Klebsiella pneumoniae infection inhibits autophagy by alveolar type II epithelial cells

ZHIYUN SHI1,2, GANG LI1,2, LIN ZHANG3, MIAO MA3 and WEI JIA1,2

1Medical Experimental Center, General Hospital of Ningxia Medical University; 2Key Laboratory of Ningxia Clinical Pathogenic Microorganisms; 3Department of Laboratory, Institute of Clinical Medicine, Ningxia Medical University, Yinchuan, Ningxia 750004, P.R. China

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Abstract. To investigate the molecular mechanism underlying the interaction between autophagosomes of alveolar type II epithelial (A549) cells and Klebsiella pneumoniae, an in vitro model of K. pneumoniae-infected A549 cells was established. Western blot analysis and immunofluorescence staining were used to detect the distribution of microtubule-associated protein 1A/1B-light chain 3 (LC3) and the expression of the LC3-phosphatidylethanolamine conjugate (LC3-II). K. pneumoniae-infected A549 cells were treated with different concentrations of an autophagy inhibitor or promoter for different time periods to assess the level of autophagy. Western blot analysis and immunofluorescence staining showed that K. pneumoniae could induce autophagy by A549 cells. With an increase in bacterial concentration and time of infection, autophagy gradually increased. The autophagy inhibitor significantly downregulated, while the promoter upregulated, expression of the autophagy-related protein LC3-II. Autophagy plays an important role in the resistance of alveolar type II epithelial (A549) cells to K. pneumoniae infection.

Introduction

Klebsiella pneumoniae is a Gram-negative enteric bacillus that is a member of the Enterobacteriaceae family. In immunocompromised patients, K. pneumoniae can infect the lungs, blood, and urinary tract. In recent years, the incidence of nosocomial infections of carbapenem-resistant K. pneumoniae (CRKP) has continued to increase, resulting in exceptionally high mortality rates (1,2). In earlier studies, our group investigated the resistance mechanism and conducted homology analysis and molecular epidemiological studies of 88 clinical isolates of penicillin- and carbapenem-resistant Enterobacteriaceae (25 CRKP) collected in Ningxia, China, from 2011 to 2016. The results of these studies revealed that 100% of carbapenem-resistant K. pneumoniae were also resistant to imipenem. Furthermore, the NDM-1 and KPC-2 genes were found to code for the carbapenemase enzyme. Drug-resistant strains can pass resistant plasmids to sensitive strains of Enterobacteriaceae, although the distribution varied (3-6).

Since there have been relatively few studies of the host innate immune response to K. pneumoniae infection, the virulence and pathogenic mechanism of this species remain unclear. Autophagy is an important component of natural immunity and plays key roles in the host immune response, including inflammation, to infections of pathogenic microbes. Microorganism-mediated autophagy has been shown to clear the host cells of Salmonella sp., Pseudomonas aeruginosa, and group A streptococcus (7-11), and may contribute to the long-term survival and replication in the host cell of Brucella melitensis (12) and Mycobacterium tuberculosis (13). Previous studies have confirmed that autophagy also protects type II alveolar epithelial cells (AECIs) from M. tuberculosis infection (14) and furthermore conveys important immunomodulatory effects in the lung against microbial infections (15). For example, AECIs can rapidly phagocytize and kill invading Aspergillus fumigatus (16). The ability of AECIs to phagocytize M. tuberculosis is 60-70% greater than that of macrophages (17), suggesting that autophagosomes may play a role in the ability of K. pneumoniae to infect AECIs. In this study, human adenocarcinoma alveolar basal epithelial (A549) cells were used to study the mechanism of autophagy of K. pneumoniae in order to establish a novel approach for effective intervention and treatment of K. pneumoniae infection.

Materials and methods

Bacterial strain and cell type. The reference strain K. pneumoniae K6 (ATCC 700603) was obtained from the American Type Culture Collection and preserved in our laboratory. A549 cells are hypotriploid alveolar basal epithelial
cells that are widely used as a model of lung adenocarcinoma, as well as an in vitro model of type II pulmonary epithelial cells, were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Reagents and instruments. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and trypsin were purchased from HyClone Laboratories, Inc. Cell culture plates were obtained from Axogen Scientific, Inc. Pierce™ Immunoprecipitation Lysis Buffer and the Pierce™ Bicinchoninic Acid (BCA) kit were purchased from Thermo Fisher Scientific, Inc. An enhanced chemiluminescence kit was obtained from Beyotime Institute of Biotechnology. Lysogeny broth (LB) solid medium was obtained from Oxoid Ltd. Rapamycin and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich; Merck KGaA. Antibodies against microtubule-associated protein 1A/1B-light chain 3 (LC3) and β-actin were acquired from Abcam PLC. Alexa Fluor™ 488 goat anti-rabbit secondary antibodies against immunoglobulin G were purchased from Invitrogen; Thermo Fisher Scientific, Inc. An apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a wet electroblotting system were acquired from Bio-Rad Laboratories, Inc. A laser confocal fluorescence microscope was purchased from Olympus Corporation.

Bacterial count. Activated single K. pneumoniae colonies were inoculated on conventional LB solid medium and incubated at 37°C for 24 h. Then a typical single colony was cultivated in LB liquid medium to the logarithmic phase at 37°C while rotating at 250 rpm. The bacterial culture was diluted in sterile physiological saline to a concentration of 1.2x10^11 bacteria/ml.

Cell culture. A549 cells were inoculated into a Petri dish and cultured in a DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin under an atmosphere of 5% CO₂/95% air at 37°C. At approximately 80% confluence, the medium was discarded and the cells were washed one or two times with phosphate-buffered saline (PBS). The cells were digested by trypsin for 1-3 min by the addition of 1 ml of trypsin. Then, 3 ml of complete medium was added to terminate the digestion and the cells were transferred to a 15-ml centrifuge tube and centrifuged 300 x g for 5 min at room temperature. After the supernatant was discarded, the cells were resuspended in 3 ml of medium and passaged (1:3) in a petri dish.

Establishment of a cellular infection model. A549 cells were seeded in T25 flasks and grown to a confluence of 80-90%. Next, the cells were digested with trypsin and plated in the wells of 6-well plates at a concentration of 2x10⁴ cells, as determined with a hemocytometer. After culturing overnight, the cells were infected with bacteria at a multiplicity of infection (MOI) of 100:1, 50:1, 10:1, 5:1, or 1:1 and incubated for 3 h. Then, the medium was removed and the cells were washed three times with PBS and then incubated in complete DMEM containing gentamycin for 24 h. The resulting cells were used for immunofluorescence staining and western blot analysis.

Detection of autophagic changes to LC3 by confocal microscopy. A549 cells were cultured in DMEM containing 10% fetal bovine serum. At 80 to 90% confluence, the culture medium was removed and the cells were washed twice with PBS, fixed with 4% paraformaldehyde at room temperature for 10 min, and then washed three times with pre-chilled PBS. Following the addition of PBS containing 0.5% Triton X-100, the cell membranes were lysed on ice for 10 min. The cells were then washed three times with pre-chilled PBS-Triton X-100. PBS containing 3% bovine serum albumin (BSA) was added and blocked at room temperature for 30 min. LC3 antibody was diluted to 1:100 in PBS-1% BSA solution. After removal of the blocking solution, the primary antibody was added and incubated at room temperature for 2 h at 4°C overnight. The primary antibody was removed by washing three times with PBS-0.35% Tween-20. Fluorescent secondary antibodies were diluted to 1:400 in PBS-1% BSA solution. After removal of PBS-T, the secondary antibody was added and the samples were incubated for 1 h at room temperature in the dark. For removal of the secondary antibody, the samples were washed three times with PBS-0.35% Tween-20. Following the addition of 100 ng/ml of 4’,6-diamidino-2-phenylindole (DAPI) solution, the samples were incubated at room temperature in the dark for 10 min. Then, the DAPI solution was removed and the samples were washed three times with PBS-0.35% Tween-20. Following the addition of anti-fluorescence quenching solution, the samples were placed in the dark at 4°C or imaged using a laser confocal fluorescence microscope.

Western blot detection of the autophagic protein LC3-II. Triplicate samples of A549 cells were infected with K. pneumoniae at multiple MOIs for various times. Then, the expression levels of LC3-II and LC3-II/LC3-I ratios were detected. The BCA assay was used to detect the concentrations of proteins extracted from K. pneumoniae-infected A549 cells. After separation by vertical electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels, the proteins were transferred to polyvinylidene fluoride membranes by semi-dry electrophoration. The membranes were sealed and incubated overnight with the primary antibody. The next day, the membranes were washed and then incubated with the secondary antibody. After a final washing, the protein bands on the membranes were visualized using a gel imager. Quantity One 1-D Analysis Software (Bio-Rad Laboratories) was used to quantify the protein bands with β-actin as a reference.

Induction and inhibition of autophagy. Induction and inhibition of autophagy were assessed using four experimental groups: An infection group (Pneu), autophagy inhibition group (Pneu + 3-MA), autophagy induction group (Pneu + rapamycin), and negative control group. At a confluence of 80-90%, the A549 cells were infected at a bacteria:cell ratio of 100:1 and incubated for 3 h. After the medium was removed, the cells were washed three times with PBS and then incubated in medium containing gentamicin. At the same time, trimethylpurine (3-MA), rapamycin, purine (3-MA), and rapamycin were added to the medium at final concentrations of 5 and 10 µM, respectively. At 24, 48, and 72 h, the cells...
were stained with immunofluorescent markers and subjected to western blot analysis.

Statistical analysis. Statistical analysis was performed using SPSS software for Windows, version 15.0 (SPSS, Inc.). The data of experiments repeated three times were used for analysis and are presented as the mean ± standard deviation. One-way analysis of variance was used for comparisons between groups. The Tukey test was used to identify differences among three or more groups. At $\alpha=0.05$, a probability (p) value of <0.05 was considered statistically significant.

Results

Morphologies of K. pneumoniae-infected cells. The bacteria were added to the cell cultures at ratios of 100:1, 50:1, 10:1, 5:1, and 1:1. As shown in Fig. 1, there were no significant changes to the morphology of A549 cells.

Confocal microscopy of autophagic changes to LC3 in A549 cells. As compared with the control group of cells that were not infected with K. pneumoniae, the autophagosomes of LC3 in the infected group were significantly changed. The immunofluorescence results showed that LC3 content in autophagosomes was lowest at a bacteria:cell ratio of 10:1. As shown in Fig. 2, K. pneumoniae inhibited autophagy and weakened the resistance of A549 cells, resulting in increased necrosis and proliferation of K. pneumoniae.

Western blot detection of the autophagic protein LC3-II. The western blot results showed that LC3-II protein expression was significantly higher in infected A549 cells than in the control uninfected group and the LC3-II/LC3-I ratio was significantly increased (P<0.05), indicating that K. pneumoniae promoted autophagy. As the proportion of bacterial cells (MOI) increased, autophagy also increased. However, as shown in Fig. 3, at a bacteria:cell ratio of 10:1, the level of autophagic

Figure 1. Morphological changes to K. pneumoniae-infected A549 cells as observed under an optical microscope.

Figure 2. Confocal microscopy observation of A549 cells infected with K. pneumoniae at bacteria:cell ratios of 100:1, 50:1, 10:1, 5:1, and 1:1. LC3 autophagy changes in K. pneumoniae-infected A549 cells. The black arrow indicates the LC3 fluorescent fusion protein.
cells had relatively decreased as compared with that of the other infection groups.

**Immunofluorescence results.** The immunofluorescence results revealed significant morphological changes to the LC3 autophagosomes of infected cells as compared with those of the uninfected control cells. With the extension of time and under the action of gentamycin, the invasive abilities of *K. pneumoniae* were weakened (autophagy increased). As shown in Fig. 4, 3-MA inhibited autophagy, while rapamycin promoted autophagy.

**Western blot results.** The western blot results (Fig. 5) showed that as compared with the control uninfected group, autophagy was increased in cells infected with *K. pneumoniae*. Moreover, with time, LC3-II protein expression gradually increased. The addition of 100 nM rapamycin to induce autophagy further upregulated LC3-II protein expression. Moreover, the addition of 10 mM 3-MA to inhibit autophagy significantly downregulated LC3-II protein expression. LC3-II protein expression levels were significantly higher in the infection and autophagy induction groups as compared with the control group (P<0.05). In addition, LC3-II protein expression was significantly lower in the infection group treated with 3-MA (P<0.05).

**Discussion**

Over the past two decades, *K. pneumoniae* has surpassed *Escherichia coli* as the important pathogen isolated from patients with purulent liver abscesses, and has tended to spread globally (18). With the large number and irrational abuse of antibacterial drugs, the drug resistance rate of *K. pneumoniae* continues to increase (19). The US Centers for Disease Control and Prevention listed KrK-resistant pneumococcus pneumoniae (CRKP) as the highest urgent grade in 2013 (20). Moreover, the World Health Organization mentioned CRKP in the global drug resistance surveillance report (21). Therefore, elucidating the pathogenic mechanism underlying *K. pneumoniae* infection and finding effective control measures is of great value for the treatment and prevention of *K. pneumoniae* infection.

The role of autophagy in bacteria-infected cells has attracted increasing attention and has been shown to play an important role in host defense, especially in immune cells (22,23).
Autophagy can directly affect the immune and inflammatory responses throughout the body. For example, autophagy participates in the clearance of invading bacteria via degradation of autophagic lysosomes and also plays an effective regulatory role in the immune response against pathogen invasion (24). Autophagy and bacterial infection restrict and promote one another. In addition, microbe-mediated autophagy can help the body to clear Salmonella sp., P. aeruginosa, and group A streptococcus (7-11), thereby promoting long-term survival and replication in host cells. For example, M. tuberculosis can survive in macrophages in latent infections (25). Recently, Ato et al found that miR-129-3p can inhibit autophagy through Atg4b, thus contributing to the survival of M. tuberculosis (26). Some Shigella and Listeria species have evolved various mechanisms to disrupt the growth and survival of autophagy systems, and to destroy autophagosomes (27), while others, such as Legionella pneumophila, regulate intracellular transport and inhibit autophagosome formation (28).

LC3 is an autophagosomal membrane protein that is considered to be a specific autophagosome marker. The expression and transformation of LC3-II (LC3-II/LC3-I) are important indicators to evaluate the level of intracellular autophagy. AECIIIs serve as the first line of defense against pulmonary exposure to exogenous pathogens and play an extremely important defensive function in lung infections. Hence, in-depth studies of the molecular mechanism underlying the interactions of AECIIIs, autophagosomes, and K. pneumoniae are warranted to further elucidate the mechanism underlying the unique immune function of AECIIIs for the prevention and treatment of K. pneumoniae infection.

In the present study, human alveolar type II epithelial (A549) cells were cultured in vitro and infected with K. pneumoniae at ratios of 100:1, 50:1, 10:1, 5:1, and 1:1 to establish an infection model. The cells were then collected at 0, 24, 48, and 72 h after infection for detection of relevant indicators. At the same time, A549 cells infected with K. pneumoniae were treated with the autophagy inhibitor 3-MA and the autophagy inducer rapamycin. The immunofluorescence and western blot results showed that in A549 cells, LC3 autophagosome expression, LC3-II protein expression, and the LC3-II/LC3-I ratio were significantly increased relative to the control uninfected cells, indicating that K. pneumoniae promotes autophagy by A549 cells. As the proportion of K. pneumoniae-infected A549 cells had increased, autophagy also increased. However, at a bacteria:cell ratio of 10:1, autophagy relatively decreased. At this ratio, K. pneumoniae can inhibit autophagy and weaken the resistance of A549 cells, resulting in increased necrosis, thereby promoting the proliferation of K. pneumoniae. With prolonged infection, cell autophagy gradually increased. In response to the addition of the autophagy inducer rapamycin, LC3-II protein expression was further upregulated, which promoted the autophagic effect of bacteria in the infected cells. On the contrary, in response to the addition of the autophagy inhibitor 3-MA, LC3-II protein expression was downregulated, which inhibited the autophagic effect of bacteria. These results further confirm that K. pneumoniae can induce autophagy in A549 cells in vitro.

There were some limitations to this study that should be addressed. Due to the COVID-19 pandemic, we were unable to perform proliferation/viability assays to quantitatively assess the responses of A549 cells or to repeat the experiments in vitro and in vivo with other cell lines in order to illustrate the hypothesis of the article.

Autophagy and inflammation are hot topics in the study of infectious diseases. Recent studies have shown that Notch signaling can serve as a substrate and participates in the process of autophagy (29). Autophagy is involved in the regulation of the inflammatory response, since the upregulation and absence of autophagy are closely related to the development of infectious diseases. In future studies, our group plans to use molecular biology techniques, both in vitro and in vivo, to further study autophagy-related targets and related molecular signaling pathways involved with inflammation. The results of the present study provide theoretical and experimental evidence of the pathogenic mechanism for the prevention and treatment of K. pneumoniae infection.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZS wrote the manuscript and performed western blot analysis. GL and LZ were responsible for the cell culture and transfection. MM and WJ contributed to the analysis of the observation indexes. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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