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IL-17A promotes the formation of deep vein thrombosis in a mouse model

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ABSTRACT

Deep venous thrombosis (DVT) is a significant problem in the health care industry worldwide. However, the factors and signaling pathways that trigger DVT formation are still largely unknown. In this study, we investigated the role of interleukin-17A (IL-17A) in DVT formation, focusing on the role of platelet aggregation, neutrophil infiltration, and endothelium cell (EC) activation. Notably, IL-17A levels increased in DVT patients as well as in a mouse DVT model. The DVT model mice were injected with recombinant mouse-IL-17A (rIL-17A) or anti-IL-17A monoclonal antibody (mAb) to further evaluate the effects of this cytokine. We found that rIL-17A promotes DVT formation, while IL-17A mAb represses DVT formation. Furthermore, platelet activation, highlighted by CD61 and CD49β expression, and aggregation were enhanced in platelets of rIL-17A-treated mice. rIL-17A also enhanced neutrophil infiltration by regulating the expression of macrophage inflammatory protein-2 (MIP-2) and the release of neutrophil extracellular traps (NETs). IL-17A mAb treatment inhibited both platelet activation and neutrophil activity. Moreover, rIL-17A appears to promote vein EC activation, while IL-17A mAb deters it. Taken together, these data suggest that IL-17A promotes DVT pathogenesis by enhancing platelet activation and aggregation, neutrophil infiltration, and EC activation and that anti-IL-17A mAb could be used for the treatment of DVT.

1. Introduction

Deep venous thrombosis (DVT) is a common peripheral vascular condition that is closely linked with pulmonary embolism (PE) and post-thrombotic syndrome, both of which lead to death in a substantial fraction of patients each year [1,2]. DVT formation has traditionally been thought to be caused by blood stagnancy, endothelial injury of the vein, and/or hypercoagulability [3]. However, recent studies suggest that DVT is also closely related to inflammatory processes. In support of this, several inflammatory markers have been shown to be increased in DVT patients, and acute infection appears to play a catalytic role in DVT development [4–6]. Unfortunately, the inflammation-related molecular mechanisms underlying DVT formation have not been investigated comprehensively.

Interleukin-17A (IL-17A) is a pleiotropic pro-inflammatory cytokine that modulates the release of a broad range of cytokines and inflammatory molecules in a variety of cell types, including epithelial cells, endothelial cells, and fibroblasts [7]. Furthermore, a recent study demonstrated that increased serum IL-17 levels in rats are positively correlated to DVT formation [8]. IL-17A has also been recognized as an important contributor to platelet aggregation as platelets express the IL-17A receptor (IL-17RA) [9,10]. Moreover, our recent studies indicate that IL-17A facilitates platelet activation and function through the extracellular signaling-regulated kinase 2 (ERK2) signaling pathway in humans [11,12]. However, whether these IL-17A-mediated changes in platelet function, activation, and aggregation play a role in DVT formation has not been established.

Platelets are important in various thrombotic processes, particularly in arterial thrombosis [13,14]. They are also thought to be involved in the initiation of venous thrombosis [15–18]. Indeed, recent evidence demonstrates that platelet activation participates in the development of venous thrombosis by enhancing the generation of thrombin, a potent platelet agonist [19]. In addition, activated platelets promote the formation of neutrophil extracellular traps (NETs), which can in turn promote DVT and modulate endothelial cell (EC) activation [4,20,21]. Thus, the apparent association of IL-17A with activated platelets and the related changes in NETs and EC function suggest that IL-17A could have a key role in DVT initiation.

In the current study, we established a mouse model of DVT that imitates the formation of thrombus via reduced blood flow and used it to explore the role of IL-17A. Furthermore, we sought to evaluate the effects of IL-17A overexpression and silencing during DVT-related

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changes in platelet function, neutrophil infiltration, and EC activation. To our knowledge, this is the first time the specific function of IL-17A has been investigated in depth during DVT formation.

2. Materials and methods

2.1. Patients

A total of 55 DVT patients (27 males, 28 females) and 45 healthy volunteers (21 males, 24 females) were recruited from the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The participants were divided into two groups: (a) Control group (n = 45) (Herein, a healthy person refers to anyone in good health in terms of physical, mental, social, and other aspects) and (b) DVT group (n = 55). The DVT patients were diagnosed by vascular ultrasound. None of the participators were administered anti-platelet, anticoagulant, or anti-inflammatory agents for 7 days prior to blood sampling. Written informed consent was obtained from every participant, and the research fully complies with the guidelines stipulated in the Declaration of Helsinki and its amendments.

2.2. Mice

Male BALB/c mice (6–8 weeks old) were purchased from the Experimental Animal Research Center (Wuhan, Hubei province, China). All of the animals were kept in standard pathogen-free conditions at the Experimental Animal Center (Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China). The animal experiments were carried out in accordance with the guidelines for the Care and Utilization of Laboratory Animals (Huazhong University of Science and Technology, China). This study was approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology and was carried out according to the regulations established for animal experimentation in the Hubei province of China as well as the constitution of the experimental animal ethics committee at Huazhong University of Science and Technology ([2014] IACUC Number: 661).

2.3. Murine DVT model and treatment grouping

The DVT model was established as described previously [22]. In brief, mice were anesthetized with 4% chloral hydrate at 12 ml/kg. Then, a 2-cm midline incision was made, and the small bowel was exteriorized onto a piece of sterile gauze pre-soaked with normal saline. The inferior vena cava (IVC) was isolated under a stereoscope with a suture needle conjugated to a knife handle. A neurosurgical vascular clip was applied to the infrarenal IVC twice at an interval of 30 s, each session lasting 15 s, to induce damage to the intestinal endothelia. A 5-0 Prolene suture was placed longitudinally along the ventral surface of the IVC, and then a 4-0 Prolene suture was tied around both the IVC and Prolene suture. The 5-0 Prolene suture was then removed, allowing blood flow to resume, thereby causing stenosis (~90%). No branches were ligated. The bowel was returned to the abdominal cavity. All mice that were observed to have bleeding during the surgery were excluded from further analysis.

Mice were randomly divided into five groups: (1) Control group, no treatment or surgical intervention; (2) Sham group, no treatment and sham operation; (3) DVT group, each mouse received 100 μl of PBS containing 0.1% albumin (R&D System, Minneapolis, MN) and underwent the surgical operation for DVT described above; (4) rIL-17A group, each mouse was i.v. injected with 50 μg/kg of recombinant mouse IL17A (rIL-17A) (PeproTech, Cot: 210–17, USA) diluted in 100 μl PBS containing 0.1% albumin and underwent the DVT surgical operation; (5) Anti-IL-17A group, each mouse was i.v. injected with 5 mg/kg of IL17A monoclonal antibody (mAb) (eBioscience, clone eBioMM17F3, lgG1, San Diego, CA) diluted in PBS containing 0.1% albumin and underwent the DVT surgical operation. All mice were given the specified treatments via tail vein injection 30 min before undergoing the DVT or sham surgery. All animals were sacrificed 12 or 24 h after the DVT/sham operation. Vascular ultrasounds were performed, and the blood from each mouse was collected by retro-orbital bleeding. Fresh thrombi were collected to measure their length and weight, and were then fixed with 4% paraformaldehyde (PFA) for further analysis.

2.4. Isolation and preparation of platelets

In each treatment group, blood was collected via retro-orbital bleeding and then drawn into a vacationer tube containing 400 μl of 3.2% sodium citrate and HEPES-Tyrode buffer (145 mM NaCl, 10 mM HEPES, 5 mM D-glucose, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, pH 6.5). To obtain platelet-rich plasma (PRP), the blood was centrifuged at 100 ×*g* for 30 min [12]. Then, the supernatant was harvested and suspended in 700 μl of HEPES-Tyrode buffer for the platelet aggregation assay. Afterwards, the remaining blood was centrifuged at 2000 ×*g* for 10 min to acquire platelet-poor plasma (PPP). The PRP was further assayed after being rinsed twice with HEPES-Tyrode buffer, followed by centrifugation at 500 ×*g* for 5 min. The washed platelets were resuspended in HEPES-Tyrode buffer before being counted and adjusted to 10^7 platelets/ml.

2.5. Platelet aggregation assay

PRP samples isolated at 12 h post-operation were pre-warmed to 37 °C in a resting state. Adenosine diphosphate (ADP, 2.5 mM; Helena Laboratories, Texas. USA) was then added to the samples to induce platelet aggregation. Platelet aggregation was turbidimetrically detected with an aggregation-monitoring device (AggRAM, Helena Laboratories, Texas, USA) and was evaluated in terms of the maximal percentage of platelet aggregation [12]. PPP samples were used to set the baseline for each treatment group in this assay.

2.6. Flow cytometry

The PRP samples were washed in HEPES-Tyrode buffer, followed by incubation with PE-conjugated anti-CD49β antibody (eBioscience, Cot: 12-5971, USA) and FITC-conjugated CD61 antibody (eBioscience, Cot: 11-0611, USA) for 30 min at room temperature. Then, after washing with HEPES-Tyrode buffer and subsequent centrifugation (500 ×*g*, 5 min), platelets were finally resuspension in 200 μl of HEPES-Tyrode buffer, and the mean fluorescence intensity (MFI) was analyzed by FACS Calibur flow cytometry (BD LSRFortessa™ X-20, USA).

2.7. Immunohistochemistry and immunofluorescence

To analyze the effect of DVT and IL-17A treatment on histology, thrombi from each treatment group were embedded in paraffin and 4 μm sections were stained with hematoxylin and eosin (H&E). Deparaffinized sections were boiled for 10 min for antigen retrieval, followed by treatment with endogenous peroxidase-blocking reagent (clone NP57, Dako) for 5 min and protein block (clone NP57, Dako) for 10 min at room temperature. After incubation with the specified antibodies overnight at 4 °C, horseradish peroxidase-labeled polymer-conjugated secondary antibodies (ENVISION: Dako) were added. Immunoreactivity was visualized with 3, 3′-diaminobenzidine tetrahydrochloride, and the sections were counterstained with Mayer's hematoxylin.

Neutrophil infiltration was evaluated by assessing the localization of myeloperoxidase (MPO)-positive cells. For this analysis, deparaffinized sections of thrombus were blocked in 0.3% hydrogen peroxide for 20 min before being incubated overnight at 4 °C with MPO antibody (1:100, Santa Cruz Biotechnology, Dallas, TX, USA). Then, the samples were incubated with biotinylated secondary antibody and a