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Coronavirus Disease 2019 (COVID-19) Coronary Vascular Thrombosis

Correlation with Neutrophil but Not Endothelial Activation

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Severe coronavirus disease 2019 (COVID-19) infection increases the risk of myocardial injury that contributes to mortality. We used multiparameter immunofluorescence to examine extensively heart autopsy tissue of 7 patients who died of COVID-19 compared to 12 control specimens, some with and some without cardiovascular disease. Consistent with prior reports, we found no evidence of viral infection or lymphocytic infiltra
tion indicative of myocarditis but did observe frequent and extensive thrombosis in large and small vessels in the hearts of the COVID-19 cohort, findings that were infre
dent in controls. The endothelial lining of thrombosed vessels typically lacked evidence of cytokine-mediated endothelial activation, assessed as nuclear expression of transcription factors p65 (RelA), pSTAT1, or pSTAT3 or evidence of inflammatory activation assessed by expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), tissue factor, or von Willebrand factor (VWF). Intimal EC lining was also generally preserved with little evidence of cell death or desquamation. In contrast, there were frequent markers of neutrophil activation within myocardial thrombi of COVID-19–infected patients including neutrophil-platelet aggregates, neutrophil-rich clusters within macrothrombi, and evidence of neutrophil extracellular trap (NET) formation. These findings point to alterations in circulating neutrophils rather than the endothelium as contributors to the increased thrombotic diathesis in the hearts of COVID-19 patients. (Am J Pathol 2021, ■: 1–9; https://doi.org/10.1016/j.ajpath.2021.09.004)

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autoantibody-mediated injury through complement activation and/or antibody-dependent cellular cytotoxicity.\textsuperscript{11} However, numerous autopsy studies have rarely detected viral protein or RNA within the heart despite evidence of abundant virus presence in the lungs of the same patients.\textsuperscript{12} Post mortem examination has also rarely revealed multifocal or diffuse myocarditis along with complement and antibody deposition.\textsuperscript{12–14} Rather, thrombosis of micro and macro coronary vessels has most consistently characterized the heart of individuals who succumbed to coronavirus disease 2019 (COVID-19).\textsuperscript{14–16} The underlying cause of this thrombotic diathesis in the hearts of severe COVID-19 patients is unsettled but has been attributed to either alterations in blood composition and/or pathological changes of the endothelial lining of the vasculature.\textsuperscript{17} This study examined heart tissues from seven autopsies of COVID-19 patients performed early in the pandemic before anticoagulation was commonly administered and compared these specimens to twelve COVID-19 negative controls, with and without heart disease, using multiparameter fluorescence microscopy to analyze the composition of thrombosed vessels. Our data show that alterations in endothelium do occur but are uncommon and do not generally colocalize with sites of thrombosis, whereas the presence of activated circulating neutrophils are routinely observed in the thrombi of patients with severe SARS-CoV-2 disease.

Materials and Methods

COVID-19 Cohort Characteristics

The COVID-19 cohort consists of 7 patients, 4 males and 3 females, who were positive for SARS CoV-2 infection by RT-PCR with ages ranging from 50 to 94 (Table 1). All patients had severe pneumonia. One patient had a sudden cardiac arrest outside of hospital, two patients developed sepsis, and one patient had recurrent acute leukemia with thrombocytopenia. Length of hospitalization ranged from 2 to 13 days (Supplemental Table S1). The COVID-19 negative controls consist of 12 patients, 8 males and 4 females, including 6 patients with pre-existing cardiac disease. Multiple sections from 2 randomly selected areas of the left ventricle were examined for each patient and control.

Immunofluorescence of FFPE Tissues

Formalin fixed, paraffin-embedded de-identified sections of heart tissues were obtained from autopsies performed at Brigham and Women’s Hospital and at Yale New Haven Hospital under IRB approved protocols. Slides were deparaffinized by graded washes in xylene and ethanol. Antigen retrieval was then performed in sodium citrate buffer (Vector, H-3300) for 30 minutes. Sections were incubated with blocking solution containing 1% BSA, 5% donkey serum in PBS for at least 1 hour at RT. Primary antibodies reactive with the following antigens were added overnight at 4°C: Cleaved Caspase 3 (1:300, Cell Signaling Technology (CST) 5A1E), ICAM-1 (1:300, Novus Biologicals (NB) BBA17), myeloperoxidase (1:200, CST E1E7I), CD45 (1:100 Abcam (ab) ab10558), CD42b (1:100, Thermo Fisher (TF) 42C01; 1:150, TF PA5-109282), VE-Cadherin (1:300, SantaCruz (sc) sc-6458), Fibrin (1:200, Dako A0080), IgM AF647 (1:200 SouthernBiotech (SB) 2020-31), CD11b (1:100 TF M1/70), myeloperoxidase AF647 (1:100, ab252131), CitH3 (1:100, ab5103), p65 (1:400, CST D14E12), pSTAT1 (1:100, CST 58D6), pSTAT3 (1:100, CST D3A7), tissue factor (1:200, CST E9M6T; 1:200 NB2-61641), VWF (1:50, ab201336), VCAM-1 (1:100, NB 6G9), E-selectin (1:100, TF PA5-96091), SARS CoV-2 spike (1:200, GeneTex GTX632604), SARS CoV-2 nucleocapsid (1:200, CST HL344), and C4d (1:100, Quidel A213). Samples were washed three times in PBS and then incubated with appropriate AlexaFluor conjugated secondary antibodies and Ulex Europaeus Agglutinin I — FITC/Rhodamine (1:200, Vector RL-1061, 1062) in same blocking solution for 1 hour at room temperature. All antibodies were validated using human tonsils or autopsy heart tissues with myocarditis, sepsis, or myocardial infarction. Secondary only controls were used to ensure specificity of primary antibody binding.

Cyclic IF of FFPE Tissue

In some instances to colocalize multiple fluorescent signals on the same field we employed a cyclic IF method that involves repeated staining and imaging of the same tissue section with quenching of the fluorescent signal between cycles and using directly conjugated antibody. Briefly, coverslips were placed on slides using PBS with 10% glycerol. After imaging, coverslips were removed by placing slides in container of PBS for 30 minutes. Sections were then bleached in PBS with 3% H2O2 and NaOH to quench fluorescence. Slides were washed 3 times, then conjugated antibodies were added overnight. Images were recorded at each step and then brought into spatial alignment for colocalization of staining patterns using ImageJ software version 2.1.0/1.53c (Fiji. https://fiji.sc, last accessed August 23, 2021).\textsuperscript{19}

Quantification of Immunofluorescent Signals

Random single and large (100+) tiled images were taken at 20× or, when possible, the whole tissue imaged at 10×. Fluorescence was normalized to vessel or tissue area. Ulex and DAPI colocalization was used to determine ROIs of EC nuclei for quantification of pSTAT1, pSTAT3, and p65 fluorescence. ImageJ was used to process and measure fluorescence of tissue.
COVID-19 Coronary Vascular Thrombosis

Table 1  Patient Demographics and Clinical Information

| COVID-19 patients (n = 7)* | Age | Sex | Cardiac history | Medications | Cause of death |
|---------------------------|-----|-----|-----------------|-------------|---------------|
| Patient 1                 | 94  | F   | CAD, NSTEMI     | Remdesivir  | Pneumonia     |
| Patient 2                 | 65  | F   | CAD, DVT, MI    | Prednisone, Rivaroxaban | Pneumonia w/DAD leading to ARDS |
| Patient 3                 | 77  | M   | None            |             | Pneumonia and pulmonary thrombosis in setting of end stage renal disease |
| Patient 4                 | 57  | M   | None            |             | Pneumonia leading to cardiac arrest, followed by shock |
| Patient 5                 | 90  | M   | CAD, MI         |             | Pneumonia w/DAD |
| Patient 6                 | 68  | F   | CAD, MI         |             | Pneumonia in setting of CV disease and recent MI |
| Patient 7                 | 50  | M   | None            | Batinatumomab | Pneumonia w/DAD in setting of B cell ALL relapse |

| Controls (n = 12)          |     |     |                 |             |               |
| Control 1                 | 89  | M   | CAD, MI         |             | Atherosclerotic and hypertensive CV disease |
| Control 2                 | 47  | M   | CAD, MI         |             | CHF           |
| Control 3                 | 79  | M   | Atrial Flutter, CKD |             | CHF           |
| Control 4                 | 58  | F   | None            |             | Respiratory failure from pulmonary adenocarcinoma |
| Control 5                 | 58  | M   | None            |             | GI hemorrhage and shock from cirrhosis |
| Control 6                 | 46  | F   | HOCM            |             | Metastatic breast cancer in setting of HOCM-induced heart failure |
| Control 7                 | 68  | M   | None            |             | Pulmonary squamous cell carcinoma leading to hemorrhage and desanguination |
| Control 8                 | 65  | M   | CAD             |             | Interstitial lung disease complicated by bronchopneumonia |
| Control 9                 | 71  | F   | None            |             | ILD leading to pneumonia |
| Control 10                | 62  | F   | None            |             | Metastatic breast cancer |
| Control 11                | 68  | M   | CAD             |             | Cirrhosis leading to esophageal varices and bleeding |
| Control 12                | 53  | M   | CAD             |             | GI bleeding from cirrhosis |

*All COVID-19 patients positive for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by RT-PCR.

Statistics

Data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism software. The U-test was used to make statistical comparisons between two groups. A P value of less than or equal to 0.05 was considered statistically significant.

Results

Thrombosis Is the Most Common Pathologic Finding in Heart Autopsy Specimens of COVID-19 Cohort

Consistent with prior reports, we observed extensive micro and macrothrombi in 6/7 of the COVID-19 cohort (Figure 1) and did not detect SARS-CoV-2 spike or nucleocapsid antigen indicative of active infection, evidence of multifocal lymphocytic infiltrates indicative of myocarditis, or complement deposition on endothelium indicative of antibody-mediated vasculitis (Supplemental Figure S1). The only patient without evident thrombosis had a diagnosis of acute lymphocytic leukemia with progressive thrombocytopenia. To quantify the extent of thrombosis, we stained slides of FFPE heart samples using antibodies reactive with platelet protein CD42b and/or fibrinogen. Based on these stains, COVID-19 patients had a greatly elevated frequency of platelet rich microthrombi per 20× field (Figure 1A) and total number of macrothrombi involving both intramyocardial arteries and veins (Figure 1B) in tissues compared to our COVID-19 negative controls. We also found several multinucleated giant cells that stained with CD42b, consistent with megakaryocytes, within the cardiac vasculature of three patients; such cells were not seen in any controls (Figure 1C). Despite the widespread evidence of thrombosis, we did not see evidence of myocyte death or acute inflammation typically associated with myocardial infarction.
ECs Show Infrequent Evidence of Injury or Activation in Heart Tissues

To assess if endothelial alterations occur in the context of COVID-19 associated cardiac vascular thrombosis, we examined the vessels of the heart for signs of endothelial cell (EC) injury or activation. EC injury by apoptosis can promote thrombosis through release of microparticles containing pro-coagulative tissue factor or by EC sloughing that can expose platelet-activating collagen. Cytokine-activated endothelium may also become procoagulant, eg, through synthesis of tissue factor, or platelet adhesive by release of high molecular weight von Willebrand factor.

Evaluation of intimal injury revealed only rare markers of cleavage/activation of caspase 3 in endothelial cells in 1/7 patients (Figure 2A) unrelated to sites of thrombosis. One patient showed both rare foci of EC denudation with adherent platelets and rare VE-Cadherin+ cell fragments colocalized with platelets (Figure 2, B and C). Another patient manifested rare foci of tissue factor expression within the vascular lumen but not colocalized with sites of thrombosis (Figure 2D). There was an increased percentage of vessels with VWF luminal staining in three COVID-19 patients, but primarily not coincident with sites of thrombosis (Figure 2E). The COVID-19 patient with the highest level of VWF expression, found in approximately 5% of
Figure 2  ECs lining heart vasculature show minimal signs of injury or activation. A–C: Images show rare evidence of EC injury in COVID-19 patients with (A) cleaved caspase 3 (CC3) expression on a few ECs, (B) denuded and sloughed off ECs at a site of platelet aggregation, and (C) circulating VE-Cadherin⁺ cell fragments bound to platelets (arrow). D: Tissue factor on vessel lumen and sites of thrombi was also rarely detected. E–I: Quantification and representative images of EC activation markers. The percentage of vessels with lumenal VWF in control and COVID-19 patients (E). The percentage of EC nuclei that showed (F) p65 nuclear localization (arrow), pSTAT1 (G), or pSTAT3 in patients and controls (H). I and J: ICAM-1 and VCAM-1 fluorescence intensity on vessels was calculated and normalized to area of tissue sampled. Data represent means ± SEM. Scale bars: 35 μm (A and B); 50 μm (C and D); 100 μm (E–J).
vessels, was patient 6 who had recently suffered a MI. The sample with the second most frequent localization of VWF was from the patient with ALL who showed no signs of thrombosis.

Next, we sought signs of cytokine-activated signaling pathways in the ECs by assessing both nuclear p65 (Rel A) translocation and STAT protein tyrosine phosphorylation. Nuclear p65, a trigger of IL-1 or TNF signaling, was limited and of no greater frequency in ECs of COVID-19 patients versus controls (Figure 2F). Endothelial pSTAT1 expression, indicative of IFN signaling, was absent in all but one patient who exhibited expression on 0.6% of ECs and none at sites of thrombosis (Figure 2G). Similarly, the percentage of ECs with pSTAT3 expression, indicative of responses to IL-6 family cytokines, did not differ significantly between COVID-19 patients and controls with the notable exception of one COVID-19 patient who showed elevated levels of pSTAT3 expression on 17.5% of ECs (Figure 2H). This patient had suffered a cardiac arrest one day before death. Consistent with a paucity of evidence for endothelial cell responses to cytokines, there was no statistically significant increase in the expression of ICAM-1 (Figure 2I) or VCAM-1 (Figure 2J) in patients compared to controls along with E-selectin expression (Supplemental Figure S2).

Figure 3 Neutrophils contribute to thrombosis in patients. A: The number of neutrophil-platelet aggregates per 20× field was quantified in patients and controls. B and C: Patient tissue images of neutrophil-platelet aggregates and a neutrophil during NETosis, capturing platelets. D: Neutrophil rich macrothrombi composed of more than 30% neutrophils were counted in entire tissue at 10×. Values were normalized by tissue area. E and F: Images showing neutrophil macrothrombi and neutrophil aggregates that are CitH3+ in circulation in COVID-19 heart tissue. G: Image shows neutrophils with increased p65 expression (arrows) in COVID-19 tissue. Myeloperoxidase (MPO) was used to identify neutrophils and Citrullinated histone H3 (Cith3) to identify NET formation. Data represent means ± SEM. *P < 0.05. Scale bars: 50 μm (B, C, E and F); 100 μm (G).
one COVID-19 patient that showed increased VCAM-1 expression was the same patient described above that suffered a recent cardiac arrest. Supplemental Table S2 summarizes several of these histological findings.

Activated Intravascular Neutrophils Feature Prominently in Thrombi in Hearts of COVID-19 Decedents

Thrombosis can also occur due to changes in the composition or activation of various components in the blood. In contrast to the limited findings of endothelial alterations, the composition of thrombi revealed the frequent presence of platelets associated with neutrophils (Figure 3A and B) and many of these neutrophils stained positive for Citrullinated Histone 3 (CitH3), an early step in neutrophil extracellular trap (NET) formation. Eventually, these neutrophils will undergo NETosis which is the expulsion of NETs or structures composed of chromatin, histones, and other internal neutrophil molecules that can bind pathogens or serve as sites of thrombosis. We found evidence of NET formation in neutrophils at sites of thrombi in 4 of the 6 COVID-19 patients with thrombi. Some images suggest NETs that appear to be directly associated with platelets (Figure 3C). Neutrophil rich macrothrombi composed of 30% or more neutrophils were also common in COVID-19 but not control specimens (Figure 3D and E). CitH3+ neutrophil only aggregates also localized in vessels (Figure 3F). Many of these neutrophils appeared to have elevated expression levels of p65 (RelA), the significance of which is uncertain but, like NET formation, may reflect an activated state (Figure 3G).

Discussion

This study reports findings that agree with and extend results of prior autopsy series of hearts from patients dying of COVID-19. We found thrombosis of micro and macro coronary vessels with little evidence of overt cardiac injury and an absence of direct infection, myocarditis, or antibody and complement binding. It is not clear if extensive thrombosis in the absence of overt myocardial injury compromises cardiac function, but low levels of circulating cardiac troponin concentrations correlate with morbidity and mortality in severe COVID-19 infections, indicative of low level and perhaps diffuse myocardial injury. To understand the likely causes of cardiac thrombus, we performed an extensive multiparameter IF analysis of COVID-19 autopsy heart tissue using advanced morphologic methodology to discern the most likely causes of the COVID-19 coronary thrombotic diathesis. Despite the limited sample size and patient heterogeneity that makes drawing conclusions from individual patients difficult, we saw no consistent evidence that any of the markers for EC activation tested, including ICAM-1, VCAM-1 and p65 correlated with thrombosis, and we saw only rare and isolated evidence of vessel injury or activation. Our data instead favor a hypothesis that the thrombotic diathesis results primarily from hypercoagulability of the blood caused by activated neutrophils.

Platelets and neutrophils during COVID-19 infection exhibit highly activated states with an increase in P-selectin surface expression on the former and Factor V, G-CSF, IL-8 expression on the latter.24–26 Reports have also documented increased numbers of circulating neutrophil platelet aggregates27 and CitH3+ neutrophils in the blood and at sites of thrombus in the lung.28 Additionally, Blasco et al29 recently reported a case series on COVID-19 patients who died of MI showing NETs within thrombi of the heart. Our findings agree with and extend these reports and suggest that neutrophils play a very prominent role in thrombosis of the heart in these COVID-19 patients.

Neutrophils may be activated to release NETs by recognition of infection by virus or bacteria, activation by cytokines, or interactions with platelets, complement and/or antibodies.30 Our data in the heart favor a role of cytokines and platelets in promoting NETs as we found CitH3+ neutrophils in thrombi that were bound to platelets in the absence of virus, antibody, or complement. Minimal endothelial activation in the heart may be preventing neutrophils from entering the heart parenchyma unlike lungs of the same patient. Interestingly, we also found neutrophil aggregates, which may serve as an additional thrombotic stimulus for platelet aggregation leading to larger thrombi.

Heparin therapy commonly administered to COVID-19 patients to block coagulation may also prove to be beneficial in reducing NET-dependent thrombus formation in patients. This negatively charged polymer can interact with positively charged histones released by NETs, rendering them unable to activate platelets and potentiate thrombosis.31 Furthermore, heparin can activate DNase I and bind P-selectin and ICAM-1 to reduce the interaction and binding of platelets and neutrophils to each other and to endothelial cells.30,32

This study has several limitations. First, postmortem tissue samples provide only insights toward the end of the disease course and a final snapshot of possible mechanisms. Earlier events may have a different pattern, but cardiac tissue is not generally accessible for analysis before death. Second, the nature of multidimensional cysic IF restricts the number of slides that can be examined, providing only limited sampling that may miss other areas with pathologic findings and is restricted to a small number of specimens. Nevertheless, we believe we are the first to apply multiparameter analyses, revealing the absence of vessel wall changes at the actual sites of thrombosis. Our data thus challenge the view that alterations in the vessel wall are the primary cause of COVID-19 cardiac thrombosis, a finding with implications for prophylaxis. Analysis of further samples will be required to validate this conclusion. We also recognize that thrombosis in hearts, which do not appear to be sites of viral infection, may be different from the processes occurring in other sites, such as lung or the gut, in which virus and abundant inflammation is present.
In conclusion, our multiparameter analysis of cardiac vessel thrombosis points to activation of circulating neutrophils as the most likely causal event. Alterations in the vessel wall, although seen in some instances, are relatively rare. In light of these findings, reducing neutrophil responses could be an important target for therapeutic intervention.

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Supplemental Data

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