

Original Article

Activation of circulated immune cells and inflammatory immune adherence are involved in the whole process of acute venous thrombosis

Le-Min Wang¹, Qiang-Lin Duan¹, Fan Yang², Xiang-Hua Yi³, Yu Zeng³, Hong-Yan Tian⁴, Wei Lv¹, Yun Jin¹

¹Department of Cardiology, Tongji Hospital of Tongji University School of Medicine, Shanghai 200065, China;

²Department of Lab Medicine, Tongji Hospital of Tongji University School of Medicine, Shanghai 200065, China;

³Department of Pathology, Tongji Hospital of Tongji University School of Medicine, Shanghai 200065, China; ⁴Department of Cardiology, First Hospital of Xi'an Jiaotong University School of Medicine, Xi'an, China

Received January 20, 2014; Accepted February 20, 2014; Epub March 15, 2014; Published March 30, 2014

Abstract: Objective: To investigate localization and distribution of integrin subunit $\beta 1$, $\beta 2$ and $\beta 3$ and morphological changes of ligand-receptor binding in thrombi of acute pulmonary embolism (PE) patients and explore activation of circulated immune cells, inflammatory immune adherence and coagulation response in acute venous thrombosis. Methods: Thrombi were collected from patients with acute PE. Immunohistochemistry was done to detect the expression and distribution of integrin $\beta 1$, $\beta 2$ and $\beta 3$ in cells within thrombi, and ligands of integrin subunit $\beta 1$, $\beta 2$ and $\beta 3$ were also determined by immunohistochemistry within the thrombi. Results: 1) Acute venous thrombi were red thrombi composed of skeletons and filamentous mesh containing large amounts of red blood cells and white blood cells; 2) Integrin subunit $\beta 1$, $\beta 2$ and $\beta 3$ were expressed on lymphocytes, neutrophils and platelets; 3) No expression of integrin $\beta 1$ ligands: Laminin, Fibronectin, Collagen I or Collagen-II on lymphocytes; integrin $\beta 2$ ligands including ICAM, factor X and iC3b are distributed on neutrophils, and ligand fibrinogen bound to neutrophils; integrin $\beta 3$ was expressed on platelets which form the skeleton of thrombi and bound to fibrinogen to construct mesh structure; 4) Factor Xa was expressed on the filamentous mesh; 5) Filamentous mesh was fully filled with red blood cell dominant blood cells. Conclusion: Acute venous thrombosis is an activation process of circulated lymphocytes, neutrophils and platelets mainly, and a whole process including integrin subunit $\beta 2$ and $\beta 3$ binding with their ligands. Activation of immune cells, inflammatory immune adherence and coagulation response are involved in the acute venous thrombosis.

Keywords: Pulmonary embolism, venous thrombosis, integrin

Introduction

Venous thromboembolism (VTE) includes pulmonary embolism (PE) and deep vein thrombosis (DVT). PE has a high morbidity, mortality and misdiagnosis rate and has been an important health problem worldwide [1]. American College of Chest Physicians (ACCP) has published Guidelines for Diagnosis, Treatment and Prevention of VTE, and some risk factors are also proposed in the guideline including advanced age, infection, malignancy, and autoimmune diseases. Moreover, risk stratification is also introduced in this guideline [2]. Although this guideline has been used to guide the clinical practice, the preventive efficacy of VTE is

still unsatisfactory [3]. In clinical practice, early, rapid and specific diagnosis of PE/DVT is crucial for its prognosis, and indicators for guidance of individualized anti-coagulation is required to determine the duration of anti-coagulation therapy. However, no specific objective indicators have been identified, due to the unclear understanding of the pathogenesis of acute venous thrombosis.

The authors have reported that the main component of acute venous thrombi is fibrinogen [4]. In our previous study, the thrombi were collected from patients with acute PE, and tandem mass spectrometry and bioinformatics were employed to determine that integrin subunit $\beta 1$,

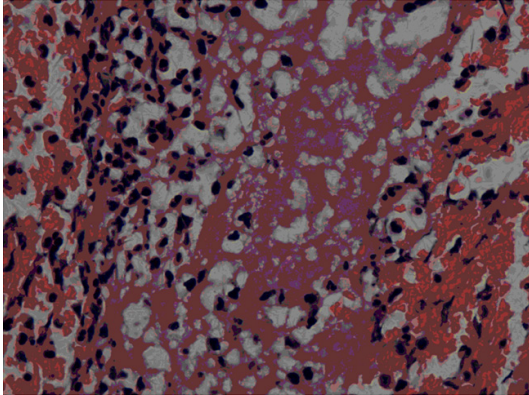


Figure 1. H&E staining of thrombus. HE staining of thrombus showed that venous thrombus was red thrombus in which cord-like structure, massive red blood cells and white blood cells with dark-brown nuclei aggregated (H&E staining, $\times 400$).

$\beta 2$ and $\beta 3$ are core proteins of acute venous thrombi. In the present study, immunohistochemistry was done to investigate the expression and cell distribution of integrin $\beta 1$, $\beta 2$ and $\beta 3$ in acute venous thrombi and the binding with different ligands in these cells. We aimed to explore role of immune cells in the process of acute venous thrombosis.

Patients and methods

Samples

Thrombi ($n=5-8$; 5-15 mm in length and 10-20 g in weight) were collected from the pulmonary artery of a patient with acute PE. Preparation of samples for pathological examination was done.

Immunohistochemistry and observation under a light microscope

After preparation of thrombus samples, HE staining, immunohistochemistry and Masson staining were performed. Following reagents were used in this study: integrin $\beta 1$: 1: 50 (Abcam B3B11), integrin $\beta 2$: 1: 150 (Abcam MEM-48), integrin $\beta 3$: 1: 150 (Abcam PM6/13); ligand anti-Laminin antibody 1: 50 (Abcam14055), ligand anti-Fibronectin antibody 1: 50 (Abcam2413), ligand anti-Collagen I antibody 1: 50 (Abcam34710), ligand anti-Collagen II antibody 1: 50 (Abcam34712), ligand anti-fibrinogen antibody 1: 100 (Abcam ab34269), ligand anti-Factor X antibody 1: 50 (Abcam ab11871), ligand anti-Factor Xa

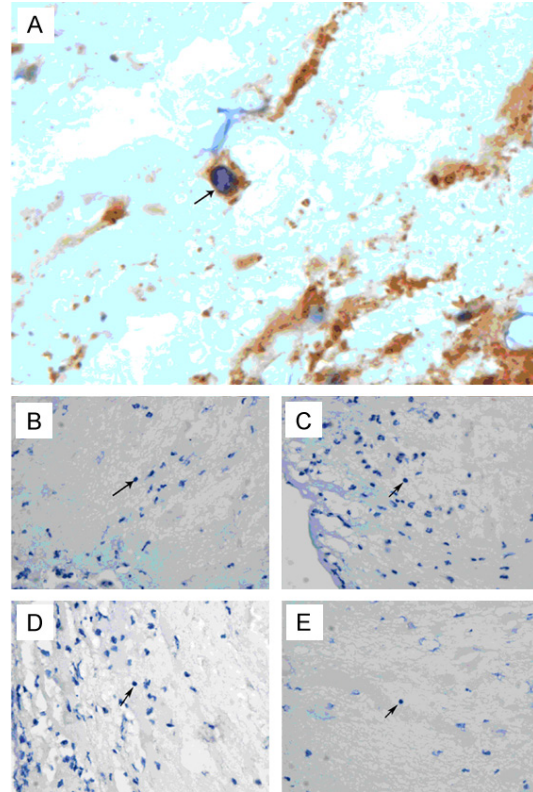


Figure 2. Immunohistochemistry. Arrow: dark-brown integrin $\beta 1$ was expressed on the lymphocytes (A, $\times 1000$). Expression of integrin $\beta 1$ ligands (Laminin, B, $\times 400$; Fibronectin, C, $\times 400$; Collagen I, D, $\times 400$; Collagen-II, E, $\times 400$) was not observed on the lymphocytes.

heavy chain antibody 1: 50 (Abcam ab140-112), ligand anti-C3/C3b antibody 1: 50 (Abcam ab11871), ligand anti-ICAM1 antibody 1: 50 (Abcam124759), ligand Von Will brand Factor antibody 1: 50 (Abcam11713), ligand anti-Vitronectin antibody 1: 50 (Abcam28023).

Results

1) Acute venous thrombi were red thrombi in which there are cord-like structures, and the spaces were filled with a large amount of aggregated red blood cells and nucleated blood cells (**Figure 1**).

2) Immunohistochemistry showed integrin $\beta 1$ was expressed on the lymphocytes (**Figure 2A**), but no expression of Laminin, Fibronectin, Collagen-I and Collagen-II (receptors of integrin $\beta 1$) was observed on the lymphocytes (**Figure 2B-E**).

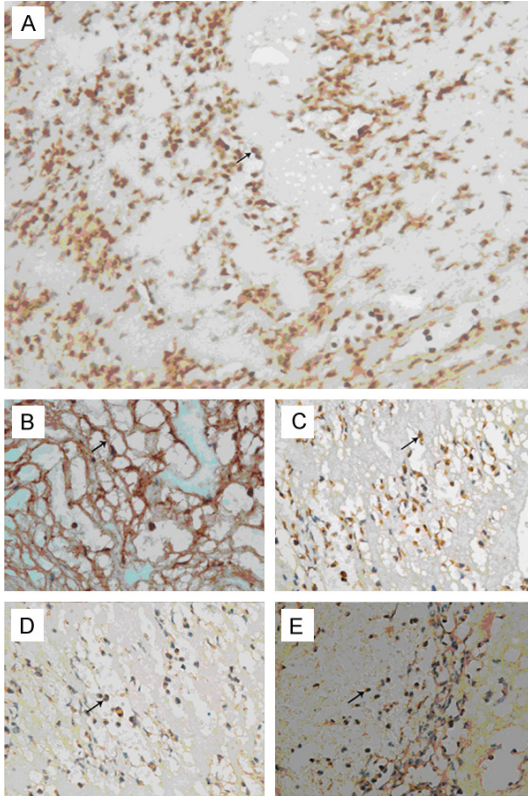


Figure 3. Immunohistochemistry. Arrow: dark-stained integrin $\beta 2$ was expressed on the neutrophils (A, $\times 400$) and bound fibrinogen (B, $\times 400$). ICAM (C, $\times 400$), factor X (D, $\times 400$), and C3b (E, $\times 400$) were expressed on neutrophils.

3) Immunohistochemistry showed integrin $\beta 2$ was expressed on the neutrophils (**Figure 3A**) which bound to fibrinogen (**Figure 3B**). The ICAM, factor X and iC3b were expressed on neutrophils (**Figure 3C-E**).

4) Immunohistochemistry showed integrin $\beta 3$ was expressed on platelets which aggregated to be thrombotic skeleton (**Figure 4A**) and coral-like structure (**Figure 4B**); these platelets bound fibrinogen to construct mesh structure (**Figure 4C**). No expression of Fibronectin, Vitronectin or vWF was observed on the platelets (**Figure 4D-F**).

5) The thrombi had mesh-like structure (**Figure 5A**, Masson staining) in which a large amount of red blood cell dominant blood cells filled (**Figure 5C**, Masson staining). In colon cancer tissues, there widely presented with dark-brown mesh-like structure in venules (**Figure 5B** anti-fibrinogen antibody 1: 100) in which a variety of cancer cells filled (**Figure 5D**).

6) Dark-brown Factor Xa was distributed on the mesh-like structure which was composed of fibrin/fibrinogen (**Figure 6A, 6B**).

Discussion

Integrin family was initially recognized as adhesion molecules mediating the adhesion between cells and extracellular matrix, which leads to the integration of cells. Integrins are widely distributed in human body. A kind of integrin can be distributed in a variety of types of cells, and one cell may have the expression of several integrins. The expression of integrins varies from activation status and differentiation status of cells [5].

Integrin is a transmembrane heterodimer composed by α and β subunits at a ratio of 1: 1 via the non-covalent bond. A total of 8 β subunits ($\beta 1$: $\beta 8$) have been identified in human. Under the quiescent condition, β subunit covers α subunit, and thus the integrin fails to bind ligand. After activation of integrin, the β subunit extends and then α subunit is exposed. α subunit mainly mediates the reversible binding of integrin to its ligand. The β subunit is responsible for signal transduction and regulation of integrin's affinity [6].

Integrin $\beta 1$ is mainly expressed on lymphocytes [7], and its ligands include laminin, fibronectin, collagen, thrombospondin and VCAM-1 [8]. The binding of Integrin $\beta 1$ and ligands is involved in immune cell adherence, which can provide costimulation for activation of T cells. Integrin $\beta 2$ is mainly expressed on the neutrophils and monocytes [9], and its ligand includes Fibrinogen, ICAM, factor X and C3b [10]. The binding of Integrin $\beta 2$ and ligands is involved in immune cell adherence, inflammation and phagocytosis. Integrin $\beta 3$ is expressed on the platelets [11] and its ligand includes fibrinogen, fibronectin, vitronectin, VWF and thrombospondin [12]. The binding of Integrin $\beta 3$ and ligands is involved in activation and aggregation of platelets.

Many cells are involved in inflammatory immune response, including cells locating in tissue, lymphocytes, neutrophils and platelets. Light microscopy showed the thrombi in acute pulmonary thromboembolism were red thrombi. Immunohistochemistry revealed that integrin $\beta 1$ was distributed on lymphocytes. Laminin,

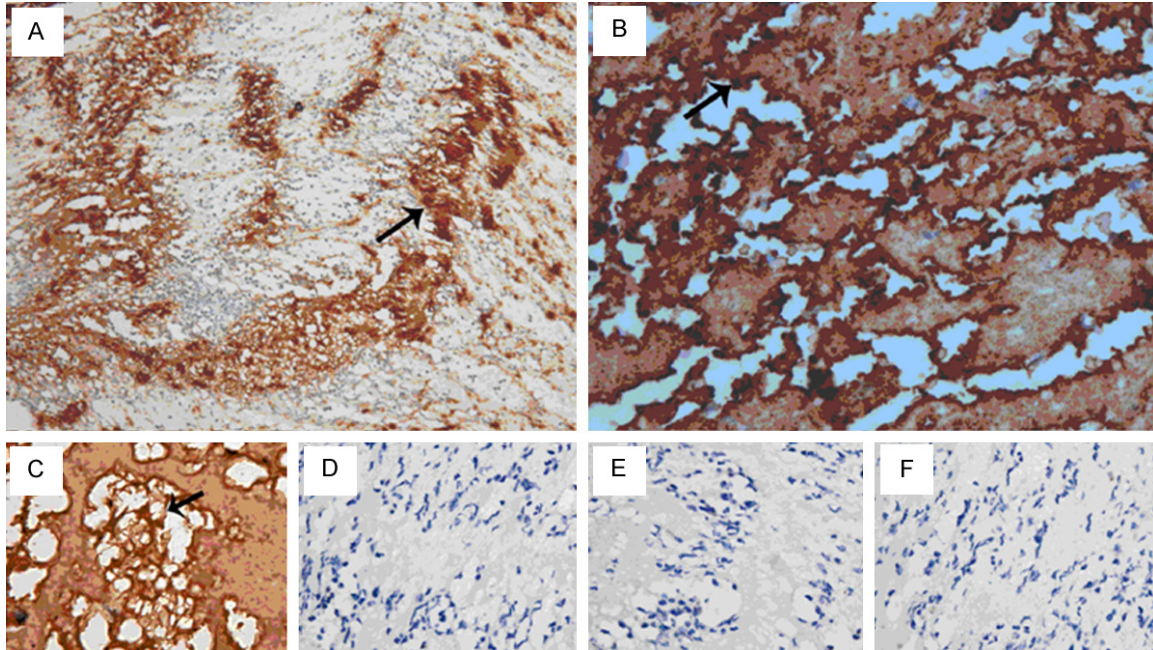


Figure 4. Immunohistochemistry. Arrow: dark-brown integrin $\beta 3$ was expressed on platelets (A, $\times 200$) and on the coral-like skeleton formed by platelets (B, $\times 400$). Platelets and neutrophils bound fibrinogen to construct mesh-like structure (C, $\times 400$). No expression of Fibronectin (D, $\times 400$), Vitronectin (E, $\times 400$), vWF (F, $\times 400$) was observed on these cells.

fibronectin, Collagen I and Collagen II, ligands of integrin $\beta 1$, were not expressed on these cells. Integrin $\beta 2$ was mainly distributed on neutrophils. The binding of activated integrin $\beta 2$ with fibrinogen results in the formation of filamentous mesh. The ligands of integrin $\beta 2$ (ICAM, factor X and C3b) were expressed on neutrophils, suggesting the binding of integrin $\beta 2$ with the ligands is involved in the thrombosis. Integrin $\beta 3$ was distributed on platelets gathered in different shapes, which bound with Fibrinogen to construct the filamentous mesh. No expression of fibronectin, vitronectin and vWF was observed on the platelets. The main protein component of acute venous thrombi is fibrinogen [4]. The result indicates the binding of platelet integrin $\beta 3$ and neutrophil integrin $\beta 2$ with ligand fibrinogen in thrombi is the early form of venous thrombosis.

In the thrombi, neutrophils and platelets are activated and bind to corresponding ligands, leading to inflammatory immune adhesion, which finally constructs filamentous mesh, a framework of venous thrombus. When the filamentous mesh was fully filled with red blood cell dominant blood cells, red thrombus formed. In the circulation, except for red blood cells,

platelets and neutrophils have the largest amount. The binding of integrins on membrane of platelets and neutrophils and their ligands is directly involved in the formation of acute venous thrombus. The binding of neutrophils and factor X can trigger the coagulation process, and activated factor X converted to Xa and distributed on the fibrinogen, promoting soluble fibrinogenic thrombi transformed to fibrinic thrombi. Acute venous thrombosis is a main activation process of circulated neutrophils and platelets, and it is a whole process of integrin subunit $\beta 2$ and $\beta 3$ binding with their ligands, and a process of inflammatory immune adherence triggering coagulation reaction.

Thirty years ago, investigators developed and applied transient or permanent inferior vena cava filter in clinical practice to block the flow back of venous thrombi into the pulmonary artery, which may prevent the occurrence of PE [13]. In the study, the mesh-like structure in thrombi is similar to a biological filter, and what is the function of this mesh-like structure?

We have reported that virus-like microorganisms were observed in cytoplasm and intercel-

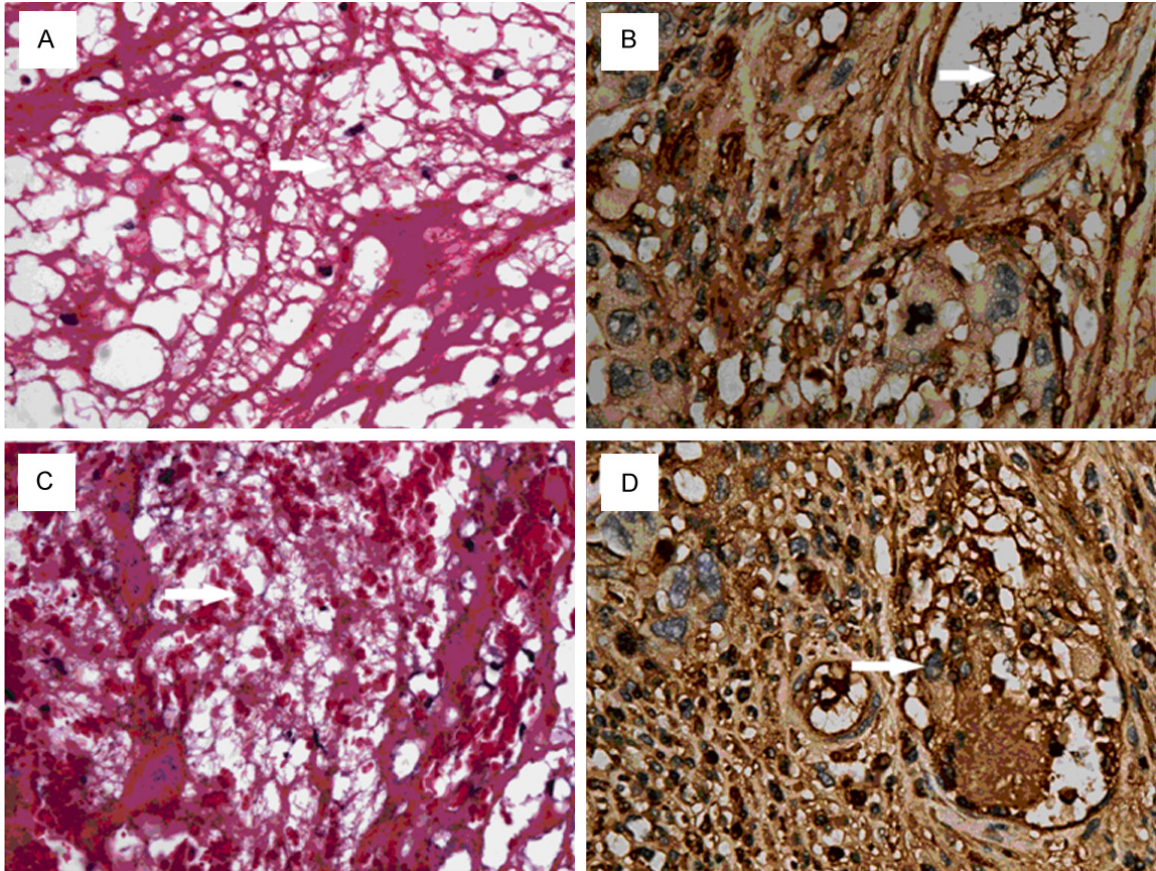


Figure 5. Masson staining of mesh-like structure. Arrow: Mesh-like structure was nest-like biological filter (A, $\times 400$, Masson staining), in which red blood cell dominant blood cells filled (C, $\times 400$, Masson staining). In colon cancer, massive mesh-like structure (anti-fibrinogen antibody, 1: 100, $\times 400$) was observed in venules, and cancer cells were also observed in this mesh-like structure (anti-fibrinogen antibody, 1: 100, D, $\times 400$).

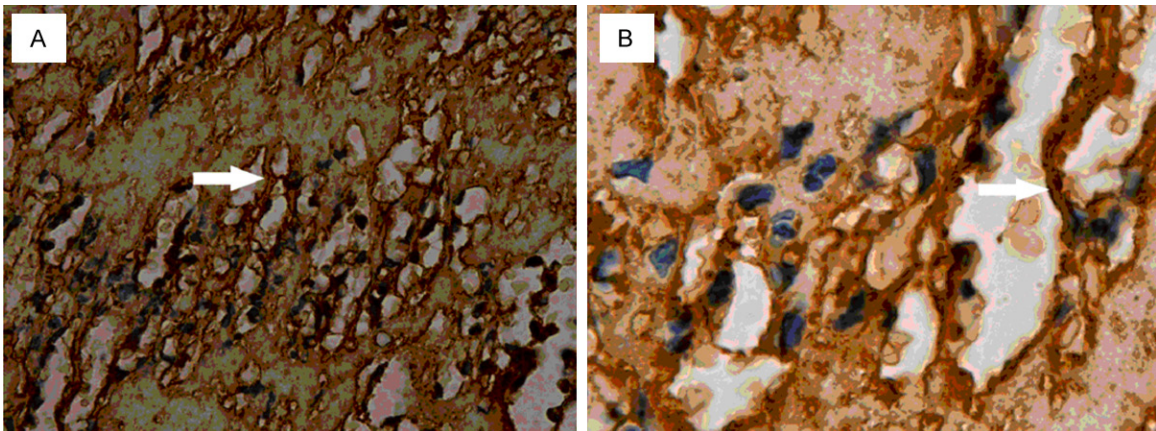


Figure 6. Factor Xa in mesh-like structure. Arrow: dark-brown factor Xa was found on the surface of mesh-like structure (A, $\times 400$; B, $\times 1000$). This suggests factor Xa acts on the fibrinogen/fibrin.

ular substance of lymphocytes from peripheral venous blood of VTE patients with pulmonary

hypertension and T cell immune dysfunction/disorder [14]. We also observed rod-shaped

bacteria like microorganisms in apoptotic phagocytes from peripheral venous blood of patients with repeated PE/DVT and T cell immune dysfunction/disorder [15], We also found DVT in the veins of multiple organs (such as pulmonary artery, kidney, liver and pancreas) of a patient who died of SARS [16]. These findings indicate that the onset of VTE has the involvement of infection of microorganisms. Moreover, the mRNA expression of T cells and NK cells was significantly down regulated in patients with symptomatic VTE, as demonstrated by human genomics. The amount of CD3+, CD8+ and CD16+56+ cells reduced significantly, the increased CD4 level in patients with symptomatic VTE, which was consistent with findings in genomics [17]. The increased level of integrin subunit $\beta 1$ in this study indicates the activation of lymphocytes, suggesting the regulatory function of lymphocytes enhanced. Malignancy is a disease related to immune dysfunction. **Figure 5B, 5D** showed mesh-like structure in the veins of cancer tissue was similar to that in venous thrombi. Furthermore, a variety of cancer cells were observed in this mesh like structure of veins in cancer tissue.

Acute venous thrombosis is an activation process of circulated lymphocytes, neutrophils and platelets, and it is a whole process of integrin subunit $\beta 1$, $\beta 2$ and $\beta 3$ binding with their ligands, and a process of immune adherence, generating biological sieve and triggering coagulation reaction. Thus, we hypothesize that, when the infected cells or cancer cells can not be effectively and timely cleared in the presence of immune dysfunction/disorder, activated neutrophils and platelets bind to their ligands to construct biological filamentous mesh-like structure, which acts as a barrier to block the flow of infected cells or cancer cells. When the filamentous mesh-like structure was fully filled with red blood cell dominant blood cells, red venous thrombi occurred. The defensive biological filamentous mesh-like structure causes venous thrombosis.

Acknowledgements

This study was supported by “12th five year” National Science and Technology Supporting Program (2011BAI11B16).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Le-Min Wang, Department of Cardiology, Tongji Hospital of Tongji University School of Medicine, Shanghai 200065, China. E-mail: wanglemin@tongji.edu.cn; Dr. Fan Yang, Department of Lab Medicine, Tongji Hospital of Tongji University School of Medicine, No.389, Xincun Road, Putuo district, Shanghai 200065, China. Tel: +86 21 66111329; Fax: +86 21 66111329; E-mail: yangfanyang@hotmail.com

References

- [1] Cardiovascular Disease Educational and Research Trust; Cyprus Cardiovascular Disease Educational and Research Trust; European Venous Forum; International Surgical Thrombosis Forum; International Union of Angiology; Union Internationale de Phlébologie. Prevention and treatment of venous thromboembolism. International Consensus Statement (guidelines according to scientific evidence). *Int Angiol* 2006; 25: 101-161.
- [2] American College of Chest Physicians. Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 2012; 141: e1S-801S.
- [3] Shackford SR, Rogers FB, Terrien CM, Bouchard P, Ratliff J and Zubis R. A 10-year analysis of venous thromboembolism on the surgical service: the effect of practice guidelines for prophylaxis. *Surgery* 2008; 144: 3-11.
- [4] Wang L, Gong Z, Jiang J, Xu W, Duan Q, Liu J and Qin C. Confusion of wide thrombolytic time window for acute pulmonary embolism: mass spectrographic analysis for thrombus proteins. *Am J Respir Crit Care Med* 2011; 184: 145-146.
- [5] Dorner M, Zucol F, Alessi D, Haerle SK, Bossart W, Weber M, Byland R, Bernasconi M, Berger C, Tugizov S, Speck RF and Nadal D. beta1 integrin expression increases susceptibility of memory B cells to Epstein-Barr virus infection. *J Virol* 2010; 84: 6667-6677.
- [6] Van Der Flier A and Sonnenberg A. Function and interactions of integrins. *Cell Tissue Res* 2001; 305: 285-298.
- [7] Cavers M, Afzali B, Macey M, McCarthy DA, Irshad S and Brown KA. Differential expression of beta1 and beta2 integrins and L-selectin on CD4+ and CD8+ T lymphocytes in human blood: comparative analysis between isolated cells, whole blood samples and cryopreserved preparations. *Clin Exp Immunol* 2002; 127: 60-65.
- [8] Fiorilli P, Partridge D, Staniszewska I, Wang JY, Grabacka M, So K, Marcinkiewicz C, Reiss K, Khalili K and Croul SE. Integrins mediate adhesion of medulloblastoma cells to tenascin and

Immune and acute venous thrombosis

- activate pathways associated with survival and proliferation. *Lab Invest* 2008; 88: 1143-1156.
- [9] Rezzonico R, Chicheportiche R, Imbert V and Dayer JM. Engagement of CD11b and CD11c beta2 integrin by antibodies or soluble CD23 induces IL-1beta production on primary human monocytes through mitogen-activated protein kinase-dependent pathways. *Blood* 2000; 95: 3868-3877.
- [10] Schwarz M, Nordt T, Bode C and Peter K. The GP IIb/IIIa inhibitor abciximab (c7E3) inhibits the binding of various ligands to the leukocyte integrin Mac-1 (CD11b/CD18, alphaMbeta2). *Thromb Res* 2002; 107: 121-128.
- [11] Fang J, Nurden P, North P, Nurden AT, Du LM, Valentin N and Wilcox DA. C56ORbeta3 caused platelet integrin alphaIIb beta3 to bind fibrinogen continuously, but resulted in a severe bleeding syndrome and increased murine mortality. *J Thromb Haemost* 2013; 11: 1163-1171.
- [12] Coburn J, Magoun L, Bodary SC and Leong JM. Integrins alpha(v)beta3 and alpha5beta1 mediate attachment of lyme disease spirochetes to human cells. *Infect Immun* 1998; 66: 1946-1952.
- [13] Athanasoulis CA, Kaufman JA, Halpern EF, Waltman AC, Geller SC and Fan CM. Inferior vena caval filters: review of a 26-year single-center clinical experience. *Radiology* 2000; 216: 54-66.
- [14] Wang L, Gong Z, Liang A, Xie Y, Liu SL, Yu Z, Wang L and Wang Y. Compromised T-cell immunity and virus-like structure in a patient with pulmonary hypertension. *Am J Respir Crit Care Med* 2010; 182: 434-435.
- [15] Wang L, Zhang X, Duan Q, Lv W, Gong Z, Xie Y, Liang A and Wang Y. Rod-like bacteria and recurrent venous thromboembolism. *Am J Respir Crit Care Med* 2012; 186: 696.
- [16] Xiang-Hua Y, Le-Min W, Ai-Bin L, Zhu G, Riquan L, Xu-You Z, Wei-Wei R and Ye-Nan W. Severe acute respiratory syndrome and venous thromboembolism in multiple organs. *Am J Respir Crit Care Med* 2010; 182: 436-437.
- [17] Duan Q, Gong Z, Song H, Wang L, Yang F, Lv W and Song Y. Symptomatic venous thromboembolism is a disease related to infection and immune dysfunction. *Int J Med Sci* 2012; 9: 453-461.