Anti-phosphatidylserine/prothrombin Complex Antibodies in Patients With Cutaneous Vasculitis: Possible Involvement in the Pathogenesis

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Abstract

Objective. It was previously demonstrated that cutaneous vasculitis, including IgA vasculitis and cutaneous arteritis (CA), is associated with the presence of IgM antibodies (Abs) against the phosphatidylserine/prothrombin complex (PS/PT). Recently, novel enzyme-linked immunosorbent assay kits for the detection of IgG and IgM anti-PS/PT (aPS/PT) Abs have become commercially available.

Methods. The prevalence of serum IgG and IgM aPS/PT Abs in both cutaneous and systemic vasculitis was determined using these kits. In addition, to examine whether aPS/PT Abs were involved in the pathogenesis of cutaneous vasculitis, inbred wild-type rats were intravenously administered with a rat IgM class aPS/PT monoclonal Ab established previously or with rat immunoglobulins as controls. To express PS on the surface of vascular endothelium, these rats were given a subcutaneous injection of cell-free histones (250 µg/ml, 300 µl/site) 2 hours in advance.

Results. Serum IgM aPS/PT Ab levels were elevated in patients with systemic vasculitis with skin involvement and CA compared to those in patients with systemic vasculitis without skin involvement and healthy controls. There was no significant difference in the serum levels of IgG aPS/PT Abs between the patients and healthy controls. Correspondingly, inbred wild-type rats intravenously administered with the aPS/PT monoclonal IgM Ab after appropriate priming—subcutaneous histone injection—developed cutaneous vasculitis. Some rats given rat IgM instead of the aPS/PT monoclonal Ab also developed cutaneous vasculitis, whereas vasculitis did not occur in rats given IgG or only priming by histones.

Conclusion. IgM aPS/PT Abs could be involved in the pathogenesis of cutaneous vasculitis.

Introduction

Phosphatidylserine (PS) is a regular constituent of the inner leaflet of the cell membrane, which are only exposed on the outside of the cell membrane during apoptosis or by damaged endothelial cells [1]. Some studies have shown that prothrombin (PT) binds specifically to the surface of apoptotic cells [2, 3]. Anti-prothrombin antibodies (aPT) belong to the family of anti-phospholipid antibodies (aPL). aPL are the serological markers of anti-phospholipid syndrome, a clinical entity characterized by vascular thrombosis and/or obstetric complications. We previously suggested that cutaneous vasculitis such as IgA vasculitis and cutaneous arteritis (CA) could be dependently associated with the presence of IgM antibodies (Abs) against phosphatidylserine/prothrombin complex (PS/PT) [4, 5]. We also proposed that cutaneous vasculitis might be related to the increased IgM aPS/PT Abs production as a common pathological background [6–8]. A new IgG and IgM QUANTA Lite™ aPS/PT screen enzyme-linked immunosorbent assay (ELISA) kit (INOVA Diagnostics, San Diego, USA) is commercially available and has become widely used over the last several years [9]. We determined the prevalence of serum IgG and IgM isotypes of aPS/PT Abs in both cutaneous and systemic vasculitis using QUANTA Lite™ aPS/PT (INOVA Diagnostics).

In addition, to examine whether aPS/PT Abs were involved in the pathogenesis of cutaneous vasculitis, inbred wild-type rats were intravenously administered with a rat IgM class aPS/PT monoclonal Ab established previously [10] or with rat immunoglobulins as controls. Then, the development of cutaneous vasculitis was determined by histopathological analyses. Because PS is expressed on the surface of apoptotic or damaged vascular endothelial cells, a specific treatment is required before the administration of aPS/PT Abs. Recent studies have demonstrated that cell-free histones induce apoptosis of vascular endothelial cells in mice [11] and rats [12]. In this study, a subcutaneous injection of cell-free histones was employed as priming to express PS on the surface of vascular endothelial cells.

Methods

Patients

We reviewed the records of vasculitis patients with cutaneous manifestations who underwent skin biopsies in our department between December 2018 and February 2020. All tissue specimens were obtained by skin biopsy, fixed in 10% formalin, step-sectioned and stained with hematoxylin and eosin (HE) and elastica van Gieson staining. A diagnosis of vasculitis in skin tissue specimens required the presence of necrotizing vasculitis, such as fibrinoid degeneration, nuclear dust, neutrophilic infiltration and erythrocyte extravasation in the dermis and subcutaneous fat tissue, and leukocytoclastic vasculitis, such as nuclear dust, neutrophilic infiltration and erythrocyte extravasation in the upper and mid-dermis. The patients were divided into two groups based on skin biopsy findings. Group 1 consisted of eight patients who had histopathological necrotizing and/or leukocytoclastic vasculitis in their skin biopsy specimens. Group 2 consisted of seven patients who were diagnosed as systemic vasculitis but had no evidence of histopathological leukocytoclastic and/or necrotizing vasculitis in their skin biopsy specimens. Ten healthy persons of comparable sex and age distributions were recruited as normal controls. Sera were collected at the same time as the skin biopsies. For sera preparation, blood was centrifuged at 1500 g for 15 minutes. Sera were subsequently aliquoted and stored frozen at -80°C until use.

ELISA for aPT/PT Abs

aPS/PT Ab levels in serum samples were tested using IgG and IgM QUANTA Lite™ aPS/PT (INOVA Diagnostics). Briefly, the serum samples diluted to 1:101, and then added to 96-well plates coated with PS/PT where they were incubated for overnight at 4°C . Add 400 μl of 1:40 diluted horseradish peroxidase (HRP) wash buffer plus to each well then discard all liquid. Repeat this step for a total of three washes. Add 100 μl of polyclonal goat anti-human IgG or IgM Abs labelled with HRP conjugate solutions to each well, and incubate for 1 hour then wash four times. Add 100 μl of 3, 3', 5, 5'-tetramethylbenzidine chromogen to each well and incubate in the dark for 45 minutes at room temperature. After 100 μl of HRP stop solution addition, measure the absorbance at 450 nm immediately. Each patient serum sample was run in triplicate. Standard calibrators were prepared as the instruction manual.

Rats

Inbred wild-type Wistar rats (4 weeks old) were purchased from Sankyo Laboratory (Sapporo, Japan) and maintained under specific pathogen-free conditions.

Experimental protocols

1. Rats ($n=4$) were given a subcutaneous injection of 0, 2.5, 25, and 250 $\mu\text{g/ml}$ cell-free histones (calf thymus-derived histones containing unfractionated whole histones; Sigma-Aldrich, St. Louis, MO, USA) on the back (300 $\mu\text{l/site}$). Two hours later, the skin was resected for immunostaining using a rat IgM class aPS/PT monoclonal Ab [10]. Formalin-fixed, paraffin-embedded skin tissues were cut into 4 μm sections, and the sections were allowed to react with 1 $\mu\text{g/ml}$ aPS/PT monoclonal Ab (rat IgM) followed by 2 $\mu\text{g/ml}$ Alexa Fluor 594-conjugated goat anti-rat IgM Ab (Abcam, Cambridge, UK). Thereafter, the sections were mounted using a solution containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA).
2. Rats ($n=19$) were given a subcutaneous injection of 250 $\mu\text{g/ml}$ cell-free histones and then divided into four groups: Group A with an intravenous administration of 1.25 $\mu\text{g/g}$ weight of the rat IgM class aPS/PT monoclonal Ab 2 hours after priming by histones ($n=5$; histone isc with aPS/PT iv), Group B with rat IgM (eBioscience, San Diego, CA, USA; $n=4$; histone isc with IgM iv), Group C with rat IgG (eBioscience; $n=5$; histone isc with IgG iv), and Group D without immunoglobulin administration ($n=5$; histone isc). One week later, all rats were euthanized for histopathological analyses.

Statistics

Statistical analysis was performed using the Mann-Whitney U test for the comparison of Ab levels, Fisher's exact probability test for the comparison of frequencies, and Bonferroni's test for multiple comparisons was also used. A p -value of less than 0.05 was considered to be statistically significant. All data are expressed as mean $\pm$ standard deviation.

Results

aPS/PT Abs in Patients

Patients in Group 1 comprised 5 females and 3 males with a mean age of 48.9 ± 18.70 years. They consisted of 3 patients with IgA vasculitis, 2 patients with eosinophilic granulomatosis with polyangiitis (EGPA), and 1 patient each with CA, microscopic polyangiitis (MPA), and granulomatosis with polyangiitis (GPA). Patients in Group 2 comprised 5 females and 2 males with a mean age of 67.71 ± 21.37 years. They consisted of 2 patients with MPA, 2 patients with EGPA, and 1 patient each with polyarteritis nodosa (PAN), GPA, and rheumatoid vasculitis. Healthy persons comprised 6 females and 4 males with a mean age of 58.5 ± 18.73 years. There was not a significantly higher presence of IgG aPS/PT Abs among Group 1, Group 2, and the normal controls (11.26 ± 1.78 U/ml vs. 10.75 ± 1.64 U/ml vs. 10.48 ± 0.65 U/ml; Fig. 1a). In contrast, the mean IgM aPS/PT Ab level in Group 1 was significantly higher than that in Group 2 (21.52 ± 5.64 U/ml vs. 12.85 ± 1.86 U/ml, $p < 0.01$; Fig. 1b). There was a significantly higher presence of IgM aPS/PT Abs in Group 1 compared to the normal controls (21.52 ± 5.64 U/ml vs. 9.74 ± 1.03 U/ml, $p <$

0.001). There was not a significantly higher presence of IgG aPS/PT Abs between Group 2 and the normal controls.

Binding of aPS/PT Ab with vascular endothelium

To bind aPS/PT Abs in the serum with vascular endothelium, endothelial cells have to express PS on the surface. In the preceding study, cell-free histones ($\geq 12.5 \mu\text{g/ml}$) induced apoptosis of cultured rat vascular endothelial cells [12]. To determine the concentration of histones necessary for the cell surface expression of PS on vascular endothelium in the skin, inbred wild-type rats were given a subcutaneous injection of histones at varied concentrations, and then the skin was subjected to immunostaining using an aPS/PT monoclonal Ab (Fig. 2a). Although the aPS/PT monoclonal Ab bound with the subcutaneous connective tissues of rats given a histone injection ($\geq 2.5 \mu\text{g/ml}$), the binding of aPS/PT monoclonal Ab with vascular endothelium was observed only when 250 $\mu\text{g/ml}$ histones were injected. These findings suggested that the subcutaneous injection of histones (250 $\mu\text{g/ml}$) induced PS expression on vascular endothelial cells and subsequent formation of the PS/PT complex with PT in the serum. Based on these findings, this condition was employed as priming for the following experiments.

Development of cutaneous vasculitis in rats given an intravenous administration of aPS/PT Ab

To determine the pathogenicity of aPS/PT Abs, inbred wild-type rats were intravenously administered with or without 1.25 $\mu\text{g/g}$ weight of a rat IgM class aPS/PT monoclonal Ab 2 hours after priming by histones. For controls, rat IgM and IgG were administered instead of the aPS/PT monoclonal Ab. The systemic organs, including the skin, were subjected to histopathological analyses. Four of five rats in Group A (histone isc with aPS/PT iv) developed cutaneous vasculitis, showing a prominent inflammatory cell infiltration in and around the small vessel walls (Fig. 2b). Some rats in Group B (histone isc with IgM iv) also developed cutaneous vasculitis, whereas no rat in Group C (histone isc with IgG iv) and Group D (histone isc) did. Vasculitis did not occur in the systemic organs other than the skin. The occurrence of vasculitis in Group A was significantly higher than in other groups (Table 1).

Table 1
Frequency of cutaneous vasculitis in rats

Group	Treatment	Vasculitis		<i>p</i> -value
		Presence	Absence	
A	Histone isc with aPS/PT iv	4	1	0.0067
B	Histone isc with IgM iv	2	2	n.s.
C	Histone isc with IgG iv	0	5	n.s.
D	Histone isc	0	5	n.s.
n.s., not significant				

Discussion

We assessed the IgG and IgM prevalence of aPS/PT Abs in patients with vasculitis using a novel commercial ELISA kit. Serum IgM aPS/PT Ab levels were elevated in patients with cutaneous vasculitis and systemic vasculitis, confirmed by the presence of histopathological vasculitis in their skin biopsies, compared to those in systemic vasculitis patients who showed no histopathological vasculitis in their skin biopsy specimens. While the need for routine testing for aPS/PT Abs is still under debate, we believe that there is overwhelming serological evidence that the Abs are strongly related to histopathological leukocytoclastic and/or necrotizing vasculitis. Based on these findings, we speculate that aPS/PT Abs, especially IgM aPS/PT Abs, will become widely recognized as an important factor when diagnosing cutaneous vasculitis.

These clinical findings were supported by the results of experiments using rats. aPS/PT Abs are possibly involved in the pathogenesis of cutaneous vasculitis, and IgMs might be meaningful. Because IgMs are present as pentamers in the plasma, they may be easily trapped on the vascular endothelium, where the endothelial cell membrane is damaged and splintery. IgM can strongly activate the complements [13], and monoclonal gammopathy is associated with high blood viscosity [14]. These properties of IgM can augment the pathogenicity of aPS/PT Abs. In contrast, the pathogenicity of aPS/PT IgG Abs could not be assessed in this study. However, all five aPS/PT monoclonal Abs established from a rat model of cutaneous vasculitis were IgM Abs [10]. This itself suggests the importance of IgM class aPS/PT Abs in the pathogenesis of cutaneous vasculitis.

CA is characteristically limited to the skin, and shows a chronic relapsing benign course [15–17]. In contrast, systemic polyarteritis nodosa (PAN), which has very high morbidity and mortality rates, is characterized by fulminant deterioration or rapid progression and is associated with intermittent acute exacerbations. It is often difficult to establish diagnosis because the histological findings in CA and PAN are identical. Several authors have described differing clinical presentations between patients with CA and PAN [18]. However, the distinction remains controversial. Our survey of Japanese dermatological vasculitis specialists on cases of CA demonstrated that it is important for dermatologists to detect the presence of vasculitis-related symptoms early in order to establish an accurate diagnosis and a timely treatment [19]. We suggest that patients presenting as IgM aPS/PT Abs positive could be originally diagnosed as having CA, and should be carefully followed to ensure this condition does not progress from CA to systemic PAN. The present study has some limitations, such as the retrospective design and the small patient sample, and consequently further studies are warranted in order to prospectively establish the role of aPS/PT Abs in vasculitis.

Conclusions

Serum IgM but not IgG aPS/PT Ab levels were elevated in patients with systemic vasculitis with skin involvement and CA compared to those in patients with systemic vasculitis without skin involvement and healthy controls. Correspondingly, inbred wild-type rats intravenously administered with the aPS/PT monoclonal IgM Ab after appropriate priming—subcutaneous histone injection—developed cutaneous vasculitis. Some rats given rat IgM instead of the aPS/PT monoclonal Ab also developed cutaneous

vasculitis, whereas vasculitis did not occur in rats given IgG or only priming by histones. These findings suggest that IgM aPS/PT Abs may be involved in the pathogenesis of cutaneous vasculitis.

Abbreviations

Abs: antibodies; aPL: anti-phospholipid antibodies; aPS/PT; anti-PS/PT; aPT: anti-prothrombin antibodies; CA: cutaneous arteritis; EGPA: eosinophilic granulomatosis with polyangiitis; ELISA: enzyme immunosorbent assay; GPA: granulomatosis with polyangiitis; HE: hematoxylin and eosin; HRP: horseradish peroxidase; MPA: microscopic polyangiitis; PAN: polyarteritis nodosa; PS: Phosphatidylserine; PS/PT: phosphatidylserine/prothrombin complex; PT: prothrombin.

Declarations

Ethical approval and consent to participate

The study was approved by the ethics committee of Tohoku Medical and Pharmaceutical University (number 2018-2-0714). Serum was separated by centrifugation from peripheral blood of patients after acquisition of a written informed consent. Experiments using rats were permitted by the Animal Experimentation Committee of Hokkaido University (permission no. 15-0034) and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University.

Consent for publication

Not applicable.

Availability of data and materials

The data sheets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

None.

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Authors' contributions

T.K. and A.I. designed the study. Y.T., T.K., Y.D., M.Y., Y.N., S.M., and A.I. performed the experiments. Y.T., T.K., Y.N., S.M., U.T. and A.I. analyzed the data. T.K. and A.I. wrote the manuscript.

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Figures

Figure 1

aPS/PT Abs in the sera IgG (a) and IgM (b) aPS/PT Abs were measured with a specific ELISA (INOVA Diagnostics, Inc, San Diego, USA) in serum samples taken from patients in normal controls (n=10), Group

1 (n=8), and Group 2 (n=7).

Figure 2

Cutaneous vasculitis induced in rats Inbred wild-type rats were given a subcutaneous injection (300 $\mu\text{l/site}$) of histones at varied concentrations (0, 2.5, 25, and 250 $\mu\text{g/ml}$), and then the skin was subjected to immunostaining with an aPS/PT monoclonal Ab (a). Representative photos are shown. Red, PS/PT; blue, DNA. Arrowhead indicates vascular endothelium that is bound with the aPS/PT monoclonal Ab. Bar,

200 μm . HE staining of skin tissues of Group A (histone isc with aPS/PT iv), Group B (histone isc with IgM iv), Group C (histone isc with IgG iv), and Group D (histone isc) (b). Representative photos are shown. Bar, 200 μm .