СРПСКА АКАДЕМИЈА НАУКА И УМЕТНОСТИ

COVID-19 ПАНДЕМИЈА:

ПОРУКЕ, НОВА САЗНАЊА И ДИЛЕМЕ

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MODULATION OF AUTOPHAGY BY SARS-CoV-2 PROTEINS

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A b s t r a c t .- Autophagy is a homeostatic lysosome-dependent catabolic process that eliminates damaged organelles, dysfunctional proteins, and macromolecular aggregates. Autophagy plays an important role in host response to viral infection as it enables degradation of viruses in autophagolysosomes and regulates innate and adaptive immunity. However, some viruses, including SARS-CoV-2, have evolved a variety of mechanisms to avoid autophagic degradation and use it for their own benefit. The aim of this study is to investigate the impact of the individual SARS-CoV-2 proteins (M, E, N, NSP4, NSP5, NSP6, NSP7, NSP8, NSP10, NSP12, NSP14, and NSP15) on autophagy in human lung epithelial cells by analyzing the expression of autophagy-related proteins, LC3-II, p62, and beclin1. The immunoblot analysis revealed that intracellular expression of non-structural proteins NSP4, NSP6, and NSP8 increased the levels of autophagy markers LC3-II and beclin-1, while the structural N protein and non-structural proteins NSP5, NSP10, and NSP15, reduced the degradation of autophagy-selective target p62. These data indicate that some SARS-CoV-2 proteins induce autophagic response, while others block its completion, thus providing grounds for further investigation of the complex interaction between the virus and the autophagic pathway.

Keywords: SARS-CoV-2, autophagy, lung epithelial cells

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1. INTRODUCTION

COVID-19 is a fast-spreading respiratory disease with worldwide devastating consequences on health, society, and economy. It is caused by a novel enveloped, positive-sense single-stranded RNA (+ssRNA) betacoronavirus SARS-CoV-2. SARS-CoV-2, like other coronaviruses, possess the largest known +ssRNA which encodes structural proteins – surface (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, as well as accessory proteins involved in virus pathogenesis (1). However, two thirds of SARS-CoV-2 genome comprise genes referred to as Open Reading Frame 1a and 1b (ORF1a/b), which encode 16 non-structural proteins (NSP) to form coronavirus replicase complex (1). NSP are responsible for viral transcription, replication, proteolytic processing, suppression of host immune responses, and are thought to play the main role in virus survival (1).

While SARS-CoV-2 usually manifests with mild cold-like symptomatology, approx. 15% of cases develop severe life-threatening lung injury accompanied by a systemic inflammatory reaction. It is dysregulated hyperinflammation in response to viral infection, rather than viral replication, that causes tissue injury in these cases (2, 3). This pathology is characterized by intense, rapid stimulation of the innate immune response that triggers activation of the Nod-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome pathway, and activation of toll-like receptors (TLR), mainly TLR-7 in endosomes, and the release of the interleukin (IL)-1β, IL-18, IL-6, IL-12, TNF-α and other proinflammatory cytokines (2, 3). TNF-α and IFN-γ have the most important role in organ damage during SARS-CoV-2 infection by causing inflammatory cell death pyroptosis and necroptosis (4). Although SARS-CoV-2-triggered hyperinflammatory response appears to be a major cause of multiple organ failure and death in infected patients, its cellular and molecular mechanisms are incompletely defined, and the therapeutic approaches to combat its detrimental effects are urgently needed.

Autophagy is an evolutionarily conserved mechanism for autodigestion/recycling of cytoplasmic macromolecules and damaged organelles, involved in the maintenance of cellular and organismal homeostasis, including that of the immune system (5). In the process of macroautophagy (hereinafter autophagy), different cytoplasmic components labelled by cargo receptors, such as p62, are encompassed by a double membrane vesicle, autophagosome, which merges with lysosome to form an autophagolysosome, where the cellular content, including p62, is degraded. Autophagy proceeds through several stages that are controlled by various ATG (autophagy-related) proteins and signaling molecules (5). It is activated both transcriptionally, through the expression of ATG genes, and by sequentially regulated post-translational modifications of ATG proteins such as autophagosome formation-initiating molecule Beclin-1 (mammalian orthologue of ATG6) and autophagosome marker microtubule-associated protein 1

light chain 3 (LC3) (6). In addition to serving as a protective mechanism against energy stress and oxidative damage (5, 6), autophagy is involved in antiviral response by directly eliminating viruses (xenophagy) in autophagolysosomes, as well as by regulating innate and adaptive immune response to viral infections (7). While microbial products and proinflammatory cytokines induce autophagy, autophagy in turn can limit inflammation by removing damaged mitochondria and blocking the inflammasome activity (8). Ideally, host autophagy should be optimized to enable prompt innate immune response in the beginning, while blocking excessive inflammation in the late phases of the infection, thus preventing tissue damage. However, this fine-tuning of autophagic response might be perturbed by viruses, which have evolved mechanisms to evade or harness autophagy to ensure effective replication, thus leading to early virus spreading followed by hyperinflammation and subsequent tissue damage.

Several structural and non-structural proteins of different SARS viruses, including SARS-CoV-2-related, were reported to modulate autophagy (11). Accordingly, recent preliminary findings suggest that SARS-CoV-2 can suppress autophagy in bronchial epithelial cells (12), while pharmacological inhibition of autophagy blocks SARS-CoV-2 cytopathogenic effect in Vero-E6 cells (13). Different SARS CoV-2 proteins are apparently involved in autophagy modulation to promote virus survival and replication. NSP15 reduces the formation of autophagosomes, while ORF3a and ORF7b cause autophagosome accumulation by blocking autophagic completion (flux) through various mechanisms including inhibition of autophagosome-lysosome fusion (ORF3a) and reduction of lysosome acidity (ORF7b) (14, 15). Investigating the effects of individual SARS-CoV-2 proteins on autophagy in relevant cell types remains important, as it could provides new insights on how to combat dysregulated immune response and tissue damage in COVID-19.

In the present study, we analyzed the effect of three structural (M, E, and N) and nine nonstructural proteins (NSP4, NSP5, NSP6, NSP7, NSP8, NSP10, NSP12, NSP14, and NSP15) on three autophagy-related molecules, LC3-II, beclin-1, and p62 in human lung epithelial cell line.

2. MATERIALS AND METHODS

2.1 Cell culture

The human large lung carcinoma cell line H460 (European Collection of Animal Cell Cultures, Salisbury, UK) was a kind gift from dr Milica Pešić (Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade, Serbia). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and 20% O_2, in a HEPES-buffered RPMI

1640 cell culture medium with L-glutamine, supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate and an antibiotic/antimycotic (all from GE Healthcare, Chicago, IL).

2.2 Cell transfection

Control (empty) plasmid pTwist-CMV-Puro 6 and plasmids encoding 3 structural (M, E, and N) proteins and nine nonstructural proteins (NSP4, NSP5, NSP6, NSP7, NSP8, NSP10, NSP12, NSP14, and NSP15) were transfected by electroporation into H460 cells. The transfection was performed using the SF Cell Line 4D-Nucleofector V Kit and 4D-Nucleofector (Lonza, Basel, Switzerland), according to the manufacturer's instructions. After transfection, the cells were rested for 24 h before being used in experiments.

2.3 Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS and protease/phosphatase inhibitor cocktail; all from Merck, Darmstadt, Germany), stored on ice for 30 min, centrifuged at 14000 g for 15 min at 4°C, and the supernatants were collected. Equal protein amounts from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Rabbit anti-human antibodies against LC3B (LC3B; #2775), beclin-1 (#3495) and p62 (NBP-1-48320; Novus Biologicals, Littleton, CO) were used as primary antibodies. Peroxidase-conjugated goat anti-rabbit IgG (11-035-144, Jackson ImmunoResearch, West Grove, PA) and goat anti-mouse IgG (H+L) (115-005-003, Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies, and specific protein bands were visualized by enhanced chemiluminescence using ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA). The intensity of protein bands was measured by densitometry using Image Lab software (Bio-Rad, Hercules, CA).

3. RESULTS AND DISCUSSION

We analyzed the effects of the intracellular expression of SARS-CoV2-encoded proteins on autophagy by examining the level of lipidated, autophagosome-associated LC3 (known as LC3-II), beclin-1, which is involved in the initiation of autophagosome formation, and autophagy cargo receptor and selective substrate p62. The immunoblot analysis revealed that the levels of LC3-II and beclin-1 were increased in H460 cells expressing N, NSP5, NSP10, and NSP15, while the expression of NSP4, NSP6, and NSP8 increased the intracellular levels

of p62 (Figure 1). The ability of NSP4, NSP6, and NSP8 to increase the levels of p62, which is selectively degraded in autolysosomes, suggests that these viral proteins might block autophagic degradation. Accordingly, the blockade of autophagic flux has previously been reported for E, M, ORF3a, ORF7a, ORF3b, and NSP6 (14, 15). On the other hand, the increase in autophagosome marker LC3-II and beclin-1, the member of autophagosome initiation complex, indicate that N, NSP5, NSP10, and NSP15 might stimulate autophagosome formation. Taken together, our data suggest a complex modulation of autophagy by SARS-CoV-2, where some proteins increase the formation of autophagosomes, while other block their maturation into autolysosomes, thus preventing autophagy completion. Such strategy would presumably enable SARS-CoV-2 to utilize double membrane autophagosomes as a scaffold for replication, while escaping subsequent degradation in autolysosomes

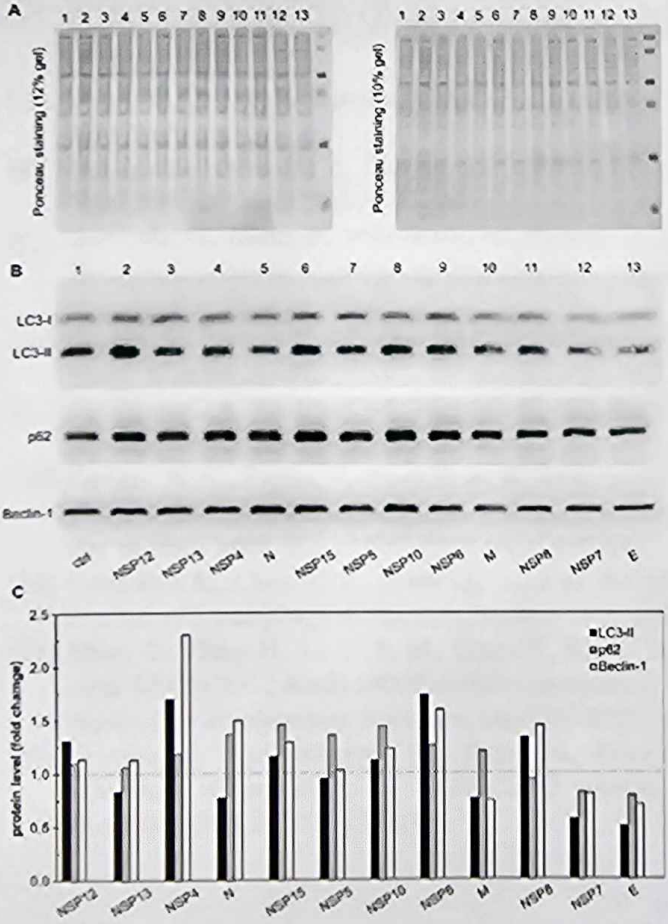

Figure 1. Modulation of autophagy by SARS-CoV-2 proteins in H460 lung epithelial cells. (A-C) H460 cells were transfected with plasmids encoding various SARS-CoV-2 proteins (the expression of SARS-CoV-2 proteins was confirmed by anti-His tag immunoblot – not shown). (A) Ponceau-S staining of proteins after electrophoresis on 12% and 10% acrylamide gels (for the detection of LC3-I/II and beclin-1/p62, respectively). (B) Immunoblot analysis of LC3-I/II, beclin-1, and p62 in cell lysates from H460 cells expressing various SARS-CoV-2 proteins. (C) Densitometry analysis of specific protein signals relative to total protein content evaluated by Ponceau-S staining in (A). The protein expression in control cells (ctrl) transfected with empty vector was arbitrarily set to 1 (dashed line).

4. CONCLUSION

The present study offers novel insights into the complex modulation of autophagic pathway by individual SARS-CoV-2 proteins. Having in mind the important role of autophagy in modulating viral replication and immune response, further investigation of the interaction between SARS-CoV-2 and autophagy, as well as the underlying mechanisms might provide means for developing autophagy-based therapeutic approaches to combat COVID-19 and the associated hyperinflammatory syndrome.

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МОДУЛАЦИЈА АУТОФАГИЈЕ SARS-COV-2 ПРОТЕИНИМА

Сажетак

Аутофагија је лизозомски посредован хомеостатски катаболички процес током којег долази до елиминисања оштећених органела, дисфункционалних протеина и макромолекуларних комплекса. Аутофагија игра важну улогу у одговору домаћина на вирусну инфекцију јер омогућава деградацију вируса у аутофаголизозомима и регулише урођени и стечени имунитет. Међутим, неки вируси, укључујући и SARS-CoV-2, су развили различите механизме како би избегли деградацију која се дешава током процеса аутофагије и подредили је у своју корист. Ова студија има за циљ да испита утицај појединачних SARS-CoV-2 протеина (M, E, N, NSP4, NSP5, NSP6, NSP7, NSP8, NSP10, NSP12, NSP14 и NSP15) на процес аутофагије који се одвија у ћелијама респираторног епитела код људи анализом експресије протеина повезаних са аутофагијом, LC3-II, p62, и беклин 1. Имуноблот анализа је показала да је унутарћелијска експресија неструктурних протеина NSP4, NSP6 и NSP8 повећала нивое експресије маркера аутофагије LC3-II и беклин-1, док су структурни N протеин и неструктурни протеини NSP5, NSP10 и NSP15 довели до смањења деградације рецептора аутофагије p62. Ови подаци указују на то да неки SARS-CoV-2 протеини индукују аутофагни одговор, док други блокирају завршетак процеса аутофагије, чиме се ствара основа за даље истраживање комплексне интеракције између вируса и процеса аутофагије.

Кључне речи: SARS-CoV-2, аутофагија, епителне ћелије плућа

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