Pneumolysin induces cellular senescence by increasing ROS production and activation of MAPK/NF-κB signal pathway in glial cells

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ABSTRACT

Senescence is an irreversible proliferation arrest that is induced by various stress stimuli including genotoxin. Pneumolysin (PLY) is a pathogenicity factor unique to *Streptococcus pneumoniae* that is important in pneumococcal-induced diseases such as otitis media, meningitis and pneumonia. However, the cell fate response to the toxin is mechanistically unclear. We investigated the effect of PLY on cellular senescence in BV-2 microglial cells. Exposure to PLY resulted in changes in the expression of phospho-p53, p21, p16, pRb and CDK2 and increased the number of senescence associated β-gal positive cells. PLY-treatment also increased PAI-1 expression and cell proliferation arrest in concentration- and time-dependent manners. PLY induced NF-κB activation and phosphorylation of SIRT-1, ERK1/2, JNK, and p38 MAPK. In addition, PLY increased the production of reactive oxygen species. Overall, the results suggest that PLY regulates microglial cellular senescence by enhancing production of reactive oxygen species, activation of MAPK and NF-κB, and phosphorylation of SIRT-1.

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1. Introduction

Cellular senescence is a state of irreversible arrest of cell growth that is accompanied by characteristic changes in cell function, morphology, and expression of various genes in response to diverse stresses including oxidative stress, ionizing radiation, and environmental toxins (Kuilman et al., 2010). Senescent growth arrest is the result of activation of the p53/p21 and p16-pRb tumor suppressor pathways which are critical in the regulation of cellular senescence (Campisi and d'Adda di Fagagna, 2007). It is also widely accepted that senescent cells are characterized by morphological alterations such as enlargement of cells and expression of several biomarkers including senescence-associated-β-galactosidase (SA-β-Gal), senescence-associated heterochromatin foci (SAHF), and plasminogen activator inhibitor 1 (PAI-1) (Kuilman and Peeper, 2009; Kurz et al., 2000).

Cellular senescence has been suggested to be related to various diseases and pathological conditions (Muller, 2009). Some types of senescence could result from damage by exposure to pathogens. *Escherichia coli* producing colibactin induces cellular senescence (Secher et al., 2013). Additionally, bacterial genotoxins such as cytolethal distending toxin and typhoid toxin, induce senescence in various cells including those of haematopoietic lineage (Grasso and Frisan, 2015).

The central nervous system (CNS) including the brain and spinal cord contains two kinds of cells, glial cells and neurons. The majority of the cells making up the brain are glial cells that influence numerous functions in the CNS (Perea and Araque, 2010). Microglial cells comprise about 10% of the total glial population in CNS parenchyma and constitute the main phagocytic population of CNS (Perry, 1998). Microglia is very sensitive to changes in their microenvironment and expresses a large variety and number of receptors, making them a relevant model to examine the effects of stimuli on CNS function. Microglia is also considered as a key cell in the initial innate immune response against CNS infections. Increasing evidence has shown the close relationship between CNS infections and senescence (Licastro and Porcellini, 2016). Additionally, it has been suggested that microglia senescence results in

functional abnormality in microglia and incorrect responses to a specific stimulus, leading to neurodegeneration (Sawada et al., 2008; Conde and Streit, 2006). However, the mechanisms underlying cellular senescence in the brain as well as how environmental factors may affect microglia function remain unclear.

Increasing evidence indicates that many pathological changes in senescent brain cells are associated with low-level chronic inflammation (Yankner et al., 2008). These senescent cells could contribute to neuroinflammation and subsequent neurodegenerative process by secreting pro-inflammatory mediators (Magistretti, 2006). Among senescent brain cells, microglial senescence might be driven by multiple factors including microbiological infection leading to altered gene expression that drives neuropathology and severe clinical morbidity. Thus, characterization of microglial senescence could provide the basis for new therapeutic targets to prevent and treat various age-related neurodegenerative diseases.

Streptococcus pneumoniae is one of the most significant human pathogens. The bacteria can cross the blood-brain barrier (Kim, 2008). The resulting barrier dysfunction is due to the cytotoxicity of pneumolysin (PLY) released by pneumococci (Zysk et al., 2001). PLY is found in clinically relevant isolates of pneumococcal meningitis and is one of the most important virulence factors of *S. pneumoniae* (Hirst et al., 2004). PLY is a member of cholesterol-dependent cytolysin family, which is oligomerized in the membrane of target cells to form a large ring-shaped transmembrane pore (Kadioglu et al., 2008). Formation of PLY pores induces cytoplasmic blebbing, mitochondrial swelling, and ultimately results in cell death (Braun et al., 2007). In addition, we and others have demonstrated that high concentrations of PLY induced microglial cell death (Braun et al., 2002; Kim et al., 2015). The choice of cell fate between apoptosis and senescence depends on various factors such as stimulus dose and damaging signals (Erol, 2011). It is possible that microglial cell senescence could be induced in response to extrinsic stimuli such as PLY at low concentration without causing apoptosis. Therefore, it will be interesting to determine whether PLY induces senescence in microglial cells.

For the present study, we used murine microglial cells (BV-2) as an in vitro model to investigate the effect of PLY on glial cell senescence, because it is known that BV-2 cells are the most frequently used substitute for primary microglia and clearly superior to primary microglia (Henn et al., 2009). In this study, we investigated the molecular mechanisms of cellular senescence in PLY-treated BV-2 microglial cells. PLY-induced cellular senescence was associated with the regulation of production of reactive oxygen species (ROS), and activation of the mitogen-activated protein kinase (MAPK), sirtuin-1 (SIRT-1), and nuclear factor-kappa B (NF-κB) signaling pathways.

2. Materials and methods

2.1. Reagents and cell cultures

Unless otherwise mentioned, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin G, and streptomycin were obtained from Life Technologies Inc. (Carlsbad, CA, USA). Dihydroethidium 2′7′-dichlorodihydro-fluorescein diacetate (H₂DCFDA) was obtained from Molecular Probes (Eugene, OR, USA). PAI-1, phosphor-p53, p53, p21, p16, CDK2, SIRT-1, phosphor-Rb, phosphor-SIRT-1, p65, ERK1/2, phosphor-ERK1/2, p38, phosphor-p38, JNK, phosphor-JNK and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). (4,5-Dimethylthiazolyl-2)-2,5-diphenylte trazolium bromide (MTT), 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), N-acetylcysteine (NAC) were purchased from Sigma Chemical Co.

The BV-2 mouse microglial cell line was kindly provided by Prof. Choon-gon Jang, Sungkyunkwan University. C6 rat glial cell and HMC3 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were proliferated in DMEM medium supplemented with 10% heat-inactivated FBS and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) in an atmosphere containing 5% CO₂ incubator at 37° C. The HMC3 cells were cultured in Eagle's minimum essential medium containing 10% FBS with 50 U/ml penicillin G sodium 50 μg/ml streptomycin sulfate, and incubated at 37 °C.

2.2. Purification of recombinant PLY

The purification of recombinant PLY was performed as described previously (Kim et al., 2015). Briefly, the PLY gene was amplified by PCR with primers of PLY (F: 5′-GGGCCCGGATCCGATGGCA AATA AAGC AGTAAATGAC-3′, R: 5′-GGCCCGCTCGAGCTAGTCATTTTC-TACCTTATCCTC-3′) that incorporated *Bam*HI and *Xho*I restriction enzyme sites. The PCR products were digested with *Bam*HI and *Xho*I enzymes and cloned into plasmid pET32b(+). After transformation, *E. coli* BL21 pET32b-PLY was grown in LB medium supplemented with kanamycin (20 μg/ml) at 37 °C in a shaking incubator until an OD600 nm of 0.6–0.8. The protein was overexpressed by providing isopropyl β-D-1-thiogalactopyranoside (final concentration of 0.5 mM) to the culture. The bacteria were incubated at 25 °C in a shaking incubator overnight. The bacteria were harvested by centrifugation at 4000 rpm for 15 min at 4 °C. The pellet was resuspended in 10 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The suspension was frozen and thawed three times, sonicated, and centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was used for histidine tagged PLY protein purification using a Ni-NTA column. The detoxified derivative of pneumolysin, pneumolysin L460D, was kindly provided by Prof. James C. Paton, University of Adelaide.

2.3. Assessment of cell viability

Glial cells (BV-2, HMC3 and C6) were seeded at a concentration of 5×10^4 cells/well in 96-well tissue culture plates (Nunc, Roskilde, Denmark) and incubated with different concentrations of PLY (100, 250, 500, 1000 ng/ml) for 12 h or 24 h. After treatment, cell viability was assessed by measurement of released lactated dehydrogenease (LDH), using the CytoTox96 LDH-release kit from Promega Co. (Fitchburg, WI, USA) according to the manufacturer's instructions. The percentage of LDH release was calculated using the following formula.

$$\text{Percentage of LDH release} = 100 \times \frac{(\text{experimental LDH release} - \text{spontaneous LDH release})}{(\text{maximal LDH release} - \text{spontaneous LDH release})}$$