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Extracellular vesicles and angiotensin-converting enzyme 2 in COVID-19 disease

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Abstract: Extracellular vesicles (EVs) are membranous vesicular structures released from almost all eukaryotic cell types under different physiological or pathological conditions. Growing evidence demonstrates that EVs can serve as mediators of intercellular communication between donor and recipient cells or microorganism-infected and noninfected cells. Coronavirus disease 2019 (COVID-19) disease is caused by infection of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) of host cells in the respiratory system and various extra-pulmonary tissue/organs, resulting in complications of multiple organ systems. As the cell surface receptor, angiotensin-converting enzyme 2 (ACE2) mediates cellular entry of SARS-CoV-2 into the host cells in patients with COVID-19. Recent studies have found that ACE2 can be released with EVs, which have been shown to interfere with the entry of the virus into host cells and thus may be involved in COVID-19 pathophysiology. In addition, ACE2, neprilysin (NEP), and thimet oligopeptidase (TOP) are the key enzymes that regulate angiotensin metabolism by converting angiotensin II or angiotensin I to angiotensin 1-7, the latter of which has protective effects in counterbalancing the harmful effects of angiotensin II in COVID-19 disease. This review summarizes the recent research progress regarding EV-associated ACE2, NEP, and TOP and the perspectives of their potential involvement in the pathophysiology of COVID-19 disease.

Introduction

Cell membrane extracellular vesicles (EVs) are subcellular membrane structures which play physiological and pathological roles in human health and diseases [1,2]. EVs are classified into three groups based on their size, including exosomes (<100 nm), microvesicles (MVs) (<1 μm), and apoptotic bodies (1–5 μm) [1,3,4]. Exosomes are formed by the exocytosis of endosomal multivesicular bodies into the extracellular milieu [1,2]. MVs are membrane vesicles derived from the cell plasma membrane surface [1,2,5]. Apoptotic bodies are large membrane vesicles released in the late stages of apoptosis and can carry nuclear fragments and mitochondria [1,2]. EVs are generated during cell activation, senescence, or programmed cell death, i.e., apoptosis, necroptosis, pyroptosis, and NETosis (a unique neutrophil death) [1,2,4].

EVs are heterogeneous in size and composition [1,4–8]. Like their parental cells of origin, EVs have double-layer lipid membranes and may contain cellular components from the nucleus, cytoplasm, or cell membrane [1,4–8]. When EVs bud off of their parental cells, they harbor a vast array of bioactive molecules, including lipids, proteins, and nucleic acids (DNA, RNA, siRNA, microRNA, and lncRNA) [1,4–8]. EVs have been found in various biological fluids, including blood, urine, bronchoalveolar lavage fluid (BALF), and other body fluids, as well as in the tissues/organs of both humans and animals [1,2,4].

Over the past few years, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has caused a global pandemic of the coronavirus disease-2019 (COVID-19), resulting in over 771 million cases with over 6.96 million deaths worldwide according to statistical information from the World Health Organization [9]. The SARS-CoV-2 virus infects host cells by binding cell membrane surface angiotensin-converting enzyme 2 (ACE2), a viral entry receptor in the respiratory system and extra-pulmonary systemic tissue/organs in patients with COVID-19 disease [10,11]. Interestingly, membrane ACE2 can also be released with EVs (EV-ACE2) from

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TABLE 1

Angiotensin metabolizing enzymes and EVs

Enzymes	Processing of angiotensin	Cellular location	Release with EVs	COVID-19 involvement	References
ACE2	Angiotensin I Angiotensin II	Plasma membrane	Yes	Yes	[11,12]
NEP	Angiotensin I	Plasma membrane	Yes	Yes	[19]
TOP	Angiotensin I	Cytosolic, Plasma membrane	Yes	Not yet investigated	[5]

Note: ACE2, angiotensin-converting enzyme 2; NEP, neprilysin; TOP, thimet oligopeptidase; EV, extracellular vesicles.

SARS-CoV-2 infected cells (Table 1) [11,12], and elevated levels of EVs have been detected in patients with COVID-19 disease [13]. Classically, ACE2 is an enzyme with activities to convert angiotensin II to angiotensin 1-7 in the renal-angiotensin system (RAS) [5,14]. Importantly, several studies have reported elevated production of angiotensin II and decreased production of angiotensin 1-7 in COVID-19 patients [15,16]. In addition, several other enzymes, i.e., neprilysin (NEP) [17] and thimet oligopeptidase (TOP) [5,18], are also involved in angiotensin metabolism and production of angiotensin 1-7 (Fig. 1). Studies from our and other groups have found that both NEP [19] and TOP [18,20,21] can be released with EVs (Table 1). Here, we summarize recent research progress regarding EVs, their association with ACE2, NEP or TOP enzymes, and their relevant effect on angiotensin metabolism, as well as their potential involvement in SARS-CoV-2 infection and pathogenesis of COVID-19 disease.

Extracellular vesicles and viral infection

Increasing evidence suggests that EVs play important roles in viral infections [1,4–6,8]. EVs can serve as vehicles for intercellular communication and the exchange of bioactive molecules between donor cells and recipient cells [1,4,8]. In addition, ligands or other cell surface molecules on EVs can bind to receptors of target cells, thus triggering intracellular signaling and resulting in inflammatory or immune responses [1,4,5,8].

EVs and viruses share similar physicochemical properties, such as small size and heterogenous size distribution [22]. Furthermore, viruses can utilize EV endocytic routes to enter noninfected cells and hijack the EV secretory pathway to exit infected cells. Thus, both share common cellular entry and biogenesis mechanisms [23,24]. Interestingly, a viral infection of cells can trigger the production of EVs through different mechanisms [25]. In turn, EVs from infected cells also play pathogenic roles in viral infectious diseases [26]. The EVs from infected cells may carry viral components or even entire virions and transfer viral components from infected cells to uninfected cells [23–25,27], thus promoting the spread of viral infection. In addition, Caobi et al. (2020) reported that EVs from virus-infected cells carry viral antigens that can be recognized by immune cells, leading to the activation of antiviral immune responses [28]. EVs from uninfected cells may contain antiviral interferons, inducing an antiviral state for restriction of viral replication [25,29].

Role of extracellular vesicles in COVID-19 disease

Like other viruses, SARS-CoV-2 can induce EV release from different cell types in infected patients [30,31]. In COVID-19 disease, the SARS-CoV-2 virus invades human host cells by infecting mucosal cells that express ACE2, the receptor for binding and entry of the SARS-CoV-2 virus into host cells [32–35]. The virus binds the host ACE2 receptor via two virus surface spike proteins [32]. As the host cell entry receptor, ACE2 likely plays a key role in determining host

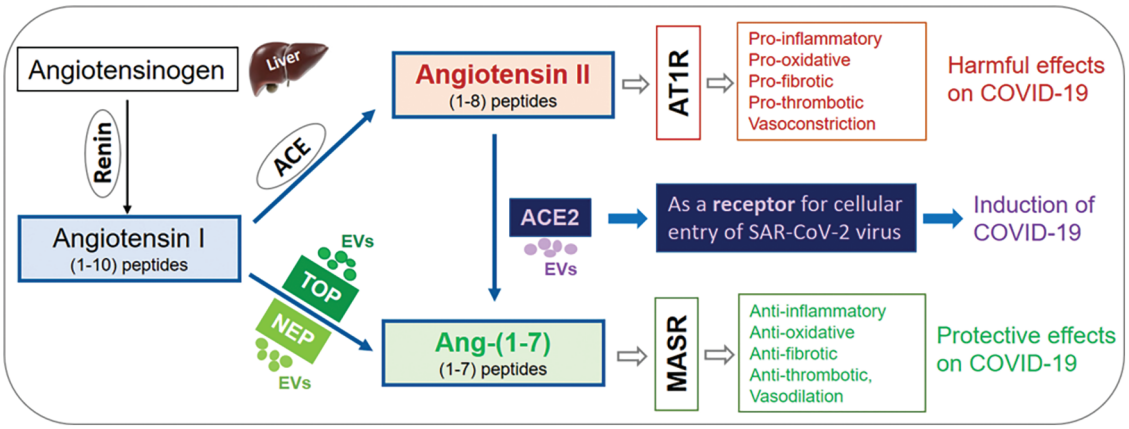

FIGURE 1. Angiotensin metabolizing enzymes, EVs, and COVID-19 disease. Schematic illustration of angiotensin metabolism and angiotensin metabolizing enzymes, ACE2, NEP, and TOP, which can be released with EVs, as well as their roles in the metabolism of angiotensin I, and angiotensin II. This figure also highlights the potential involvements of angiotensin II, ACE2, and angiotensin 1-7 in COVID-19 disease. ACE2: angiotensin-converting enzyme 2; NEP: neprilysin; TOP: thimet oligopeptidase; EV: extracellular vesicles; COVID-19: coronavirus disease 2019; Mas receptor (MASR).

cell tropism and infectivity by the virus [32]. ACE2 is abundantly expressed not only in the respiratory system [10] but also in multiple extra-pulmonary tissues, directing the viral damage of host cells towards different organ systems [10,11,36]. Some studies have reported elevated ACE2 expression in infected organs of COVID-19 patients [37,38], while other studies suggest SARS-CoV-2 binding may result in downregulation of ACE2 expression [39,40]. There is, thus, significant controversy regarding the role of ACE2 in COVID-19 pathogenesis.

As discussed earlier, SARS-CoV-2 infection occurs through interaction between the viral spike protein and the host receptor ACE2 [29,41]. ACE2 has been detected in EVs from pulmonary cells in COVID-19 BALF [42,43], airway liquid secretions for pathological evaluation of various lung diseases [44,45], including COVID-19 lung diseases. It is noteworthy that EVs containing high levels of ACE2 in BALF from patients with severe COVID-19 are associated with reduced intensive care unit (ICU) and hospitalization times [43]. Furthermore, the level of ACE2 on the ACE2-EV surface is positively correlated with its ability to block SARS-CoV-2 [46]. El-Shennawy et al. (2022) reported that EV-associated ACE2 shows 135-fold higher potency in blocking the binding of SARS-CoV-2 viral spike protein to human host cells *in vitro* as compared to vesicle-free recombinant ACE2, thus blocking viral infection of host cells by acting as decoy sites of binding [11]. Importantly, studies using ACE2-expressing EVs generated from engineered cell lines, which were transfected with ACE2-expressing plasmids for stable ACE2 expression, have shown protective potential in the treatment of COVID-19 disease [11,43,47,48]. Therefore, engineered ACE2-EVs may serve as an alternative therapeutic strategy in addition to other treatment methods, while plasma levels of ACE2-EVs may serve as a clinical biomarker for the prognosis of COVID-19 disease severity.

EVs from viral infected host cells contain viral components, including viral RNA and proteins, which may contribute to viral replication and immune evasion [30,31]. In addition, EVs also carry host proteins, i.e., ACE2, CD9, transmembrane serine protease 2 (TMPRSS2), which can mediate the viral entry process and COVID-19 infection by aiding in viral incorporation into host cells [41,49–52]. ACE2-carrying EVs can compete with host cell surface ACE2, blocking SARS-CoV-2 virus binding and infection of host cells [11]. However, ACE2-carrying EVs may also be transferred to target receptor-null cells, thus making these previously naïve cells more susceptible to viral infection [53]. In addition, EV-associated TMPRSS2 can cleave the spike protein of the SARS-CoV-2 virus, which enables the virus to bind to the receptor, and subsequently enter host cells [49,52]. Previous studies have shown that CD9 may collaborate with TMPRSS2 in cleaving viral fusion glycoproteins and facilitate quick entry of coronavirus (e.g., MERS-CoV) into lung cells [54]. Studies also showed that membrane surface CD9 may also be involved in entry and exit mechanisms in respiratory viruses, including SARS-CoV-2 virus [48,55–57].

EVs have been implicated in the progression of cytokine storm, the main mechanism for severe illness and mortality in COVID-19 patients [50]. EVs derived from immune cells or lung epithelial cells in COVID-19 patients carry pro-inflammatory cytokines, chemokines, and damage-associated molecular patterns (DAMPs) [29,58], which all enhance inflammatory responses. An array of lung cells, such as epithelial cells, endothelial cells, and alveolar macrophages, can all release EVs [50,59], contributing to elevated levels of EVs in COVID-19 patients. Higher concentrations of EVs have been detected in the pulmonary edema fluid of patients with acute respiratory distress syndrome compared to controls [60]. In lipopolysaccharide (LPS)-induced cytokine storm models, EVs in BALF can initiate inflammatory responses in the lung via enhanced expression of tumor necrosis factor-α, interleukin-6, and junction proteins [61]. Administering natural sphingomyelinase inhibitor GW4869 in LPS-induced lung inflammation mouse models significantly decreased the level of pro-inflammatory cytokines and reduced lung elastance and alveolar collapse, thus providing protection against the effects of cytokine storm [62], probably due to the inhibitory effects of GW4869 on EV generation [63]. These studies support the involvement of EVs in initiating and aggravating cytokine storm.

It has long been known that EVs carry molecules, i.e., tissue factor (TF) and phosphatidylserine (PS), with pro-coagulant activities [1,64], which is also important in propagation of the pro-coagulant state in certain serious complications of COVID-19 disease [13,65]. EVs from COVID-19 patients have been shown to carry increased levels of biologically active TF [66–68], a key initiator of the extrinsic coagulation pathway, or membrane PS [69], an essential cofactor of coagulation, on EV surfaces. In fact, TF-EV levels were markedly higher when associated with COVID-19 and demonstrated increased fibrinolytic activities as compared to coagulation processes associated with septic shock in non-COVID-19 cases [66,70]. TF-EV activity has been associated with COVID-19 disease severity and mortality [67]. In addition, levels of TF-EV activity have been found to correlate with plasma D-dimer, which is known to be associated with thrombosis in COVID-19 [67]. Furthermore, SARS-CoV-2 infection causes systemic inflammation and over-activation of the immune system along with endothelial dysfunction and platelet activation, which are the known functions of EVs in immune system-related diseases [64,71]. All of the above lead to systemic pro-thrombotic states, which result in micro-thrombosis and multi-organ damage and failure in patients with COVID-19 [70,72].

Extracellular vesicles, ACE2, angiotensin 1-7 axis and COVID-19 disease

In addition to being the viral entry receptor for SARS-CoV-2 infection [10], ACE2 classically functions as an enzyme in the renin–angiotensin system (RAS) to catalyze angiotensin metabolism and regulate the balance between angiotensin II and angiotensin 1-7 (Table 1), contributing to systemic complications of COVID-19 disease [51,73]. ACE2 is a transmembrane enzyme with a short cytoplasmic domain, a transmembrane domain, a catalytic ectodomain, and an amino-terminal signal peptide [73–75]. ACE2 can be detected as both membrane bound and solute forms [75].

Both membrane-bound and soluble forms of ACE2 can actively cleave angiotensin II [75]. Recent studies have found that ACE2 can be either released as full-length molecules with EVs (EV-ACE2) [11,12] or shed as ectodomain ACE2 exomeres (ecto-ACE2) through cleavage by ADAM10/17 [12,76]. Both forms of EV-associated ACE2 can bind to SARS-CoV-2 and inhibit its infection [12]. Levels of ACE2-positive EVs are associated with the severity of COVID-19 disease, indicating their defensive properties for maintaining homeostatic conditions in COVID-19 patients [11]. Studies have also reported the increased production of angiotensin II and decreased production of angiotensin 1-7 in COVID-19 patients [15,16,77], while the balance of angiotensin II in angiotensin 1-7 is regulated by ACE2 [5,14]. However, angiotensin 1-7 also has regulatory effects on ACE2 expression, and continuous infusion of angiotensin 1-7 has shown organ-specific downregulation effects on local ACE2 expression *in vivo* in rats [78]. Therefore, there may be a tightly regulated equilibrium between angiotensin I/II and angiotensin 1-7 as well as ACE/ACE2. In the context of COVID-19, a few studies have shown that SARS-CoV-2 binding can downregulate ACE2 expression [39,40].

As the major biologically active effector peptide of the RAS system [79–81], angiotensin II can activate AT1 (angiotensin II receptor type 1) receptor and trigger pro-inflammatory, prooxidative, pro-fibrotic, pro-thrombotic and vasoconstrictive effects, contributing to the severity of COVID-19 disease (Fig. 1) [73,79–81]. In contrast, angiotensin 1-7 has a range of anti-inflammatory, antioxidant, vasodilatory, and natriuretic protective effects through the G protein-coupled receptor (GPCR) MAS receptor (Fig. 1) [73,79,80]. Furthermore, angiotensin 1-7 also has a critical role in protecting against lung inflammation and fibrosis [79,80]. Recent studies have reported a potential link between angiotensin 1-7 and COVID-19 disease severity [82–86]. Decreased levels of blood angiotensin 1-7 have been reported in COVID-19 patients who were either severely ill or have died [83,84,86]. In contrast, higher levels of angiotensin 1-7 have been associated with reduced COVID-19 disease severity [83,84,86]. Furthermore, dysregulation of ACE2, angiotensin I/II, and angiotensin 1-7 are associated with mortality and end-organ damage in COVID-19 patients [87,88]. In fact, angiotensin 1-7 peptide replacement therapy has been proposed for the treatment of severe COVID-19 [79,85,89]. Both membrane-bound and soluble forms of ACE2 can actively cleave angiotensin II to angiotensin 1-7 [75], which has anti-inflammatory, antioxidant, and vasodilatory effects. Thus, EV-associated ACE2 may also be involved in COVID-19 by regulating the conversion of angiotensin II metabolism [75]. Although no relevant studies have yet been reported, it would be worthwhile to investigate the catalytic activities of circulating EV-associated ACE2 and its effects on the metabolism of angiotensin I/II and angiotensin 1-7, as well as their relevance to the severity of COVID-19 disease. Interestingly, several studies have reported that treatment with ACE inhibitors or angiotensin receptor blockers (ARBs) was associated with lower levels of inflammation and reduced risk of COVID-19 disease [90,91]. However, meta-analyses [92,93] and a randomized clinical trial [94] did not support the findings of these initial studies.

Extracellular vesicles, Neprilysin and Thimet Oligopeptidase, and COVID-19 diseases

In addition to ACE2, NEP and TOP are endopeptidases that can convert angiotensin I to angiotensin 1-7 by cleaving internal peptide bonds [5,17,18]. Interestingly, both NEP and TOP can be released with EVs based on recent studies from our and other publications [5,18,19]. Importantly, EV-associated NEP and TOP are enzymatically bioactive (Fig. 1) [5,18,19].

A recent study reported that NEP inhibitor sacubitril can inhibit further cleavage of angiotensin 1-7 [17], and therefore, may favor the preservation of angiotensin 1-7 [17]. Studies have shown the beneficial effects of sacubitril on the regulation of angiotensin 1-7 and the improvement of outcomes of COVID-19 disease [15,17,95–97]. Thus, several clinical trials using neprilysin inhibitor sacubitril have been proposed for the treatment of COVID-19 for a multi-targeted therapeutic approach [15,98]. In fact, sacubitril administration has shown the beneficial effects in COVID-19 patients with lung or cardiovascular complications [15,17,98]. NEP is abundantly expressed in many tissues/organs, including the lungs [99]. The above studies provide insights and also indicate the potential importance of NEP in COVID-19 pathogenesis. The roles of EV-associated NEP in the pathophysiology of COVID-19 disease, the plasma levels of NEP-positive EVs and their existence in BALF in COVID-19 patients, and their association with disease severity of COVID-19 have not yet been investigated. These are important questions to be explored in future studies.

TOP is primarily located in the cytosol, but it can also be associated with the cell membrane or secreted into the extracellular space (Table 1) [5,18]. About 20–25% of total TOP enzyme activity is associated with membrane fractions [100], and TOP has been visualized on the plasma membrane surface by confocal microscopy [5,18,101]. Interestingly, we have recently demonstrated that membrane-associated TOP can be released with EVs to the extracellular space [18,20,21]. Most importantly, EV-associated TOP exhibits considerable enzymatic activity [5,18,20,21]. Our findings suggest that EV-associated TOP might be a previously unrecognized, novel form of extracellular TOP [5,18,20,21], in addition to its soluble form in the extracellular milieu. The soluble form of TOP easily diffuses into the circulation and can be diluted by large volumes of systemic circulation. In contrast, EV-associated TOP may stay in the microenvironment in relatively higher concentrations [18,20,21]. Thus, the EV-associated format of TOP may enable the enzyme to work more potently in the local tissue/organs on its substrates, i.e., angiotensin I, and contribute to the progression of the relevant pathological conditions. Since TOP is known to convert angiotensin I *in vitro* to the biologically active peptide angiotensin 1-7 [102], one may expect that bioactive EV-associated TOP may also be able to convert angiotensin I to angiotensin 1-7. TOP converts angiotensin I to angiotensin 1-7, providing a path to side-step angiotensin II of the regular RAAS pathway, which has pro-inflammatory

effects. TOP is expressed by various tissues/organs, including the lungs [103]. TOP has been proposed to be involved in COVID-19 disease [5,104,105] due to its role in converting angiotensin I to angiotensin 1-7, being able to offset the harmful effects of angiotensin II in favor of the anti-inflammatory and lung protective effects of angiotensin 1-7. Thus, the potential involvement of TOP and EV-associated TOP in pathological roles in COVID-19 disease would be worthwhile to investigate. A better understanding of TOP and EV-associated TOP in COVID-19 disease may provide insights into their role in clinical diagnosis, prognosis, and development of new therapeutic strategies.

Conclusion

In this viewpoint, we summarized the role of EVs in the pathogenesis of COVID-19 disease, including the role that EVs may play in propagating viral infection, promoting pro-thrombotic conditions, and enhancing the progression of cytokine storm in COVID-19 disease. The current paper has discussed recent progress regarding EV-associated ACE2 and its effects on interfering with viral entry into host cells and the therapeutic potential of engineered EV-associated ACE2 on COVID-19 disease based on its function of serving as the cell surface receptor for SARS-CoV-2. Based on the enzymatic activities of ACE2, which converts angiotensin II to angiotensin 1-7, and TOP and NEP which convert angiotensin I to angiotensin 1-7, we have discussed their EV-associated forms and the perspective insights regarding their potential involvements in COVID-19 disease through their functions in angiotensin metabolism. Through their enzymatic activity on the RAAS pathway, ACE2, NEP, and TOP may be able to offset the pro-inflammatory and harmful effects of angiotensin II in favor of the anti-inflammatory and lung protective effects of angiotensin 1-7. The roles of EVs and their associated ACE2, NEP, and TOP are complicated, and the potential roles of EV-associated ACE2, NEP, and TOP would thus be novel areas worthwhile of investigation and would provide insights into therapeutic strategy, diagnosis, and prognosis of COVID-19 disease.

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Availability of Data and Materials: Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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