplemented with apyrase. I–L gap27 ( ), is indicative of coordination of the Ca²⁺ signal in the presence of apyrase ( ), but not ATPase apyrase, but not by gap27. The effect of apyrase was reversed on July 27, 2017 http://ajpcell.physiology.org/ Downloaded from

Fig. 6. Mechanically induced changes in [Ca²⁺], in cultured AT2 cells. Color maps (left to right) are of [Ca²⁺], of 7-day-old cultured AT2 cells that retained AT22 phenotypic characteristics over time in response to mechanical stimulation of a single cell (arrow). Time after stimulation is indicated in each box (lower right). White lines depict cell borders. A color bar indicating approximate [Ca²⁺] is shown (bottom). Images are of cells grown on a type I collagen/Ln-5/fibronectin matrix without KGF; however, all matrix/growth factor conditions that maintained AT22 phenotypic characteristics responded in a similar fashion. A–D: mechanical stimulation in Hanks’ balanced salt solution (HBSS) alone. E–H: mechanical stimulation in HBSS supplemented with apyrase, I–L: mechanical stimulation in HBSS supplemented with 130 μM gap27. Mechanical stimulation of a single cell resulted in an intercellular Ca²⁺ signal (A–D). The restriction of the signal in the presence of apyrase (E–H), but not gap27 (I–L), is indicative of coordination of the [Ca²⁺] response through extracellular release of nucleotide triphosphates.

Fig. 7. Mechanically induced Ca²⁺ waves in cultured AT2 cells grown under different matrix/growth factor conditions. The average number of cells participating in a mechanically induced intercellular Ca²⁺ signal is plotted for each matrix/growth factor condition tested. Error bars are standard deviation. *Significantly different number of cells displaying increases in [Ca²⁺], after mechanical stimulation (P < 0.01). In all matrix/growth factor conditions that supported AT22 cell phenotype in 7-day-old cultures, Ca²⁺ waves were blocked by the ATPase apyrase, but not by gap27. The effect of apyrase was reversible, with washout restoring control level of cell participation.

Experimental Conditions

AT1 phenotypic characteristics, respectively, is consistent with a recent report proposing that gap junctional proteins are under phenotypic control in alveolar epithelial cells (1). However, of the five connexins tested in this report, Cx46 was constitutively expressed in cells with either AT1 or AT22 phenotypic characteristics, and Cx32 and Cx40 were variably expressed in cells with AT22 phenotype characteristics dependent on the presence (Cx32) or the absence (Cx40) of KGF (Table 1). When connexin expression was compared with cultured AT22 cells in contact with neighboring cells or without neighbors, a marked reduction in connexin expression was observed in all growth conditions except for the type I collagen/Ln-5/KGF matrix/growth factor combination (Fig. 3). Under this growth condition, Cx26 (indicative for AT22), Cx32 (dependent on KGF), and Cx46 (constitutive between AT1 and AT22) showed percentages of solitary cells positive for expression that were similar to adjacent cells. Thus expression of specific connexins in alveolar epithelium may be associated with phenotype, although some connexin isoforms are independent of the differentiated state of the alveolar epithelial cell.

There is precedence for the regulation of connexin expression in response to changes in the extracellular matrix. In a recent study (20), Cx43 expression patterns were altered by the amount of fibronectin in the matrix, resulting in a cell flattening and an AT1-like phenotype. In cell matrices in which fibronectin was removed, Cx43 expression was shifted from plasma membrane expression to a juxtanuclear position, which subsequently limited cell coupling. Extracellular matrix influence on connexin expression is not limited to cultured AT22 cells; both primary liver cultures and
keratinocytes have displayed altered connexin expression patterns in response to extracellular matrix changes (26, 41). In keratinocyte cultures, Ln-5 induced a shift of Cx43 expression to the plasma membrane controlled through the binding of the α3β1-integrin; growth on type I collagen alone was not sufficient for this transformation (26). Combined, these data suggest a role for the extracellular matrix control of differentiation via connexin expression.

The connexin isoform expression patterns among cultured AT2 cells are obviously complex and are likely cellular responses to environmental cues. Recent findings that emphasize variable transfer of specific tracers or small metabolites between gap junctions made of differing connexin isoforms (16, 17, 32) raise the possibility that complex patterns of connexin isoform expression support changes in intercellular communication among or between cultured AT2 cells. AT1 cell phenotypes and all of the tested matrix/KGF combinations that supported AT2 phenotypes in 7-day-old cultures were shown to be functionally dye coupled with Lucifer yellow microinjection studies (Fig. 4). This is in agreement with previous studies on cultured AT2 cells (1, 20, 27). Conversely, the gap junction-mediated propagation of intercellular Ca\(^{2+}\) signals in 7-day-old cultured AT2 cells expressing AT1 phenotypic characteristics (25) were not observed in the 7-day-old cultured AT2 cells that retained AT2 phenotypic characteristics (Fig. 7). The cells that retained AT2 markers and morphology responded to mechanical stimulation by propagating intercellular Ca\(^{2+}\) signals via a coordinated extracellular release of nucleotide triphosphates. As previously reported, nucleotide triphosphate-mediated intercellular Ca\(^{2+}\) signals only occurred in cultured cells with AT1 phenotypic characteristics after localized wounding and subsequent compromise of the plasma membrane (25). The inconsistency between the transfer of second messenger molecules between intact cells and functional dye coupling of Lucifer yellow further suggests that permeability through gap junctions made of different connexin isoforms can be dependent on the structural features (e.g., size and charge) of the passing molecule. It also raises the physiological hypothesis that different connexin expression patterns allow for different permeabilities to second messenger molecules and cellular metabolites and thus differential cellular coordination and tissue function.

An examination of connexin expression differences from cultured AT2 cells with AT2 phenotypic characteristics and AT1 phenotypic characteristics shows an increase in Cx43 as cells take on an AT1 phenotype (Table 1) (1). It is tempting to attribute the propagation of the intercellular Ca\(^{2+}\) signals through gap junctions formed by Cx43, and there is precedence for this in cultured cell models. For example, HeLa cells intercellularly propagate Ca\(^{2+}\) signals in response to mechanical stimulation through extracellular release of ATP. However, if HeLa cells are transfected with Cx43 linked to the green fluorescent protein, they can propagate intercellular Ca\(^{2+}\) signals via gap junctions (33). Cx43 transfection has also been shown to similarly coordinate intercellular Ca\(^{2+}\) signals via gap junctions in cultured HEK-293 cells (42). These artificial increases in Cx43 and subsequent gains of function in HeLa and HEK-293 cells parallel that seen during differentiation of cultured AT2 cells (Fig. 2) (1, 20, 25) and suggest that the observed gap junctional-mediated intercellular Ca\(^{2+}\) signals in cultured AT2 cells with AT1 phenotypic characteristics is through gap junctions made of Cx43. However, we cannot rule out differential regulation of expressed connexins nor differential connexin expression of isoforms not tested in this study during AT2 cell differentiation.

Ca\(^{2+}\) signaling pathways have been shown to be important in AT2 cell physiology. For example, they are associated with extracellular matrix protein synthesis (14), differentiation from an AT2 to AT1 phenotype (21), surfactant secretion (37), and recognition of AT1 cell signaling (2). The redundant mechanisms observed in the intercellular Ca\(^{2+}\) signal also may have some physiological significance. For example, during the repair of the alveolar epithelium, AT2 cells divide, differentiate, and migrate to reform the epithelial layer (43). Coordination of AT2 cell function immediately following injury in which AT2 cells are not normally in contact with each other may require extracellular signaling, whereas migrating collections of closely packed alveolar epithelial cells observed later in wound healing may better coordinate specific messages via gap junctions. Thus both the mechanism and physiological outcome of Ca\(^{2+}\) communication may be defined by the cell phenotype and/or distance needed for communication. The establishment of extracellular matrix and/or growth factor conditions that can maintain AT2 phenotypic characteristics in cultured cells will allow for a better model to assess physiological differences, such as cell coupling and signaling mechanisms, between AT2 and AT1 cells in vivo.

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