CIRCULATING ENDOTHELIAL CELLS AS A MARKER OF ENDOTHELIAL INJURY IN SEVERE COVID -19

Christophe Guervilly[1,2], Stephane Burtey[3,4], Florence Sabatier[4,6,8], Raphaël Cauchois[4,5], Guillaume Lano[3,4], Evelyne Abdili[6], Florence Daviet[3], Laurent Arnaud[6], Philippe Brunet[3,4], Sami Hraiech[1,2], Noémie Jourde-Chiche[3,4], Marie Koubi[5], Romaric Lacroix[4,6], Léa Pietri[7], Yaël Berda[6], Thomas Robert[3,4], Clara Degioanni[8], Mélanie Velier[4,6], Laurent Papazian[1,2], Gilles Kaplanski[4,5], and Françoise Dignat-George[4,6].

Affiliations

[1] Medical Intensive Care Unit, North Hospital, APHM, Chemin des Bourrely, 13015 Marseille, France.

[2] CEReSS, Center for Studies and Research on Health Services and Quality of Life EA3279, Aix-Marseille University, 27 Bd Jean Moulin, 13010, Marseille, France

[3] Department of Nephrology and Renal Transplantation, La Conception, APHM, 147 Bd Baille, 13005, Marseille, France.

[4] Aix Marseille Univ, INSERM 1263, INRAE, C2VN, Marseille, 27 Bd Jean Moulin, 13010 France.

[5] Department of Internal Medicine and Clinical Immunology, La Conception Hospital, APHM, 147 Bd Baille, 13005 Marseille, France.

[6] Laboratory of hematology and vascular biology, La Conception Hospital, APHM, 147 Bd Baille, 13005 Marseille, France

[7] Department of Nutrition, Metabolic Diseases, Endocrinology, La Conception Hospital, APHM 147 Bd Baille, Marseille

[8]Cell Therapy Unit, INSERM CBT1409, La Conception Hospital, APHM, 147 Bd Baille, 13005 Marseille, France.

Corresponding author:

Pr. Françoise Dignat-George

Francoise.dignat-george@univ-amu.fr

Aix Marseille Univ, INSERM 1263, INRAE, C2VN, 27 Bd Jean Moulin 13005 Marseille, France.

Tel.: + 33 4 91 83 56 00

Fax: + 33 4 91 38 36 02

ABSTRACT

Beside the commonly described pulmonary expression of the coronavirus disease 2019 (COVID-19), major vascular events have been reported. The objective of this study was to investigate whether increased levels of circulating endothelial cells (CEC) might be associated with severe forms of COVID-19. Ninety-nine patients with COVID-19 were enrolled in this retrospective study. Patients in the intensive care units (ICU) had significantly higher CEC counts than non-ICU patients and the extent of endothelial injury was correlated with putative markers of disease severity and inflammatory cytokines. Altogether, these data provide *in vivo* evidence that endothelial injury is a key feature of COVID-19.

SUMMARY

Circulating endothelial cells detached from injured vessel were measured in 99 hospitalized COVID-19 patients. They were significantly higher in those admitted to ICU, correlated with inflammatory cytokines and severity criteria, indicating that a marked endothelial injury accompanied disease pathogenesis.

Key words: Circulating endothelial cells, COVID-19, endothelial injury

INTRODUCTION

The COVID-19 pandemic, caused by infection with SARS-CoV-2, has become a global threat to public health, affecting more than four million individuals worldwide. COVID-19 patients are usually mildly symptomatic, but 20% of them will evolve towards viral pneumonia, and among them, 5% will experience an atypical form of acute respiratory distress syndrome (ARDS) [1]. SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2), which is highly expressed in the lungs. ACE2 is also expressed by the endothelium [2], one of the largest organs in the human body. Moreover, cardiovascular complications [3] consisting of strokes, pulmonary embolisms, high blood pressure, vasculitis and Kawasaki-like disease [4] are emerging as severe COVID-19 symptoms, suggesting that endothelial injury could constitute a key pathogenic mechanism. Despite widespread interest in the pathophysiology of the disease, little is known about the involvement of the endothelium during the course of COVID-19.

The vascular endothelium is a dynamic organ that plays key roles in vascular homeostasis, such as maintaining vascular tone, permeability and inflammatory response, preserving the hemostatic balance. Any endothelial injury, including infections, impairs regulatory functions of the endothelium with subsequent vasoconstriction, ischemia, inflammation and activation of the coagulation cascade, ultimately leading to vessels denudation and exposure of the thrombogenic subendothelium [5].

Circulating endothelial cells (CEC) are stressed cells detached from injured vessels [6]. They are detectable at very low levels in healthy conditions. Increased CEC counts have been reported in various diseases of inflammatory, infectious or ischemic origin, where they evidence a profound vascular insult and are indicative of disease severity [6]. The objective of the present study was to measure CEC in the blood of patients with COVID-19, in relation to systemic inflammation and disease severity.

MATERIALS AND METHODS

Patients

Ninety-nine adult patients with COVID-19 were enrolled in a retrospective study approved by a local medical ethics committee of Aix-Marseille University. Written informed consent was obtained from the patients or their relatives. COVID-19 was diagnosed when patients presented with viral pneumonia associated with objective findings on lung-CT scan or chest X-ray, and infection was confirmed by positive SARS-CoV-2 RT-PCR in nasopharyngeal swab, sputum or bronchoalveolar lavage. Patients were hospitalized either in the COVID-19 medicine or nephrology units (non-ICU) or in intensive care unit (ICU), depending on the severity of their oxygen requirements. Blood samples were collected by within 48 h of patient admission.

Quantification of CEC, soluble endothelial cell adhesion molecules and inflammatory cytokines

CEC were counted according to a previously published standardized protocol [6]. Briefly, CEC were isolated by immunomagnetic separation with beads (Dynabeads M-450, Thermo Fisher, Carlsbad, CA) coated with CD146 (clone S-endo1, Biocytex, Marseille, France) and enumerated using a fluorescence microscope (Eclipse TE2000-S, Nikon, Tokyo, Japan) after acridine orange labeling. CEC were identified according to the following consensus criteria: stained rosette cells, size over 15 µm, bearing more than five beads.

Plasma (EDTA) levels of soluble Vascular Cell Adhesion Molecule (sVCAM-1) and E-Selectin (sE-Selectin) and serum levels of Interferon gamma-induced protein 10 (IP-10) and interleukin 6 (IL-6) were quantified by magnetic luminex assay (Bio-Techne, Minneapolis, MN)

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 software. Categorical variables were presented as frequencies, and continuous variables were presented as the median [25-75 percentiles]. Comparisons between groups were performed using Mann-Whitney test for quantitative variables and Fisher's exact tests for frequencies. Associations between continuous variables were analyzed using Spearman's correlation tests. Multiple linear regression was performed to determine the impact of cardiovascular risk factors and comorbidities on CEC levels. Statistical significance was defined as $p<0.05$.

RESULTS

Patients' characteristics are reported in table 1. The non-ICU patients were older and less frequently obese than the patients in the ICU. The prevalence of other comorbidities was higher in the non-ICU patients. During their stay in the ICU, all patients needed invasive mechanical ventilation. At inclusion, ICU patients had more severe lymphopenia and higher levels of leukocytes, neutrophils fibrinogen, C-reactive protein and lactate dehydrogenase than non-ICU patients.

Measurement of CEC revealed that 55% of patients had CEC counts above 20 cells/mL, considered as the upper limit of normal values. CEC count was significantly higher in ICU patients than in non-ICU patients (49 [24-103] vs 18 [6-70] CEC/mL, p=0.03) (Fig. 1A). CEC counts were negatively correlated

with platelet and lymphocyte counts (r=-0.334, p=0.0008 and r=-0.336, p=0.0007, respectively) (Figure 1B), previously identified as COVID-19 severity markers [7]. Interestingly, a positive correlation between the CEC counts and the length of hospital stay (r=0·261, p=0·02) was observed.

The plasma concentrations of the inflammatory cytokines IL-6 and IP-10, and of two inflammation-induced endothelial adhesive molecules, E-Selectin and sVCAM-1, were significantly higher in ICU patients than non-ICU patients (table 1). CEC counts were correlated with sVCAM-1 (r=0.374, p=0.0002) and IP-10 concentrations (r=0.487, p<0.0001) (Figure 1C) but not with sE-Selectin and IL-6 concentrations.

Finally, because we analyzed the relationship between CEC count, comorbidities and cardiovascular risk factors in patients with COVID-19. In univariate analysis, increased CEC levels were associated with CKD but not with high blood pressure, diabetes, age, obesity or CVD. Multivariate analysis integrating age, obesity, hypertension, CKD, cardiovascular disease - and ICU hospitalization, indicated that among comorbidities factors only CKD could be identified as independently associated with CEC counts (p= 0.011). Admission in ICU was also associated with high CEC levels independently of all comorbidities that differ between ICU and non ICU patients (p=0.046). Consequently, after the exclusion of patients with CKD, the difference between CEC levels in ICU patients compared to non-ICU patients was even more pronounced (p=0.002) (Figure 1A).

DISCUSSION

This is the first observation of increased CEC counts in the blood of patients with COVID-19. CEC counts were significantly higher in patients admitted to the ICU, positively correlated with the length of hospital stay and were inversely correlated with platelet and lymphocyte counts. Increased CEC levels were also associated with increased concentrations of the cytokine IP10 and sVCAM-1. These

results provide *in vivo* evidence that a marked and widespread endothelial injury occurred in the course of proinflammatory vessel activation in the more severe forms of COVID-19.

CEC have been previously reported to be increased in patients with cardiovascular risk factors [6]. Although our cohort showed a high prevalence of various comorbidities, CKD was the only condition identified as a major determinant of elevated CEC counts in patients with COVID-19. Consistently, previous studies indicated that increased CEC counts behave as a marker of endothelial dysfunction with pejorative prognosis value in hemodialysis patients [8]. Thus, more than reflecting preexisting vascular dysfunction associated to typical risk factors, a rise in CEC counts in COVID-19 patients could be indicative of the acute deleterious impact of the disease process itself. However, CKD, which is increasingly recognized as a risk factor for severe COVID-19 development, may favor endothelial vulnerability.

Endothelial involvement across different vascular beds was shown in recent postmortem histopathological observations of COVID-19 patients with multisystem organ failure. Ultrastructural damage, including disruption of intercellular junction, cell swelling and a loss of contacts with the basal membrane, were associated with accumulation of inflammatory cells together with the presence of apoptotic bodies and viral inclusion structures in endothelial cells, recapitulating features of endotheliitis [9,10]. This suggests that both viral infection of endothelial cells and inflammation-induced microangiopathy could contribute to cell detachment and elevated CEC counts. Indeed, other coronaviruses are cytopathic [11] and potent inducers of the intracytoplasmic NLRP3 inflammasome, known to control pyroptosis and to play a role in endothelial detachment in Kawasaki Disease [12], a disease recently associated with the COVID-19 spectrum [4]. Excessive activation of the NLRP3 inflammasome, triggering IL-6 and Interferon-γ production may also mediate a severe inflammatory endothelial cell response. Consistent with the “cytokine storm” involved in the severe forms of COVID-19 [13], we found that CEC counts were positively correlated with the

concentrations of IP-10, and sVCAM-1, known to be induced by Interferon-γ. This suggests that the cytokine storm plays an important role in the endothelial cell monolayer disruption during SARS-CoV-2 infection, as already reported in other viral diseases [14]. Loss of adhesive contact of infected endothelial cells, involving inflammatory cytokines or leukocytes-derived proteases has also been documented during HSV or CMV infection [6, 15].

Markers allowing to stratify the progression of COVID-19 are helpful to improve the management of patients. Interestingly, we found that the CEC levels in COVID-19 patients were inversely correlated with lymphocytes and platelets counts, recognized as prognostic factors for early identification of severe COVID-19 cases [8]. CEC counts were also correlated with the length of hospital stay. Thus, CEC could be proposed as a noninvasive and specific biomarker to quantify endothelial injury associated with the exacerbated inflammatory host response that characterizes severe COVID-19.

There are some limitations to this study. The small sample size and the overall low mortality rate limited the evaluation of the prognostic value of CEC, notably for the prediction of cardiovascular complications. In addition, because serial blood samples could not be available, future studies are required to investigate the kinetics of CEC and its relationship with COVID 19 evolution and therapeutic interventions.

In conclusion, increased CEC counts provide a direct proof of endothelial damage during the course of COVID-19 and a clinically informative biomarker of disease severity. The data may also bring rationale for therapies that stabilize the endothelium in addition to limiting inflammation in vulnerable patients. Future longitudinal studies are required to define the usefulness of CEC as a non-invasive vascular biomarker for risk stratification and response to treatment.

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Potential conflicts of interest

F. DG and R.L disclose grants from Stago and a patent on microvesicle fibrinolytic activity licensed to Stago. C.G reports personal fees from XENIOS FMC Inc. Similarly, S.B reports personal fees from Otsuka, Bayer and from Sanofi. All these disclosures are outside the submitted work.

Legends

Table 1: Baseline demographics, treatments, biological parameters and outcomes of the study patients

Figure 1: CEC enumeration in patients with COVID-19. (A) CEC/mL values in Non-ICU patients (n=80), ICU patients (n=19), Non-ICU Non-CKD patients (Non-ICU CKD -, n=52), patients with CKD (Non ICU CKD +, n=28). **(B)** Correlations between CEC count and platelets and lymphocytes. **(C)** Correlations between CEC count and IP-10 and sVCAM-1.

CEC: circulating endothelial cells. COVID-19=coronavirus disease 2019. ICU: intensive care unit. IP-10: interferon gamma-induced protein 10. sVCAM-1: soluble vascular cell adhesion molecule-1.

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	Non-ICU Patients	ICU Patients	*p*-value
Demographic information			
Total No·	80	19	
Age[a] (years)	68.2 (57.7-80.3)	55.3 (43.3-60.7)	0.0001
Sex M/F	44/36	12/7	ns
Cardiovascular risk factors			
Obesity[b], n(%)	15 (19)	9 (47)	0.01
Chronic Kidney Disease[c], n(%)	28 (35)	0 (0)	0.001
stage 3 n(%)	9 (11)	0 (0)	
stage 4 n(%)	1 (1)	0 (0)	
stage 5D n(%)	18 (23)	0 (0)	
Hypertension, n(%)	43 (54)	4 (21)	0.01
Diabetes, n(%)	20 (25)	4 (21)	ns
Cardiovascular disease, n(%)	24 (30)	0 (0)	0.005
Chronic respiratory disease			
Asthma, n(%)	1 (1)	2 (11)	ns
Chronic Obstructive pulmonary disease, n(%)	3 (4)	0 (0)	ns
Clinical data at admission and treatments received during stay			
Oxygen Saturation[a], %	96 (94-97)	86·5 (81-93)	<0·0001
Received supplemental oxygen, n(%)	64 (80)	19 (100)	0·04
Invasive mechanical ventilation, n(%)	NA	19 (100)	
Extra Corporeal Membrane Oxygenation, n (%)	NA	8 (42)	

Anti-viral treatment				
	Hydroxychloroquine + azithromycine, n (%)	58 (73)	12 (63)	ns
	Lopinavir + ritonavir, n (%)	0 (0)	5 (26)	0.0002
	None, n (%)	22 (28)	2 (11)	ns
Outcomes				
	28 days mortality, n (%)	3 (3)	0 (0)	ns
	Transferred into ICU, n (%)	13 (16·3)	NA	
	Total length of hospital stay at study end point of alive discharged patients (days)	14 (9-18)	27 (23-34)	0.0001
	Hospital discharged at 28 days, n (%)	70 (88)	6 (31)	<0.0001
Laboratory results at inclusion				
	White blood cell count[a] ($x10^9$/L)	5.7 (4.6-7.3)	6.7 (5.6-12.0)	0.04
	Neutrophil[a] ($x10^9$/L)	3.8 (2.8-5.3)	6.1 (4.2-10.1)	0.002
	Lymphocyte[a] ($x10^9$/L)	1.2 (0.8-1.8)	0.9 (0.7-1.1)	0.02
	Platelet count[a] ($x10^9$/L)	272 (195-317)	240 (188-310)	ns
	Fibrinogen[a] (g/L)	5.7 (4.8-7.5)	6.8 (6.4-7.8)	0.04
	D-dimer[a] (ng/ml)	1.6 (0.8-2.1)	2.5 (1.4-4.7)	ns
	C-Reactive Protein[a] (mg/L)	49 (19-89)	125 (91-179)	0.004
	Lactate deshydrogenase[a] (U/L)	291 (224-344)	473 (376-640)	<0.0001
	Creatinine[a, d] (µmol/L)	71 (51-102)	66 (57-80)	ns

Circulating Endothelial Cells				
	CEC (n/ml)[a]	18 (6-70)	49 (24-103)	0.03
Soluble endothelial markers				
	sVCAM-1[a] (ng/mL)	1700 (1135-2992)	2532 (1902-4795)	0.04
	sE-Selectin[a] (ng/mL)	25 (20-36)	39 (31-58)	0.008
Cytokines				
	IP-10[a] (pg/mL)	89 (53-211)	227 (134-336)	0.001
	IL-6[a] (pg/mL)	25 (13-48)	93 (44-142)	0.0003

[a] median (25-75 percentile);[b]Obesity was defined as an body mass index> 30kg/m²; [c]Chronic kidney disease was defined as an estimated glomerular filtration rate <60mL/min/1.73m² or requirement for hemodialysis for more than 3 months; [d] hemodialyzed patients were excluded; M. F, male/female; NA, not applicable; ns, non significant; ICU : intensive care unit; CEC : circulating endothelial cells; sVCAM-1: soluble vascular cell adhesion molecule 1;sE-Selectin: soluble E- Selectin; IP-10: Interferon gamma-induced protein 1; IL-6: interleukin 6.

Table 1: Baseline demographics, treatments, biological parameters and outcomes of the study patients.

Figure 1