Relationship between lysosomal-associated membrane protein-2 and anti-phosphatidylserine/prothrombin complex antibody in the pathogenesis of cutaneous vasculitis

T. Kawakami[1], A. Kikuchi[2], C. Miyabe[1], T. Ikeda[1], S. Takeuchi[2], Y. Tamura[2], Y. Nishibata[2], S. Masuda[2], D. Nakazawa[3], U. Tomaru[4], A. Ishizu[2]

[1]Division of Dermatology, Tohoku Medical and Pharmaceutical University, Sendai; [2]Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido University, Sapporo; [3]Department of Rheumatology, Endocrinology and Nephrology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo; [4]Department of Pathology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan.

Tamihiro Kawakami, MD, PhD
Ayaka Kikuchi, MT
Chie Miyabe, MD, PhD
Takaharu Ikeda, MD, PhD
Sora Takeuchi, MD, PhD
Yuto Tamura, MT
Yuka Nishibata, MT
Sakiko Masuda, MT, PhD
Daigo Nakazawa, MD, PhD
Utano Tomaru, MD, PhD
Akihiro Ishizu, MD, PhD

Please address correspondence to:
Akihiro Ishizu,
Faculty of Health Sciences,
Hokkaido University,
Kita-12, Nishi-5, Kita-ku,
Sapporo 060-0812, Japan.
E-mail: aishizu@med.hokudai.ac.jp

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ABSTRACT

Objective. *We investigated the relationship between lysosomal-associated membrane protein-2 (LAMP-2) and anti-phosphatidylserine/prothrombin (PS/PT) antibody in the pathogenesis of cutaneous vasculitis.*

Methods. *Cell surface LAMP-2 expression of human neutrophils was measured using flow cytometry. Twenty inbred wild-type Wistar-King-Aptekman-Hokudai (WKAH) rats were divided into four groups: Group 1, rabbit IgG injection only as negative control (n=5); Group 2, both histone and rabbit IgG injection (n=5); Group 3, anti-LAMP-2 antibody injection only (n=5); and Group 4, both histone and anti-LAMP-2 antibody injection (n=5). Ten WKAH rats were divided into two groups: Group A, histone, anti-PS/PT antibody, and anti-LAMP-2 antibody injection (n=5), and Group B, histone, anti-PS/PT antibody, and rabbit IgG injection as control (n=5).*

Results. *LAMP-2 expression on human neutrophils was induced by cell-free histone exposure in a dose- and time-dependent manner. Histopathological examination revealed the recruitment of neutrophils in cutaneous small vessels in all Group 4 rats. These observations were not evident in systemic organs other than the skin. LAMP-2 expression on the surface of vascular endothelial cells was evident in Group 2, exclusively in the skin, but not in Group 1. Thrombi were detected in various organs in all Groups A and B rats. However, no apparent thrombi were observed in the skin.*

Conclusion. *Anti-PS/PT and anti-LAMP-2 antibodies are responsible for independent effector mechanisms in the rats given intravenous injection of cell-free histones. We considered that undetermined factors other than cell-free histones could be required for the induction of cutaneous vasculitis by anti-PS/PT and anti-LAMP-2 antibodies.*

Introduction

Lysosomal-associated membrane protein-2 (LAMP-2) is a highly glycosylated protein and an abundant constituent of the lysosomal membrane involved in lysosomal biogenesis and phagocytosis (1-5). LAMP-2 is critical for autophagy and presentation of intracellular antigens (6). Recent research has suggested that LAMP-2 could be one of the target antigens in the pathogenesis of vasculitides. We previously reported that LAMP-2 could play some role in the pathogenesis of cutaneous vasculitis (7). This corresponds to the recent report showing that serum LAMP-2 levels are increased in small and medium-vessel vasculitis (8). In addition, we found that injected anti-LAMP-2 antibody in premorbid cutaneous vasculitis model rats led to the recruitment of neutrophils in cutaneous small vessels (9).

We generated a rat monoclonal antibody that could recognise rat phosphatidylserine/prothrombin (PS/PT) complex (10, 11). We previously reported that small-vessel cutaneous vasculitis could be dependently associated with the presence of anti-PS/PT antibodies (12, 13).

In the present study, we investigated the relationship between LAMP-2 and anti-PS/PT antibody in the pathogenesis of cutaneous vasculitis. As both LAMP-2 and PS are intracellular molecules in principle, a certain priming is needed if anti-LAMP-2 and anti-PS/PT antibodies bind to their antigens in vivo. For this purpose, we employed histones that have been shown to induce apoptosis of vascular endothelial cells *in viv*o (14). Histones have been regarded as important endogenous cytotoxic mediators released from immune cells, including

neutrophils, under inflammatory conditions (15).

Methods

Rats

Inbred wild-type Wistar-King-Aptekman-Hokudai (WKAH) rats were maintained at the Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine. These rats are seronegative for autoantibodies, including anti-neutrophil cytoplasmic antibody. Experiments using rats were done in accordance with the Guidelines for the Care and Use of Laboratory Animals in Hokkaido University (Permission no. 15-0034).

Reagents

Calf thymus-derived histones that contained unfractionated whole histones were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-human LAMP-2 polyclonal antibody (rabbit IgG) that can react with rat and mice LAMP-2 was purchased from abcam (Cambridge, UK). Anti-PS/PT monoclonal antibody against rat PS/PT complex was generated using the conventional hybridoma method in our laboratory (10). Rabbit IgG (eBioscience, San Diego, CA, USA) and rat IgM (Invitrogen, Tokyo, Japan) were employed as controls for anti-LAMP-2 and anti-PS/PT antibodies, respectively.

Cell surface LAMP-2 expression of human neutrophils

Blood samples were obtained from heathy persons after acquisition of written informed consent. The use of human samples was permitted by the Ethics Committee of the Faculty of Health Sciences, Hokkaido University (Permission No. 18-34). After enrichment of polymorphonuclear cells using Polyporphprep (Axis-Shield, Dundee, Scotland), the samples were re-suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum. These samples were exposed to calf thymus-derived histones (0, 12.5, 25, 50, and 100 μg/ml) for 2 h at 37°C. Alternatively, the samples were exposed to 25 μg/ml calf thymus-derived histones for 0, 1, 2, and 4 h at 37°C. After washing with phosphate-buffered saline, the samples were

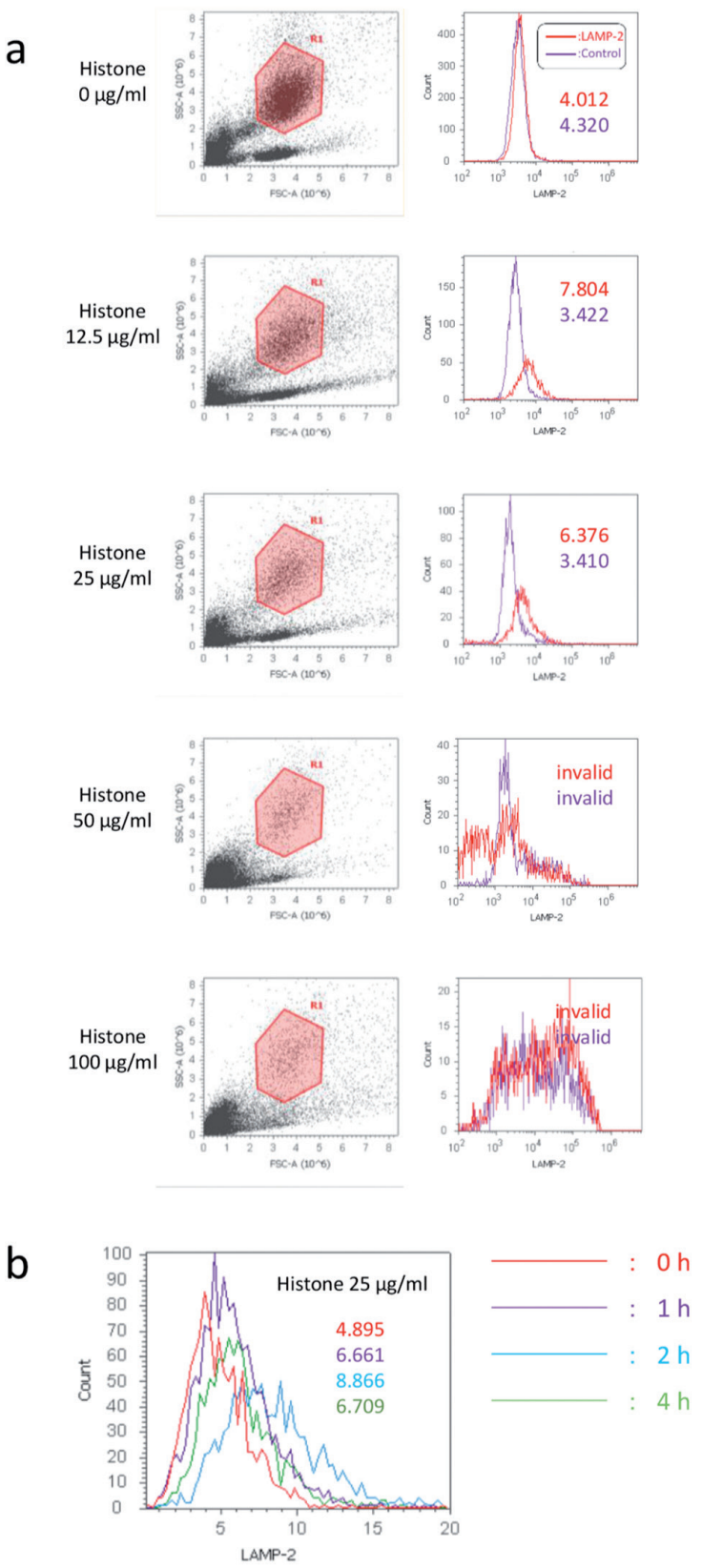

Fig. 1. Cell surface LAMP-2 expression of human neutrophils induced by histone exposure. (**a**) LAMP-2 expression on the cell surface of neutrophils was induced by histones in a dose-dependent manner up to 25 μg/ml. At more than 50 μg/ml, the neutrophil count declined. (**b**) When neutrophils were exposed to 25 μg/ml histones for 0, 1, 2, or 4 h, LAMP-2 expression on the cell surface of neutrophils was increased time-dependently up to 2 h. The numbers in the histogram panels represent the mean fluorescent intensity of each histogram.

reacted with anti-LAMP-2 antibody followed by Alexa 488-conjugated anti-rabbit IgG antibody (Invitrogen). These samples were subjected to flow cytometry (FCM; Attune® Acoustic Focusing Cytometer; Applied Biosystems, Foster City, CA). Neutrophils were gated according to the characteristic forward and side scatter profiles, and then histograms of LAMP-2 expression were displayed.

Injection of cell-free histones and anti-LAMP-2 antibody into rats

WKAH rats (4–5 weeks old) were given a single injection of calf thymus-derived histones (10 μg/g weight) on day 0 with two times injection of anti-LAMP-2 antibody (1 μg/g weight) on days 0 and 7 via the tail vein. Dosing of histones and anti-LAMP-2 antibody was determined according to our previous studies (9, 11). Twenty rats were divided into four groups: Group 1, rabbit IgG injection only as negative control (n=5); Group 2, both histone and rabbit IgG injection (n=5); Group 3, anti-LAMP-2 antibody injection only (n=5); and Group 4, both histone and anti-LAMP-2 antibody injection (n=5). On day 14, histopathological examination was conducted on the rats.

LAMP-2 expression on cutaneous vascular endothelial cells after intravenous injection of cell-free histones

Formalin-fixed paraffin-embedded tissue sections of the skin, brain, and heart of Groups 1 and 2 rats were subjected to immunofluorescent staining for LAMP-2 using Alexa 594-conjugated anti-rabbit IgG antibodies (Invitrogen) as a secondary antibody.

Injection of cell-free histones, anti-PS/PT antibody, and anti-LAMP-2 antibody into rats

WKAH rats (4-5 weeks old) were given intravenous injection of calf thymus-derived histones (10 μg/g weight). Two hours later, intravenous injection of anti-PS/PT antibody (1 μg/g weight) with or without anti-LAMP-2 antibody (1 μg/g weight) was added. Dosing of histones and antibodies was determined according to our previous studies (9, 11). Ten rats were divided into two groups: Group A, histone, anti-PS/PT antibody, and anti-LAMP-2 antibody injection (n=5), and Group B: histone, anti-PS/PT antibody, and rabbit IgG injection as control (n=5). Three days later, all rats were killed for histopathological examination.

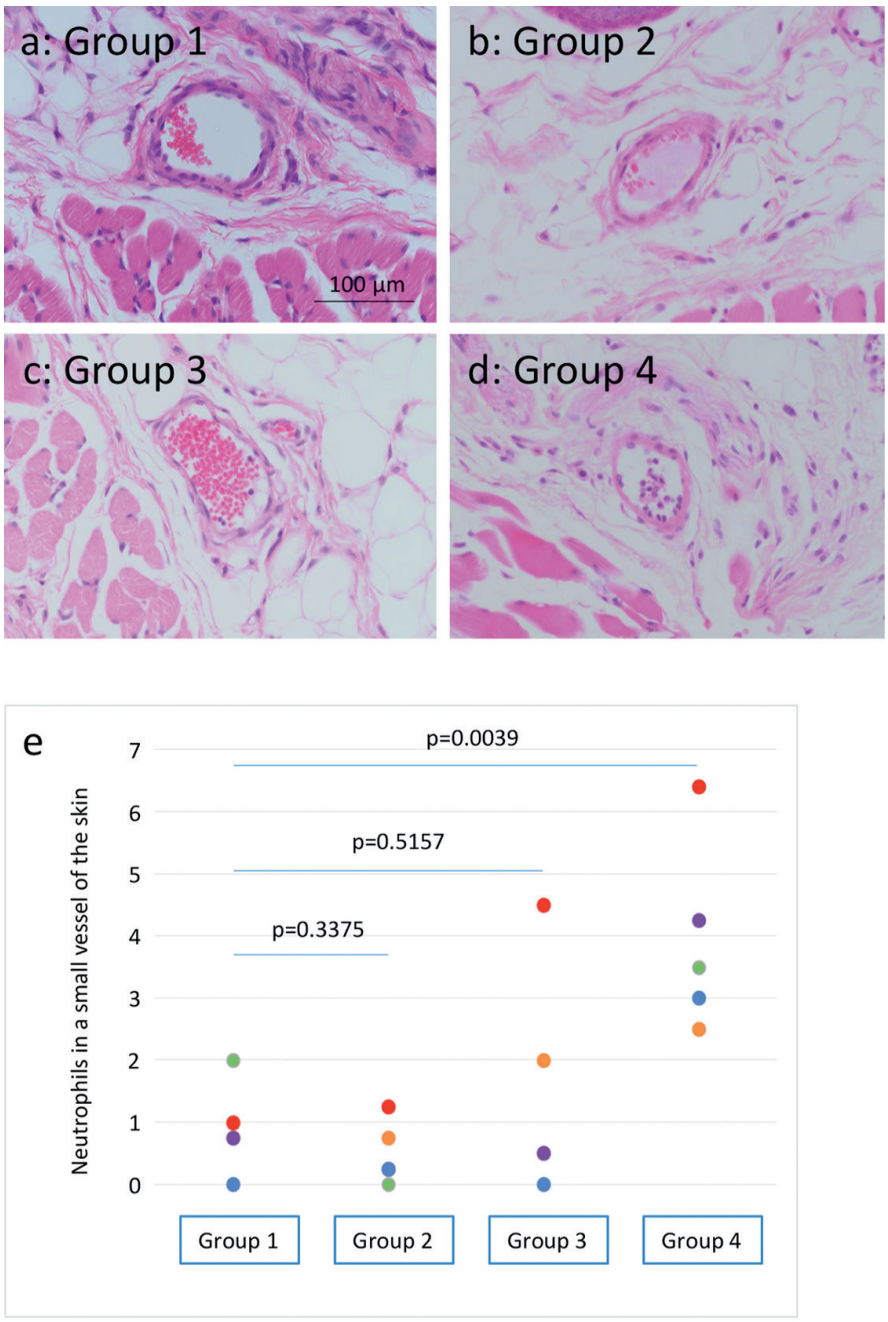

Fig. 2. Recruitment of neutrophils in cutaneous vessels induced by histone exposure and subsequent anti-LAMP-2 antibody injection.
Histopathological examination revealed the recruitment of neutrophils in cutaneous vessels in all Group 4 rats. (**a-d**) Representative microphotographs of skin specimens. (**e**) Number of neutrophils in cutaneous vessels.

Statistical analyses

Student's t-test and Mann-Whitney U-test were applied for comparison of the two *in vitro* and *in vivo* groups, respectively. $p<0.05$ was regarded as statistically significant.

Results

Cell surface LAMP-2 expression of human neutrophils induced by histone exposure

To verify the cell surface LAMP-2 ex-

pression of neutrophils induced by histones, human blood samples were exposed to 0, 12.5, 25, 50, or 100 μg/ml calf thymus-derived histones for 2 h. LAMP-2 expression on the cell surface of neutrophils was increased by histone exposure dose-dependently up to 25 μg/ml (Fig. 1a). At histone exposure of more than 50 μg/ml, the neutrophil count declined. When neutrophils were exposed to 25 μg/ml histones for 0, 1, 2, or 4 h, LAMP-2 expression on the cell surface of neutrophils increased time-dependently up to 2 h (Fig. 1b). After more than 4 h, the neutrophil count declined.

Injection of histones and anti-LAMP-2 antibody recruited neutrophils in cutaneous vessels

Histopathological examination revealed the recruitment of neutrophils in cutaneous small vessels in all Group 4 rats (Fig. 2a-d). These observations were not evident in systemic organs other than the skin. We counted the number of neutrophils in cutaneous small vessels in each group (Fig. 2e). The number in Group 4 was significantly higher compared to that in Group 1 (p=0.0039). The number in Group 3 was higher compared to that in Group 1 but was not statistically significant.

To confirm LAMP-2 expression on the cell surface of vascular endothelial cells, immunofluorescent staining for LAMP-2 was performed on the tissue sections of the skin, brain, and heart of Groups 1 and 2 rats. Results showed that LAMP-2 expression on the cell surface of vascular endothelial cells was evident in Group 2, exclusively in the skin, but not in Group 1 (Fig. 3a-d).

Injection of histones, anti-PS/PT antibody, and anti-LAMP-2 antibody into rats

Haematoxylin and eosin staining was administered to sections of the cerebrum, cerebellum, thymus, salivary glands, eyes, heart, lungs, liver, pancreas, kidneys, intestine, and skin of Groups A and B rats. We detected thrombi in various organs in all rats from both groups. However, no apparent thrombi were observed in the skin. We counted the number of vessels where thrombi were

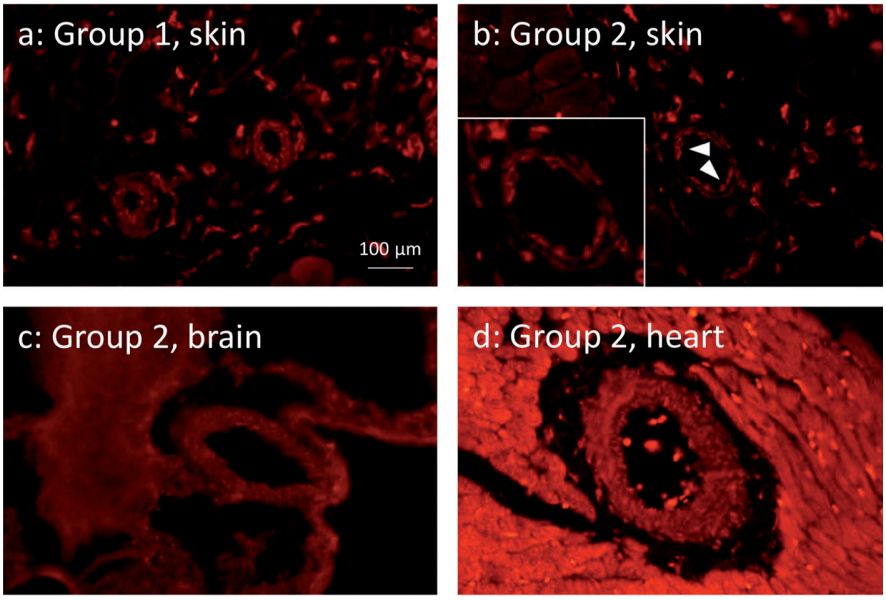

Fig. 3. Immunofluorescent staining for LAMP-2 of tissue sections of Groups 1 and 2 rats. LAMP-2 expression on the cell surface of vascular endothelial cells was evident in the skin of Group 2 (**b**, arrowheads) but not in the skin of Group 1 (**a**) or the brain and heart of Group 2 (**c** and **d**). Insert in (b): high power field of the vessel.

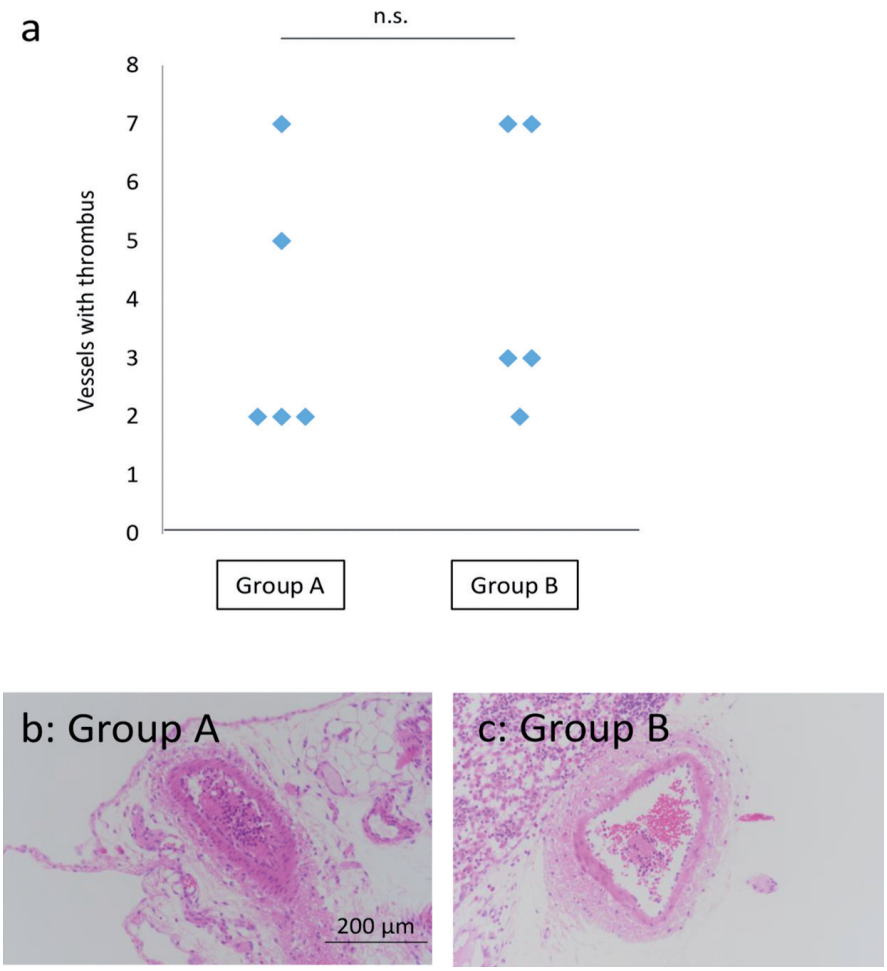

Fig. 4. Thrombus formation in Groups A and B rats. (**a**) Total number of vessels with thrombi in Groups A and B rats. (**b** and **c**) Haematoxylin and eosin staining in pancreatic sections. Thrombi were present in Group A (b) and Group B (c).

present and noted that there were no significant differences between Groups A and B (Fig. 4a-c). In contrast, we found recruitment of neutrophils in cutaneous small vessels in all Group A rats. However, the finding was not evident in any of the Group B rats.

Discussion

Histopathological examination revealed no significant recruitment of neutrophils in cutaneous vessels in Group 3, anti-LAMP-2 antibody injected alone. FCM demonstrated that LAMP-2 expression on the cell surface of neutrophils was induced by cell-free histone exposure in a dose- and time-dependent manner.

A considerable number of neutrophils in cutaneous vessels in Group 4, which were not apparent in the other groups, were observed when we injected cell-free histones before injection of anti-LAMP-2 antibody into rats. In addition, immunofluorescent staining showed that LAMP-2 expression on the cell surface of cutaneous vascular endothelial cells was induced by histone injection into Group 2 rats. 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 (16). Willcocks *et al*. (17) demonstrated cell surface expression of LAMP-2 on vascular endothelial cells and suggested that anti-LAMP-2 antibody could bridge LAMP-2 on vascular endothelial cells and neutrophils via Fcγ receptors. We proposed that the introduction of histones could move LAMP-2 to the cell surface of neutrophils and that anti-LAMP-2 antibody could bridge vascular endothelial cells and neutrophils through antigen-specific binding in cutaneous vessels. Based on the present results, this appears to be a skin-specific phenomenon, although we cannot currently explain the basis for this result.

We found thrombi in diverse organs, including the cerebrum, heart, and liver, but not in the skin of rats given intravenous injection of cell-free histones followed by anti-PS/PT monoclonal antibody, regardless of additional anti-LAMP-2 antibody injection, as seen in Groups A and B. We proposed that anti-PS/PT and anti-LAMP-2 antibodies are responsible for independent effector mechanisms in the rats given intravenous injection of cell-free histones. However, we have reported that both anti-LAMP-2 and anti-PS/PT antibodies could play some role in the common pathogenesis of cutaneous vasculitis in humans (13). We considered that undetermined factors other than cell-free histones could be required for the induction of cutaneous vasculitis by anti-PS/PT and anti-LAMP-2 antibodies.

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