Improving Pneumovirus Isolation Using a Centrifugation and AZD1480 Combined Method

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The isolation of respiratory viruses, especially from clinical specimens, often shows poor efficiency with classical cell culture methods. The lack of suitable methods to generate virus particles inhibits the development of diagnostic assays, treatments, and vaccines. We compared three inoculation methods, classical cell culture, the addition of a JAK2 inhibitor AZD1480, and centrifugation-enhanced inoculation (CEI), to replicate human respiratory syncytial virus (HRSV) and human metapneumovirus (HMPV). In addition, a combined method using AZD1480 treatment and CEI was used on throat swabs to verify that this method could increase virus isolation efficiency from human clinical specimens. Both CEI and AZD1480 treatment increased HRSV and HMPV genome replication. Also, the combined method using CEI and AZD1480 treatment enhanced virus proliferation synergistically. The combined method is particularly suited for the isolation of interferon-sensitive or slowly growing viruses from human clinical specimens.

Keywords: Virus isolation, centrifugation, AZD1480, human respiratory syncytial virus, human metapneumovirus

Introduction

The emergence of new infectious respiratory viruses has increased owing to climate change, environmental change, zoonosis, rise in human population, and globalization over the decades [1–3]. Infectious diseases caused by these viruses affect not only personal health but also whole societies, especially economies and public health systems [1, 4]. With an increase in emerging and re-emerging infectious diseases worldwide, it is crucial to identify the etiologic agents in order to prevent the spread of new infectious diseases. Currently, most laboratories employ detection methods ranging from traditional assays, including culturing, microscopy, serological assays, and PCR, to new methods such as metagenomics [5–7]. Among these methods, virus isolation using cell culture is critical to confirm pathogenicity and to analyze epidemiological findings. The isolated etiologic agents have been used to develop diagnostic assays, treatments, and vaccines.

However, it is still challenging to isolate viruses from clinical specimens using a classical cell culture method. This method is restricted by the condition of the clinical specimen and the ability of the virus to propagate in only permissive cell lines. Some viruses also require a long incubation period before the cytopathic effect (CPE) is observed [6]. Several reports have shown virus isolation approaches for replicating the enhanced respiratory virus, including the use of centrifugation-enhanced inoculation (CEI) [8], co-cultivated cells [9], interferon (IFN) inhibitors [10, 11], and well-differentiated pseudostratified human airway epithelium (HAE) [12, 13].

Human respiratory syncytial virus (HRSV) and human metapneumovirus (HMPV) that belong to the family *Pneumoviridae* are the main causes of lower respiratory illness among young children and the elderly [14, 15]. These viruses have been divided into subgroups and evolve continuously to new genotypes. However, there are no available vaccines or therapeutics, except palivizumab

prophylaxis for HRSV. One of the major hurdles in understanding the pathogenesis of these viruses is the difficulty of isolating HRSV and HMPV.

In this study, we used recombinant HRSV and HMPV expressing fluorescence in Vero cells and HRSV A2 strain in HEp-2 cells to analyze virus replication efficiency using CEI and AZD1480. AZD1480 is a novel ATP-competitive JAK2 inhibitor that blocks JAK-STAT signaling pathways involved in immunity [16]. Here, we used AZD1480 as an inhibitor of the IFN and IL-6 systems that cause antiviral response in innate immunity [10, 17]. We also compared the efficiencies of classical cell culture and the combined method of CEI and AZD1480 treatment to isolate HRSV-A, HRSV-B, and HMPV from human clinical specimens.

Materials and Methods

Cells, Viruses, and Reagents

Recombinant human respiratory syncytial virus expressing red fluorescent protein (HRSV-RFP, Korea University College of Medicine, Korea) [18], recombinant human metapneumovirus expressing green fluorescent protein (HMPV-GFP, National Institute of Infectious Diseases, Japan) [19], and HRSV A2 strain (ATCC VR-1540) were used.

Vero cells and HEp-2 cells (ATCC, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml). HRSV-RFP was propagated in Vero cells with DMEM supplemented with 2% FBS, while HMPV-GFP was propagated in Vero cells with DMEM supplemented with 2 µg/ml N-acetyl trypsin and without FBS. HRSV A2 strain was cultured using HEp-2 cells with DMEM containing 2% FBS. The IFN inhibitor, AZD1480 ((S)-5-chloro-N²-(1-(5-fluoropyrimidin-2-yl)ethyl)-N⁴-(5-methyl-1H-pyrazol-3-yl)pyrimidine-2,4-diamine, SelleckChem, USA) was dissolved in DMSO at 5 mM concentration, aliquoted, and stored in the dark at -70°C.

RNA Extraction and RT-PCR

At various time points post-infection, viral RNA was extracted from the cell cultures using Qiagen viral RNA mini kit and QiAamp 96 Virus Qiacube HT kit (Qiagen, Germany). The number of virions in the culture was estimated (genome copies/ml) using PowerChek HRSV A&B/HMV real-time PCR kit (KogenBiotech, Korea), using the standard genome copies provided in the kit. All experiments were performed in triplicate.

Classical Inoculation and Centrifugation-Enhanced Inoculation

Vero cells (2×10^5 per well) were cultured in two 24-well plates in 200 µl DMEM with 2% FBS and infected with HRSV-RFP at a multiplicity of infection (MOI) of 0.05. One plate underwent classical inoculation, which involved gentle shaking of the plate every 15 min for 2 h at 37°C in a 5% CO₂ incubator. The other plate

was centrifuged at 259 ×g for 30 min and incubated for 90 min at 37°C in a 5% CO₂ incubator. After inoculation, the media with virus were removed and 500 µl fresh DMEM with 2% FBS was added to the cells. For HMPV-GFP inoculation, the same protocol was followed using serum-free DMEM with 2 µg/ml N-acetyl trypsin. Likewise, the HRSV A2 strain was used to infect 2×10^5 HEp-2 cells using the same protocol. The cells and media were harvested 12, 24, 48, 72, 96, and 120 h post-infection. The infected cells were imaged using a fluorescence microscope (Axiovert 200, Carl ZEISS, Germany).

AZD1480 Treatment

Virus growth kinetics were determined in Vero cells infected with HRSV-RFP or HMPV-GFP at an MOI of 0.001 and grown in the presence or absence of 2 µM AZD1480. For the HRSV A2 strain, DMEM with 2% FBS and 0.25 µM AZD1480 were used. After treatment, the cells and media were harvested every 12 h. On the other hand, classical cultures were incubated at 37°C in a 5% CO₂ incubator for 2 h after infection, and the medium was substituted with media without AZD1480.

Combination of Centrifugation-Enhanced Inoculation and AZD1480 Treatment

HRSV-RFP or HMPV-GFP at MOI of 0.01 was used to infect 2×10^5 Vero cells per well in two 24-well plates. One plate was used for classical inoculation and the other plate was centrifuged, and fresh media with 2–4 µM AZD1480 was added. The viral culture was harvested 12, 24, 36, 48, 60, 72, 84, and 96 h post-infection.

Clinical Specimen Isolation

Ethical approval (approval no. 2013-08EXP-03-5C and 2014-08EXP-6C-A) for this study was obtained from the Institutional Review Board at the Korea Centers for Disease Control and Prevention (KCDC). Clinical samples were obtained from 36 sentinel hospitals located nation-wide, through the Korea Influenza and Respiratory Viruses Surveillance System (KINRESS, KCDC, Korea) [20]. Samples positive for HRSV or HMPV were selected. HRSV was isolated from clinical samples using HEp-2 cells, while Vero cells were used for isolating HMPV. Then, 100 µl of the clinical sample was inoculated by the classical or combined method, as described previously. For HMPV isolation, serum-free DMEM with 2 µg/ml N-acetyl trypsin was used. The cells and media were harvested 72 h post-infection and the viral genome copies were estimated.

Statistics

Statistical analyses were performed using GraphPad Prism version 6.07. The differences among the four methods were analyzed using 2-way analysis of variance (ANOVA). The other data were analyzed by 2-tailed unpaired *t*-test. *P* < 0.05 was considered significant in all analyses.

Fig. 1. Centrifugation-enhanced inoculation accelerated virus replication.

Vero cells were infected with (A) HRSV-RFP and (B) HMPV-GFP, and HEp-2 cells were infected with (C) HRSV A2 by the classical inoculation method (full line) and centrifugation-enhanced inoculation method (dotted line). $*p \leq 0.05$; $***p \leq 0.0001$ compared with classical method.

Results

Centrifugation-Enhanced Inoculation Accelerates Virus Replication

Recombinant human respiratory syncytial virus expressing red fluorescent protein (HRSV-RFP), recombinant human metapneumovirus expressing green fluorescent protein (HMPV-GFP), and HRSV A2 were inoculated by classical and CEI methods. Using cell culture supernatant and pellet, RNA was extracted and used for quantitative RT-PCR. All three viruses inoculated by CEI replicated more rapidly than the ones inoculated by the classical method (Fig. 1). At 12 h post-infection, the genome copies of HRSV-RFP (Fig. 1A), HMPV-GFP (Fig. 1B), and HRSV A2 (Fig. 1C) were 3.7-, 6.9-, and 6.3-fold higher, respectively, by CEI compared to classical inoculation. At 24 h post-infection, the number of HMPV-GFP genome copies was 11.3-fold higher with CEI than with classical inoculation.

In plaque morphology analysis using fluorescence

microscopy, HRSV-RFP and HMPV-GFP inoculated by CEI appeared to form large and more plaque (Figs. 3A and 3B). These results suggest that CEI enhances respiratory virus replication efficiently at an early infection time. However, there were no differences in the peaks of virus genome copies between CEI and classical inoculation, which were 8.98 and 8.89 log copies/ μL for HRSV-RFP, 7.42 and 7.30 log copies/ μL for HMPV-GFP, and 9.05 and 8.93 log copies/ μL for HRSV A2, respectively (Fig. 1). These results show the possibility that CEI could reduce virus culture time of slow-growing viruses.

IFN Inhibitor AZD1480 Increases Virus Replication

We selected AZD1480 following a screen of several IFN inhibitors and determined its cell-line-dependent inhibitory concentration (unpublished data). The optimal non-toxic concentration was identified as 0.25 μM for HEp-2 cells and 2 μM for Vero cells.

We observed that genome copies of HRSV-RFP, HMPV-GFP, and HRSV A2 were higher in the presence of

Fig. 2. AZD1480 treatment increased replication of HRSV-RFP, HMPV-GFP, and HRSV A2.

Vero cells were infected with (A) HRSV-RFP and (B) HMPV-GFP, and HEp-2 cells were infected with (C) HRSV A2 by the classical inoculation method (full line) and AZD1480 treated inoculation method (dotted line). $***p \leq 0.0001$ compared with classical method.

Fig. 3. Effect of centrifugation-enhanced inoculation and AZD1480 on HRSV-RFP and HMPV-GFP plaque formation in Vero cells. Vero cells were infected with (A) HRSV-RFP at MOI 0.05 and (B) HMPV-GFP at MOI 0.05, and the cells were observed using fluorescence microscopy according to post-infection (hr) ($\times 100$ magnification). Vero cells were infected with (C) HRSV-RFP at MOI 0.001 and (D) HMPV-GFP at MOI 0.001 with $4 \mu\text{M}$ AZD1480. The cells were treated with DMSO as a negative control. The cells were taken pictures on post-infection 6 days using fluorescence microscopy ($\times 40$ magnification).

AZD1480 than in its absence (Fig. 2). There were 6.1-, 4.2-, and 12.9-fold more genome copies of HRSV-RFP, HMPV-GFP, and HRSV A2, respectively with AZD1480 treatment at 72 h post-infection, than with negative control treatment (DMSO treatment). At 96 h post-infection, the genome copies of HRSV A2 increased dramatically to 71.8-fold higher than those obtained by the classical inoculation. The peaks for genome copies using AZD1480 treatment and classical inoculation were 10.5 and 9.85 log copies/ μL for HRSV-RFP, 8.60 and 8.11 log copies/ μL for HMPV-GFP, and 10.87 and 9.01 log copies/ μL for HRSV A2, respectively (Fig. 2).

In fluorescence microscopy analysis (Figs. 3C and 3D), AZD1480 treatment increased the plaque size of HRSV-RFP and HMPV-GFP, and show the high number of infected neighboring cells before fusion. These results

suggest that AZD1480 treatment enhances virus proliferation and spread by inhibiting signaling pathways of innate immunity. Thus, AZD1480 treatment during virus culture could improve and facilitate isolation of IFN-sensitive viruses.

Combined Inoculation Enhanced Respiratory Virus Propagation

We investigated whether combined inoculation with CEI and AZD1480 treatment would enhance virus replication even more than either of the methods alone. We observed that the combined method significantly increased genome copies of HRSV-RFP, HMPV-GFP, and HRSV A2 by 4.6-, 15.2-, and 91.2-fold, respectively at 72 h post-infection (Fig. 4 and Table 1). HRSV A2 in particular propagated dramatically in IFN-responsive HEp-2 cells with AZD1480

Fig. 4. Synergetic increase in virus replication by the combined method. The graph compares virus replication by classical inoculation (black bar), AZD1480 treatment (horizontal bar), CEI (vertical bar), and the combined method (blank bar). **p* ≤ 0.05; ***p* ≤ 0.001; ****p* ≤ 0.0001.

supplementation, while HRSV-RFP and HMPV-GFP propagated more efficiently in Vero cells with CEI than with AZD1480 treatment. This combined strategy of CEI and AZD1480 treatment could aid virus isolation from clinical specimens, which is vital for pathogen research and the development of diagnostics, vaccines, and therapeutics.

Table 1. Comparison of viral replication after application of AZD1480 treatment, CEI, and the combined method normalized against replication using the classical method.

Relative ratio	AZD1480	Centrifugation	Combined
HRSV-RFP	1.98 ± 0.18 ^a	2.66 ± 0.11 ^b	4.55 ± 0.14 ^c
HMPV-GFP	2.38 ± 0.21 ^a	7.69 ± 1.31 ^b	15.21 ± 4.77 ^a
HRSV-A2	12.87 ± 1.57 ^b	2.70 ± 0.65 ^a	91.18 ± 3.49 ^a

^a*p* ≤ 0.05; ^b*p* ≤ 0.001; ^c*p* ≤ 0.0001

Combined Inoculation Facilitates Proliferation of HMPV and HRSV from Clinical Specimens

To evaluate whether the combined inoculation with CEI and AZD1480 treatment would enhance virus isolation from clinical specimens, we used throat swabs diagnosed with HRSV and HMPV infection. A total of 46 HRSV specimens containing 26 HRSV-A and 20 HRSV-B subgroups and 48 HMPV specimens were used. Among these, viral cultures could not be detected using RT-PCR in 7 HRSV and 5 HMPV specimens. We analyzed the remaining 39 HRSV and 43 HMPV specimens by classical

Fig. 5. Isolation of HMPV and HRSV from patient specimens. The graph compares genome copy numbers of virus cultures obtained with classical or combined inoculation methods using (A) 43 HMPV (*p* = 0.0905) and (B) 39 HRSV (21 HRSV-A and 18 HRSV-B type) (*p* = 0.0341) specimens. The results were divided into four groups (I, mock > combined; II, mock < combined with copies difference < 10; III, mock < combined with copies difference > 10; IV, virus detected only in the combined method).

or combined inoculation methods (Fig. 5). The combined inoculation method significantly increased HMPV genomic copies 72 h post-infection (Fig. 5A). Thirty-eight (88.4%) of the 43 HMPV-diagnosed samples showed higher viral copies with the combined method, and the remaining five samples (group I) showed more copies with the classical inoculation method. Of the 38 samples, 25 (65.8%) of group II showed less than 10-fold, and nine (23.7%) of group III showed over 10-fold higher viral copies with the combined method. The remaining four (10.5%) of group IV showed viral copies only in the combined inoculation with no copies in the classical inoculation method. However, there was no statically difference between the combined inoculation and the classical inoculation of 43 HMPV ($p = 0.0905$) specimens.

Higher viral copies were observed for 17 (81.0%) of 21 HRSV-A and 17 (94.4%) of 18 HRSV-B samples with the combined method (Fig. 5B). In group III, the genome copies of six HRSV-A (28.6%) and eight HRSV-B (44.4%) samples with the combined method were more than log10 of those obtained by the classical inoculation at 72 h post-infection. For samples HRSV-A20 and A21, there was a 10,000-fold increase between the combined and classical methods of inoculation. In group II, there was an approximately 3 to 9-fold increase in the genome copies of 11 HRSV-A (52.4%) and four HRSV-B samples (22.2%) in HEp-2 cells with the combined method. There were only four HRSV-A samples and one HRSV-B sample in group I where the genome copies were higher with the classical method. Notably, only five HRSV-B (27.8%) samples in group IV showed virus genome copies and CPE with the combined method. Unlike HMPV specimens, the combined inoculation of HRSV showed a significant difference with the classical as $p < 0.05$.

Discussion

There is an ever-increasing need for efficient virus isolation methods, as unknown infectious viruses have emerged continuously worldwide. Based on several reports of inoculation methods, we selected CEI and AZD1480 treatment to examine if either or both methods could enhance replication of HRSV and HMPV and aid their isolation from clinical specimens. The isolation of HRSV and HMPV from clinical samples has been reported to be difficult because of their poor cytopathic effects on cell lines and long incubation time [9, 21, 22]. In this study, we found that recombinant respiratory viruses (HRSV-RFP, HMPV-GFP) and wild viruses (HRSV-A, HRSV-B,

and HMPV) propagated better with the CEI and AZD1480 combined method, compared to the classical inoculation method.

The CEI has been used to enhance infections of various viruses for many years. However, the mechanisms of CEI remain unknown [23]. The most reasonable explanation is that virus particles are sediment to cell surface by centrifugal forces to promote the physical contact to cellular receptors for virus entry [24, 25]. However, CEI did not affect the attachment of bluetongue virus to cells, even though the virus was replicated more in the culture with centrifugation than without centrifugation [26]. Another possibility is that centrifugation may affect the expression of cellular genes relating to viral entry, such as the increase of viral receptors [27]. However, this explanation is also controversial [23]. Despite the unclear mechanism of CEI for virus infection, CEI is a useful technique for efficient virus infection in vitro cell systems.

The combined method for controlling culture time and innate immunity synergistically increased virus replication, compared to either CEI or AZD1480 treatment alone. Also, virus isolation from clinical samples was more efficient with the combined method, and increases of 81–94.4% were observed in viral genome copies compared to those by the classical inoculation method. Interestingly, in some clinical samples, the viruses could only be replicated with the combined method and the combined method more efficiently enhanced the replication of HRSV-B strains than HRSV-A strains.

AZD1480 inhibits JAK2 with IC_{50} of 0.26 nM and has a lesser inhibitory activity against JAK1 [16]. It also blocks STAT3 signaling, affecting signaling pathways regulating innate immunity downstream of interferon and IL-6 [28, 29]. IL-6 is a pleiotropic cytokine, secreted when pathogenic pathogens cause respiratory infections, and it induces JAK2/STAT3 through the IL-6R receptor to inhibit the replication of various viruses [30, 31]. Therefore, although Vero cells lack IFN expression because of the deletion of IFN genes [32], replication of recombinant HRSV-RFP and HMPV-GFP increased approximately 2-fold in Vero cells when cultured in media supplemented with 2 μ M AZD1480, through inhibiting IL-6 signal pathway. In HEp-2 cells, which contain the complete IFN signaling pathway, treatment with AZD1480 was more efficient than that in Vero cells. While Vero cells are generally used for growing IFN-sensitive viruses, various cells such as HEp-2 and MRC5 are often utilized to isolate respiratory viruses, including HRSV and human coronaviruses from clinical samples, as some viruses show

tissue tropism. AZD1480 treatment could enhance virus replication much more efficiently in IFN-responsive cells. However, the sensitivity of cells to AZD1480 should also be considered. In our study, Vero cells were resistant to 4 μ M AZD1480, while HEp-2, a human hepatocarcinoma cell line, was only resistant to 0.25 μ M AZD1480. This disparity originates from the fact that this reagent was developed as an anti-cancer drug [33]. Therefore, the optimal concentration of AZD1480 should be determined for each cell line to avoid toxic effects.

In conclusion, our findings demonstrate that the combined method with CEI and AZD1480 treatment allows the efficient isolation of human respiratory syncytial virus and human metapneumovirus from clinical specimens. This method could be practically applied for the isolation and identification of newly emerging viruses or unidentified viruses for virus production, vaccine development, virus diagnostics, and antiviral therapeutics.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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