Increased Serum MMP-9 in Long-COVID May Reflect Activation of Microglia by SARS-CoV-2 Spike Protein

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Short Report

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Abstract

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 was elevated in patients with COVID-19. MMP-9 was measured in the serum of long COVID patients and healthy controls, as well as in the supernatant fluid of cultured human SV-40 microglia, by commercial ELISA. Results were analyzed with one-way ANOVA. MMP-9 in the serum of Long-COVID patients and supernatant fluid from cultured human microglia stimulated by recombinant SARS-CoV-2 Spike protein was assayed by ELISA. MMP-9 was significantly elevated in the serum of Long-COVID patients compared to healthy controls. Moreover, cultured human microglia released MMP-9 when stimulated by Spike protein.

In conclusion, MMP-9 may contribute to the development of Long-COVID and serve both as a prognostic biomarker and as target for treatment.

Introduction

Long COVID has been considered the “Next National Health Disaster” in the US (Phillips and Williams, 2021). As many as 50 per cent of those infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may develop Long-COVID (Thaweethai et al., 2023), especially Neuro-COVID (Ali et al., 2022; Almulla and Al-Hakeim, 2023) characterized cognitive dysfunction (Hadad et al., 2022). Long COVID may last up to two years. (Shanley et al., 2022) However, the precise pathogenesis of Long COVID has yet to be fully elucidated (Proal and VanElzakker, 2021). SARS-CoV-2 enters cells via its coronavirus spike protein binding to its cell surface receptor, angiotensin-converting enzyme 2 (ACE2) (Tai et al., 2020). SARS-CoV-2 Spike protein may enter the brain from the nose through the nasal neural mucosa following the olfactory nerve tract (Meinhardt et al., 2021). While the exact brain pathogenetic mechanisms remain unclear, evidence points to the involvement of neuroinflammation (Sodagar et al., 2022; Tremblay et al., 2022), especially perivascular inflammation (Lee et al., 2021) and blood-brain barrier (BBB) disruption (Bonetto et al., 2022; Lee et al., 2021), leading to neuronal damage (Zingaropoli et al., 2022).

Autopsy studies of patients with COVID-19 showed severe neuronal loss in the capillaries of the choroid plexus (Yang et al., 2021), as well as neuronal necrosis and glial cell hyperplasia (Xu et al., 2024). A two-year longitudinal study using plasma proteomics to probe Long-COVID reported that pathways related to neuron generation and differentiation were persistently suppressed (Gu et al., 2023).

A critical component of neuronal connectivity is the extracellular matrix (ECM) that can be disrupted by matrix metalloproteinases (MMPs). MMP-9 has emerged an important molecule in neuropsychiatric (Kaczmarek et al., 2023; Lopez-Navarro and Gutierrez, 2022) and neurodegenerative disorders (Beroun et al., 2019). MMP-9 can disrupt the polysaccharide scaffolding of the brain matrix and digest tight junction proteins, thus disrupting neuronal connectivity (Stawarski et al., 2014). MMP-9 can cause vascular
inflammation and increase BBB permeability (Dhanda and Sandhir, 2018). MMP-9 levels were elevated in the serum of COVID-19 patients and were associated with disease severity (Ding et al., 2023; Savic et al., 2022).

We investigated serum levels of MMP-9 in Long-COVID patients, and whether recombinant SARS-CoV-2 Spike protein could stimulate release of MMP-9 from cultured human microglia and mast cells.

Methods

Patients

Patients (n = 15, 6 female and 9 male, mean age were 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 (FDOH) 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-Co-V-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-year-old and were able to consent to the phenotyping study. The Unrecovered group had moderate to severe illness as indicated by PROMIS 29 score of 45 or lower on the physical sub score, and fatigue that does not resolve with rest and one additional symptom from the CDC SI screener. Individuals were excluded from the study if they had medical or psychiatric conditions diagnosed prior to testing positive for SARS-CoV-2. Examples of exclusions included: 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 visit and patients were excluded if there was evidence of abnormal diastolic function or cardiomyopathy and/or O2 saturation of 92% or below on the 6-min exercise. Serum from healthy subjects (n = 20, 8 female and 12 male, mean age were 52 years old) was purchased from Biolvt Elevating Science (Hicksville, NY).

Human Microglia Cell Culture

The immortalized human microglia-SV40 cell line 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 (FBS) and 1% penicillin/streptomycin in type I collagen-coated T25-flasks (BD PureCoat™ ECM Mimetic Cultureware Collagen I peptide plates, Becton Dickinson, Bedford, MA). Microglia-SV40 maintains their phenotype and proliferation rates for over 10 passages, during which all experiments were performed using multiple microglia thaws and sub-cultured...
cells. Experiments were carried out in type I collagen coated plates (BD). Cell viability was determined by trypan blue (0.4%) exclusion.

**Cell Treatments**

SV40 microglia (2.5 x 10^5 cells) were stimulated with recombinant full-length SARS-CoV-2 Spike protein (from Abcam, Waltham, MA, 10 ng/ml for 24 hr) and MMP-9 was measured in the supernatant fluid by ELISA (BioTechne, Minneapolis, MN) according to the manufacturer's instructions. Control cells were treated with equal volume of culture medium in all experiments.

**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, followed by post-hoc analysis by Dunnett's Multiple Comparison Test or the Wilcoxon post-hoc paired rank sum test. Comparisons among groups were performed with one-way analysis of Variance (ANOVA). All statistical analyses were performed by using GraphPad Prism 9.4.1.

**Results**

Here we show significantly increased (*p < 0.05, t-test) levels of MMP-9 in the serum of Long-COVID patients compared to healthy control subjects (Fig. 1).

We also investigated whether SARS-CoV-2 Spike protein (1, 5, and 10 ng/ml for 24 hr) could stimulate release of MMP-9 from SV-40 microglia. The Spike protein at 1, 5 and 10 ng/ml significantly (*p < 0.05) increased release of MMP-9 from microglia (Fig. 2). Neurotensin (NT) used as control significantly increased MMP-9 release compared to unstimulated control cells.

**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 and were associated with severity (Ding et al., 2023; Savic et al., 2022). MMP-9 polymorphisms were also reported to increase the susceptibility to COVID-19, especially when accompanied by neurologic symptoms (Bonetto et al., 2022). MMP-9 has also been associated with reduced BBB integrity (Bonetto et al., 2022; Rempe et al., 2016). 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 protein S100B, associated with brain damage (Tsilioni and Theoharides, 2023). Additional evidence indicates that the Spike protein can directly activate microglia (Jeong et al., 2022; Olajide et al., 2022; Samudyata et al., 2022) leading to proinflammatory effects.
The neurological issues of Long-COVID may be attributed to the SARS-CoV-2 Spike protein (Theoharides and Kempuraj, 2023) since SARS-CoV-2 has not been shown to infect brain cells. In particular, a recent study demonstrated that SARS-CoV-2 has limited potential to proliferate in the brain and was unable to transmit between synaptic axons neurons in a human stem cell-derived neuronal culture system (Luczo et al., 2024). Perivascular inflammation with lymphocytic and microglial infiltration was noted in the brains of 52 deceased patients with COVID-19 (Wierzba-Bobrowicz et al., 2021). 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 (Patterson et al., 2021) and inside extracellular vesicles for up to one year (Craddock et al., 2023; Peluso et al., 2022). Recent papers reported that the SARS-CoV-2 Spike protein could be detected in Long-COVID patients for 6–12 months (Swank et al., 2023) and be present in “reservoirs” (Proal et al., 2023) including the brain. Microglia have been considered key players in the development of neuroinflammatory (Bachiller et al., 2018) and neurodegenerative disorders (Hickman et al., 2018; Perry et al., 2010). A recent meta-analysis of serum and plasma proteomic data indicated a significant association of COVID-19 with neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease (Mahin et al., 2024).

There are limitations in this study since we do not know the original severity of COVID-19 in the patients studied. The source of MMP-9 is also not known. SARS-CoV-2 could stimulate release of MMP-9 from cultured macrophages (Murphy et al., 2023). Another paper reported that cultured human mast cells can also produce MMP-9 in response to an ionophore (Kimata et al., 2006).

In conclusion, these results indicate that MMP-9 may possibly serve as a prognostic biomarker for development of Long-COVID and potential target for treatment. In fact, MMP-9 inhibitors have been considered for the treatment of traumatic brain injury (Sunny et al., 2024). In particular, the natural flavonoid nobiletin has been reported to inhibit MMP-9 (Kim et al., 2014).

**Declarations**

On behalf of all authors, the corresponding author states that there is no conflict of interest. All human studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments; and the specific national laws have been observed.

**Author Contribution**

KD performed the serum measurements. IT performed the in vitro studies and the supernatant fluid measurements and analyzed the results. KKA collected and stored the serum samples. NGK designed the clinical study, obtained HIRB approval and evaluated the patients.

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References


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Figures

**Figure 1**

Serum levels of MMP-9. Scattergram of serum values of MMP-9. MMP-9 was measured in the serum of Long-COVID (n=15, mean age 55 years) and healthy control subjects (n=20, mean age 52 years) using ELISA kit. *p<0.05; t-test compared to control subjects.
Figure 2

Recombinant SARS-CoV-2 Spike protein stimulates release of MMP-9 from human microglia. SV-40 microglia (2.5 x 10^5 cells) were stimulated with recombinant full-length SARS-CoV-2 Spike protein (1, 5, 10 ng/ml for 24 hr) and MMP-9 was measured in the supernatant fluid by ELISA. NT used as control significantly increased MMP-9 release compared to unstimulated control cells. C=control. All conditions were performed in triplicate for each dataset and repeated 3 times (n=3). Results are presented as mean ± standard error of the mean (SEM). One-Way ANOVA showed significant difference among means (p=0.0007) listed here as p<0.05.