Lysosomal-associated membrane protein-2 plays an important role in the pathogenesis of primary cutaneous vasculitis

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Abstract

Objectives. Recent research suggests that lysosomal-associated membrane protein-2 (LAMP-2) could be one of the target antigens in the pathogenesis of vasculitides. We established a transgenic rat model, env-pX rats, with various vasculitides including cutaneous vasculitis. Human primary cutaneous vasculitis includes cutaneous polyarteritis nodosa (CPN) and Henoch–Schönlein purpura (HSP). We measured serum anti-LAMP-2 antibody levels in morbid env-pX rats and injected anti-LAMP-2 antibody into premorbid env-pX rats. We further measured serum anti-LAMP-2 antibody levels in patients with CPN and HSP.

Methods. Cutaneous vasculitis was observed in 30% of 6-month-old morbid env-pX rats. In contrast, these findings were rare in premorbid env-pX rats under 3 months old. We also examined 85 patients with CPN and 36 adult patients with HSP. Serum anti-LAMP-2 antibody levels were determined using ELISA. Premorbid env-pX rats under 3 months old were given an i.v. injection of anti-LAMP-2 antibody at day 0 and day 7. At day 14, these rats underwent histopathological and direct immunofluorescence examination. Cell surface LAMP-2 expression of rat neutrophils was examined by flow cytometry.

Results. Serum anti-LAMP-2 antibody levels were significantly higher in morbid env-pX rats than in wild-type normal rats. In addition, the levels in the cutaneous vasculitis group of morbid env-pX rats were significantly higher than the no cutaneous vasculitis group. Intravenous anti-LAMP-2 antibody injection into premorbid env-pX rats under 3 months old induced infiltration of neutrophils into cutaneous small vessels. Anti-LAMP-2 antibody-binding neutrophils were detected there. LAMP-2 expression on the cell surface of neutrophils in premorbid env-pX rats under PMA stimulation was higher compared with controls. Serum anti-LAMP-2 antibody levels in CPN and HSP were significantly higher than those of healthy controls.

Conclusion. These data support a positive relationship between anti-LAMP-2 antibody and cutaneous vasculitis.

Key words: cutaneous vasculitis, lysosomal-associated membrane protein-2, cutaneous polyarteritis nodosa, Henoch–Schönlein purpura.

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 suggests that LAMP-2 could be one of the target antigens in the pathogenesis of vasculitides.

We established a transgenic rat model, the env-pX rat, which carries the env-pX gene of human T lymphocyte...
virus type I (HTLV-I). The env-pX gene was introduced into the germline of Wistar-King-Aptekman-Hokudai (WKAH) rats under the control of the HTLV-I long terminal repeat promoter [7–9]. The env-pX transgene is expressed constitutively in the systemic organs of env-pX rats. We have previously reported that various types of vasculitis, such as necrotizing arteritis of medium-sized arteries and small vessel vasculitis without fibrinoid necrosis, occurred in env-pX rats [7]. They were negative for MPO-ANCA and PR3-ANCA. Necrotizing arteritis frequently occurred in the thymus, salivary glands, testes, pancreas and systemic connective tissues. In contrast, small vessel vasculitis without fibrinoid necrosis mainly occurred in the skin. Previous studies revealed that small vessel vasculitis in the skin but not systemic necrotizing arteritis could be transferred by env-pX bone marrow cells into wild-type WKAH rats [8, 9]. These findings suggested that diverse vasculitides were involved in env-pX rats. In this study, we focused on cutaneous small vessel vasculitis in env-pX rats.

Human primary cutaneous vasculitis includes cutaneous polyarteritis nodosa (CPN) and Henoch–Schoenlein purpura (HSP). CPN is a necrotizing vasculitis of small to medium-sized arteries within the skin [10, 11]. A diagnosis of CPN requires the presence of histopathological necrotizing vasculitis in the lower dermis and subcutaneous fat. HSP is characterized by non-thrombocytopenic palpable purpura over the lower extremities and IgA-containing immune complexes within the leucocytoclastic vasculitis [12, 13]. Histologically, leucocytoclastic vasculitis is seen in the upper to mid-dermis. We previously reported that LAMP-2 could play some role in the pathogenesis of adult HSP and CPN [14, 15].

In the present study, we measured serum anti-LAMP-2 antibody levels in morbid env-pX rats and injected anti-LAMP-2 antibody into premorbid env-pX rats to investigate whether anti-LAMP-2 antibody leads to the development of cutaneous vasculitis. We further measured serum anti-LAMP-2 antibody levels in patients with CPN and HSP.

Materials and methods

Rats

Inbred WKAH rats and WKAH rats bearing the env-pX gene of HTLV-I (env-pX rats) were maintained at the Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine. Experiments using rats were done in accordance with the guidelines for the care and use of laboratory animals in Hokkaido University.

Patients

Eighty-five patients (27 men and 58 women; 44.4 ± 16.9 years) with CPN and 36 adult patients (12 men and 24 women; 46.1 ± 19.7 years) with HSP seen at the Department of Dermatology, St Marianna University School of Medicine, were included in the study. A diagnosis of CPN requires the presence of histological necrotizing vasculitis, such as fibrinoid degeneration, nuclear dust, neutrophilic infiltration and erythrocyte extravasation in the lower dermis and subcutaneous fat. No evidence of vasculitis was revealed in other organs including kidneys, lungs and salivary glands. The following tests were negative or within the normal range: ANAs and virus serology including hepatitis A, B and C. These patients were diagnosed according to the Japanese criteria [11] and the KAWAKAMI algorithm [16]. The HSP patients had clinically palpable purpura over the lower extremities, and histopathology revealed leucocytoclastic vasculitis with vascular deposition of IgA-dominant immune complexes based on DIF staining according to standard procedures. The patients were diagnosed according to the criteria defined by the ACR [17] and Chapel Hill Consensus Conference (CHCC) definitions [18]. These patients were compared with 51 age- and gender-matched healthy controls. This study was approved by the St Marianna University ethics committee, and informed consent was obtained from all patients.

Sera and skin samples

Serum samples from both the rats and patients were collected and immediately centrifuged at 1500g for 30 min at 4 °C. Serum samples were stored at –80 °C until analysis. Serum was obtained from each patient when the disease was active and the patient was not on prednisolone or immunosuppressive therapy. Skin tissue specimens were fixed in 10% formalin, step-sectioned and stained with haematoxylin–eosin.

Immunohistochemistry for rat LAMP-2

Immunohistochemistry (IHC) for rat LAMP-2 was performed using anti-LAMP-2 antibody diluted at 1:100 (T-19; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Detection of serum anti-LAMP-2 antibody levels in rats and humans

Serum anti-LAMP-2 antibody levels were determined using an ELISA kit (Nipro, Kusatsu, Shiga, Japan). Microtitre plates (Nunc Immunoplate, Roskilde, Denmark) were coated with 100 μl of LAMP-2 antigen (PEP-039; Thermo Scientific, Waltham, MA, USA) in coating buffer overnight at 4 °C. The antigen was applied at 4.0 μg/ml per well. The plates were then washed three times and kept at room temperature for 3 h. A total of 100 μl of rat or human serum, diluted 1:50 in phosphate-buffered saline (PBS), was added to each well. The plates were incubated at 25°C for 1 h; after washing, anti-rat IgG-alkaline phosphatase antibody produced in rabbit (Sigma, St Louis, MO, USA) diluted 1:5000 for rat samples or anti-human IgG-alkaline phosphatase antibody produced in goat (Sigma) diluted 1:10000 for human samples was added. Incubation resumed for 1 h at 25°C. 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma) in substrate buffer was used as a substrate, and colour development was measured spectrophotometrically at 405 nm.
Injection of anti-LAMP-2 antibody into rats

Premorbid env-pX rats under 3 months old were given an i.v. injection of 200 µg/ml of anti-LAMP-2 antibody (T-19; Santa Cruz Biotechnology) at day 0 and day 7. At day 14, histopathological examination was performed on the rats. As a control, premorbid env-pX rats under 3 months old were given an i.v. injection of 200 µg/ml of goat IgG (CS120172A; Cell Sciences Inc., Canton, MA, USA) similarly.

DIF staining

Skin tissues were obtained from premorbid env-pX rats that had been given the anti-LAMP-2 antibody. Formalin-fixed paraffin-embedded skin sections were subjected to DIF staining, using FITC-conjugated donkey anti-goat IgG antibodies (A11055; Invitrogen, Carlsbad, CA, USA), 4',6-diamidino-2-phenylindole (F6057; Sigma-Aldrich, St Louis, MO, USA) was used in mounting solution for nuclear staining.

Cell surface LAMP-2 expression of rat neutrophils

Blood samples were obtained from premorbid env-pX rats and WKAH healthy rats. After removal of erythrocytes by treatment with ammonium chloride, residual samples were resuspended in PBS. These samples were reacted with unlabelled anti-LAMP-2 antibody (goat IgG; Santa Cruz Biotechnology) and PE-labelled anti-CD11b antibody (BD Biosciences, Franklin Lakes, NJ, USA), with or without stimulation by phorbol-12-myristate-13-acetate (PMA, 20 or 200 nM for 2 h at 37°C), for 30 min on ice. After removal of unbound antibodies, the samples were next allowed to react with FITC-conjugated donkey anti-goat IgG antibodies (Invitrogen) for 30 min on ice. These samples were subjected to flow cytometry (FCM). Neutrophils were gated as CD11b+ cells, and then histograms of LAMP-2 expression were displayed. As a control for the anti-LAMP-2 antibody, goat IgG was employed.

Statistical analyses

Differences between quantitative parameters between groups were assessed using the Mann-Whitney U test. To investigate the cut-off value of serum anti-LAMP-2 antibody, a receiver operating characteristic (ROC) curve was constructed using statistical analysis software (JMP 8.0.2). All data are expressed as mean ± S.D. All analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered to be statistically significant.

Results

Cutaneous vasculitis in env-pX rats

Cutaneous vasculitis was observed in ~30% of the 6-month-old morbid env-pX rats. Histopathological analysis revealed small vessel vasculitis with cellular infiltrates containing neutrophils in the dermis (Fig. 1A and B). These findings were typically not seen in premorbid env-pX rats under 3 months old. IHC demonstrated that neutrophils infiltrating the dermal small vessels expressed LAMP-2 (Fig. 1C).

Serum anti-LAMP-2 antibody levels in env-pX rats

We determined serum anti-LAMP-2 antibody levels in eleven 6-month-old morbid env-pX rats and 25 WKAH wild-type normal rats. Serum anti-LAMP-2 antibody
levels differed significantly between the env-pX rats (0.527 U/ml, median 0.459, quartile 0.385) and WKAH wild-type rats (0.268 U/ml, median 0.244, quartile 0.055) ($P < 0.0001$; Fig. 2A). ROC analysis was performed to obtain the area under the curve (AUC) and optimal cut-off point of serum anti-LAMP-2 antibody for env-pX rats. The AUC of serum anti-LAMP-2 antibody was 0.920. The optimal cut-off point (sensitivity; specificity) of serum anti-LAMP2 antibody was 0.308 U/ml (1.000; 0.760).

These rats were categorized into two groups: a cutaneous vasculitis group and a no cutaneous vasculitis group. Serum anti-LAMP-2 antibody levels in the cutaneous vasculitis group (0.654 U/ml, median 0.731, quartile 0.209) were significantly higher than those of the no cutaneous vasculitis group (0.376 U/ml, median 0.358, quartile 0.083) ($P = 0.045$; Fig. 2B).

Neutrophil infiltration into cutaneous small vessels induced by anti-LAMP-2 antibody

Anti-LAMP-2 antibody was intravenously injected into premorbid env-pX rats under 3 months old. Histopathological examination revealed infiltration of neutrophils into cutaneous small vessels in all rats examined (Fig. 3). These observations were not evident in systemic organs other than skin. Conversely, these findings were not seen in age-matched env-pX rats with i.v. injection of control IgG.

**Fig. 2** Serum anti-LAMP-2 antibody levels in env-pX rats and WKAH normal rats.

Serum anti-LAMP-2 antibody levels in 11 env-pX rats were significantly higher than those of 25 WKAH wild-type rats (A). Significant differences in serum anti-LAMP-2 antibody levels observed between the presence and absence of cutaneous vasculitis (B). **$P < 0.01$, *$P < 0.05$.**

Infiltration of anti-LAMP-2 antibody-binding neutrophils into the small vessels

In order to determine whether the anti-LAMP-2 antibody-binding neutrophils infiltrated the skin, DIF staining for goat IgG was conducted on skin specimens obtained from the premorbid env-pX rats that had been given anti-LAMP-2 antibody. DIF staining revealed the presence of anti-LAMP-2 antibody-binding neutrophils within the small vessels.

**Fig. 3** Neutrophil infiltration into cutaneous small vessels induced by anti-LAMP-2 antibody.

Infiltration of neutrophils into the cutaneous small vessels was observed after i.v. injection of anti-LAMP-2 antibody into premorbid env-pX rats (haematoxylin–eosin stain; original magnification $\times400$; A). Neutrophil infiltration was seen in and around the small vessels of the dermis (haematoxylin–eosin stain; original magnification $\times400$; B). Arrows indicate infiltrated neutrophils.
of IgG-positive neutrophils in cutaneous small vessels (Fig. 4). Because the anti-LAMP-2 antibody belongs to goat IgG, these finding showed that anti-LAMP-2 antibody-binding neutrophils infiltrated cutaneous small vessels in rats injected with anti-LAMP-2 antibody.

Cell surface LAMP-2 expression of neutrophils in premorbid env-pX rats

In order to verify the cell surface LAMP-2 expression of neutrophils in premorbid env-pX rats, blood samples were subjected to FCM. LAMP-2 expression on the cell surface of neutrophils was increased by PMA dose dependently (Fig. 5). Expression levels were higher in premorbid env-pX rats than normal WKAH rats at any PMA dose.

Serum anti-LAMP-2 antibody levels in CPN patients and HSP patients

Serum anti-LAMP-2 antibody levels differed significantly between the 85 patients with CPN (0.270 U/ml, median 0.236, quartile 0.104), the 36 patients with adult HSP (0.284 U/ml, median 0.251, quartile 0.0938) and the 51 healthy controls (0.233 U/ml, median 0.222, quartile 0.121; Fig. 6). Serum anti-LAMP-2 antibody levels in CPN were significantly higher than those of the healthy controls ($P=0.0353$). Serum anti-LAMP-2 antibody levels in HSP were also significantly higher than those of the healthy controls ($P=0.0308$).

Discussion

In the present study, we examined serum anti-LAMP-2 antibody levels in model rats and humans with cutaneous vasculitis and injected anti-LAMP-2 antibody into the model rats. Serum anti-LAMP-2 antibody levels were significantly higher in morbid env-pX rats compared with WKAH healthy rats based on ELISA. Furthermore, we found that the presence of cutaneous vasculitis in these env-pX rats was closely related to the elevated anti-LAMP-2 antibody levels. Histopathological findings of skin specimens obtained from premorbid env-pX rats injected with anti-LAMP-2 antibody revealed infiltration of neutrophils into cutaneous small vessels. DIF studies further demonstrated binding of the injected anti-LAMP-2 antibody in these neutrophils. We demonstrated that cell surface LAMP-2 expression of neutrophils was higher in premorbid env-pX rats than in wild-type rats. Based on these findings, we considered that anti-LAMP-2 antibody could bind neutrophils, and then the anti-LAMP-2 antibody-binding neutrophils could infiltrate the cutaneous small vessels in env-pX rats. Willcocks et al. [19] showed 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 Fc$\gamma$ receptors. These mechanisms and subsequent abundant neutrophil degranulation could initiate cutaneous vasculitis in env-pX rats.

Although original env-pX rats develop systemic vasculitis, the possible association of anti-LAMP-2 antibody seems to be limited to cutaneous vasculitis. This may be explained by the particularity of cutaneous vasculitis. In our previous work on bone marrow engraftment of env-pX rats, cutaneous vasculitis was exclusively transferred to wild-type rats by bone marrow cells from env-pX rats [8, 9]. Thus, we suggest that anti-LAMP-2 antibody might be closely related to the pathogenesis of cutaneous vasculitis but not systemic vasculitis in env-pX rats.

Human primary cutaneous vasculitis also differs from systemic vasculitis. Patients are of two types: those who present with medium-sized vessel vasculitis such as CPN and those with small vessel vasculitis such as HSP, based on cutaneous biopsy findings. CPN is defined as an exclusive clinical cutaneous vasculitic entity with benign and

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**Fig. 4** Infiltration of anti-LAMP-2 antibody-binding neutrophils into cutaneous small vessels.

A. Skin specimens from premorbid env-pX rats intravenously injected with anti-LAMP-2 antibody (haematoxylin–eosin stain; original magnification ×400; A). Serial sections were subjected to DIF staining for goat IgG (yellow; goat IgG; blue: nuclei; B). Goat-IgG positive neutrophils (anti-LAMP-2 antibody-binding neutrophils) were seen in the small vessels (arrows; B).
chronic course without systemic involvement. Small-vessel vasculitides can be divided into pauci-immune vasculitides associated with ANCAs and immune deposit-associated vasculitides. HSP is representative of the immune deposit-associated small-vessel vasculitides and all patients have cutaneous vasculitis. In contrast, ANCA-associated vasculitis mainly involves the kidneys and lungs, although the skin is sometimes involved as a systemic manifestation of the disease. Previous studies have reported no evidence of MPO-ANCA in serum samples from HSP and CPN patients by direct ELISA or by capture ELISA [14, 15]. Roth et al. [20] suggested that anti-LAMP-2 antibody might exist at very low titres in a minority of patients with ANCA-associated systemic vasculitis.

In the present study, we found significantly elevated serum anti-LAMP-2 antibody levels in CPN patients compared with healthy controls. Serum anti-LAMP-2 antibody levels differed significantly between patients with HSP and healthy controls. We previously investigated possible correlations between CPN and anti-phosphatidylserine-prothrombin complex (anti-PSPT) antibodies [21]. We also reported that small vessel vasculitis including HSP could be dependently associated with the presence of anti-PSPT antibodies [22]. Based on these data, we suggested that anti-LAMP-2 antibody and anti-PSPT antibodies could play a role in the common pathogenesis of CPN and HSP. Previous studies have suggested that molecular mimicry between LAMP-2 and bacterial adhesion protein FimH could cause antibodies to LAMP-2 in susceptible individuals after infection with fimbriated...
bacteria, and in turn cause medium to small vessel vasculitis [19]. Streptococcal infection is known to be a frequent trigger for CPN and HSP [23, 24]. We propose that antecedent bacterial infection could induce overproduction of anti-LAMP-2 antibody, which reacts with LAMP-2 in neutrophils and vascular endothelial cells, and lead to cutaneous vasculitis. Further investigations are needed to further explore the potential roles of LAMP-2 in cutaneous vasculitis.

**Rheumatology key messages**

- Anti-LAMP-2 antibody levels were related to the presence of cutaneous vasculitis in env-pX rats.
- Anti-LAMP-2 antibody could initiate cutaneous vasculitis in env-pX rats.
- Anti-LAMP-2 antibody could be related to the pathogenesis of human cutaneous vasculitis, including CPN and HSP.

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

**References**

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