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Pathway of hepatic and renal damage through non-classical activation of the renin-angiotensin system in chronic liver disease

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COVID-19 AND LIVER CIRRHOSIS: FOCUS ON THE NON-CLASSICAL RENIN-ANGIOTENSIN SYSTEM AND IMPLICATIONS FOR THERAPY

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List of abbreviations. ACE, angiotensin converting enzyme. ACE2, angiotensin converting enzyme type 2. ACEi, angiotensin converting enzyme inhibitor. ADAM-17, a disintegrin and metalloproteinase domain-17. ALT, alanine aminotransferase. Ang II, angiotensin II. Ang1-7, angiotensin 1-7. ARB, angiotensin type 1 receptor antagonist. ARDS, acute respiratory distress syndrome. AST, aspartate aminotransferase. AT_1R, angiotensin type 1 receptor. AT_2R, angiotensin type 2 receptor. BDL, bile duct ligation. des-Arg1-BK, des-arginine bradykinin. ERK, extracellular signal-regulated kinase. HCC, hepatocellular carcinoma. IL, interleukin. MasR, Mas receptor. MRGD, Mas-related G protein-coupled receptor member D. NAFLD, non-alcoholic fatty liver disease. NEP, neprilysin. RAS, renin-angiotensin system. sACE2, soluble ACE2. SARS, severe acute respiratory syndrome. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. TNFα, tumor necrosis factor-α.

Keywords: ACE2, Angiotensin 1-7, inflammation, SARS-CoV-2.

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ABSTRACT

Angiotensin-converting enzyme type 2 (ACE2) is the cell receptor of SARS-CoV-2, the viral agent of COVID-19. ACE2 and a network of further enzymes and receptors constitute the non-classical renin-angiotensin system. ACE2 cleaves angiotensin II, which promotes vasoconstriction, oxidative stress, liver and lung inflammation and fibrosis, into angiotensin 1-7 (Ang1-7), which binds to Mas receptors (MasR), resulting in arterial vasodilatation, natriuresis, anti-inflammatory and anti-fibrotic effects in tissues. Viral binding to ACE2 allows viral entry into human cells including hepatocytes, followed by viral replication and host cell depletion of ACE2. The coronavirus-dependent demise of ACE2 and its product (Ang1-7) leads to cytokine activation and cytokine-induced hepatocyte apoptosis and necrosis, which in turn decreases liver reserve and induces hepatic failure. Several studies have shown that approximately one third of patients with cirrhosis, especially those with decompensation, die after a median of 10 days from COVID-19 diagnosis, and nearly two-thirds of deaths occur before intensive care unit admission. Liver and kidney functions deteriorate rapidly after hospital admission for COVID-19, suggesting that these patients die mostly from worsening of liver cirrhosis, rather than from pulmonary insufficiency. Pharmacological interventions which may provide novel strategies to counter liver cirrhosis decompensation due to COVID-19 include non-peptidic MasR agonist AVE0991, which replaces the anti-inflammatory and anti-fibrotic effects of Ang1-7, and metallopeptidase neprilysin inhibitor candoxatrilat, which reduces Ang1-7 clearance and causes portal pressure reduction with increased natriuresis in experimental cirrhosis. Moreover, SF2809E, an inhibitor of serine protease chymase (an enzyme generating most tissue angiotensin II) may also block TMPRRS2, a host serine protease that primes SARS-CoV-2 spike glycoprotein before adhesion to ACE2. These and further drugs deserve consideration in patients with COVID-19 and hepatic comorbidities.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive-sense single-stranded RNA virus with a crown-like appearance of spike proteins that project from its envelope. The syndrome caused by SARS-CoV-2 was named **CO**rona**VI**rus **D**isease outbreak or COVID-19, which was first diagnosed in the year 20**19**. To date, more than one million patients have died from the illness worldwide. The ramifications of this pandemic are far and wide, with economies at stake in all continents.

In severe cases, SARS-CoV-2 infects type II pneumocytes and leads to acute respiratory distress syndrome (ARDS). Comorbidities, including chronic liver diseases, arterial hypertension, obesity, diabetes, cardiovascular and pulmonary dysfunctions, cancer and cerebrovascular diseases worsen the prognosis of patients with COVID-19 (1). This is because the cell membrane-bound metallopeptidase angiotensin-converting enzyme type 2 (ACE2), the primary entry receptor for SARS-CoV-2, is ubiquitous, leading to multiple organs being involved in COVID-19 (2, 3).

SARS-CoV-2 and the liver

In the liver, hepatic endothelial cells, occasional bile duct cells, and perivenular hepatocytes express ACE2, and therefore are susceptible to SARS-CoV-2 damage (4). After viral entry, SARS-CoV-2 RNA is found in liver tissue and direct liver cell damage ensues. This manifests as microvacuolar steatosis, syncytial multinuclear hepatocytes, lobular and portal activity (5). However, SARS-CoV-2 can also cause liver damage from the COVID-associated cytokine storm with consequent hepatocyte apoptosis and necrosis that mainly lead to hepatic failure and the demise of patients with cirrhosis. Clinically, transaminase levels are increased in up to 62% of patients with severe COVID-19 even in the absence of pre-existing liver disease (6). Most infected patients show

hypoalbuminemia and elevation of gamma-glutamyl transferase (6). In patients with underlying liver disease, >50% of cases of COVID-19 are severe, with 17% mortality (7). Liver cirrhosis is an independent risk factor of mortality in COVID-19: these patients die from ARDS, as well as from complications of cirrhosis. The combined COVID-HEP and SECURE-Cirrhosis registries from the European Association for the Study of the Liver confirm that 38% of cirrhotic patients with COVID-19 had worsening ascites, encephalopathy, or acute kidney injury, and 40% of those patients died (7). In a group of cirrhotic patients with COVID-19 from North America, mortality was 30%, while that in patients with COVID-19 alone was 13% (8). Among 50 cirrhotic patients with COVID-19 from Italy, 30-day mortality was 34% (9). Other contributors to increased mortality during the pandemic include delayed screening for varices and hepatocellular carcinoma (HCC), or cancellation of elective therapeutic procedures such as resections or ablative therapies for HCC. Drug-induced liver injuries related to the drugs used to treat COVID-19 have been described.

The non-classical RAS

To understand why the combination of chronic liver disease, especially cirrhosis, and COVID-19 is potentially deadly, one needs to draw on the knowledge of the non-classical or tissue renin-angiotensin system (RAS) (Figure 1), a network of systemic and local production of angiotensins involved in the regulation of inflammation, fibrogenesis and tissue blood perfusion. The major player is ACE2, which is a transmembrane protein, with an extracellular N-terminal domain containing a mono-carboxypeptidase site and a short transmembrane C-terminal tail. ACE2 N-terminal domain is the SARS-CoV-2 binding site (2, 3). Ubiquitous ACE2 cleaves the Pro_7-Phe_8 bond of angiotensin II (Ang II) to form Ang 1-7. Ang 1-7 binds to specific cell membrane G protein-coupled receptors called Mas receptors (MasRs) (2, 10) (Figure 1). This leads to vasodilatation and increased renal sodium excretion through the production of arachidonic acid and

increased cell levels of cyclic GMP (11). The ACE2-Ang1-7-MasR axis also modulates the expression of pro-inflammatory cytokines (TNF-α, interleukin [IL]-1β, IL-6, and transforming growth factor-β) in most tissues (12), leading to anti-inflammatory effects.

Ang1-7 can be transformed into heptapeptide alamandine by an aspartate decarboxylase that converts Asp_1 of Ang1-7 into Ala_1 of alamandine, which binds to Mas-related G protein-coupled receptor member D (MRGD) (Figure 1), leading to arterial vasodilatation. Ang II may be converted by the same aspartate decarboxylase into octapeptide angiotensin A, an agonist of vasodilator angiotensin type 2 receptors (AT_2Rs) (13).

Genetic ACE2 deficiency causes upregulation of inflammatory mediators and elevated inflammatory responsiveness to proinflammatory stimuli. Membrane bound ACE2 protects mice against experimental acute lung injury (14). Finally, ACE2 metabolizes des-arginine bradykinin (des-Arg1-BK), which promotes inflammation and pulmonary edema in COVID-19 via stimulation of B1 receptors in lung endothelial cells (15).

SARS-CoV-2 and the Non-Classical RAS

SARS-CoV-2 has spikes on its envelope, which are primed by cellular trypsin-like serine protease TMPRSS2 and, to a lesser extent, cysteine proteases cathepsin B and L for viral entry into cells. The spikes of SARS-Cov-2 has a S glycoprotein which contains two functional domains: an S1 ACE2-binding domain and an S2 domain essential for fusion of viral envelope and cell membrane (16). Proteolytic digest by TMPRSS2 between S1 and S2 is critical for viral entry into cells. SARS-CoV-2 S glycoprotein may also be primed, at a site different from TMPRSS2 cleavage site, by furin, an enzyme of the subtilisin-like proprotein convertase family that is upregulated in heart

failure patients (3), or by plasmin, a furin-like protease (17) (Figure 2). Once primed, spike proteins interact with the extracellular domain of ACE2, followed by clathrin-dependent endocytosis of the complex, SARS-CoV-2 RNA release inside cells, viral replication, and shedding of new progenies into the bloodstream (16) (Figure 2).

SARS-CoV-2 infection prompts inflammatory responses that depend on cellular depletion of ACE2. Firstly, internalization of ACE2 bound to the infecting virions reduces the availability of the enzyme on the cell surface (Figure 2). Secondly, unknown viral mediators induce gene expression of metallosecretases such as a disintegrin and metalloproteinase domain-17 (ADAM-17). ADAM-17, whose activity is increased by hyperglycemia, functions as a "sheddase" and releases anchored ACE2, TNF-α, IL-4 and IFNγ from the cell membranes (Figure 3). Finally, the cell is further depleted of ACE2 function because ACE2 is downregulated by free IL-4 and IFNγ, released by ADAM-17 (Figure 3). TMPRSS2, which primes the S spikes of SARS-Cov-2 for ACE2 attachment, also cleaves ACE2 and competes with ADAM-17 for ACE2 extracellular shedding (16-18) (Figure 4). Thus, this functions like an internal feedback loop to control SARS-Cov-2 cell entry.

In the cirrhotic liver, ACE2 protein expression increases >90-fold compared to controls (4). ACE2 is upregulated in areas of active liver fibrogenesis in bile duct ligated (BDL) rats (4); recombinant ACE2 has hepatic anti-inflammatory and anti-fibrogenic effects in BDL and CCl_4-treated rats (19). Thus, ACE2 is a negative regulator of the RAS and limits liver fibrosis. Ang1-7 opposes the Ang II-AT_1R axis through binding to the C-terminal domain of ACE, thereby inhibiting Ang II generation (12). Ang II, on the other hand, activates extracellular signal-regulated kinase (ERK)1/ERK2 and reduces ACE2 cell expression (14). Thus, it would appear that having excess ACE2 is advantageous to the patient with liver cirrhosis. However, overexpression of ACE2 in liver cirrhosis may facilitate

infection by SARS-CoV-2. Once infected, the presence of ACE2 is needed to maintain Ang 1-7 production, as the loss of ACE2 after cell entry means that there is a shift of the RAS to a higher Ang II and lower Ang1-7 tone. Ang II promotes acute lung injury, vasoconstriction, oxidative stress, liver inflammation and fibrosis (2, 10). Indeed, in COVID-19 patients, plasma Ang II levels are higher than normal and correlate with the viral load in bronchoalveolar lavage fluid. This contributes to widespread inflammation (1). Therefore, any compound that blocks Ang II production or action such as ACE inhibitor (ACEi) lisinopril and angiotensin type 1 receptor blockers (ARBs) should increase ACE2 activity. The conundrum is that ACEis and ARBs may improve outcomes in patients with ARDS through reduced Ang II content or function, but both classes of drugs potentially increase susceptibility to SARS-CoV-2 infection through cellular upregulation of ACE2 (2). Thus, the use of ACEis and ARBs remains a matter of great debate in COVID-19 patients without liver cirrhosis. At present, the European Society of Cardiology, the American Heart Association, the Heart Failure Society of America, and the American College of Cardiology state there is no clinical evidence to suggest discontinuation of ACEis or ARBs because of COVID-19 (20), as ACEis also reduce the cleavage of Ang1-7 into angiotensin 1-5 (Figure 1), thereby maintaining Ang 1-7 levels. In patients with cirrhosis and ascites, these drugs are not recommended because available trials show that ACEis and ARBs increase the risk of hypotension and renal failure (10, 21).

High plasmin levels are also found in cirrhotic patients, related to increased tissue-type plasminogen-activator activity relative to plasminogen-activator-inhibitor activity and decreased levels of alpha 2-antiplasmin (22). Targeting plasmin with inhibitors (aprotinin, tranexamic acid) may be an option to reduce hyper-fibrinolysis (critical to disease progression in all patients with severe COVID-19) (1) and priming of viral spike glycoprotein in infected cirrhotic patients.

Overexpression of endothelial bradykinin B1 receptors occurs in livers of rats with experimental cirrhosis (23). Endothelial bradykinin B1 receptors bind to des-Arg1-BK, which promotes inflammation. The relative lack of ACE2 following internalization after viral attachment means that des-Arg1-BK is no longer cleared by ACE2 during infection, further fueling the inflammation. However, currently we have no drugs to modulate des-Arg1-BK action.

Other drugs in common use that could potentially increase ACE2 levels are listed in Table 1. Chronic exercise is another important stimulus of ACE2 expression in the brain and the periphery (2). It is still unclear for individual patients, whether increased cellular ACE2 levels is an open gate to viral infection or a guarantee that tissues will be more protected against inflammation.

Lessons from the non-classical RAS

Many drugs in development that affect the non-classical RAS (10) could also be beneficial to patients with liver cirrhosis and COVID-19. These include:

- Metallopeptidase inhibitor MLN-4760 (Millennium Pharmaceuticals Incorporated, Cambridge, Massachusetts, U.S.A.) causes a conformational change in the catalytic domain of ACE2 that mimics the move of a closing clamshell. This could hinder SARS-CoV-2 binding to its receptor (24).

  In patients with early infection with COVID-19, MLN-4760 could potentially reduce viral spread to vital organs. In isolated and perfused BDL livers of rats, intraportal MLN-4760 infusion surprisingly increased Ang1-7 production from angiotensin I, an effect abolished by neprilysin (NEP) inhibition, showing that, when liver ACE2 is blocked by MLN-4760, NEP converts angiotensin I into Ang1-7 (25) (Figure 1). This upsurge of Ang1-7 could prove to be protective in cirrhotic patients with COVID-19.

- AVE0991 (Sanofi-Aventis, Frankfurt, Germany) is a nonpeptidic Ang1-7 analog. In experimental murine models of acute lung injury, intragastric administration of AVE0991 reduced lung edema, myeloperoxidase activity, and histological lung injury score, without arterial hypotensive effects (26). AVE0991 reduces intrahepatic resistance to portal flow and portal pressure in rats with BDL- or CCl_4-induced experimental cirrhosis (27).

  Alamandine is a potential candidate therapy for COVID-19. It is related chemically to Ang1-7 and might be associated to AVE0991. The latter is an agonist of MasRs, while alamandine exerts its effects through binding with MRGDs (13).

- Decreased metabolic clearance of Ang1-7 may be helpful in patients with COVID-19. Candoxatrilat (Pfizer Central Research, Sandwich, Sussex, UK) is a specific inhibitor of metallopeptidase neprilysin, which cleaves Ang1-7 into angiotensin 1-4 (Figure 1), thereby increasing Ang 1-7 concentrations. In experimental liver cirrhosis, candoxatrilat reduced portal pressure and increased urinary excretion of sodium and atrial natriuretic peptide without effects on arterial pressure and plasma renin activity (28, 29).

- Trypsin-like serine protease inhibitors (camostat and nafamostat mesylates) (11) may prevent host TMPRSS2 from priming the viral spike glycoprotein. Chymase, another trypsin like serine protease, is the main source of pro-fibrogenic Ang II in human tissues. Therefore, chymase inhibitor SF2809E (Meiji Seika Pharma Co., Ltd., Yokohama, Japan) reduces portal pressure and liver fibrogenesis in experimental CCl_4 cirrhosis (30) and might prevent TMPRSS2 from priming the viral spike glycoprotein.

- Soluble ACE2 (sACE2) converts Ang II into Ang1-7 in extracellular fluids and antagonizes binding of coronavirus spike glycoprotein to transmembrane ACE2. Recently, in early COVID-19, recombinant ACE2 has been delivered intranasally to reduce systemic viral spread (20).

It is clear that the mechanisms of SARS-CoV-2 infection have unveiled the key role of the non-classical RAS in the pathogenesis of COVID-19. This has implications for patients with underlying liver cirrhosis and COVID-19. Drugs able to modify the pathophysiological impact of the virus exist and, therefore, are worthy of consideration in this complex clinical context.

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Figure legends

Figure 1. Diagram depicting pathways of synthesis and degradation of angiotensins in classical and non-classical RAS, with respective receptors for each bioactive peptide. Classical RAS is defined as the ACE-Ang II-AT_1R axis; the non-classical RAS is composed primarily of the ACE2-Ang1-7-MasR axis and the Angiotensin 2-8/Angiotensin 3-8 pathway. The main degradative pathway for Ang II in normal humans is through the sequential actions of plasma aminopeptidases A and N, not through ACE2; serine endopeptidase chymase, in heart, renal tubules and ubiquitous mast cells, converts Ang I into Ang II as efficiently as ACE does in the vascular endothelium; Zn-metallo-endopeptidase neprilysin (NEP) cleaves angiotensin I into Ang1-7 and, finally, the latter vasodilator and natriuretic peptide into the inactive by-product angiotensin 1-4. In other words, inside RAS, NEP generates Ang1-7 but continues to metabolize Ang1-7 at the Tyr_4-Ile_5 bond to finally form angiotensin 1-4. Aspartate decarboxylase converts Asp_1 of Ang II and Ang1-7 into Ala_1 to generate angiotensin A and alamandine, respectively. ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme type 2; $AT_{1\text{-}2\text{-}4}Rs$: angiotensin type 1-2-4 receptors; MasR: Mas receptor; MRGD: Mas-related G protein-coupled receptor member D.

Figure 2. Mechanisms of Covid-19 binding to cell membrane ACE2, viral spike glycoprotein priming into S1 and S2 subunits by host proteases furin, plasmin and TMPRRS2, and viral entry along with ACE2 into human cells. Protease furin may be found as free-floating in the extracellular fluids or as membrane-bound enzyme. N-terminal domain of ACE2 is the extracellular viral binding site, while ACE2 C-terminal tail anchors the enzyme to plasma membranes.

Figure 3. SARS-CoV-2 causes ACE2 cellular depletion through ADAM-17 upregulation, and ADAM-17-mediated IL-4 and IFNγ cellular shedding into extracellular fluids. In turn, free IL-4 and IFNγ downregulate ACE2 cellular expression. N-terminal domain of ACE2 is the extracellular viral

binding site, while ACE2 C-terminal tail anchors the enzyme to plasma membranes. sACE2: soluble ACE2.

Figure 4. Arginine and lysine residues within ACE2 amino acids 697 to 716 are essential for ACE2 cleavage by TMPRSS2; ADAM-17 requires arginine and lysine residues within ACE2 amino acids 652 to 659 for cleavage. A C-terminal ACE2 fragment of 13 kDa results from TMPRSS2 processing of the enzyme. Soluble ACE2 (sACE2), obtained through action of ADAM-17 on cellular ACE2, is the complete N-terminal ectodomain of the enzyme, binds SARS-CoV2 virions and converts Ang II into Ang1-7 in the extracellular space.