Lung (2014) 192:781–791
DOI 10.1007/s00408-014-9620-7

AIRWAY BIOLOGY

Peptide Regulation of Gene Expression and Protein Synthesis in Bronchial Epithelium

V. Kh. Khavinson · S. M. Tendler · B. F. Vanyushin · N. A. Kasyanenko · I. M. Kvetnoy · N. S. Linkova · V. V. Ashapkin · V. O. Polyakova · V. S. Basharina · A. Bernadotte

Received: 8 April 2014 / Accepted: 22 June 2014 / Published online: 12 July 2014

Abstract
Introduction Some studies have shown that peptides have high treatment potential due to their biological activity, harmlessness, and tissue-specific action. Tetrapeptide Ala-Asp-Glu-Leu (ADEL) was effective on models of acute bacterial lung inflammation, fibrosis, and toxic lung damage in several studies.
Methods We measured Ki67, Mcl-1, p53, CD79, and NOS-3 protein levels in the 1st, 7th, and 14th passages of bronchoepithelial human embryonic cell cultures. Gene expression of NKX2-1, SCGB1A1, SCGB3A2, FOXA1, FOXA2, MUC4, MUC5AC, and SFTPA1 was measured by real-time polymerase chain reaction. Using the methods of spectrophotometry, viscometry, and circular dichroism, we studied the ADEL–DNA interaction in vitro.
Results Peptide ADEL regulates the levels of Ki67, Mcl-1, p53, CD79, and NOS-3 proteins in cell cultures of human bronchial epithelium in various passages. The strongest activating effect of peptide ADEL on bronchial epithelial cell proliferation through Ki67 and Mcl-1 was observed in "old" cell cultures. ADEL regulates the expression of genes involved in bronchial epithelium differentiation: NKX2-1, SCGB1A1, SCGB3A2, FOXA1, and FOXA2. ADEL also activates several genes, which reduced expression correlated with pathological lung development: MUC4, MUC5AC, and SFTPA1. Spectrophotometry, viscometry, and circular dichroism showed ADEL–DNA interaction, with a binding region in the major groove (N7 guanine).
Conclusions ADEL can bind to specific DNA regions and regulate gene expression and synthesis of proteins involved in the differentiation and maintenance of functional activity of the bronchial epithelium. Through activation of some specific gene expression, peptide ADEL may protect the bronchial epithelium from pulmonary pathology. ADEL also may have a geroprotective effect on bronchial tissue.

Keywords Tetrapeptide · Bronchial epithelium · Signal molecule · Gene expression · Peptide–DNA interaction · Cell culture · Geroprotection · Geroprotector · Anti-aging · Peptide · COPD · ADEL

V. Kh. Khavinson
Pavlov Institute of Physiology of RAS, St-Petersburg, Russia

V. Kh. Khavinson · S. M. Tendler · I. M. Kvetnoy · N. S. Linkova · V. O. Polyakova · V. S. Basharina · A. Bernadotte
Saint-Petersburg Institute of Bioregulation and Gerontology, St-Petersburg, Russia

S. M. Tendler
Karolinska University Hospital, Solna, Stockholm, Sweden

B. F. Vanyushin · V. V. Ashapkin
Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia

N. A. Kasyanenko
Saint-Petersburg State University, St-Petersburg, Russia

I. M. Kvetnoy · V. O. Polyakova
D.O. Ott Research Institute of Obstetrics and Gynecology, St-Petersburg, Russia

A. Bernadotte (✉)
Department of Neurobiology, Care Sciences and Society, Division for Neurogeriatrics, Karolinska Institutet, Stockholm, Sweden
e-mail: alexandra.bernadotte@ki.se

Abbreviations

AEDG	Ala-Glu-Asp-Gly-peptide
BAL fluid	Bronchoalveolar fluid
CD	Circular dichroism
CD79	Cluster of differentiation 79, CD79a and CD79b types are members of immunoglobulin superfamily and both activate B cells
CD79A	CD79a molecule, immunoglobulin-associated alpha (gene)
cDNA	Complementary DNA
COPD	Chronic obstructive pulmonary disease
GAPD	Glyceraldehyde-3-phosphate dehydrogenase
Hoxa3	Homeobox protein
FoxA2	Forkhead box protein A2
FOXA1	Forkhead box A1 gene
FOXA2	Forkhead box A2 gene
KEDW	KEDW-NH2, Lys-Glu-Asp-Trp-NH2–peptide
KI-67	Antigen KI-67 protein
Mcl-1	Induced myeloid leukemia cell differentiation protein, inhibits apoptosis and enhances cell survival
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related) gene
miRNA 365	Small noncoding RNAs, regulates NKX2-1
MKI67	Marker of proliferation Ki-67 gene
MUC4	Mucin 4, cell surface associated (gene)
MUC5AC	Mucin 5AC, oligomeric mucus/gel-forming (gene)
NOS-3	Nitric oxide synthase 3
NOS3	Nitric oxide synthase 3 gene
Nkx2.1	NK2 homeobox 1 protein also known as thyroid transcription factor 1
NKX2-1	Homeobox protein Nkx-2.1, isoform 2 (gene)
Notch1	Transmembrane protein of the Notch family, member 1, promotes differentiation
Scgb3A2	Secretoglobin, family 3A, member 2
SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin) (gene)
SCGB3A2	Secretoglobin, family 3A, member 2 (gene)
SFTPA1	Surfactant protein (gene)
SP-A1	Pulmonary surfactant-associated protein A1
p53	Cellular tumor antigen p53, phosphoprotein p53 or tumor suppressor p53
TP53	Tumor protein p53 gene
TNF-a	Tumor necrosis factor alpha

Introduction

Disturbance in the bronchial epithelium function is observed in various lung diseases such as chronic obstructive bronchitis, lung emphysema, bronchial asthma, and others. The lung diseases are especially debilitating for the elderly population and are one of the leading causes of death worldwide [1]. Therefore, the main goal of new pharmaceuticals is to restore bronchial epithelium function.

Some studies have shown that peptides have high treatment potential due to their biological activity [2], harmlessness, and tissue-specific action. [2–5]. It has also been shown that peptides affect gene expression [2,6–8].

Peptide ADEL is a tetrapeptide (Ala-Asp-Glu-Leu), which restores the lungs' function in various pathologies, which has been shown in several studies [9]. Moreover, the peptide ADEL was effective on models of acute bacterial lung inflammation, chronicle fibrosis, and sub-lethal toxic lung damage [9]. All the above-listed pathologies are characterized by significant changes in lung morphology as well as in cell composition of bronchoalveolar fluid (BAL fluid) with increased neutrophil and lymphocyte quantity, as well as a reduced number of alveolar macrophages. The model of acute lung inflammation on rats showed that the peptide ADEL normalized the BAL fluid composition on the 6th day of the study, which evidenced the anti-inflammatory effect of the tetrapeptide.

As mentioned above, the peptide ADEL normalizes the cell composition of the bronchial tissue in various pathological processes as well as induces the expression of genes encoding some markers of proliferation and activity in bronchial epithelium [10]. However, the molecular mechanisms of this process have not been thoroughly studied at this time. Hence, this work aimed to show the ADEL's influence on the gene expression and synthesis of proteins—such as MKI67, MCL1, TP53, CD79A, NOS3 genes and Ki67, Mcl-1, p53, CD79, NOS-3 proteins—involved in normal functioning of bronchial epithelium.

These proteins were chosen for their abilities to be reflective of functional activity and cell renewal processes in lung tissue. Ki67 protein is a non-specific marker of cell proliferation, which for instance is reduced in COPD [11]. Mcl-1 protein is an anti-apoptotic factor of Bcl-2 family, which in bronchial epithelium is reduced in case of hypoxia. Factor p53 is a pro-apoptotic protein, the expression of which increases in bronchial epithelium under the influence of adverse environmental factors (such as air pollution) and lung pathology (COPD, cancer) [12–14]. Membrane-crossing glycoprotein CD79 was chosen as a marker of inflammation and local immunity index due to its expression on some epithelial cells as well as on B lymphocytes [15], when its reduced expression correlated with an autoimmune pathology [16].

It was also interesting to investigate the NO-synthase enzyme (NOS-3), since it is known to act as a catalyst for nitrogen oxide elaboration, which is a cell response

modulator in various tissues, including bronchial epithelium. Being formed and discharged from endothelium, NO inhibits thrombocyte aggregation, proliferation, cell migration, regulates apoptosis, and maintains the function of the endothelial cell barrier [17].

To study the peptide ADEL's influence on the gene expression, we chose the genes that regulate the differentiation and activity of bronchial epithelial cell, such as NKX2-1, SCGB1A1 and SCGB3A2, and FOXA1 and FOXA2.

Nkx2.1 protein, encoded by NKX2-1 gene, is known as a differentiation factor of bronchial epithelial burst-forming cells [18], and the disturbance of NKX2-1 expression is associated with miRNA 365 pathology, which can lead to lung cancer [19, 20]. The SCGB1A1 and SCGB3A2 genes encode corresponding secretoglobins produced by lung cells in all mammals. The lack of expression of SCGB1A1 and SCGB3A2 has been shown to aggravate chronic lung inflammation. Besides that, Scgb3A2 protein serves as a marker of burst-forming cells of the respiratory tract. Its expression is regulated by Notch1 and Nkx2.1 proteins [21, 22]. FOXA1 gene product is a factor of terminal differentiation of alveolar epithelium and affects SCGB1A1 secretoglobin expression [23]. Another gene of this group—FOXA2—encodes a factor of embryonic tissue differentiation in lungs, pancreas, liver, and nervous tissue. It is important to note that the FoxA2 protein regulates gene activity through its interaction with histones [24, 25]. The MUC4, MUC5AC, and SFTPA1 genes take part in a functional activity of bronchial epithelium. Mucin 4 and mucin 5AC are secreted by the respiratory tract epitheliocytes as a protective mucin formation. Disturbance in the proper expression of these genes leads to inflammation. Apart from that, reduced MUC4 expression serves as a potential marker and potential target for treatment of non-small cell lung carcinomas [26]. The SP-A1 protein (SFTPA1 gene product) stimulates phagocytosis, TNF-a production, and inhibits surfactant secretion. The level of SP-A1 secretion correlates with respiratory function indices (pulmonary vital capacity and expiratory volume in 1 s) in patients with COPD, stage 1–2. The transcription aberration of this gene facilitates the development of lung adenocarcinoma [27].

Considering that one of the possible mechanisms of peptides action is their ability to penetrate the cell nucleus and to interact with DNA [3, 7, 8], we decided to study the interaction between peptide ADEL and DNA in vitro.

Materials and Methods

Cell Culture

We investigated the 1st, 7th, and 14th passages of bronchoepithelial human embryonic cell cultures, FLECH line (from the Research Institute of Influenza of the Ministry of Healthcare of the Russian Federation). The 1st passage was treated as a "young" culture, 7th passage as "mature" culture, 14th as "old" culture in compliance with recommendations of the International Association for Cultural studies (USA, San Francisco, 2007).

For the cell-based immunocytochemical assay, we divided cell cultures into 3 groups: 1—control (with saline solution), 2—with control tetrapeptide (Lys-Glu-Asp-Trp, KEDW) [10, 28], and 3—with studied peptide ADEL.

The embryonic cell cultures were cultivated in Petri dishes (3.5 cm in diameter) and treated with gelatin solution ("Biolot", Russia), 15 % of Fetal Bovine Serum, 82.5 % of iMEM, 1.5 % of HEPES buffer with L-glutamine, and 1 % penicillin–streptomycin solution under standard conditions (5 % CO_2, 37 °C). We used the Trypsin–Versen solution for cell subcultivation in the ratio 3:1. We then added peptide Lys-Glu-Asp-Trp (KEDW) and the peptide ADEL at the concentration of 20 ng/ml to the 2nd and 3rd cell cultures, respectively.

Immunohistochemistry

For immunocytochemistry, we used the primary monoclonal antibodies against Ki67 (1: 50, Dako), Mcl-1 (1:40, Novocastra), p53 (1:25, Dako), CD79 (1:150, Vectorlab), and NOS-3 (1:40, Leica) and secondary antibodies—biotinylated anti-mouse immunoglobulins (Novocastra). The permeabilization was carried out using 0.1 % Triton X-100. Visualization of the reaction was performed using horseradish peroxidase diaminobenzidine (EnVision Detection System, Peroxidase/DAB, Rabbit, and Mouse).

We used microscope Nikon Eclipse E400, digital camera Nikon DXM1200, and "Videotest Morphology 5.2" software to evaluate the immune cytochemical coloration.

In each case, we analyzed 10 visual fields (x200). The area of expression was estimated as the ratio of immune-positive cell area to the total area of cells viewed, and was expressed in percentage. The index characterized the intensities of the studied Ki67, Mcl-1, p53, CD79, NOS-3 levels and secondary antibodies—biotinylated anti-mouse level in cells. In each series of four experiments, we studied five samples of each cell group in each passage.

Real-Time Polymerase Chain Reaction Analysis

Gene expression was measured by real-time polymerase chain reaction (PCR-RT). In this case, we divided the cultures into experimental and control groups, where a salin solution was added to control cultures, while a solution with the peptide ADEL (20 ng/ml) was added to the experimental group cultures.

We performed RNA stabilization on obtained cell cultures, as well as following the total RNA purification using RNA Protect Cell Reagent and RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's recommendations. The obtained RNA samples were used to synthesize the first strand of complementary DNA using oligo $(dT)_{18}$ (Sintol, Moscow) and reverse transcription kit–Omniscript RT Kit (Qiagen, Germany) according to the kit manufacturer's recommendations. 1 μg of purified total RNA was used as a matrix in each reverse transcription reaction. The obtained reaction mixture was used as a matrix for polymerase chain reaction (PCR) in an amount of 1 μl of mixture for 25 μl volume reactions. Quantitative PCR with the fluorescent dye SYBR Green I was performed by means of QuantiFast SYBR Green PCR Kit (Qiagen, Germany) and thermocycler CFX96 Real-Time PCR Detection System (BioRad Laboratories, Inc., USA).

For statistical analyses and diagram building, we used CFX Manager Software. The GAPDH mRNA was taken as an inner standard (its concentration was taken as the unit in all samples). We used three independent cell samples from each group (biological parallels) and conducted at least three parallel studies in nearby wells for each cDNA sample.

Construction of oligonucleotide primers was performed by means of online service NCBI Primer-Blast. We chose those pairs of primers, where one spanned an exon-exon junction. For our experiment, we used oligonucleotides, synthesized by Sintol (Moscow, Russia).

Physical Methods

Using the methods of spectrophotometry, viscometry, and circular dichroism, we studied the interaction of the peptide ADEL with DNA in vitro with the help of a spectrophotometer SF-56 (Experimental Design Office "Spectrum", Russia) and autodichrograph Mark IV (Jobin–Yvon, France). Solution viscosity was measured with low-gradient viscometer of original construction on the base of Zimm–Crothers model [29]. For our study, we chose the calf thymus DNA (Sigma) with molecular mass 9×10^6 determined from the intrinsic viscosity value at 0.15 M NaCl. After 4 days of storage at 4 °C, the stock of DNA solution, which dissolved in distilled water, was mixed with NaCl solution up to the required salt concentration (usually 0.005 M) and then filtered. A high salt concentration (1 M NaCl) was used to suppress the polyelectrolyte expansion of high molecular DNA in the solution and to reveal the role of nonelectrostatic interactions in the process of complex formation.

DNA concentration in a solution was defined from the difference in the absorption at two wavelengths (270 and 290 nm) after hydrolysis at 100 °C with $HClO_4$. This method allowed us to estimate the real extinction coefficient E260 (P) and to control DNA nativity during interaction by hyperchromism at 260 nm. The DNA and peptide solutions were mixed together and examined after one day of storage at 4 °C.

Statistical Analysis

Statistical analysis of the experimental data of immunocytochemical studies of gene expression included the calculation of an arithmetic mean, a standard deviation from the mean, and a confidence interval for each sample. The statistical analysis was performed in the program "Statistica 7.0". For the species distribution analysis and the null hypothesis test, we used the Student's *t* test for independent samples.

To assess statistical homogeneity among multiple samples, we used the non-parametric ANalysis Of VAriance (ANOVA) (Kruskal–Wallis test). Differences between groups were considered statistically significant at $p < 0.05$. Graphics and tables were performed by "EXEL".

Results of physical methods (spectrophotometry, viscometer, and circular dichroism) are qualitative and not a subject to statistical analysis. To assess the reproducibility of the results, each experiment was repeated eight times.

Results

Peptide ADEL Influenced the Cell Confluency in Old Culture

The study of the peptide ADEL's influence on the cell culture of the human bronchial epithelial cells showed that the confluency of cells in "old" control cultures (14th passage) was significantly lower than that in the similar cultures (Fig. 1). In both intact control cultures and cultures under the effect of ADEL, the confluency of "young" (3rd passage) and "mature" (7th passage) cells was statistically similar. This fact can be explained by the ADEL's ability to stimulate cell proliferation in the aging cultures, when the aging control cell cultures lose their ability to proliferate and to form the monolayer during passages. According to a recent study, the peptide AEDG (tetrapeptide Ala-Glu-Asp-Gly) caused fibroblasts to overcome the Hayflick limit [5]. Perhaps ADEL has similar effects on the culture of bronchial epithelium, but this requires further study.

Peptide ADEL Influenced the Protein Level and Cell Culture

The study of protein level in the bronchial epithelial cells and their respective gene expression allowed us to establish

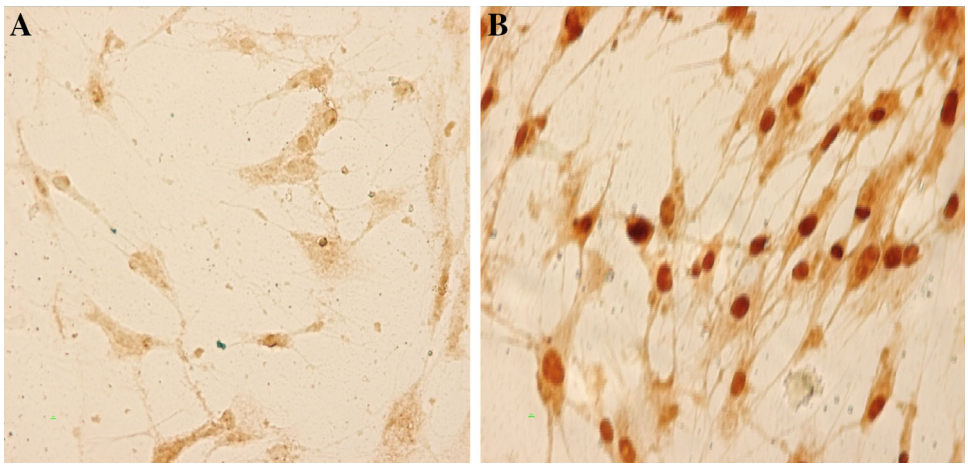

Fig. 1 The peptide ADEL's influence on the "old" cell confluency in culture of the human bronchial epithelial cells. FLECH line, 14th passage, immunocytochemistry, x200: **a** control, **b** peptide ADEL

Table 1 The tetrapeptides' influence on levels of proteins, connected with the functional activity of bronchial epithelium

Marker	Group	Area of expression (%)		
		3rd passage	7th passage	14th passage
Ki67	Control	4.21 ± 0.05	4.15 ± 0.07	4.07 ± 0.12
	Peptide Lys-Glu-Asp-Trp (KEDW)	4.32 ± 0.10	4.23 ± 0.08	4.16 ± 0.12
	Peptide Ala-Asp-Glu-Leu (ADEL)	6.52 ± 0.07*	10.12 ± 0.34*	13.92 ± 0.41*
Mcl-1	Control	2.63 ± 0.07	2.41 ± 0.06	2.38 ± 0.09
	Peptide Lys-Glu-Asp-Trp (KEDW)	3.57 ± 0.11*	3.05 ± 0.10*	2.29 ± 0.14
	Peptide Ala-Asp-Glu-Leu (ADEL)	3.72 ± 0.06*	4.55 ± 0.13*	4.51 ± 0.11*
p53	Control	4.82 ± 0.09	5.31 ± 0.13	5.96 ± 0.23
	Peptide Lys-Glu-Asp-Trp (KEDW)	4.88 ± 0.15	5.43 ± 0.20	5.22 ± 0.18*
	Peptide Ala-Asp-Glu-Leu (ADEL)	3.01 ± 0.07*	4.14 ± 0.11*	4.07 ± 0.17*
CD79	Control	0.61 ± 0.05	0.33 ± 0.06	0.54 ± 0.07
	Peptide Lys-Glu-Asp-Trp (KEDW)	0.62 ± 0.06	0.38 ± 0.07	0.49 ± 0.06
	Peptide Ala-Asp-Glu-Leu (ADEL)	0.72 ± 0.09*	0.61 ± 0.07*	0.76 ± 0.06*
NOS-3	Control	8.72 ± 0.06	9.04 ± 0.07	9.13 ± 0.08
	Peptide Lys-Glu-Asp-Trp (KEDW)	9.02 ± 0.12	9.10 ± 0.12	9.09 ± 0.11
	Peptide Ala-Asp-Glu-Leu (ADEL)	8.65 ± 0.09	9.11 ± 0.10	8.31 ± 0.09*

*$p < 0.05$—as compared to control

that peptide ADEL regulated the expression of MKI67, MCL1, TP53, CD79A, NOS3 genes and levels of Ki67, Mcl-1, p53, CD79, NOS-3 markers, compared to the peptide KEDW, which regulated the level of only one studied marker—Mcl-1 (Table 1).

Peptide ADEL increased the level of Ki67, the inactivation of which leads to an inhibition of cellular proliferation and ribosomal RNA synthesis. The Ki67 level in "young" (3rd passage), "mature" (7th passage), and "old" (14th passage) bronchial epithelial cell cultures increased by 1.55-fold, 2.44-fold, and 3.42-fold, respectively (Table 1). We showed that both ADEL and KEDW peptides increase the anti-apoptotic protein Mcl-1 level; however, ADEL was more efficient.

The peptide ADEL increased the level of Mcl-1, which inhibits apoptosis and enhances cell survival under the influence of ADEL. The Mcl-1 level in "young" cultures increased by 41 %, while in "mature" and "old" cells it increased by 89 % (Table 1). KEDW on the other hand stimulated the expression of these genes in cells of the "young" and "mature" cultures significantly less by 36 and 27 %, respectively (Table 1). It is an intriguing fact that the strongest activating effect of peptide ADEL on bronchial epithelial cell proliferation through Ki67 and Mcl-1 was observed in "old" cell cultures. This may indicate a geroprotective effect of this peptide on bronchial tissue.

Besides that, peptide ADEL reduced the level of apoptosis in bronchial epithelial cell culture, through the level of p53. This index was reduced under the influence of ADEL by 38, 28, and 32 % in "young", "mature", and "old" cell cultures, respectively (Table 1). During cellular senescence (during the increase of passage from 3 to14), the level of p53 increased in the control cultures.

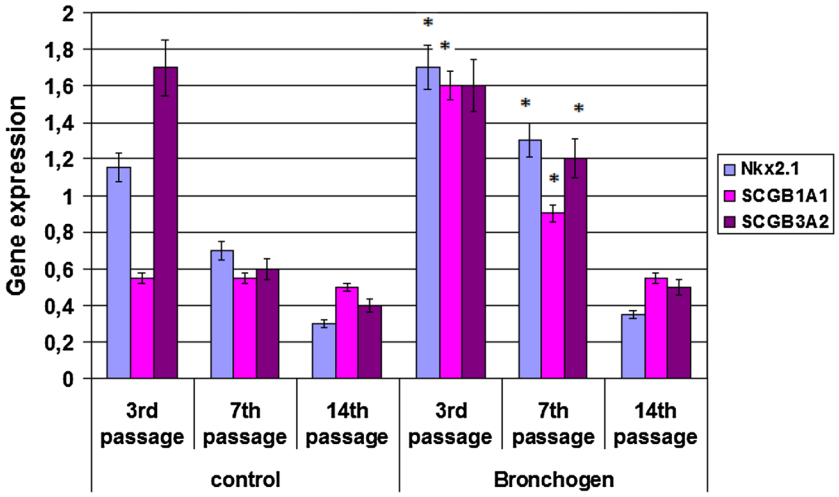

Fig. 2 The influence of peptide ADEL on the gene expression of bronchial epithelium. *$p < 0.05$—as compared to control

ADEL also increased gene expression and level of membrane glycoprotein CD79, which activates local immunity reactions. Under the influence of ADEL, the level of CD79 marker rose in "young", "mature", and "old" bronchial epithelial cell cultures by 18, 85, and 41 %, respectively (Table 1).

Peptide ADEL considerably reduced the level of NOS-3, which generated free radical monoxide (NO), when the level of NOS-3 increased in "old" control cell cultures of bronchial epithelium (Table 1).

Peptide ADEL Influenced the Gene Expression

Under the effect of peptide ADEL, the expression of NKX2-1 and SCGB1A1 genes in "young" cell cultures showed, respectively, 1.54-fold and 2.67-fold increase, compared to the control group (Fig. 2). In "mature" cell cultures, the influence of peptide ADEL contributed to the increased expression of all studied genes such as NKX2-1, SCGB1A1, and SCGB3A2 by 62.5, 33, and 77 %, respectively (Fig. 2). Hence, the peptide ADEL stimulated epigenetically the early stages of bronchial epithelial cell differentiation.

More evident stimulating effect on the gene expression ADEL is found in "mature" and "old" bronchial epithelial cells. Thus, in "young" cell cultures, ADEL increased the expression of FOXA2 gene 15.5-fold, while in "mature" cell cultures, it stimulated the expression of FOXA1 and FOXA2 genes by 53 % and 2.2-fold, respectively (Fig. 3). In the "old" cell cultures, ADEL contributed to the increase of FOXA1 and FOXA2 gene expression by 2.25- and 3.33-fold, respectively (Fig. 3).

ADEL also activated the expression of genes, whose reduced activity correlated with the development of various lung pathologies [26, 27]. Under the influence of ADEL in "young" cell cultures, the expression of MUC5AC and SFTPA1 genes increased by 50 and 65 %, respectively (Fig. 4). ADEL also stimulated the expression of MUC4 and SFTPA1 genes in "adult" cell cultures by 2.23-fold and 1.67-fold, respectively (Fig. 4). ADEL contributed to increased gene expression of MUC4 and SFTPA1 by 1.83- and 1.71-times in the "old" cell cultures, respectively (Fig. 4).

Thus, in the "old" cell cultures, ADEL has an advantageous effect on genes involved in terminal differentiation and functional activity of bronchial epithelial, while ADEL's effect on early differentiation genes can be realized only in "young" and "mature" cultures.

The Peptide ADEL–DNA Interaction

Figure 5 represents the absorption spectra of DNA solution, the peptide, and their mixture. Concentrations of the components expressed in moles (for DNA—in phosphate group moles) are equal in all systems. In the solution of little ionic strength (0.005 M NaCl), the interaction made itself evident in a hypochromic change in the DNA absorption spectrum in the wavelength region less than 272 nm, while at long wavelengths an "arm" appeared (Fig. 5). Another hypochromic change on the DNA absorption band was being registered in 1 M NaCl solution. It could not be connected to the increase in optical density which occurred from dissemination due to aggregation, because outside the absorption bands of the components the optical density of the solution equaled zero. It is important to underline that the absorption at wavelengths more than 240 nm was practically absent. These results indicate the distinction in ADEL–DNA interaction depending on NaCl concentration. In both cases, the interaction involved nitrogenous bases, as they are

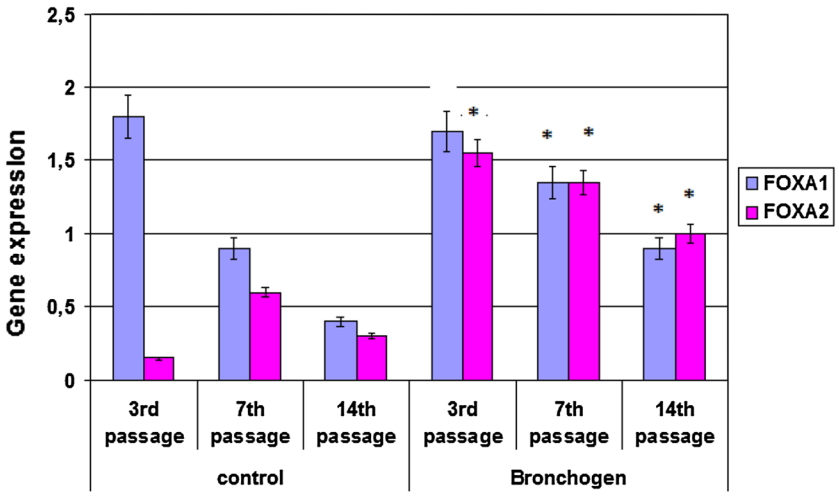

Fig. 3 ADEL's influence on gene expression of late differentiation of bronchial epithelium. *$p < 0.05$—as compared to control

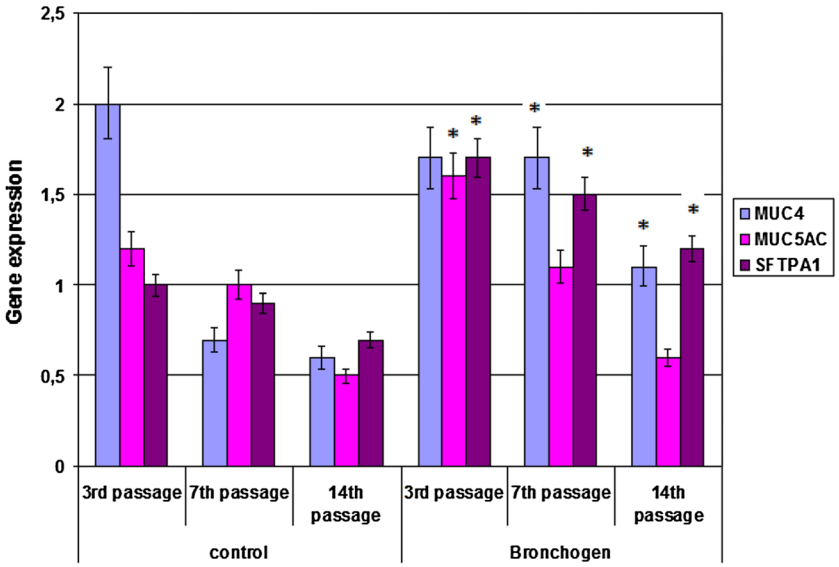

Fig. 4 ADEL's influence on the expression of bronchial epithelium functional activity gene. *$p < 0.05$—as compared to corresponding control

particularly responsible for DNA spectral properties. The binding destabilized DNA secondary structure when the charge was shielded (1 M NaCl). In the solution of little ionic strength (0.005 M NaCl) (at the same concentrations), we observed the alteration of DNA absorption spectrum, which is typical for ligand binding upon the position N7 and intact double helix structure.

The spectra of circular dichroism (CD) of the compounds and their complex at two NaCL concentrations have also shown (Fig. 6) that the peptide ADEL exerted a specific effect on secondary DNA structure, particularly in a solution of small ionic strength (it is known that the CD spectra of the DNA secondary structure are not changed over insignificant destabilization). The peptide ADEL had a CD spectrum in the wavelength region of less than 240 nm; however, it did not cause any changes in the positive spectral band of the CD spectrum.

Due to the fact that the CD spectrum of the DNA is dependent on the peptide concentration in a solution, it indicates the significant influence of the peptide concentration in DNA solution on the binding process (Fig. 7). The type of spectral changes coincides with the one observed in protonation of double helix DNA on guanine position N7 [30], which confirmed the conjectured peptide binding on this position. It is worth noticing that no such changes have been observed in 1 M NaCl. Another characteristic of CD spectra of DNA at high concentrations of the peptide ADEL was a significant increase of negative zone intensity in wavelength area below 240 nm (in the zone of the peptide absorption). This tendency in a spectrum could only be observed during alpha-helical structure formation. Arguably during the binding of DNA with some of peptides, a macromolecule can serve as a matrix to form the structure that appears in the peptide CD spectrum [31].

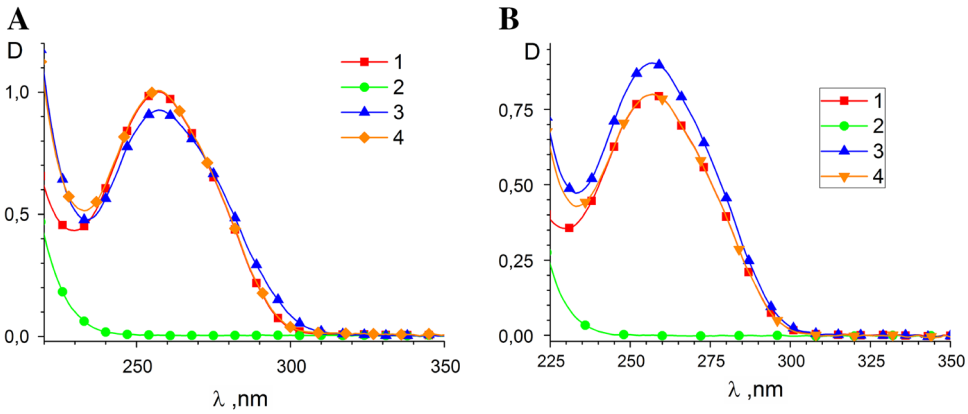

Fig. 5 DNA absorption spectrum (*1*), ADEL absorption spectrum (*2*), DNA–ADEL complex absorption spectrum (*3*), calculated amount of interaction components (*4*) in 0,005 M NaCl (**a**) and 1 M NaCl (**b**). Designations: λ wavelength, *D* optic density of the solution

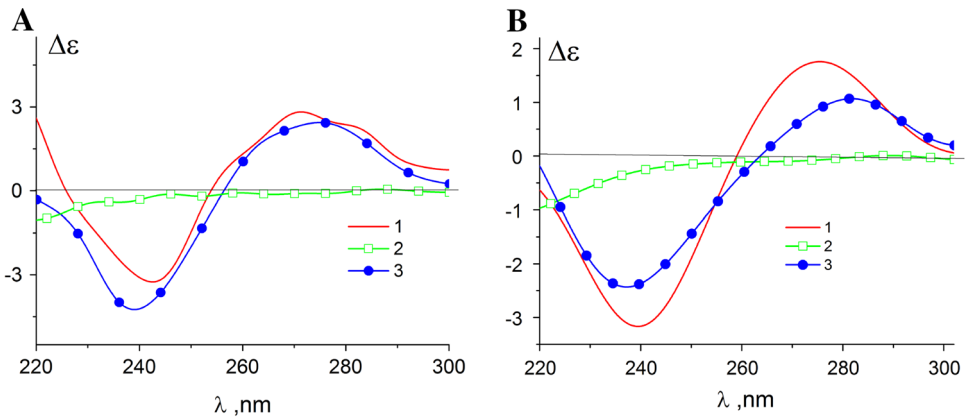

Fig. 6 CD spectrum of DNA (*1*), CD spectrum of ADEL (*2*), and CD spectrum of ADE–DNA complex (*3*) in 1 M (**a**) and 0.005 M (**b**) NaCl. Designations: λ wavelength, $\Delta\varepsilon$ differential of molar extinction coefficients of right- and left-polarized light

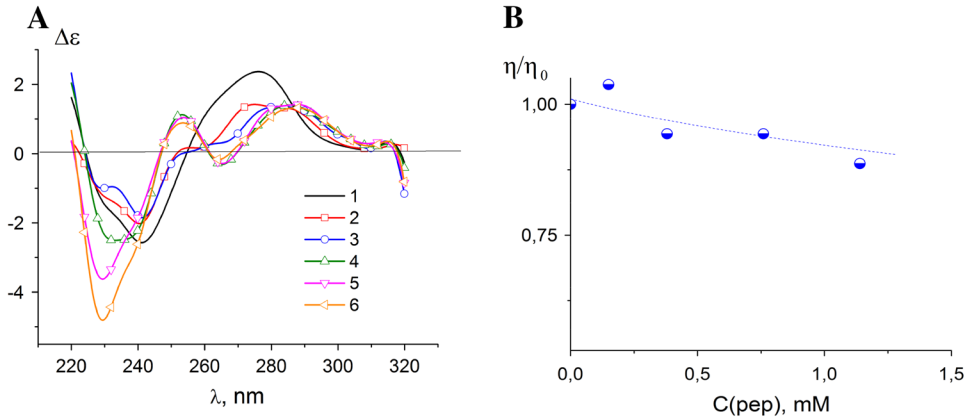

Fig. 7 CD spectra of ADEL–DNA interaction (**a**) relative alteration of reduced viscosity of DNA solution (**b**) depending on the peptide concentration. C(DNA) = 0.005 %. For **a** C (pep) = 0(*1*); 0.15 mM (*2*); 0.3 mM (*3*); 0.9 mM (*4*); 1,5 mM (*5*); 2.25 mM (*6*). Designations: λ wavelength, $\Delta\varepsilon$ differential of molar extinction coefficients of right- and left-polarized light

This points out that the orientation of peptide chain lengths is parallel to the DNA groove. It is apparently related to the major groove where the atom N7 guanine is located, and where the DNA solution viscosity reduces insignificantly. This shows no intercalation of any peptide group into double helix and coheres with the absence of double helix destabilization during the interaction. Such interaction did not appear in 1 M NaCl, possibly due to the certain role also played by electrostatic and ion–dipole interactions in the system.

Discussion

In our data ADEL has confirmed the notion of previous studies that it has an ability to regulate a wide variety of proteins in human bronchial epithelium, and therefore have an influence on treatment of acute and chronic lung inflammation [9]. In a previous study, ADEL was found to have a 2- to 3-fold increased expression of Hoxa3 gene transcription factor in cell cultures of human bronchial epithelium during the ageing process [10]. We suggested

that since Hoxa3 protein is involved in the regulation of proliferation, differentiation and apoptosis of various epithelial cells, it may indicate the influence of ADEL on gene expression of various regulatory proteins [32]. And our study confirmed this suggestion indeed.

We have studied some proteins that effect proliferation, differentiation, and cell activation. ADEL specifically showed to regulate the cell renewal processes for the bronchial epithelium. ADEL also changed the functional cell state by acting on CD79 and NOS-3 proteins. It has been suggested that the increased expression of the glycoprotein CD79, observed in bronchial epithelium by the influence of ADEL, may indicate an increased local immunity of the bronchopulmonary system. This may be used as a prognostic marker to assess a reduced risk of the autoimmune disorders [15].

ADEL increased functional activity and proliferation by enhanced level of proteins, such as proliferation markers-Ki-67 and anti-apoptotic protein Msl-1 (the syntheses of which were reduced in patients with COPD and hypoxia), with a simultaneous reduced p53 apoptotic factor's level [11, 13, 14].

We found it intriguing that some effects of ADEL were more pronounced in the "old" cell cultures compared with the "young" ones. In the "old" cell cultures, ADEL regulates the cell renewal process more strongly by increasing the level of proliferation markers, Ki-67, and anti-apoptotic marker, Msl-1. The Ki-67 level decreased in the "old" cell cultures of the control group and it correlates with the data of other studies suggesting that a decrease in Ki67 synthesis is a marker of cellular senescence [33]. Therefore, it can be concluded that ADEL can be regarded as a geroprotective substance in the bronchial epithelium.

ADEL was found to epigenetically regulate the expression of genes involved in the differentiation of bronchial epithelium: NKX2-1, SCGB1A1, SCGB3A2, FOXA1, and FOXA2. Thus, the regulation of gene expression of NKX2-1 and CGB3A2 showed an induced differentiation of bronchial epithelial cells [18, 34], and coinciding increased expression of SCGB1A1 and SCGB3A2 pointed to the reduction of chronic inflammatory processes in the lung tissue [21, 22]. The absent effect on NKX2-1, SCGB1A1, and SCGB3A3 expression in the "old" cell cultures compared with the "young" and "mature" cell cultures probably can be explained by a sharp decline of the gene expression in the control cultures. Perhaps the decreased expression can be explained by the methylation process, which can block those genes from reacting to ADEL's regulatory function. However, this assumption is debatable and requires further investigation.

Increased expression of FOXA1 and FOXA2 under the influence of ADEL indicates a regulation of cell differentiation in the bronchial epithelium on the initial and final stages. Furthermore, it is known that the FOXA1 product regulates the expression of SCGB1A1, while the protein Nkx2 regulates synthesis of secretoglobin (family 3A) [23]. However, ADEL is likely to influence only FOXA1, and the increased SCGB1A1 expression is supposed to be a secondary effect. On the other hand, it is quite possible that ADEL activates both mentioned genes directly. Previously obtained data showed an ability of ADEL to bind with cortical histones, some of which interact with FOXA2 [6, 7, 35]. These data suggest that the mechanism of peptide regulation of the gene expression and protein synthesis involves signal cascades, and the process may have several stages of realization at the genetic, sub-cell, and cellular levels. Furthermore, the epigenetic regulation of FOXA2, involved in differentiation and development of diverse tissues, may touch upon the fundamental basis of ontogenesis.

Through activating the expression of markers of bronchial cells' functional activity, including MUC4, MUC5AC, and SFTPA1 [26], peptide ADEL may protect the bronchial epithelium from pulmonary pathology. The received results are especially important since the reduced expression of MUC4 gene was verified in cancer diseases and reduced MUC5AC expression causes insufficient mucin synthesis as well as a disturbance in defense mechanisms in lung tissue. While changes in SP-A1 protein (product of SFTPA1 gene) synthesis correlated with breathing function are seen in patients with COPD.

By showing that synthetic alkaline and amphiphilic peptides, comprising some lysine residues, are able to penetrate the cell and form complexes with DNA and RNA, we could evaluate the efficiency of ADEL mechanism. Moreover, it was found that the binding with the peptides and DNA led to hardening of the DNA structure [36]. We based our hypothesis on a short peptide-DNA interaction [3, 8] and aimed to appraise the possibility of ADEL–DNA interaction, since the molecular modeling has shown that short peptides interact with specific sequences of nucleotides in DNA [3, 8].

ADEL consists of 4 amino acids: alanine, glutamic acid, aspartic acid, and leucine. Apart from the opposite charges of terminal groups at neutral pH, the charges of amino acid residues—Ala (0), Glu (-1), Asp (-1), Leu (0)—provided total electronegativity of the peptide, which supposedly should have prevented its binding to a highly negative DNA molecule in solutions of little ionic strength. Nevertheless, as it appears from spectral and hydrodynamic data, the interaction actually occurred.

Physical methods showed that the most likely point of ADEL binding is guanine atom N7 in a DNA major groove, and the ADEL–DNA interaction depends on the peptide concentration. Nowadays, ADEL–DNA interaction is widely discussed in the scientific literature [37]. It is not

surprising, since at the end of the last century, it was found that short peptides based on Tat-protein (a transcriptional activator of human immunodeficiency virus HIV-1) are able to penetrate cells [38]. These cell-penetrating peptides (CPP) are able to transport into the cell proteins, nucleic acids, and liposomes [39]. Therefore, there are different suggestions to the mechanism of how peptides penetrate the cytoplasmic and nuclear membrane [40, 41].

To sum up the results, ADEL can bind to specific DNA regions and epigenetically regulate the gene expression and synthesis of proteins involved in the differentiation and maintenance of functional activity of the bronchial epithelium.

Conclusion

The ADEL peptide was concluded to regulate the synthesis of a wide range of proteins in human bronchial epithelial cells, which confirmed results previously obtained [9, 10]. ADEL specifically regulates the processes of cell renewal in bronchial epithelium through its influence on levels of Ki67, Mcl-1, p53 proteins, and cells' functional condition through its influence on CD79 and NOS-3 proteins. The reason why some of the effects of ADEL were more pronounced in "young" cell cultures and others in the "old" ones can be explained by non-simultaneous reduction in the levels of mentioned protein synthesis during cellular senescence.

It is established that ADEL epigenetically regulates the expression of genes involved in bronchial epithelium differentiation: NKX2-1, SCGB1A1, SCGB3A2, FOXA1, and FOXA2. Considering that some of their products mutually regulate each other's expression and, apart from that, interact with histones (peptide ADEL targets as well), it can be suggested that the peptide has one key- or several independent-points of application in corresponding signal cascades.

By activation of the expression of MUC4, MUC5AC, and SFTPA1 genes—markers of bronchial epithelial cells' functional activity—ADEL is able to prevent lung pathology development. This suggests the prospects of further investigation of ADEL as a substance for normalizing the function of the respiratory system. It may be assumed that the influence of ADEL, connected to the regulation of gene expression and protein synthesis of bronchial epithelium, can be caused by ADEL–DNA interaction. Physical methods suggest that the most likely point of ADEL binding is guanine atom N7 in a DNA major groove and that the ADEL–DNA interaction depends on the peptide concentration.

Conflict of interest None of the authors has any conflict of interest related to this manuscript.

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