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COVID-19 Therapy Based on Soluble ACE2: The Use of Receptor-Fc Fusion Proteins

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ABSTRACT

After two years of the first outbreak, COVID-19 is still a health issue for all the world. This deadly and highly transmissive disease have caused millions of deaths, challenging the sanity systems all over the globe. Despite the unquestionable success of vaccines, the emergence of new variants of SARS-CoV-2, the etiologic agent of this disease, menaces their effectiveness due to the appearance of resistant strains. This reinforces the importance of exploring therapeutic approaches, some of which focus on interfering the binding of the virus to ACE2 receptor in the host cells. This may contribute to reduce the viral load of patients, but also could serve as a prophylactic tool to be used in highly exposed people. The administration of soluble ACE2 is a strategy aiming to serve as a decoy receptor, trapping the virus, and to take advantage of tissue protective and anti-inflammatory properties associated to carboxypeptidase activity of this molecule. The fusion to Fc regions could potentially ameliorate the positive impact of exogenous ACE2 supply in COVID-19 patients by increasing half-life and avidity for the virus, among other practical benefits. In the present article, we review several strategies to address the optimization of these Fc fusion proteins through the discussion on the different formats, design principles, benefits and concerns and the opportunities of implementing combinations.

KEYWORDS: ACE2; COVID-19; Fusion protein; SARS-CoV-2; Neutralization

ABBREVIATIONS: ACE2: Angiotensin Converting Enzyme-2

INTRODUCTION

During the last two years the humanity has been hit by Covid-19 pandemic, whose etiologic agent is SARS-CoV-2 virus. Covid-19 is a very complex disease able to produce respiratory distress, hypertension, heart failure and/or systemic inflammation, among other manifestations that may conduce to death [1]. The high transmissibility of SARS-CoV-2 and high mortality respect to other coronaviruses has gained the attention of all scientific community who is intensely seeking for a way to impede its expansion. Many efforts have been concentrated in the development of vaccines, with undeniable success [2,3]. However, the emergence of new variants menaces the triumph of the active immunotherapy. As the virus spread hasn't been controlled novel strains are continuously being reported, and some of them, like beta and omicron, have shown a clear reduction in the protection conferred by the vaccines currently approved [4-6]. This situation encourages further investigation on therapeutic approaches that work at different infection stages. Several strategies have been explored to block the binding of the virus to its receptor. Some of them relies on mAbs but are also susceptible to alterations in the SARS-CoV-2 structure [7,8]. Thus, the use of a decoy receptor has been a more straightforward approach that would act independently of the mutations occurring in the virus. Herein, we review the use of receptor-based traps aiming to block the virus's accessibility to the cell, with a special emphasis in the Fc fusion derivatives of these proteins.

ACE2 As Target for Covid-19 Therapy

SARS-CoV-2, with an RNA genome, belongs to the *Coronaviridae* family and infects a wide spectrum of animal species like birds and mammals, including humans [9]. The virus contains four structural

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proteins: the spike, envelope, membrane and nucleocapsid [10]. The spike is responsible for the binding to the receptor in human host plasma membrane, through its receptor-binding domain (RBD) and mediates the entry. The virus interacts with the host receptor angiotensin-converting enzyme 2 (ACE2) and internalizes into the cells, by using mostly the host cell endocytic pathways [11,12]. This receptor has been also the gateway for entry of other coronaviruses like SARS-CoV and NL63 [13]. The affinity of ACE2 binding to SARS-CoV-2 is 10~20-fold higher than that of SARS-COV, which may be associated to the severity of COVID-19 [14]. The binding of SARS-CoV spike to the receptor conduces to cleavage of the spike by proteases such as TMPRSS2/4, furin, and/or cathepsins, and further mediates the fusion of the viral membrane to the plasma membrane [11]. Moreover, new reports describe the existence of additional factors that can modulate or promote the entry such as AXL, Neuropilin-1 (NRP-1), Basigin (CD147), AT1 (Angiotensin II receptor type 1), and AVPR1B (Vasopressin V1b receptor) proteins [15-17]; in any case, ACE2 is widely recognized as the primary receptor.

On the other hand, ACE2, a type I transmembrane protein, is broadly distributed throughout the human body. It is expressed in the kidney, testis, intestine, lung, retina, cardiovascular system, adipose tissue and central nervous system [18,19]. However, several works unveil a gradient of ACE2 expression in the upper and lower airways and terminal airspace compartments [20-23]. More specifically, the highest expression has been observed in ciliated cells of the proximal airway and the alveolar type II cells of lung epithelium [24]. The human ACE2 protein (805 amino acids), consists of an extracellular N terminal claw-like protease domain (PD) and a C-terminal collectrin-like domain with a cytosolic tail [19,25]. The PD can bind RBD of SARS-COV-2, enabling virus entry [13,26]. ACE2 helps transporting of amino acids across the membrane [27]. Nevertheless, the most important physiological role is associated to its carboxypeptidase function, by converting angiotensin I (Ang I) to Ang 1-9 or Ang II to Ang I 1-7. Additionally, it is able to process other vasoactive peptides like neurotensin, kinetensin, and des-Arg bradykinin [28]. These actions oppose to those of its homolog ACE and are critical in the regulation of renin angiotensin system (RAS) (Figure 1).

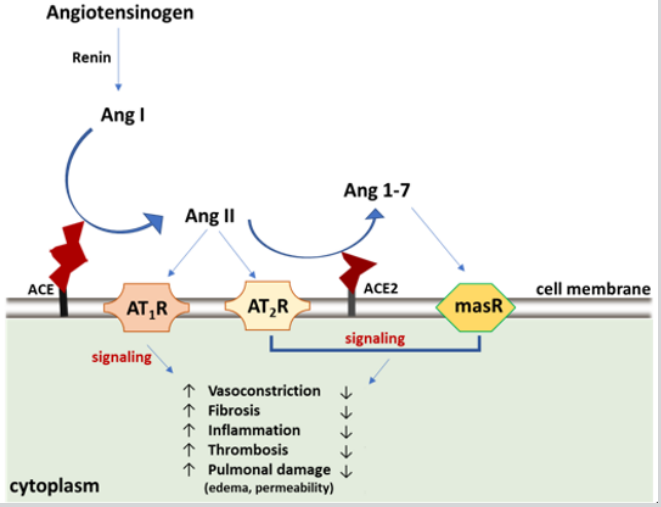

Figure 1: Role of ACE2 in the regulation of renin-angiotensin system (RAS).

Previous evidences point out that reinforcing the ACE2-Ang I (1-7)-MasR axis decreases cytokine release and protects against organ injury in many human diseases, including cardiovascular disease, obesity, chronic kidney disease, liver diseases and lung injury [29-32] (Figure 2).

Therefore, the use of soluble ACE2 for COVID-19 therapy is sustained on its capability of acting as a trap for SARS-CoV-2 virus, facilitating its neutralization, and also the possibility of contributing to protect from lung injury and acute respiratory distress syndrome through restoring the balance of RAS. The latter effect would be the consequence of counteracting viral-induced ACE2 protein shedding and ACE2 protein decreased expression, upon the spike protein binding [33]. In line with this, it is noteworthy to highlight that the subsequent recovery of pulmonary ACE2 is shown to be critical to prevent excessive neutrophil infiltration and protect from the lung inflammation progression [34].

Design of ACE2-Fc fusion proteins

Different formats and composition of ACE2-Fc have been described in the literature, which differ fundamentally in:

a. The isotype of Fc

b. Carboxypeptidase activity,

c. Binding to RBD and

d. The length of ACE2.

Excepting isolated reports [35,36], most of the referenced recombinant proteins have been expressed in mammalian cells, consistent with the complexity of these large glycoproteins. Ultimately, the design features of each variant not only must respond to a functional need but also meet the requirements to guarantee a good manufacturability.

Fc Region

While soluble ACE2-based therapies have exhibited promising effects for the treatment of COVID-19 [37] the short half-life time in the blood of this protein makes it difficult to use as a treatment [38]. On this subject, ACE2 molecules fused to Fc domains of human immunoglobulins have been generated given the role of Fc region in prolonging the half-life of the fused proteins upon interaction with FcRn [39-42]. In addition, fusion to Fc regions has other benefits such as enhanced avidity, increased protein expression and easy purification of recombinant molecules by protein A chromatography [43,44]. Moreover, the coupling to Fc

region allow taking advantage of the biological effector functions of this domain [45]. That is why several constructs use the constant region of an IgG1 antibody. Thus, the recruitment of dendritic cells, macrophages, and natural killer cells through the CD16 receptor engagement by the Fc region against viral particles or infected cells may facilitate the elimination of the virus via phagocytocis and ADCC [46]. A recent work of [47] showed that ACE2-Fc was able to activate *in vitro* NK cell degranulation after coincubation with Spike-expressing H1975 cells, which may be a possible mechanism for clearance of infected cells *in vivo*.

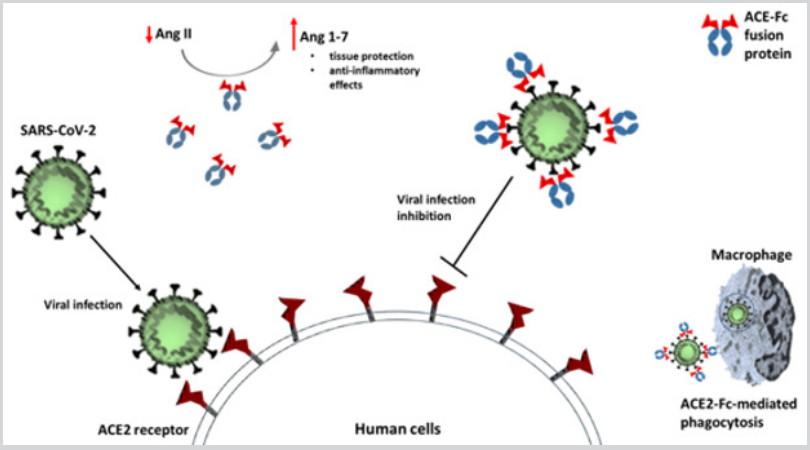

Figure 2: ACE2-Fc as a therapeutic agent to inhibit SARS-CoV-2 viral infection: Spike protein on the SARS-CoV-2 surface binds to ACE2 on plasma membrane, leading to viral uptake by host cell and the initiation of infection. The soluble ACE2 extracellular domain with a prolonged half-life because of fusion to Fc region, blocks this interaction and prevents infection. For certain designs, the presence of functional Fc domain may contribute to virus clearance upon the engagement with Fc gamma receptor on innate immune cells (e.g., Macrophages). Catalytically active ACE2 moiety can provide protection through the reduction of Angiotensin II (AngII) and, in hence, the consequences associated to activation of ATR1 signaling. This effect is reinforced by the increase of Ang 1-7 and triggering of MasR axis (Figure 1).

However, a potential concern relies on the possibility that Fc interaction with CD16 receptor turns into an alternative way of binding to virus and further entry of the pathogen to the cell. This problem has prompted the use of IgG4 isotype domain to largely reduce Fc binding to CD16 [48]. This strategy would also minimize the risk of provoking a disease enhancement by the Fc functions such as complement dependent cytotoxicity (CDC) and antibody dependent cytotoxicity (ADCC), according to accumulated knowledge with neutralizing antibodies [49,50]. The introduction of mutations in Fc region has also served to this purpose, being L434A L435A (LALA) one of the most frequently used [51].

Carboxypeptidase Activity

It is well known that lung injury, heart and renal failure and hypertension depict the severe forms of COVID-19 and that exacerbated inflammation is determinant in the progression and outcome of the disease. This fact supports the rationale of using the enzymatic activity of soluble ACE2 receptor, considering its important role in the regulation of inflammatory responses, cardiovascular system function and tissue protection. This concept has been followed by several research groups who have proposed an ACE2 therapeutic agent with peptidase activity. For that reason, many constructs are based on the full-length ectodomain of native human ACE2 comprising residues 18-740 (Q9BYF1, Uniprot). In order to facilitate the manufacturability of the molecule some approaches contemplate the use of truncated forms of ACE2 portion [47,48] without compromising the enzymatic activity. The administration of competent ACE-2 based biologics has been backed up by a good safety profile in a study of repeated doses of ACE2-Fc in mice for up to two months and Phase I and Phase II clinical trials for human pulmonary arterial hypertension and acute respiratory distress syndrome (ARDS) (ClinicalTrials.gov identifier: NCT04335136).

Despite these assuring evidences, there is a latent concern associated to the unknown effects of high extracellular levels of ACE2 in the organism resulting from a therapy based on a functional peptidase receptor. The catalytic activity of therapeutic recombinant soluble ACE2 (hrsACE2) hydrolyses a broad range of vasoactive hormonal substrates including AngII, apelin-13, bradykinin metabolites, important mediators of RAS that ultimately may have an impact in several organs such as the heart, the blood vessels, the kidney and the lung. To date, it is not well demonstrated the harmlessness of this additional carboxypeptidase activity in the context of a multiorgan related disease like COVID-19. This scenario has defined a strategy in ACE2-Fc design characterized by the introduction of mutations in the receptor moiety to diminish or completely abrogate the enzymatic activity to potentially avoid adverse events (Table I). One example is the work of [51] that demonstrated the protective effect *in vivo* against SARS-CoV-2 of a variant of ACE2-Fc with no catalytic function.

Binding to RBD

Since virus neutralization is the main goal of ACE2 based therapeutics in COVID-19, the increase of affinity for SARS-CoV-2 spike has been undertaken with molecules bearing or not Fc regions. To this end it has been also assayed the engineering of trimeric ACE2 proteins aiming to compete more efficiently with dimeric ACE2 on the host cell membrane because of their higher avidity. These trimeric ACE2 variants (some of them with inserted mutations) have translated into enhanced binding to spike and, in turn, improved antiviral effect [52,53]. Among the Fc fusion proteins highlights the case of MDR504 hACE2-Fc, containing one mutation intended to modify ACE2 activity that resulted in an increased binding to RBD and spike of SARS-CoV-2, and a higher neutralization potential, as measured by pseudovirus assay [51].

Table 1: ACE2-Fc fusion proteins for the treatment of COVID-19.

Therapeutic Agent	Expression System	Fc Isotypes	Carboxypeptidase Activity	RBD Binding	Biological Observations	Refs.
ACE2-Fc	Mammalian HEK293 cells	Human IgG1	Three mutants with inactive enzyme activity (R273A, H378A and E402A mutants)	Similar levels of binding to viral RBD compared to wild type ACE2. EC50 (ng/mL) of 12.6, 26.9 and 21.8 for the R273A, H378A and E402A mutants	All variants showed similar levels of efficacy to block pseudoviral transduction. Wild-type ACE2-Fc had a leading IC50 of 0.13 μg/mL, followed by H378A, R273A and E402A with their IC50s of 0.16 μg/mL, 0.19 μg/mL and 0.25 μg/mL, respectively	45
ACE2-Fc	Mammalian Expi293 cells	Human IgG1	Molecule with two catalytic inactivation mutations (H374N and H378N)	Lower binding to SARS-CoV-2 compared to wild type ACE2. Kd of 8.23 nM and 0.161 nM for spike RBD and Spike extracellular domain	Neutralizations of various SARS-CoV-2 strains including D614G and V367F mutations, as well as the emerging SARS-CoV-2 variants, B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.1 (Kappa), and B.1.617.2 (Delta). Prophylactic and therapeutic hACE2-Fc treatments effectively protected mice from SARS-CoV-2 infection	62
ACE2-IgG4-Fc	Mammalian FreeStyle 293-F Cells	Human IgG4/ kappa isotype with a S228P sequence alteration	maintains enzymatic activity of ACE2	Kd of 4nM	Exhibit promising pharmaceutical properties and a broad antiviral activity at single digit nanomolar concentration.	48
ACE2-Fc	Plant cells (glycoeng-ineered *Nicotiana benthamiana*)	Human IgG1	maintains enzymatic activity of ACE2	Kd of 10.1 ± 0.2 nM	The produced dimeric ACE2-Fc variant is glycosylated with mainly complex human-type N-glycans and functional with regard to enzyme activity. It showed wild-type virus neutralization.	35
ACE2-Fc	Plant cells (*Nicotiana benthamiana*)	Human IgG1	maintains enzymatic activity of ACE2	Retains binding to RBD	Exhibited potent antiSARS-CoV-2 activity in vitro. Treatment with ACE2-Fc fusion protein after viral infection dramatically inhibit SARS CoV-2 infectivity in Vero cells Moreover, treatment with ACE2-Fc fusion protein at the pre-entry stage suppressed SARS-CoV-2 infection with an IC50 of 94.66 μg/ml.	36
MDR504 hACE2-Fc	Mammalian HEK293T cells	Human IgG1 with LALA mutations	completely abrogated catalytic activity (unspecifed mutation, called MDR504)	Significantly increased binding to SARS-CoV2 spike protein compared to the wild type ACE2	Parenteral administration of the protein showed stable serum concentrations with a serum half-life of ~ 145 hours. Prophylactic administration of ACE2-Fc significantly attenuated SARS-CoV-2 infection in a murine model.	51
SI–F019	Mammalian ExpiCHO cells (transient expression). CHOZN cell (stable cell line)	Human IgG1 (Fc null: LALA, K322A mutation)	maintains enzymatic activity of ACE2	Kd of 21.8nM	SI–F019 binds with increased affinity to variant forms of RBD (corresponding to variants of concern) relative to wild-type RBD, in contrast to monoclonal antibodies which tended to show weaker binding to some variants. SI–F019 shows good neutralization efficacy for the original strain and even better neutralization for variants.	63

From monotherapy to combined treatments

The introduction of soluble ACE2 to fight COVID-19 repurposed these receptor-based biologics, previously used with mild success in ARDS but with favorable results regarding safety and tolerability [54]. The first evidences of the clinical use in COVID-19 were reported in 2020. [37] and colleagues demonstrated that hrsACE2 treatment in a severe patient induced a rapid viral clearance in plasma and respiratory system. This effect was accompanied by a marked reduction of Angiotensin II, IL-6 and IL-8 levels. Further results from a Phase II clinical trial showed benefits of hrsACE2 for severely ill COVID-19 patients, as confirmed by the improvement in mechanical ventilator-free days and reduction in viral RNA load [55]. On the other hand, receptor-Fc fusion proteins have already reached the clinics. An ACE2-Fc fusion protein developed by Shanghai Henlius Biotech, Inc. (HLX71), started to be evaluated in healthy individuals and the recruitment of volunteers is now completed (ClinicalTrials.gov Identifier: NCT04583228). Although scarce results are available, the experience with one subject demonstrated that it was safe and well tolerated [56]. Another phase I clinical trial is being conducted in healthy participants with SI-F019, a recombinant Human Bivalent ACE2-Fc Fusion (ClinicalTrials.gov Identifier: NCT04851444). In order to increase the success rate in a very complex and multifactorial disease like COVID-19, combined treatments have also been pursued, yet in preclinical studies. The combination of hrsACE2 with other therapeutic modalities, mainly RNA polymerase inhibitor or antisense single-stranded DNA, resulted in effectively reduced cell infection with SARS-CoV2 *in vitro*

[54,57]. Additionally, Liu et al. proposed the combination of HLX71 (ACE2-Fc) with HLX70, a therapeutic neutralizing antibody (NAb) [45]. The two recombinant proteins cocktail elicited synergy in blocking the binding of RBD to hACE2 on HEK293T, and in antiviral activity [45]. Furthermore, it was demonstrated that the combined treatment significantly enhanced N501Y virus mutant clearance, *in vivo*. Moreover, HLX70+HLX71 could effectively neutralize *in vitro*, 45 virus mutants including the HLX70-resistant variants and the widely circulated mutants (N501Y, P.1, B.1.1.7, B.1.429, B.1.526-1, B.1.617, and 501Y.V2-1) [45]. Other combination therapy aimed targeting two different stages of virus cycle: cell entry via its receptor ACE2 and intracellular viral RNA replication. In this sense, the combinatorial treatment of hrsACE2 and remdesivir enhanced the reduction of viral load and viral progeny *in vitro* [54].

A bispecific fusion protein based on the ACE2 enzyme has also been reported. This is ACE-MAB, a chimeric molecule composed by a full-length human antibody against the SARS-CoV-2 Spike fused to a truncated version of the ACE2 [58]. In summary, the therapeutic possibilities of ACE2 for COVID-19 are still underexploited and a wide spectrum of approaches remain to prove its efficacy in the clinical setting. The selection of the ACE2-Fc construct and proper ACE2 concentrations, treatment schedule and stage of the disease have to take into account several considerations to get better results with minimal toxicity. Recent studies have informed that sACE2 can also act as the receptor of SARS-COV-2, mediating viral entry into the cell [59,60]. However, it has been proved that rhACE2 concentrations (~10–200 mg/mL), much higher than the physiological ones, block SARS-COV-2 infection rather than facilitating the virus entry, which is optimal at concentrations near the physiological range (i.e., ng/mL level) [60]. Anyhow, further studies need to be performed to elucidate the optimal ACE2 concentrations to use in patients. The knowledge accumulated on the pathophysiology of COVID-19 suggest that the administration of exogenous ACE2 at initial stages of the disease, to inhibit viral infectivity, should be better than more advanced stages where the intensified inflammation is established and independent of active viral driver [61-63].

CONCLUSION

Exogenous administration of ACE2 represents a promising therapeutic approach to combat COVID-19. The use of ACE2-Fc fusion proteins may improve this receptor-based treatment by increasing the half-life and, in turn, the bioavailability of the molecule, among other advantages. Novel designs have been proposed to potentiate the functionality of these virus traps and, in some cases, to reduce the potential negative impact associated to extra ACE2 peptidase activity, all of them to be validated in the clinical setting. Anyway, the outstanding results of ACE2-Fc biologics in preclinical investigations uncover the great opportunities of this therapy, less likely to be menaced by the emergence of SARS-CoV-2 new variants.

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