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Original Article

Modulation of cellular transport characteristics of the human lung alveolar epithelia

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Abstract

Among the drug delivery and targeting (DDT) routes, lung alveolar epithelium has been given enormous attentions in terms of the delivery of a wide range of macromolecules such as gene- or protein-based nanopharmaceuticals. However, little is known about cellular modulation of lung transport characteristics by endogenous and/or exogenous agents. Thus, in the current study, impact of dexamethasone (DEX), which is a customary additive to the culture media of alveolar epithelial cells, was assessed with respect to some transport properties of the human adenocarcinoma A549 cells, which is a well-known cell-based *in vitro* model for lung epithelia. To achieve such goal, we studied the trans epithelial electrical resistance (TEER) and barrier restrictiveness using the paracellular marker, mannitol. Further more, the effect of DEX on the expression of clathrin, the main integral protein of clathrin coated pits, as well as dynamin (GTP binding protein essential for the vesicle budding off process) were assessed. A549 cells treated with DEX displayed distinct phenotypic and growth changes, also resuling in higher TEER values (~ 100 Ω.cm2) compared to the untreated cells (~ 45 $\Omega.cm^2$). Such paracellular tightness properties were also confirmed by mannitol permeability assay, showing a marked difference in the permeability coefficient between untreated and DEX-treated A549 cells, i.e., 7.83± 0.3.7 ($\times 10^{-6}$ $cm.s^{-1}$) and 3.46 ± 0.22 ($\times 10^{-6}$ $cm.s^{-1}$), respectively. Although DEX exerted little impact on expression of the clathrin mRNA, it elicited a significant increase in dynamin protein expression. Therefore, it is proposed that DEX supplementation of alveolar epithelial A549 cells culture media may confer a better model for pulmonary DDT investigations. However, more extensive work is needed to be carried out in order to clarify the precise role of DEX in cellular transport of macromolecules via receptor-mediated endocytosis/transcytosis.

Keywords: Alveolar epithelial; A549 cells; Cell culture; Lung delivery; Nanopharmaceutical targeting.

Introduction

Pulmonary route is of interest for both localized and systemic delivery of therapeutics, particularly biopharmaceuticals. Several anatomic/physiologic features make the lung suited to such implementations, including the large surface area (about 110-140 m^2 in adult human lung), thin barrier (1), high density of plasmalemmal vesicles (2) that may be involved in transport, extensive vascularization, relatively

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less enzymatic activity and a high level of perfusion which is equal to the total cardiac output (3, 4).

However, it is now well-known that the alveolar region in the intact lung is not easily accessible, thus numerous attempts have been conferred to utilize *ex vivo* and *in vitro* models. *In vitro* cell culture models, obtained from either continuous cell lines or isolated primary cultures, provide a useful tool for pulmonary DDT of a wide range of small to large molecules. Among these models, nevertheless, the simplicity of continuous cell lines in comparison to primary cell cultures, override its use despite pros with primary cultures that represent closer characteristics to that of *in vivo*.

Lung epithelium is mainly composed of two types of cells, extremely flat squamous alveolar epithelial type I (ATI) and small cuboidal type II (ATII) cells. ATI pneumocytes contain non-coated plasmalemmal invagination (caveolae in addition to clathrin) that can be a potential feature for removal of endogenous and exogenous solutes from alveolar airspace to blood (5, 6). ATII cells possess numerous lamellar bodies and play a major role in production of the alveolar surfactants (7). Furthermore, ATII cells serve as progenitor for the alveolar epithelium (8), which can divide and also differentiate to type I phenotype.

At both tissue and cellular level, glucocorticoids (GCs) are known to modulate a large variety of differentiation and growth responses of cells (9-12). It seems that GCs exert their beneficial effects on lung maturation and development through influence upon lung structure, alveolar-capillary permeability and the surfactant system.

Based upon available literature, using rat alveolar cell culture, a very intriguing observation is that in experiments for transport purposes, DEX is included within the culture medium, while investigators looking at the plasticity of alveolar cell differentiation tend not to include any glucocorticoid in their culture medium. However, surprisingly, no substantial information is available in published literature about the role of GC with respect to modulating transport characteristics of alveolar epithelial cell monolayers. Given all these statements above, we found it reasonable to propose the overall influence of DEX, an intrigue supplement, on the barrier properties of alveolar epithelial cells. In the current study, utilizing the human adenocarcinoma cell line (A549), which is considered to display the characteristics of type II phenotype (13), the effects of DEX (a synthetic GC) on the barrier property of alveolar epithelium have been studied. Further more, we looked at the expression of clathrin transcriptome along with dynamin protein in A549 cells, upon treatment with DEX. These two cellular elements are primarily involved in the cellular endocytosis and/or transcytosis trafficking of endogenous/exogenous macromolecules such as gene/protein-based nanoparticles (14).

Experimental

Materials

A549 cell line was obtained from the European Collection of Animal Cell Culture (ECACC) (Salisbury, UK). All cell culture plates and trans-wells were from Corning Costar (High Wycombe, UK). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin, Moloney Murine Leukemia Virus (MMLV) reverse-transcriptase, and Deoxy nucleotide triphosphate monomers (dNTPs) were all ordered from Invitrogen (Paisley, UK). Molecular weight DNA 100 bp ladder (hyper ladder IV) was from New England Biolabs (Herts, UK). Forward and reverse oligonucleotide primers were ordered from MWG-Biotech (Milton Keynes, UK). Dexamethasone, Sodium cacodylate trihydrate and [^{3}H]-manitol and Tri-reagent were purchased from Sigma-Aldrich (Poole, UK). Glutaraldehyde and osmium tetroxide were both from TAAB (Aldermaston, UK). Rainbow molecular weight marker was obtained from Amershem (Little Chalfont, Bucks, UK). Super Signal Ultra detection kit was from Pierce (Chester, UK). Fat-free milk powder was ordered from Marvel (London, UK). Mouse anti-dynamin II was from Transduction Laboratories (Oxford, UK). Rabbit anti-mouse Horseradish peroxidase (HRP) labeled with secondary antibody obtained from Dako (Cambridge, UK). All other chemicals not denoted specifically

were obtained from commercial sources in the highest quality available.

Cell culture

A549 cells were cultivated in DMEM containing, 10% FBS and penicillin G (100 units per ml) and streptomycin sulfate (100 µg per ml) at a seeding density of 4.0×10^4 cells /cm^2 onto 25 or 75 cm^2 T-flasks. Cultured cells were maintained in a water saturated atmosphere at 37°C, 5% CO_2 and 95% air, with medium replaced every 48 h and cells being passaged once a week. Prior to the experiment, cultured cells on T-flasks, were washed with pre-warmed phosphate buffer saline (PBS), 3 times, and cells detached from the flask using trypsin-EDTA with incubation at 37º C for 2-3 min, followed by addition of culture media (containing 10% FBS) to neutralize the excess trypsin activity. The resulting suspension was centrifuged; the cell pellet re-suspended in fresh media and counted using a hemocytometer. Cells were seeded at a density of 4.0×10^4 cells /cm^2 (with or without 0.1 µM DEX) onto the appropriate plate or polycarbonate membrane Transwell. Cells were fed every 24 h with pre-warmed fresh media.

(LM) and scanning electron microscopy (SEM)

The morphology of alveolar epithelial cells during culture period was monitored by LM and SEM. For LM, A549 cells were cultured on 24 well tissue culture treated plates with or without DEX supplementation. After confluency (96 h), photographs were captured using an Olympus IX50 inverted microscope, fitted with an Olympus DP10 automated digital processor for image capture (Olympus, Japan).

For SEM, A549 cells were grown on 13 mm cover-slips in plastic Petri-dishes, with or without Dex for 96 h. Prior to fixation, the monolayers were washed twice using PBS to remove all proteins, after which the fixation solution (comprised of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and 2% sucrose) was instantly added to cover the monolayer. The dishes were fixed for 30 min at room temperature. The post fixation process was carried out using incubation of samples with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 30 min at room temperature. Samples were then dehydrated using a graded ethanol solution (50, 70, 90 and 100%) and 30 min incubation with each concentration. The fixed samples were freeze-dried, sputter-coated with gold and observed under a scanning electron microscope (Steroscan 120, Cambridge Instruments, Cambridge, UK).

Trans epithelial electrical resistance

The tightness of the alveolar cell monolayer was investigated by measurement of the bioelectrical resistance properties. Briefly, A549 cells were cultured on 6.5 mm polycarbonate Transwell membranes, with or without DEX (0.1 µM) supplementation. TEER measurement was begun 48 h following cell seeding, using an Evom ™ epithelial voltohmmeter (World Precision Instrument, Sarasta, USA). Before TEER measurement, the electrode was kept in culture medium (37° C) for 10 min. As the electrical resistance displayed on Evom is based on ohm (Ω), TEER (Ω. cm^2) was calculated according to equation 1.

$$TEER\ (\Omega\ cm^2) = reading\ (\Omega) \times area\ (cm^2)$$

Equation 1

Mannitol permeability across A549 cells

The Para-cellular restrictiveness of the A549 cell monolayers was further examined by assessment of mannitol (a hydrophilic paracellular marker) permeability through the monolayer. Briefly, A549 cells (in the presence or absence of DEX) were cultured on Trans-wells for 4 days. Prior to permeability test, [^{3}H]-mannitol was added to a DMEM dosing solution at a concentration of 0.25 µCi /ml. The cell monolayers were washed twice with PBS, the media at the basolateral side (1 ml) of the Transwell replaced with DMEM and the mannitol dosing solution added to the apical side. The cells were incubated at 37ºC on an orbital shaker during the experiment. Sampling from basolateral side (100 µl each time) was carried out for designated time points (i.e. 15, 30, 45, 60, 90 and 120 min) with an equal volume of warm DMEM added to replace the volume. Analysis of the radiolabeled probes was undertaken by liquid scintillation counting (Wallac 1409, PerkinElmer

Life Sciences, Boston, MA, USA). Permeability coefficient was calculated according to equation 2:

$$\frac{dM}{dt} = \rho \times A \times C_d$$

Equation 2

Where, M is the cumulative amount of solute transported to basolateral side (nM); ρ is permeability coefficient ($cm.sec^{-1}$); A is the Transwell surface area (cm^2); C_d is the concentration of radioactive material in the apical compartment ($nM.cm^{-3}$) and t is time (sec).

RT-PCR detection of clathrin

To assess the transcriptomic expression of clathrin in the A549 cells upon treatment with DEX, we exploited a standard RT-PCR methodology. Total RNA was harvested (using Tri-reagent extraction solution) and reverse transcribed using MMLV-rt with pdN6 random primers and the RNase inhibitor, Rnasin. Aliquots of cDNA were subjected to PCR. The primers were designed, using the internet-based primer design program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/). The forward and reverse primers for clathrin were 5'-tgaagttggcacaccaccta-3' and 5'-agttcttcagcaccggctaa-3', respectively, and for beta-actin were 5'-ggcatgggtcagaaggatt-3' and 5'-ggggtgttgaaggtctcaaa-3', respectively. The PCR thermocycling program was as follows: denaturing at 94°C for 30 sec, annealing at 57°C for 45 sec and extension at 72°C for 45 sec through a total of 30 cycles. The PCR recipe (for 25 µl reaction) consisted of: 2.5 µl Taq 10× buffer, 1-3 µl $MgCl_2$ (25 mM), 2 µl dNTPs (5 µM), 2 µl cDNA (100 ng/1µl), 0.25-1 µl forward and reverse primers (10 pmol/1µl), and 0.1µl Qiagen Taq polymerase enzyme (5 Units/1µl) and RNase/DNase free ddH_2O (up to 25 µl). The PCR products were electrophoresed through a 1% agarose gel containing ethidium bromide (0.1 µg/1ml) and visualized under UV light. The density of expressed bands was measured using the GS-700 densitometer and molecular analysis software (Bio-Rad, Hempstead, UK). The density of each spot was subjected to local background density subtraction and normalized to the density of the house keeping gene, β-actin. The ratios of treated over untreated samples were performed for 2 independent replicates.

Western blot analysis of Dynamin

A549 cells were cultured for 96 hrs with or without Dex and subjected to western bloting analysis. Cells were washed (×3) with ice cold phosphate buffer saline (PBS), then, the total protein was harvested using 100-200 µl lysis buffer (50mM Tris, 5mM EGTA, 150mM NaCl and 1% Triton) containing protease inhibitors (NaVO4, NaF, PMSF, phenylarsine oxide, sodium molybdate, leupeptin and aprotinin). After 15 min incubation in ice, cell debris was removed by centrifugation at 12,000 ×g at 4°C for 15 min. The supernatant was analyzed for protein content using a standard BSA assay by means of DC protein assay kit (Bio-Rad, Hempstead, UK). Total protein concentration was measured at 750 nm using a UV/visible spectrophotometer (Ultraspec 3100 pro, (Amersham Biosciences, Cambridge, UK). After mixing with the loading buffer [60 mM Tris, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) bromophenol, and 250 mM DTT], samples were boiled for 10 min at 100°C and equal amounts of protein (30 µg/lane) along with rainbow molecular weight marker loaded onto an SDS-PAGE (5% stacking gel and 8% resolving gel) and run at 100 V. Protein patterns were transferred onto Protran nitrocellulose membrane and the free sites blocked, using the blocking buffer [5% (w/v) marvel dried skimmed milk (DSM) in the Tris buffered saline (TBS)/Tween (0.05%)] for 2 h at room temperature. The membrane was incubated in a 1:250 dilution of anti dynamin II antibody-primary and a 1:1250 dilution of HRP conjugate IgG.

Statistical analysis

For statistical analysis, the unpaired two tailed student t-test or analysis of variance (ANOVA) followed by a post-hoc multiple comparison test were exploited using Graphpad Prism software. A p-value less than 0.05 was assumed to represent significant differences.

Results and discussion

The DDT via lung confers a distinctive pathway for delivering of macromolcules, however there is no substantial information about modulation of the cellular transport characteristics of alveolar epithelium by glucocorticodies (e.g., dexamethasone), as additive agent for cell culture. Hence, we investigated the impact of DEX on cellular transport potential of the human A549 cells through paracellular and endocytic routes. To study the morphological effect of DEX on the cellular integrity and architecture of A549 cells, LM and SEM examinations were undertaken. Figure 1 represents the LM and SEM images of the A549 cells in the absence and presence of DEX (panels A, B, C and D, respectively). A clear difference could be seen in Figure 1, between the treated and untreated A549 cells. Panels A and C demonstrate the cuboidal type II morphology of the A549 cells grown in the absence of DEX, while panels B and D represent more flattened and elongated phenotypes, following culture in the presence of DEX. A similar phenomenon was observed during the culture of isolated alveolar type II cells obtained from the rat lung (our un-

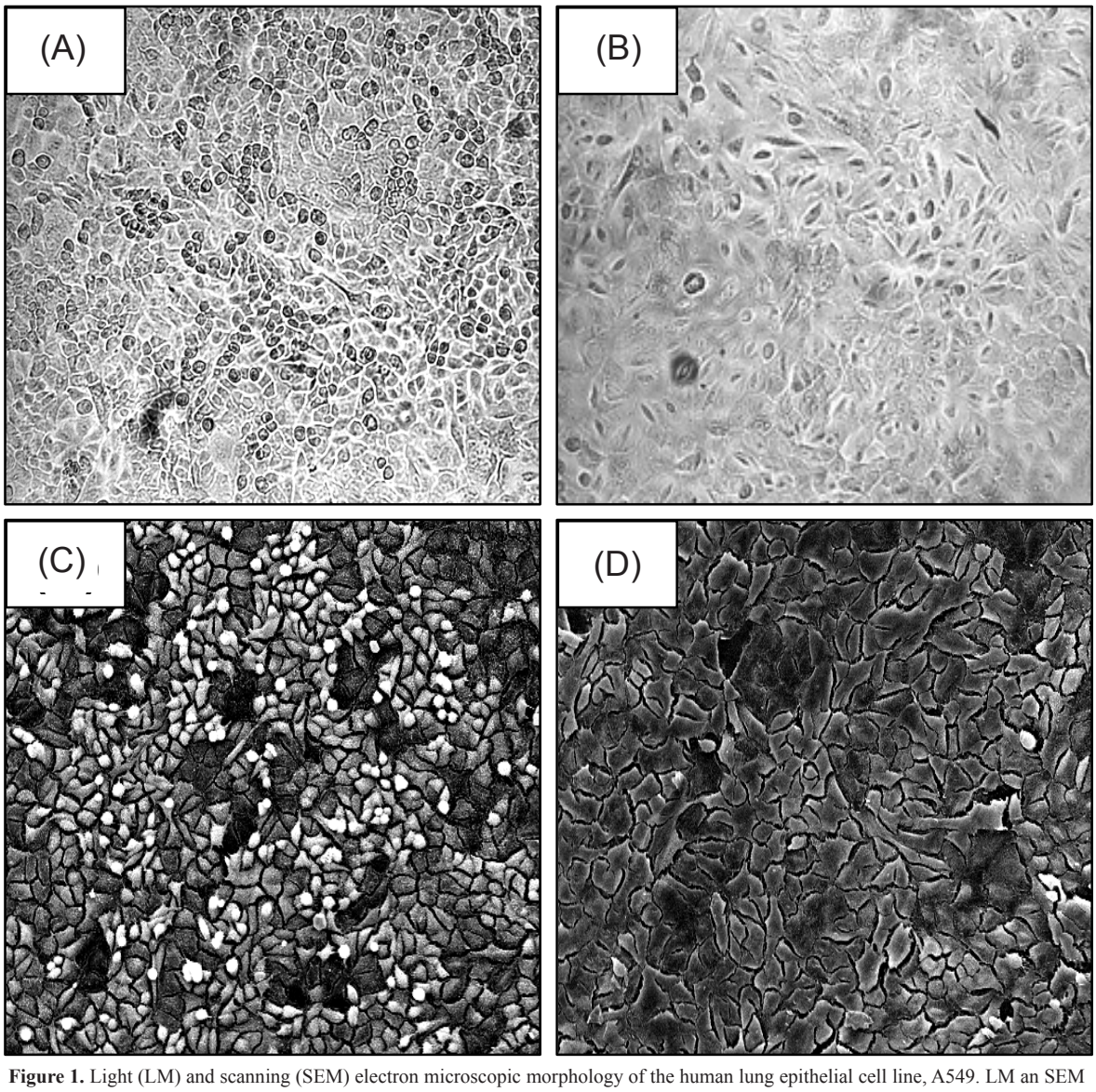

Figure 1. Light (LM) and scanning (SEM) electron microscopic morphology of the human lung epithelial cell line, A549. LM an SEM images were prepared 96 h post seeding. Panels A and C show morphology (LM and SEM, respectively) of the cultured cells in the absence of DEX, while panels B and D present the morphology (LM and SEM, respectively) of A549 cells cultured in the presence of DEX (0.1 μM). Cultured cells in the absence of DEX (panels A and C) represent the cuboidal morphology, whilst panels B and D (cultured in the presence of DEX) demonstrate a more elongated phenotype.

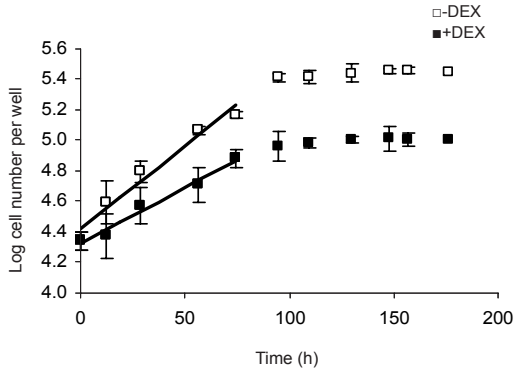

Figure 2. The effect of Dexamethasone on the growth profile of A549 cells. Cultured cells in exponential phase reveal different doubling times of 31 and 47.8 (hrs) in the absence and presence (0.1 mM) of Dexamethasone, respectively. Data represents the mean ± s.d. of 6 replicates. Two-tailed student's t-test with a p-value less than 0.05 was used for statistical significance.

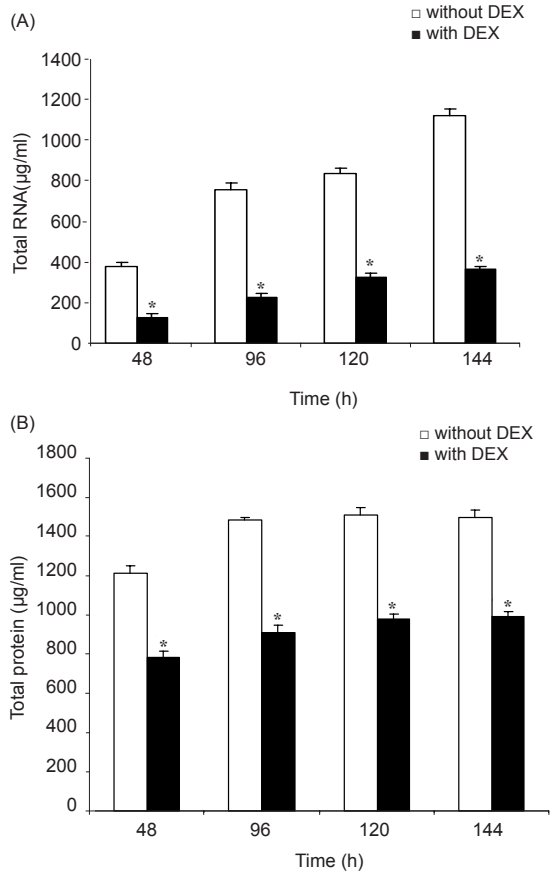

Figure 3. Effect of Dexamethasone on the production of total RNA (panel A) and protein (panel B) in A549 cells. Dexamethasone 0.1 (µM) significantly inhibits the production of total RNA and protein in A549 cells (Panels A and B, respectively). * represents significant difference ($p<0.05$) compared to the control sample (no Dexamethasone).

published data). These phenotypic changes may be in consensus with the observations made by Cheek *et al.* (1989) and Campbell *et al.* (1999), during the differentiation of rat ATII towards a more type I-like phenotype. These researchers also showed morphological changes, such as extended thin cytoplasm and protruding nuclei, as a result of differentiation process. These annotations could collectively result in ability of DEX, to somehow pursue the differentiation process.

The possible role of DEX to mediate differentiation process could well be associated with anti-proliferatory mechanisms. Different groups have reported the cell growth inhibitory role of DEX in different cells, for example the human lung adenocarcinoma (A549) (15), human corneal epithelial (16), mouse fibroblast (17) and human osteosarcoma (18) cell lines.

Figure 2 represents the growth profile of A549 cells in the absence and presence (0.1 µM) of DEX. This finding clearly reveals a lower proliferation of A549 cells in the presence of DEX. Logarithmic cell number versus time (h) plots showed linear exponential phases of growth, with doubling times (DT) of 31 and 48 h in the absence and presence of DEX, respectively. A significant ($p<0.05$) difference was observed in DT and ultimate plateau cell numbers, indicating the growth inhibitory effect of DEX (Figure 2), which is consistent with that of *in vivo* investigations. Luyet *et al.* (2002) reported that DEX treatment (0.1-0.01 µg/g body weight) on days 1-4 of early post- neonatal rats could reduce the rate of cell proliferation, at the expected peak day (day 4) for cell proliferation, to a basic level (19). Similarly, Okajima and colleagues (2001) administrated DEX (0.4mg/kg maternal body weight per day, intra-peritoneal) on day 20 and 21 of gestation period to the rats. They showed that DEX treatment lowered the numerical density of alveoli, but increased the mean alveolar radius (20).

Parallel with the cell number in the culture, figure 3 represents the synthesis of total RNA and protein (Panels A and B, respectively) in A549 cells grown with or without DEX (0.1 µM). The amount of harvested RNA from cells in the presence of DEX, show a significant reduction compared to the control cells. DEX reduces RNA

synthesis at least 3 folds after 48 h in culture. This pattern is followed through to exposure times of 144 h (Figure 3 A). Furthermore, Figure 3B demonstrates the rate of protein production in A549 cells cultured with DEX. The effect on post-transcriptional and translation step is obvious and subsequently the amount of total protein shows a significant difference between + and – DEX cultures. DEX reduced the amount of total protein by approximately 60-70% of control levels. Reduction of protein synthesis has been reported previously in DEX treated A549 cells (21). The authors noticed a lower protein level in the confluent A549 monolayer exposed to 0.25 μM DEX for 72 hours. The inhibitory effect of GCs on the growth could be via its influence on cell metabolism and growth (22). In cell culture, glucocorticoids may induce the synthesis of inhibitory proteins or affect the uptake of some substrate for cell growth, such as glucose or amino acids (22).

Trans-epithelial electrical resistance across A549 cell monolayer was examined as shown in Figure 4 (panel A). We noted that A549 cells responded to DEX, with an increase in TEER, although not as profound as those we have seen in rat cells (i.e., 1000 *vs* 200 $\Omega.cm^2$ for DEX-treated and untreated, respectively) (from our yet un-published data). In A549 cells, TEER was significantly increased by exposure to 0.1 μM DEX, with a maximum TEER of about 100 $\Omega.cm^2$ compared to 45 $\Omega.cm^2$ for untreated cells.

This relationship between DEX in culture medium and TEER across primary monolayer cultures of alveolar cells has not been explicitly reported in the literature. Although as mentioned previously, almost in all reports using primary rat alveolar monolayers for permeability assessments, DEX is exclusively added to the culture medium (23, 24).

Based on to our findings, GCs appear to modulate tight junctional characteristics within alveolar epithelial cultures. Substantial evidence shows similar phenomenon in other cellular barriers. For example, brain capillary endothelial cells, which comprise the blood-brain barrier, are responsive to glucocorticoids, in particular hydrocortisone, which improves tight junction integrity within isolated primary

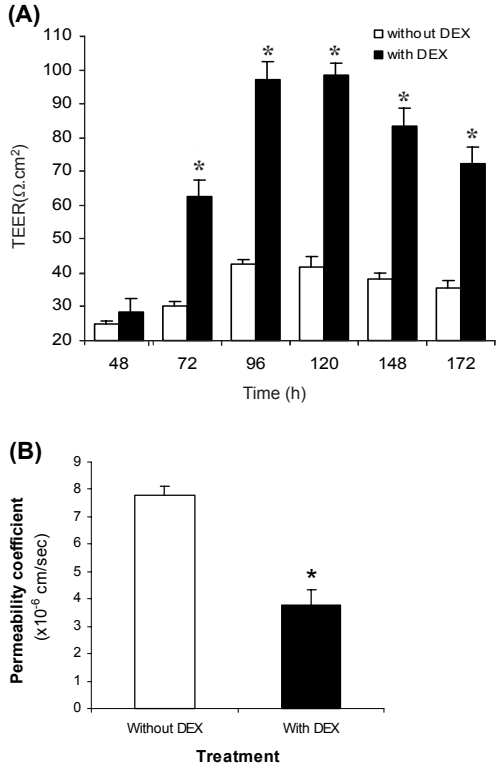

Figure 4. Trans epithelial electrical resistance (TEER) (panel A) and paracellular permeability of mannitol (panel B) in A549 cells. Significant ($p<0.05$) increase in TEER values for A549 cells in the presence of 0.1 (μM) Dexametasone on day 3 and after wards was observed (panel A). Significant ($p<0.05$) permeability coefficient values for A549 cells in the presence or absence of 0.1 (μM) Dexamethasone was observed. * represents significant difference ($p<0.05$) compared to the control sample (without Dexamethasone).

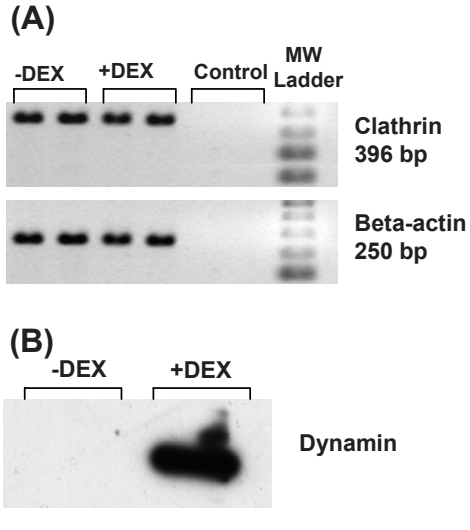

Figure 5. RT-PCR analysis of clathrin mRNA level (panel A) and western blot analysis of dynamin (panel B) in A549 cells, following 96 h culture in the presence (+DEX) or absence (-DEX) of dexamethasone.

porcine brain microvessel endothelial cells (25). Hydrocortisone (500 nM) increases TEER up to 1000 $\Omega.cm^2$ (from 300 $\Omega.cm^2$) and decreases cell monolayer permeability to sucrose down to 0.5×10^{-6} ($cm.sec^{-1}$).

Permeability coefficient of paracellular marker is in close association with the generation of high TEER within cell monolayer. Isolated rat ATII cells on polycarbonate filters acquire type I-like phenotype and exhibit low or high TEER (533±37 or 2111 ±144, respectively) (26). These cells showed different mannitol permeability coefficients depending on the TEER values, i.e. 7.83± 0.3.7 ($\times 10^{-6}$ $cm.sec^{-1}$) for low TEER versus 3.46 ± 0.22 ($\times 10^{-6}$ $cm.sec^{-1}$) for high TEER monolayers. To assess the permeability properties of human alveolar epithelial cells, transport of mannitol was monitored. The A549 cells were cultured on polycarbonate trans-wells with or without DEX (0.1 μM) for 4 days. Permeability coefficients of mannitol from apical to basolateral compartments have been shown infigure 4 (panel B). A significant ($p<0.05$) difference was observed between the untreated and DEX-treated A549 cells. DEX treatment of A549 cells resulted in a higher permeability coefficient value ($5.35 \pm 0.1 \times 10^{-6}$ $cm.sec^{-1}$), compared to 4.66 ± 0.1 ($\times 10^{-6}$ $cm.sec^{-1}$) for the untreated cells. The mannitol permeability data together with TEER results clearly indicate that DEX contributes to the generation of higher trans-epithelial resistance and a more restrictive barrier property, possibly via modulation of tight junctional elements. However, it should be evoked that A549 cells displayed a lower response to DEX modulation, compared to the primary alveolar epithelial cells.

In an attempt to assess the effect of DEX on endocytosis pathway involved in delivery of macromolecules, we looked at the expression of two elements of this route (i.e., clathrin and dynamin), exploiting RT-PCR and western blot approaches, as seen in Figure 5. A significant effect was observed on the expression of dynamin, but not clathrin. Pronounced expression of dynamin, which belongs to the large GTPase family (27) and supports the receptor mediated endocytosis through its action to mediate vesicle fission from the plasma membrane, was observed in the A549 cells upon treatment with DEX (Figure 5B). In A549 cells cultured in the absence of DEX, dynamin could not be detected despite its presence in these cells. However its' expression was dramatically increased in the presence of Dex, as seen in Figure 5B. Dexamethasone induction of dynamin has previously been reported (27). The authors showed that dynamin expression increases the Dex-drived pancreatic acinar cell differentiation. Dynamin is known to wrap around the neck of budding vesicles and facilitates the fission of vesicles (28). The role of dynamin in caveolae and clathrin mediated transport is well documented (29-31). For instance, these researchers have reported that endocytic pathway through clathrin-coated pits or caveolae, both were inhibited following inducible synthesis of mutant dynamin (K44A). Further more, the uptake of albumin by alveolar epithelium was reported to be increased in response to the DEX treatment.

Thus, upon our findings, we propose that the presence of DEX in the culture medium of ATII cells provide a versatile model for alveolar epithelia, that may be considered as an appropriate platform for pulmonary DDT investigations. In addition, because of the pronounced expression of dynamin and small changes in the clathrin mRNA, we speculate that dexamethasone may induce a non-clathrin coated endocytic pathway in alveolar cells, that is yet to be examined.

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