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ORIGINAL ARTICLE

Generation and Characterization of Recombinant Influenza A(H1N1) Viruses Resistant to Neuraminidase Inhibitors

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Abstract
Objectives: To examine the effect of neuraminidase (NA) mutations on the NA inhibitor (NAI) resistance phenotype, the recombinant influenza A/Chungbuk/4448/2008(H1N1) virus isolated in South Korea during the 2008–2009 season was generated by reverse genetics.
Methods: Site-directed mutagenesis was introduced on the *NA* gene of A/Chungbuk/4448/2008(H1N1) virus, and a total of 23 single, double, and triple mutants were generated. Resistance phenotype of these recombinant viruses was determined by NA-inhibition (NAI) assays based on a fluorometric method using two NAIs (oseltamivir and zanamivir).
Results: NA-inhibition assays showed that all the single and double mutants containing the Y275 except the single Y275-E119V mutant conferred important levels of resistance to oseltamivir, whereas all the single, double, and triple mutants containing the E119V mutation were associated with the resistance to zanamivir.
Conclusion: Considering the effect of mutations in *NA* gene on the resistance to NAIs, it is important to monitor the possible emergence and dissemination of multidrug-resistant variants in the human population due to amino acid changes at *NA* gene as well as to develop novel NAIs.

1. Introduction

Influenza infects approximately 20% of the world's population, and more than half a million individuals die every year of influenza-associated complications [1]. In 2009, the pandemic influenza A(H1N1) virus spread quickly among humans worldwide to cause the first influenza pandemic of the 21st century [2].

Vaccination and antiviral treatment are essential for the prevention and control of influenza infection. With the use of antiviral drugs for the clinical management of influenza, neuraminidase (NA) inhibitors (NAIs) have

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been proved to be very effective against influenza A and B viruses [3]. However, NAI-resistant strains have been reported frequently [4,5], in case of the emergence during the treatment of drug or dissemination of drug-resistant variants.

Several subtype-specific mutations in framework or catalytic residues of NA that confer resistance to NAIs have been reported [1,6–8]. The dominant change conferring oseltamivir resistance in the current seasonal influenza viruses is a mutation in the *NA* gene, H275Y (N1 numbering). The frequency of isolates with the H275Y mutation has increased with each flu season, including in countries where oseltamivir is not prescribed regularly. Particularly, most isolated seasonal influenza A(H1N1) viruses during the 2008–2009 season were found to encode the H275Y substitution in the *NA* gene, conferring resistance to oseltamivir [9]. Other NA mutations (N2 numbering: E119G, H274Y, R292K, and N295S) that have been reported to confer resistance to NAIs were each introduced into recombinant A/Vietnam/1203/04(H5N1) influenza virus [10].

Here we selected A/Chungbuk/4448/2008(H1N1) virus isolated in South Korea during the 2008–2009 season, which was one of the viruses isolated from throughout the country by the Korean Influenza Surveillance Scheme [11]. To investigate the effect of different NA mutations on the NAI resistance phenotype of A/Chungbuk/4448/2008(H1N1) virus, we generated recombinant A/Chungbuk/4448/2008(H1N1) virus using a reverse genetics system, introduced the different NA mutations into the background of the recombinant A/Chungbuk/4448/2008(H1N1) virus, and compared their NAI resistance phenotypes.

2. Materials and methods

2.1. Cells and viruses

Madin Darby canine kidney (MDCK) cells and human embryonic kidney cells transformed with large T antigen (293T cells) were obtained from American Type Culture Collection (Manassas, VA, USA). A/Chungbuk/4448/2008(H1N1) virus isolated in South Korea during the 2008–2009 season was used for the generation of recombinant viruses.

2.2. Antiviral compounds

Tartrate salt of oseltamivir carboxylate (active form of Tamiflu) was provided generously by F. Hoffmann-La Roche, Inc. (Basel, Switzerland); it was dissolved in distilled water such that the final oseltamivir carboxylate concentration was 10 mM. Zanamivir (Relenza), which was provided by GlaxoSmithKline (Stevenage, UK), was dissolved in distilled water to a final zanamivir concentration of 10 mM. Aliquots were stored at −20 °C until use.

2.3. Generation of recombinant viruses and site-directed mutagenesis

Recombinant viruses were generated using the eight-plasmid reverse genetics system with pHW2000 (the vector was provided kindly by Robert Webster, St. Jude Children's Research Hospital, Memphis, TN, USA) [12]. The eight genes from the cultured A/Chungbuk/4448/2008(H1N1) virus were amplified by reverse transcription polymerase chain reaction (RT-PCR) and incorporated into the pHW2000 plasmid. All eight plasmids were transfected into a coculture of 293T and MDCK cells for 3 days, and then further prepared in 10-day-old embryonated chicken eggs. The collected supernatant from the transfected cells was injected into the allantoic cavities of the eggs and incubated at 37 °C for 3 days to amplify the rescued viruses. The viruses generated in eggs were stored at −80 °C until use.

To investigate mutations at NA key residues (N1 numbering: I117V, I117M, E119V, I223V, Y275H, R293K, N295S, and S334N), site-directed mutagenesis was conducted on the *NA* gene cloned plasmid using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA). All recombinant plasmids were sequenced to ensure the absence of undesired mutations. The eight plasmids were then cotransfected to 293T mixed with MDCK cells. Supernatants were collected at 3 days post-transfection and used to inoculate specific pathogen-free (SPF) eggs. Introduced mutations of the recombinant wild type virus as well as the NA mutants were confirmed by sequencing.

2.4. NA activity assay and NA inhibition assay

The NA activity of each virus sample was determined by a modified fluorometric assay that measured 4-methylumbelliferone released from the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma-Aldrich, St. Louis, MO, USA) by the enzymatic activity of the influenza virus NA [13,14].

The drug resistance phenotype was determined by NA inhibition assays using the MUNANA (Sigma) substrate, with minor modifications [13–15]. The viruses were tested for susceptibility to oseltamivir and zanamivir. To determine the drug concentration required to inhibit 50% of the NA activity [50% inhibitory concentration (IC_{50})], 50 μL of virus, diluted according to the NA activity assay, was mixed with various concentrations of inhibitor in microtiter plates (FluoroNunc plates; Nalge Nunc International, Penfield, NY, USA). The final reaction mixture concentration of the NAIs ranged from 0.01 nM to 10,000 nM. The virus–inhibitor mixture was incubated at room temperature for 45 minutes prior to the addition of 50 μL of the MUNANA substrate (0.3 mM) and then incubated at 37 °C for 60 minutes. The reaction was terminated by the addition of 100 μL of the stop solution. The IC_{50} for each drug was calculated from the dose–response curve using GraphPad Prism software, version 5 (San Diego, CA, USA). The sensitive viruses