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REVIEW ARTICLE

Tissue factor expression, extracellular vesicles, and thrombosis after infection with the respiratory viruses influenza A virus and coronavirus

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Abstract

Tissue factor (TF) is induced in a variety of cell types during viral infection, which likely contributes to disseminated intravascular coagulation and thrombosis. TF-expressing cells also release TF-positive extracellular vesicles (EVs) into the circulation that can be measured using an EVTF activity assay. This review summarizes studies that analyze TF expression, TF-positive EVs, activation of coagulation, and thrombosis after infection with influenza A virus (IAV) and coronaviruses (CoVs), including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), SARS-CoV, and Middle East respiratory syndrome CoV (MERS-CoV). The current pandemic of coronavirus disease 2019 (COVID-19) is caused by infection with SARS-CoV-2. Infection of mice with IAV increased TF expression in lung epithelial cells as well as increased EVTF activity and activation of coagulation in the bronchoalveolar lavage fluid (BALF). Infection of mice with MERS-CoV, SARS-CoV, and SARS-CoV-2 also increased lung TF expression. Single-cell RNA sequencing analysis on the BALF from severe COVID-19 patients revealed increased TF mRNA expression in epithelial cells. TF expression was observed in peripheral blood mononuclear cells infected with SARS-CoV. TF was also expressed by peripheral blood mononuclear cells, monocytes in platelet-monocyte aggregates, and neutrophils isolated from COVID-19 patients. Elevated circulating EVTF activity was observed in severe IAV and COVID-19 patients. Importantly, EVTF activity was associated with mortality in severe IAV patients and with plasma D-dimer, severity, thrombosis, and mortality in COVID-19 patients. These studies strongly suggest that increased TF expression in patients infected with IAV and pathogenic CoVs contributes to thrombosis.

KEYWORDS
influenza A virus, SARS-CoV-2, thrombosis, tissue factor

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1 | INTRODUCTION

Infection with viruses, such as influenza A virus (IAV) and coronaviruses (CoVs), activates the coagulation system and can lead to disseminated intravascular coagulation and thrombosis.[1] The current pandemic of coronavirus disease 19 (COVID-19), which is caused by infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is associated with a high rate of thrombosis.[2,3]

Single-stranded (ss) RNA respiratory viruses, such as IAV, SARS-CoV, SARS-CoV-2, and Middle East respiratory syndrome CoV (MERS-CoV), are detected by a variety of receptors, including endosomal toll-like receptors (TLRs).[4] For instance, ssRNA activates TLR7 and TLR8 whereas double-stranded (ds) RNA formed during the replication of ssRNA viruses activates TLR3.[1] The dsRNA mimetic polyinosinic-polycytidylic acid (poly I:C) is used experimentally to activate TLR3.

Tissue factor (TF) is the receptor for factor (F) VII/VIIa.[5] It is constitutively expressed by adventitial fibroblasts and pericytes around blood vessels and plays an essential role in hemostasis.[6–9] TF expression can also be induced in vascular cells, such as monocytes and endothelial cells.[10,11] Indeed, the TF-FVIIa complex has been shown to contribute to the coagulopathy and mortality in a baboon model of sepsis.[12] TF-expressing cells also release TF-positive extracellular vesicles (EVs) into the circulation that can activate coagulation and platelets.[13–15] EVs can be isolated from plasma and levels of EVTF activity can be measured using a functional assay called an EVTF activity assay.[16] Interestingly, poly I:C induced TF expression in human endothelial cells but not in human monocytes *in vitro*.[17] We also found that poly I:C induces TF expression in human endothelial cells.[18] Intraperitoneal injection of poly I:C into mice also activates coagulation in a TLR3-dependent manner[17] (S Antoniak and N Mackman, University of North Carolina at Chapel Hill, unpublished data).

Induction of TF expression is likely to contribute to the activation of coagulation and thrombosis during viral infections. For instance, Ebola virus induced TF expression in peripheral blood mononuclear cells (PBMCs) in vitro and TF was expressed by PBMCs isolated from Ebola-infected monkeys.[19] We recently showed that plasma from monkeys infected with Ebola virus had elevated levels of EVTF activity.[20] Based on earlier studies, we speculate that the majority of these TF-positive EVs are derived from monocytes. Importantly, inhibition of the TF-FVIIa complex reduced mortality in monkeys infected with Ebola virus.[21] HIV infection is also associated with activation of coagulation and increased monocyte TF expression.[22,23] One study found that inflammatory monocytes isolated from HIV patients expressed TF.[24] Similarly, infection of pigtail macaques with Simian immunodeficiency virus induced TF expression in inflammatory monocytes and a coagulopathy that was reduced by inhibition of the TF-FVII complex.[24] These studies indicate that TF expression induced during viral infection plays a central role in the activation of coagulation.

2 | TF EXPRESSION AND IAV

Influenza viruses cause seasonal and pandemic respiratory infections.[25] IAV is an enveloped ssRNA virus. IAV/H1N1 patients with severe acute respiratory distress syndrome (ARDS) have an activated coagulation system and an increased risk of thrombosis. In hospitalized IAV/H1N1 patients, elevated D-dimer was associated with a higher risk of disease progression.[26] One study found that 5.9% of 119 hospitalized H1N1 patients had thrombotic vascular events.[27] Another study found a higher rate of venous thromboembolism (VTE) in hospitalized H1N1 patients with ARDS compared with non-H1N1 patients with ARDS (44% vs. 29%).[28]

We found that patients with primary IAV/H1N1 in the intensive care unit had increased levels of EVTF activity as well as markers of activation of coagulation and fibrinolysis (thrombin-antithrombin complexes and D-dimer) in their plasma compared with healthy controls.[29] Furthermore, EVTF activity was significantly higher in non-survivor patients compared with survivors. At present, we do not know the cellular origins of the TF-positive EVs in the circulation of severe IAV patients. These data suggest that circulating TF-positive EVs may contribute to VTE in IAV patients and could be used as a prognostic marker in IAV/H1N1 patients in the intensive care unit.

Tissue factor expression has also been analyzed in mouse models of IAV infection (Table 1). An early study reported an increase in TF mRNA expression in the lungs of mice infected with IAV/1918 H1N1.[30] We found that infection of mice with IAV (mouse-adapted PR8/H1N1 strain) led to a transient increase in lung TF mRNA and TF

TABLE 1 Analysis of TF expression in samples from mice infected with different viruses

Virus	Type of analysis	Findings	Ref
IAV	Lung mRNA	Increased TF	[30]
IAV	Lung mRNA and BALF protein	Increased TF mRNA and protein, Increased BALF EVTF No increase in mice lacking TF in epithelial cells	[31]
MERS-CoV	Lung mRNA	Increased TF	Unpublished data, T. Sheahan
SARS-CoV	Lung mRNA	Increased TF	[30]
SARS-CoV-2	Lung mRNA	Increased TF	Unpublished data, L. Gralinski

Abbreviations: IAV, influenza A virus; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TF, tissue factor.

activity with a peak of expression 4 days after infection.[31] In addition, bronchoalveolar lavage fluid (BALF) of infected mice contained high levels of EVTF activity and thrombin-antithrombin complexes compared with uninfected mice.[31] Infection of mice with a high dose of virus led to an increase in EVTF activity in the plasma (K Tatsumi, S Antoniak and N Mackman, University of North Carolina at Chapel Hill, unpublished data 2015). Importantly, mice with the TF gene deleted in lung epithelial cells but not mice with the TF gene deleted in myeloid cells had significantly lower basal lung TF expression compared with wild-type mice and no induction of TF expression after IAV infection.[31] Mice with the TF gene deleted in epithelial cells also had reduced activation of coagulation after IAV infection.[31] These results indicate that lung epithelial cells are the major site of both basal and induced TF expression in the lung after IAV infection.

IAV/H1N1 infection of mice with the TF gene deleted in lung epithelial cells had increased lung hemorrhage and death compared with infected controls.[31] This indicated that TF expression in lung epithelial cells is also required for hemostasis. Therefore, TF has a dual role during respiratory virus infection. On the one hand, it protects from hemorrhage incurred during infection, but on the other hand excessive TF expression may cause thrombosis.

3 | TF EXPRESSION AND CORONAVIRUSES

3.1 | MERS-CoV

MERS-CoV appeared first in 2012 in Saudi Arabia and is limited to the Middle East.[32] Disseminated intravascular coagulation is one of the major complications in fatal MERS-CoV patients.[33] Lung TF expression is increased in mice infected with mouse-adapted MERS-CoV [34] (Table 1) (T Sheahan, University of North Carolina at Chapel Hill, unpublished data 2020).

3.2 | SARS-CoV

SARS-CoV emerged in 2002 and is associated with ARDS and death.[35] Pathologic studies indicate that SARS-CoV infection results in denudation of airway epithelial cells and small vessel thrombosis in the lung.[36–38] Infection with SARS-CoV is associated with increased plasma D-dimer and thrombosis.[39,40] One study found that SARS-CoV was able to infect and replicate in PBMCs.[41] In addition, TF expression was increased in PBMCs infected with SARS-CoV compared with mock infection.[41] This suggests that TF expression by PBMCs may contribute to thrombosis in patients infected with SARS-CoV. At present, there are no studies of TF expression during SARS-CoV infection of humans.

A mouse model was developed using a mouse-adapted SARS-CoV MA15. Mice infected with this virus reproduced many pathologic features of patients infected with SARS-CoV.[42] Global lung gene expression patterns in mice infected with SARS-CoV MA15 were analyzed for up to 7 days.[30] TF mRNA expression was strongly increased at day 2 after infection and remained elevated at days 4 and 7 after infection (Table 1).[30] This result indicates that TF expression is increased in the lung after SARS-CoV infection.

3.3 | SARS-CoV-2

SARS-CoV-2 infection is also associated with a high rate of thrombosis.[43] VTE was observed in 0.9% to 6.5% of noncritically ill COVID-19 patients versus 8% to 69% in critically ill patients.[2,3] A recent study compared the rates of both VTE and arterial thrombotic events in 13,217 hospitalized influenza patients versus 579 hospitalized COVID-19 patients.[44] The rates of thrombosis were higher in in the COVID-19 patients compared with the influenza patients (11% vs. 3.3%). Interestingly, this difference was driven by differences in the rates of VTE; the rate of VTE in influenza patients was 3.6% (95% CI: 2.7–4.6) compared with 23% (95% CI: 16–29) in COVID-19 patients. In contrast, arterial thrombotic events were slightly higher in influenza patients (7.5%; 95% CI: 6.3–8.8) compared with COVID-19 patients (4.4%; 95% CI: 1.9–8.8).

Levels of plasma D-dimer are highly elevated in COVID-19 patients.[45–49] Several studies found an association between D-dimer and mortality.[50–52] D-dimer was also found to be associated with thrombosis.[52]

It is likely that increased TF expression contributes to thrombosis in COVID-19 patients.[3] TF is constitutively expressed by lung epithelial cells, which are a primary target of SARS-CoV-2.[53] The effect of SARS-CoV-2 infection on gene expression has been analyzed using transcriptomics. One study performed bulk RNA-sequencing analysis on BALF and PBMCs from 3 COVID-19 patients from Wuhan and three controls.[54,55] Another study performed single cell (sc) RNA sequencing analysis on BALF from three moderate and six severe COVID-19 patients and three controls.[55] FitzGerald et al.[56] analyzed these datasets of SARS-CoV-2 infection to identify changes in the expression of genes involved in coagulation. Analysis of bulk RNA-sequencing data of BALF revealed increased TF mRNA expression.[56] In contrast, another study with five COVID-19 patients from Wuhan did not observe an increase in TF expression in bulk RNA-sequencing of BALF samples compared with controls.[57] This difference may be due to the severity of disease in the COVID-19 patients in the two studies. ScRNA sequencing analysis can be used to identify the cell type-specific mRNA expression profiles in BALF from COVID-19 patients and controls. Importantly, severe COVID-19 patients had increased TF expression in epithelial cells in the BALF compared with epithelial cells present in BALF from moderate COVID-19 patients and healthy controls.[56] Interestingly, epithelial cells in the severe BALF of COVID-19 patients but not monocyte-derived macrophages were found to express increased TF.[56] These data indicate that in severe COVID-19 patients the major source of TF in BALF are epithelial cells.

One small study analyzed TF mRNA and protein expression by *in situ* hybridization and immunofluorescence, respectively, in lungs

of COVID-19 patients with ARDS, patients with ARDS, and normal controls.[58] TF mRNA expression was 2-fold higher in the lungs of COVID-19 patients with ARDS compared with non-COVID-19 patients with ARDS. The level of TF protein in the lungs of COVID-19 patients was 2.1-fold higher than non-COVID-19 patients with ARDS and 11-fold higher than normal controls.[58] TF expression was increased in endothelial cells but not in epithelial cells in COVID-19 lungs compared with controls lungs. In contrast, another study reported upregulation of TF predominantly associated with the alveolar epithelium in a COVID-19 patient.[59] This finding is more consistent with data from animal models.

Bulk RNA-sequencing analysis on PBMCs from three COVID-19 patients from Wuhan and three controls was performed.[54,55] PBMCs from one of the three COVID-19 patients exhibited higher TF expression compared with no TF expression in PBMCs from the three controls (Table 2).[56] This suggests that PBMCs can express TF during SARS-CoV-2 infection. Another study observed increased platelet-monocyte aggregates in severe COVID-19 patients and TF expression on the monocytes but not platelets in these aggregates.[60] Interestingly, platelets from severe COVID-19 patients induced TF expression in monocytes isolated from healthy controls.[60] Monocyte TF expression was associated with D-dimer in COVID-19 patients. TF was also expressed by neutrophils isolated from COVID-19 patients and associated with neutrophil extracellular traps.[61] Platelet-rich plasma from COVID-19 patients induced TF expression in neutrophils from healthy individuals.[61] One study reported a significant increase in TF-positive platelets and granulocytes and a trend toward increased TF-positive monocytes in COVID-19 patients compared with healthy controls.[62] One problem with this study is that it is unclear if platelets are expressing TF or simply acquiring TF-positive EVs from other cells.[63] Similar to studies with Ebola virus and HIV, these studies suggest that circulating PBMCs are a major source of TF expression and activation of coagulation during SARS-CoV-2 infection.

Other studies have measured levels of circulating EVTF activity in COVID-19 patients (Table 2). We found that two cohorts of COVID-19 patients have elevated levels of EVTF activity compared with healthy controls.[64,65] In the larger cohort of COVID-19 patients, the level of EVTF activity correlated with D-dimer and was associated with severity and mortality.[64] Another study also found an increase in EVTF activity in COVID-19 patients compared with controls.[66] Similar to our study, EVTF activity was higher in severe COVID-19 patients compared with patients with moderate disease.[66] Levels of EVTF activity were also correlated with D-dimer and were associated with an increased thrombotic risk.[66] Another study reported an increase in TF protein on EVs and TF activity in COVID-19 patients.[67] Finally, a recent study found that EVTF activity was increased in the plasma of severe but not moderate COVID-19 patients compared with controls.[59] This study used the commercial ZYMUPHEN MP-TF assay to measure levels of EVTF activity, which is less sensitive than the EVTF activity assay.[68] Taken together, these studies demonstrate increased levels of circulating TF-positive EVs in COVID-19 patients. These TF-positive EVs may contribute to thrombosis in COVID-19 patients and may be useful as a biomarker of thrombotic risk.

At present, we do not know the cellular origins of the TF-positive EVs present in the circulation of COVID-19 patients. Although some investigators have used flow cytometry to determine the cellular origin of circulating TF-positive EVs, we feel that this technique is not sensitive enough to simultaneously measure levels of cell type-specific markers and TF on EVs because of the low levels of TF.[69] We speculate that a likely source of circulating TF-positive EVs is activated monocytes because these cells have been shown to express TF in COVID-19 patients. Indeed, depletion of leukocyte-derived EVs significantly decreased the level of EVTF activity in the plasma of COVID-19 patients, which suggests that the majority of these TF-positive EVs are derived from activated monocytes (F Dignat-George, Aix-Marseille Universite, unpublished data 2021). However,

TABLE 2 Analysis of TF expression in samples from COVID-19 patients

Sample	Type of analysis	Findings	Ref
BALF	Bulk RNA-sequencing	Increased TF	54,56
BALF	Bulk RNA-sequencing	No change in TF	57
BALF	Single cell RNA-sequencing	Increased TF in epithelial cells	55,56
PBMC	Bulk RNA-sequencing	Increased TF (1/3 samples)	54,56
Whole blood	Protein	Monocyte TF expression, not platelets	60
Whole blood	Protein	Platelet, granulocyte, and monocyte TF expression	62
Plasma	Activity	Increased EVTF activity associated with D-dimer, severity, and survival	64
Plasma	Activity	Increased EVTF activity	65
Plasma	Activity	Increased EVTF activity associated with D-dimer, severity, and thrombosis	66
Plasma	Activity	Increased EVTF activity in severe patients	59
Serum	Protein + activity	Increased EVTF activity	67

Abbreviations: COVID-19, coronavirus disease 2019; EVTF, extracellular vesicle tissue factor; PBMC, peripheral blood mononuclear cell; TF, tissue factor.

it is possible that other cell types, such as endothelial cells, neutrophils, and epithelial cells, also release TF-positive EVs into the circulation in COVID-19 patients.

Tissue factor mRNA and protein expression has also been studied in primary normal human bronchial epithelial cells (NHBECs). TF mRNA expression was significantly increased in cells infected with SARS-CoV-2 compared with mock-infected cells.[56,70] In addition, SARS-CoV-2 infection of NHBECs increased TF protein expression.[56] Surprisingly, PR8 IAV infection of NHBEC did not increase TF expression.[56,70] However, one must be cautious in interpreting results from NHBECs studies because these experiments were performed with basal cells in submerged culture and not with differentiated cells in air–liquid interface culture. The receptors of IAV and SARS-CoV-2 are expressed in differentiated epithelial cells, including goblet and ciliated cells.[71–73] Thus, the air–liquid interface culture system is a better model for studying pathologic processes during viral infection.[71]

A mouse model of COVID-19 has been established using a mouse-adapted virus called SARS-CoV-2 MA.[74,75] Infection of mice with SARS-CoV-2 increased lung TF expression (L Gralinski, University of North Carolina at Chapel Hill, unpublished data 2021). The model will enable future mechanistic studies to determine the protective and pathologic contribution of TF expression by different cell types, such as epithelial cells, monocytes, neutrophils, and endothelial cells, in the setting of SARS-CoV-2 infection.

Viral infection of cells releases sphingomyelinases into the outer leaflet of the plasma membrane that breaks down sphingomyelin.[76] Sphingomyelin maintains TF in an encrypted state.[77] Interestingly, a recent study found that infection of human monocyte-derived macrophages with a pseudovirus expressing the SARS-CoV-2 spike protein increased TF activity without increasing TF protein expression.[78] Infection of the cells induced the translocation of acid sphingomyelinase to the outer leaflet of the plasma membrane, where it degraded sphingomyelin and relieved the encryption of TF. The pseudovirus infection of the cells also increased the release of TF-positive EVs.[78] This provides an additional mechanism to increase TF activity during viral infections.

4 | CONCLUSIONS

Tissue factor expression is induced in the lungs of mice infected with IAV, MERS-CoV, SARS-CoV, and SARS-CoV-2. In the case of IAV infection, this induction occurs in epithelial cells. Similarly, BALF samples from severe COVID-19 patients had increased TF expression in epithelial cells. In COVID-19 patients, PBMCs, monocytes, and neutrophils express TF. Finally, the level of circulating EVTF activity was increased in severe IAV/H1N1 infection and SARS-CoV-2 infection. EVTF activity was associated with D-dimer, severity, and thrombosis in COVID-19. EVTF activity was associated with mortality in both IAV patients and COVID-19 patients. These studies strongly suggest that increased TF expression in patients infected with IAV/H1N1 and highly pathogenic CoVs contributes to thrombosis. Targeting pathologic TF expression in patients infected with respiratory viruses, including IAV and SARS-CoV-2, may reduce thrombosis.

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CONFLICT OF INTERESTS

None.

AUTHOR CONTRIBUTIONS

Nigel Mackman drafted the manuscript and Steven P. Grover and Silvio Antoniak provided comments.

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