A Novel soluble ACE2 variant with prolonged duration of action neutralizes SARS-CoV-2 infection in Human Kidney Organoids

Short title: Novel ACE2 protein neutralizes SARS-CoV-2 infection

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Supplemental Material

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Methods

Design, production and purification of the short human ACE2 1-618 and of the human ACE2 1-618 with an Albumin Binding Domain (ABD) tag (continuation of methods)

The short variant of human ACE2 protein consisting of 618 amino acids (hACE2 1-618) and hACE2 1-618 fused albumin-binding domain (ABD) were generated and tested as follows. The cDNA of human *ace2* was generated by PCR amplification using as a template human kidney cDNA library (Milipore). We used specific primers that determine the length of the short *ace2 1-618* cDNA to be amplified compatible with the expression vector restriction sites (pcDNA, Invitrogen, Carlsbad, CA, USA). The absence of mutations in the amplified cDNA was verified by sequencing. The plasmid with the inserted cDNA of the short ACE2 variant 1-618 was then expressed by stable transfection in human embryonic kidney cells (293 cell line) (see below).

An artificial gene encoding ABDCon, a variant of ABD with improved albumin binding affinity (fM range), favorable biophysical characteristics and improved stability[12], 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 short *ace2 1-618* cDNA using a "sewing" PCR to produce the fusion chimera (*hace2 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*, HEK293 cells were then transfected with the plasmid construct.

The presence and size of the over-expressed proteins were verified in Western blot using a specific ACE2 antibody (Abcam, Ab38888). Enzyme activity was confirmed by the detection of fluorescence formation using an artificial ACE2 substrate Mca-APK (Dnp) in the culturing medium. For large scale production and purification of the naked and ABD-tagged ACE2-1-618 in stably transfected HEK cells, single clones were selected and expanded to large culture flasks. Conditioned serum-free medium from the selected clones of

stably transfected 293 cells overexpressing the naked and ABD-tagged ACE2-1-618 proteins was concentrated on Centricon 70 centrifugal devices (cut off 30 kDa). The resulting retentate was cleared by centrifugation 23,000 g for 10 min at 4C, subjected to anion exchange Q-sepharose followed by size exclusion chromatography on Superdex 200 pg. Eluted fractions were applied to SDS-PAGE, transferred to PVDF membrane and stained with Brilliant Blue to assess protein and screened for ACE2 activity using Mca-APK-Dnp substrate. Protein concentration in fractions containing ACE2 activity was determined using BCA assay (Pierce).

Generation of human kidney organoids (continuation of methods)

For staining studies, we used a set of human kidney organoids, derived from H9 human embryonic stem cells (ESCs, female WA09 cell line from WiCell, NIH approval number NIHhESC-10-0062) as previously described (26). On day 3, once culture became ~50% confluent, culture medium was replaced with basic differentiation medium Advanced RPMI 1640 (ThermoFisher Scientific) and 1× GlutaMAX (ThermoFisher Scientific) containing 8 μM CHIR99021(Reprocell). On day 4, culture medium was replaced with basic differentiation medium, containing 10 ng/ml Activin A (R&D Systems). On day 7, medium was replaced with basic differentiation medium containing 10 ng/ml FGF9 (R&D Systems). On day 9, differentiated kidney progenitors form renal vesicle like clusters in the wells of six-well plate. For asynchronous mixing of progenitors, directed differentiation was performed on two batches of H9 cells staggered 2 days apart. On day 9, the first batch of kidney progenitors were harvested with TrypLE Express (ThermoFisher Scientific) and resuspended at a density of 2.5×10^5 cells/μl in organoid initiation medium containing APEL2 (Stemcell Technologies), 1.5% PFHM-II (ThermoFisher Scientific), 100 ng/ml FGF9, 100 ng/ml BMP7 (R&D Systems), and 1 μg/ml Heparin (Sigma-Aldrich). In a 24-well plate, organoid initiation medium was added (1ml/well) and an air-liquid interface was created by suspending isopore membranes (EMD Millipore) at the surface of the medium. Resuspended kidney progenitors were aggregated on top of the filter (2 μl/aggregate). On day 11, aggregated cells were gently dissociated into small cell clusters with a 200 μl micropipette and mixed with second batch of newly differentiated kidney progenitor cells. One dissociated aggregate was mixed with 5×10^5 kidney progenitor cells in 4 μl of organoid initiation medium and re-aggregated again in two aggregates at the air-liquid interface. Medium was changed every 48 h or when it turned yellow. On day 13, all growth factors were removed from the medium and organoids were cultured for the next 5 days with APEL2 containing 1.5% PFHM-II.

In vitro assay for detection of hACE2-RBD interaction

Using an artificial ACE2 substrate, Mca-APK-Dnp (10 μM) (Bachem), ACE2 activity of hrACE2 1-740 aa was not affected by the presence of recombinant glycosylated RBD (aa 319-541, RayBiotech) in a 0.001 to 100 ng/ml concentration range. Purified His-tagged RBD protein (aa 319-541, RayBiotech) was dissolved in Tris-buffered saline (TBS) pH 7.4 and loaded into 96-well Ni-coated black plate (ThermoFisher) (100μL/well) for

binding. TBS alone (100 ul/well) was loaded to blank wells. After incubation for 1 hr at room temperature, five washes were done using 200µL of TBS supplemented with 0.05% Tween20 (wash buffer). 100µL of hrACE2 1-740 (positive control), and mouse rACE2 (negative control) were added to the wells and incubated for 1 hour at room temperature at concentrations ranging from 1000ng/ml to 6.25 ng/ml. Afterwards, wells were washed 5 times using 200µL of wash buffer for each wash. Finally, Mca-APK-Dnp substrate (1 µM final concentration) was added and fluorescence formation was measured in microplate fluorescence reader FLX800 at 320 nm using excitation and 400 nm emission filters.

Dose dependent binding of enzymatically active human rACE2 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 - RFU) from the cleavage of the Mca-APK-Dnp ACE2 substrate. There was no increase in RFU in negative control wells, i.e. coated with mrACE2 at any tested concentration (from 1000ng/ml to 6.25 ng/ml) as well as in wells not coated with RBD (TBS only).

We then used lysates of isolated cytosol, nuclear or membrane fractions (Invent Biotechnologies, Inc, Plymouth, MN, USA) 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 (aa 319-541, RayBiotech) was dissolved in Tris-buffered saline (TBS) pH 7.4 and loaded into 96-well Ni-coated black plate (ThermoFisher) (100µL/well) for binding for 1 hour. After five washes with 200µL of wash buffer, the described above organoid tissue lysate samples (10uL) were diluted in 90 µL TBS, pH 7.4, and applied in a total volume of 100 µL loaded onto the plate and left for 1 hr at room temperature. Afterwards, wells were washed 5 times using 200µL of wash buffer for each wash. Finally, Mca-APK-Dnp substrate (1 µM final concentration) was added and fluorescence formation was measured in microplate fluorescence reader FLX800 at 320 nm using excitation and 400 nm emission filters. The organoid tissue lysates on wells without His-tagged RBD protein coating did not produce any measurable fluorescence at otherwise the same assay conditions. The measured fluorescence in organoid lysates was corrected for total protein content (measured using BCA Protein Assay Kit, ThermoFisher) 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.

Results

Catalytic Efficiency of Human ACE2 1-618 and Human ACE2 1-618 with an ABD tag

The catalytic efficiency to form Phenylalanine (Km/Kcat M-1*s-1) from Ang II was essentially the same for ACE2 1-618 (n=4) and ACE2 1-618-ABD (n=4) (1.44±0.09 and 1.63±0.36, respectively) and slightly lower but not significantly than that of ACE2 1-740 (n=4) (2.00±0.31).

Using the same assay, we found that the three ACE2 proteins were able to generate Phenylalanine from des-9Arginine. Catalytic efficiency was slightly but significantly higher for ACE2 1-618-ABD (n=6) (1.45±0.14 M-

1*s-1), than for ACE2 1-740 (n=6) (0.69±0.15 M-1*s-1 (p=0.002)) or the naked ACE2 1-618 (n=6) (0.94±0.15 M-1*s-1) (p=0.023).

Prolonged in vivo activity of human ACE2 Variant 1-618-ABD

The elimination phase half-life and the mean residence time were markedly longer for the ABD-tagged ACE2 1-618 (25.7±4.1) compared to the naked ACE2 1-618 protein (3.2±0.6) and the native ACE2 1-740 protein (6.8±1.0) ($p<0.001$). This shows a marked extension of in vivo half-life of ACE2 1- 618 achieved by adding the ABD tag.