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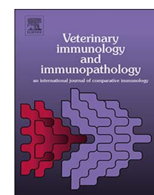

Research paper

A ROS-dependent and Caspase-3-mediated apoptosis in sheep bronchial epithelial cells in response to *Mycoplasma Ovipneumoniae* infections

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A R T I C L E I N F O

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A B S T R A C T

Mycoplasma Ovipneumoniae (*M. ovipneumoniae*) is a primary etiological agent of enzootic pneumonia in sheep and goats. It can enter and colonize ovine respiratory epithelial cells to establish an infection, which leads a serious cell death of epithelial cells. However, the nature of the interaction between pathogen of *M. ovipneumoniae* and host cells in the cell injury is currently not well understood. In this study, we investigated the epithelial cell apoptosis caused by an infection of *M. ovipneumoniae* in sheep primary air-liquid interface (ALI) epithelial cultures. The results showed that *M. ovipneumoniae* could specifically bind to ciliated cells at early stage of infection. Flow cytometric analysis demonstrated that an infection of *M. ovipneumoniae* induced a time-dependent cell apoptotic cell death, accompanied with an increased production of extracellular nitric oxide (NO), intracellular reactive oxygen species (ROS) production and activation of caspase-3 signaling in sheep bronchial epithelial cells. The induced cell apoptosis was further confirmed by a transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) assay. Interestingly, the *M. ovipneumoniae*-induced apoptosis and activation of caspase-3 were correlated with the production of ROS but not NO. Mechanistically, *M. ovipneumoniae*-induced cell apoptosis was mediated by a mechanism by increasing the expression of phosphorylation of p38 and pro-apoptotic proteins, and activating caspase-3, caspase-8 and poly ADP-ribose polymerase (PARP) cleavage. These results suggest a ROS-dependent and caspase-3-mediated cell apoptosis in sheep bronchial epithelial cells in response to *M. ovipneumoniae* infections.

1. Introduction

Mycoplasma Ovipneumoniae (*M. ovipneumoniae*) is an etiological agent of enzootic pneumonia of ovine, which causes considerable economic losses in ovine industry worldwide. This tiny organism is able to bind to the cilia of epithelial cells in airways and elicit the dysfunction of cilia, leading to impaired ciliary clearance (Besser et al., 2013; Buddle et al., 1984; Niang et al., 1998). Previous studies have demonstrated that *M. pneumoniae* and other *Mycoplasma* can cause an efflux of potassium, damage of mitochondria, release of mitochondria DNA, increase of ROS production and intracellular calcium, which are involved in an activation of caspases signaling. Functionally, these signals are able to target components involved in regulations of both innate and adaptive immune responses (Damte et al., 2011; Hwang et al., 2008; Muneta et al., 2008; Woolley et al., 2012; Xu et al., 2013).

Apoptosis is an essential process underlying multicellular organism development and function, which also plays a crucial role in the regulation of the immune response upon an external insult. A compelling body of evidence suggests that the metabolic products of mycoplasma cells could induce significant oxidative damage. However, under pathological circumstances, oxidative stress caused by excessive oxygen free-radicals may lead to cell injury by mechanisms involved in mitochondrial dysfunction and reduction of activities of antioxidant enzymes (Choi et al., 2012; Li et al., 2016; Sun et al., 2008). In this context, both nitric oxide (NO) and reactive oxygen species (ROS) have been implicated in the process of cell apoptosis (Blaser et al., 2016; Liu et al., 2015).

The colonization on epithelia of respiratory tract is a key step for establishment of infections for many respiratory pathogens, including mycoplasma infections (Evans and Koo, 2009; Fahy and Dickey, 2010; Johnson, 2011; Matsui et al., 1998). With respect to a mycoplasma infection, previous colonization models of *M. pneumoniae* infection

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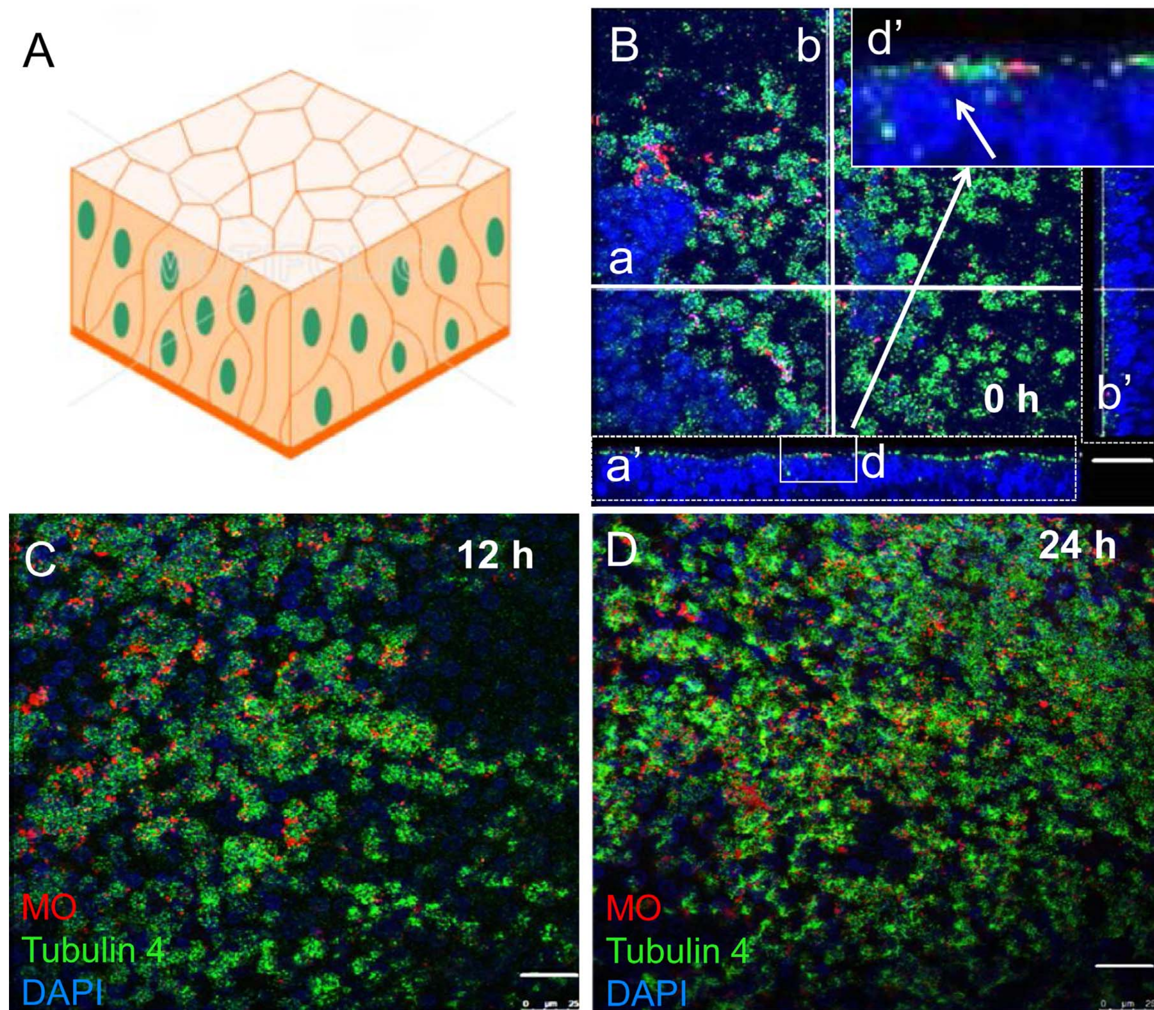

Fig. 1. M. ovipneumoniae binds to ciliated cells of primary of ALI sheep bronchial epithelia in vitro.6-week well-differentiated epithelial cells were infected apically with *M. ovipneumoniae*. The culture was stained with antibody against *M. ovipneumonias* bacteria (red) and anti-tubulin 4 antibody (green). A, a diagram showed a model of epithelial layer of the ALI cultures with pseudostratified epithelium. B, a representative image of confocal microscopy for ALI culture after binding of *M. ovipneumonias* for 20 min and cultured for additional 0 h lines a and b indicated two crossed scanned sections, and a' and b' were the respective stacked images of a and b sections. d' in B was the enlarged inset of selected area d in stacked image a, which showed a co-localized *M. ovipneumonias* and cilia of cells (yellow fluorescence, produced by superimposed green and red signal, arrow). C, a representative image of confocal microscopy for ALI culture after binding of *M. ovipneumonias* for 20 min and cultured for additional 12 h. D, a representative image of confocal microscopy for ALI culture after binding of *M. ovipneumonias* for 20 min and cultured for additional 24 h. Reconstructed LSCM (laser scanning confocal microscope) image stacks indicated the presence of cilia (green) and *M. ovipneumoniae* (red). Bars in B-D: 50mm.

employed with submerged organ and/or tissue cultures have contributed to our current understanding of interactions of pathogen-host cells, however these models are limited in their ability to accurately reflect the environment of the airway mucosa and functions of ciliated cells of respiratory epithelial cells (Krause and Chen, 1988; Lipman and Clyde, 1969; Lipman et al., 1969; Matsui et al., 1998; Powell et al., 1976; Wilson and Collier, 1976).

Our previous studies have demonstrated an induction of apoptotic cell death and a MyD88-dependent TLR signaling of sheep airway epithelial cells in response to *M. ovipneumoniae* infection using a fully differentiated ALI culture model generated with primary sheep bronchial epithelial cells (Li et al., 2016; Xue et al., 2015). In order to further characterize mechanisms underpinning immune responses sheep airway epithelial cells induced by an *M. ovipneumoniae* infection, we thus extend to interrogate the apoptotic cell death epithelial cells caused by *M. ovipneumoniae* infections using ALI cultures of sheep airway epithelial cells. Our results revealed that *M. ovipneumoniae* could interact with sheep epithelial cells by binding to ciliated cells of ALI epithelial cells, induce the production of ROS and cell apoptosis that accompanied with an increased caspase activity in these cells.

2. Materials and methods

2.1. Propagation of M. ovipneumoniae

The *M. ovipneumoniae* Queensland Strain Y98 (Jones et al., 1976) was purchased from the China Institute of Veterinary Drug Control (Beijing, China). The mycoplasma bacterial strain was cultured and propagated in a mycoplasma broth containing mycoplasma broth base CM403, supplement-G SR59 (OXOID, Hampshire, UK), 0.5% glucose, and 0.002% phenol red at 37 °C in 5% CO_2 (Jones et al., 1976). The titer of *M. ovipneumoniae* culture was determined by colony-forming assay and presented as the colony-forming unit (CFU)/mL (Hardy et al., 2002).

2.2. In vitro ALI culture of sheep bronchial epithelia and infection

This study was approved by the ethics committee for use and care of animals at Ningxia University. The generation of ALI cultures of sheep bronchial epithelial cells were derived from slaughter-house material and described in our previous study (Xue et al., 2015). For infection, 6-weeks ALI cultures were infected with *M. ovipneumoniae* by applying

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