HUMAN ANGIOTENSIN-CONVERTING ENZYME 2 (ACE2) IS A RECEPTOR FOR HUMAN RESPIRATORY CORONAVIRUS NL63

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1. INTRODUCTION

Until 2003, only two human coronaviruses, HCoV-OC43 (group 2) and HCoV-229E (group 1), were well adapted to growth in tissue culture. These HCoVs have generally been associated with mild upper respiratory disease, although HCoV-229E can cause pneumonia in immunocompromised patients.[5] In contrast, animal coronaviruses (CoVs) have been associated with a wide variety of diseases, many of them severe, in multiple animal and avian species. From the animal coronaviruses, a great deal about CoV biology and pathogenesis is known. The variety of animal hosts and diseases among CoVs are due in large part to differences in the spike glycoprotein.[1,13,6,8,10] CoV spikes are large type 1 membrane glycoproteins that are the major determinants of receptor specificity as well as virulence.

The recent discoveries of HCoV-NL63 (group 1) and its association with a variety of respiratory tract infections, including pneumonia and croup in young children,[15] and HKU1 (group 2), isolated from a patient with pneumonia,[16] shows that coronaviruses are emerging as important pathogens of the human lower respiratory tract.

The previously known group 1 CoVs use aminopeptidase N (APN) glycoprotein from their respective host species as their principal receptor.[11] In addition, feline APN (fAPN) is a receptor for all of these group 1 coronaviruses (Table 1).[12] HCoV-NL63 is most closely related to the group 1 coronaviruses. Indeed, the spike glycoprotein of HCoV-NL63 shares 55% amino acid identity with the spike of HCoV-229E. We therefore tested whether this new virus would also utilize hAPN as its cellular receptor.

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Table 1. Group 1 coronavirus receptors.[11]

Virus	hAPN[a]	pAPN[b]	cAPN[c]	fAPN[d]
HCoV-229E	+	-	-	+
PRCoV	-	+	-	+
TGEV	-	+	-	+
CCoV	-	-	+	+
FCoV	-	-	-	+

[a] Human aminopeptidase N.
[b] Porcine aminopeptidase N.
[c] Canine aminopeptidase N.
[d] Feline aminopeptidase N.

2. METHODS AND RESULTS

2.1. NL63 Infection of Cell Lines

LLC-MK2 cells, Vero E6 cells, and a human lung fibroblast-like cell line, MRC-5 (ATCC # CCL-171), were inoculated with NL63 and then observed for signs of CPE for 96 hours postinfection. Mild and transient CPE, consisting mostly of rounding and the appearance of vacuoles, was observed at 36 hours postinfection in all three cell types. HCoV-NL63 viral antigens were detected by immunofluorescence with a cross-reactive polyclonal goat antibody raised against HCoV-229E virions followed by fluorescein isothionate (FITC)-conjugated donkey anti-goat IgG as previously described.[2] Viral antigen was detectable from 24 to 96 hours postinfection, with a maximum of about 25% of cells positive for antigen at 72 hours postinfection.

We inoculated LLC-MK2 and Vero E6 cells, which are permissive for HCoV-NL63 infection,[14] as well as NIH-3T3 cells stably expressing hAPN (NIH-3T3/hAPN), BHK-21 cells stably expressing fAPN (BHK/fAPN), which are permissive for HCOV-229E infection, with either HCoV-NL63 or HCoV-229E at an MOI of 0.07. HCoV-229E and HCoV-NL63 viral antigens were both detected in susceptible cells with immunofluorescence. Viral antigens were detected in LLC-MK2 and Vero E6 cells inoculated with HCoV-NL63. No infection was detected in LLC-MK2 or Vero E6 cells inoculated with HCoV-229E. No viral antigens were detected in NIH-3T3/ hAPN or BHK/fAPN cells inoculated with HCoV-NL63, but robust infection was apparent in these cells after inoculation with HCoV-229E (Table 2).

The observations that both SARS-CoV and HCoV-NL63 grow in LLC-MK2 and Vero E6 cells led us to test the hypothesis that HCoV-NL63 uses the same receptor as SARS-CoV, angiotensin-converting enzyme 2 (ACE2).[9] BHK-21 cells were transiently transfected with a plasmid encoding full-length human ACE2 (hACE2, kindly provided by Michael Farzan), and inoculated 48 hours after transfection with HCoV-NL63 or HCoV-229E. HCoV-NL63 antigen was detected in hACE2-transfected BHK-21 cells, but not in non-transfected controls. No HCoV-229E antigen was detected in either transfected or hACE2-negative control BHK-21 cells. ACE2-transfected cells were permissive for HCoV-NL63 replication, with peak yields of 10^5 TCID$_{50}$/ml as assayed on LLC-MK2 cells.

To determine which receptor HCoV-NL63 uses to infect MRC-5 cells, we tested MRC-5s for surface expression of hACE2 by flow cytometry. MRC-5 cells expressed a small amount of ACE2, relative to LLC-MK2 and Vero E6 cells (Table 3).

Table 2. Susceptibility of cultured cells to HCoV-NL63 and HCoV-229E infection.

	Virus	
Cell type	HCoV-229E	HCoV-NL63
LLC-MK2	-	+
VERO E6	-	+
MRC-5	+	+
BHK	-	-
BHK/hACE2	-	+
BHK/fAPN	+	-

3. DISCUSSION

In this paper, we show that infectious HCoV-NL63 used human ACE2 as an efficient receptor and that this group 1 coronavirus cannot use feline APN, the receptor for all other group 1 coronaviruses tested, or human APN, the principal receptor for HCoV-229E. Hoffmann et al., also showed that human ACE2 served as a receptor for HCoV-NL63 using retrovirus pseudotyped with HCoV-NL63 spike glycoprotein.[4]

The finding that HCoV-NL63 uses ACE2 as a receptor is surprising in that HCoV-NL63 is most closely related to HCoV-229E, and its genome structure is most similar to that of PEDV in group 1, while SARS-CoV, which also uses human ACE2 as a receptor, is a distantly related member of group 2 coronaviruses. Although the group 1 spikes studied to date share no more than 48% amino acid sequence identity, all but HCoV-NL63 are able to use fAPN as a receptor.

Interestingly, although the spike glycoprotein of HCoV-NL63 shares only 25% amino acid sequence identity with that of SARS-CoV (Urbani strain), the two viruses use the same receptor. It is possible that the two viruses have evolved independently to bind to ACE2. The finding that HCoV-NL63 and SARS-CoV utilize the same human receptor glycoprotein has important implications for coronavirus evolution. The genomes of all group 2 coronaviruses except SARS-CoV contain a gene encoding an HE glycoprotein that binds to O-acetylated sialic acids,[7] apparently derived by recombination of the mRNA encoding HE from influenza C with the genome of an ancestral group 2 coronavirus.[7] Group 1 coronaviruses including HCoV-NL63 lack this HE gene. Perhaps both HCoV-NL63 and SARS-CoV descended from a common ancestral coronavirus that used ACE2 as its receptor, and this lineage split from the ancestral group 2 coronaviruses before the HE gene was acquired.

Understanding HCoV-NL63 S glycoprotein and how its interactions with its receptor, hACE2, will elicudate how pathogenic coronaviruses emerge and may suggest new strategies for prevention and therapy.

Table 3. Expression of ACE2 on Vero E6, LLC-MK2, and MRC-5 cells.

	Percent cells gated	
Cell type	Control antibody	Anti-ACE2
Vero E6	3.1	77.6
LLC-MK2	17.7	70.8
MRC-5	7.7	27.0

4. ACKNOWLEDGMENTS

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