Communication

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Long COVID elevated MMP-9 and release from microglia by SARS-CoV-2 Spike protein

Abstract

Objective – Long COVID is a major health concern because many patients develop chronic neuropsychiatric symptoms, but the precise pathogenesis is unknown. Matrix metalloproteinase-9 (MMP-9) can disrupt neuronal connectivity and be elevated in patients with long COVID.

Methods – In this study, MMP-9 was measured in the serum of long COVID patients and healthy controls, as well as in the supernatant fluid of cultured human microglia cell line stimulated by recombinant severe acute respiratory syndrome coronavirus 2 Spike protein, as well as lipopolysaccharide (LPS) and neurotensin (NT) used as positive controls. MMP-9 was measured by commercial enzyme-linked immunosorbent assay.

Results – MMP-9 was significantly elevated in the serum of long COVID patients compared to healthy controls. Moreover, there was significant release of MMP-9 from a cultured human microglia cell line stimulated by LPS, NT, or Spike protein. We further show that pretreatment with the flavonoids luteolin and tetramethoxyluteolin (methlut) significantly inhibited the release of MMP-9 stimulated by the Spike protein.

Conclusion – MMP-9 from Spike protein-stimulated microglia could contribute to the development of long COVID and may serve as a target for treatment including the use of luteolin.

Keywords: brain inflammation, long COVID, luteolin, mast cells, microglia, MMP-9

Abbreviations

CDC	Centers for Disease Control and Prevention
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
LPS	lipopolysaccharide
MMP-9	matrix metalloproteinase-9
NT	neurotensin
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2

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1 Introduction

Long COVID has been considered the "Next National Health Disaster" in the United States [1]. As many as 50% of those infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may develop long COVID [2], especially neuropsychiatric symptoms [3] known as Neuro COVID [4,5] that may last up to 2 years, [6], and maybe more common in patients with other chronic neurologic diseases [7]. However, the precise pathogenesis of long COVID has yet to be fully elucidated [8].

SARS-CoV-2 Spike protein may enter the brain from the nose through the nasal neural mucosa following the olfactory nerve tract [9]. While the exact brain pathogenetic mechanisms remain unclear, evidence points to the involvement of neuroinflammation [10,11], especially

perivascular inflammation [12] and blood–brain barrier (BBB) disruption [12,13], leading to neuronal damage [14]. Autopsy studies of patients with COVID-19 showed severe neuronal loss in the capillaries of the choroid plexus [15], as well as neuronal necrosis and glial cell hyperplasia [16]. A 2-year longitudinal study using plasma proteomics to probe long COVID reported that pathways related to neuron generation and differentiation were persistently suppressed [17].

A critical component of neuronal connectivity is the extracellular matrix (ECM) that can be disrupted by matrix metalloproteinases (MMPs). MMPs are important in tissue formation, neuronal network remodeling, and BBB function [18]. Matrix metalloproteinase-9 (MMP-9) has emerged as an important molecule in neuropsychiatric [19,20] and neurodegenerative disorders [21]. MMP-9 can disrupt the polysaccharide scaffolding of the brain matrix and digest tight junction proteins, thus disrupting neuronal connectivity [22]. MMP-9 can cause vascular inflammation and increase BBB permeability [23]. MMP-9 levels were elevated in the serum of COVID-19 patients and were associated with disease severity [24,25].

We investigated serum levels of MMP-9 in long COVID patients, whether recombinant SARS-CoV-2 Spike protein could stimulate the release of MMP-9 from cultured human microglia and whether the flavonoid luteolin could inhibit this process.

2 Methods

2.1 Patients

Patients (n = 13, 6 females and 7 males, mean age was 57 years old) were recruited from Southern Florida. Study participants were recruited from a companion longitudinal study of residents of South Florida who tested positive for SARS-CoV-2. Nova Southeastern University IRB No: 2020-590 (approved January 6, 2021, expires January 11, 2025). Individuals were recruited from those who tested positive for COVID-19 in Broward County and were included in the Florida Department of Health Bureau of Epidemiology COVID-19 surveillance data, or in the records of Community Health of South Florida Inc. (CHI), a Federally Qualified Health Center in Miami-Dade County or in the records of participating community-based provider offices. The inclusion/exclusion criteria for unrecovered individuals were fatigue, as well as one additional symptom that began after positive SARS-CoV-2 test and that the participant self-reported experiencing "a good bit of the time," "most of the time," or "all of the time" during the past month. Individuals were 18–65 years old and were able to consent to the phenotyping study. The unrecovered group had moderate to severe illness as indicated by Patient-Reported Outcomes.

Measurement Information System-29 [26] score of 45 or lower on the physical sub-score, fatigue that does not resolve with rest and one additional symptom from the Centers for Disease Control and Prevention (CDC) SI screener. Individuals were excluded from the study if they had medical or psychiatric conditions diagnosed before testing positive for SARS-CoV-2. Examples of exclusions were: severe chronic obstructive pulmonary disease, organ failure, chronic infection, rheumatic and chronic inflammatory disease, chronic lung disease, or major neurologic disease. In addition, the following were assessed during clinical visits and patients were excluded if there was evidence of abnormal diastolic function or cardiomyopathy and/or O_2 saturation of 92% or below on the 6 min exercise. Serum from healthy subjects (n = 13, 6 females and 7 males, mean age was 57 years old) was purchased from BioIvt Elevating Science (Hicksville, NY).

2.2 Human microglia cell culture

The immortalized human microglia-SV40 cell line (hTERT; T0251) derived from primary human microglia was purchased from Applied Biological Materials Inc. (ABM Inc.; Richmond, BC, Canada) and cultured in Prigrow III medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in type I collagen-coated T25-flasks (BD PureCoat™ ECM Mimetic Cultureware Collagen I peptide plates, Becton Dickinson, Bedford, MA) as recommended by the supplier. Microglia-SV40 maintains their phenotype and proliferation rates for about ten passages, during which all experiments were carried out using multiple thaws and sub-cultured cells. Experiments were carried out in type I collagen-coated plates (Becton Dickinson). Cell viability was determined by trypan blue (0.4%) exclusion.

2.3 Cell treatments

SV40 microglia (2.5×10^5 cells) were stimulated with recombinant full-length SARS-CoV-2 Spike protein (Abcam, Waltham, MA, USA) at 10 ng/mL or lipopolysaccharide (LPS) and neurotensin (NT) (from Sigma-Aldrich, St. Paul, MN, USA) at 10 ng/mL and 10 nM, respectively, for 24 h. MMP-9 was measured in the supernatant fluid by enzyme-linked immunosorbent assay

(ELISA) (BioTechne, Minneapolis, MN) kits according to the manufacturer's instructions. Control cells were treated with an equal volume of culture medium in all experiments. We pre-incubated microglia with either luteolin or methlut (50 μM from CAS Biosciences, NY, USA) in dimethylsulfoxide (<1% final concentration) for 10 min before incubation with Spike protein for 24 h and then measured MMP-9 in the cell culture supernatant by ELISA.

2.4 Statistical analysis

All experimental conditions were performed in triplicate and all experiments were repeated at least three times ($n = 3$). Results from cultured cells are presented as mean ± SD. Comparisons between control and stimulated cells were performed using either parametric tests (unpaired 2-tailed, Student's t-test, for independent samples) or Mann–Whitney non-parametric test depending on the normality of distribution that was checked with the Shapiro–Wilk's test. Comparisons among groups were performed with one-way analysis of variance (ANOVA) followed by *post-hoc* analysis by Dunnett's Multiple Comparison Test or the Wilcoxon *post-hoc* paired rank sum test. All statistical analyses were performed by using GraphPad Prism version 10.0.3 (275) (GraphPad Software, Boston, MA, USA).

Ethical statement: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee; and the specific national laws have been observed.

Informed consent: Informed consent has been obtained from all individuals included in this study.

3 Results

We first measured serum MMP-9. There were significantly increased levels of MMP-9 in the serum of long COVID patients compared to healthy control subjects (Figure 1).

We then investigated whether SARS-CoV-2 Spike protein could stimulate the release of MMP-9 from cultured human SV-40 microglia cell line. Incubation with the Spike protein (1, 5, and 10 ng/mL) for 24 h significantly increased the release of MMP-9 from microglia (Figure 2). LPS and NT used as "positive" triggers also significantly increased MMP-9 release compared to unstimulated control cells (Figure 2).
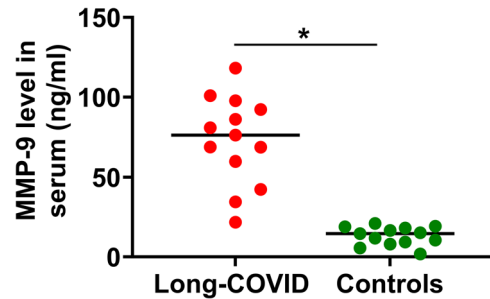

Figure 1: Scattergram of serum values of MMP-9. MMP-9 was measured in the serum of long COVID patients ($n = 13$, mean age 57 years) and healthy control subjects ($n = 13$, mean age 57 years) using commercial ELISA kit. *$p < 0.05$; t-test compared to control subjects.

We then investigated the possible inhibitory effect of the flavonoids luteolin (3′,4′,5,7-tetrahydroxyflavone) and tetramethoxyluteolin (3′,4′,5,7-tetramethoxyflavone, methlut). Pre-incubation of the microglia with either flavonoid (50 μM) for 10 min significantly inhibited the release of MMP-9 stimulated by the Spike protein (Figure 3).
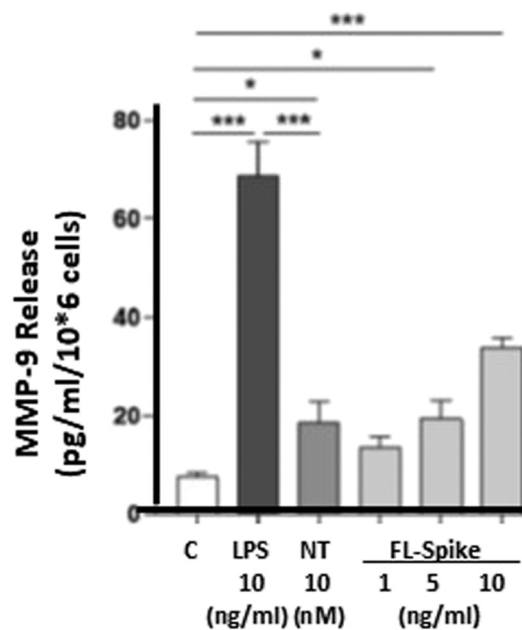

Figure 2: MMP-9 release from human microglia stimulated by SARS-CoV-2 Spike protein. SV-40 microglia (2.5 × 10⁵ cells) were stimulated with recombinant full-length SARS-CoV-2 Spike protein (FL-Spike, 1, 5, 10 ng/mL), LPS (10 ng/mL), or NT (10 nM) for 24 h, and MMP-9 was measured in the supernatant fluid by ELISA. LPS and NT were used as "positive triggers." C = control. All conditions were performed in triplicate for each dataset and repeated three times ($n = 3$). Results are presented as mean ± standard error of the mean (SEM). Statistical significance is indicated as *$p < 0.05$ and ***$p < 0.001$.
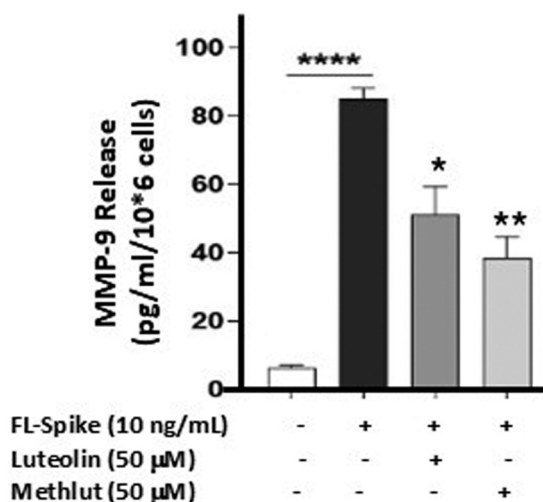
Figure 3: Inhibition of SARS-CoV-2 Spike protein-stimulated release of MMP-9 from human microglia by flavonoids. SV-40 microglia (2.5×10^5 cells) was stimulated with recombinant full-length SARS-CoV-2 Spike protein (FL-Spike, 10 ng/mL) for 24 h after pre-incubation (10 min) with either luteolin or methlut (50 μM). MMP-9 was measured in the supernatant fluid by ELISA. All conditions were performed in triplicate for each dataset and repeated three times ($n = 3$). Results are presented as mean ± SEM. Conditions with flavonoids were compared to the one with FL-Spike protein. Statistical significance is indicated as $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$.

4 Discussion

Here we show that MMP-9 is elevated in the serum of long COVID patients. Elevated serum MMP-9 levels have been reported in COVID-19 [27] and were associated with symptom severity [24,25]. MMP-9 polymorphisms were also reported to increase the susceptibility to COVID-19, especially when accompanied by neurologic symptoms [13]. Blood MMP-9 levels were also reported to be higher in the acute phase of Neuro COVID patients [13]. MMP-9 has been associated with reduced BBB integrity [13,18].

We also show that microglia release MMP-9 when stimulated by SARS-CoV-2 Spike protein. We had previously reported that SARS-CoV-2 Spike protein stimulated cultured human microglia to secrete IL-1β, IL-18, and S100β, associated with brain damage [28]. Additional evidence indicates that the Spike protein can directly activate microglia [29–31] leading to proinflammatory effects.

SARS-CoV-2 has not been shown to infect brain cells [32–34]. The neurological issues of long COVID may, therefore, be attributed to the SARS-CoV-2 Spike protein [35] stimulating microglia [36,37]. Perivascular inflammation with lymphocytic and microglial infiltration was noted in the brains of 52 deceased patients with COVID-19 [38]. Microglia have been considered key players in the development of neuroinflammatory [39] and neurodegenerative

disorders [40,41]. The duration of long COVID may depend on the length of antigen presence since it was reported that Spike protein was detected in CD16^{+} monocytes in long COVID patients up to 15–24 months post-infection [42] and inside extracellular vesicles for up to 1 year [43,44]. Recent papers reported that the SARS-CoV-2 Spike protein could be detected in long COVID patients for 6–12 months [45]. One study reported the presence of antibodies against Spike protein in the cerebrospinal fluid of 12 patients with COVID-19 [46], as well as its presence in "reservoirs" [47] including the "skull–meninges–brain axis" [48].

Our results also show that the flavonoids luteolin and methlut could inhibit the release of MMP-9 stimulated by the Spike protein. We had previously shown that these flavonoids could inhibit cultured human microglia stimulated by NT to release IL-1β [49] and reduce "brain fog" associated with long COVID [50]. In particular, nobiletin (hexamethoxyflavone) was reported to inhibit MMP-9 [51]. Another paper reported that methoxylated flavones inhibited tumor necrosis factor-mediated induction of MMP-9 [52].

Others have reported that polyphenolic compounds can lower MMP-9 levels *in vivo* and *in vitro* [53–55]. In particular, resveratrol prevented ischemic brain injury in a mouse model by inhibiting the activation of proinflammatory microglia via the MMP-9 pathway [56]. Interestingly, minocycline has been reported to reduce MMP-9 effects in rodents [57–59]. Statins have also been reported to have a beneficial effect by reducing hippocampal MMP-9 levels in a rat model of cognitive decline [60] and patients with acute ischemic stroke [61]. In fact, MMP-9 inhibitors have been considered for the treatment of traumatic brain injury [62].

There are limitations in this study: (a) we do not know the original severity of COVID-19 in the patients studied, (b) the results were compared to healthy controls and not recovered long COVID patients, and (c) the source of MMP-9 in the serum of long COVID patients is also not exactly known since SARS-CoV-2 could also stimulate the release of MMP-9 from cultured macrophages [63] and several cell types in the brain [64]. Another paper reported that cultured human mast cells can also produce MMP-9 [65].

We believe MMP-9 from Spike protein-stimulated microglia could contribute to the development of long COVID and may serve as a target for treatment including the use of luteolin, especially in a liposomal form in olive pomace oil to increase absorption (PureLut[R]).

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