A Novel Soluble ACE2 Variant with Prolonged Duration of Action Neutralizes SARS-CoV-2 Infection in Human Kidney Organoids

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ABSTRACT

Background There is an urgent need for approaches to prevent and treat SARS-CoV-2 infection. Administration of soluble ACE2 protein acting as a decoy to bind to SARS-CoV-2 should limit viral uptake mediated by binding to membrane-bound full-length ACE2, and further therapeutic benefit should result from ensuring enzymatic ACE2 activity to affected organs in patients with COVID-19.

Methods A short variant of human soluble ACE2 protein consisting of 618 amino acids (hACE2 1–618) was generated and fused with an albumin binding domain (ABD) using an artificial gene encoding ABDCon, with improved albumin binding affinity. Human kidney organoids were used for infectivity studies of SARS-CoV-2 in a BSL-3 facility to examine the neutralizing effect of these novel ACE2 variants.

Results Whereas plasma ACE2 activity of the naked ACE2 1–618 and ACE2 1–740 lasted about 8 hours, the ACE2 1–618-ABD resulted in substantial activity at 96 hours, and it was still biologically active 3 days after injection. Human kidney organoids express ACE2 and TMPRSS2, and when infected with SARS-CoV-2, our modified long-acting ACE2 variant neutralized infection.

Conclusions This novel ACE2 1–618-ABD can neutralize SARS-CoV-2 infectivity in human kidney organoids, and its prolonged duration of action should ensure improved efficacy to prevent viral escape and dosing convenience.


Angiotensin-converting enzyme 2 (ACE2) has been primarily studied for its properties as a monocarboxypeptidase that efficiently cleaves Angiotensin (Ang) II to form Ang 1–7.1–7 ACE2 also cleaves several substrates of interest in cardiovascular and kidney disease, such as apelins 13 and 36 and des 9Arg bradykinin.2–15 Early in 2020, it was recognized that ACE2 is the main receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).16–20 After binding of SARS-CoV-2 S spike to membrane-bound full-length angiotensin-converting enzyme 2 (FL-ACE2), there is priming by a protease TMPRSS2 that is needed for the fusion and internalization of the ACE2-viral spike complex.16 Earlier into 2020, we21 and others22 proposed that administration of soluble ACE2 protein acting as a decoy to bind to SARS-CoV-2 should limit viral uptake mediated by membrane-bound FL-ACE2. Consequently, SARS-CoV-2 entry into the cells and viral replication should be prevented. In addition, further therapeutic benefit should result from restoring decreased ACE2 activity and therefore, correct the altered balance of Ang II and bradykinins as reviewed in ref. 21. These two distinctive actions expected from administering soluble ACE2 protein are both important and need to be examined experimentally by approaches that cannot be easily applied in human research. The promise of soluble ACE2 therapy is illustrated by a recent case report of a patient with coronavirus disease 2019 (COVID-19) who appeared to respond favorably to the administration of...
We have recently generated short recombinant mouse ACE2 variants that exhibit full ACE2 activity. Here, we report the generation of a human short ACE2 variant that has 618 amino acids. This ACE2 variant moreover was fused with the albumin binding domain (ABD) as a strategy to increase its duration of action. We reasoned that when administered in vivo, the feature of enhanced duration of action should provide a more favorable profile in terms of intercepting the SARS-CoV-2 than the native soluble protein that has a short half-life of only a few hours.

To test whether our novel soluble ACE2 proteins can reduce SARS-CoV-2–mediated infectivity, we use human kidney organoids as a convenient model; studies in rodents are limited by the resistance to SARS-CoV-2 infectivity because the ACE2 of rodents lacks the receptor binding domain (RBD) for the S spike of SARS-CoV-2, which is present in human ACE2. Short of in vivo studies in animals, human kidney organoids provide a convenient model to study certain aspects of kidney injury, including inherited kidney diseases, development disorders, and nephotoxicity. In a recent study, human kidney organoids were used to demonstrate the neutralizing effect of native soluble ACE2 1–740 on SARS-CoV-2 infectivity. We, therefore, utilized kidney organoids to demonstrate the efficacy of our novel ACE2 variants in neutralizing SARS-CoV-2 infectivity.

**METHODS**

**Generation of Human ACE2 1–618 and a Fused Chimera with an ABD Tag**

A C-terminally truncated recombinant human ACE2 protein of 618 amino acids that we termed ACE2 1–618 was generated using an approach similar to that used previously by us to generate a short mouse rACE2 variant 1–619 (Supplemental Material). This truncated ACE2 variant was found to be as enzymatically active as the longest soluble intact mouse rACE2 1–740. Human ACE2 1–618 was then fused with a small (5-kD) ABD in order to prolong in vivo duration of action (as described in more detail in Supplemental Material). The cDNA encoding ABDCon, a variant of ABD with improved albumin binding affinity (femtomolar range), favorable biochemical characteristics, and improved stability, was synthesized along with a flexible linker (G4S3), which was placed at the N terminus from the ABDCon cDNA (IDT). The cDNA of the G4S3-ABDCon construct was fused to the C terminus of ACE2 1–618 cDNA using a “sewing” PCR to produce the fusion chimera (human ACE2 1–618-ABD). The cDNA of the fusion chimera ACE2 1–618-ABD was then inserted into pCDNA6 plasmid (Invitrogen) using custom synthesized complementary primers (IDT) and the Gibson assembly kit (NEB). After verifying the DNA sequence of the pCDNA6 fused with the ACE2 1–618-ABD, HEK-293 cells were then transfected with the plasmid construct. Both proteins (the naked ACE2 1–618 and ACE2 1–618-ABD) were expressed in human kidney embryonic cell line HEK-293 and purified using Fast Protein Liquid Chromatography on Q-Sepharose followed by size exclusion chromatography on Superdex 200 pg to >90% purity.

**Enzyme Activities and Kinetic Constants of Purified Short Recombinant ACE2 Variants**

The ability of the two purified short human recombinant ACE2 variants to cleave two natural substrates of ACE2 was examined. ACE2 cleaves Ang II (1–8), to form Ang-(1–7), as well as des Arg bradykinin [bradykinin-(1–8)] to form bradykinin-(1–7). ACE2 is known to remove the C-terminal amino acid phenylalanine from the cleavage reaction with either substrate. An assay quantifying phenylalanine formation from ACE2 substrates was used to evaluate the relative enzymatic potency of the two ACE2 variants as compared with equivalent molar amounts of the standard ACE2 1–740, which was used as a benchmark. For assessment of activity levels, the Michaelis–Menten model was used to derive the parameters of catalytic kinetics, such as $K_m$ and $K_{cat}$.

**Plasma ACE2 Activity**

The pharmacokinetic profiles of human ACE2 1–618-ABD were assessed in wild-type mice as compared with those of naked human ACE2 1–618 and the native ACE2 1–740. The mice received a single intraperitoneal injection of each purified rACE2 variant at a dose of 1 mg/g body wt. Blood samples were collected by tail bleeding either before or at several indicated time points after injection. Blood samples were collected in heparinized capillaries, and plasma was isolated by centrifugation at 1850 × g for 10 minutes at 4°C. Mca-APK (Dnp) substrate (Bachem, Bubendorf, Switzerland) was used to measure ACE2 enzyme activity in plasma. The pharmacokinetic parameters on the basis of plasma ACE2 activity measurements were calculated using Prism 8 software (GraphPad, La Jolla, CA).

**Significance Statement**

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a pandemic for which currently there are limited preventative and therapeutic approaches. The use of soluble ACE2 protein has been recently proposed as a way to intercept the SARS-CoV-2 S spike from binding to the full-length membrane-bound ACE2 receptor. We have developed a newly bioengineered soluble ACE2 protein of shorter molecular size and modified by fusing it with an albumin binding domain (ABD) tag to extend its duration of action. Here, we demonstrate in studies conducted in a BSL-3 facility that human kidney organoids can be infected by SARS-CoV-2 as they possess the human full-length ACE2 receptor and transmembrane serine protease 2, which are needed for infectivity. Using this model, we demonstrate that our novel soluble ACE2 variant can inhibit SARS-CoV-2 infection, suggesting its potential preventative and therapeutic use.
BP Response to Ang II–Induced Hypertension
To demonstrate the biologic enzymatic activity in vivo of our ACE2 1–618 variants and the benchmark ACE2 1–740, we used a model of Ang II–induced hypertension.9,15 The effects of the three ACE2 proteins on acute Ang II–induced hypertension were examined as previously described by us.9,15,31 We injected 10- to 20-week old male C57bl/6 mice intraperitoneally with vehicle (PBS), human ACE2 1–618, ACE2 1–618-ABD, or ACE2 1–740 (all at 1 mg/kg body wt). After 1 hour, 3 days, or 7 days, mice were anesthetized with an intraperitoneal injection of ketamine (150 mg/kg body wt) and then placed on a temperature-controlled platform immediately. Systolic BP (SBP) was monitored noninvasively every 30 seconds for a period of 20 minutes, including 5 minutes of baseline SBP recording, after which acute hypertension was induced with an intraperitoneal bolus injection of Ang II (0.2 mg/kg body wt).

Generation of Human Kidney Organoids and Culture Conditions
Human kidney organoids were generated using a previously published protocol,26 with slight modifications. H9 human embryonic stem cells (female WA09 cell line from WiCell, National Institutes of Health approval number NIHESC-10–0062) were plated at a density of 1.7×10⁴ cells per cm² in mTeSR1 medium (STEMCELL Technologies) on a geltrex-coated (ThermoFisher Scientific) six-well plate. After cells became approximately 50% confluent, differentiation was started with the treatment of 8 μM CHIR99021 (Reprocell) in the basic differentiation medium (advanced RPMI 1640 containing 1× 1-glutamine supplement). On day 4, medium was replaced with basic differentiation medium supplemented with 10 ng/ml Activin A, and on day 7, medium was replaced with basic differentiation medium supplemented with 10 ng/ml FGF9. On day 9, differentiated kidney progenitors form renal vesicle–like clusters and were fed with the basic differentiation medium supplemented with 10 ng/ml FGF9 and 1 μM CHIR99021. On day 11, the medium was changed to the basic differentiation medium containing 10 ng/ml FGF9. From day 14, the cells were fed with the basic differentiation medium every 2–3 days until harvest on day 21. On day 21, some organoids were transferred to a six-well plate or collected for isolation of cellular fractions. Several days later, using sterile tips, organoids from the six-well plate were plated onto a sterile 96-well plate with fresh medium (five organoids per well) for subsequent tissue infectivity experiments.

Another set of human kidney organoids was generated from H9 cells as previously described32 and used for staining studies. Briefly, highly functional kidney cells were generated by keeping an optimal balance between static traditional tissue culture and three-dimensional organ culture. For directed differentiation of H9 cells to kidney progenitors, cells were plated at a density of 1.7×10⁴ cells per centimeter² in StemFit (ams-bio) on a geltrex-coated six-well plate (Supplemental Material). Staining was done using anti-human ACE2 (AF933; R&D Systems) and anti-TMPRSS2 antibodies (NBP-2 38263; Novus) to localize expression of ACE2 and TMPRSS2.

In Vitro Assay for Detection of hACE2-RBD Interaction
Using an artificial ACE2 substrate Mca-APK-Dnp (10 μM), ACE2 activity of ACE2 1–740 was not affected by the presence of recombinant glycosylated RBD in a 0.001- to 100-ng/ml concentration range. On the basis of this finding, we developed a method that measures interaction between viral S1 RBD and human ACE2 using as a readout ACE2 activity. Purified His-tagged RBD protein was dissolved in Tris-buffered saline (TBS; pH 7.4) and loaded into 96-well Ni-coated black plate for binding. TBS alone was loaded to blank wells. After incubation for 1 hour at room temperature, five washes were done. One hundred milliliters of human ACE2 1–740 (positive control) and mouse ACE2 (negative control) were added to the wells and incubated for 1 hour at room temperature at concentrations ranging from 1000 to 6.25 ng/ml. Afterward, wells were washed five times. Finally, Mca-APK-Dnp substrate (1 μM final concentration) was added, and fluorescence formation was measured in a microplate fluorescence reader (a detailed description is in Supplemental Material). Dose-dependent binding of enzymatically active human ACE2 protein to the RBD-His protein immobilized onto the Ni-coated microplate was confirmed by concentration-dependent increase in fluorescence formation (expressed in relative fluorescence unit) from the cleavage of the Mca-APK-Dnp ACE2 substrate.

We then used lysates of isolated cytosol, nuclear, or membrane fractions from human kidney organoids to assess binding of hACE2 from those isolated fractions to the RBD domain of SARS-CoV-2 S1 protein. Purified His-tagged RBD protein was dissolved in TBS and loaded into 96-well Ni-coated black plates (100 μl per well) for binding for 1 hour. After five washes, the organoid tissue lysate samples (10 μl) were diluted in 90 μl TBS, loaded onto the plate, and left for 1 hour at room temperature. Afterward, wells were washed five times before Mca-APK-Dnp substrate (1 μM final concentration) was added, and fluorescence formation was measured in a microplate fluorescence reader (a detailed description is in Supplemental Material). The measured fluorescence in organoid lysates was corrected for total protein content and converted to a concentration of ACE2 protein by reference to a standard curve of recombinant ACE2 (R&D Systems) assayed under the same conditions.

SARS-CoV-2 Neutralization Assay
Under Biosafety Level 3 conditions, 0 and 200 μM indicated ACE2 proteins (mouse ACE2 1–740, human ACE2 1–740, human ACE2 1–618, and human ACE2 1–618-ABD) in PBS were mixed with 400 PFU of SARS-CoV-2
Figure 1. Pharmacokinetics of soluble human recombinant ACE2 variants in vivo after intraperitoneal injection. Mice were injected intraperitoneally with ACE2 1–618-ABD (red), the naked ACE2 1–618 (green), or the native ACE2 1–740 (blue). Plasma ACE2 activity was measured at various time points, starting 1 hour postinjection. The ACE2 1–618-ABD variant resulted in higher-peak plasma ACE2 activity as compared with the naked ACE2 1–618 and the native ACE2 1–740. Whereas the activity of the naked ACE2 1–618 and ACE2 1–740 had decreased markedly by 8 hours, the ACE2 1–618-ABD resulted in persistent activity at 72 and 96 hours, therefore demonstrating a marked extension of in vivo t1/2 of ACE2 1–618 achieved by adding the ABD tag. RFU, relative fluorescence unit.

RESULTS

Catalytic Efficiency of Human ACE2 1–618 and ACE2 1–618-ABD as Determined by Phenylalanine Assay

Information is in Supplemental Material.

Prolonged In Vivo Activity of Human ACE2 Variant 1–618-ABD

The peak plasma ACE2 activity for the naked ACE2 1–618 and the native ACE2 1–740 was between 2 and 4 hours (Figure 1). The ACE2 1–618-ABD variant resulted in a higher peak plasma ACE2 activity as compared with the naked ACE2 1–618 and the native ACE2 1–740. Whereas the activity of the naked ACE2 1–618 and ACE2 1–740 had decreased markedly by 8 hours, the ACE2 1–618-ABD resulted in persistent activity at 72 and 96 hours (Figure 1). The t1/2 of the ACE2 proteins is in Supplemental Material.

BP-Lowering Effect of Human ACE2 1–618 and ACE2 1–618-ABD during Acute Ang II Infusion

SBP was monitored noninvasively every 30 seconds for a period of 20 minutes. This approach is suitable for acute studies, whereas for chronic studies, radiotelemetry is the preferred approach. In one set of experiments, mice were pretreated with vehicle (PBS), ACE2 1–740, ACE2 1–618, or ACE2 1–618-ABD by a single intraperitoneal injection (at 1 mg/kg body wt) 1 hour before receiving a bolus injection of Ang II (0.2 mg/kg body wt). The injection of Ang II 1 hour later resulted in a rapid increase in BP in the control group that had received PBS as preinjection vehicle, followed by its decline. In contrast, the increase in BP was markedly blunted and normalized to about the same extent by the three proteins (Figure 2, A–C).

To examine their duration of action, the same injection protocol was used except that Ang II was injected 3 days after injection of ACE2 1–740, naked ACE2 1–618, and ACE2 1–618-ABD. Three days after their injection, neither ACE2 1–740 nor the naked ACE2–618 were effective in normalizing the BP after Ang II injection (Figure 2D). By contrast, ACE2 1–618-ABD, when given 3 days prior to Ang II injection, still had a significant attenuation of BP (Figure 2E). When ACE2 1–618-ABD was given 7 days prior to Ang II injection, however, the response was no longer different from the PBS-treated control (Figure 2F).

(nCoV/Washington1/2020; provided by the National Biocontainment Laboratory, Galveston, TX) in DMEM for 1 hour at 37°C and then used to infect kidney organoid cells for 3 days. Advanced RPMI medium without ACE2 was used as the not treated reference. Cells were washed and lysed in RNA extraction buffer and purified using a NucleoSpin 96 RNA kit following the manufacturer’s instructions (Macherey-Nagel). SARS-CoV-2 RNA was quantified by qRT-PCR using the SuperScript III Platinum One-Step qRT-PCR Kit with ROX (ThermoFisher Scientific) and normalized using Eukaryotic 18S rRNA Endogenous Control (VIC/MGB probe; 29Applied Biosystems) via a StepOnePlus real-time PCR system (Applied Biosystems). All reactions were performed in a dual-plex qRT-PCR using the Centers for Disease Control and Prevention recommended primers for N1. Primer and probe sequences are as follows: 2019-nCoV_N1 Forward Primer (2019-nCoV_N1-F), GACCCCAATACTAGCCAAT; 2019-nCoV_N1 Reverse Primer (2019-nCoV_N1-R), TCTGGTACTGCGAGTTGAATCTG; Probe (2019-nCoV_N1-P), FAM-ACCCCGCATCCGTGTTGTTGGACC-BHQ1.

Human Kidney Organoids Express ACE2, Which Can Bind to the RBD Domain of the SARS-CoV-2 S Spike

The kidney organoids were generated as described in Methods.26,32 A representative photomicrograph of the kidney organoid showed tightly packed tubular clusters on day 19 (Figure 3A). Lotus Tetragonolobus Lectin staining confirmed that kidney organoids were filled with proximal tubules (Figure 3B). We then examined the presence of ACE2 and TMPRSS2, a protease needed for SARS-CoV-2 S spike and ACE2 activation.16 We found by immunofluorescence and confocal microscopy that ACE2 was expressed in tubular-like structures mainly in what appears to represent the apical membrane (Figure 3D). Staining for the protease TMPRSS2 was also present in these structures, and
Human kidney organoids were used to isolate cytosolic, nuclear, and membrane fractions for subsequent evaluation of ACE2 activity. Consistent with immunostaining, in the membrane fraction ACE2 activity was clearly detectable (0.55 ± 0.18 ng Eq rACE2 per microgram of total protein, n = 3). ACE2 activity was also found in the nuclear fraction, but none could be detected in the cytosolic fraction (not shown).

To establish whether ACE2 from kidney organoids is capable of binding the RBD of the SARS-CoV-2 S1 spike protein, the same isolated cell fractions were tested in an in vitro pull-down assay capable of detecting human ACE2-RBD interaction (see Methods). The interaction of the SARS-CoV-2 S1 spike protein and ACE2 was found to take place mainly in the membrane fraction of the kidney organoids. This shows that the ACE2 that is present in the human kidney organoids plasma membrane can bind the SARS-CoV-2 S1 spike protein (Figure 3C). Negligible ACE2-RBD interaction was measured in the nuclear fraction and in the cytosolic fraction, consistent with low ACE2 activity measured in these fractions.

Neutralization of SARS-CoV-2 in Kidney Organoids Exposed to Soluble ACE2 Proteins

Naked and ABD-tagged human ACE2 1–618 were used at a concentration that was previously found effective in maximally neutralizing SARS-CoV-2 (200 μg/ml) by soluble native human ACE2 1–740.22 Soluble human ACE2 1–740 was used as a positive control, and mouse ACE2 1–740 (lack of binding to SARS-CoV-2 S1 spike) was used as negative control at the same concentration. The ACE2 proteins were mixed with SARS-CoV-2 particles (MOI 0.02 for 30 minutes at 37°C) in an advanced RPMI medium.

Kidney organoids were then infected for 1 hour at 37°C and washed with PBS, and a new medium was added. On the third day after infection, levels of viral RNA were assessed by qRT-PCR. As expected, mouse ACE2 1–740 did not prevent viral infectivity, as shown by similar high viral mRNA levels as in the untreated group (Figure 4). By contrast, both naked and ABD-tagged ACE2 1–618 markedly reduced viral replication to the same extent as human ACE2 1–740 used as positive control (Figure 4).

DISCUSSION

Here, we report the generation of a shorter truncate of human soluble ACE2 with 618 amino acids and its fusion with ABD to extend its duration of action. Both forms are as active as the native human soluble ACE2 (740 amino acids) as demonstrated by their catalytic efficiency and biologic activity. The latter

Figure 2. BP-lowering effect of human ACE2 proteins in Ang II–induced hypertension in mice. In (A–C), the ACE2 proteins were given intraperitoneally 1 hour prior to Ang II injection. The increase in SBP was blunted and the recovery was enhanced by (A) ACE2 1–740 (blue, n = 6; P = 0.002), (B) ACE2 1–618 (green, n = 5; P = 0.01), and (C) ACE2 1–618-ABD (red, n = 5; P = 0.007). Each protein was examined with a group of separate controls (n = 6 for [A] and n = 4 for [B and C] each). In (D–F), the ACE2 proteins were given intraperitoneally 3 days prior to Ang II injection. (D) Both the naked ACE2 1–618 (green, n = 5) and the ACE2 1–740 (blue, n = 5) were no longer effective in normalizing the SBP after Ang II injection as compared with controls (n = 4). (E) In contrast, ACE2 1–618-ABD (red, n = 4) significantly attenuated Ang II–induced hypertension as compared with controls (n = 9; P = 0.008). In (F), ACE2 1–618-ABD protein (red, n = 5) was given intraperitoneally 7 days prior to Ang II injection and was no longer effective in lowering the SBP after Ang II injection like the controls that received PBS (n = 9).
was demonstrated by the pharmacokinetic profile of plasma ACE2 activity (Figure 1) and by the response of BP to infused Ang II (Figure 2). In this model of acute Ang II–induced hypertension, the BP-lowering effect was sustained for 3 days with the fused ACE2 1–618-ABD chimera, consistent with the enzymatic activity in plasma shown in the pharmacokinetic analysis. We then went on to demonstrate that the novel ACE2 1–618-ABD variant was capable to neutralize SARS-CoV-2 infectivity in human kidney organoids. This approach was recently used by Monteil et al.\textsuperscript{22} to show that the native human soluble ACE2 protein (ACE2 1–740) can neutralize SARS-CoV-2 at a dose comparable with that used by these authors to achieve a maximal neutralizing effect. By contrast, the mouse counterpart protein mACE2 1–740 used as a negative control did not reduce infectivity whatsoever.

We also demonstrated that human kidney organoids have membrane-bound ACE2 that can interact with the RBD of the SARS-CoV-2 S1 spike protein. This is in support of the findings of a recent preprint, also demonstrating ACE2 expression in a three-dimensional renal epithelial tubuloid system, similar to our human kidney organoids but derived from human kidney tissue.\textsuperscript{33(preprint)} Of interest, this preprint also reports the novel finding that Kidney injury molecule-1 is an alternative receptor for SARS-CoV-2.\textsuperscript{33(preprint)} Moreover, we show the presence of TMPRSS2 with areas of colocalization with ACE2 in proximal tubule–like structures. This protease is critically needed for activation of the ACE2–SARS-CoV-2–spike complex and subsequent internalization into the cell.\textsuperscript{16,34} Because the lungs are the known port of entry of SARS-CoV-2, studies in lung tissue, preferably lung organoids, are desirable to demonstrate efficacy of ACE2 proteins to neutralize SARS-CoV-2 infectivity. The kidney organoids we used were of female origin, and ideally, one should examine this model originating from

Figure 3. Human kidney organoids express ACE2, which can bind to the RBD domain of the SARS-CoV-2 S2 spike protein. Kidney organoids were generated from asynchronous mixing of two differentiating human embryonic stem cell–derived kidney progenitors at the air-liquid interface. (A) Representative photomicrograph of kidney organoid showing tightly packed tubular clusters on day 18, and (B) Lotus Tetragonolobus Lectin (LTL) staining showing that kidney organoids were filled with proximal tubules. (C) In silico pull-down assay showing binding of ACE2 to the RBD domain of SARS-CoV-2 S1 spike protein. ACE2 from cytosol, nuclear, or membrane fractions of kidney organoids (n=3 per group). (D) Immunofluorescence staining showing high expression of ACE2 and TMPRSS2 in the proximal tubules of kidney organoids and areas of colocalization in the apparent apical membrane.
Effect of recombinant soluble ACE2 proteins on SARS-CoV-2 infection of human kidney organoids. Kidney organoids were infected with a combination of infectious viral particles (400 PFU) and soluble mouse (m) or human (h) ACE2 proteins (n=3 per each group) for 1 hour. Three days postinfection, levels of viral RNA were assessed by qRT-PCR. All three human ACE2 variants markedly reduced RNA levels of SARS-CoV-2, whereas the mouse ACE2 1–740 counterpart did not. Single data points and means are shown for each group.

Figure 4. Effect of recombinant soluble ACE2 proteins on SARS-CoV-2 infection of human kidney organoids. Kidney organoids were infected with a combination of infectious viral particles (400 PFU) and soluble mouse (m) or human (h) ACE2 proteins (n=3 per each group) for 1 hour. Three days postinfection, levels of viral RNA were assessed by qRT-PCR. All three human ACE2 variants markedly reduced RNA levels of SARS-CoV-2, whereas the mouse ACE2 1–740 counterpart did not. Single data points and means are shown for each group.

In addition, after viral binding, the ABD-tagged ACE2 could direct the SARS-CoV-2–ACE2 complex to a different cell sorting pathway as compared with naked ACE2. ACE2-ABD, when bound to albumin, might direct the complex into the FcRn-mediated recycling pathway, which would be away from the lysosome-endosome pathway that seems crucial for viral processing and replication.42 This may provide a theoretical advantage for our ACE2 1–618 protein fused with ABD over the naked ACE2 1–740 protein.

Infection with SARS-CoV-2 may result in internalization and loss of membrane-bound FL-ACE2, similar to what was reported for SARS-CoV.43 As a result of this loss in membrane-bound FL-ACE2, the degradation of its substrates, namely Ang II and des Arg bradykinin, would be impaired.21 Without ACE2 acting as a guardian to prevent excessive levels of peptides with proinflammatory effects, the lungs but also, other organs, like the kidneys, are prone to local injury.21,43,44 If the hypothesis is correct, it is logical to expect that administration of ACE2 in the form of soluble protein should have therapeutic benefit, not only due to its decoy effect but also, by restoring the altered balance of Ang II and bradykinins that ensues with depletion of this enzyme. In this regard, it is important to perform studies in vivo using animal models that are sensitive to SARS-CoV-2 infection, like hamsters or ferrets.45,46 We plan such studies in a transgenic mouse model rendered susceptible to SARS-CoV-2 infectivity by expressing human FLACE2 (k18-hACE2 mice).47 In such studies, the effect of soluble ACE2 proteins on mortality and organ injury should be examined.

Of note, the native form of soluble ACE2 1–740 is currently being studied in a clinical trial (NCT04335136) and was also successfully administered intravenously twice a day for compassionate use in a single patient case report recently.23 Our data presented here suggest that the prolonged duration of action of our novel ACE2 1–618–ABD variant should offer a better way to prevent viral
escape and afford more convenient dosing schedules. The potential protective effect on the kidneys of our shorter long-acting soluble ACE2 and its prolonged \textit{in vivo} \textit{t}_{1/2} are important differences as compared with the native human recombinant soluble ACE2. Comparative studies in permissive animal models of SARS-CoV-2 infection should determine the efficacy and relative advantages of each ACE2 protein.

**DISCLOSURES**

D. Batlle and J. Wysocki are coinventors of the issued patent “Active Low Molecular Weight Variants of Angiotensin Converting Enzyme 2” and provisional patents “Active low molecular weight variants of Angiotensin Converting Enzyme 2 (ACE2) for the treatment of diseases and conditions of the eye” and “Shorter soluble forms of Angiotensin Converting Enzyme 2 (ACE2) for treating and preventing coronavirus infection.” D. Batlle is founder/owner of Angiotensin Therapeutics Inc. D. Batlle has received consulting fees from AstraZeneca, Relypsa, and Tricida, all unrelated to this work. During the conduct of these studies, D. Batlle received unrelated support from National Institute of Diabetes and Digestive and Kidney Diseases grant R01DK104785, as well as AstraZeneca and Feinberg Foundation; received honoraria from AstraZeneca, Relypsa, and Tricida; and was a scientific advisor member with Relypsa and Tricida, G. Randall reports consultancy agreements with Optikira. J.A. Wertheim reports consultancy agreements with Miromatrix Medical; ownership interest in Bristol-Myers Squib and Merk & Co.; honoraria from Ann and Robert H. Lurie Children’s Hospital of Chicago, Dartmouth College, Miromatrix Medical, National Institutes of Health, Springer Publishing Company, and Taylor and Francis; patents and inventions via a patent that is not receiving any royalties; and being a scientific advisor or member with Standards Coordinating Body, Organogenesis, Alliance for Regenerative Medicine. J. Wysocki reports scientific advisor capacity for Angiotensin Therapeutics Inc. All remaining authors have nothing to disclose.

**SUPPLEMENTAL MATERIAL**

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.20200101537/-/DCSupplemental.

**ACKNOWLEDGMENTS**

J. Wysocki designed and performed many of the experiments and wrote parts of the paper. M. Ye did the staining studies. L. Hassler helped with the writing of the manuscript and data analysis. J.A. Wertheim oversaw the generation of kidney organoids by A.K. Gupta and Y. Wang, A.K. Gupta, Y. Wang, and J.A. Wertheim edited the manuscript. G. Randall supervised the infectivity studies in the Biosafety Level 3 facility done by V. Nicoleascu. D. Batlle designed experiments and wrote most of the paper.

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