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THE DYSREGULATION OF SURFACTANT-ASSOCIATED PROTEINS' HOMEOSTASIS IN CAVERNOUS TUBERCULOSIS OF LUNGS

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Abstract

Despite the progress and development of socially important scientific areas in medicine, tuberculosis remains one of the global problems. The surfactant system is one of the key mechanisms in the formation of immune response expression. In this regard, the aim of our study was investigation of surfactant apoproteins' state both in the foci of specific destruction and in the surrounding intact lung tissue to assess its functional activity, the degree of respiratory failure and possible dissemination of tuberculosis inflammation.

The materials for the study were fragments of human lungs taken from patients died or operated about of cavernous pulmonary tuberculosis (n=163) with an active bacterial discharge (n=89) and with clinical abacillation (n=74). A complex morphological study revealed the stereotypical dynamic depression of the surfactant-associated proteins A, B and C in all the samples both in cavernous areas of destruction and pericavitic areas, and in intact lung tissue. The maximum intensity of immunohistochemical expression was recorded in alveolar macrophages, which indicated intensive recycling and utilization of surfactant components. At the same time, the synthetic activity of type II alveolocytes was activated mainly to the SP-A and to SP-B in the lower extent. The most sensitive was SP-C, the amount of which was significantly reduced in intact lung tissue. At that, this collectin totally disappeared both in the area of cavernous destruction and in pericavitic area. Minimizing of surfactant components' production and its active utilization in intact lung tissue leads to disorganization of the monolayer on the inner surface of the alveoli with their subsequent collapse and progressive respiratory failure.

Keywords *tuberculosis, lungs, surfactant, proteins, immunohistochemistry, morphology.*

Introduction

Despite the progress and development of socially important scientific areas in medicine, tuberculosis (TB), remains one of the global problems **[***WHO, 2017*]. High rates of both morbidity and mortality, absence of specificity in clinical manifestation at the early stages of the disease, a variety of clinical and morphological forms, recurrent course, torpidity to therapy – it is only a small list of unresolved problems associated with tuberculosis process **[***Fox G. et al., 2016*].

Modern science carefully details the individual pathogenic aspects of the TB, but is still unable to give a full answer to the main question formulated by North et al. in 2004: "why the immune system of susceptible people is not able to cope with pulmonary infection and thus to stop the development of the disease?"**[***North R. et al., 2004*]. One of the reasons for the inadequate functioning of the innate immunity in the progression of specific inflammation is the dysregulation of cellular and humoral mechanisms with the formation of a "vicious circle". Thus, one of the key mechanisms in the development of the most common pulmonary forms of secondary tuberculosis is the surfactant system **[***Ince L. et al., 2018*].

Surfactant is a protein-lipid complex and consists of 80% phospholipids, 8% neutral lipids (cholesterol and free fatty acids) and 12% proteins. Hydrophilic (SP-A and SP-D), represents the protein component of the surfactant and hydrophobic (SP-B and SP-C) types of apo-

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proteins synthesized by type II alveolocytes (A2), nonciliated bronchial epithelial cells and Clara cells.

SP-A is quantitatively the most significant surfactant-associated hydrophilic collectin, the molecular weight of which, depending on the degree of glycosylation, varies within 28-36 kDa **[***Kankavi O. et al., 2004*]. Functionally, it is necessary for lipid binding and regulation of surfactant homeostasis. The complex action of SP-A and SP-D (hydrophilic protein, ≈ 39-43 kDa) has both immunomodulatory effects and antibacterial activity by stimulating alveolar macrophages (AM) and increasing opsonization followed by phagocytosis of bacteria, including *Mycobacteria tuberculosis.* **[***Vieira. F. et al., 2017*]. In addition, they can inactivate allergens and reduce allergen-induced lymphocytes' activity, as well as promote the release of histamine, cytokines, immunoglobulins and free radicals by immune cells **[***Cabre E. et al.,2018*].

Two other proteins, SP-B and SP-C, are hydrophobic apoproteins and provide surface activity of phospholipids **[***Yang L. et al., 2009*]. SP-B is a surfactant-associated protein (SAP) with a molecular weight of 8 *kDa*. It fixes phospholipids of the monomolecular surfactant film at the interface between the two air-liquid phases. SP – C (molecular weight-4 *kDa*) participates in the formation of tubular myelin, promotes the creation of a surfactant monolayer, preventing the collapse of the alveoli **[***Augusto L. et al., 2003*]. It should be noted that most studies of the role of surfactant in the development of bronchopulmonary pathology are based on the analysis of experimental data, immunological reactions in vivo and in vitro and do not reflect the morphological specificity of destructive forms of TB **[***Jiramethee N. et al., 2017*].

In this regard, the aim of our investigation was to study the state of surfactant apoproteins not only in the focus of specific destruction, but also in the surrounding intact lung tissue to assess its functional activity, the degree of respiratory failure and possible dissemination of tuberculosis inflammation.

MATERIAL AND METHODS.

The materials for the study were the fragments of the lungs from patients died or operated about fibrous-cavernous tuberculosis (FCT) of the lungs (n=163). All patients were divided into 2 main groups:

Group 1 – Pulmonary FCT with active bacterial excretion (MBT+) – n=84;

Group 2 – Pulmonary FCT without excretion (MBT-) – n=79.

Fragments of 30 patients' lungs died of the diseases not associated with lung disease (myocardial infarction, acute violation of cerebral circulation) have been used as a control group for comparison of morphological indicators.

Criteria for inclusion of patients in the study were age from 18 to 65 years, negative clinical and laboratory data on the presence of comorbid pathology (viral hepatitis B, C and HIV), exacerbation of chronic diseases of other organs/systems and informed consent.

For standard histological examination fragments of the walls of the cavity, pericavitic area and macroscopically intact lung tissue was fixed in 10% neutral formalin, followed by waxing and formation of sections with thickness of 4-5 mm. Visualization of pathological changes was carried out by staining with hematoxylin and eosin **[***Yanin V. et al., 2015*]. In addition, to determine acid-stable bacilli histochemical study on the method of Ziehl-Nielsen was carried out, and staining with picrofuchsin by van Gieson method according to standard protocols to detect collagen fibers was performed **[***Yanin V. et al., 2015*]. For the assessment of the SAP conducted immunohistochemical (IHC) study using markers SP-A (AB3420; ChemiconIntern.Inc., USA; rabbitanti-human, 1:250), SP-B (AB 3436; ChemiconIntern. Inc., USA; rabbit anti-human; 1:2000), SP-C (AB 3428; ChemiconIntern. Inc., USA; rabbit anti-human; 1:500). Visualization system StreptABComplex/APDako-Cytomation. **[***Dabbs D., 2006*]

In connection with the expression of the SAP as within the various cellular elements and the inner surface of the alveoli and bronchioles evaluated the intensity of reaction semiquantitative method taking into account the localization (Table 1).

Viewing and photographing of micropreparations was carried out with the help of a light microscope "OlympusCX-41", OLYMPUS DIGITAL CAMERA C5050 ZOOM (USA).

Statistical analysis of the obtained data was carried out by methods of variation statistics using non-parametric criteria by the software package Statistica for Microsoft Windows, version 10.0., StatSoft Inc., USA. Statistical analysis included the construction of variation series of quantitative

Table 1.

Assessment of the intensity of expression of surfactant-associated proteins

Staining	The degree of reaction	Assessment, point
Absents	Absents	0
Red stripes on the edge of the cytoplasm (discontinuous membrane contour) and low intensity of cytoplasmic staining	Weak (low level of expression)	1
Red grains in the cytoplasm (dot-like expression), discontinuous strip lining the surface of the alveoli	Moderate (medium level of expression)	2
Diffuse red cytoplasmic color and uniform strip lining the surface of the alveoli	Expressed (high level of expression)	3

data, the calculation of the arithmetic mean, standard deviation, and error of the average value, the coefficient of variation and the value of the deviation of the indicator from the control and intergroup values. The significance of the differences in the compared values was determined using the nonparametric Kruskal-Wallis test. The critical level of significance of differences between the groups was assumed to be p=0.05. Descriptive statistics are presented as M±SE, where M – mean value, SE – standard error.

Results

The study showed that the maximum expression of SP-A was determined in the zone of caseous necrosis of the FCT-MBT+ group (2,90±0,03), among leukocyte-necrotic detritus free-lying in the lumen or fixed to the underlying layer of specific granulation tissue. This indicator was statistically significant ($p<0.05$) different from the control group (0.00) and FCT-MBT -group (0.06±0.03).In the area of specific granulation tissue, characterized by proliferation of a defective capillaries and a prominent diffuse infiltration by the cells of histiocytic and lymphoid origin (predominantly CD4+ T-cells), the presence of a few CD68+ macrophages with intensely red granular inclusions in the cytoplasm was recorded **[***Sorokina I. et al., 2013*]. In the group of patients with clinical abacillation (FCT-MBT-) against the background of the missing pyogenic layer, the number of such macrophages progressively increased as it approached the cavity. In histologically verified foamy epithelioid cells, certain cells with dormant Mycobacterium (bright red intracellular fragments of acid-unstable bacilli visualized by a method of Ziehl-Nielsen). Negative expression was recorded in all zones (Fig. 1).

The fibrous layer of the cavity with a slightly expressed diffuse background staining of the red color without committing to any cellular elements. This expression pattern was recorded in all cases regardless of active discharge, which is likely related to the total fibrotic transformation of the lung parenchyma due to confirmed histochemically proliferation of collagen fibers (visualized by van Gieson method),with the purpous of specific tuberculous inflammatory focus separation **[***Golubinskaya E. et al., 2017a*].

In the pericavitic zone, regardless of the MTB excretion, intensely colored A2 and AM were visualized, which were compactly grouped in partially collapsed alveolar lumens, being located in two rows on the surface of the alveolar wall.

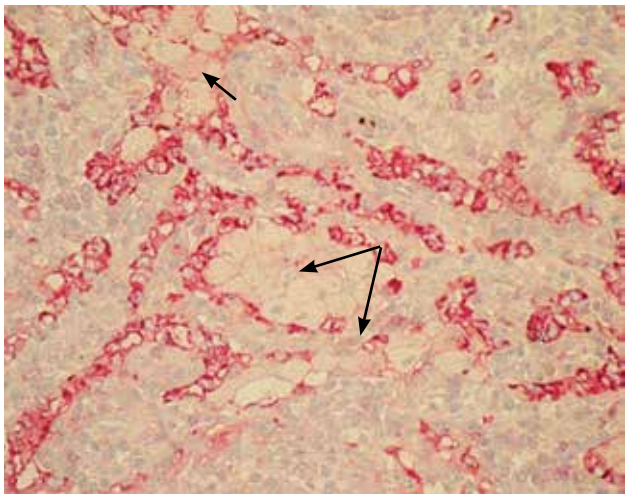

Figure 1. *FCT-MBT+. IHC expression of SP-A in foamy macrophages (arrow). Total magnification x40.*

In the areas of emphysematous changes in the alveoli, the expression of SP - A is in the form of a continuous thin strip of red lining the alveolar wall. At the same time, the amount of A2 with synthetic activity of surfactant and AM components, freely lying in the lumen of the alveoli and, accordingly, their eliminating, was reduced. In the interalveolar septa, the increased number of tissue macrophages with granular inclusions of red color in the cytoplasm is recorded, which may be the result of the intensification of SP-A phagocytosis by these cells for their subsequent polarization.

Changes in the expression of SP-A were also determined in the area of the draining bronchus, with significant intergroup differences depending on the activity of bacterial discharge ($p<0.05$) (Fig. 2).

In FCT-MBT+, a light degree of expression in the form of a linear lining of red color is localized on the apical surface of ciliary epithelium in the draining bronchus and in the bronchial exudate (1.94±0.03). In bronchi with signs of nonspecific chronic bronchitis remote from the cavity, in the cytoplasm of ciliary epithelium and in the bronchial epithelial lining of the alveoli, particularly in A2 and AM high intensity of expression of SP-A

Table 2.

Assessment of the intensity of expression of surfactant-associated protein A

Locatization	SP-A		
	Control	MBT+	MBT-
Pyogenic layer	0.00	2.90±0.03*	0.06±0.03**
The granulation layer	0.00	2.82±0.04*	2.81±0.04*
Area of fibrosis	0.00	1.17±0.04*	1.05±0.04**
Areaof distelectase	0.00	2.90±0.03*	2.27±0.06**
Area of emphysema	0.00	1.07±0.03*	0.99±0.05**
Area of draining bronchus	1.03±0.32	1.94±0.03*	1.09±0.03**
Type 2 alveolocytes	1.06±0.25	1.92±0.03*	1.07±0.04**
Alveolar macrophages	1.07±0.25	1.96±0.03*	2.03±0.04*

Notes: *- $p\leq0.05$relative to the control group; ** - $p\leq0.05$ relative to MBT+

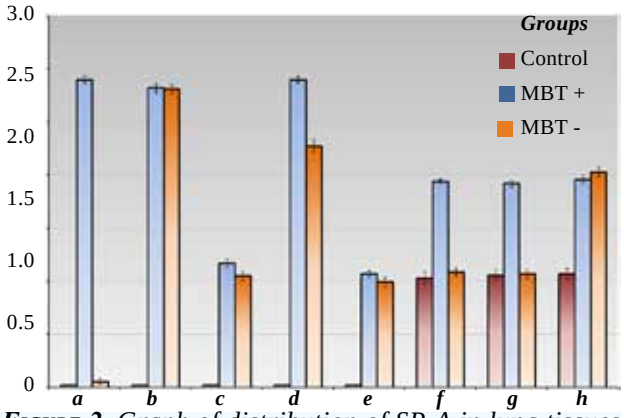

Figure 2. *Graph of distribution of SP-A in lung tissues in FCT, depending on the activity of bacterial excretion.* ***Notes: a*** *- pyogenic layer,* ***b*** *- granulation layer,* ***c*** *- fibrous lauer,* ***d***- *area of distelectase,* ***e***- *area of emphysema,* ***f*** *- area of draining bronchus,* ***g*** *- type 2 alveolocytes,* ***h*** *- avleolar macrophages*

was registered (Table 2).

In the group of patients with FCT-MBT-, the expression of SP-A (1.09±0.29) was determined as fine-grained cytoplasmic inclusions (dot-like) in atrophic cells of the ciliated epithelium, due to the prominent hyperplasia of goblet cells producing mucosal secretion in the bronchial lumen (Fig.3). In addition, the presence of single positively colored tissue macrophages in the peribronchial space was recorded

In assessing the intensity of expression of SP-B and SP-C directly in the focus of cavernosal deformation in both cases with MBT+ and MBT - in the zones of caseous necrosis, specific granulation and fibrous tissue, a negative reaction was recorded (0 points) (Table 3).

In active FCT, the expression of SP-B in the

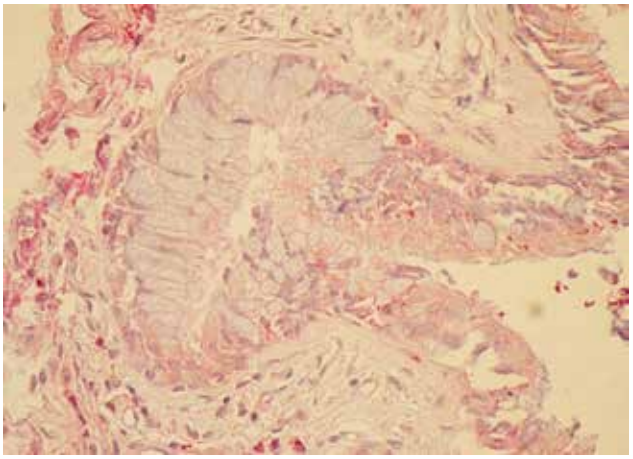

Figure.3. *FCT-MBT-. Zone of nonspecific panbronchitis. Dot-like expression of SP-A in the ciliated epithelium. Focus of hyperplasia of goblet cells with negative expression of SP-A.x400.*

TABLE 3.

Assessment of the intensity of expression of surfactant-associated proteins B and C

Locatization	SP-B			SP-C		
	Control	MBT+	MBT-	Control	MBT+	MBT-
Pyogenic layer	0.00	0.00	0.00	0.00	0.01±0.012	0.00
The granulation layer	0.00	0.00	0.00	0.00	0.00	0.01±0.012
Area of fibrosis	0.00	0.00	0.00	0.00	0.00	0.00
Area of distelectase	0.00	2.90±0.03*	2.92±0.03*	0.00	0.95±0.02*	0.95±0.02*
Area of emphysema	0.00	0.93±0.03*	0.95±0.02*	0.00	0.00	0.00
Area of draining bronchus	1.03±0.18	0.06±0.03*	0.05±0.02*	0.00	0.01±0.012	0.01±0.012
Type 2 alveolocytes	2.93±0.25	0.95±0.02*	0.96±0.02*	1.93±0.20	0.95±0.02*	0.95±0.03*
Alveolar macrophages	2.00±0.06	1.06±0.03*	1.06±0.03*	1.00±0.26	0.00*	0.00*

NOTES: **- p≤0.05 relative to the control group; ** - p≤0.05 relative to MBT+*

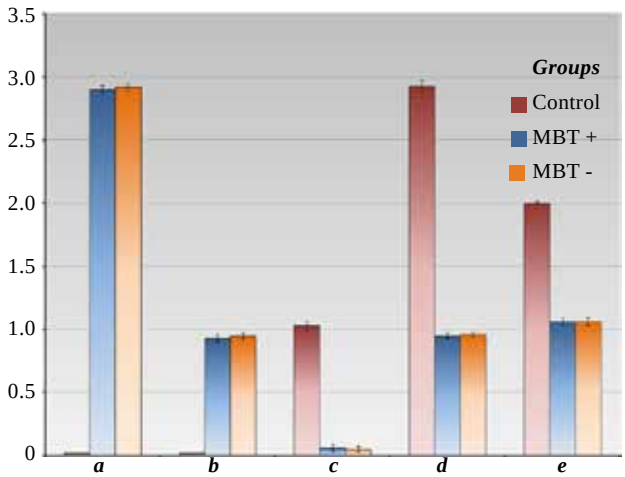

FIGURE 4. *Graph of distribution of SP-B in lung tissues in this area depending on the activity of bacterial excretion.*
NOTES: ***a*** *- area of distelectase,* ***b*** *- area of emphysema,* ***c*** *- area of draining bronchus,* ***d****- type 2 alveolocytes,* ***e*** *-avleolar macrophages*

area of the draining bronchus was negative, but in the area of distelectase near the fibrous wall of the cavity there was its' high level (2,90±0,03) (Fig. 4), indicating proliferation clusters A2 and AM in diselectatic alveoli (Fig.5A). Similar reaction in A2 expressing SP-C (0.95±0.02). (Fig.5B).

It is important to note that positively stained macrophages in all studied areas were found in exclusive cases and localized in the lumen of the alveoli only. Tissue macrophages in interalveolar septa, peribronchial and intact pulmonary tissue demonstrated negative expression.

In areas of emphysema beyond the cavity, SP-B expression was sharply decreased, acquiring intermittent contour in all observations, regardless of the activity of bacterial discharge. The number of

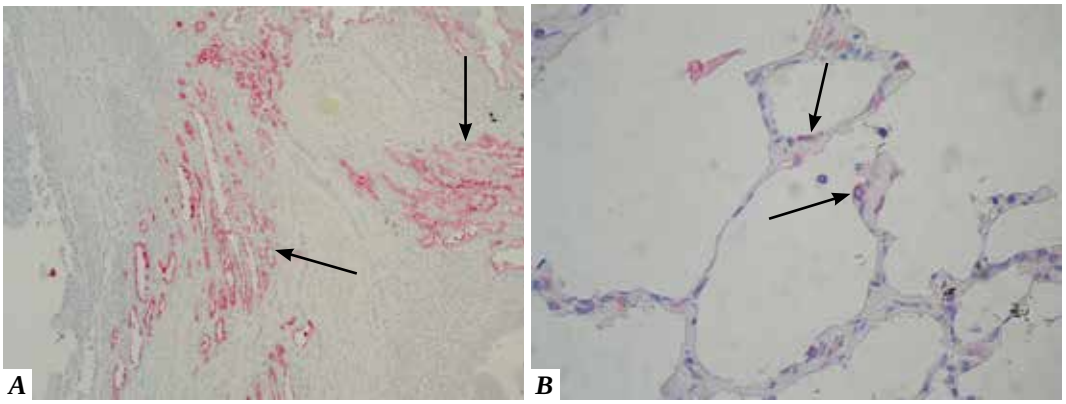

FIGURE 5. *FCT-MBT+. IHC reaction with markers SP-B and SP-C.*
A *- IHC expression of SP-B in a zone of specific inflammation: negative reaction in the fibrous wall of the cavity, focal positive reaction in pericavitic area (arrow). X10.*
B*- IHC, the expression of SP-C in the area of emphysema. Single positively stained alveolocytes (arrow). X40.*

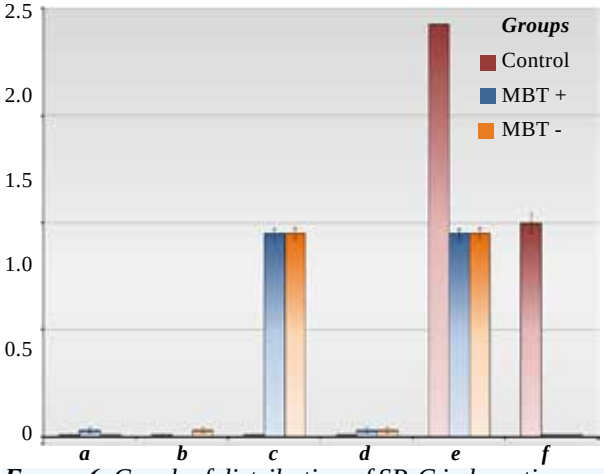

Figure 6. *Graph of distribution of SP-C in lung tissues in this area depending on the activity of bacterial excretion* ***Notes: a*** *- pyogenic layer,* ***b*** *- granulation layer,* ***c*** *- area of distelectase,* ***d*** *- area of draining bronchus,* ***e*** *- type 2 alveolocytes,* ***f*** *- avleolar macrophages*

epithelial cells synthesizing this protein decreased dramatically. Given features of localization indicated the damage of the A2 and the decrease in the function of surfactant because of the progressive and apparently irreversible distension of the alveoli. In this case, the expression of SP-C in the specified zone is absent (0 points), determining in single A2 cells or completely disappearing (Fig.6).

Discussion

The discovery of the lung surfactant in the 50s of the XX century marked the beginning of numerous studies of its physical and chemical properties, metabolism, and role in physiological conditions and in various pathological states of the lungs. There is a large number of research proving the role of surfactant deficiency in the development of various bronchopulmonary pathology. However, most of them are based on the study of quantitative indicators of SAP in broncho-alveolar lavage and do not reflect the nature of intercellular interactions. Some studies were conducted on experimental animals'tissue samples, which does not always allow extrapolating the results to patients with secondary forms of tuberculosis, including destructive cases. **[***Gordon S. et al., 2014***]**.

In our morphological study of the expression of SAP A, B and C in the lung fragments of patients with cavernous tuberculosis, a significant decrease in their overall level was found in comparison with the control samples of the lungs in people who died of a pathology not related to the respiratory system. In addition, the intergroup differences in the localization nature of collectin A, manifested as far as the distance from the focus of cavernous deformation and depending on the activity of bacterial discharge.

The maximum expression of SP-A and SP-B in the zone of caseous necrosis in patients of FCT-MBT+ group was established directly in the cavity wall. This reaction may be a consequence of the residual antigenic activity of necrotic A2 and AM, taking an active part in the production, recycling and disposal of surfactant components.

The subsequent zonal depression of the reaction intensity from the layer of specific granulation tissue to the fibrous one did not show significant intergroup differences. At the same time, it significantly differed from the control group due to the increase in the number of active CD68+macrophages infiltrated the specific granulations'growth fields. It is important to note that the foamy macrophages and giant Pirogov-Langhans cells in all cases were characterized by a negative expression, which is a consequence of the congestion by the lipid inclusions and the hyperactivation of macrosialine protein, manifested in the fusion of late endosome and lysosomes of the phagocyte **[***Golubinskaya E. et al., 2018***]**.

One of the fundamental histological differences between FCT-MBT+ and FCT-MBT- is the presence of a draining bronchus, usually containing caseous masses with a large number of mycobacteria. In our study, the intensification of SP-A expression on the surface of the ciliated epithelium and in peribronchial macrophages with the activation of bacterial discharge was established. This reaction may be associated with the activation of the opsonizing action of histiocytes due to the significant number of mycobacteria, which are located not only in the bronchial lumen, but also in the nearest peribronchial tissue **[***Golubinskaya E. et al., 2018***]**. Expressed bacterial insemination explained the appearance of positively colored tissue macrophages, possibly polarizing into pro-inflammatory macrophages of type 1 with high cytokine activity, including VEGF products for activation of neoangiogenesis and remodeling of intercellular matrix **[***Golubinskaya E, Kramar T, 2018***]**.

In general, in pericavernous area largely regardless of active discharge there is a redistribution of all surfactant-associated proteins. The ac-

cumulation of apoproteins in areas of distelectase can have a dual meaning. One of the reasons for this expression is the mechanical conglomeration of alveolocytes and AM in connection with the partial collapse of the alveoli. At the same time, on the other hand, it may be the result of increased proliferative and synthetic activity of A2 as a compensatory – adaptive reaction in response to deep destructive and prominent inflammatory processes, which is associated with the activation of the main functions: immunomodulatory and anti-inflammatory **[***Lock M. et al., 2015*]. The increased expression in AM shows the intensive recycling of components of surfactant abled to stimulate macrophages to phagocytosis **[***Glasser J. et al., 2012*].

IHC study of hydrophobic apoproteins SP-B and SP-C has allowed establishing stereotyped reactions, regardless of the bacteria's discharge activity, which was manifested in their significant redistribution to the affected, but preserved the ability of gas exchange alveoli in the area of distelectase. In our opinion, this type of expression is also associated with the compensatory-adaptive reaction A2 to stabilize the monolayer of the surfactant and ensure the stability of the alveoli. **[***Nandkumar M. et al., 2014*]. Critical depression of SP-C, up to total disappearance in destructive and dystrophic processes in the lungs is associated with either the most pronounced sensitivity of this collectin, which leads to accelerated degradation in the tuberculosis process, or the consequence of high sensitivity to catabolic destruction in non-viable, including operated tissues.

Thus, in the formation of destructive forms of secondary tuberculosis, particularly itscavernous forms, is determined by a complex deficiency and imbalance of proteins of the surfactant homeostasis inthe lungs with vectorial functional synthetic SP-A redistribution of A2 to intensify immunomodulatory and antibacterial functions by stimulating alveolar macrophages and increasing opsonization and subsequent phagocytosis of Mycobacteria tuberculosis. Minimizing of surfactant components' production and its active utilization in intact lung tissue lead to disorganization of the monolayer on the inner surface of the alveoli, their collapse and progression of respiratory failure.

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