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Citation	Rheumatology, 56(6), 1013-1018 https://doi.org/10.1093/rheumatology/kew477
Issue Date	2017-06
Doc URL	https://hdl.handle.net/2115/66949
Rights(URL)	https://creativecommons.org/licenses/by-nc/4.0/
Type	journal article
File Information	Rheumatology2017 kew477.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

RHEUMATOLOGY

Rheumatology 2017;56:1013–1018
doi:10.1093/rheumatology/kew477
Advance Access publication 10 January 2017

Concise report

Establishment of a rat model of thrombosis induced by intravenous injection of anti-phosphatidylserine–prothrombin complex antibody

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Abstract

Objective. Recent studies have suggested that aPS-PT antibody is one of the most relevant autoantibodies to APS. This study aimed to demonstrate the pathogenicity of aPS-PT antibody *in vivo*.

Methods. At first, cultured rat vascular endothelial cells (RECs) were exposed to calf thymus-derived histones. Two hours later, lactate dehydrogenase release from the RECs and expression of PS on the cell surface were assessed. Next, we administered an i.v. injection of calf thymus-derived histones into Wistar rats (12.5 μg/g weight of 8-week-old female rats), and 2 h later they were given an i.v. injection of aPS-PT mAb (1.25 mg/g weight, n = 6) or an equal dose of rat IgM as controls (n = 5). Three days later, histological examination was conducted.

Results. Calf thymus-derived histones (>12.5 μg/ml) could injure RECs *in vitro*. Simultaneously, annexin V could bind to the RECs; thereby, this result indicated that cell-free histone exposure of vascular endothelial cells induced cell surface expression of PS, which is naturally present inside the plasma membrane. Thrombosis developed with higher frequency in the rats given an i.v. injection of aPS-PT mAb than in controls.

Conclusion. We established a rat model of thrombosis induced by i.v. injection of aPS-PT mAb.

Key words: anti-phosphatidylserine–prothrombin complex antibody, anti-phospholipid syndrome, thrombosis, animal model

BASIC SCIENCE

Rheumatology key messages

- Thrombosis was induced in rats by i.v. injection of aPS-PT complex antibodies.
- The pathogenicity of aPS-PT antibodies was demonstrated *in vivo*.
- The inducible thrombosis model could be a useful tool to investigate the pathogenesis of APS.

Introduction

APS is an autoimmune disease characterized by recurrent thrombosis and pregnancy morbidity attributable to production of aPL in the serum [1]. aPLs recognize phospholipid-binding plasma proteins, such as β2-glycoprotein I and prothrombin. Recent studies have suggested that aPS-PT is one of the most relevant autoantibodies to APS [2]. Although the prothrombotic property of aPS-PT antibodies has been demonstrated *in vitro* [3], the *in vivo* pathogenicity of aPS-PT antibodies has not been determined.

Anionic phospholipids, including PS, are not naturally expressed on the surface of viable cells, but they are

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Submitted 29 July 2016; revised version accepted 30 November 2016

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translocated to the surface of the plasma membrane of cells during apoptosis [4]. Administration of cell-free histone in mice induces apoptosis of vascular endothelial cells [5]. Based on these findings, we hypothesized that exposure of vascular endothelial cells to cell-free histone could induce PS expression on the cell surface. In the first part of this study, we observed this phenomenon *in vitro*.

We recently generated a rat mAb that could recognize the rat phosphatidylserine–prothrombin complex [6]. This mAb was derived from autoimmune-prone rats, which developed diverse CTDs with production of various autoantibodies, such as anti-nuclear, anti-DNA and aCL [7]. In the second part of this study, we determined that administration of sequential i.v. injection of cell-free histones and aPS-PT mAb could develop thrombosis in normal rats.

Methods

Cells and reagents

The rat inferior vena cava-derived vascular endothelial cells (RECs) were established in our laboratory [8]. The RECs were maintained in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum and 2×10^{-8} M 2-mercaptoethanol. Calf thymus-derived histones that contain unfractionated whole histones were purchased from Sigma-Aldrich (St Louis, MO, USA).

aPS-PT mAb

The rat mAb against rat phosphatidylserine–prothrombin complex was generated in our laboratory [6]. For this purpose, the conventional hybridoma method was used. In brief, spleen cells derived from the autoimmune-prone rats were fused with mouse myeloma cells, P3U1, and screening for the production of antibody against rat phosphatidylserine–prothrombin complex and cell cloning were carried out. Finally, five hybridoma clones that produced aPS-PT monoclonal antibody were established. Isotyping revealed that their immunoglobulin class was all IgM. Among the five hybridoma clones, the best-grown one was used for mass production of rat aPS-PT monoclonal antibody. Rat IgM (eBioscience, San Diego, CA, USA) was used as the control.

Lactate dehydrogenase release assay

The RECs (2.5×10^4/well of 96-well round-bottomed plates) were exposed to 0, 6.25, 12.5, 25, 50 or 100 μg/ml of calf thymus-derived histones. Two hours later, lactate dehydrogenase (LDH) release from the RECs was determined using the Cytotoxicity LDH Release Assay Kit (Dojindo, Kumamoto, Japan).

Flow cytometry

The RECs (2.5×10^5) were exposed to 0 or 25 μg/ml of calf thymus-derived histones and then incubated with or without FITC-labelled annexin V according to the instructions of the apoptosis detection kit (BD Biosciences, Tokyo, Japan). Samples were analysed using a flow cytometer (Attune Acoustic Focusing Cytometer, Thermo Fisher Scientific, Yokohama, Japan).

Injection of cell-free histones and aPS-PT mAb into rats

Eight-week-old female Wistar rats maintained in the facility for animal experiments in Hokkaido University were used (n = 11). These rats were given an i.v. injection of calf thymus-derived histones (12.5 μg/g weight), and 2 h later, they were given an i.v. injection of 1.25 mg/g weight of aPS-PT mAb (n = 6) or rat IgM (n = 5). Three days later, all rats were killed for histological examination. Haematoxylin and eosin staining was performed on sections of the cerebrum, cerebellum, eyes, salivary glands, thymus, heart, lungs, liver, pancreas, spleen, kidneys, intestine, skin and muscles. Experiments using animals in this study were approved by the Ethics Committee of Hokkaido University (permission no. 15-0034).

Immunohistochemistry

Histological sections of the rats given an i.v. injection of aPS-PT mAb, in which thrombi were present, were subjected to immunohistochemistry for rat IgM. For this purpose, we used horseradish peroxidase-conjugated goat anti-rat IgM antibodies (Abcam, Cambridge, UK) and the diaminobenzidine substrate kit (Sigma-Aldrich). Sections of the rats given an i.v. injection of calf thymus-derived histones only were stained simultaneously as controls.

Statistics

Student's *t*-test and the Mann–Whitney *U*-test were used to compare the two *in vitro* and *in vivo* groups, respectively. A value of $P < 0.05$ was regarded as statistically significant.

Results

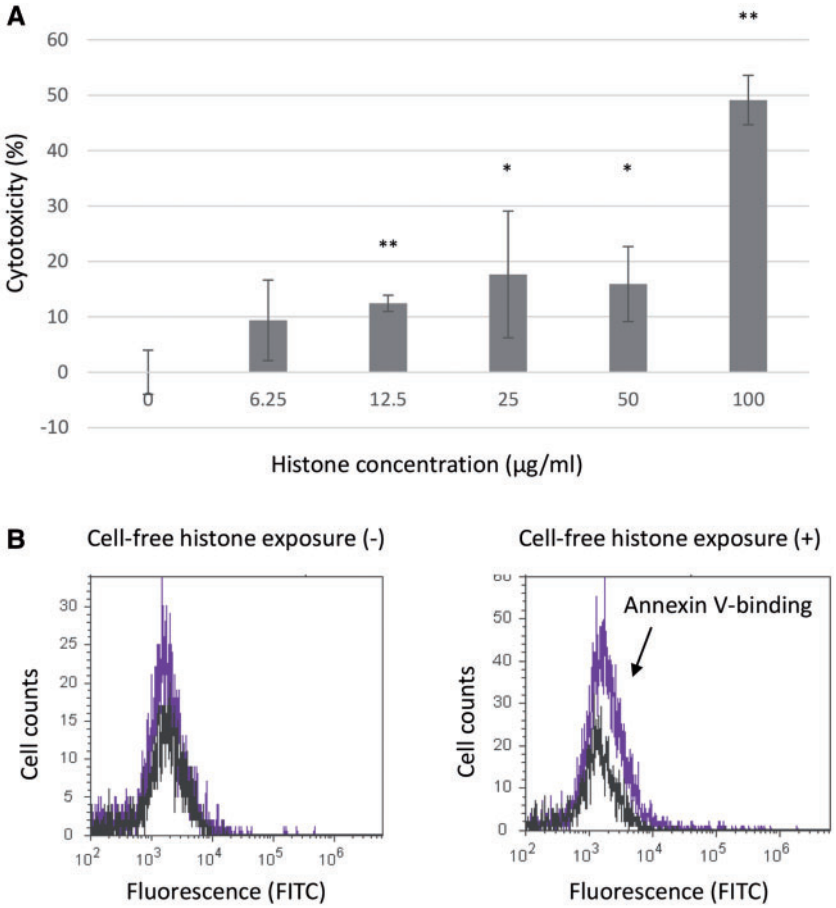
Cytotoxicity of cell-free histones to vascular endothelial cells

In order to elucidate our hypothesis that cell-free histones could injure vascular endothelial cells and induce PS expression on the cell surface, RECs (2.5×10^4/well of 96-well round-bottomed plates) were exposed to 0, 6.25, 12.5, 25, 50 or 100 μg/ml of calf thymus-derived histones. Two hours later, LDH release from the REC was determined. Cell-free histone exposure induced LDH release from the REC in a dose-dependent manner, and the degree was statistically significant when the histone concentration was 12.5 μg/ml or more (Fig. 1A).

Expression of PS on the surface of vascular endothelial cells induced by cell-free histone exposure

The RECs (2.5×10^5) were exposed to 0 or 25 μg/ml of calf thymus-derived histones, and the binding of annexin V was determined by flow cytometry. Higher fluorescence attributable to the binding of FITC-labelled annexin V was found on the cell-free histone-treated RECs compared

FIG. 1 Effects of cell-free histone exposure on vascular endothelial cells



(**A**) RECs (2.5×10^4/well of 96-well round-bottomed plates) were exposed to 0, 6.25, 12.5, 25, 50 or 100 μg/ml of calf thymus-derived histones. Two hours later, LDH release from the RECs was determined. LDH release was represented as the percentage cytotoxicity. Data are displayed as the mean (S.D.) of three independent experiments. Asterisks indicate the statistical difference from the background cytotoxicity (histone concentration: 0 μg/ml): *$P < 0.05$, **$P < 0.01$. (**B**) RECs (2.5×10^5) were exposed to 0 or 25 μg/ml of calf thymus-derived histones, and then the binding of annexin V was determined by flow cytometry. Left: RECs without cell-free histone-treatment. Right: cell-free histone-treated RECs. Purple bars represent annexin V binding. Black bars represent background. Results were reproduced in three independent experiments, and representative results are shown. LDH: lactate dehydrogenase; RECs: rat vascular endothelial cells.

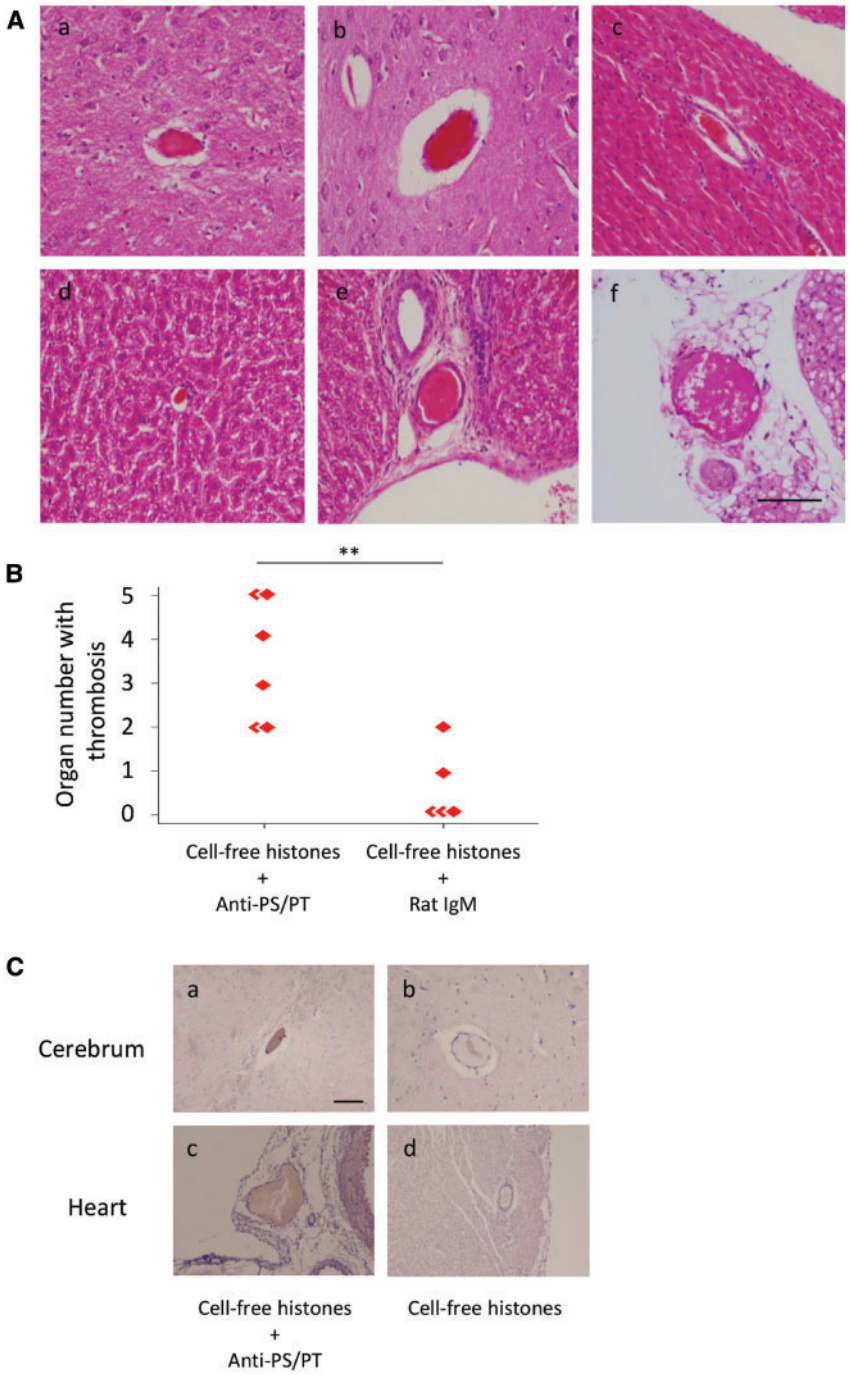
with those without histone treatment (Fig. 1B). Previous studies have demonstrated that annexin V could bind to PS [9]. These findings suggested that PS could be expressed on the vascular endothelial cell surface by cell-free histone exposure.

Establishment of a rat model of thrombosis induced by i.v. injection of aPS-PT mAb

It has been demonstrated that mice died after i.v. injection of 60–80 μg/g weight of cell-free histones [10]. Likewise, Wistar rats (8 weeks old, female) given a single tail vein injection of calf thymus-derived histones (62.5 μg/g weight) died in our preliminary experiments. Autopsy revealed that pulmonary bleeding was fatal to these rats. In order to create a clinical condition wherein vascular endothelial cells are injured but allow survival of the rats, Wistar rats (8 weeks old, female, n = 11) were given an i.v. injection of 12.5 μg/g weight of calf thymus-derived histones. The dose was equivalent to 100 μg/ml of histone concentration in the blood, which was enough to injure RECs *in vitro*. Two hours later, the rats were given an i.v. injection of 1.25 mg/g weight of aPS-PT mAb (n = 6) or rat IgM as controls (n = 5).

Three days later, all rats were killed for histopathological examination. We found thrombi in diverse organs, including the cerebrum, heart and liver, of the rats given the i.v. injection of aPS-PT mAb (Fig. 2A). On the contrary, only a few thrombi were observed in controls. We counted the number of organs with thrombosis in each rat. Thrombosis developed with higher frequency in the rats given the i.v.

Fig. 2 Thrombosis induced by i.v. injection of aPS-PT mAb



(**A**) Eight-week-old female Wistar rats (n = 11) were given an i.v. injection of calf thymus-derived histones (12.5 μg/g weight), and 2 h later, they were given an i.v. injection of 1.25 mg/g weight of aPS-PT mAb (n = 6) or rat IgM (n = 5). Three days later, all the rats were killed for histological examination. Thrombi were observed in diverse organs, including the cerebrum (**a** and **b**), heart (**c**) and liver (**d** and **e**), of the rats given the i.v. injection of aPS-PT mAb (bar = 100 μm). In controls, only a few thrombi were observed (**f**, connective tissue around the thymus, bar = 100 μm). (**B**) The number of organs with thrombosis in each rat was plotted. **$P < 0.01$. (**C**) Presence of rat IgM in thrombi induced by aPS-PT mAb. Sections from the rats given an i.v. injection of aPS-PT mAb, in which thrombi were present, were subjected to immunohistochemistry for rat IgM. Sections shown are as follows: the cerebrum (**a** and **b**) and heart (**c** and **d**) of the rats given an i.v. injection of aPS-PT mAb (**a** and **c**) or an i.v. injection of cell-free histones only (**b** and **d**). Results were reproduced in three independent experiments, and representative photomicrographs are shown. bar = 100 μm.

injection of aPS-PT monoclonal antibody than in controls (Fig. 2B).

Presence of rat IgM in thrombi induced by aPS-PT mAb

Immunohistochemistry revealed that rat IgM was involved in the formation of thrombi in the rats given the i.v. injection of aPS-PT mAb (Fig. 2C). The collective evidence suggests that aPS-PT can accelerate thrombogenesis through the exposure of cell-free histones *in vivo*.

Discussion

The roles of both vascular endothelial cells and haematopoietic cells, including platelets and monocytes, are not negligible in thrombogenesis, in addition to the factors of coagulation and fibrinolysis. However, studies to date on the pathogenicity of aPS-PTs have not fully paid attention to vascular endothelial cells. In the present study, we focused on the involvement of vascular endothelial cells in thrombogenesis related to aPS-PTs.

We have demonstrated that exposure to cell-free histones could induce phosphatidylserine on the cell surface of vascular endothelial cells. Although the mechanism of aPS-PT production has not been unrevealed, this is the initial step of aPS-PT binding to the cells. Cell-free histones have been shown to bind to and penetrate through the plasma membrane of some cell types and induce the formation of pores that alter the permeability of the plasma membrane [11]. The most important cells that donate cell-free histones *in vivo* are neutrophils. Activated neutrophils form extracellular DNA traps called neutrophil extracellular traps (NETs) [12] and release histones. Histones derived from NET-forming neutrophils have been recognized as important activators of the coagulation cascade, as well as integral components of thrombi [13, 14].

The possibility that the aPS-PT mAb could bind to phosphatidylserine directly *in vivo* should be considered. However, the possibility is unlikely because we have demonstrated that the aPS-PT mAb does not bind to phosphatidylserine directly in the enzyme-linked immunosorbent assay [6].

Prothrombin in the serum has been shown to bind to phospholipids, such as phosphatidylserine [15]. Phosphatidylserine is not naturally expressed on the surface of viable cells but is translocated to the surface of the plasma membrane of apoptotic cells [4]. We previously suggested that prothrombin bound to apoptotic vascular endothelial cells could be immunogenic and could cause aPS-PT production [16, 17]. Moreover, recent studies have demonstrated that aPL from patients with APS could enhance NET formation by neutrophils [18]. Although further studies are needed, the collective findings suggest that a vicious cycle via aPS-PT and NETs is involved in the pathogenesis of APS. Not only an undetermined anti-PS/PT antibody-mediated mechanism, but also a cell-free histone-mediated mechanism may be implicated in the thrombogenesis in APS.

This is the first demonstration of *in vivo* thrombogenesis induced by aPS-PT mAb. The inducible thrombosis model established in this study could be a useful tool to investigate the pathogenesis of APS *in vivo*.

Funding: This study was supported by a grant for Research on Rare and Intractable Diseases, including intractable vasculitis, from the Ministry of Health, Labour and Welfare of Japan and a grant from the Japan Agency for Medical Research and Development (15ek0109121).

Disclosure statement: The authors have declared no conflicts of interest.

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