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Clarithromycin suppresses induction of monocyte chemoattractant protein-1 and matrix metalloproteinase-9 and improves pathological changes in the lungs and heart of mice infected with influenza A virus

Etsuhisa Takahashi, Irene L. Indalao, Takako Sawabuchi, Keiko Mizuno, Satoko Sakai, Takashi Kimoto, Hyejin Kim, Hiroshi Kido*

Division of Enzyme Chemistry, Institute for Enzyme Research, Tokushima University, Kuramoto-cho 3-18-15, Tokushima 770-8503, Japan

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Abbreviations:
BBB
blood-brain barrier
IAV
influenza A virus
MC
methyl cellulose
MOF
multiple organ failure
pfu
plaque-forming units
PID
post-infection day
PR8
influenza A/Puerto Rico/8/34(H1N1)

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ABSTRACT

The influenza A virus (IAV)–cytokine–trypsin/matrix metalloproteinase-9 (MMP-9) cycle is one of the important mechanisms of multiple organ failure in severe influenza. Clarithromycin, a macrolide antibiotic, has immune modulatory and anti-inflammatory effects. We analyzed the effects of clarithromycin on the induction of chemokines, cytokines, MMP-9, trypsin, vascular hyper-permeability and inflammatory aggravation in mice with IAV infection. IAV/Puerto Rico/8/34(H1N1) infection increased the levels of monocyte chemoattractant protein-1 (MCP-1) and cytokines in serum, and MMP-9 and trypsin in serum and/or the lungs and heart. Clarithromycin significantly suppressed the induction of serum MCP-1 and MMP-9 and vascular hyperpermeability in these organs in the early phase of infection, but did not suppress the induction of trypsin, IL-6 or IFN-γ. Histopathological examination showed that clarithromycin tended to reduce inflammatory cell accumulation in the lungs and heart. These results suggest that clarithromycin suppresses infection-related inflammation and reduces vascular hyperpermeability by suppressing the induction of MCP-1 and MMP-9.

1. Introduction

Influenza A virus (IAV) is the most common infectious pathogen in humans and causes significant morbidity and mortality, particularly in infants and the elderly population [1,2]. Multiple organ failure (MOF) with vascular hyperpermeability is reported in the progressive stage of seasonal influenza virus pneumonia, particularly in patients with underlying risk factors [3] and is also common in infection with the highly pathogenic avian influenza viruses [4]. We reported previously that the "influenza–cytokine–trypsin/matrix metalloprotease-9 (MMP-9)" cycle is one of the key pathogenic mechanisms that interact with IAV multiplication, vascular hyperpermeability and MOF in severe influenza [5–8].

Marked increases in the levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) (i.e., cytokine storm) affect cell adhesion, permeability, apoptosis, mitochondrial energy metabolism and reactive oxygen species, and can potentially result in vascular dysfunction, hyperpermeability and MOF [9,10]. These cytokines upregulate cellular trypsin [5–8] and MMP-9 through the activation of nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) [11,12]. Induced and secreted trypsin potentiates viral entry and replication in various organs and vascular endothelial cells, because IAV has no viral hemagglutinin processing protease in its genes and post-transcriptional hemagglutinin cleavage by cellular trypsin-type proteases is indispensable for viral membrane fusion activity, virus entry into cells and multiple replication

* Corresponding author.
E-mail address: kido@tokushima-u.ac.jp (H. Kido).

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cycles [7,13–15]. In addition, secreted trypsin induces cytokine release via proteinase-activated receptor (PAR)-2 [16] and also plays a role in blood-brain barrier (BBB) destruction by increasing intracellular calcium concentrations and loss of tight-junction protein, zonula occludens-1, via PAR-2 [5]. Furthermore, the secreted trypsin efficiently converts proMMPs to active MMPs [18,19]. Among the activated MMPs, upregulation of MMP-9 promotes endothelial hyperpermeability and enhances inflammatory cell migration through the degradation of type IV collagen in the vascular basement membrane [17,20].

There is growing evidence that macrolides with a 14-member ring, such as erythromycin and clarithromycin, are bifunctional drugs; they have an anti-inflammatory [21–24] and immune-modulatory properties [25–28], in addition to their antimicrobial effects. We reported previously that clarithromycin enhances secretory IgA production and its neutralizing activities through the induction of IgA class switching recombination and upregulation of B-cell-activating factor of the tumor necrosis factor family molecule in mucosal dendritic cells as an adjuvant or immunomodulatory compound in IAV-infected mice [28]. Thus, oral administration of clarithromycin in pediatric patients with influenza enhanced anti-viral mucus and systemic immunity and reduced the re-infection rate in the subsequent year [27]. While previous studies described the immunomodulatory effects of clarithromycin [26–28], the anti-inflammatory effects of clarithromycin on vascular hyperpermeability in the pathogenesis of MOF in severe influenza are not fully understood.

Vascular permeability is controlled by a “barrier” comprised of the micro-vascular wall, which includes the endothelial glycocalyx, endothelium, basement membrane, and all accessory cells wrapped around the outer surface of the vessel [29]. Though each of these components contributes to vascular wall permeability, we focused on disruption of the basement membrane and up-regulation of MMP-9, since MMP-9 is one of the important factors in the cycle of “influenza–cytokine–trypsin/MMP-9” described above.

The present study was conducted to determine the anti-inflammatory effects of clarithromycin on the induction of MMP-9, cytokines and chemokines in serum, and MMP-9, trypsin, vascular hyperpermeability and pathological changes in the lungs and heart of mice infected with IAV.

2. Materials and methods

2.1. Animals and virus infection

Four-week-old Balb/c female mice just after weanling were purchased from Japan SLC (Shizuoka, Japan) and used in all experiments. Influenza A/Puerto Rico/8/34(H1N1) (PR8) was kindly provided by The Research Foundation for Microbial Diseases of Osaka University (Kagawa, Japan). All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996), and the study was approved by the Tokushima University Animal Research Ethics Committee (#T27-37).

Under ketamine and xylazine anesthesia, we administrated intranasally 25 plaque-forming units (pfu) of PR8 in 15 μl of saline, which induces cytokine storm but does not cause death up to post-infection day 9 (PID-9). Saline alone was instilled intranasally in the control mice. Immediately after infection, each group of animals were treated orally with a solution of clarithromycin at 150 μg/head in 100 μl methyl cellulose (MC) or MC as the vehicle every 12 h for 9 days.

2.2. Histological analysis

The isolated perfused lungs and heart of each group of animals (n = 3) were fixed with 10% buffered formalin, pH 7.2, and then embedded in paraffin. The paraffin blocks were cross-sectioned at 5 μm thickness using microtome (Leica Instruments, Nusslosh, Germany). The sections were subjected to hematoxylin-eosin staining (H&E staining). Microscopic images of the cranial lobe of the right lung and heart were acquired with an all-in-one Fluorescence Microscope (BZ-X710; Keyence Corporation, Osaka, Japan). Evans’ blue-treated tissue sections were also analyzed by a Fluorescence Microscope BZ-X710. The hypercellular areas with infiltrating inflammatory cells (shown in red) and the hypocellular areas (shown in green) in three different areas of the lung and heart of each animal were quantified by BZ-X analyzer software (Keyence Corporation), as described previously [31,32], and presented as percentages of the hypercellular area in the lung and heart section areas.

2.3. Evans’ blue treatment

To examine changes in vascular permeability in the lungs and heart during the early phase of IAV infection, each non-infected and infected mouse (n = 3) received intraperitoneal injection of 0.2% Evans’ blue in 100 μl of phosphate-buffered saline (PBS) on post-infection days (PIDs) 3, 6 and 9, as described previously [30]. One hour after the injection, the entire body was perfused through the left ventricle with 30 ml of saline, and the heart and lungs were excised. The tissues were homogenized with 500 μl of saline, and then centrifuged at 6000 × g for 20 min at 4 °C to collect the supernatant. The concentration of Evans’ blue dye in the supernatant was measured at a wavelength of 590 nm with a SPECTRA max PLUS (Molecular Devices, Sunnyvale, CA).

2.4. Western immunoblotting

The lungs and heart of each group of animals treated with clarithromycin or MC (n = 5) were homogenized with extraction buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP 40, 0.5% deoxycholate, 0.4 mM EDTA, and 0.5 mM sodium orthovanadate, and centrifuged at 12,000 × g for 20 min at 4 °C. The protein concentration of the supernatant was measured by a BCA assay kit (Thermo Scientific, Rockford, IL), according to the protocol provided by the manufacturer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10–20% gradient gels (Multigel II mini, COSMO BIO, Tokyo, Japan) under reducing conditions. Proteins separated by SDS-PAGE were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and incubated overnight with anti-MMP-9 or anti-β-actin antibodies at 4 °C. After washing, the membranes were incubated with peroxidase-conjugated anti-mouse (Invitrogen, Carlsbad, CA) or −rabbit (Sigma, St. Louis, MO) IgG antibodies for one hour at room temperature, and proteins were detected using enhanced chemiluminescence detection reagent (GE Healthcare Biosciences, Uppsala, Sweden).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Blood samples collected from the mouse heart of each group of animals treated with clarithromycin or MC (n = 5) were centrifuged at 2000 × g for 10 min at 4 °C and the serum was used for measurement of MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), IL-6, interferon-γ (IFN-γ), and TNF-α by ELISA kits (R&D Systems, Minneapolis, MN), using the method recommended by the manufacturer. The isolated lungs and heart were minced and homogenized with 30 vols of PBS, and then centrifuged at 12,000 × g for 20 min at 4 °C to collect the supernatant for measurement of MMP-9 and trypsin levels. Trypsin levels were measured with mouse Trypsin ELISA kit (MyBioSource, San Diego, CA) according to the protocol provided by the manufacturer.

2.6. Statistical analysis

The results are expressed as mean ± standard deviation (SD). Differences between groups were examined using Student’s t-test. P