Expression analysis of mRNA of cathelicidin (LL-37) in bronchial epithelial cells infected with *Mycoplasma pneumoniae*, *Escherichia coli* and *Staphylococcus aureus*

Ocena ekspresji mRNA katelicydyny LL-37 w komórkach nabłonka oskrzeli zakażonych *Mycoplasma pneumoniae*, *Escherichia coli* i *Staphylococcus aureus*

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Streszczenie

Wprowadzenie: Katelicydyna LL-37 wydzielana jest przez szereg komórek, m.in. przez komórki nabłonka oskrzeli. Prezentuje szeroki zakres aktywności. Odgrywa istotną rolę w regulacji funkcji komórek, w tym komórek nabłonka. Posiada właściwości przeciwdrobnoustrojowe i immunomodulujące. Niszczy drobnoustroje w sposób bezpośredni, jak również poprzez aktywację kolejnych procesów immunologicznych. LL-37 działa między innymi na bakterie Gram-dodatnie i Gram-ujemne, natomiast w zakażeniach *Mycoplasma pneumoniae* rola LL-37 nie jest dokładnie zbadana.

Cel: Celem pracy była ocena ekspresji mRNA katelicydyny LL-37 w komórkach nabłonka oskrzeli pod wpływem zakażenia *Mycoplasma pneumoniae*, *Escherichia coli* i *Staphylococcus aureus.*

Materiał i Metody: Przeprowadzono hodowle linii komórek nowotworowych oskrzeli Beas2B ATCC CRL-9609 zakażonych *Mycoplasma pneumoniae* ATCC 15531, *Escherichia coli* ATCC 25922 i *Staphylococcus aureus* ATCC 29213 i niezakażonych (hodowla spontaniczna). Oznaczono ekspresję mRNA dla LL-37.

Wyniki: W przypadku hodowli zakażonej *Mycoplasma pneumoniae* zaobserwowano istotny statystycznie wzrost ekspresji LL-37 w 24 h w porównaniu do hodowli spontanicznej i pozostałych hodowli zakażonych. W hodowli zakażonej *Escherichia coli* i *Staphylococcus aureus* istotny statystycznie wzrost ekspresji w porównaniu do hodowli spontanicznej zaobserwowano w 72 h.

Wnioski: Uzyskane wyniki potwierdziły rolę katelicydyny w obronie przeciwdrobnoustrojowej w zakażonych komórkach nabłonka oskrzeli. Stwierdzono wpływ zakażenia *Mycoplasma pneumoniae* na zwiększoną ekspresję katelicydyny oraz szybsze pobudzenie jej ekspresji w porównaniu z *Escherichia coli* i *Staphylococcus aureus*.

Słowa kluczowe: Katelicydyna, LL-37, *Mycoplasma pneumoniae,* peptydy przeciwdrobnoustrojowe

Summary

Introduction: Cathelicidin LL-37 is secreted by a number of cells, including bronchial epithelial cells and also neutrophiles, monocytes, lymphocytes and keratinocytes. It plays an important role in regulating the functions of cells, including epithelial cells. It has antimicrobial and immunomodulating properties. It destroys microorganisms directly as well as by activating subsequent immunological processes. LL-37 is active against Gram-positive and Gram-negative bacteria, while the role of LL-37 in *Mycoplasma pneumoniae* infections has not been thoroughly investigated.

Aim: The aim of the study was to evaluate the expression of mRNA of cathelicidin LL-37 in bronchial epithelial cells infected with *Mycoplasma pneumoniae*, *Escherichia coli* and *Staphylococcus aureus*.

Materials and methods: The bronchial tumor cell lines Beas2B ATCC CRL-9609 infected with *Mycoplasma pneumoniae* ATCC 15531, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 and uninfected (spontaneous) were cultured. LL-37 mRNA expression was determined.

Results: In the case of culture infected with *Mycoplasma pneumoniae*, a significant increased LL-37 expression was observed in 24 h compared to the spontaneous culture and the remaining infected cultures. In cultures infected with *Escherichia coli* and *Staphylococcus aureus* a significant increased expression was observed after 72 h as compared to spontaneous culture.

Conclusions: The obtained results confirmed the role of cathelicidin in antimicrobial defense in infected bronchial epithelial cells. The influence of *Mycoplasma pneumoniae* infection on the increased expression of cathelicidin was found and a faster stimulation of LL-37 expression was observed in comparison to *Escherichia coli* and *Staphylococcus aureus*.

Key words: Cathelicidin, LL-37, *Mycoplasma pneumoniae,* antimicrobial peptides

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1. Introduction

Cathelicidin LL-37 belongs to antimicrobial peptides (AMPs), which are key cationic peptides involved in the regulatory processes during infection and inflammation. Cathelicidin is an important, conservative component of the mechanisms of innate immunity and exhibits antimicrobial properties against various microorganisms, such as Gram-positive and Gram-negative bacteria, fungi and viruses [1].

The cells of the innate immune response, as well as epithelial cells, express various receptors, including TLRs (Toll-like receptors), which inform about the invasion of pathogens [2]. TLRs are able to react with exogenous infectious ligands, such as cell wall elements: LPS (lipopolysaccharide) of Gram-negative bacteria, and LTA (lipoteichoic acids) of Gram-positive bacteria, components of bacterial motility apparatus, single- and double-stranded RNA and DNA sequences characteristic only for microorganisms. TLRs have different locations and ligands: TLR1 (including: TLR1, 2, 6 and 10), TLR4 and TLR5 subfamilies, are found mainly in the cytoplasmic membrane and recognize the elements of cell walls, capsule and components of flagella; TLR3, TLR7-9 and TLR11-13 subfamilies are found in intracellular organelles, such as endosomes, and are mainly involved in the recognition of pathogen nucleic acids. The ligand binding to TLR leads to the activation – via different kinase cascades – of transcription factors, such as NFκB (Nuclear Factor κ-light chain-enhancer of activated B cells), or AP-1 (Activator Protein-1) [3].

Unlike all other prokaryotes, *Mycoplasma pneumoniae* (*M. pneumoniae*) has no cell wall and not contain LPS endotoxin. Despite this, the bacterial dipalmitoylated lipoprotein activates NF- κB via TLR1, TLR2 and TLR6 [4]. Moreover, the cytadherent property of *M. pneumoniae* (i.e., its ability to adhere closely to epithelial cells) also induces an inflammatory response via TLR2-independent pathway: TLR4 has been shown to be involved in this TLR2-independent induction of inflammatory responses [5].

The above processes lead to the stimulation of the expression and secretion of proinflammatory cytokines, such as TNF-α, chemokines, but also antimicrobial peptides, including cathelicidin, which are quickly released from cells. LL-37 further stimulates inflammatory cells to chemotaxis and to produce inflammatory cytokines [6]. In turn, proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β can also induce expression and release of cathelicidin [7]. Respiratory epithelial cells can also be activated by leukotrienes B4 (LTB4) to produce LL-37 [6]. Thus, the expression of the gene encoding cathelicidin is regulated during the inflammatory process, but also directly by microorganisms [8]. Moreover, LL-37 regulates the inflammatory response, e.g., activation of phagocytes and production of pro-inflammatory cytokines, by blocking binding of LPS and LTA to TLRs [9, 10, 11].

An antimicrobial, stimulating and immunomodulating function of LL-37 is activated after peptide secretion [5, 9]. Cathelicidin is synthesized as a non-active propeptide, and proteolytic cleavage in the region joining the signal part to the C-terminal domain produces an active 37 amino acid peptide, called human cationic antimicrobial peptide (hCAP)-18 [8]. The genes encoding LL-37 (locus on 3p21) are constitutively expressed in neutrophil precursors and/or show induced expression in a variety of cells, such as monocytes, mast cells, natural killer cells (NK), B cells, T cells and epithelial cells [8]. The active form of LL-37 shows bactericidal activity: it interacts with bacterial cell membranes, leading to their destruction and microbial cell lysis [12, 13]. However, its role in infections caused by atypical bacteria, such as *M. pneumoniae* is not fully understood.

Mycoplasma pneumoniae and two other studied bacteria, *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) can be the etiological factors of lower respiratory tract infections. During such infections the numerous immunological processes, like secretion of inflammatory and regulatory mediators, such as cytokines, prostaglandin, leukotriens, proteins, peptides, including LL-37, are activated.

The atypical bacterium, *Mycoplasma pneumoniae*, most often causes community-acquired atypical pneumonia in young adults. It can also cause asymptomatic infections. Due to the mild course of such pneumonia, it is called "walking pneumonia", because the patient feels so well that he does not have to stay at home. Such infections are usually without fever or coughing. In turn, *E. coli* and *S. aureus* are less likely to cause community-acquired lower respiratory tract infections and the course of infections caused by them is more severe. *E. coli* infections are most often found in people staying in nursing homes, in patients who have been hospitalized many times and in elderly patients. *S. aureus* is rarely the cause of acute community-acquired lower respiratory tract infections. It most often causes infections in the upper respiratory tract. It is also the causes of mixed and chronic infections, as well as following viral infections, especially those caused by flu virus (pneumonia).

The aim of the study was to evaluate the expression of mRNA of LL-37 in bronchial epithelial cells infected with *M. pneumoniae*, *E. coli* and *S. aureus*.

2. Material and methods

Bronchial tumor cell line Beas2B ATCC CRL-9609 was used to set up the culture. The following bacterial strains were used to infect individual colonies: *M. pneumoniae* ATCC 15531, *E. coli* ATCC 25922 and *S. aureus* ATCC 9213.

The Beas2B ATCC CRL-9609 cells were cultured in Eagle's Minimum Essential Medium in tissue culture plates and after reaching confluent monolayer of cells were infected with 100µl of 0.5 Mc Farland's suspension with *M. pneumoniae* ATCC 15531, *E. coli* ATCC 25922, *S. aureus* ATCC 29213. One cell culture was left uninfected (spontaneous culture). After 5 min, 30 min, 2 h, 24 h, 48 h, and 72 h the cells were collected (with a tripsine) and after isolation of RNA with GeneMATRIX DNA/RNA/Protein Purification Kit (Eurx), LL-37 mRNA expression was determined with duplex real Time-PCR method using TaqMan probes. Expression was determined with CFX96 Real-Time System (BioRad). GAPDH was used as a reference gene.

M. pneumoniae ATCC 15531 (ATCC) strain was cultured on the biphasic Mycoplasma Agar Base CM 401 (Oxoid) and Mycoplasma Broth Base CM 403 (Oxoid) with addition of the selective supplement P SR 60 (Oxoid). Bacteria were incubated on the medium with a pH of 7.4-8.0 in a moist chamber under microaerophilic conditions, at 35°C for 14 days. Molecular real-time PCR method with TaqMan probes was used to confirm the growth of *M. pneumoniae*. PCR testing was performed using BD MAX (Becton Dickinson).

E. coli ATCC 25922 and *S. aureus* ATCC 29213 strains were cultured in Columbia Agar with 5% Sheep Blood (Becton Dickinson) at 35°C in an oxygen atmosphere for 18 hours. The second passage was used for the study.

The experiment was set 4 times. Chosen significance level for this study was 0.05. Statistical analysis was performed using Statistica version 13.

3. Results

Expression of LL-37 mRNA was analyzed at all time points in both infected and non-infected cells. Cells infected with *M. pneumoniae* showed significant increase of LL-37 expression after 24h of culture, as compared to the non-infected culture (the Independent Samples t-Test, p=0.014) and also significant increase as compared to the cultures infected with *E. coli* and *S. aureus* (the Independent Samples t-Test, p=0.009 and p=0.033). Cells infected with *E. coli* and *S. aureus*, showed statistically significant increase in LL-37 expression after 72h of culture (the Independent Samples t-Test, p=0.018, p=0.001) as compared to the non-infected culture (see Table 1, Figure 1).

The growth of *M. pneumoniae* in Beas2B ATCC CRL-9609 cell culture was observed in cells collected after 24h, 48h and 72 h (see Figure 2, 3).

Table 1. Expression of LL-37 gene in non-infected cells and the cells infected with *Mycoplasma pneumoniae*, *Escherichia coli* and *Staphylococcus aureus.*

Time of incubation	Expression of LL-37 gene (2-ΔΔCt)			
	Non-infected cells	Cells infected with *M. pneumoniae*	Cells infected with *E. coli*	Cells infected with *S. aureus*
5 min	1.29 ± 0.62	1.36 ± 0.40	1.81 ± 0.62	1.56 ± 0.46
30 min	1.39 ± 0.98	0.96 ± 0.37	1.01 ± 0.41	1.86 ± 0.97
2 h	1.06 ± 0.38	1.23 ± 0.59	0.76 ± 0.35	2.06 ± 0.69
24 h	1.08 ± 0.49	3.51 ± 1.33	0.92 ± 0.31	1.51 ± 0.57
48 h	3.26 ± 1.81	3.35 ± 1.44	1.83 ± 0.66	1.59 ± 1.19
72 h	0.89 ± 0.34	3.29 ± 2.71	4.00 ± 1.90	4.45 ± 0.61

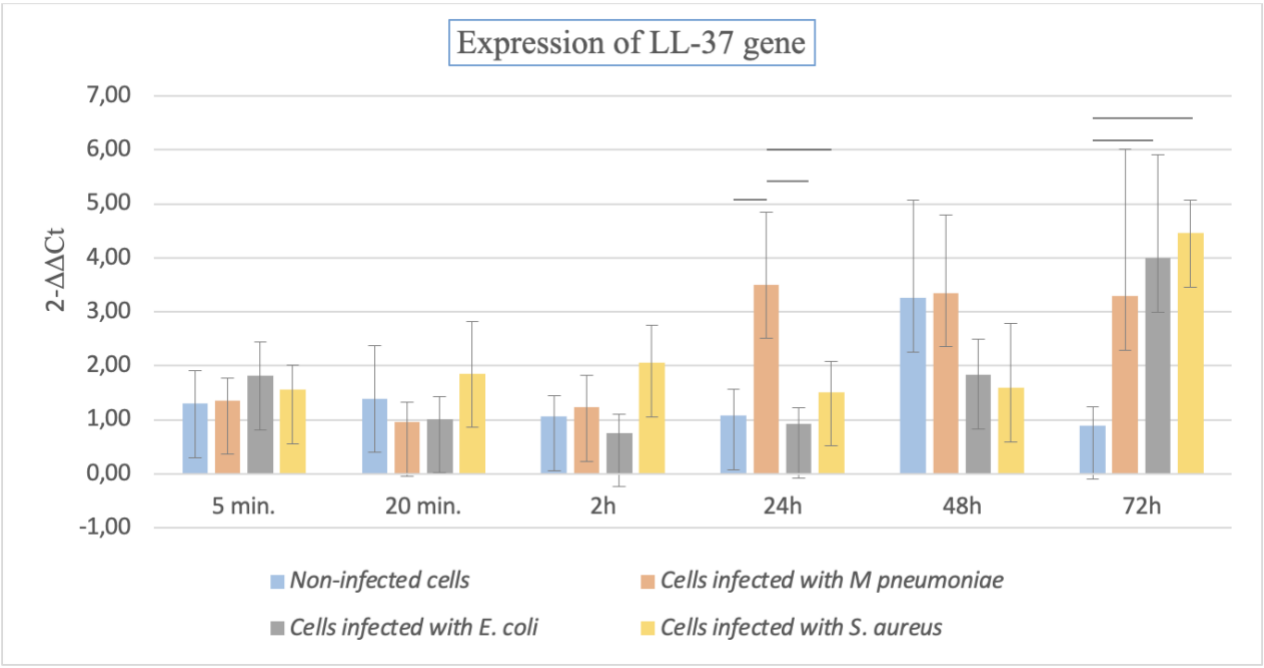

Figure 1. Expression of LL-37 gene in non-infected cells and the cells infected with *Mycoplasma pneumoniae*. *Escherichia coli* and *Staphylococcus aureus*. Statistical differences: p=0.014. p=0.009. p=0.033. p=0.018. p=0.001.

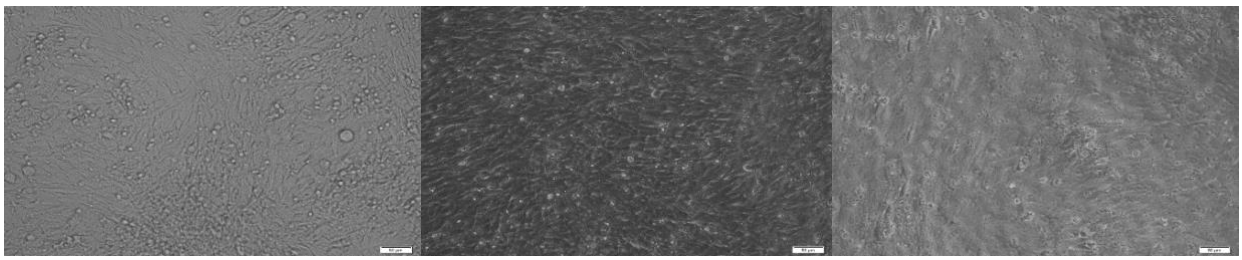

Figure 2. Non-infected Beas2B cells culture after 24h, 48h and 72h.

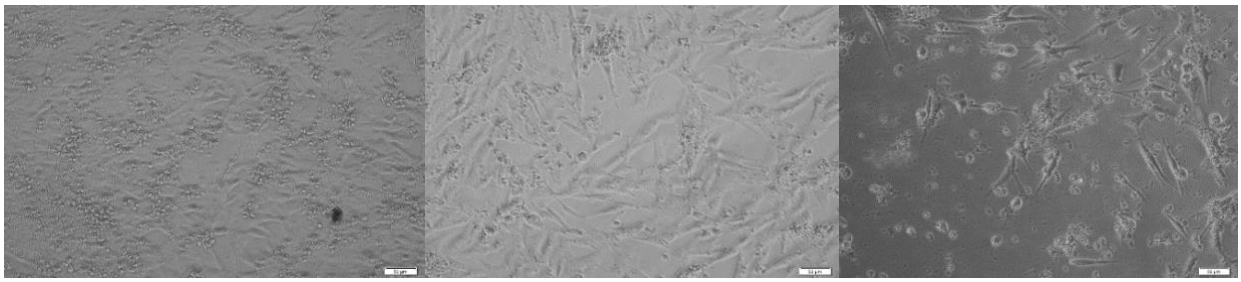

Figure 3. Beas2B cells culture infected with *M. pneumoniae* after 24h, 48h and 72h.

4. Conclusions and Discussion

The obtained results confirmed the role of cathelicidin in antimicrobial defense in infected bronchial epithelial cells. The influence of *M. pneumoniae* infection on the increased expression of cathelicidin was found and, additionally, its stimulation of LL-37 expression was observed earlier than in case of *E. coli* and *S. aureus*.

The faster activation of LL-37 expression by atypical bacteria such as *M. pneumoniae* compared to *E. coli* and *S. aureus* may be caused by the specific way of binding of this bacterium to the cilia base of respiratory epithelial cells. *M. pneumoniae* adheres closely to epithelial cells. It binds to sialic glycoproteins through P1 adhesin, which is the part of a special adhesive structure on the cell surface of mycoplasma. Infection of the surface of the epithelium caused by mycoplasmas results in penetrating the mucus. They adhere to the ends of the cilia and quickly migrate to the base of the cilia [23]. This may contribute to faster activation of epithelial cells than in the case of infection with typical bacteria such as *E. coli* and *S. aureus* and next faster activation of the expression of the LL-37 cathelicidin gene.

The faster response of the innate immune mechanism resulting in the secretion of LL-37 ameliorates the course of the disease. Indeed, the course of infection with *M. pneumoniae* is milder compared to *E. coli* and *S. aureus* infections. As found by others, the amount of secreted caltelicidin is also important in the course of *M. pneumoniae* infection. A study by Tani K. et al. showed that the production of CRAMP (cathelin-related antimicrobial peptide) by mouse neutrophils activated by thioglycolate was augmented 10-fold by the addition of *M. pneumoniae* [14]. Rivas-Santiago B. et al. investigated the role of cathelicidin LL37 in the innate immune response to *Mycobacterium tuberculosis* (*M. tuberculosis*), which is an intracellular parasite. They studied the induction and production of the LL-37 in A549 epithelial cells, alveolar macrophages, neutrophils, and monocyte-derived macrophages after infection with *M. tuberculosis*. They showed that mycobacterial infection induced the expression and production of LL-37 in all of the metioned cells, including epithelial cells, but with alveolar macrophages being the most efficient [15]. In recent years, there has been the growing interest in designing new peptides derived from LL-37. Based on the antimicrobial region of LL-37, such peptides have potent antimicrobial, antibiofilm, and immunomodulatory activities. In the near future, it will likely be possible to use synthetic antimicrobial peptides in the prevention and treatment of bacterial infections [16]. The vitamin D pathway directly induces the expression of LL-37 and it contributes to increasing its expression. It is very important in the lungs. Pulmonary epithelial cells are able to change the inactive form of vitamin D to its active form. Next, the active form of vitamin D locally produces an active form of LL-37, which has not only a huge immunomodulatory role but also direct antimicrobial activity. The supplementation of vitamin D could increase the expression of LL-37 and has the potential to be critical for recovery in pulmonary infectious diseases, including those caused by *M. pneumoniae*. [17,18].

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