

Thrombosis in small and medium-sized pulmonary arteries in Wegener's granulomatosis: A confocal laser scanning microscopy study*, **

Trombose em artérias pulmonares pequenas e médias em granulomatose de Wegener: Um estudo com microscopia confocal a laser

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Abstract

Objective: Wegener's granulomatosis (WG) can cause endothelial cell damage and thromboembolic events. Nevertheless, there have been few studies on the pulmonary microcirculation—small and medium-sized pulmonary arteries (SMSPA)—in patients with WG. The objective of this study was to quantify fibrin thrombi in the SMSPA of patients with WG. **Methods:** We analyzed 24 SMSPA samples collected from six patients with WG and 16 SMSPA samples collected from four patients without WG. In all samples, we used the endothelial cell marker CD34 and confocal laser scanning microscopy in order to detect intravascular fibrin thrombi. We calculated the total vessel area, the free lumen area, and the thrombotic area. **Results:** The mean total vessel area was similar in the WG and control groups ($32,604 \mu\text{m}^2$ vs. $32,970 \mu\text{m}^2$, $p = 0.8793$). Thrombi were present in 22 (91.67%) of the 24 WG group samples and in none of the control group samples ($p < 0.0001$; OR = 297; 95% CI: 13.34–6,612). The mean thrombotic area was greater in the WG group samples than in the control group samples ($10,068 \mu\text{m}^2$ vs. $0.000 \mu\text{m}^2$; $p < 0.0001$). In contrast, the mean free lumen area was smaller in the WG group samples than in the control group samples ($6,116 \mu\text{m}^2$ vs. $24,707 \mu\text{m}^2$; $p < 0.0001$). **Conclusions:** Confocal laser scanning microscopy revealed a significant association between pulmonary microvascular thrombosis and WG. This suggests a possible role of microvascular thrombosis in the pathophysiology of pulmonary WG, evoking the potential benefits of anticoagulation therapy in pulmonary WG. However, further studies are needed in order to confirm our findings, and randomized clinical trials should be conducted in order to test the role of anticoagulation therapy in the treatment of patients with pulmonary WG.

Keywords: Vasculitis; Antibodies, antineutrophil cytoplasmic; Wegener granulomatosis; Thrombosis; Lung; Microscopy, confocal.

Resumo

Objetivo: A granulomatose de Wegener (GW) pode causar dano nas células endoteliais e fenômenos tromboembólicos. Entretanto, poucos estudos analisaram a microcirculação pulmonar — artérias pulmonares de pequeno/médio calibre (APPMC) — em pacientes com GW. O objetivo deste estudo foi quantificar trombos de fibrina em amostras de APPMC de pacientes com GW. **Métodos:** Analisamos 24 APPMC de seis pacientes com GW e 16 APPMC de quatro pacientes controles sem GW. Utilizamos CD34 para a marcação do endotélio em todas as amostras e microscopia confocal a laser para detectar trombos de fibrina intravasculares. Calculamos a área total do vaso, a área livre do lúmen e a área trombótica. **Resultados:** A média da área total do vaso foi similar no grupo GW e no grupo controle ($32.604 \mu\text{m}^2$ vs. $32.970 \mu\text{m}^2$, $p = 0,8793$). Trombos foram identificados em 22 das 24 APPMC (91,67%) no grupo GW, e em nenhuma do grupo controle ($p < 0,0001$; OR = 297 (IC95%: 13,34–6.612). A média da área trombótica foi maior no grupo GW do que no grupo controle ($10.068 \mu\text{m}^2$ vs. $0.000 \mu\text{m}^2$, $p < 0,0001$). Em contraste, a média da área livre do lúmen foi menor no grupo GW que no grupo controle ($6.116 \mu\text{m}^2$ vs. $24.707 \mu\text{m}^2$, $p < 0,0001$). **Conclusões:** A microscopia confocal a laser mostrou uma associação significativa entre trombose microvascular pulmonar e GW. Isso sugere um possível papel da trombose microvascular na fisiopatologia da GW pulmonar, evocando o potencial benefício da anticoagulação na GW pulmonar. Entretanto, novos estudos são necessários para confirmar nossos achados, assim como um ensaio clínico randomizado a fim de testar o papel da anticoagulação no tratamento de pacientes com GW pulmonar.

Descritores: Vasculite; Anticorpos anticitoplasma de neutrófilos; Granulomatose de Wegener; Trombose; Pulmão; Microscopia confocal.

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Introduction

Lung function can be affected by various clinical conditions and diseases. The pathophysiology of this effect can be divided into four large groups: neuromuscular/chest wall; airway; parenchyma; and circulation.⁽¹⁻⁴⁾ The pulmonary circulation is difficult to evaluate in clinical practice, requiring the use of right heart catheterization, ventilation-perfusion scanning, contrast-enhanced CT of the chest, or lung biopsy. Pulmonary circulation disorders include the various types of pulmonary vasculitis—Wegener's granulomatosis (WG), Behçet's disease, and Takayasu's arteritis—all of which can cause vessel wall inflammation, aneurisms/stenosis, and thrombosis.^(4,5)

The features of WG, which is one of the forms of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, include inflammation/necrosis of small and medium-sized arteries and severe clinical presentations, such as acute kidney injury and alveolar hemorrhage.⁽⁵⁻⁷⁾ Recently, thromboembolic events have also emerged as a novel clinical conundrum in WG.^(6,8,9) However, there have been few histopathological descriptions of thrombi in the pulmonary microcirculation of ANCA-associated vasculitis, probably due to a lack of awareness of this histopathological finding, considering that the question of thromboembolic events in WG was not raised until 2005.^(8,10-12) Therefore, we conducted this confocal laser scanning microscopy-based study in order to determine the presence of fibrin thrombi and to quantify them in small and medium-sized pulmonary arteries.

Methods

The patients were selected with a computer-aided search tool in order to identify all of the adults (≥ 18 years of age), during a six-year period (from January 1, 2000 to December 31, 2005), who had undergone surgical lung biopsy at the University of São Paulo School of Medicine *Hospital das Clínicas*, located in the city of São Paulo, Brazil, for the diagnosis of pulmonary/systemic diseases and who fulfilled the clinical and pathological criteria for the diagnosis of WG.⁽⁵⁾ The search identified six patients with WG, whose chest CT revealed non-excavated nodules/masses, but no findings suggesting alveolar

hemorrhage. In addition, the patients had no renal involvement, which was expected since, if they had had renal alterations, the biopsy would have been performed in the kidneys and not in the lungs, considering that lung biopsies have much higher morbidity and mortality risks than do renal biopsies. Furthermore, the patients were not receiving prednisone/immunosuppressants prior to the lung biopsy. The characteristics of these patients are presented in Table 1. The study design was approved by the review board of the institution.

The surgical lung biopsy slides of the six patients with WG were obtained and independently reviewed by two pulmonary pathologists, who were blinded to the diagnosis of WG. In all of the cases, both pathologists made the diagnosis of WG in accordance with previously outlined criteria, and no areas of alveolar hemorrhage were encountered.^(7,10-12) In addition, four normal lung specimens, derived from autopsies of four individuals with fatal cranioencephalic trauma but without lung disease, were used as the control group. Tissue samples were embedded in paraffin, cut into 20-mm sections, deparaffinized, and heated in 10 mM citrate buffer (pH = 6.0) for 50 min in order to unmask antigens. The tissue sections were incubated at high temperature with monoclonal mouse anti-human antibody against CD34 (Clone QBEnd/10, 1:80; Novocastra Laboratories Ltd., Newcastle, UK). Subsequently, the sections were revealed with a secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse antibody (1:40; Sigma Chemical, St. Louis, MO, USA), and were mounted with an aqueous mounting medium. A confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) equipped with three laser sources—Argon (excitation wavelength: 488 nm), HeNe1 (543 nm), and HeNe2 (633 nm)—and an inverted fluorescence microscope (Axiovert 100 M; Carl Zeiss) were used in order to obtain series of images, considering the ease of conducting a morphometric study with the latter type of microscope. For image processing, we used the LSM 510 software (Carl Zeiss). The examination under confocal laser scanning microscopy was performed at a magnification of $\times 40$, and 1-mm thick serial images were obtained for each vessel (resulting in a total of 20 images per vessel) in order to avoid autofluorescence problems related

Table 1 – Characteristics of the six patients with Wegener’s granulomatosis.

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Gender	Female	Female	Female	Male	Male	Female
Age, years	30	42	52	36	37	44
Nasal or oral inflammation	P	P	P	P	P	P
Abnormal chest radiograph	P	P	P	P	P	P
Abnormal urinary sediment	A	A	A	A	A	A
Lung biopsy: granulomatous inflammation of an artery or perivascular area	P	P	P	P	P	P
ANCA	P	P	P	P	P	P
Other organs involved	A	A	P (eye)	A	P (skin)	A
Good response to cyclophosphamide	P	P	P	P	P	P

P: present; A: absent; and ANCA: antineutrophil cytoplasmic antibody.

to the thickness of the tissue sections (20 mm). In the vascular analysis, we used only the first image (1-mm thick) of the serial images for each artery.

For each individual, we analyzed four small or medium-sized pulmonary arteries, evaluating a total of 24 WG group samples and 16 control group samples. These arteries had external diameters of 20-300 µm, the ratio of the smallest external diameter divided by the largest external diameter being greater than 0.6, which assured a transversal cut of the vessels.⁽¹³⁻¹⁵⁾ In the WG group, for each case (tissue section), we initiated the search for pulmonary arteries in the center of the area with WG-related pathological involvement, and continued the search in a spiral fashion until encountering four arteries. In the control group, for each case (tissue section), we initiated the search for pulmonary arteries in the center of the slide and continued the search in a spiral fashion until encountering four arteries. Subsequently, we used only the first image

(1-mm thick) of the serial images related to each artery in order to identify thrombi within the vessels and to calculate the areas. The search for thrombi was visual, as previously described.⁽¹³⁻¹⁸⁾ In addition, the vessels were divided into the following components (Figure 1): total vessel area (the entire area of the vessel, corresponding to the area within circle 1); lumen area (the area within circle 2); thrombus area (area occupied by the thrombus, corresponding to the area within circle 3); vessel wall area (corresponding to the area within circle 1 area minus the area within circle 2); and free lumen area (lumen area of the vessel with no thrombus, corresponding to the area within circle 2 minus the area within circle 3). Furthermore, we created the thrombotic index, calculated as the ratio of the thrombus area divided by the total vessel area and expressed as a percentage.

The statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). Data were expressed as



Figure 1 – Confocal laser scanning microscopy images. In a), a normal pulmonary artery with the endothelium marked in green (control group). In b), a normal pulmonary artery showing circle 1 (delineating the total vessel area) and circle 2 (delineating the lumen area). In c), a pulmonary artery from a patient with Wegener’s granulomatosis, showing circle 1 (total vessel area), circle 2 (lumen area), and circle 3 (thrombus area).

Table 2 – Areas of small and medium-sized pulmonary arteries in the Wegener's granulomatosis group and in the control group.^a

Area, μm ²	WG group	Control group	p
	(n = 24)	(n = 16)	
Total vessel	32,604 ± 17,830	32,970 ± 11,356	0.8793
Thrombus	10,068 ± 9,525	0.00 ± 0.00	< 0.0001
Lumen	16,184 ± 10,592	24,707 ± 8,717	0.0135
Free lumen	6,116 ± 5,956	24,707 ± 8,717	< 0.0001
Vessel wall	16,419 ± 10,016	8,263 ± 3,932	0.0047

WG: Wegener's granulomatosis. ^aData presented as mean ± SD.

means ± SDs for the continuous variables and as frequencies and percentages for the categorical variables. The level of significance was set at $p < 0.05$. We tested the normal distribution of the data within the groups with the Kolmogorov-Smirnov test, and we used the Mann-Whitney test for the comparison between nonparametric, unpaired continuous variables. In addition, we used Fisher's exact test to compare the categorical variables, and the OR was calculated.

Results

The mean age was 40.1 ± 7.6 years in the WG group and 38.2 ± 8.2 years in the control group ($p > 0.05$).

Thrombi were present in 22 (91.67%) of the 24 WG group samples but in none of the control group samples ($p < 0.0001$; OR = 297.00; 95% CI: 13.34–6,612). The mean total vessel area of the arteries was similar in the WG and control groups ($32,604 \pm 17,830 \mu\text{m}^2$ vs. $32,970 \pm 11,356 \mu\text{m}^2$, $p = 0.8793$), as intended in the study design. The mean thrombus area showed a significant difference between the WG and control groups ($10,068 \pm 9,525 \mu\text{m}^2$ vs. $0.000 \pm 0.000 \mu\text{m}^2$; $p < 0.0001$; Table 2). This remarkable finding of pulmonary microvascular thrombosis in the patients with WG was corroborated by the statistically significant difference between the two groups in terms of the mean thrombotic index ($31.86 \pm 20.40\%$ vs. $0.00 \pm 0.00\%$; $p < 0.0001$). In addition, the mean lumen area and the mean free lumen area were significantly smaller in the WG group than in the control group ($16,184 \pm 10,592 \mu\text{m}^2$ vs. $24,707 \pm 8,717 \mu\text{m}^2$; $p = 0.0135$; and $6,116 \pm 5,956 \mu\text{m}^2$ vs. $24,707 \pm 8,717 \mu\text{m}^2$; $p < 0.0001$, respectively), showing obstruction of the vascular bed by the thrombi. Furthermore, the mean vessel wall area was significantly larger in the WG group than

in the control group ($16,419 \pm 10,016 \mu\text{m}^2$ vs. $8,263 \pm 3,932 \mu\text{m}^2$; $p = 0.0047$).

Discussion

The major finding of the present study was the high prevalence of thrombosis, as observed under confocal laser scanning microscopy, in small and medium-sized pulmonary arteries of patients with WG. This finding opens a new avenue of research in pulmonary vascular pathology, raising the question of whether in situ microvascular thrombosis plays a role in the pathophysiology of WG. In addition, one should consider the likely participation of the in situ microvascular thrombosis in WG-related stroke, cutaneous necrosis, deep vein thrombosis (DVT), and glomerulonephritis, corroborated by the description of renal failure that is reversed by heparin in WG.⁽¹⁹⁾

The high prevalence of in situ pulmonary microvascular thrombosis and the elevated incidence of DVT/pulmonary embolism (PE) in WG might be a consequence of endothelial damage. Such endothelial damage might correlate with various alterations detected in the serum of patients with this kind of vasculitis, such as those seen in the levels/concentrations of ANCA, proteinase 3 (PR3), apoptotic endothelial cells, and thrombomodulin.^(20–23) First, ANCA causes premature neutrophil activation, resulting in disrupted neutrophil-endothelium interaction and, consequently, in endothelial damage.⁽²⁰⁾ However, vascular endothelial injury is one element of Virchow's triad, which proposes the mechanisms responsible for DVT/PE. Second, the PR3 produced by activated neutrophils correlates with the disease activity and causes apoptosis of endothelial cells.⁽²¹⁾ Third, apoptotic endothelial cells in the serum derive from organs such as the lungs and kidneys.⁽²⁴⁾ Therefore,

if serum levels of apoptotic endothelial cells are high (as in active WG), the apoptosis of endothelial cells is occurring in lung arteries, with a consequent increase in tissue factor levels and a decrease in thrombomodulin levels on the endothelial surface, contributing to the prothrombotic state and, possibly, to in situ pulmonary microvascular thrombosis.^(22,23) Finally, serum thrombomodulin levels represent inactive fragments of thrombomodulin (with no anticoagulant activity) released from the endothelial surface following cell damage.⁽²²⁾ Consequently, if the serum thrombomodulin level is high (as in active WG), the pool of active thrombomodulin on the endothelial surface, which has anticoagulant properties, is low, once again predisposing to in situ thrombosis.⁽²³⁾

In view of the abovementioned data, it is reasonable to consider the possible role of anticoagulation in patients with WG-related lung involvement, especially during the activity of the disease (except in those with WG-related hemorrhagic manifestations, especially considering that hemorrhage contraindicates the use of anticoagulants).⁽²⁵⁾ However, this idea would need to be addressed in a randomized clinical trial.⁽²⁵⁾ In addition, the potential benefits of anticoagulation especially consist of four points. First, the anticoagulation therapy in WG could be used to treat in situ thrombosis, considering that anticoagulation is beneficial in other diseases with in situ pulmonary microthrombosis, such as idiopathic pulmonary arterial hypertension, and that the frequency of in situ thrombosis is lower in idiopathic pulmonary arterial hypertension than in WG (20–50% vs. 91%).^(14,16,18) In addition, the thrombotic lesions in this ANCA-associated vasculitis were addressed in one report of renal failure in patients with WG that was reversed with only the use of heparin.⁽¹⁹⁾ Second, the anticoagulation could be used for the prophylaxis of thromboembolism in WG, considering that the prophylactic use of warfarin is an effective therapy in patients with previous idiopathic venous thromboembolism, and that these patients have an incidence of DVT/PE similar to that of WG (7.2 vs. 7.0 cases/patient-year).^(25–27) Third, anticoagulation could be used as anti-inflammatory therapy, based on platelet activation facilitating leukocyte activation, with the consequent endothelial cell damage/thrombosis in situ; 10–20% of WG patients

present with inflammation that is refractory to immunosuppression.⁽²⁸⁾ Fourth, there was a recent report of one patient with pulmonary hypertension caused by ANCA-associated vasculitis that showed clinical improvement after anticoagulation therapy, underscoring the importance of in situ thrombosis/endothelial dysfunction in the pulmonary microcirculation of patients with WG and the potential benefit of anticoagulation in this type of vasculitis.⁽²⁹⁾

Our study has some limitations. First, studies involving a population of patients with WG and pulmonary involvement should be conducted in order to exclude a possible bias in our case series, considering that the causes and the pathophysiology of WG are not fully understood. Second, our sample size was small. However, this is common in other studies involving lung biopsies in WG, considering that lung biopsies are much less common than are renal biopsies in WG.⁽³⁰⁾ One group of authors studied the expression of the chemokine CCL5 in the lung biopsies of six patients with WG (the same number of patients evaluated in the present study).⁽³⁰⁾ Third, the correct histopathological diagnosis of in situ thrombosis is not simple, due to the difficulty in differentiating between the morphology of pulmonary thromboembolism and that of thrombi formed in situ. However, it is accepted that large thrombi in major pulmonary arteries are embolic and that the primary thrombi (in situ) are found in small and medium-sized pulmonary arteries.^(13,14) Considering this fact, we analyzed only small and medium-sized pulmonary arteries to study in situ thrombosis in the present study; in addition, our patients did not have DVT/PE. Fourth, in order to achieve a better understanding of in situ microvascular thrombosis in patients with WG, it would be interesting to evaluate for an underlying coagulation disorder. Although this evaluation was not performed in our study, we believe that (in situ) pulmonary microvascular thrombosis mainly occurs due to WG-related endothelial dysfunction and serum markers of prothrombotic activity. This idea is corroborated by a group of authors who reported that almost all of the cases of DVT/PE in the WG patients they studied occurred during the active form of the disease, and that the coagulation disorders were evaluated, but were not present, emphasizing the

connections among disease activity, endothelial cell damage, and thrombosis in WG.^[9]

In conclusion, we demonstrated a high prevalence of microvascular thrombosis among our patients with WG, as well as a significant association between pulmonary arterial thrombosis and WG. Consequently, it would be interesting to add these findings to the existing data regarding the pathophysiological pathway of this type of vasculitis. However, further studies are needed in order to confirm our findings, and randomized clinical trials should be conducted in order to investigate the possible role of anticoagulation therapy in the management of patients with WG and lung involvement (except for hemorrhagic manifestations), especially in patients with active disease, because anticoagulation therapy itself also has anti-inflammatory effects.

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