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Morphological and functional alterations in endothelial colony-forming cells from recovered COVID-19 patients

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More than 197 million people have been infected by Severe Acute Respiratory Syndrome Coronavirus 2, with more of 4.2 millions of deaths and more of 177 millions have overcome the disease COVID-19 [1]. Complications observed in deceased patients as well as those who have survived the disease, are related to damage of the vascular endothelium and the consequent severe dysfunction of the coagulation system, resulting in a wide variety of potentially devastating thrombotic events characterized by high levels of D-dimer and fibrinogen, suggesting a direct relationship with endothelial dysfunction [2]. Endothelial dysfunction describes various pathophysiological conditions, including an activation state that generates a pro-inflammatory environment as well as the synthesis of inflammatory cytokines and the formation of thrombus [3]. As a result, an abnormal endothelial function increased, mobilization or recruitment of circulating endothelial cells (CEC), endothelial progenitor cells (EPCs), or endothelial colony-forming cells (ECFCs) are present. ECFCs with high potential for inducing angiogenesis, may be the result of detachment of endothelial cells from the vascular wall or bone marrow [4]. Previously, it was informed that an abnormal number of EPCs was present in patients with moderate and severe COVID-19 and in those recovered from this disease [5].

We analyzed the frequency, function and morphology of ECFCs as well as their response to convalescent plasma in 15 COVID-19 recovered male patients, without a history of major comorbidities matched by age (20–50 years) with 10 controls. All had subacute, moderate disease in the previous 4 weeks as confirmed by means of a positive real time-polymerase chain reaction (RT-PCR) test and all of them received prophylactic anticoagulant therapy with enoxaparin (40 mg SC, OD). At the time of peripheral blood (PB) sampling, all patients were negative for COVID-19 (by real-time RT-PCR) and all treatments had been stopped. An evaluation of D-dimer, C reactive protein and fibrinogen was performed. The clinical manifestations, the laboratory markers and their controls was normal (data not shown). Mononuclear cells (MNCs) of PB were obtained according to Alvarado-Moreno et al. [6] and colonies of endothelial cells (ECs) of ECFCs (ECFC-ECs) were identified, counted and expanded to get homogeneous cultures of ECs.

To compare the likely endothelial effects of a prothrombotic state such as that found in our COVID-19 recovered patients with a chronic thrombotic disease, we used ECFC-ECs previously obtained from five COVID-19-negative patients with recurrent venous thromboembolic disease (VTD) cryopreserved in our laboratory. Results are expressed as mean ± standard deviation. Student *t*-test and Mann-Whitney *U* test were used to analyze the results and $P \leq 0.05$ was considered significant. Data were analyzed with Sigma Stat statistical software (v.3.5, Sigma

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Stat, San Jose, CA, USA). The study protocol was approved by the National Ethics Committee of the Instituto Mexicano del Seguro Social (IMSS-R-2020-785-167 and IMSS-R-2015-785-091) and informed consent was obtained from each individual. This study fulfilled the principles of the Declaration of Helsinki.

We analyzed the content of MNCs in all samples and observed an increase in total cell number of recovered COVID-19 patients as compared with controls (350×10^6 vs. 150×10^6, respectively; $P \leq 0.001$), which represents a 2.3-fold increase cell count (data not shown). This finding correlates with previous reports where an increase of CD14 and CD16 monocytes up to 50% was found, specifically monocytes and monocyte-derived macrophages capable of secreting IL-6, TNF-α, and IL-10, related with the cytokine storm observed in COVID-19 patients and the endothelial dysfunction associated with immunothrombosis [7]. Our study focused on patients with a 30 year-old median age and a subacute moderate disease because in previous studies with rhesus macaque and older and younger human adults infected with SARS-CoV-2 no significant differences in the percentage and number of monocytes were found [8].

Noteworthy, we found an increase of ECFCs that also appeared in a shorter period of time in samples from COVID-19 recovered patients as compared with controls; however, the highest number of ECFCs was obtained from samples from VTD patients (Fig. 1A and B), a fact that suggests a faster and constant mobilization of ECFCs in a chronic prothrombotic disease [6] as compared with a subacute disease. It is important to emphasize that, because our research was performed in PB samples obtained four weeks after the onset of symptoms, it would be important to evaluate the biological behavior of ECFCs during the very acute phase of COVID-19.

We evaluate the morphology of ECFCs obtained from controls (Fig. 1C and D) and from recovered COVID-19 patients, and the immature characteristics of these last cells show that, they exhibit profound abnormalities in size namely elongations resembling pseudopods, and large cytoplasm with prominent nuclei highly compatible with a senescent state (Fig. 1E), showed no proliferative capacity when they were sub-cultured (Fig. 1F). In contrast, immature ECFCs from donors and VTD patients depict the same characteristics and proliferative activity as previously demonstrated by our working group [6] (Fig. 1G and H). Our results strongly suggest an abnormal functional endothelial status determined by an absence or reduction of endothelial regeneration in subacute, moderate COVID-19 patients. We may speculate that all disturbances found in ECFCs from COVID-19 patients are worse during the acute phase of a severe disease, a fact that may help to explain the highly significant increase of thrombotic episodes observed in these patients [9].

Interestingly, when immature ECFCs from either recovered COVID-19 patients and controls were cultured for 48 h in presence of plasma from recovered COVID-19 patients, we observed in cultures control a cell grouping, effect similar to the formation of tubular structures, an event that has been associated with angiogenesis and vascular regeneration (Fig. 2A and C) (Fig. 2B and D). These facts suggest that ECFCs-ECs from COVID-19 recovered patients lose their regenerative and angiogenic capacity as well as their ability to properly respond to the stimulation with plasma and that, as a consequence, they are unable to restore or maintain an appropriate endothelial function. [7].

The analysis of the response of mature ECFC-ECs (in passage 3), obtained from controls and VTD patients during 48 h in presence of control and COVID-19 plasma, shows similar effects as those found in immature ECFCs, with similar tubular structures when cells were incubated in the presence of control and COVID-19 plasma (Fig. 2E, F and G). These results show the ability of normal cells to respond to plasma components and to restore the endothelial function. In contrast, when we evaluate the effect of COVID-19 plasma on mature ECFC derived from VTD, a low ability to form tubular structures was observed when compared with controls (Fig. 2H, I and J). This fact suggests that there is also a poor response to plasma stimulation which may be likely associated with the endothelial dysfunction detected in subjects with comorbidities [6].

In our study, we used convalescent COVID-19 plasma free of SARS-CoV-2 but perhaps with increased concentrations of inflammatory cytokines. As consequence, addition of such highly inflammatory milieu to dysfunctional cells may easily potentiate the damage and explain the results observed in our report. It is important to emphasize that this research could not performed in ECFC-ECs from recovered COVID-19 patients because their cells were not able to proliferate and stay in subsequent cultures. Thrombosis is, perhaps, the leading cause of morbidity and mortality in COVID-19 patients and endothelial cell dysfunction seems to be the most important factor directly causing these complications [10]. Our results strongly suggest that mature and immature ECFCs obtained from subacute, moderate COVID-19 patients have functional abnormalities that impede them to proliferate in vitro and respond to some components of plasma obtained from individuals with subclinical inflammation.

Apparently, these abnormalities reduce their ability to form tubular structures having a negative impact on their recovery. All these events may be even worse with more deleterious consequences in severely ill COVID-19 patients. Of course, it is important to determine whether the observed damage of the ECFCs is directly associated with a cytopathic effect of the virus or secondary to an uncontrolled immune response as occurs during COVID-19 disease which is characterized by huge amount of inflammatory molecules released to the environment [7].

It should me mentioned that CFCEs have been widely described in various diseases where abnormal molecular mechanisms as well as morphological and functional characteristics of these cells have been identified: in the mechanisms of angiogenesis associated with von Willebrand Disease [11]; chronic myeloid leukemia, may show an increased number of colonies of ECFCs [12]; hereditary haemorrhagic telangiectasia, in which a mutation in endoglin leads to a disorganized cytoskeleton with abnormal angiogenic abilities [13]; patients with ischemic heart disease may have an increased number or colonies of ECFCs [14]; in chronic obstructive pulmonary disease, ECFCs may exhibit an accelerated senescence and abnormal angiogenic ability [15]; in idiopathic pulmonary fibrosis it has been shown low numbers of colonies of ECFC, abnormal gas transfer, and increased cell proliferation [16]; gestational diabetes mellitus is characterized by an altered proliferation and premature senescence while in type 2 diabetes mellitus, neovascularization and proliferation are both reduced [17]; pulmonary arterial hypertension has been associated with an increased proliferative potential with a deficiency in ability to form vascular networks [18].

A major limitation of our study is that patients included had a moderate, subacute variant of the disease and, consequently, our findings may not represent what may occur in the more severe or acute phases of the disease. Moreover, we cannot underestimate the fact that all patients included in this research received prophylactic anticoagulant therapy, a variable that could likely modify the results obtained.

In conclusion, we demonstrated for the first time that ECFCs from patients recovered from COVID-19 show both, morphological characteristics of dysfunctional endothelial cells and almost no proliferation and angiogenic capability. These findings may help to understand the pathophysiology of dysfunctional endothelium in COVID-19 patients. Of course, as we have shown here, this dysfunctional status of the endothelium may help to explain the highly frequent presentation of thrombotic episodes in patients with COVID-19 even at the sub-acute phase and the likely need to prolong the anticoagulant therapy in some specific populations affected with this virus.

Authorship contributions

AC-G, AM-C A and JAA-M: design of study. JAA-M, JD-M, VD-R, RA-D and II-S: perform the assays of the study. AC-G, AM-C and JAA-M: writing of the manuscript.

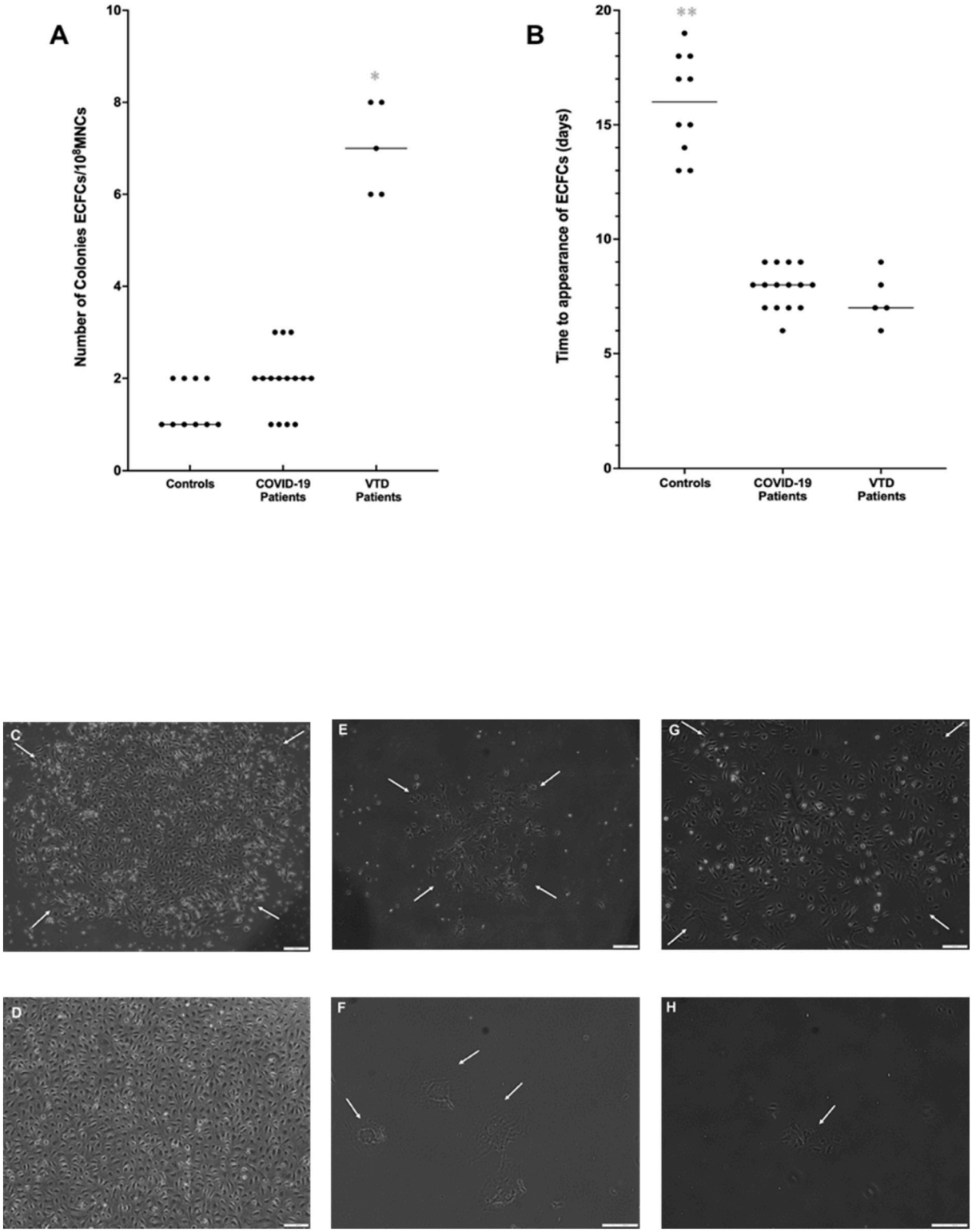

Fig. 1. Frequency and identification of ECFCs in PB from recovered COVID-19 patients, healthy donors (Controls) and VTD patients. (A) The figure shows the number of ECFCs colonies obtained per 100 mL of MNCs seeded in culture plates. (B) Time to detection (days) of ECFCs per 1×10^8 MNCs seeded in culture plates. Results are expressed as mean ± SD of 15, 10 and 5 independent samples, respectively. *: $P < 0.001$, Controls and recovered COVID-19 patients vs. VTD patients. **: $P < 0.001$, Controls vs. recovered COVID-19 patients and VTD patients. Representative phase-contrast photomicrographs from 3 experiments in triplicate (4× magnification) of ECFCs colonies detected in culture from: Controls before (C) and after expansion (D); recovered COVID-19 patients before (E) and after expansion (F) and VTD patients before (G) and after expansion (H). Arrows indicate colony boundary, the typical morphology of a normal immature colony and after reseeding, they present a robust proliferative potential that leads to cell confluence. VTD patients present the same characteristics but with slow proliferation; in recovered COVID-19 patients, the colonies show obvious morphological alterations without proliferative capacity. Scale bar represents 200 μm.

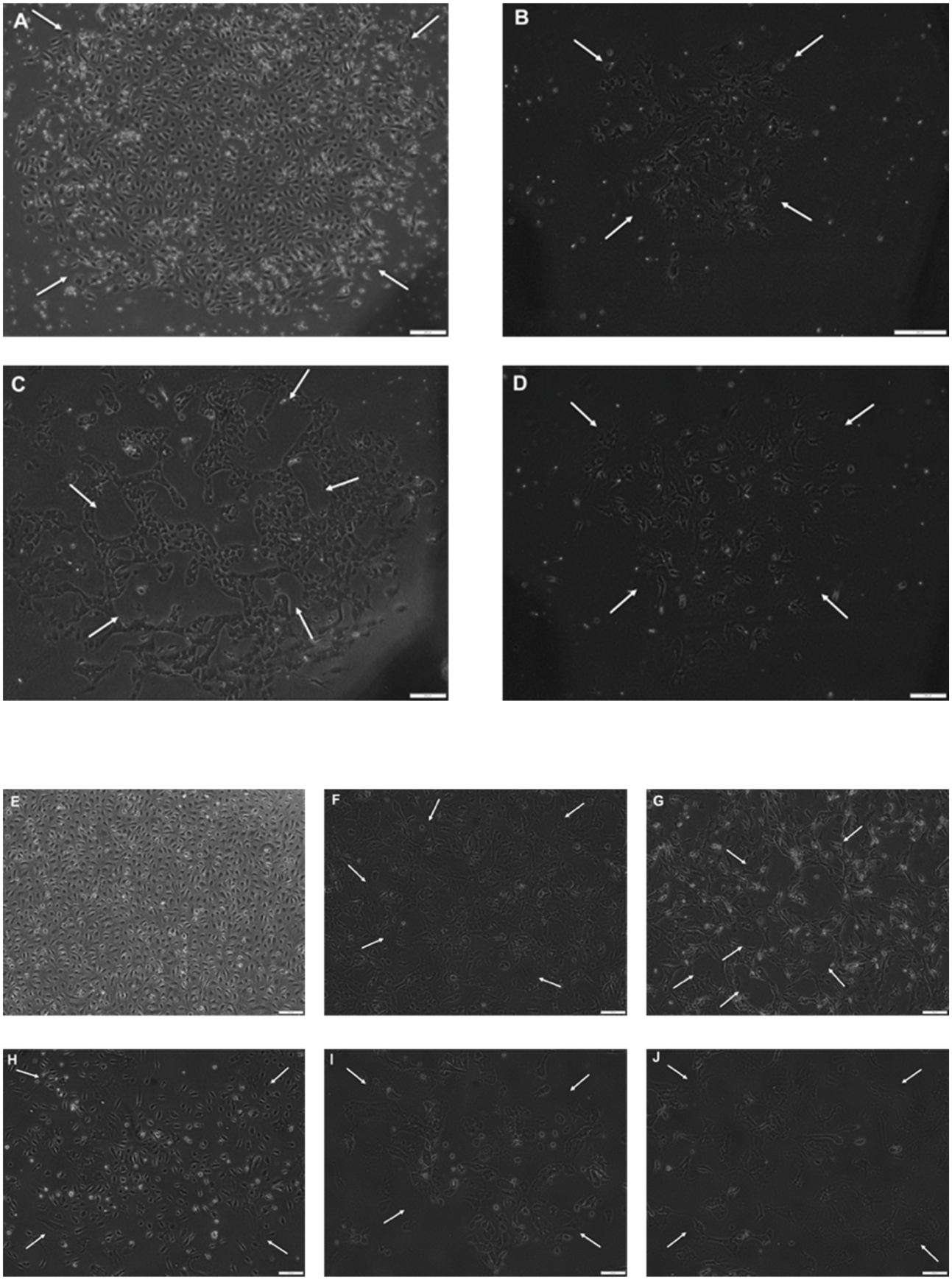

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Fig. 2. Controls but not COVID-19 immature and VTD mature ECFCs form tubular structures in vitro. Representative phase-contrast photomicrograph from 3 experiments in triplicate (4× magnification) of an immature ECFCs culture in normal medium for Controls (A), and recovered COVID-19 patients (B); arrows indicate colony boundary. To evaluate angiogenic ability, were incubated 48 h with plasma from recovered COVID-19 patients, tubular structures with characteristics similar to vascular lumen were detected in Controls (C), arrows show these structures but not in recovered COVID-19 patients (D). Mature ECFCs (passage 3) from Controls (E) or VTD patients (H) were incubated 48 h in presence of plasma from healthy donors in Controls (F) and VTD patients ECFCs (I) or plasma of recovered COVID-19 patients in Controls (G) and VTD patients ECFCs (J). Presence of control and COVID-19 plasma, shows in ECFCs of Controls, similar effects as those found in immature ECFCs, with similar tubular structures when cells were incubated in the control and COVID-19 plasma (F) and (G). Arrows show structures similar to vascular lumen. In mature ECFC derived from VTD, control and COVID-19 plasma show a low ability to form tubular structures (I) and (J). Arrows show structures with deficiencies to form vascular structures. Scale bar represents 200 μm.

Declaration of competing interest

Authors declare no conflicts of interest.

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References

[1] World Health Organization, COVID-19 Weekly Epidemiological Update. Edition 51, August 3, 2021 published.
[2] F.A. Klok, M.J.H.A. Kruip, N.J.M. van der Meer, et al., Confirmation of the high cumulative incidence of thrombotic complications in critically ill ICU patients with COVID-19: an updated analysis, Thromb. Res. 191 (2020) 148–150, https://doi.org/10.1016/j.thromres.2020.04.041.
[3] J. Thachil, N. Tang, S. Gando, et al., ISTH interim guidance on recognition and management of coagulopathy in COVID-19, J. Thromb. Haemost. 18 (2020) 1023–1026, https://doi.org/10.1111/jth.14810.
[4] P.J. Critser, Yoder., Endothelial colony forming cells role in neoangiogenesis and repair, Curr. Opin. Organ. Transplant. 15 (2010) 68–72, https://doi.org/10.1097/MOT.0b013e32833454b5.
[5] P. Mancuso, A. Gidaro, G. Gregato, et al., Circulating endothelial progenitors are increased in Covid-19 patients and correlate with SARS-CoV-2 RNA in severe cases, J. Thromb. Haemost. 6 (2020), https://doi.org/10.1111/jth.15044.
[6] J.A. Alvarado-Moreno, J.R. Hernández-López, A. Chávez-González, et al., Endothelial colony-forming cells: biological and functional abnormalities in patients with a history of recurrent, unprovoked venous thromboembolic disease, Thromb. Res. 137 (2016) 157–168, https://doi.org/10.1016/j.thromres.2015.11.005.
[7] Y. Zhou, B. Fu, X. Zheng, et al., Pathogenic T cells and inflammatory monocytes incite inflammatory storm in severe COVID-19 patients, Natl. Sci. Rev. (2020), https://doi.org/10.1093/nsr/nwaa041.
[8] P. Yu, F. Qi, Y. Xu, et al., Age-related rhesus macaque models of COVID-19, Anim. Model Exp. Med. 3 (2020) 93–97, https://doi.org/10.1002/ame2.12108.
[9] J.A. Alvarado-Moreno, A. Majluf-Cruz, Covid-19 and dysfunctional endothelium: the Mexican scenario, Arch. Med. Res. 6 (2020) 587–588, https://doi.org/10.1016/j.arcmed.2020.05.004.
[10] B. Buijsers, C. Yanginlar, A. de Nooijer, et al., Increased plasma heparanase activity in COVID-19 patients, Front. Immunol. 11 (2020), 574047, https://doi.org/10.3389/fimmu.2020.575047.
[11] S.N. Selvam, L.J. Casey, M.L. Bowman, et al., Abnormal angiogenesis in blood outgrowth endothelial cells derived from von Willebrand disease patients, Blood Coagul. Fibrinolys. 28 (2017) 521–533, https://doi.org/10.1097/MBC.0000000000000635.
[12] L. Teofili, M. Martini, M.G. Iachininoto, et al., Endothelial progenitor cells are clonal and exhibitthe JAK2(V617F) mutation in a subset of thrombotic patients with ph-negative myeloproliferative neoplasms, Blood 117 (2011) 2700–2707, https://doi.org/10.1182/blood-2010-07-297598.
[13] L.A. Hernandez, F. Sanz-Rodriguez, R. Zarrabeitia, et al., Blood outgrowth endothelial cells from hereditary haemorrhagic telangiectasia patients reveal abnormalities compatible with vascular lesions, Cardiovasc. Res. 68 (2005) 235–248, https://doi.org/10.1016/j.cardiores.2005.06.009.
[14] M. Massa, R. Campanelli, E. Bonetti, et al., Rapid and large increase of the frequency of circulating endothelial colony-forming cells (ECFCs) generating late out growth endothelial cells in patients with acute myocardial infarction, Exp. Hematol. 37 (2009) 8–9, https://doi.org/10.1016/j.exphem.2008.09.007.
[15] K.E. Paschalaki, R.D. Starke, Y. Hu, et al., Dysfunction of endothelial progenitor cells from smokers and chronic obstructive pulmonary disease patients due to increased DNA damage and senescence, Stem Cells 31 (2013) 2813–2826, https://doi.org/10.1002/stem.148.
[16] D.M. Smadja, L. Mauge, H. Nunes, et al., Imbalance of circulating endothelial cells and progenitors in idiopathic pulmonary fibrosis, Angiogenesis 16 (2013) 147–157, https://doi.org/10.1007/s10456-012-9306-9.
[17] S.F. Leicht, T.M. Schwarz, P.C. Hermann, et al., Adiponectin pretreatment counteracts the detrimental effect of a diabetic environment on endothelial progenitors, Diabetes 60 (2011) 552–561, https://doi.org/10.2337/db10-0240.
[18] M. Toshner, R. Voswinckel, M. Southwood, et al., Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension, Am. J. Respir. Critic. Care Med. 180 (2009) 780–787, https://doi.org/10.1164/rccm.200810-1662OC39.