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The role of oxidized phospholipids in COVID-19-associated hypercoagulopathy

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Abstract. – OBJECTIVE: There is more pronounced hypercoagulation in COVID-19 infection than in other viral lung infections. Oxidized phospholipids (OxPLs) appear in COVID-19-infected lungs due to oxidative stress, after which they promote the induction of tissue factor (TF) expression and inflammatory programmers in monocytes, as well as activate endothelial cells to recruit and bind to monocytes. Therefore, we aimed to demonstrate the role of OxPLs in inflammatory and procoagulant responses in COVID-19 infection.

PATIENTS AND METHODS: Patients with a positive SARS-CoV-2 polymerase chain reaction test and ten healthy donors were included in the study. Peripheral blood was drawn at inclusion for OxPAPC, IFN-γ, and CCL2 serum level measurements. Clinical data were collected from electronic patient medical files. The serum levels of OxPAPC, IFNγ, and CCL2 were measured by immune assays.

RESULTS: Seventy-two patients were included in the study. OxPAPC and CCL2 were higher in the patients than in the controls (<0.003 and 0.011, respectively). INF-γ did not significantly differ between groups. There was no difference between the patients with lung involvement and those without CCL2, INF-γ, and OxPAPC. D-dimer, CRP, and ferritin were higher in the patients with lung involvement. Serum levels of INF-γ and CCL2 were positively correlated with each other (r:0.757, p<0.0001), but no correlation was detected between OxPAPC and INF-γ or CCL2. There was no correlation between OxPAPC and hematologic or biochemical parameters.

CONCLUSIONS: OxPAPC, which is thought to contribute to hypercoagulability, was found to be high in the patients with Covid-19 infection. The role of OxPLs in COVID-19-associated hypercoagulopathy should be investigated further in experimental models and in larger patient groups.

Key Words:
COVID-19, Thrombosis, Oxidative stress injury, Lung

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel enveloped RNA betacoronavirus that emerged in December 2019 in Wuhan, China and is the cause of coronavirus disease 2019 (COVID-19)[1]. This virus has a tropism for the lungs, causing pneumonia in a significant portion of infected patients. Some patients with pneumonia suddenly deteriorate into severe respiratory failure, which is characterized by adult respiratory distress syndrome (ARDS)[2].

COVID-19 infection begins with an initial phase of viral replication that can be followed by a second phase of immunopathology driven by a hyperinflammatory response[1]. It is hypothesized that delayed induction of antiviral interferon responses is related to other virus escape mechanisms, such as production of interferon inhibitory proteins, which perpetuate viral damage, lead to an excessive accumulation of monocytes, macrophages, and neutrophils in the lungs, and ultimately cause cytokine storm[1,3,4]. Similar to other severe infections, cytokine storm and systemic inflammatory response after endothelial cell damage promote coagulation in COVID-19 infection[5-7]. In severe COVID-19 infection, microvascular thrombosis is observed in the lungs and other organs, contributing to the morbidity and mortality observed in this disease[1,8-11].

Proinflammatory cytokines, especially interleukin (IL)6, are thought to trigger procoagulant

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phenotype and cause microvascular thrombosis in COVID-19 infection by inducing tissue factor (TF) expression in mononuclear cells and by interacting monocytes with the endothelium[1,7]. However, the presence of more pronounced hypercoagulation in COVID-19 infection than in other viral infections of lung tissue suggests that additional mechanisms contribute to the process. It has been proposed that oxidized phospholipids (OxPLs), which have been detected in the lungs of patients infected with SARS-CoV-2, contribute to the process[1,12]. OxPLs appear in the virus-infected lungs due to oxidative stress, and they promote the induction of TF expression and inflammatory programmers in monocytes as well as activate endothelial cells to recruit and bind to monocytes[12,13]. In experimental acute lung injury, OxPLs, such as oxidized 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (OxPAPC), triggered macrophage activation through the TLR4–TRIF–TRAF6-NF-κB pathway[12].

Interferon (IFN)-γ is produced by cells of the immune system after viral sensing[14]. INF-γ signalling plays a major role in establishing cellular immunity, and it also induces gene products that prime the Type I IFN response[15,16]. Likewise, Type I IFN signalling primes IFN-γ signalling[17]. Overall, IFN-γ plays a pivotal role in regulating immune function and bridging the innate and adaptive responses. CC-chemokine ligand 2 (CCL2), which is also referred to as monocyte chemoattractant protein 1 (MCP1), is a chemokine that plays a major role in the recruitment of monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by SARS-CoV-2 and other viral infections[1,18,19]. Therefore, the serum levels of INF-γ and CCL2 can be used as a marker of effective inflammatory and immune responses to Covid-19 infection.

In this study, we studied the serum levels of OxPAPC, IFN-γ, and CCL2 in patients with Covid-19 infection. We aimed to investigate the role of OxPLs in the inflammatory and procoagulant responses observed in Covid-19 infection.

Patients and Methods

Patients and Controls

Patients with a positive SARS-CoV-2 polymerase chain reaction test that were hospitalized in our department between 01 May 2020 and 30 June 2020 and were included in the study. Ten healthy donors in the same age and gender groups were also included. The Ethics Committee of Tekirdag Namik Kemal University approved the study. All patients were treated according to national guidelines regarding COVID-19 infection during the study period. Famciclovir was given to all the patients for 5 or 10 days, according to disease severity. Appropriate thromboprophylaxis with subcutaneous low-molecular weight or unfraxionated heparin was given. Clinical and laboratory data were collected from the patients' electronic medical files. Peripheral blood was drawn at inclusion for measurement of OxPAPC, IFN-γ, and CCL2 serum levels.

Immunoassays

Serum samples were obtained by centrifugation of 4 mL whole blood samples and were stored at -80°C until subsequent analysis. All samples were thawed immediately prior to immune assay tests. The serum level of OxPAPC was measured by using the commercially available OxPAPC ELISA kit (MyBiosource Inc., San Diego, CA, USA) with a 1.0 pg/mL sensitivity. Serum levels of IFNγ and CCL2 were assayed by sandwich immunoassays using commercially available kits (BT Lab, Shanghai, China). The intra- and inter-assay coefficients of variation for both were <8% and <10%, respectively. All measurements were performed in duplicate and in accordance with manufacturer's instructions.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics (Version 25.0; IBM Corp., Armonk, NY, USA). The Mann-Whitney U test was used to compare two independent groups. A chi-square test was used for comparison of categorical variables. Correlations between continuous variables were determined by the Pearson method. All p-values were two-sided, and $p<.05$ was considered statistically significant.

Results

Table I displays the demographic and clinical characteristics of the patients and healthy donors included in this study (n=72). Median age was 42.5 years and 54.2% were women. OxPAPC (Figure 1A) and CCL2 (Figure 1B) serum levels were higher in patients than in controls. However, the study groups did not show a statistically significant difference regarding INF-γ serum levels (Figure 1C).

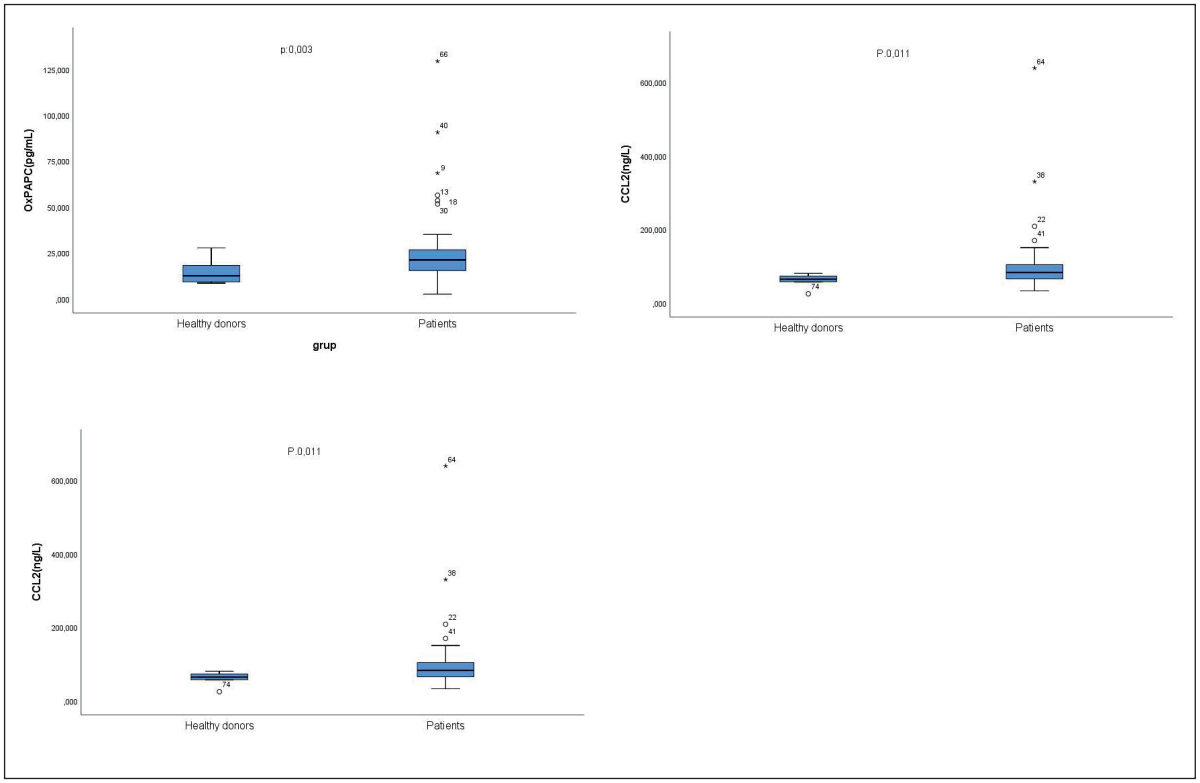

Figure 1. Comparison of OxPAPC (a), CCL2 (b), and INF-γ (c) serum levels of the patients and the healthy donors.

There was no difference in CCL2, INF-γ, or OxPAPC serum levels between patients with lung involvement and patients without (Table II). D-dimer, CRP, and ferritin were higher in patients with lung involvement. Moreover, patients with lung involvement were generally older than those without lung involvement (Table II). We had only six patients who needed intensive care support; there were no differences between these and other patients regarding CCL2, INF-γ, and OxPACS levels (data not shown).

Serum levels of INF-γ and CCL2 were positively correlated (*r*:0.757, *p*<0.0001). However, no correlation was detected between OxPAPC and INF-γ or CCL2 *(r*:-0.007, *p*:0.95 and *r*:-0.141, *p*:0.23, respectively). There was a positive correlation between INF-γ and CCL2 serum levels and platelet count (Table III). We did not observe any

Table I. Demographic and clinical characteristics of the patients and the healthy donors.

	Patients	Healthy donors	*p*
Age, median (range)	42.5(18-89)	44.5 (31-63)	0.65
Gender, Fn (%)	39(54.2%)	4(40%)	0.4
Lung involvement, n (%)	46 (63.9%)	-	
Transfer to ICU n (%)	6 (8.3%)	-	
Leucocyte count (x10^9/L)	5.51±2.32	7.60±1.41	**0.002**
Lymphocyte count (x10^9/L)	1.46±0.70	2.22±0.50	**0.001**
Hgb, gr/dl	13.26±2.06	14.13±0.91	0.77
Platelet count (x10^9/L)	212±56	238±115	0.51
CRP (mg/L)	18.68±421.38	3.21±2.96	**0.041**
Ferritin (ng/ml)	151.4±254.0	65.9±33.2	0.23
Creatinine (mg/dl)	0.95±0.48	0.80±0.20	0.24

Data are shown as mean±SD if not specified.

Table II. Demographic and clinical characteristics of the patients with and without lung involvement.

	Lung involvement (+), n:44	Lung involvement (-), n: 26	p
Age, median (range)	48(18-89)	31.5 (18-71)	0.002
Gender, F/M n (%)	23/23(50/50)	16/10 (61.5/38.5)	0.46
Leucocyte count (x10^9/L)	5.45±2.63	5.63±1.67	0.37
Lymphocyte count (x10^9/L)	1.39±0.70	1.59±0.70	0.17
Hgb (g/dl)	13.33±1.95	13.13±2.26	0.56
Platelet count (x10^9/L)	209±59	219±51	0.30
CRP (mg/L)	19.83±44.50	16.67±39.03	**0.037**
Ferritin (ng/ml)	287.74±441.6	81.27±54.33	**0.009**
Creatinine (mg/dl)	1.0±0.58	0.85±0.18	0.15
INF-γ (ng/ml)	31.40 ± 28.53	24.26 ± 12.48	0.30
CCL2 (ng/L)	106.9 ± 93.88	77.96 ± 27.71	0.11
OxPAPC (pg/L)	25.05 ± 21.03	25.31 ± 13.23	0.32
D-dimer (mg/L)	604.8 ± 1347.5	237.2 ± 493.0	**0.023**

Data are shown as mean±SD if not specified.

correlation between OxPAPC levels and hematologic or biochemical parameters (Table III).

Discussion

Our study shows that serum OxPAPC levels increased in patients with COVID-19 infection. In accordance with previous studies, CCL2 levels also increased in patients with COVID-19 infection, although we did not observe a statistically significant increase in the INF-γ levels in those patients. CCL2 and INF-γ serum levels were positively correlated with each other. On the other hand, the increase in CCL2 and INF-γ levels was not correlated with increase in OxPAPC. No correlation could be shown between OxPAPC level and inflammatory parameters, such as CRP, ferritin, and D-dimer. There was only a positive correlation between the increase in INF-γ and CCL2 levels and the increase in platelet count.

COVID-19 is associated with a thrombogenic coagulopathy with a range of presentations[7]. Patients frequently present with mild thrombocytopenia and increased D-dimer levels that correlate with disease severity[20,21]. In line with this, thromboses can occur at all severity levels of COVID-19 presentation and can range from small to large vessel clots; reports have documented DIC, microvascular thromboses, pulmonary emboli, aorto-iliac and mesenteric thrombi, and large vessel strokes[7,9,22,23]. Furthermore, severe thrombotic complications occur in up to 85% of intensive care patients[24]. SARS-CoV-2 can infect and damage endothelial cells, causing cytokine storm and systemic inflammatory syndromes that promote coagulation[6,25]. Nevertheless, the increased rates of coagulation activation and thrombotic complications in COVID-19 infection have not been fully explained. We hypothesized that OxPLs that occur in the pulmonary vasculature after viral sensing may contribute to the process.

The lungs, which have a large surface area that is exposed to the aerobic environment, are highly susceptible to oxidative stress, and the lipids at the air-liquid interface are an ideal substrate for oxidation. Imai et al., with their mice model, demonstrated that *in vivo* acid challenge caused

Table III. Correlation analysis between OxPAPC, INF, and CCL2 serum levels and hematologic or biochemical parameters of the patients.

	WBC	LYM	PLT	CRP	Ferritin	D-dimer	Creatinine
OxPAPC	-0.056*	-0.074*	-0.003*	-0.038*	-0.060*	-0.016*	-0.046*
	0.64**	0.54**	0.97**	0.76**	0.65**	0.89**	0.70**
INF	0.198*	-0.003*	**0.345***	-0.016*	0.060*	0.042*	0.009*
	0.09**	0.97**	**0.003****	0.89**	0.65**	0.72**	0.93**
CCL2	0.152*	-0.083*	**0.252***	0.008*	0.034*	0.046*	0.042*
	0.20**	0.49**	**0.034****	0.95**	0.79**	0.70**	0.73**

*: r; **: p; LYM: lymphocyte; PLT: platelets.

production of reactive oxygen species (ROS) in alveolar macrophages and triggered production of OxPLs[12]. They also found higher production of ROS in alveolar macrophages isolated from mice treated with inactivated H5N avian influenza virus. Moreover, they showed that inactivated H5N1 viruses can induce ROS formation and TLR4 surface expression on primary human monocytes. When Imai et al[12] analysed lung samples from nine individuals who had developed ARDS following SARS-CoV-1 infection, they observed marked production of OxPLs in the inflammatory exudates lining the injured air spaces, in the pneumocytes and in the alveolar macrophages. Oxidized phospholipids are recognized as danger-associated molecular patterns (DAMPs) by cell scavenger receptors, inducing a cascade of intracellular signalling events that culminate in inflammasome activation and endothelial cell dysfunction[26]. It has been shown that OxPAPC induces lung injury and cytokine production by lung macrophages via TLR4-TRIF[12]. Therefore, OxPLs can contribute to the formation of a procoagulant phenotype both by increasing the secretion of cytokines, such as IL6, through the TRAF-TRIF pathway and by activating cell scavenger receptors.

In our study, it was observed that OxPAPC level increased in patients with COVID-19 infection. However, no relationship was found between OxPAPC level and various inflammatory response indicators, including INF, CCL2, CRP, and ferritin, nor between OxPAPC level and markers of coagulation activation, such as D-dimer. No relationship was found between OxPAPC and the severity of the disease, such as lung involvement or the need for transfer to intensive care. Although the rate of lung involvement was high in our study, most of the patients were in the early period of the disease and had mild clinical findings. Only six of the patients had ferritin above 500 ng/ml; D-dimer values of over 1000 mg/L were observed in eight patients. This might explain why OxPL levels were not statistically significantly correlated with ferritin and D-dimer in our study.

CC-chemokine ligand 2 (CCL2) plays a key role in the recruitment of macrophages, memory T cells, and dendritic cells to sites of inflammation[18,19]. IFN-γ directly inhibits viral replication and, more importantly, has immunostimulatory and immunomodulatory effects after viral sensing[27,28]. Therefore, we measured serum levels of CCL2 and IFN-γ to demonstrate the inflammatory and immune responses developed by the host against SARS-CoV-2. CCL2 levels were high; IFN-γ levels, though elevated in patients infected with COVID-19, did not reach statistical significance. The increase in these two substances showed a certain level of immune response in patients with COVID-19 infection. However, this response did not correlate with OxPAPC levels. The fact that the OxPAPC increase is independent of IFN-γ and CCL2 levels suggests that any hypercoagulability in COVID-19 infection that is due to OxPLs might be independent of antiviral response. However, this hypothesis needs to be demonstrated with more direct findings.

Conclusions

Our findings suggest that OxPAPC elevation occurs independently of the effective immune response required for disease control. The role of OxPLs in the hypercoagulopathy that occurs in COVID-19 infection should be investigated in experimental models and in larger patient groups in order to draw firm conclusions on this issue.

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