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Surface Changes in Type II Pneumocytes Isolated from Rats during the Cultivation

Z. KOVÁČIKOVÁ[1], E. TÁTRAI[2], Z. ADAMIS[3]

[1]Institute of Preventive and Clinical Medicine, Bratislava, Slovak Republic, [2]National Institute of Occupational Health, Budapest and [3]National Institute of Chemical Safety, Budapest, Hungary

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Summary

Type II cells isolated from the rat lung were maintained in culture for 8 days. The activity of alkaline phosphatase and lectin binding properties were studied. The alkaline phosphatase activity and the number of lamellar bodies were continually decreasing during the studied time period. The profile of lectin binding (*Maclura pomifera* and *Ricinus communis*) did not change during the cultivation.

Key words

Pneumocytes type II • Lectin binding • Alkaline phosphatase • Rat

Introduction

The alveolar epithelial surface comprises two distinct epithelial cell types, type I and type II cells. Type I cells cover about 95 % of the alveolar surface area and act as a protective barrier and gas exchange surface. These cells are highly sensitive to injury, because they are incapable of mitosis and cellular repair. In response to the loss of type I cells, the mitotic activity of type II cells increases and this is followed by differentiation of daughter cells into new type I cells (Schneeberger 1991). Type II cells, which cover only 5 % of the surface area, are of cuboidal shape. The synthesis and secretion of pulmonary surfactant that is stored in unique cytoplasmic lamellar bodies belong to the main functions of these cells (Leikauf and Driscoll 1993). Histochemical staining revealed that alkaline phosphatase (AP) is associated only with the apical portion of alveolar type II cells in the bronchoalveolar region of the lung (Miller *et al.* 1987) and it is therefore used as marker for this type of cells.

As the lung is a very complex organ, as far as cell diversity is concerned, the isolation and culture of its individual cell types enables a better understanding of the cell responses to various toxic agents. There exist various techniques for isolation of alveolar type II cells from different species (Kikkawa and Yoneda 1974, Dobbs *et al.* 1980, Richards *et al.* 1987).

It has been reported that alveolar type I and II cells differ in their lectin binding properties. *Maclura pomifera* (MPA), a lectin binding α-D-galactose residues, binds to the apical surface of type II but not type I cells, while *Ricinus communis* I (RCA), a lectin specific for β-D-galactose residues (Brandt 1982) binds to the surface of type I but not type II cells. According to Dobbs *et al.* (1985), a loss of *Maclura pomifera* binding occurred during the cultivation of type II cells after two days and an increase was observed in *Ricinus communis* binding.

Fax +420 2 293 894
E-mail physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

On the basis of the above results we aimed to clarify the following questions: 1). whether there is any connection between the decrease in alkaline phosphatase activity and changes in the pattern of lectin binding in the membranes of type II pneumocytes during 8 day cultivation, and 2) whether the decreased number of lamellar bodies runs in parallel with the changes of the above parameters.

Methods

Animals

Male Wistar rats (Velaz, Prague, Czech Republic) weighing 180-220 g were used in these experiments. The animals were housed under standard laboratory conditions and were given a conventional laboratory diet (MOK, Velaz, Prague, Czech Republic) and tap water *ad libitum*.

Reagents and media

Durcupan ACM was purchased from Fluka, DMEM from Pansystem GmbH (Aidenbach, Germany), fetal calf serum from SEBAK GmbH (Aidenbach, Germany). All other chemicals were supplied by Sigma.

Cell isolation

Rat alveolar type II pneumocytes were isolated according to the method of Richards *et al.* (1987) and Hoet *et al.* (1994). Briefly, after a intraperitoneal sodium pentobarbital injection (60 mg/kg), the lung was perfused *via* the pulmonary artery with sterile saline and was mechanically ventilated. The lung with trachea were removed and bronchoalveolar lavage was performed, the lung were partially trypsinized, chopped and the cell mixture purified by centrifugation on a discontinuous Percoll gradient (density 1.089 and 1.04, 250 x *g* for 20 min). The interface was collected and after rewashing the cells were resuspended in the medium and plated in a Petri dish. After 1 h incubation in an atmosphere of 95 % air/5 % CO_2, the unattached cells were collected and sedimented. Several preparations were made by cytospin for AP, MPA and RCA staining. The remaining cells were used for further experiments.

Cell culturing

The cells were cultured on 96-well plates (Falcon) in DMEM supplemented with 10 % FCS at 37 °C in an atmosphere of 95 % air/5 % CO_2. The plating density was 100 000 cells/well. For testing the ability to bind lectins 500 000 cells were plated in Petri dishes (3.5 cm diameter). The medium was changed for the first time 20 h after plating and then every 24 h. After finishing the cultivation the cells were washed twice with PBS.

Cell identification

Both cultured cells and cytospin preparations were stained for AP by the method of Bingle *et al.* (1990). Lamellar inclusion bodies were counted using phase contrast microscopy. Only cells with at least four lamellar bodies were considered as type II.

Electron microscopy

$2x10^6$ cells were fixed in 2 % glutaraldehyde in 0.1 % cacodylate buffer at pH 7.4. Cells were sedimented (500 x *g*), postfixed with cacodylate buffered OsO_4, dehydrated through a graded ethanol series and embeded in Durcupan ACM. Sections were cut with a Reichert Jung ultramicrotome, stained by uranyl acetate-lead citrate and examined using a JEOL JEM 100C electron microscope. Thousand cells were counted and the cell distribution was expressed in percentage. Cells without nucleus and dead cells were excluded.

Biochemical evaluation of alkaline phophatase

120 µl of equal volume of 300 $mmol.l^{-1}$ TRIS-HCl pH 8.5 and 10 $mmol.l^{-1}$ p-nitrophenyl phosphate were added to each well. After incubation for 60 min at 37 °C the reaction was stopped with 60 µl of 0.5 $mmol.l^{-1}$ NaOH. The absorbance was read at 405 nm using an automatic microplate reader (Dynatech MR 7000).

Lectin binding

For studying lectin binding, the Type II cells were cultured in 35 mm plastic Petri dishes. The lectin histochemical evaluation was performed immediately after isolation and after 2, 4, 6 and 8 days of cultivation. *Maclura pomifera* agglutinin (MPA, Sigma, product number L2013) was used at the concentration 20 $\mu g.ml^{-1}$ and *Ricinus communis* agglutinin (RCA, Sigma, product number L2516) at 5 $\mu g.\ ml^{-1}$. After fixation in a buffered neutral formalin (pH 7.4), the cells were incubated with 0.1 % H_2O_2 in methanol for 20 min at room temperature. The cells were incubated with biotinylated lectins for 20 min after washing with PBS. After rinsing with PBS they were incubated for 30 min with a streptavidin-biotin-peroxidase complex. Finally, they were treated with a DAB-H_2O_2 mixture for 15 min. To detect endogenous peroxidase activity the cells were incubated with DAB-

H_2O_2 alone. Non-specific binding of the reagent was checked by its incubation with the streptavidin-peroxidase reagent and with DAB-H_2O_2. To confirm the specificity of lectin staining, the cells were preincubated with appropriate hapten sugars (α-D-galactose, β-D-galactose). Lectin binding was completely blocked or significantly weakened by hapten treatment. In each sample 300 cells were counted, the RCA and MPA positive cells were expressed in percentage of the total cell number. Experiments were repeated 3-6 times (3 animals per experiment). The data are means ± S.E.M.

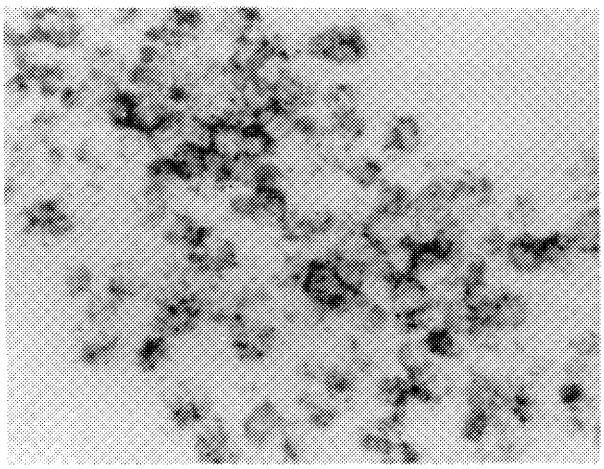

Fig. 1. *Type II pneumocytes isolated from adult rat lung. Alkaline phosphatase reaction after differential attachment. type II cells show a very intense reaction. Magnification: x 480.*

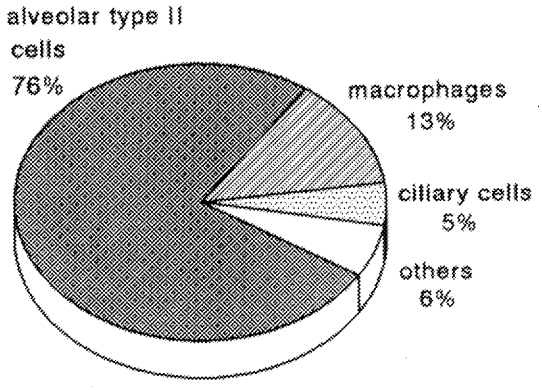

Fig. 2. *Cell distribution in the suspension of freshly isolated cells.*

Results

Freshly isolated type II cells exhibited intense AP activity (Fig. 1). Thousand cells were counted and their purity was checked. As type II cells only those were considered that contained at least four lamellar bodies. Their average purity was 76 %. The cell distribution in the sample of isolated cells is shown in Figure 2. The electron microscopy proved that the isolated cells remained intact (Fig. 3).

During cultivation, the AP activity was evaluated (both histochemically and biochemically) and the cells were counted by phase contrast microscopy. The number of type II cells decreased gradually and no AP positive cells with four lamellar bodies could be detected after 6 days of cultivation. A similar trend was seen when AP was determined biochemically. As the AP activity was evaluated in the wells directly by the microplate reader, it was impossible to measure the activity in fresh isolated cells. We started to measure this after 2 days of cell cultivation. In 8-day cultures, the activity was about 15 % of that measured in 2-day cultures.

Twenty-four to 48 h after plating, cells became attached either in clusters of two or more cells, or as scattered single cells with RCA and MPA positivity (Figs 4a,b). These cells contained lamellar inclusion bodies in the cytoplasm and showed AP positivity (Table 1). By the end of day 4, the cells formed a confluent monolayer in the wells. After 4 days, there was a progressive loss of both lamellar inclusion bodies and AP positivity. On day 6 and 8, only few scattered cells remained with 3 or less lamellar bodies, or without any lamellar bodies, and they exhibited alkaline phosphatase negativity. The cells were stained to the same extent both with RCA and MPA (Fig. 4c,d). Trypsin and DNase digestion (these procedures were used in the process of cell isolation) did not influence the lectin binding capacity (unpublished data).

Discussion

After differential attachment, the results of AP activity were in good agreement with the results of transmission electron microscopy (TEM). The number of AP-positive type II pneumocytes containing 4 or more lamellar bodies rapidly decreased as a function of cultivation time and completely disappeared by day 6. The biochemical evaluation of the enzyme showed the same trend, but 15 % of the original activity could be traced after 8 day's of cultivation. The difference between the results of histology and biochemistry was clear. The biochemical method, which is more sensitive, reflected the amount of the enzyme localized on the cell surface, but did not provide any information about the number of lamellar bodies.

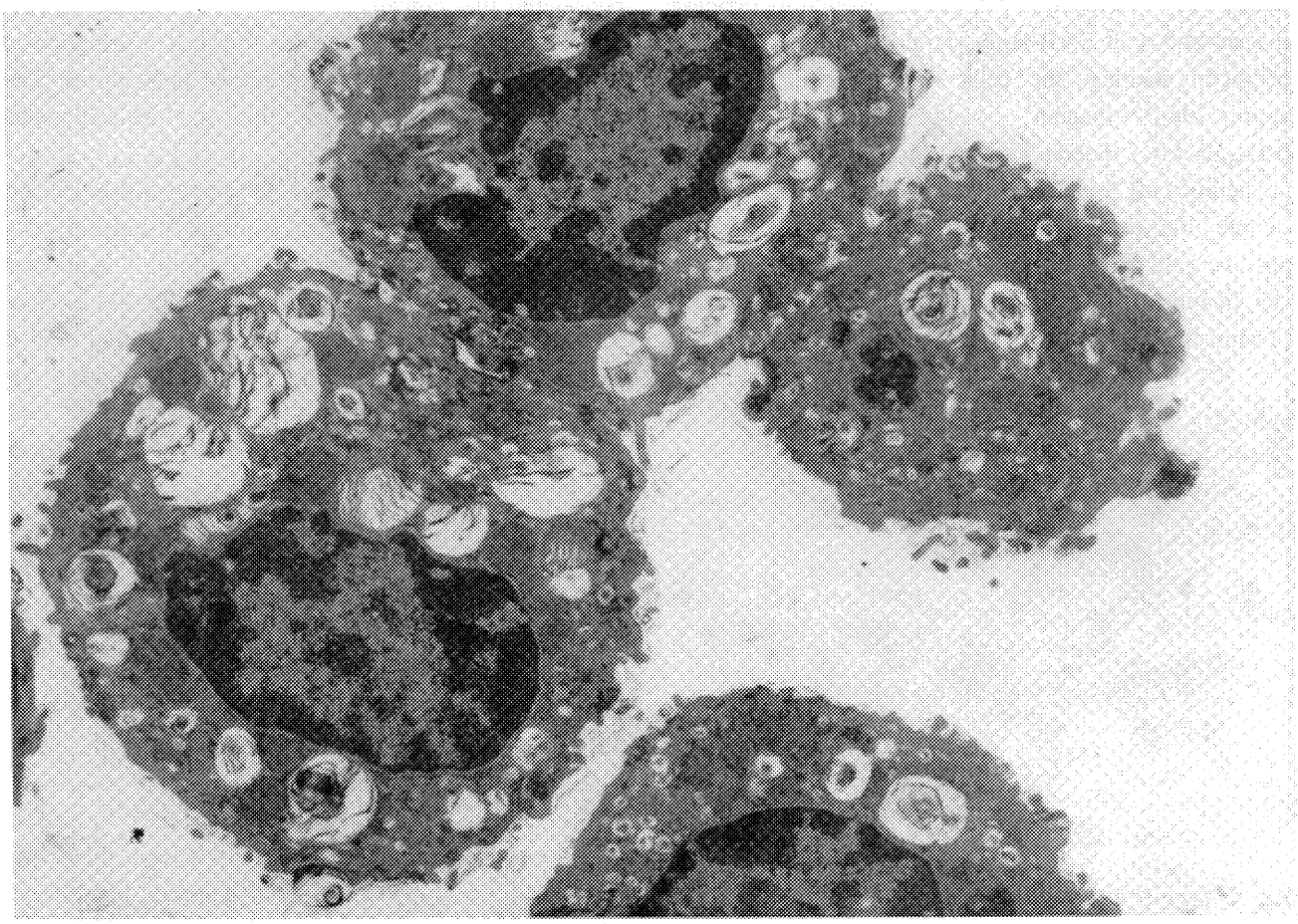

Fig. 3. *Electron micrograph of isolated type II cells from rat lung. The membranes and organelles in type II pneumocytes remained intact after differential attachment. Original magnification: x 5000.*

Table 1. Cytochemistry of type II cells at different periods of cultivation

Time (day)	RCA positivity (%)	MPA positivity (%)	AP positivity (%)
0	97.17±1.90	96.83±1.90	63.67±9.84
1	72.00±12.64	72.33±3.49	50.83±3.49
2	85.33±10.93	86.67±9.39	53.67±4.64
4	93.00±2.52	95.33±2.40	42.67±4.81
6	42.67±7.51	44.67±0.88	n.d.
8	42.33±2.85	44.00±2.65	n.d.

Values represent means ± S.E.M. n.d. - not detected, RCA - Ricinus communis, MPA - Maclura pomifera. 0 day represents characteristics of isolated cells

At early stages, after attachment, RCA and MPA positivity did not differ in repeated experiments (n=6). The positivity for lectin binding did not change concomitantly with AP positivity and the number of lamellar bodies. After the fourth day of cultivation, type II pneumocyte cells reacted in the same manner with both RCA and MPA, which is contrary to the data in current literature (Dobbs *et al.* 1985).

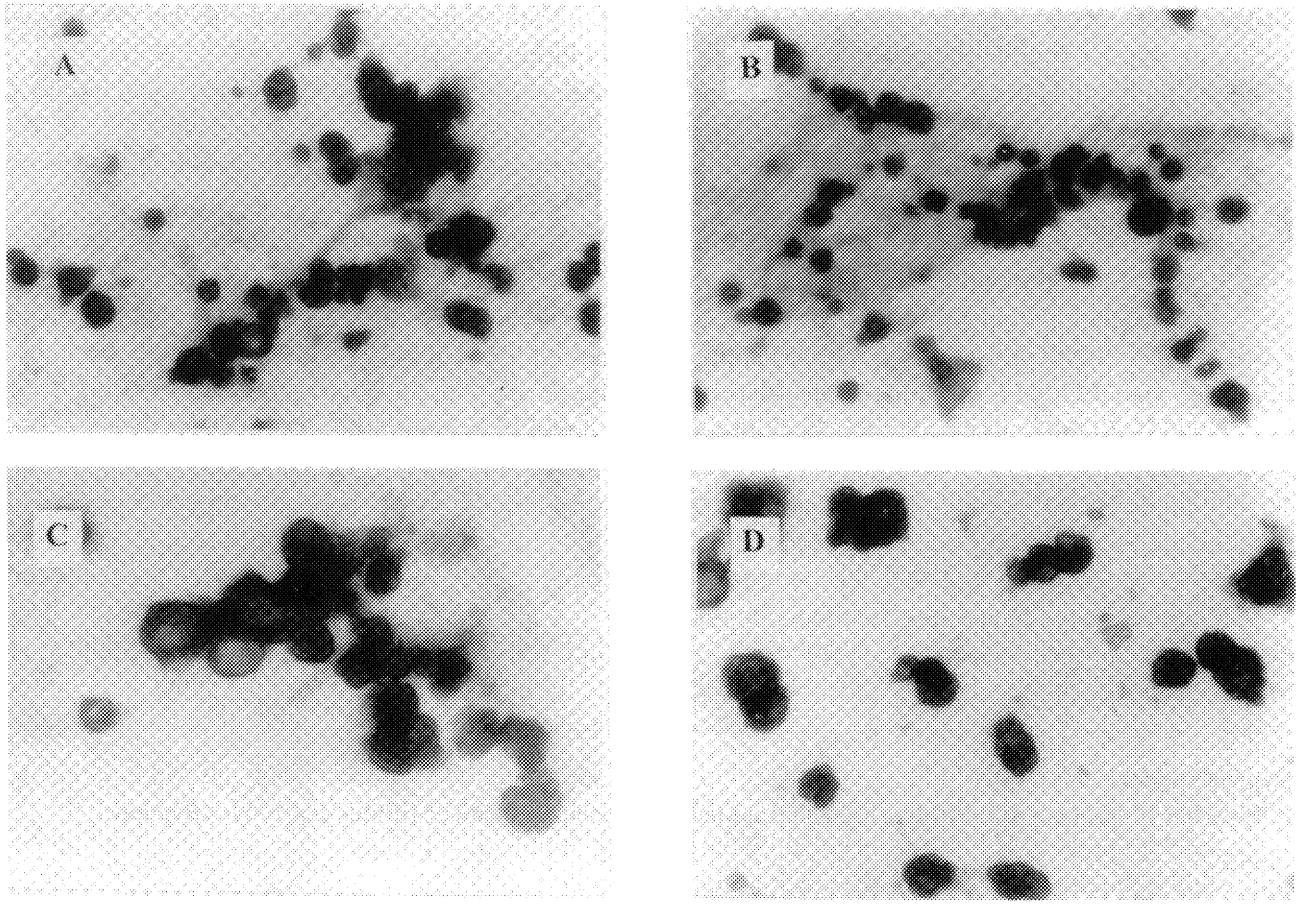

Fig. 4. *Cultured type II cells from rat lung. A. Reaction with RCA in two-day culture: type II cells react intensively with RCA. B. Reaction with MPA in two-day culture: type II cells show strong positivity with MPA. C. Reaction with RCA in eight-day culture: the cells are very strongly stained with RCA. D. Reaction with MPA in eight-day culture: the cells exhibit intensive staining with MPA. Magnification: x 360.*

It can thus be summarized that the major change in the cell cultures occurred between days 4 and 6. At that time, we observed both a decrease in the number of AP positive cells and lamellar bodies and changes on the cell surface which could be related to the lower binding of RCA and MPA.

Although the cells form a confluent layer from day 4, their membranes may be changed. It is well-known from *in vivo* studies that under some pathological conditions, e.g. fibrosing alveolitis (Corin *et al.* 1985) the „transitional epithelium" contains lamellar bodies with MPA positivity (Kasper *et al.* 1993). It seems likely that the transitional epithelium already develops *in vitro* at a very early stage of cultivation. In addition, the above results point to the fact that it is necessary to compare the observations made on isolated cells to those of cells examined *in vivo*.

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Reprint requests

Z. Kováčiková, Institute of Preventive and Clinical Medicine, Limbová 14, 833 01 Bratislava, Slovak Republic.