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Mouse lung slices: An *ex vivo* model for the evaluation of antiviral and anti-inflammatory agents against influenza viruses

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ABSTRACT

The influenza A virus is notoriously known for its ability to cause recurrent epidemics and global pandemics. Antiviral therapy is effective when treatment is initiated within 48 h of symptom onset, and delaying treatment beyond this time frame is associated with decreased efficacy. Research on anti-inflammatory therapy to ameliorate influenza-induced inflammation is currently underway and seems important to the impact on the clinical outcome. Both antiviral and anti-inflammatory drugs with novel mechanisms of action are urgently needed. Current methods for evaluating the efficacy of anti-influenza drugs rely mostly on transformed cells and animals. Transformed cell models are distantly related to physiological and pathological conditions. Although animals are the best choices for preclinical drug testing, they are not time- or cost-efficient. In this study, we established an *ex vivo* model using mouse lung slices to evaluate both antiviral and anti-inflammatory agents against influenza virus infection. Both influenza virus PR8 (H1N1) and A/Human/Hubei/3/2005 (H3N2) can replicate efficiently in mouse lung slices and trigger significant cytokine and chemokine responses. The induction of selected cytokines and chemokines were found to have a positive correlation between *ex vivo* and *in vivo* experiments, suggesting that the *ex vivo* cultured lung slices may closely resemble the lung functionally in an *in vivo* configuration when challenged by influenza virus. Furthermore, a set of agents with known antiviral and/or anti-inflammatory activities were tested to validate the *ex vivo* model. Our results suggested that mouse lung slices provide a robust, convenient and cost-efficient model for the assessment of both antiviral and anti-inflammatory agents against influenza virus infection in one assay. This *ex vivo* model may predict the efficacy of drug candidates' antiviral and anti-inflammatory activities *in vivo*.

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1. Introduction

Influenza A virus (IAV) is still a threat to human health and poses a global concern due to its unpredictable, pandemic potential and pathogenesis. As high evolutionary rates of the influenza virus makes vaccination strategies difficult, anti-influenza drugs are crucial for the control of influenza pandemics. Antiviral therapy is generally licensed for use within 48 h of influenza illness onset, and delaying treatment is associated with decreased drug efficacy and increased morbidity and mortality (Kandun et al., 2008). Although influenza-induced pathology is still unclear, the uncontrolled immune response may be the major contributor to influenza virus-induced mortality (de Jong et al., 2006; Iwasaki and Medzhitov, 2011; Kobasa et al., 2007). Thus, studies on the modulation of the host immune response are currently underway. Several animal studies have shown that anti-inflammatory agents

Abbreviations: TNF-α, tumour necrosis factor alpha; IL-6, interleukin 6; RANTES, regulated on activation, normal T cell expressed and secreted; MIP-3α, macrophage inflammatory protein 3 alpha; IP-10, interferon-gamma induced protein 10; IL-10, interleukin 10; IL-1β, interleukin 1 beta; IFN-γ, interferon gamma; PPAR-γ, peroxisome proliferator-activated receptor gamma; EGCG, epigallocatechin gallate; CXCR3, chemokine (C-X-C motif) receptor 3; TNFR1, tumour necrosis factor alpha receptor 1; IL-1R, interleukin 1 receptor; MIP-1, macrophage inflammatory protein 1; CCR2, C-C chemokine receptor type 2; GM-CSF, Granulocyte–macrophage colony-stimulating factor; i.p., intraperitoneal; BALF, bronchoalveolar lavage fluid; RIG-I, retinoic acid-inducible gene I; TLR 7/8, toll-like receptor 7/8; TLR 3, toll-like receptor 3; MUNANA, 2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid.

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can protect mice from death against influenza infection. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) has been shown to protect 79% of mice from death against lethal influenza infection through manipulation of the PPAR-γ pathway, whereas it does not inhibit virus replication (Cloutier et al., 2012). The P38 inhibitor significantly protects mice from lethal influenza infection without affecting virus replication (Borgeling et al., 2014). Statins not only reduce the levels of LDL-cholesterol, but they also counteract the inflammatory changes associated with acute coronary syndrome and improve survival in patients with influenza. Similarly, in patients hospitalised with laboratory-confirmed seasonal influenza, statin treatment is associated with a 41% reduction in 30-day mortality (Fedson, 2013). Therefore, strategies targeting aberrant host immune responses may be good complements for existing antiviral drugs.

The routine strategies for anti-influenza drug development rely primarily on cell-based assays in primary screening followed by animal studies. However, the cost of *in vivo* studies is very high due to the use of animals and a large quantity of investigational compounds. Lung slices, which provide a bridge between single cells and whole animals, are broadly used in physiology and toxicity studies (Morin et al., 2013; Sanderson, 2011). Different from single cell lines, lung slices possess multiple cell types and preserve the physiological and functional cellular relationships within the body. Cell–cell and cell–matrix interactions result in lung slices closely resembling the morphology and functionality of the lung. Whereas the evaluation of the efficacy of a drug based on animal studies is expensive and time-consuming, the lung slice model may serve as a valuable tool for efficacy tests of compounds for the treatment of influenza. With the development of tissue slicers, which produce slices rapidly and reproducibly, an increasing number of studies have employed lung slices to explore the interaction of hosts and pathogens (Chakrabarty et al., 2007; Londt et al., 2013; Punyadarsaniya et al., 2011; Seehase et al., 2012; Van Poucke et al., 2010; Wu et al., 2010). Recently, several studies have shown that pig lung slices can support influenza virus replication (Londt et al., 2013; Van Poucke et al., 2010). Another study demonstrated that influenza virus infection can induce robust cytokine and chemokine responses in human lung slices (Wu et al., 2010). Theoretically, the three-dimensional lung slice culture system can be used to evaluate the potency of both antiviral and anti-inflammatory agents against influenza virus infection.

In the current study, we showed that influenza viruses can efficiently replicate in mouse lung slices and induce significantly elevated levels of cytokines and chemokines. A panel of antiviral and anti-inflammatory agents were tested for their antiviral activities and/or anti-inflammatory effects in the mouse lung slices. The results from the lung slice model are consistent with those from mouse studies. Our results showed that the lung slice model provides a robust, convenient and cost-economical method for the screening and evaluation of both antiviral and anti-inflammatory agents against influenza virus infection in one assay.

2. Material and methods

2.1. Virus strains and animals

6–8-week old BALB/c mice were purchased from Changsha Laboratory Animal Center (Hunan province, China) and were housed under specific-pathogen-free condition. All experiments were conducted according to the protocol approved by the Animal Care and Use Committee of Wuhan Institute of Virology, Chinese Academy of Sciences (WIVA08201201).

Mouse adapted A/PuertoRico/8/34 (H1N1) and mouse adapted A/Human/Hubei/3/2005 (H3N2) were propagated in the allantoic cavity of 10-day-old specific-pathogen-free embryonated chicken eggs for 48 h. The allantoic fluids were collected and filtered with 0.22 μm filter and stored at −80 °C. The virus strains were provided by the virus collection at Wuhan Institute of Virology, Chinese Academy of Sciences, China.

2.2. Chemicals

15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), ribavirin and the neuraminidase substrate 2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid (MUNANA) were purchased from Sigma–Aldrich. EGCG was purchased from Sichuang Weikeqi Biological Technology Co. Ltd. (China). Glycyrrhizin was purchased from Shanghai Hanxiang Biological Technology Co. Ltd. (China). The P38 pathway inhibitor SB203580, the ERK pathway inhibitor U0126 and the SAPK/JNK pathway inhibitor SP600125 were purchased from Beyotime Institute of Biotechnology (China). Oseltamivir carboxylate (GS 4071) was obtained from Toronto Research Chemicals (Canada). All compounds were initially dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich).

2.3. Preparation of the mouse lung slices

Mouse lung slices were prepared using a modification of a protocol that has been previously reported (Bauer et al., 2010). After anesthetisation by intraperitoneal injection of sodium pentobarbital (75 mg/kg), the mouse was bled through the abdominal aorta. Then, the trachea was exposed, dissected from surrounding tissues and was cannulated with an 18-gauge needle. Through the cannula, the lung was inflated with 1.3 ml of 2% low-melting agarose (BIO-RAD) dissolved in Hank's buffered saline solution (HBSS) solution. The whole animal was cooled with ice for 10 min to solidify the agarose and, thereby, the lung. Then, the lung was taken out *en bloc* from the thoracic cavity and placed in the slice culture medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, DMEM/F-12, GIBCO) at 4 °C for an additional 15 min to completely solidify the agarose. The culture medium was supplemented with 100 units/ml of penicillin, 100 μg/ml streptomycin and 250 ng/ml of amphotericin to avoid contamination. The lung lobe was afterwards dissected and cut to create a flat surface at the end of the primary bronchus. Another flat surface was cut approximately 0.8 cm from the first surface. The cube was maintained in the pre-chilled slice culture medium prior to or during the slicing. The cube was cut into slices of desired thickness using a vibratome slicer (Leica, VT1200S). Each mouse lung cube generated at least 24 250-μm slices. The slices were then transferred into a 48-well cell culture plate and covered with 250 μl of slice culture medium in each well. The medium was changed every hour at least three times before virus infection to remove cell debris.

2.4. Slice viability

The lung slice viability was assessed by bronchoconstriction and live/dead staining. The bronchoconstriction was monitored under a microscope when adding or removing 10^{-4} M acetyl-ß-methylcholine chloride (Sigma–Aldrich). The photos were taken using a Nikon Inverted Research Microscope Ti ECLIPSE. For live/dead staining experiments, the slices were incubated with Calcein AM (1 μM) and Propidium Iodide (PI, 1 μg/ml) for 20 min at room temperature. A Nikon Multiphoton Confocal Microscope A1 MP^+ was used to record the images.

2.5. Infection of the lung slices with influenza viruses

The lung slices were infected with 200 μl of 10^5 PFU/ml influenza viruses for 2 h. Virus diluent was used as a negative control.

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