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INFLUENCE OF EXTRACELLULAR MATRIX AND COLLAGEN COMPONENTS ON ALVEOLAR TYPE 2 CELL MORPHOLOGY AND FUNCTION

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SUMMARY

The effects of various extracellular matrices and collagenous components on the morphology, growth, and function of cultured alveolar type 2 cells is examined. Cells grown on an endothelial matrix (EC) showed the greatest adherence, some cell division, and spreading to reach confluence sooner than cells grown on an epithelial matrix or on various types of collagen. The attenuated cells from all cultures were not true type 1 cells because, on trypsinization, they detached as sheets, reverted immediately to a cuboidal shape held together by junctional complexes, and showed an apparently normal content of lamellar bodies. The greatest synthesis of disaturated phosphatidylcholine (DSPC) was seen in cells grown on EC soon after confluence, but all cultures showed reduced but equal levels of DSPC-DNA by Day 4. This occurred whether cells were attenuated or cuboidal in shape. The results suggest that some component(s) of the endothelial matrix at the alveolar basement membrane facilitates epithelial cell growth. However, over longer culture periods the matrix preparations had little effect on type 2 cell proliferation whereas function diminished. This suggests that maintenance of these cells as normal type 2 cells or their further differentiation to the type 1 form requires some additional cell derived factor(s).

Key words: lung; pulmonary surfactant; cell culture; cell division; differentiation.

INTRODUCTION

The type 2 alveolar epithelial cell has two main functions: It is the site of synthesis of pulmonary surfactant (18), and it is the regenerative cell of the alveolus whereby epithelial injury is repaired by type 2 cell division with subsequent transformation of some cells to the type 1 form (1). Various attempts have been made to study type 2 cells in culture with respect to control mechanisms of surfactant synthesis and secretion as well as proliferation and differentiation to type 1 cells. However, even freshly isolated cells cultured in plastic dishes lose functional activity with time, do not proliferate, and show morphologic changes as cells spread out to resemble type 1 epithelium (6,11).

These cellular changes may be due to culturing on an inappropriate substrate because various studies have indicated that the properties of cellular growth and differentiation may be dependent on the underlying matrix (12,17). In the lung, type 1 epithelium predominantly overlies capillaries where the matrix is mostly type IV collagen and laminin, whereas type 2 cells are usually found in alveolar corners where there is an additional matrix component of type I collagen (13). It is possible that the underlying collagen may influence the epithelial cell structure and function, and we have previously shown in developing lung that inhibition of fibrillar collagen production is associated with retarded surfactant synthesis by the type 2 cells (10).

A number of published studies suggest a role for a specific matrix or a single component in maintaining type 2 cell morphology or function (5,8,15,21), but it is difficult to make comparisons among the various experimental conditions. Our aim in the present studies is to compare the morphology, growth, and differentiation of type 2 cells from the same fresh preparation grown on culture dishes coated with various collagen types, and with extracellular matrices of epithelial and endothelial cells. These complete matrices were used since the basement membrane of the air-blood barrier at the capillary level is composed of fused epithelial and endothelial basement membrane components (25).

MATERIALS AND METHODS

Type 2 cell preparation. Type 2 cells were isolated from rat lungs using the method of Dobbs et al. (7). Briefly, the lungs were excised from 150-g, male Sprague-Dawley rats and, after perfusion, 40 ml solution containing elastase at 4.3 U/ml was instilled into the trachea over a 20-min period. Subsequently the lungs were minced and filtered through successive nylon meshes down to 7 μm, to obtain a cell suspension. Inasmuch as type 2 cells lack Fc receptors, the mixed cell suspension was incubated in a petri dish coated with IgG to which other lung cells and leukocytes attached. Nonadherent cells were collected, spun down, and rinsed. We have examined pellets of these cells by electron microscopy and found that >94%

of cells were typical type 2 cells containing lamellar lipid inclusions. Viability by trypan blue was >90%. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for counting by hemacytometer and seeding into culture dishes.

Usually cells were isolated from two rats then pooled for each experimental run. Cells were seeded into wells of a 24-well culture plate (Falcon) at 2×10^5 cells in 1 ml of DMEM with FBS. Alternatively (*see* below), cells were seeded into 35-mm petri dishes, which have 5 times the surface area, at 1×10^6 cells in 2 ml DMEM. Cells were cultured for periods up to 7 d for a series of morphologic and biochemical studies comparing behavior on different substrates.

Substrate preparation. Cells were seeded directly onto the plastic (PL) surface of 24-well culture plates or into plastic wells coated with various extracellular matrix components. Collagens type 1 from calf skin and type IV from human placenta (Sigma Chemical Co., St. Louis, MO) were dissolved by stirring overnight at 4° C in sterile 20 m*M* acetic acid. A stock solution of 4 mg/ml was diluted with cold, sterile 100 m*M* NaCl in 100 m*M* tris pH 7.6. The dilution varied so that a final volume of 0.4 ml was pipetted into each well of a culture plate to give a final collagen content of 100 or 400 μg. The plates were incubated at 37° C for 1 h to form collagen gels. Similarly, a mixture of type I and IV solutions were combined in equal quantities to give a mixed gel of the same collagen content. In addition to the collagen gels, amorphous collagen layers of 50 and 200 μg were prepared by pipetting the acetic acid solutions into wells, then drying at room temperature in a laminar flow hood.

The epithelial basement membrane material, Matrigel (Collaborative Research, Inc., Bedford, Mass.), was also coated onto culture plates. This matrix is derived from the EHS mouse tumor and is rich in collagen type IV and laminin. The concentrate was diluted 1:1 with DMEM then 0.1 ml (about 0.5 mg protein) was coated onto each culture well at 4° C. A gel formed when culture plates were incubated at 37° C for 1 h before seeding the cells.

A commercially available endothelial cell-derived matrix was also used. Culture dishes (35 mm) were purchased with a coating of extracellular (EC) matrix secreted by bovine corneal endothelial cells (Cedarlane Labs, Hornby, Ontario). These cells were removed by 30 min treatment with 0.5% Triton X-100. The remaining amorphous extracellular layer is predominantly collagens III and IV with fibronectin (9). This matrix is similar to that produced by vascular endothelial cells and promotes the growth of both human and bovine cells (9). The EC matrix remains and is firmly adherent to the plastic surface. Each dish was rinsed with media at 37° C before seeding cells.

Cell adherence. One day after seeding type 2 cells onto the various substrates, the medium was removed and the cell layers washed with serum-free DMEM, then incubated with a trypsin-EDTA solution at 37° C for 5 min to remove the adherent cells. After adding DMEM with FBS, the solutions were collected and the cells spun down, resuspended, and counted in a hemacytometer. Because there was equally low DNA synthesis in all cell preparations at 1 d (*see* below) the cell number was expressed as a percentage of the seeded cell number to give percent cell adherence. This was done in four experiments with at least four wells or three 35-mm dishes used for each substrate.

Morphology. At daily intervals during the culture period, 35-mm dishes of cells were photographed using a phase contrast microscope, and a dish was prepared at each time for in situ electron microscopy. The medium was removed, and the cells attached to the petri dish were rinsed in phosphate buffer before fixing with 2% glutaraldehyde for 1 h. Subsequently, this solution was replaced with 1% osmic acid followed by a graded series of alcohols to absolute, then Spurr plastic mixture. At this point, the ends were cut off embedding capsules, which were inverted over the cells in the petri dish. The plastic rose about 2 mm inside the capsules, and the dishes were placed in a 65° C oven to polymerize the plastic. Next day, additional liquid plastic was added to each capsule to make a 1-cm pillar on polymerizing. At this point the

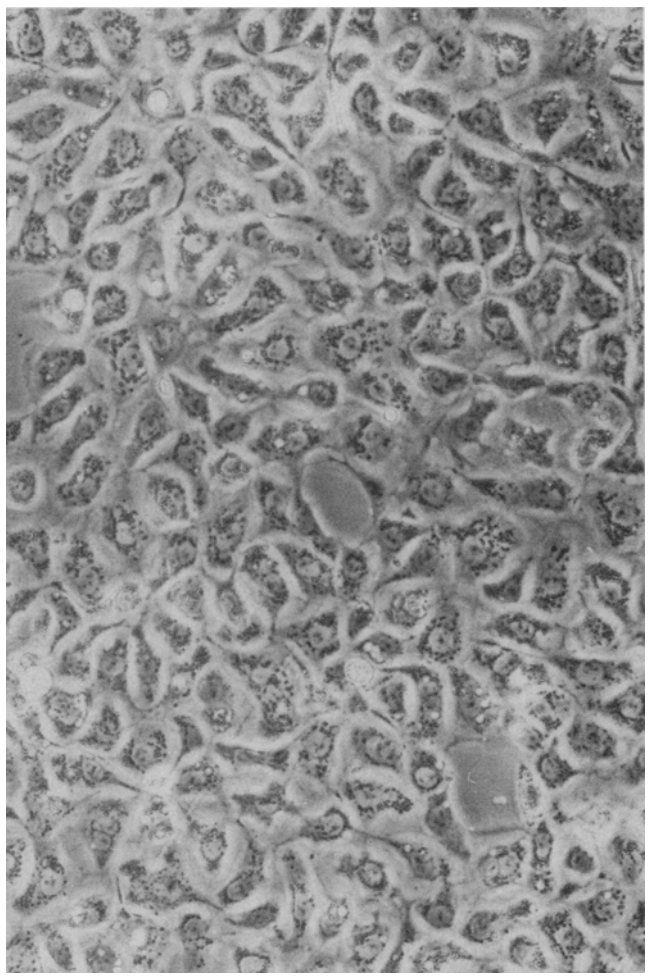

FIG. 1. Phase microscopy, type 2 cell cultures after 2 d growth on EC matrix. Cells are confluent and still contain dark inclusions. ×260.

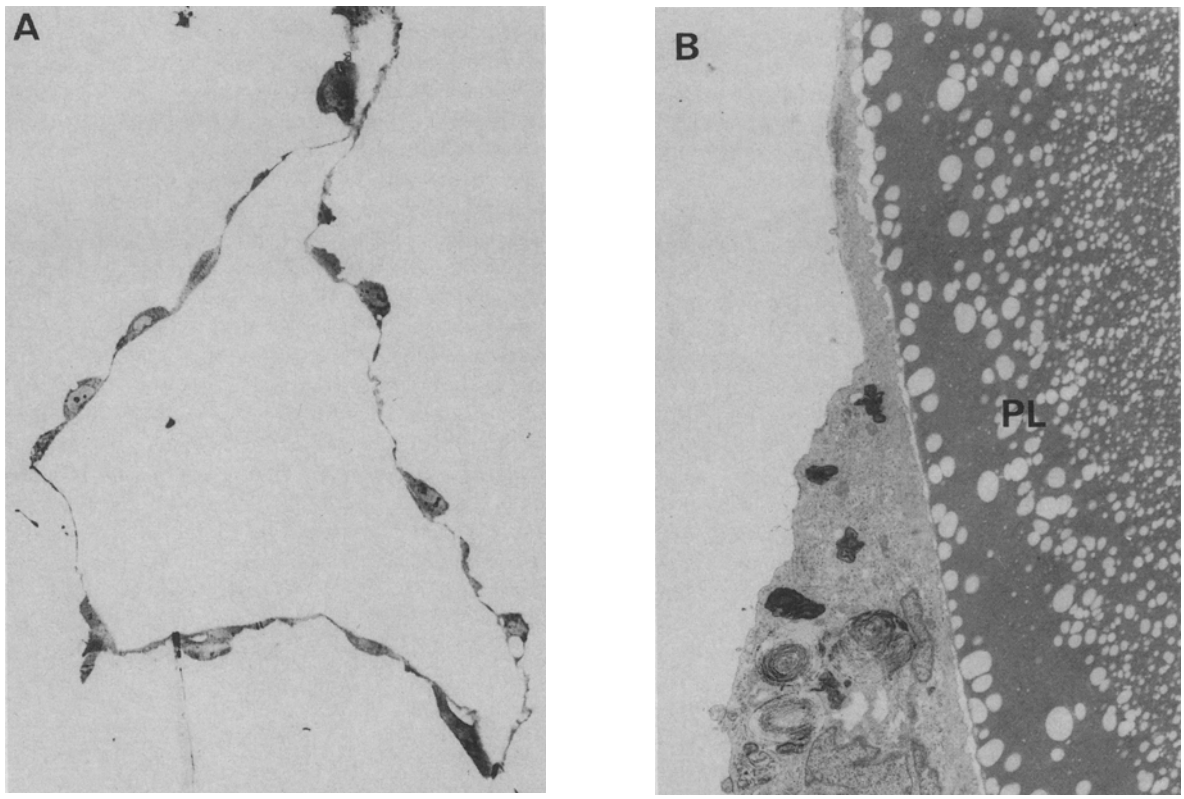

FIG. 2. Type 2 cell cultures after 3-d growth on plastic. *A*, in situ embedding shows a monolayer of attentuated epithelial cells with areas of very thin cytoplasm; 0.5-μm section, toluidine blue stain. ×683. *B*, electron microscopy shows an epithelial cell with apparently few lamellar bodies spreading over the plastic surface. ×4550.

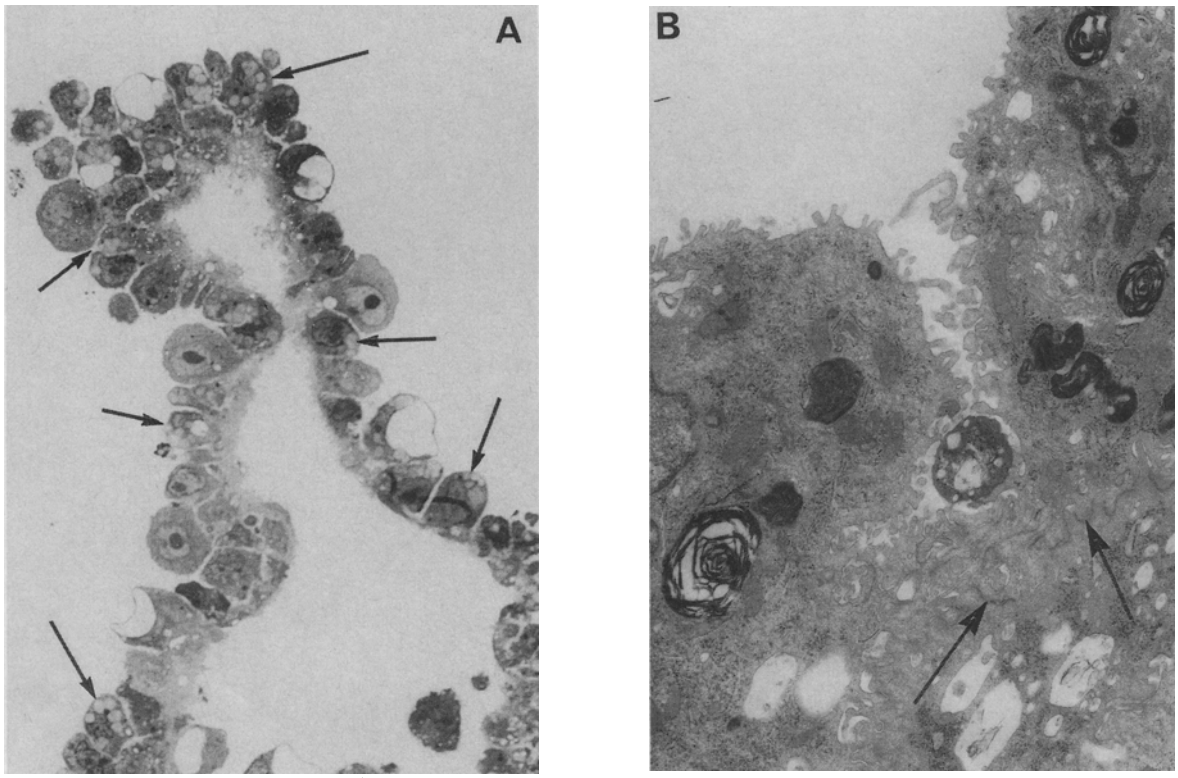

FIG. 3. Sections of cells from 3-d cultures removed from plastic. *A*, cells remain joined together as a monolayer reverting to cuboidal form with many inclusions (*arrows*); 0.5-μm section, toluidine blue stain. ×975. *B*, EM of the same pellet shows cuboidal type 2 cells with dark lamellar bodies; cells are connected by junctional complexes (*arrows*). ×12350.

capsule was broken off or cut way, leaving a plastic block with the cell monolayer at one end still attached to the surface coating from the removed petri dish. From these blocks, 0.5-μm sections were cut for light microscopy, and ultrathin sections cut for electron microscopy. In addition, type 2 cells were prepared as pellets for electron microscopy. Cells cultured as above were removed by trypsin, spun down, and rinsed. After 1 d they tended to remain as clumps or sheets but could be spun into a pellet for embedding in Spurr plastic. Sections were examined by light and electron microscopy after various days of culture.

DNA synthesis. Because of the difficulty in achieving a single cell suspension after 1 d of culture, cell counts were inaccurate, so we chose to evaluate cell proliferation by total DNA levels and by uptake of [^{3}H]thymidine. Cells were cultured for various periods before adding [^{3}H]thymidine at 0.1 μCi/ml for the final 4 h of culture. Cells were removed as above from three wells or dishes per time, and duplicate cytospin preparations made from each suspension. Slides were subsequently dipped in Kodak NTB2 emulsion in the dark room under a red filter. After 2 wk in total darkness, they were developed and stained with toluidine blue. For each time period and substrate, the percentage of cells labeled was calculated after counting 500 cells per slide.

Because of possible DNA synthesis without cell division, cells at Days 2, 3, and 4 were given 0.01 μg colchicine for the final 4 h, to arrest dividing cells in metaphase. Cells were removed and cytospin preparations made, stained for light microscopy, and the number of metaphases counted.

The total DNA of the cell cultures at each time was also measured biochemically. Cells pooled from three wells per plate or one 35-mm dish were washed with 5% trichloroacetic acid (TCA) then scraped off into TCA. The total DNA was measured by fluorimetry (22). The DNA was determined in duplicate for each of four experimental series, and was standardized to a seeding density of 2×10^5 cells. Means ± SE were calculated at each time and compared to DNA in cells grown on plastic by the Student's *t* test.

Disaturated phosphatidycholine (DSPC) levels. As a marker of type 2 cell function, the incorporation of [^{3}H]palmitic acid into DSPC, the major phospholipid component of pulmonary surfactant, was measured at various times of culture on the different substrates. A dose of 1.0 μCi/ml [^{3}H]palmitic acid (potassium palmitate complexed to albumin) was added for the final 4 h of culture. The medium was removed, and the cells were washed then scraped into cold water. Lipids were extracted (4) then [^{14}C]DSPC was added as a tracer to correct for losses, and unlabeled DSPC was added as a carrier. The lipids were reacted with osmic acid and, after separation on an alumina column, DSPC was purified by thin layer chromatography (19). The spot was scraped off and dpm counted. Total incorporation into DSPC was determined and DSPC-DNA calculated for each culture

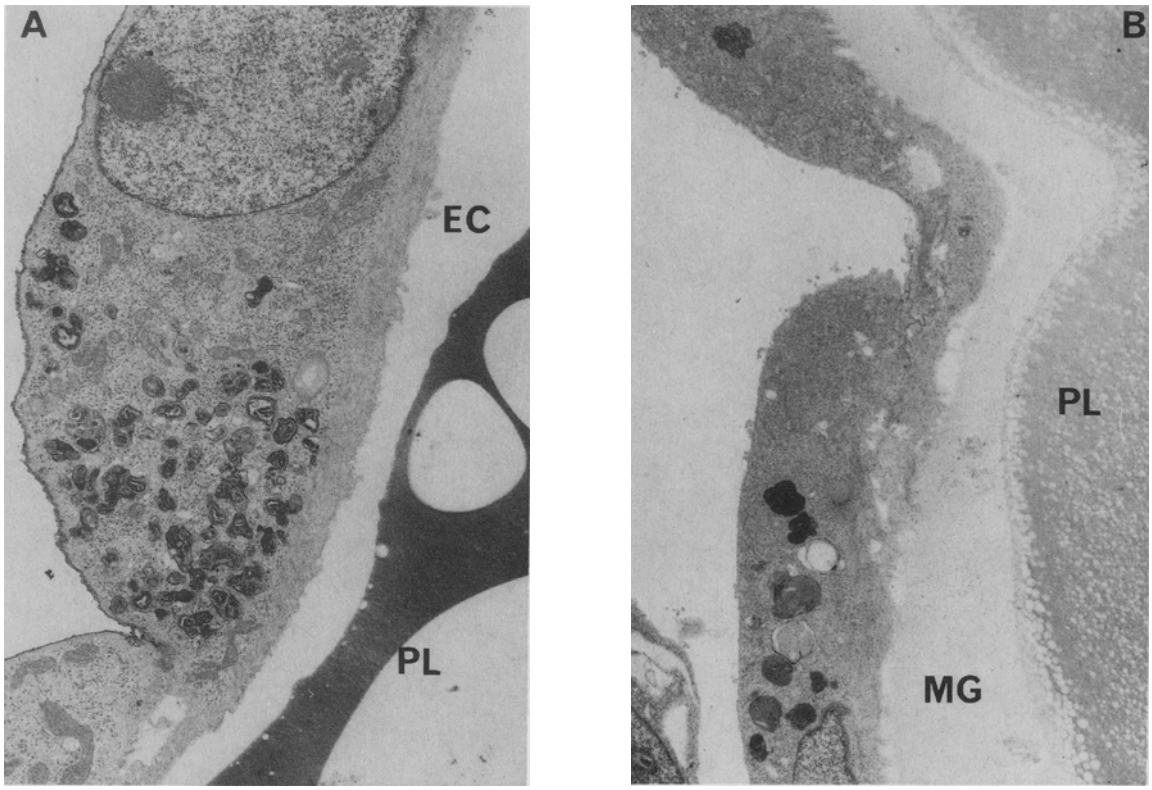

FIG. 4. Electron micrographs of type 2 cells in situ grown for 3 d on different matrices. *A*, extracell ×7800. *B*, Matrigel ×5200. In each case the cells spread over the matrix and PL, but these still contain dark lipid inclusions.

condition at Days 2, 3, and 4. When the culture medium was saved, negligible amounts of [^{3}H]DSPC were detected.

Results

Morphology. Although all dishes were seeded at the same cell density, the type 2 cells reached confluence at 2 to 3 d on EC compared to 4 d or more for cells grown on all other matrices (Fig. 1). Cells on type I collagen and Matrigel (MG) tended to stay in clusters initially then spread to give larger cells at later times. In all preparations examined by phase microscopy, dark inclusions typical of lamellar bodies were seen in decreasing numbers with time.

Cell morphology was seen in more detail in a 0.5-μm section and by electron microscopy. Cells grown directly on plastic spread over the surface and formed junctions with neighboring cells. This resulted in an attenuated cytoplasm, the cells resembling type 1 epithelium with apparently few lamellar bodies, as seen by light or electron microscopy (Fig. 2). However, when these fresh cell cultures at 2 to 4 d were briefly exposed to trypsin, intact epithelial sheets were removed from the surface as the cells remained joined together as a single cell layer. Sections of these preparations showed that the cells did not become round and separate but reverted immediately to the original cuboidal shape with junctional complexes between cells. The size and lamellar body content was similar to normal in vivo type 2 cells (Fig. 3).

Cells grown on gels of type IV collagen and the IV-with-I mixture resembled those grown on plastic, and no differences were seen when cells were grown on different concentrations of collagen. Cells cultured on the EC matrix also seemed to spread over the dish but still contained lipid inclusions when the in situ preparations were examined (Fig. 4 *A*). Cells maintained on MG were more rounded initially and did not spread as rapidly, but by Days 4 and 7 they resembled cells grown on plastic (Fig. 4 *B*). In the case of cultures grown on EC, MG, or gels, when the cells were removed with trypsin, they became a sheet of cuboidal cells and retained intercellular junctions.

Electron microscopy of these collagen gels and the commercial matrices showed that the coatings usually contained little or no fibrillar collagen, whereas a normal type 2 cell morphology was best maintained when the underlying matrix contained a higher proportion of type I collagen fibers. This situation was found at some areas of the MG but most often when a dried type I collagen sample was used as substrate. By phase microscopy and in situ sectioning, the cells did not attenuate but remained cuboidal, often in aggregates with prominent lipid inclusions (Fig. 5 *A*). By electron microscopy, the basal surface of these type 2 cells seemed anchored to the collagen fibrils (Fig. 5 *B*).

In all cultures it seemed that fewer cells were present at 7 d, and they were more spread out with fewer lipid

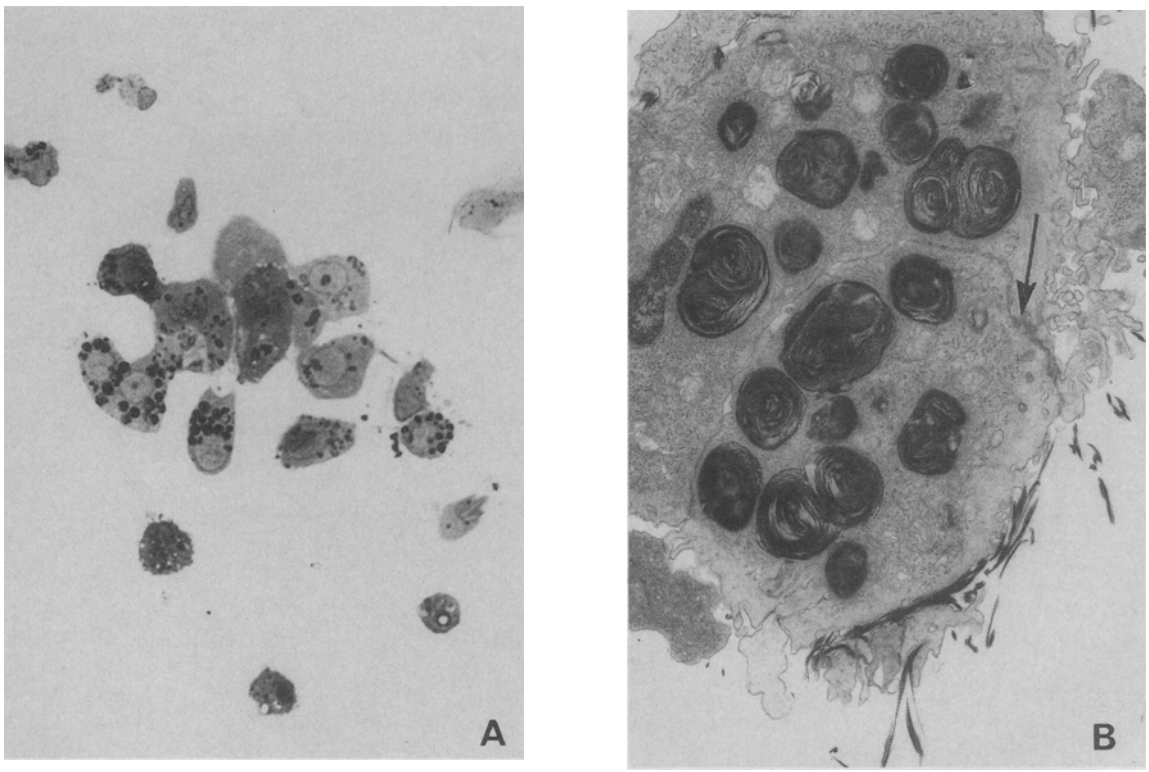

FIG. 5. Cells in situ grown 2 d on plastic dish coated with a dry collagen layer. *A*, clusters of cells maintain a shape and morphology similar to in vivo type 2 cells; 0.5-μm section. ×975. *B*, electron microscopy of this area shows typical type 2 cells with lamellar bodies. The cells are anchored at the base to collagen fibers and are linked by junctional complexes (*arrow*). ×13650.

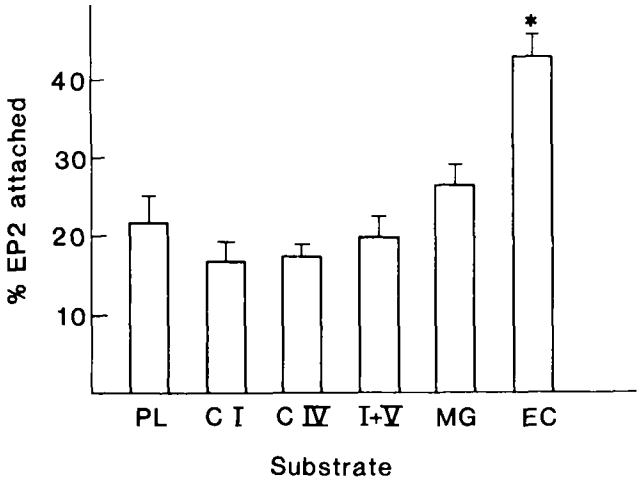

FIG. 6. Mean ± of initial seeded cells that were recovered 1 d after incubation in plastic wells coated with different substrates. *PL* = plastic; *CI* = collagen type I; *CIV* = collagen type IV; I & IV = equal mixture of collagen types I and IV; *MG* = matrigel; EC = extracel. ★ $P < 0.01$ significantly greater than plastic.

inclusions, although intercellular junctional complexes were still seen.

Attachment and growth. Cells were seeded at the same relative density on the different coated dishes, and the number removed after 1 d was counted. Very little DNA synthesis occurred over the first day (*see* below) so that the cell number recovered reflects adherence. The mean number of cells attached at 1 d was 20 to 25% of the original on all surfaces except EC, which gave a significantly greater rate of cell attachment at 43% (Fig. 6).

Cell proliferation could not be measured accurately by cell counts because these epithelial cells, once removed from the culture dishes, remained in clumps held together by cell junctions; if these were disrupted, many cells disintegrated. For these reasons we assessed cell growth by total DNA and DNA synthesis over the 7-d culture period. There was little change in DNA levels of cells grown on plastic or on the collagen preparations (Table 1). There seemed to be a higher trend for DNA values of cells on MG, which probably reflects the slightly higher attachment initially. The cultures on EC gave the highest initial DNA level. This increased at Day 2 and was always significantly higher than all other cell cultures ($P < 0.01$).

Pulse incorporation of [^{3}H]thymidine was also examined. There was very low labeling of cells in the first day then about 2% of cells were in DNA synthesis under the various conditions to 7 d, with the notable exception of the EC preparations. At Day 2 only there was significantly higher DNA synthesis in type 2 cells grown on the EC (Fig. 7, Table 2). Inasmuch as the higher labeling of cells grown on EC may be a function of the greater plating efficiency, in a few trials type 2 cells were seeded at twice the usual number onto plastic and MG-coated dishes. At Day 2, no increase in growth rate was seen, the cells reached confluence by spreading, and [^{3}H]thymidine uptake was unchanged from that given in Table 2.

Colchicine was added to some experimental cultures to determine if cell division actually occurred. Mitotic figures were not seen in any group, except for the cells grown on EC for 2 d. At this time of maximal DNA synthesis, a small percentage of cells (0.83 ± 0.1) were arrested in metaphase. In 0.2% of cells, a labeled metaphase was seen (Fig. 7 *B*).

Surfactant synthesis. As a measure of surfactant synthesis, the incorporation of palmitic acid into DSPC was measured and standardized to the DNA level. After 2 d of culture the values obtained were about 45% of those measured in freshly isolated type 2 cells. In all cases, surfactant levels declined over 4 d; however at Day 3 the cells grown on EC contained significantly higher DSPC levels than any other set of type 2 cells (Fig. 8). Some of the cultures were maintained to 7 d, when in each case DSPC values had declined further. No differences in DSPC levels were found among the cell preparations grown on different types of matrix, whether gel or amorphous, at various concentrations.

DISCUSSION

Studies of type 2 alveolar cells in culture have focused on the proliferative capacity of the cells in relation to

TABLE 1

DNA CONTENT OF CULTURES (MG ± SE/2 × 10^5 SEEDED TYPE 2 CELLS) GROWN ON DIFFERENT SUBSTRATES

	Days of Culture, $n = 5$				
Surface	1	2	3	4	7
Plastic	0.15 ± .02	0.17 ± .02	0.18 ± .03	0.23 ± .03	0.22 ± .03
Collagen I	0.16	0.14	0.18	0.19	0.21
IV	0.14	0.15	0.18	0.17	0.18
I & IV	0.14	0.14	0.18	0.19	0.19
Matrigel	0.20 ± .02	0.23 ± .03	0.24 ± .03	0.24 ± .03	0.25 ± .04
Extracell	0.28[a] ± .02	0.40[a] ± .04	0.42[a] ± .03	0.49[a] ± .03	0.51[a] ± .05

[a] $P < 0.05$, value > plastic on same day.

alveolar repair, and on phospholipid metabolism in relation to the synthesis of pulmonary surfactant (6,11,14,20). From the results of these and other studies it seems that even when maintained in primary culture over several days, lipid metabolism is reduced or altered, fewer lamellar bodies characteristic of type 2 cells are seen, and the cells flatten out over the plastic surface. The combination of reduced surfactant synthesis and flattened shape has been used as evidence that cellular transformation from type 2 to type 1 epithelium cells occurs in culture. In the present study, cells grown on plastic show the same characteristics; they spread out, attach by intercellular junctions, show a flattened appearance when fixed in situ (Fig. 2 *A*), and in sections seem to have few lamellar bodies. However, when these cells are removed from surface contact the junctions hold the cells together as sheets, the cuboidal shape is immediately restored (Fig. 3 *A*), and they seem similar to normal type 2 cells. This occurs to some extent with cells removed from the various substrates, and suggests that, as cells spread out and maintain epithelial cell-to-cell contact, there is a reduction in lamellar bodies that may not be accurate if quantitated per cell. Thus it seems that the culture of type 2 cells results in a spreading of the cells with somewhat poorer function rather than a transformation to a type 1 epithelium

The changes in type 2 morphology and function seen in culture led to trials of various substrates in attempts to improve the maintenance of these cells. In many culture systems, using different cell types, components of the extracellular matrix have been shown to influence cell proliferation and differentiation (12,17). In the present study, using complete matrices or collagen components, type 2 cell preparations all reach confluence, most rapidly when grown on EC. Total DNA is highest in EC cultures at 1 d and at each subsequent day examined. Inasmuch as thymidine incorporation is equally low in every group at 1 d, it seems that the initial high DNA in the EC group reflects cell adherence. This was confirmed in a separate cell attachment experiment where most seeded type 2 cells adhered to the EC matrix. Other components were not better than plastic, a finding similar to one study of type 2 cell adhesion in which no difference was found when separate components of alveolar matrix or fibroblast secreted matrix were used (3). We used EC because it is an endothelial matrix and may have a structure or composition similar to that lying beneath type 1 alveolar cells, which are in direct contact with a fused epithelial and endothelial basement membrane in the lung. Attachment and spreading are important properties in situations of alveolar repair where type 2 cells proliferate, then attach and migrate to repair type 1 cell injury at the capillary wall (1).

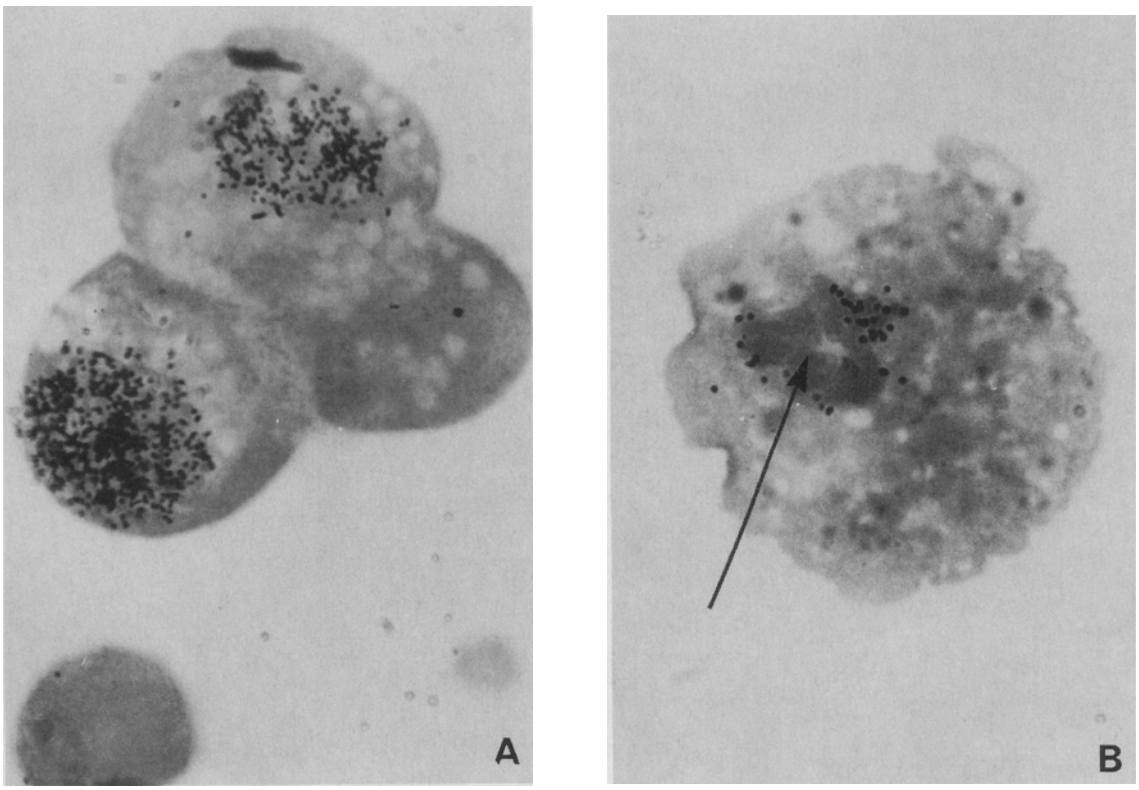

FIG. 7. Type 2 cells grown on EC for 2 d with a [^{3}H]thymidine pulse and 0.1 μg/ml colchicine added for the final 4 h. *A*, autoradiograph shows heavily labeled nuclei. *B*, some cells show labeling and are also arrested in metaphase (*arrow*); 0.5-μm sections. ×1235.

TABLE 2

PERCENTAGE (± SE) OF [³H]THYMIDINE-LABELED TYPE 2 CELLS GROWN ON DIFFERENT SUBSTRATES

Surface	Days of Culture, $n = 4$				
	1	2	3	4	7
Plastic	0.8 ± .8	2.6 ± .5	2.0 ± .4	3.1 ± .7	1.8 ± .4
Collagen I	0.2	2.2	2.7	3.2	2.0
IV	0.4	1.0	1.8	2.8	1.7
I & IV	0.5	1.1	1.8	2.1	1.8
Matrigel	0.2 ± .1	2.3 ± .4	1.7 ± .2	2.2 ± .4	1.9 ± .3
Extracell	1.0 ± .2	11.6[a] ± 2.0	2.5 ± .5	2.5 ± .4	2.0 ± .3

[a] $P < 0.01$, value > plastic at same day.

From Days 1 to 7 in culture, the DNA level increased only in the cultures grown on EC. On the other substrates, pulse thymidine labeling showed a consistent 2% of cells labeled and a small increase in total DNA. In EC-coated dishes, the consistently higher DNA levels could be attributed to the initial higher adherence plus a large increase in DNA synthesis at Day 2. Because of earlier reports of increased thymidine incorporation without cell division in cells grown on EC (14), and in cultures with growth factors added (5,20), we carried out a colchicine experiment to arrest cells in metaphase. Only cells grown on EC were seen in mitosis, although the relatively low percentage suggests that not all cells synthesizing DNA subsequently undergo division. It is likely that the cells cultured on EC reach confluence fastest due to a combination of greater cell attachment, followed by some cell division and later spreading.

At culture Day 3, cells grown on the EC matrix had a significantly higher level of DSPC synthesis per microgram of DNA than all other cell preparations. These cells were already confluent at Day 2, so that it is unlikely that DSPC associated with membrane synthesis makes a major contribution to this value. Otherwise, all values for DSPC synthesis showed a steady decline with time to Day 4 of culture. During this period, cell morphology varied from very attenuated cells with apparently few lamellar bodies in sections of cells grown on plastic, type IV, or mixed collagens to less spread-out morphology on EC and MG. The morphology most like type 2 cells in vivo was found in cultures where there was most collagen fibers (Fig. 5). However, in spite of the varied morphology in these cultures, the DSPC-DNA was equivalent at Day 4. Other investigators have shown that floating collagen cells (8), the use of MG (21,23), or culture on amnion basement membrane (16) improves type 2 cell morphology, but it does not seem that normal function is maintained over time in these various systems.

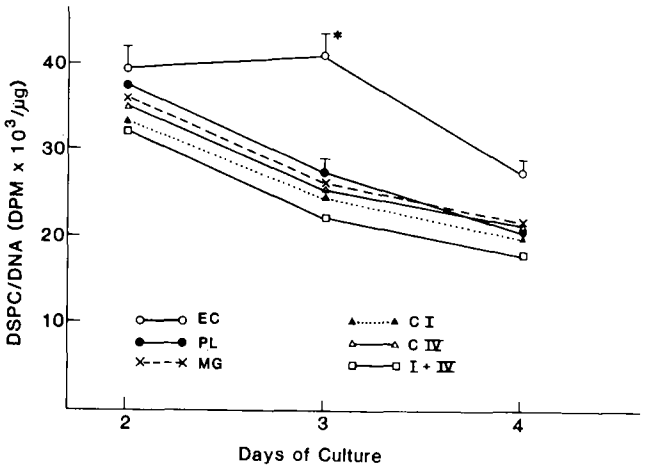

FIG. 8. Incorporation of [³H]palmitic acid into DSPC at various times, standardized to the amount of DNA/sample. Symbols as in Fig. 6.

The present study comparing type 2 cell behavior on various substrates shows that an endothelial-cell-derived matrix, which may have similarities to the alveolar basement membrane, allows better adherence of type 2 cells and promotes limited cell division in addition to cell spreading, properties that are useful in epithelial repair processes. Although epithelial cells become attenuated in culture, differentiation to a type 1 epithelial form does not occur on any of the substrates and probably requires some other factor not present in this culture system. However, the cultured cells do not remain as normal functioning type 2 cells. This may also indicate the lack of a specific factor, such as that secreted by fibroblasts during fetal lung development, that is required to maintain normal type 2 cell morphology and function (2,24).

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