contrast, internalized GFP-BP180 did not colocalize with either clathrin or caveolin-1 (Figure 4, B and C). These results suggested that BP-IgG—induced BP180 internalization occurs via a clathrin- and caveolae-independent endocytic pathway.

We next examined the effect of NEM, hypertonic sucrose, nystatin, and genistein. We confirmed the activity of these inhibitors of general, clathrin-dependent, and caveolae-dependent endocytosis in 804G cells by analyzing the localization of EGFR, which undergoes clathrin-dependent endocytosis, and CTB, which undergoes caveolae-dependent endocytosis (Supplemental Figure S4).[24]

NEM dramatically inhibited both GFP-BP180 internalization and the morphological changes in 804G cells induced by BP-IgG treatment, suggesting that BP-IgG—induced BP180 internalization is mediated through an endocytic pathway (Figure 5A). In contrast, pre-incubation with hypertonic sucrose, a clathrin-dependent endocytosis inhibitor, failed to prevent BP180 internalization (Figure 5B). Similarly, nystatin (Figure 5C) and genistein (Figure 5D) did not inhibit BP-IgG—induced BP180 internalization. Because nystatin is a caveolae-dependent endocytosis inhibitor and genistein is a tyrosine kinase inhibitor, these results confirmed that BP180 endocytosis is mediated through clathrin-, caveolae-, and tyrosine kinase—independent mechanisms.

BP-IgG Induces BP180 Internalization via a Macropinocytic Pathway

We next investigated whether BP-IgG—induced BP180 internalization involved macropinocytosis. Macropinocytosis can be distinguished by an involvement of actin dynamics and Na^+/H^+ exchanger and non-specific fluid uptake.[25,26] In this study, GFP-BP180—expressing 804G cells were treated with

BP-IgG in the presence of either cytochalasin D (Figure 5E) or EIPA (Figure 5F). These macropinocytosis inhibitors blocked GFP-BP180 internalization in 804G cells. Moreover, in NHEKs, GFP-BP180 internalization was inhibited by EIPA (Figure 5G). We further confirmed that some of the internalized BP180 colocalized with 10-kDa dextran—AF 594 in 804G cells (Figure 6A). The internalization of the fluid-phase marker was inhibited by pre-incubation with cytochalasin D and EIPA (Figure 6, B and C). These results demonstrated the participation of macropinocytosis in BP-IgG—induced BP180 internalization.

Macropinocytosis Inhibitor Rescues BP-IgG—Induced Reduction in the Adhesive Strength of the Cells

A previous study has shown that BP-IgG treatment reduces the adhesive strength of the cells to their substrate.[15] Hence, we assessed whether EIPA inhibits this phenomenon. Non-treated, normal IgG—treated, and BP-IgG—treated NHEKs were vortex mixed after pre-incubation with or without EIPA, and then the numbers of adherent cells were counted. BP-IgG treatment decreased the adhesive strength of the cells, which was consistent with the result of the previous study.[15] In contrast, after pre-incubation with EIPA, BP-IgG—treated NHEKs did not exhibit a reduction in the adhesive strength, compared with normal IgG-treated or non-treated cells (Figure 7).

BP180 Is Internalized along with BP-IgG

We next examined whether BP-IgG is internalized along with its antigen, BP180. For this purpose, 804G cells expressing GFP-BP180 were incubated with HiLyte Fluor 647—conjugated BP-IgG for 30 minutes, washed, and then observed. HiLyte Fluor 647—conjugated BP-IgG was clustered along the cell substratum—attached surface, together
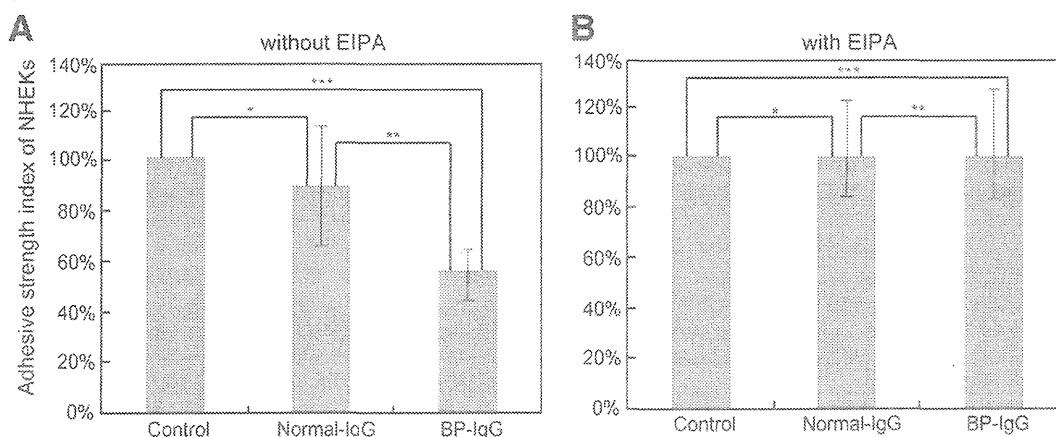

Figure 7   BP-IgG treatment increases the detachment of NHEKs from their substrate after vortex mixing, and the effect is inhibited by pre-incubation with EIPA. After pre-incubation with (B) or without (A) EIPA, NHEKs were treated with or without normal IgG and BP-IgG for 6 hours. The cells were vortex mixed, and the numbers of adherent cells were counted. The y axis depicts the percentage of adherent cells after agitation. The control index value without IgG addition was calculated as 100%. A: Without pre-incubation with EIPA, the adhesive strength of BP-IgG—treated cells was reduced to approximately 60% compared with normal IgG-treated cells. *P = 0.38 (not significant); **P = 0.0017; ***P = 0.0061. B: After pre-incubation with EIPA for 30 minutes, the adhesive strength of BP-IgG—treated cells was comparable to normal IgG-treated and non-treated cells. *P = 0.96; **P = 1.00; ***P = 0.96 (no significant differences).

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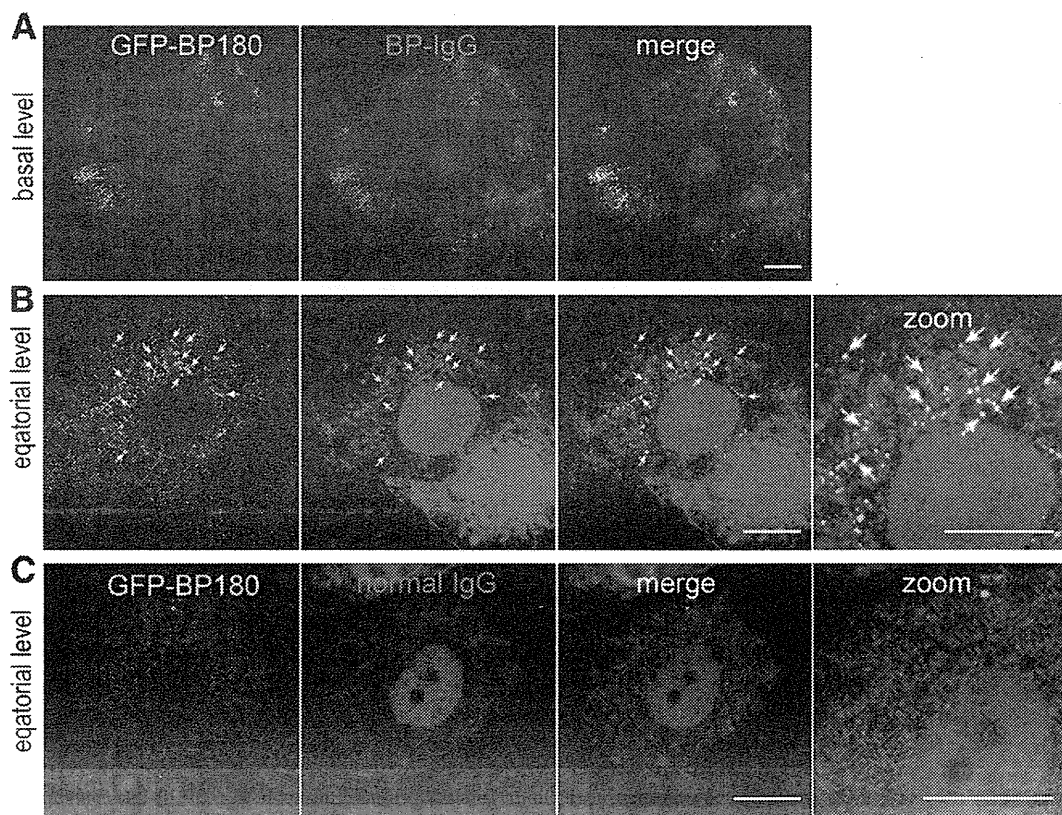
Figure 8 HiLyte Fluor 647—conjugated BP-IgG is internalized along with BP180. HiLyte Fluor 647—conjugated BP-IgG (**A** and **B**) and HiLyte Fluor 647—conjugated normal IgG (**C**) were observed in GFP-BP180—expressing 804G cells after incubation for 30 minutes. **A:** HiLyte Fluor 647—conjugated BP-IgG colocalized with GFP-BP180 into hemidesmosome-like structures at the cell substratum—attached surface. **B:** At the equatorial plane, internalized BP-IgG was visible and colocalized with internalized GFP-BP180 (**arrows**). **C:** HiLyte Fluor 647—conjugated normal IgG failed to induce internalization of both IgG and GFP-BP180. Scale bars: 10 μm.

with GFP-BP180 (Figure 8A), and distributed as cytoplasmic spots (Figure 8B). Some of the internalized BP-IgG spots colocalized with GFP-BP180. In contrast, after incubation with HiLyte Fluor 647—conjugated normal IgG, neither GFP-BP180 nor normal IgG was internalized (Figure 8C). These results suggested that a complex of BP-IgG bound to its BP180 antigen is internalized.

Intracellular and Extracellular Domains of BP180 Are Internalized Simultaneously

BP180 undergoes proteolytic cleavage during migration and other pathological conditions.[27–30] The precise cleavage sites are unclear but may exist in the NC16A domain.[27,29,30] To examine whether proteolytic cleavage of BP180 occurs during BP-IgG—induced internalization, BP-IgG—treated GFP-BP180—expressing 804G cells were stained with antibody mAb233, which recognizes the extracellular domain of BP180. BP-IgG—treated untransfected NHEKs were doubly stained with mAb233 and antibody J17, which recognizes the cytoplasmic domain of BP180. These studies showed that both intracellular and extracellular domains of internalized BP180 colocalized as cytoplasmic spots in both cell types (Figure 9, A and B).

Discussion

The aim of the present study was to assess the role of BP-IgG in the initiation of noninflammatory blister formation in BP in an *in vitro* system. Our results indicate the following: i) BP-IgG causes BP180 internalization and morphological changes by an FcR-independent mechanism, which is consistent with the results of the previous studies,[15,16,31,32] ii) BP-IgG—induced BP180 internalization and BP-IgG—induced reduction in the adhesive strength of the cells require macropinocytosis, iii) BP180 is internalized, along with BP-IgG, and iv) both intracellular and extracellular domains of BP180 are internalized after binding with BP-IgG.

In subconfluent cultures of both 804G cells and NHEKs, BP180 internalization induced by BP-IgG occurs rapidly, within <30 minutes. Interestingly, there are some differences in the fates of BP180 and the morphological characteristics between 804G cells and NHEKs. The 804G cells showed more prominent GFP-BP180 internalization and cell rounding than in NHEKs. This may reflect the different amino acid sequences between rat BP180 and human BP180, specifically in the binding site to α6 integrin.[33] This raises the possibility that, in 804G cells, GFP-human BP180 may not be constrained at the plasma
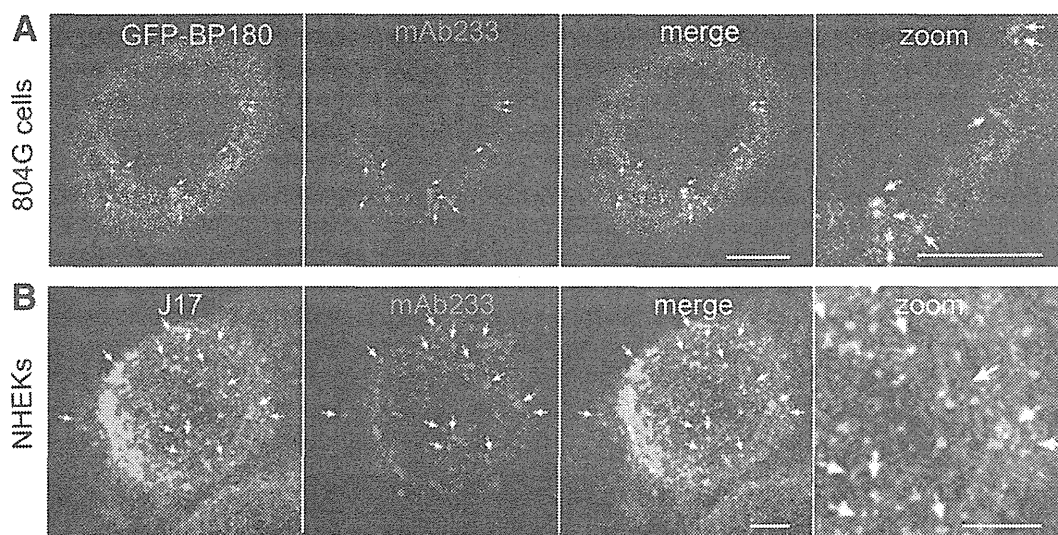
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Figure 9 The intracellular domain of BP180 is internalized, along with the extracellular domain of BP180. **A:** GFP-BP180—expressing 804G cells were stained using anti-extracellular BP180 domain antibody mAb233, 2 hours after the incubation with BP-IgG. **B:** NHEKs were doubly stained using mAb233 and anti-cytoplasmic BP180 domain antibody J17. These studies showed that both intracellular and extracellular domains of internalized GFP-BP180 colocalized as cytoplasmic spots (**arrows**). Scale bars: 5 μm.

membrane by α6 integrin, resulting in higher internalization and less adhesion.

We confirmed that BP-IgG—induced BP180 internalization also occurs in confluent cultures, although the process appears less efficient than in subconfluent cultures. This result is important, because confluent cultures mimic more the *in vivo* situation. Less efficient internalization may reflect lower accessibility of BP-IgG to the substratum-attached surface of the cells in confluent cultures.

Based on the results of our fluid-uptake and live cell imaging assays using cytochalasin D and EIPA, which inhibit actin polymerization and transmembrane Na^+ transport systems, respectively, we conclude that BP180 internalization occurs via macropinocytosis. Moreover, macropinocytosis of BP180 reduces cell-substrate adhesion, because EIPA rescues BP-IgG—induced detachment of the cells after vortex mixing. Interestingly, we also showed that a tyrosine kinase is unlikely to be involved in BP-IgG—induced BP180 internalization. This is not contrary to the literature because the role of tyrosine kinases in macropinocytosis is controversial.[25,34—36]

Our results have an interesting parallel with those involving the pathogenesis of another blistering skin disease, pemphigus vulgaris (PV). The PV antigen, desmoglein 3 (Dsg3), undergoes endocytosis after PV autoantibody binding.[24,37,38] Such as the BP180 internalization shown herein, PV-IgG—induced Dsg3 internalization is mediated by a clathrin- and dynamin-independent mechanism.[24] However, in contrast to BP180 internalization, both nystatin and genistein inhibit PV-IgG—induced Dsg3 internalization. Therefore, Dsg3 and BP180 internalizations are considered to be controlled by distinct endocytic pathways. The difference in cytoplasmic

domains between Dsg3 and BP180 may specify the distinct endocytic pathways by which these internalizations are regulated.[24]

Some reports have suggested that ectodomain shedding of BP180 occurs in BP, although its pathogenic role is unclear.[29,30,39—41] In this study, we detected both intracellular and extracellular domains in the internalized BP180, suggesting that ectodomain shedding of BP180 does not necessarily occur in BP180 internalization and may not be essential to BP disease onset.

Some previous studies have reported that BP-IgG F(ab′) 2 and BP-IgG Fab fragments are insufficient to induce blisters in mouse models of the disease.[11,42,43] In contrast, herein, we have shown that whole IgG and BP-IgG F(ab′)2 and BP-IgG Fab fragments cause BP180 internalization and cell morphological changes. One possible explanation for these contradictory results is that BP-IgG—induced BP180 internalization is insufficient to induce blister formation, although it reduces cell-extracellular matrix adhesive strength. We speculate that blistering requires various inflammatory responses at the cell-extracellular matrix zone, where it is first weakened by BP180 internalization, further leading to a BP-specific split at the lamina lucida. In other words, we suspect that blister formation in BP requires both BP-IgG—induced BP180 internalization and FcR-independent and FcR-dependent immune responses.

In conclusion, the results of the present study suggest that BP180 internalization is an early event in disease pathogenesis of BP and occurs via a macropinocytic pathway. This new understanding of disease pathogenesis should provide us with new approaches for the treatment of BP.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2012.11.029*.

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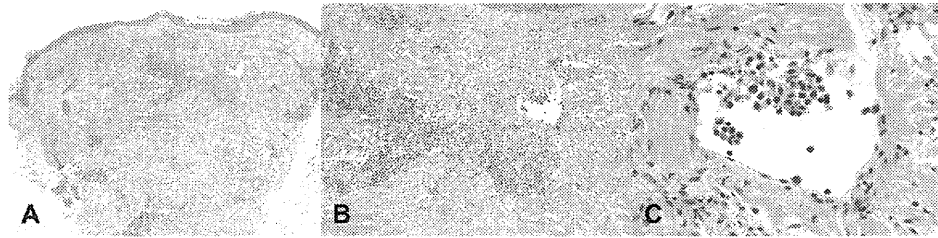
Fig 2. Angioimmunoblastic T-cell lymphoma. Skin biopsy specimen with superficial and deep perivascular and intralymphatic infiltrate of atypical lymphocytes, with scattered mitoses and admixed plasma cells. (**A** to **C**, Hematoxylin-eosin stain; original magnifications: **A**, ×4; **B**, ×20; **C**, ×40.)

Conflicts of interest: None declared.

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Successful single-cycle rituximab treatment in a patient with pemphigus vulgaris and squamous cell carcinoma of the tongue and IgG antibodies to desmocollins

To the Editor: Pemphigus can be triggered by many insults, including drugs, infections, and neoplasms. Radiation-induced pemphigus vulgaris accompanied by an internal malignancy has been rarely reported,[1] but the link between pemphigus vulgaris and malignancy still needs to be elucidated. Moreover, the treatment of pemphigus in this condition is often challenging, as the patient's immune system is already suppressed by radiation and chemotherapy.

A 54-year-old man presented to us with blisters and ulcers on the oral mucosa and face that started after radiotherapy for squamous cell carcinoma (SCC) of the tongue 2 years earlier. Blisters and ulcers first developed on the tongue and back of the throat, which affected eating and swallowing and required a tracheostomy. The ulceration was assumed to be related to the radiation but then spread to the nose and cheeks (Fig 1, *A*). Pemphigus vulgaris was diagnosed but it was recalcitrant to high-dose prednisolone, azathioprine, cyclophosphamide, and intravenous immunoglobulin. Further skin biopsy specimens of the vegetative plaque over the irradiated site on the right jaw that persisted indicated only pemphigus vulgaris and ruled out infection. IgG enzyme-linked immunosorbent assay indices of antidesmoglein-3 antibodies of 146 and antidesmoglein-1 antibodies of 3 were consistent with mucosal dominant type pemphigus vulgaris. Interestingly, IgG antibodies against desmocollins 1, 2, and 3 with relatively high titers were also detected in the sera of the patient by novel enzyme-linked immunosorbent assays using the eukaryotic recombinant proteins.

Rituximab was given as 4 weekly infusions of 375 mg/m^2 along with prednisolone at 40 mg/d (approximately 0.5 mg/kg/d). After a single cycle of rituximab treatment, the patient achieved 90% remission in 2 months. To prevent recurrence, 40 mg of prednisolone was sustained for 3 months and then tapered. Eighteen months after the initiation of treatment, he was in complete remission without any treatment (Fig 1, *B*).

This unusual case of pemphigus vulgaris highlights 2 interesting points. Clinically, this case suggests that a single cycle of rituximab is an effective treatment for recalcitrant pemphigus accompanied by an internal malignancy. In this condition, immunosuppressants such as azathioprine, mycophenolate, and cyclophosphamide are relatively contraindicated, as they can induce cancers and our patient's immune system was already suppressed
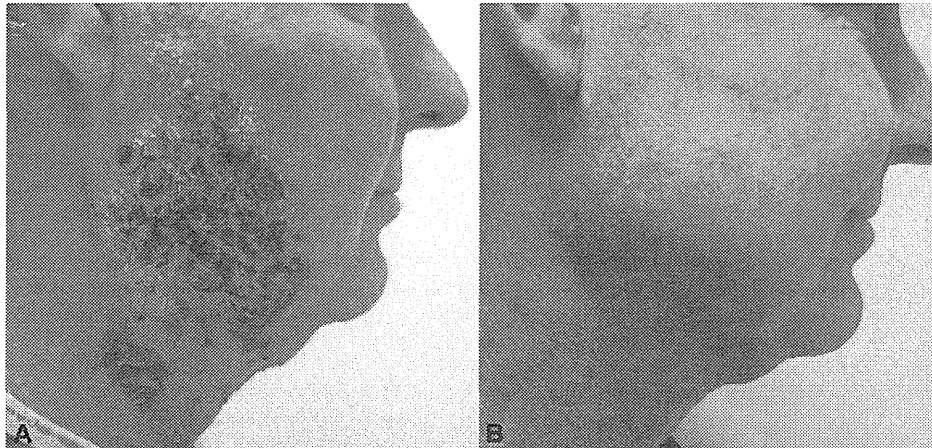
Fig 1. Clinical features. **A**, Vegetative erosions with crusts on cheeks before rituximab treatment. **B**, Complete remission without treatment 12 months after single cycle of rituximab treatment.

by radiation and chemotherapy. A single cycle of rituximab infusion could be considered in this challenging situation.

In terms of pathogenesis, the IgG antibodies that were detected against desmocollins could explain the linkage between the SCC and pemphigus vulgaris. Desmocollins are the main desmosomal transmembrane proteins that play an important role in intercellular adhesion, and connection between the cytokeratins and the cell membrane. Desmocollin 3—mediated binding is crucial for keratinocyte cohesion and is impaired in pemphigus.[2] IgG autoantibodies against desmocollin 3 also induce loss of keratinocyte adhesion.[3] As decreased cell-cell adhesion is a pathogenic feature common to both the invasive and metastatic ability of SCCs[4,5] and the development of pemphigus,[2] antibodies against desmocollins might be the pathogenically relevant factors linking SCC and pemphigus. Our case suggests the possibility that SCC produces autoantibodies against desmocollins during invasion or metastasis and the SCC-induced antidesmocollin autoantibodies could induce pemphigus vulgaris.

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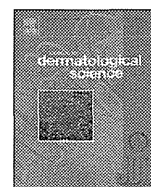
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http://dx.doi.org/10.1016/j.jaad.2012.12.967

Contents lists available at SciVerse ScienceDirect

Journal of Dermatological Science

journal homepage: www.elsevier.com/jds

An attempt to develop mouse model for anti-laminin γ1 pemphigoid

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ABSTRACT

Background: We recently reported that the autoantibodies of anti-p200 pemphigoid sera react with laminin γ1 and renamed this entity as anti-laminin γ1 pemphigoid. However, it has not been clarified whether the anti-laminin γ1 autoantibodies, particularly those to the C-terminal integrin binding site, affect the dermoepidermal junction and cause subepidermal blisters.
Objective: The aim of this study was to develop animal models for anti-laminin γ1 pemphigoid.
Methods: We attempted to produce two mouse models for anti-laminin γ1 pemphigoid; (1) a passive transfer model: injection of rabbit IgG to shorter bacterial recombinant protein of the murine laminin γ1 C-terminal 107 amino acids, and (2) an active disease model: direct immunization to mice with this recombinant protein.
Results: Immunoblotting revealed that 70% of patient sera reacted with the shorter recombinant protein of human laminin γ1 C-terminus. In the passive transfer model, rabbit IgG to the murine laminin γ1 C-terminus was deposited, without C3 deposition, at the epidermal basement membrane zone. In contrast, in the active disease model, direct immunofluorescence of mouse skin sections showed no deposition of either murine IgG or C3. Blister formation was not seen in either model both phenotypically and histopathologically.
Conclusion: In the two different mouse animal models for anti-laminin γ1 pemphigoid, although rabbit IgG to the recombinant laminin γ1 C-terminus bound to the epidermal basement membrane zone in passive transfer model, no obvious blister formation was seen. To reproduce skin lesions in mouse models for anti-laminin γ1 pemphigoid, further improvement should be needed.
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1. Introduction

Anti-laminin γ1 pemphigoid is an autoimmune subepidermal bullous disease, first described as anti-p200 pemphigoid in 1996. This disease is distinct from other subepidermal blistering diseases, including bullous pemphigoid (BP), mucous membrane pemphigoid (MMP) and epidermolysis bullosa acquisita (EBA).

Anti-laminin γ1 pemphigoid patients clinically present with tense blisters and urticarial papules and plaques resembling BP, particularly in psoriatic patients. Other patients may present with vesicular pemphigoid-like small vesicles. Histopathological examination shows subepidermal blisters and inflammatory infiltrates mainly of neutrophils in the upper dermis [1]. IgG antibodies in the patient sera react with the epidermal basement membrane zone (BMZ) of the normal skin and with dermal side of 1 M NaCl split skin by indirect immunofluorescence (IF). By immunoblotting, IgG antibodies recognize a 200 kDa protein in human dermal extracts. In 2009, we identified this protein as laminin γ1 and renamed this disease from anti-p200 pemphigoid to anti-laminin γ1 pemphigoid [2,3]. The pathogenic role of the IgG anti-laminin γ1 antibodies is a key issue to reveal the pathomechanisms of this disease.

In order to investigate the pathogenesis, animal disease models have been developed for several autoimmune bullous diseases, including pemphigus vulgaris (PV), pemphigus foliaceus (PF), Brazilian endemic pemphigus foliaceus (Fogo selvagem), BP, anti-laminin 332 type MMP and EBA, which reproduce their clinical, histopathological and immunopathological features [4–12]. However, no animal model for anti-laminin γ1 pemphigoid has been developed.

Our previous studies indicated that C-terminal domain of laminin γ1 contains main epitopes [2,13]. Furthermore, Ido et al. [14] showed that the glutamic acid residue within laminin γ1 C-terminus is

essential for binding of integrin to laminin trimers. Therefore, we hypothesized that IgG antibodies reacting with laminin γ1 C-terminus induce dermal-epidermal separation in anti-laminin γ1 pemphigoid.

In this study, to assess this hypothesis, we first confirmed that IgG antibodies in the patient sera reacted with shorter bacterial recombinant protein of human laminin γ1 C-terminal 107 amino acids. Then, we attempted to develop mouse models for anti-laminin γ1 pemphigoid both by passively injecting rabbit antibodies to a recombinant protein of murine laminin γ1 C-terminus into mice and by actively immunizing mice with this recombinant protein.

2. Materials and methods

2.1. Patients

All described studies were performed following the guidelines of the medical ethics committee of Kurume University School of Medicine. Each participant in this study provided an informed consent agreement, and all studies were conducted according to the Declaration of Helsinki Principles. Serum samples were obtained from 19 patients who were diagnosed as anti-laminin γ1 pemphigoid by the following features; (1) tense blisters on the skin, (2) linear deposits of IgG at epidermal BMZ by direct IF, (3) presence of circulating IgG autoantibodies bound to the dermal side of 1 M NaCl split human skin by indirect IF, and (4) IgG reactivity with the 200 kDa protein by immunoblotting of human dermal extracts. Normal control sera were obtained from 12 healthy volunteers.

2.2. Animals

Adult mice (Seven-week-old) of BALB/c, BALB/c$^{nu-/nu-}$ and SJL-1 strains were purchased from Japan SLC (Hamamatsu, Japan), and pregnant mice of BALB/c and C57BL/6J strains were obtained from KBT Oriental (Tosu, Japan). Five New Zealand white rabbits weighing approximately 2 kg (Charles River Japan Inc., Yokohama,

Japan) were used to obtain rabbit antisera. Each rabbit was subcutaneously immunized with 100 μg recombinant protein suspended in Freund's complete adjuvant at the first injection, and thereafter with the recombinant protein suspended in Freund's incomplete adjuvant every other week. The experimental protocols were outlined in the National Institutes of Health Guideline for the Care and Use of Laboratory Animals, and were approved by the Animal Research Committee of Kurume University.

2.3. Expression of laminin γ1 recombinant proteins

Glutathione-s-transferase (GST)-fused bacterial recombinant proteins of human laminin γ1 C-terminal 107 amino acids (GST-hLmγ1c; amino acids 1503–1609), murine laminin γ1 C-terminal 246 amino acids (GST-mLmγ1-E8; amino acids 1362–1607) and 107 amino acids (GST-mLmγ1c; amino acids 1501–1607) were produced (Fig. 1). Shorter recombinant proteins were simply designated h- and mLmγ1c. Longer murine recombinant protein was designated GST-mLmγ1-E8, because the corresponding region is called E8 fragment of laminin γ1 [2,14]. DNA sequence data were retrieved from UCSC Genome Bioinformatics. The cDNA fragments were obtained by reverse transcription PCR of total RNA from the newborn mouse skin or normal human skin using the following primers.

For cDNA to produce GST-hLmγ1c:
forward) 5′-GCCGGGATCCGCCAGAAAAGCCAAAAACTCTG-3′, and
reverse) 5′-ATAGCGGCCGCTAGGGCTTTTCAATGGACGGGGTG-3′.
For cDNA to produce GST-mLmγ1-E8:
forward) 5′-CGGGATCCAATGACATTCTCAACAACCTGAAAG-3′, and
reverse) 5′-ATAGCGGCCGCTAGGGCTTCTCGATAGACGGGGTG-3′.
For cDNA to produce GST-mLmγ1c:
forward) 5′-GCCAGAAAGGCCAAAAACT-3′, and
reverse) 5′-TTACAGCCCTCTCGCCACTA-3′.

The PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Then, the cDNA fragments were inserted into pGEX4T-1 (Amersham Biosciences, Piscataway, NJ). GST-fused recombinant proteins were induced in Escherichia coli BL21 and purified by glutathione–agarose affinity chromatogra-

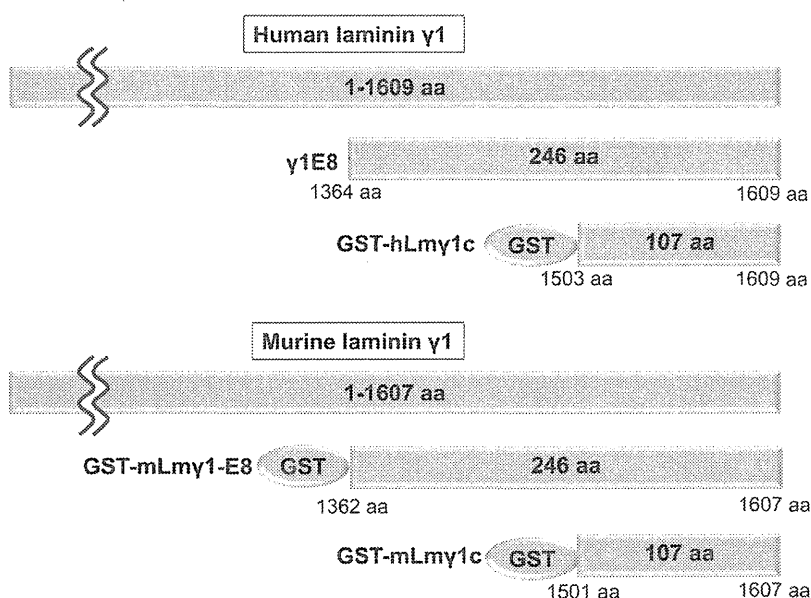

Fig. 1. A scheme depicting entire molecules and truncated recombinant proteins for C-terminal domains of both human and murine laminin γ1. In this study, we produced three GST-fused recombinant proteins, GST-hLmγ1c encoding 1503–1609 amino acids of human laminin γ1, GST-mLmγ1-E8 encoding 1362–1607 amino acids of murine laminin γ1and GST-mLmγ1c encoding 1501–1607 amino acids of murine laminin γ1. The previously prepared eukaryotic recombinant protein encoding 1364–1609 amino acids (γ1E8), as well as structures of full length human and murine laminin γ1, is also shown.

phy. Protein concentration was measured at 280 nm by spectrophotometer.

2.4. Isolation of rabbit IgG and affinity-purification of specific rabbit IgG

IgG from rabbit serum was isolated by using Protein G Agarose, Fast Flow (Millipore Corporation, Temecula, CA), as described previously [11]. Rabbit IgG specific to GST-mLmγ1c was affinity-purified by using NHS-activated Hi Trap column (Amersham Biosciences, Piscataway, NJ). Six mg GST-mLmγ1c was coupled to an NHS-activated Hi Trap column (1 ml) according to the manufacturer's instructions. Serum from rabbit immunized with GST-mLmγ1c was applied to the column and bound antigen-specific rabbit IgG was eluted, as described previously [11]. Concentration of the purified rabbit IgG was measured at 280 nm by spectrophotometer.

2.5. IF and immunoblot analysis

Frozen sections of mouse skin were examined by direct IF using FITC-conjugated anti-rabbit immunoglobulins, anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) and anti-mouse C3 (Cappel, Solon, OH) antisera diluted at 1:100. Indirect IF was performed using normal BALB/c mouse skin sections. Immunoblotting was performed as described previously [15]. Anti-laminin γ1 monoclonal antibody (B-4) (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control. The product of mouse laminins purified from cultured Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, which contain laminin-111 [16], was purchased from Millipore Corporation.

2.6. Passive transfer mouse model study

In passive transfer model study of injection of rabbit IgG, we used neonatal mice (12–24 h of age) of BALB/c (n = 4) and C57BL/6J (n = 4) strains, and adult mice of BALB/c (n = 3) and BALB/c$^{nu-/nu-}$ (n = 4) strains. Four mg rabbit IgG to GST-mLmγ1c was intraperitoneally injected into the neonatal mice of the two strains every day for 4 days. Ten mg rabbit IgG to GST-mLmγ1c was subcutaneously injected into the adult BALB/c mice every second day for 14 days. Twenty mg rabbit IgG to GST-mLmγ1c was subcutaneously injected into the BALB/c$^{nu-/nu-}$ mice every other day for 20 days.

For passive transfer study of injection of affinity-purified IgG specific to GST-mLmγ1c, we used neonatal mice (12–24 h of age) of BALB/c (n = 3) and C57BL/6J (n = 3) strains. Two mg affinity-purified IgG was intraperitoneally injected into the neonatal mice every day for 3 days. Skin and serum samples were taken one day after the last injection.

2.7. Active disease mouse model study

In active disease model study, we used adult SJL-1 mice (n = 7), which were immunized subcutaneously at their tail base with 60 μg GST-mLmγ1c emulsified in nonionic block copolymer adjuvant TiterMax (Sigma–Aldrich Corp., St. Louis, MO) every week for 12 weeks. Negative control mice (n = 3) were immunized with 60 μg GST recombinant protein emulsified in TiterMax. Skin and serum samples were taken one week after the last immunization.

3. Results

3.1. Sera from most anti-laminin γ1 pemphigoid patients recognized recombinant protein of human laminin γ1 C-terminal 107 amino acids

Two previous studies [2,13] indicated that the sera from most anti-laminin γ1 pemphigoid patients recognized 246 amino acids of human laminin γ1 C-terminus, which is called E8 fragment of laminin γ1 [2,14], suggesting that the main pathogenic epitopes reside within the region. To identify immunogenic region more precisely, we produced a smaller GST-fused recombinant protein of human laminin γ1 C-terminal 107 amino acids (GST-hLmγ1c) (Fig. 1) and tested its reactivity to patient sera by immunoblotting. Our immunoblot analysis showed a positive reactivity for sera from 13 (68%) of the 19 patients, but negative results for all 12 normal control sera (Fig. 2). This finding indicated that the major epitopes for patient sera reside within C-terminal 107 residues of laminin γ1.

3.2. Patient sera scarcely recognized the murine epidermal BMZ

The patient autoantibodies may induce blister formation in the mouse skin, if they crossreact with murine laminin γ1. To check the cross-reactivity, we examined sera from 10 patients with anti-laminin γ1 pemphigoid by indirect IF of the normal mouse skin sections. However, only 3 (30%) of the 10 sera reacted with the murine epidermal BMZ (Fig. 3). This result suggested that patient sera are not ideal for production of blister formation in mouse skin.
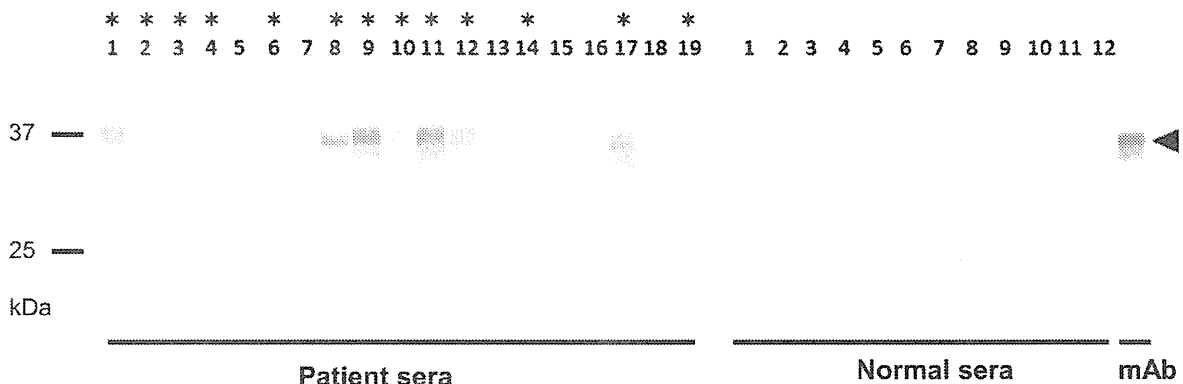

Fig. 2. Immunoblot analysis of GST-hLmγ1c. Thirteen of 19 patient sera were positive (68%), while sera of all 12 normal control sera were negative. The position of the recombinant protein is indicated by an arrow in the right. Asterisks indicate positive lanes. The positions of molecular weight markers are shown in the left. mAb, a monoclonal antibody specific to C-terminal domain of laminin γ1 (B-4).
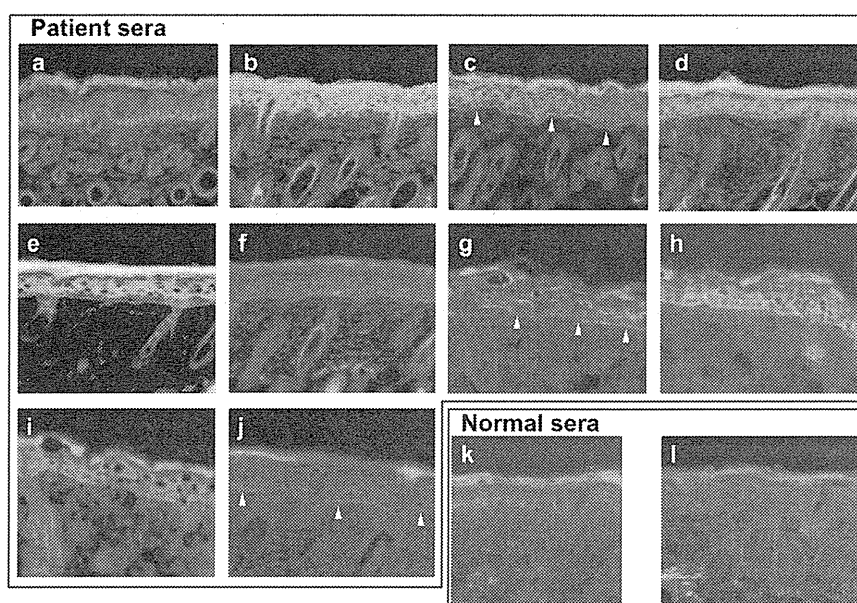
Fig. 3. Reactivity of patient sera to epidermal BMZ of the mouse skin by indirect IF. Sera from 10 patients (a–j) were analyzed at 1:10 dilution by indirect IF of mouse skin. Three patients reacted with murine epidermal BMZ (arrowheads) (c, g, and j). Two normal control sera showed no reactivity (k and l).

3.3. Rabbit antiserum to GST fused recombinant protein of laminin γ1 C-terminal 107 amino acids showed strong reactivity with murine epidermal BMZ, but that of laminin γ1 C-terminal 246 amino acids showed weak reactivity

To confirm the pathogenic role of anti-laminin γ1 C-terminus antibodies, we prepared two bacterial GST-fused recombinant proteins of murine laminin γ1 for C-terminal 246 amino acids (GST-mLmγ1-E8) and C-terminal 107 amino acids (GST-mLmγ1c) (Fig. 1). By immunizing rabbits with the recombinant proteins, we obtained two rabbit antisera. The two rabbit antisera reacted strongly with murine laminin γ1 by immunoblot analyses of GST-mLmγ1c and EHS mouse sarcoma cell derived laminins [16] (Fig. 4a).

By indirect IF of normal mouse skin sections, the rabbit anti-GST-mLmγ1c antiserum showed very high reactivity to epidermal BMZ at a titer up to 1:81,920 (Fig. 4b). In contrast, the reactivity of the rabbit anti-GST-mLmγ1-E8 IgG antiserum was much lower at a titer of 1:160–1:640, although immunization to rabbits was repeatedly performed (Fig. 4c). Therefore, we decided to use rabbit IgG to GST-mLmγ1c for further studies.

3.4. In passive transfer mouse model, rabbit IgG to GST-mLmγ1c bound to epidermal BMZ, but did not show either C3 deposition or blister formation

We attempted to establish passive transfer mouse model to determine the pathogenic activity of rabbit IgG to laminin γ1. For this purpose, rabbit IgG to GST-mLmγ1c was injected into neonatal mice of BALB/c and C57BL/6J strains, and into adult mice of BALB/c and BALB/c$^{nu-/nu-}$ strains.

By direct IF, all mice showed rabbit IgG deposition at the murine epidermal BMZ (Fig. 5b, f, j and n), and several mice also showed IgG deposition to capillary vessels in the upper dermis (arrowheads) (Fig. 5b and j). No mice showed murine C3 deposition (Fig. 5c, g, k, and o). Indirect IF of mouse skin sections detected anti-BMZ rabbit IgG antibodies in the sera of all mice at dilutions of 1:10,240–1:81,920 (Fig. 5d, h, l, and p).

However, none of these mice clinically showed any blister formation or erythema. Histopathological examination of the mouse skin did not show any blister formation or accumulation of neutrophils in the upper dermis, which are typical features in anti-laminin γ1 pemphigoid patients (Fig. 5a, e, i, and m).

3.5. In passive transfer mouse model, affinity-purified rabbit IgG specific to GST-mLmγ1c bound to epidermal BMZ, but did not show either C3 deposition or blister formation

To inject rabbit IgG to GST-mLmγ1c at higher concentration into mice, we purified rabbit IgG specific to GST-mLmγ1c by affinity column coupled with GST-mLmγ1c. By indirect IF of mouse skin sections, affinity-purified rabbit IgG specific to GST-mLmγ1c showed very strong reactivity to epidermal BMZ at a titer up to 1:81,920. We injected the affinity-purified rabbit IgG specific to GST-mLmγ1c into neonatal mice of BALB/c and C57BL/6J strains. However, none of these mice showed clinically any blister formation or erythema. Histopathological examination showed no blister formation, either (Fig. 6a and e). Similar to the results in mice injected with non-purified rabbit IgG, direct IF of the skin sections of mice injected with affinity-purified rabbit IgG showed rabbit IgG deposition at epidermal BMZ (Fig. 6b and f), but no C3 deposition was found (Fig. 6c and g). Indirect IF of mouse skin sections confirmed the presence of anti-BMZ rabbit IgG antibodies in the sera of the injected mice (Fig. 6d and h).

3.6. Active disease model showed neither deposition of murine IgG at epidermal BMZ nor any blister formation

Sitaru et al. successfully produced pathogenic autoantibodies by injecting recombinant protein of type VII collagen into mice in active disease mouse model of EBA [17]. To apply this method to anti-laminin γ1 pemphigoid, we used GST-mLmγ1c, as well as SJL-1 mice, which were shown to produce blisters at higher rate than other strains in previous EBA study [17]. SJL-1 mice were subcutaneously immunized with GST-mLmγ1c or GST recombinant protein as negative control at their tail base once every week
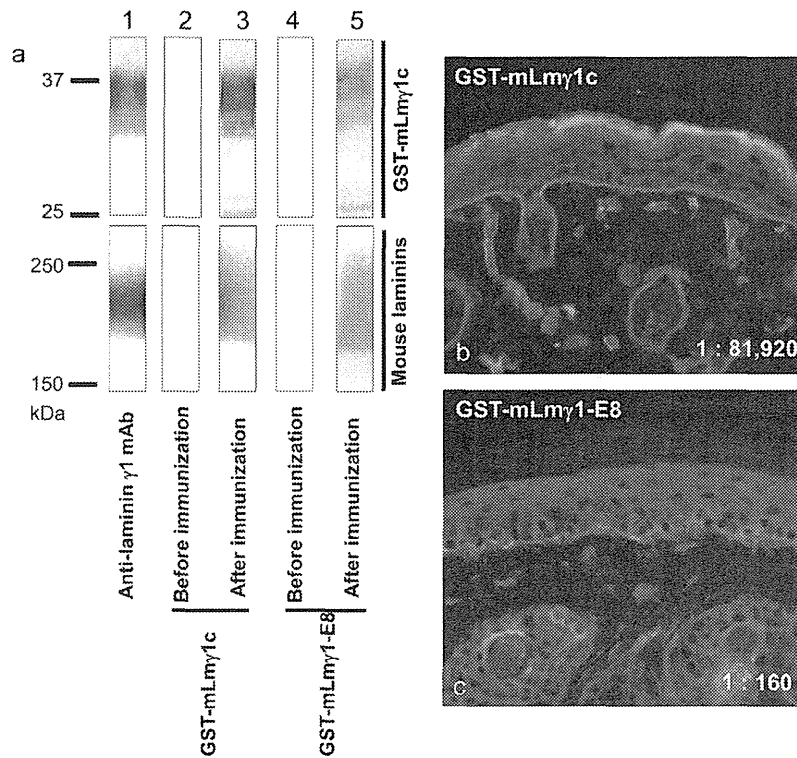
Fig. 4. Evaluation of rabbit IgGs to laminin γ1 C-terminus. We obtained two rabbit IgGs to GST-mLmγ1c and GST-mLmγ1-E8. (a) Immunoblot analyses of GST-mLmγ1c (upper panel) and mouse laminins purified from Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, containing laminin-111 (lower panel). The positions of molecular weight markers are shown in the left. Anti-laminin γ1 monoclonal antibody (B-4) strongly reacted with either the GST-mLmγ1c or laminin γ1 (lane 1). Rabbit IgG to GST-mLmγ1c (lane 3), but not preimmune rabbit IgG (lane 2), reacted with these proteins. Similarly, rabbit IgG to GST-mLmγ1-E8 (lane 5), but not preimmune rabbit IgG (lane 4), reacted with these proteins. (b and c) Indirect IF of mouse skin sections. Rabbit IgG to GST-mLmγ1c strongly reacted with the murine epidermal BMZ at a titer 1:81,920 (b), whereas rabbit IgG to GST-mLmγ1-E8 showed much lower titer (1:160–640) (c).
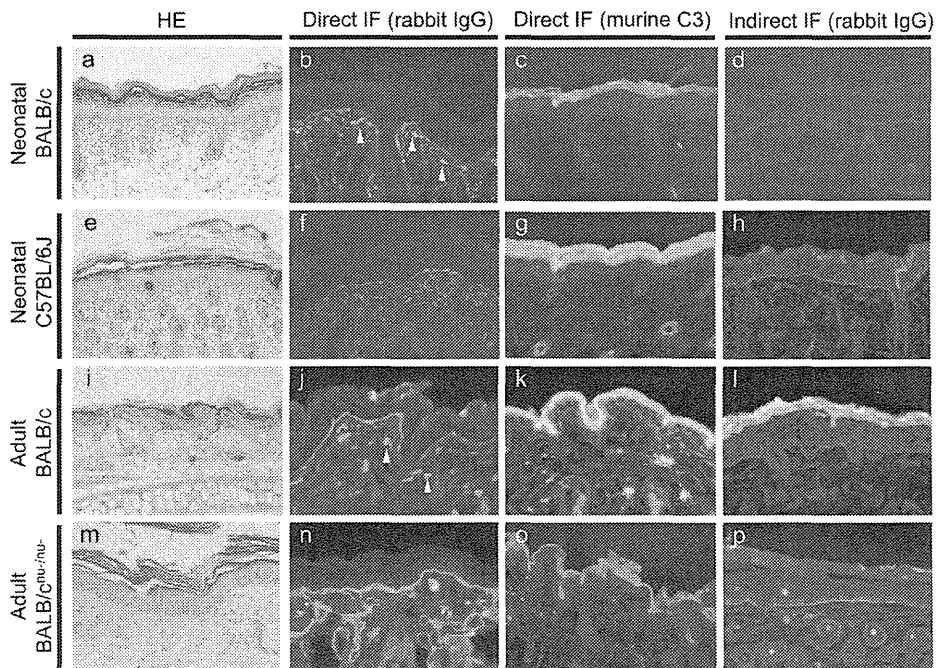

Fig. 5. Passive transfer model of injection of rabbit IgG to neonatal and adult mice of different strains. Rabbit IgG to GST-mLmγ1c was injected to neonatal mice of BALB/c (a–d) and C57BL/6J (e–h) strains, and to adult mice of BALB/c (i–l) and BALB/c$^{nu-/nu-}$ (m–p) strains. Hematoxylin and eosin staining features (a, e, i, and m). Direct IF features for rabbit IgG deposition at the epidermal BMZ (b, f, j, and n), and murine C3 deposition (c, g, k, and o). Indirect IF features of mouse skin sections for the mouse sera (d, h, l, and p). Arrowheads indicate capillary vessels in the upper dermis.
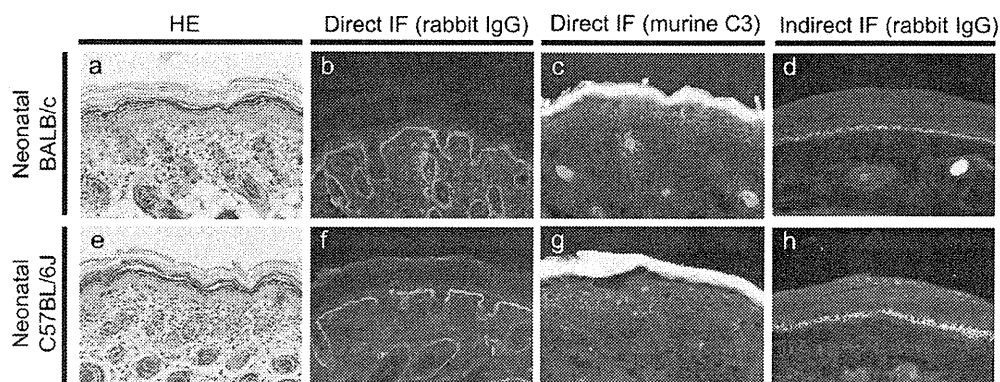
Fig. 6. Passive transfer model of injection of affinity-purified rabbit IgG specific to GST-mLmγ1c to neonatal mice of different strains. Affinity-purified rabbit IgG specific to GST-mLmγ1c was injected to neonatal mice of BALB/c (a–d) and C57BL/6J (e–h) strains. Hematoxylin and eosin staining features (a and e). Direct IF features for rabbit IgG deposition at the epidermal BMZ (b and f) and murine C3 deposition (c and g). Indirect IF features of mouse skin sections for the mouse sera (d and h).

for 12 weeks. However, no mice showed clinically either blisters or erythema on the skin. Histopathological examination showed no blisters, either (Fig. 7a and e). Direct IF showed neither murine IgG deposition (Fig. 7b and f) nor murine C3 deposition (Fig. 7c and g) at murine epidermal BMZ. The sera from mice immunized with GST-mLmγ1c did not react with epidermal BMZ by indirect IF of mouse skin sections (Fig. 7d and h). In contrast, the sera from these mice reacted strongly with mouse laminin γ1 by immunoblotting of laminins purified from EHS mouse sarcoma cells (Fig. 7i).

4. Discussion

In this study, we demonstrated that the sera from most anti-laminin γ1 pemphigoid patients reacted with the recombinant protein of human laminin γ1 C-terminal 107 amino acids, which is shorter than that used in the previous study [2,13]. Since human sera from anti-laminin γ1 pemphigoid patients scarcely recognized murine epidermal BMZ, we attempted to develop animal disease models using rabbit IgG to bacterial recombinant protein of murine laminin γ1 C-terminal domains. For passive transfer model, we injected both neonatal and adult mice with rabbit IgG raised against the bacterial recombinant protein of murine laminin γ1 C-terminal 107 amino acids. For active disease model, adult mice were injected with this recombinant protein.

For the passive transfer model, we could obtain very high titer rabbit IgG to the short recombinant protein. All neonatal and adult mice injected with the rabbit IgG showed clear rabbit IgG
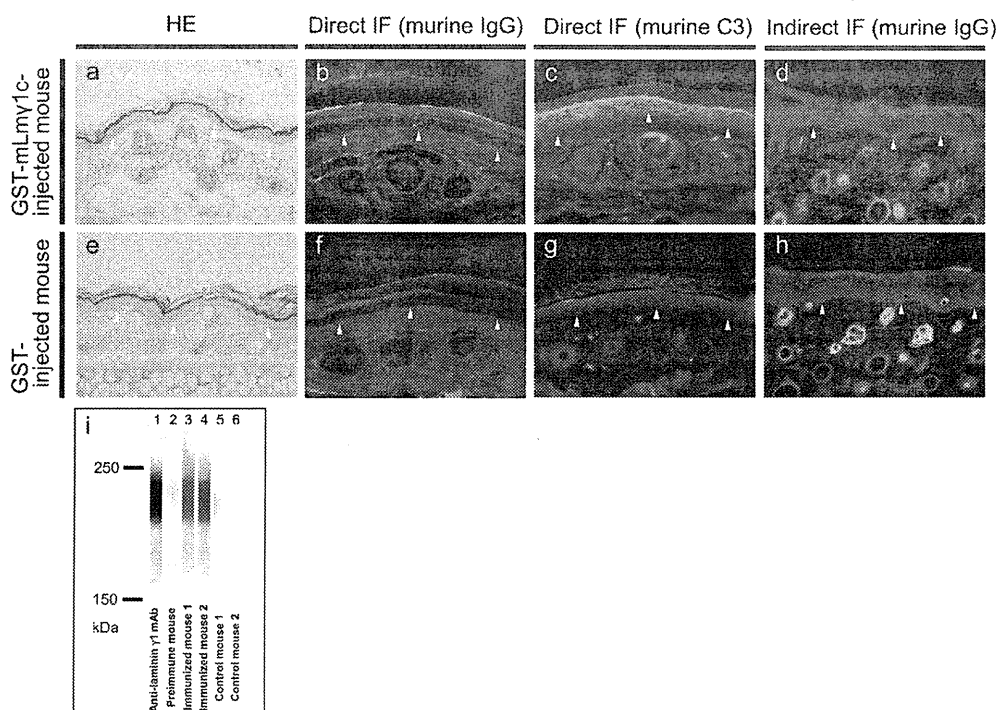

Fig. 7. Active disease mouse model. SJL-1 mice were immunized with GST-mLmγ1c (a–d) or with GST recombinant protein as negative control (e–h). Hematoxylin and eosin staining features (a and e). Direct IF features for depositions of either murine IgG (b and f) or murine C3 (c and g). Indirect IF features of mouse skin sections for the mouse sera (d and h). Arrowheads indicate murine epidermal BMZ. (i) Immunoblot analysis of mouse laminins purified from EHS mouse sarcoma cells. Anti-laminin γ1 monoclonal antibody (B-4) (lane 1) and sera from SJL-1 mice immunized with GST-mLmγ1c (lanes 3 and 4) reacted with laminin γ1, while sera from preimmune SJL-1 mouse (lane 2) and SJL-1 mice immunized with GST recombinant protein (lanes 5 and 6) showed no reactivity.

deposition at murine epidermal BMZ. However, these mice showed neither murine C3 deposition nor blister formation.

In active disease model, direct IF showed no deposition of either murine IgG or murine C3 at murine epidermal BMZ. Indirect IF of mouse skin sections did not show any reactivity of circulating IgG in the mice with epidermal BMZ. However, IgG in sera of the mice strongly reacted with laminin γ1 by immunoblotting of mouse laminins purified EHS mouse sarcoma cells.

While we have been revising our manuscript for this study, Vafia et al. reported a study of the pathogenicity of anti-laminin γ1 antibodies in anti-laminin γ1 pemphigoid [18]. In the study, they showed the results of analyses of passive transfer model and active disease model using bacterial recombinant protein of murine laminin γ1 C-terminal 246 amino acids, which were similar to the results of the present study. Particularly, in the study, neonatal and adult mice of several strains, which were injected with rabbit IgG to the recombinant protein, showed clear deposition of rabbit IgG, but not murine C3, to murine epidermal BMZ, although no blisters were seen in either passive transfer model or active disease model. These results are exactly the same as our results.

However, in their active disease model, the skins from a few mice showed faint IgG deposits at the epidermal BMZ and all mice showed circulating IgG antibodies to the basal keratinocyte at the epidermal BMZ, which were different from the results in our study. Although the real reason for these differences is unknown, the difference in size between the recombinant proteins used in the two studies may cause them. Thus, we used a shorter murine laminin γ1 recombinant protein than that used in the study by Vafia et al., because rabbit IgG to the shorter recombinant protein showed stronger reactivity to the mice epidermal BMZ than that to the longer one in our study (see Fig. 4b and c).

Thus, we could not obtain positive results in this study. However, we cannot exclude the possibility that antibodies to laminin γ1 C-terminus have pathogenic activity, because of the following reasons. The first point is that we used bacterial recombinant proteins, which differ from eukaryotic proteins in terms of the glycosylation profiles and folding patterns. Glycosylation may be essential for the function of laminins, since laminins are heavily glycosylated [19,20]. In addition, many different post-translational modifications exist between eukaryotic and bacterial expression systems [21]. This assumption is also supported by the fact that mouse model for anti-laminin 332 MMP using bacterial recombinant proteins has not been established [10].

The second point is that antigenicity of laminin γ1 may differ among the different tissues, e.g., epidermal BMZ or vascular BMZ. This assumption was suggested in a previous study [2]. In our study, the rabbit IgG to GST-mLmγ1c deposited to both epidermal and vascular BMZ, which is not commonly seen in the patient skin [2]. Thus, it is likely that pathogenic autoantibodies react only with unique epitopes of laminin γ1 C-terminus present in epidermal BMZ.

Thirdly, since laminins exist as trimers in vivo [22], it is also possible that antibodies to laminin trimer, but not antibodies to laminin monomer, are pathogenic. As many as 10 different laminin trimers contain laminin γ1 [22]. Therefore, native laminin trimers may be used to confirm the pathogenicity of anti-laminin γ1 antibodies in the future studies.

Lastly, it is important which region of laminin γ1 is used in animal disease model. Our previous study suggested that patients' sera reacted with laminin γ1 C-terminus [2]. In this study, we also confirmed this reactivity by immunoblot analysis of the recombinant protein of human laminin γ1 C-terminus. These results may support a hypothesis that anti-laminin γ1 autoantibodies exert their pathogenic activity by impairing laminin–integrin binding at laminin γ1 C-terminus. This assumption may be supported by the fact that antibodies to major epitopes within NC16a domain of BP180 can induce blister formation in BP mouse model [8]. From

these results, in this study, we used only antibodies to laminin γ1 C-terminus.

Moreover, Vafia et al. used 6 short recombinant proteins of laminin γ1 covering the whole molecule and demonstrated that only few anti-laminin γ1 pemphigoid sera reacted with outside of laminin γ1 C-terminal 246 amino acids, indicating that anti-laminin γ1 pemphigoid sera rarely react with epitope(s) in N-terminal domain [18].

In addition, they analyzed IgG affinity-purified from anti-laminin γ1 pemphigoid sera for its blister-inducing capacity by cryosection assay, an ex vivo model of autoantibody-induced dermal-epidermal separation [18]. For the affinity-columns, they used three different eukaryotic recombinant proteins; i.e., human laminin γ1 C-terminus, E8 fragment covering C-terminus of laminin 111 trimer and full length human laminin γ1 [18]. In this study, none of the three affinity-purified IgG samples induced dermal-epidermal separation. These results might indicate that pathogenic epitopes are not present even on eukaryotic recombinant proteins or trimer, although there is still possibility that pathogenic patient antibodies may react with epidermal BMZ-specific laminin γ1.

By indirect IF of normal mouse skin sections, rabbit IgG to GST-mLmγ1c strongly reacted with epidermal BMZ at titer up to 1:81,920. Unexpectedly, rabbit IgG to GST-mLmγ1-E8, which is longer than GST-mLmγ1c, showed relatively weak reactivity as titer up to 1:160–640. These results suggest that shorter recombinant protein of laminin γ1 C-terminus can effectively produce antibodies. The reason for this phenomenon is unknown. However, antibodies may efficiently be produced only to epitopes in the most C-terminal domain. Alternatively, unknown steric hindrance in longer recombinant protein may mask the epitopes. However, in this study, even high titer rabbit IgG to shorter GST-mLmγ1c could not induce blisters in mice.

In active disease model, the mice immunized with GST-mLmγ1c did not show deposition either murine IgG or murine C3, in spite of repeated immunizations. The reason is not known why rabbit IgG to recombinant protein could bind to epidermal BMZ of injected mice, while no murine IgG deposition was found in mice immunized with the same recombinant protein. However, we may speculate that immune tolerance to murine laminin γ1 in immunized mice inhibits the production of antibodies to native protein in the skin sections in active disease model, while immunized rabbit could produce antibodies to react with native epitopes of laminin γ1 in the mouse skin sections in passive transfer model.

Another discrepant result in the active disease model study was that sera from the mice recognized laminin γ1 by immunoblotting, but not by indirect IF of mouse skin sections. This may also be explained the same theory that the mouse antibodies can react with degenerated epitopes in immunoblotting, but not with native epitopes in the skin section.

Complement deposition at epidermal BMZ and neutrophilic infiltration in the upper dermis are characteristic features in anti-laminin γ1 pemphigoid [23–26]. Therefore, complement activation and neutrophilic infiltration may be critical processes in anti-laminin γ1 pemphigoid, while complement activation is not necessary for blister formation in animal model for anti-laminin 332 MMP [27]. However, in our passive transfer model, despite the clear IgG deposition at epidermal BMZ, neither complement deposition nor neutrophilic infiltration was seen. The previous passive transfer studies for BP and EBA showed deposition of both rabbit IgG and murine C3 at the epidermal BMZ [8,11]. At the present, the reason why antibodies to laminin γ1 cannot activate complements is unknown. Further studies will be required to develop animal models, in which complement activation and neutrophil infiltration occur.

In conclusion, our mouse models using bacterial recombinant protein of murine laminin γ1 C-terminal 107 amino acids did not induce blister formation, even though direct IF of mouse skin in the passive transfer model showed clear IgG deposition at the epidermal BMZ. Similar results were obtained by other group using bacterial recombinant protein of murine laminin γ1 C-terminal 246 amino acids. To overcome this difficulty, as an immunogen, we may use native laminin γ1 from mammalian skin. Identification of target laminin trimer recognized by anti-laminin γ1 pemphigoid patient sera is also one of the future research directions.

Acknowledgments

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Anti-Alpha-2-Macroglobulin-Like-1 Autoantibodies Are Detected Frequently and May Be Pathogenic in Paraneoplastic Pemphigus

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Paraneoplastic pemphigus (PNP) shows autoantibodies mainly to plakin and desmosomal cadherin family proteins. We have recently identified alpha-2-macroglobulin-like-1 (A2ML1), a broad range protease inhibitor, as a unique PNP antigen. In this study, we tested a large number of PNP sera by various methods. Forty (69.0%) of 58 PNP sera recognized A2ML1 recombinant protein expressed in COS7 cells by immunofluorescence (IF) and/or immunoprecipitation (IP)/immunoblotting (IB). IP/IB showed higher sensitivity than IF. In addition, 22 (37.9%) PNP sera reacted with A2ML1 by IB of cultured normal human keratinocytes (NHKs) under non-reducing conditions. Statistical analyses using various clinical and immunological data showed that the presence of anti-A2ML1 autoantibodies was associated with early disease onset and absence of ocular lesions. Next, to investigate the pathogenic role of anti-A2ML1 antibody, we performed additional functional studies. Addition of anti-A2ML1 polyclonal antibody to culture media decreased NHK cell adhesion examined by dissociation assay, and increased plasmin activity detected by casein zymography, suggesting that anti-A2ML1 antibody may decrease NHK cell adhesion through plasmin activation by inhibition of A2ML1. This study demonstrates that autoantibodies to A2ML1 are frequently and specifically detected and may have a pathogenic role in PNP.

INTRODUCTION

Paraneoplastic pemphigus (PNP) is an autoimmune multi-organ syndrome with intractable stomatitis and polymorphous cutaneous lesions (Anhalt et al., 1990), and underlying neoplasias are mainly lymphoproliferative tumors (Wolff

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Abbreviations: A2ML1, alpha-2-macroglobulin-like-1; AP, alkaline phosphatase; BP, bullous pemphigoid; cDNA, complementary DNA; Dsg, desmoglein; EPL, envoplakin; ESTs, expressed sequence tags; HRP, horseradish peroxidase; IB, immunoblotting; IF, immunofluorescence; IP, immunoprecipitation; IP–IB, immunoprecipitation and immunoblotting; NHK, normal human keratinocyte; PA, plasminogen activator; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PF, pemphigus foliaceus; PIC, protease inhibitor cocktail; PNP, paraneoplastic pemphigus; PPL, periplakin; PV, pemphigus vulgaris; RP, recombinant protein

et al., 1999; Hsiao et al., 2001; Kimyai-Asadi and Jih, 2001; Mimouni et al., 2002).

PNP sera recognize various autoantigens by immunoprecipitation (IP) or immunoblotting (IB) (Oursler et al., 1992; Hashimoto et al., 1995). IgG antibodies in PNP sera typically bind to plakin family proteins, including desmoplakins I and II, BP230, periplakin (PPL) (Kiyokawa et al., 1998), and envoplakin (EPL) (Ruhrberg et al., 1996; Kim et al., 1997; Kiyokawa et al., 1998), as well as desmoglein 3 (Dsg3) and/or Dsg1 (Hashimoto et al., 1995; Amagai et al., 1998). Finally, we have recently identified the 170 kDa antigen as alpha-2-macroglobulin-like-1 (A2ML1) (Schepens et al., 2010). A2ML1 is a broad range protease inhibitor belonging to alpha-2-macroglobulin family. Northern blot and reverse transcriptase-PCR studies revealed A2ML1 messenger RNA in epidermal keratinocytes.

Keratinocyte differentiation was found to associate with increased expression levels of A2ML1 (Galliano et al., 2006; Schepens et al., 2010). By immunohistochemistry, A2ML1 was detected within keratinocytes between the uppermost granular layers and the cornified cell layers of the epidermis. Specifically, A2ML1 is secreted into the extracellular space, and inhibits in vitro various proteases, including chymotrypsin, papain, thermolysin, and subtilisin A (Galliano et al., 2006). In addition, A2ML1, in analogy with alpha-2-macroglobulin, is thought to have a role in defense mechanisms,

maintenance of epidermal homeostasis, and regulation of cytokine and growth factor releases (Galliano *et al.*, 2006).

However, the prevalence of autoantibodies to A2ML1 was not studied in large series of PNP sera, and clinical and immunological significance and pathogenic role of anti-A2ML1 autoantibodies in PNP are still unknown (Schepens *et al.*, 2010). In this study, we have systematically examined 58 PNP sera by various techniques to search for the presence of anti-A2ML1 autoantibodies, and performed statistical analysis between the presence of anti-A2ML1 antibodies and clinical and immunological characteristics. Furthermore, we performed dissociation assay and protease assay to investigate the pathogenic role of anti-A2ML1 antibody.

RESULTS

A2ML1 was expressed in the normal epidermis but not in the rat bladder

Expression profile of A2ML1 in the skin and rat bladder was verified by immunofluorescence (IF) using anti-A2ML1 mouse polyclonal antibody (pAb). A2ML1 was expressed in the granular layers of the normal human epidermis (Figure 1a), as described previously (Galliano *et al.*, 2006; Schepens *et al.*, 2010). In contrast, A2ML1 was not expressed in the rat bladder, whereas IgG antibodies in PNP serum clearly reacted with transitional epithelia (Figure 1b).

COS7 cells expressing A2ML1 recombinant protein (RP) were recognized by PNP sera in IF

We then assessed the reactivity of all 58 PNP sera by IF using transiently transfected COS7 cells expressing c-Myc-tagged human A2ML1 RP. Both anti-c-Myc rabbit pAb and anti-A2ML1 pAb stained the cytoplasm of transfected COS7 cells in a granular pattern (Figure 2a). The obtained signals showed perfect colocalization with those by PNP sera, indicating that the PNP sera recognized A2ML1 (Figure 2a). In the IF study for all 58 PNP sera, 30 (51.7%) sera recognized A2ML1 RP on COS7 cells (Figure 2b).

c-Myc-tagged recombinant A2ML1 RP was detected inside of transfected COS7 cells but not in culture media

Proteins in COS7 cells transfected with A2ML1 complementary DNA (cDNA) were separated into four different fractions; i.e., culture medium, washing buffer (cold PBS (phosphate-buffered saline)) extract, IP buffer extract, and final pellet. Although protein bands corresponding to the 172 kDa A2ML1 RP (including c-Myc tag) were seen only faintly in IP buffer extract and pellet by protein staining with amido-black, IB using anti-c-Myc pAb clearly showed the RPs in IP buffer extract and pellet but not in culture medium or washing buffer extract (Figure 3a). Additional experiments confirmed that the same A2ML1 RP in IP buffer extract was detected by both anti-A2ML1 pAb and anti-c-Myc pAb (Figure 3b).

IgG anti-A2ML1 autoantibodies were detected in PNP sera by IP and IB (IP–IB) of COS7 cells transfected with A2ML1 cDNA

By preliminary IB studies of either A2ML1-expressing COS7 cell extracts or normal human epidermal extracts under standard reducing condition, none of PNP, pemphigus vulgaris (PV), pemphigus foliaceus (PF), bullous pemphigoid (BP), or normal control sera detected A2ML1 proteins (data not shown), confirming the results of our previous studies that PNP sera do not react with A2ML1 in IB under standard reducing conditions.

Then, we examined all 58 PNP sera, diseases control sera, and normal sera by IP–IB of extracts of A2ML1 RP-expressing COS7 cells. Proteins immunoprecipitated with sera were separated on SDS-PAGE and immunoblotted with anti-c-Myc pAb. In this study, 35 (60.3%) of 58 PNP sera reacted with c-Myc-tagged A2ML1 RP (Figure 3c). None of the 10 sera each from PV, PF, and BP patients and 30 normal human sera reacted with A2ML1 RP (Figure 3c).

PNP sera recognized A2ML1 in differentiated cultures of normal human keratinocytes (NHKs) by IB under non-reducing condition

To examine whether PNP sera detect disulfide bond–related conformational epitopes, we performed IB of NHK extracts

a Normal human skin   **b**   Rat bladder

A2ML1 pAb | A2ML1 pAb | PNP serum
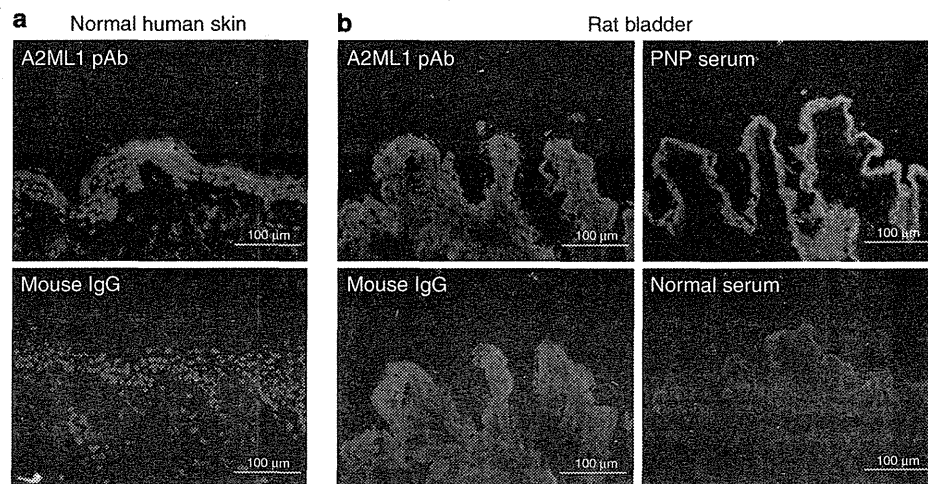
Mouse IgG | Mouse IgG | Normal serum

Figure 1. Immunofluorescence (IF) for expression of alpha-2-macroglobulin-like-1 (A2ML1) in the human skin and rat bladder. (a) IF of normal human skin sections. (b) IF of rat bladder sections. Anti-A2ML1 mouse polyclonal antibody (A2ML1 pAb), purified normal mouse IgG, paraneoplastic pemphigus (PNP) serum, and normal human serum were used. Bar = 100 μm.
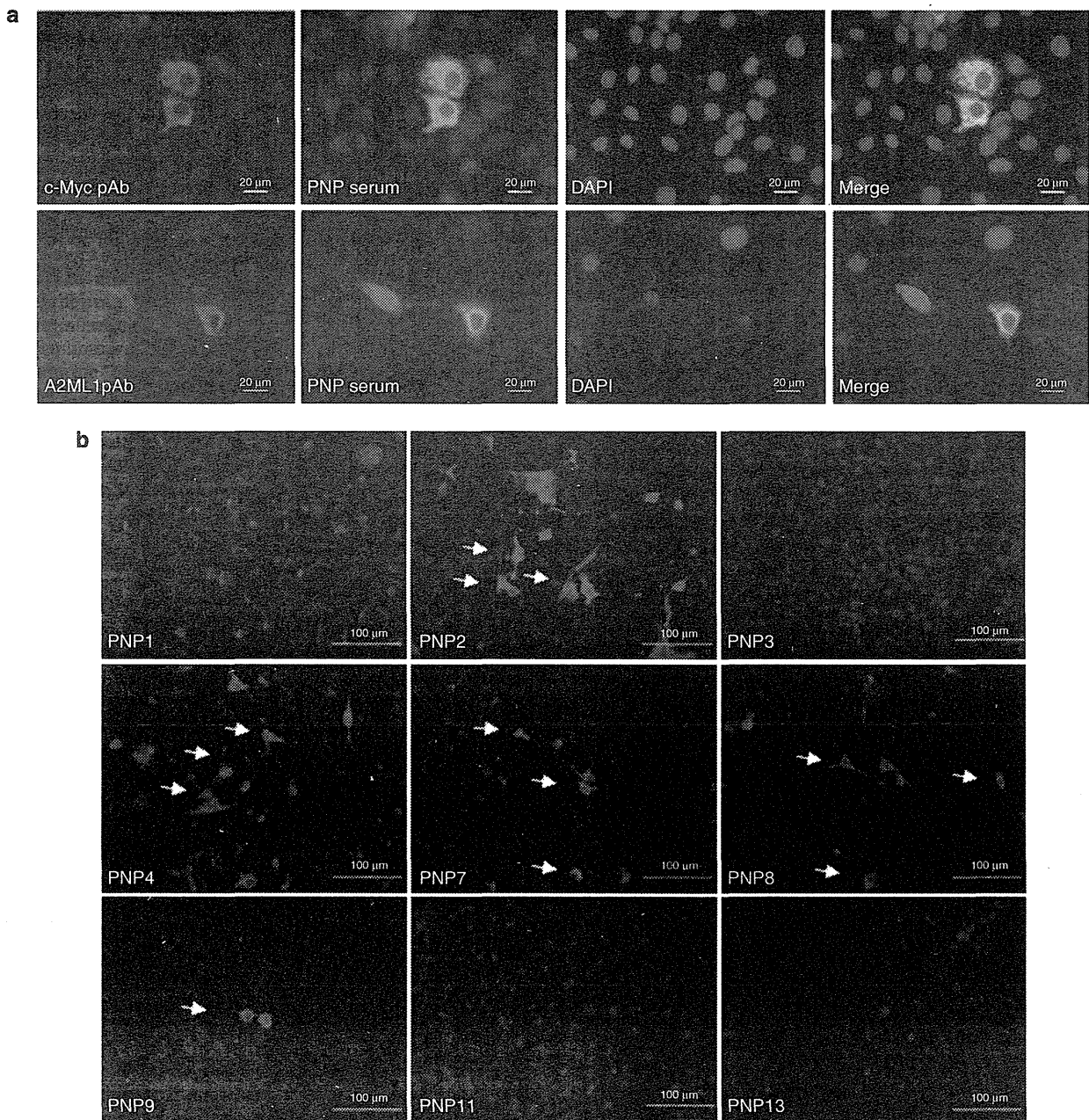
— 129 —

Figure 2. Immunofluorescence (IF) studies of alpha-2-macroglobulin-like-1 (A2ML1) complementary DNA (cDNA)-transfected COS7 cells. (a) IF of COS7 cells expressing c-Myc-tagged A2ML1, triple-stained with a representative paraneoplastic pemphigus (PNP) serum and 4',6-diamidino-2-phenylindole (DAPI), as well as with either anti-c-Myc pAb or anti-A2ML1 mouse polyclonal antibody (A2ML1 pAb). Bar = 20 μm. (b) IF of A2ML1-expressing COS7 cells, double-stained with 58 PNP sera and DAPI. The case numbers are the same as the case numbers in immunoprecipitation and immunoblotting (IP–IB) figure and Supplementary Table S1 online. PNP2, PNP4, PNP7, PNP8, and PNP9 showed positive reactivity, while PNP1, PNP3, PNP11, and PNP13 were negative. Positive cells are indicated by arrows. Bar = 100 μm.

under both reducing and non-reducing conditions for all 58 PNP sera. Twenty-two (37.9%) PNP sera recognized a protein comigrating with A2ML1 detected by anti-A2ML1 pAb under non-reducing condition (Figure 3d). None of the 5 PV sera, 5 PF sera, 5 BP sera, and 15 normal human sera showed this reactivity (Figure 3d). In contrast, under reducing condition, none of the same set of PNP and control sera recognized the

170 kDa A2ML1 protein band, which was strongly detected by anti-A2ML1 pAb (Figure 3e).

Statistical comparison of clinical and immunological conditions between anti-A2ML1 antibody–positive and –negative PNP patients
We statistically analyzed the relationship between the presence of anti-A2ML1 autoantibodies and various clinical and
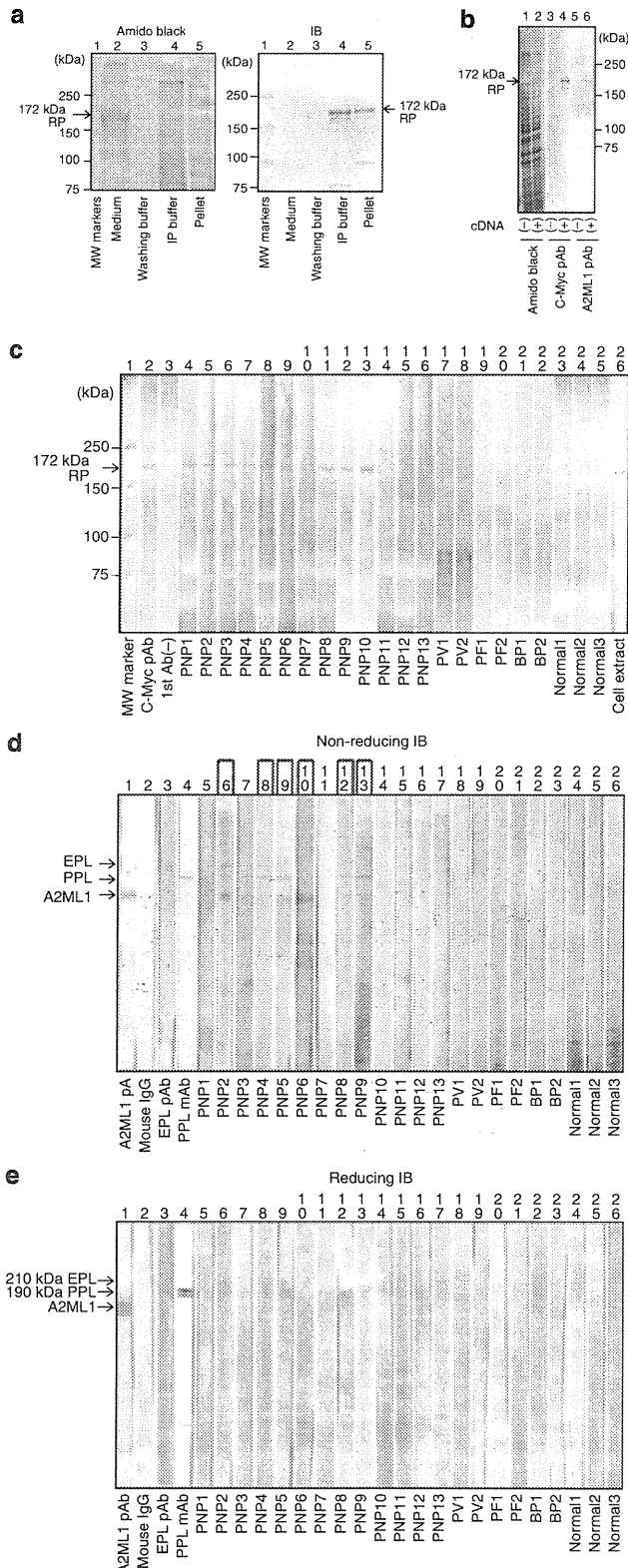
immunological findings in PNP (Figure 4 and Table 1). The 40 PNP sera (69.0%) with positive reactivity in IP–IB and/or COS7 cell IF were considered as anti-A2ML1 antibody-positive (A2ML1-positive). A2ML1-postive PNP cases had significantly earlier disease onset than A2ML1-negative PNP cases ($P=0.026$) (Figure 4). Interestingly, ocular mucous membrane lesions occurred less frequently in A2ML1-postive PNP cases ($P=0.050$) (Figure 4). There was no significant correlation of the presence of anti-A2ML1 antibodies with any other parameters (Figure 4 and Table 1). In Supplementary Table S1 online, relevant clinical parameters and the results for all studies of anti-A2ML1 antibodies are summarized.

A2ML1 was present in NHK culture medium

As the first step of functional studies of anti-A2ML1 antibody, we examined the presence of A2ML1 in NHK culture medium by IB of NHKs at low and high levels of calcium. No A2ML1 was detected in non-concentrated NHK culture media (data not shown). In 100 × concentrated culture media, A2ML1 was detected in NHKs cultured at both low and high calcium levels (Figure 5a). A2ML1 was also detected in NHK extracts weakly at low calcium and strongly at high calcium (Figure 5a), as described previously (Galliano *et al.*, 2006).

Anti-A2ML1 pAb bound to A2ML1 in NHK culture media

To examine whether anti-A2ML1 pAb actually bound to A2ML1 in culture media, we performed IB of NHK culture media treated with Protein G agarose. After IP with Protein G, A2ML1 was detected in NHK culture medium incubated with normal mouse IgG, but not with anti-A2ML1 pAb (Figure 5b).

Figure 3. Immunoblotting (IB) study of four fractions of COS7 cells, immunoprecipitation and immunoblotting (IP–IB) of alpha-2-macroglobulin-like-1 (A2ML1) complementary DNA (cDNA)–transfected COS7 cells for various sera, and IB of normal human keratinocyte (NHK) extracts under non-reducing and reducing conditions. (a) Blots of four fractions obtained from A2ML1 cDNA–transfected COS7 cells, stained with amido-black (left panel), and processed for IB for anti-c-Myc pAb (right panel). (b) IP buffer extracts of COS7 cells transfected with or without A2ML1 cDNA were stained with amido-black, and processed for IB using both anti-c-Myc pAb and anti-A2ML1 mouse polyclonal antibody (A2ML1 pAb). (c) IP buffer extracts of transfected COS7 cells were immunoprecipitated with the representative paraneoplastic pemphigus (PNP) sera, disease control sera, and normal sera, as well as anti-c-Myc pAb, and immunoblotted with anti-c-Myc pAb. The A2ML1 recombinant protein (RP) was detected by sera of PNP1–PNP10 (lanes 4–13), but not by sera of PNP11–PNP13 (lanes14–16). In lane 26, IP buffer extract of transfected COS7 cell was directly stained by IB with anti-c-Myc pAb. The positions of molecular weight (MW) markers and A2ML1 RP are shown by bars and arrows, respectively, in the left of each panel. (d) The results of IB under non-reducing condition. MWs are not shown, because MW markers cannot be used in this condition. The PNP sera with numbers surrounded by squares, but no control sera, reacted with the protein band corresponding to A2ML1. Some PNP sera also reacted with envoplakin (EPL) and periplakin (PPL). (e) IB under reducing condition. No sera reacted with the 170 kDa A2ML1, while some PNP sera reacted with EPL or PPL. Positions of EPL, PPL, and A2ML1 are shown in the left of each panel. BP, bullous pemphigoid; PF, pemphigus foliaceus.
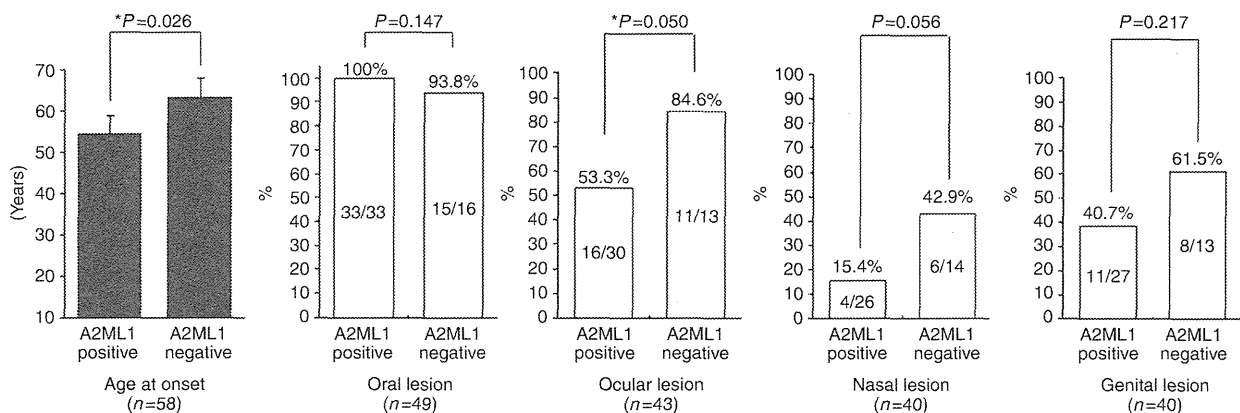
Figure 4. Influence of the presence of anti-alpha-2-macroglobulin-like-1 (A2ML1) antibodies on age at onset of paraneoplastic pemphigus (PNP) and the appearance of various mucosal lesions. Statistically significant differences were seen in age at disease onset and ocular mucosal lesion, but not in oral, nasal, or genital mucosal lesions. *P = 0.05 (after Bonferroni adjustment).

Table 1. Clinical and immunological features in 58 PNP cases, differences between anti-A2ML1-antibody–positive and –negative PNP cases, and statistical analyses

Parameters (n = cases with description)	A2ML1 positive	A2ML1 negative	Total
Gender (n = 53)			
Females	24/36 (66.7%)	11/17 (64.7%)	35/53
Males	12/36 (33.3%)	6/17 (35.3%)	18/53
Cutaneous lesions			
Trunk (n = 48)	21/33 (63.6%)	10/15 (66.7%)	31/48
Extremities (n = 49)	18/33 (54.5%)	10/16 (62.5%)	28/49
Bronchiolitis obliterans (n = 58)	7/40 (17.5%)	3/18 (22.2%)	10/58
Castleman's disease (n = 58)	6/40 (15.0%)	2/18 (11.1%)	8/58
Dsg1, indices (n = 58) cutoff index: >20	26.52±38.78	11.86±21.99	21.96±34.93
Dsg3, indices (n = 58) cutoff index: >20	86.14±76.47	75.95±70.70	82.97±74.26
Human skin indirect IF (n = 58)	30/40 (75.0%)	14/18 (77.8%)	44/58
Rat bladder indirect IF (n = 58)	29/40 (72.5%)	11/18 (61.1%)	40/58
Acantholysis (n = 43)	17/27 (63.0%)	6/16 (37.5%)	23/43
Necrotic cells (n = 43)	12/27 (44.4%)	4/16 (25.0%)	16/43
Liquefaction degeneration (n = 43)	4/27 (14.8%)	3/16 (18.8%)	7/43
Good response to therapies (n = 48)	16/32 (50.0%)	8/16 (50.0%)	24/48
Outcome (n = 43)			
Alive	11/30 (36.7%)	3/13 (23.1%)	14/43
Dead	19/30 (63.3%)	10/13 (76.9%)	29/43

Abbreviations: A2ML1, alpha-2-macroglobulin-like-1; Dsg, desmoglein; IF, immunofluorescence; PNP, paraneoplastic pemphigus.

Anti-A2ML1 pAb reduced cell–cell adhesion of cultured NHKs

We determined the effect of anti-A2ML-1 pAb on NHK cell adhesion by dissociation assay. Remarkable increase in fragmentation of culture sheets of NHKs was induced by incubation with anti-A2ML1 pAb, but not with anti-PPL mAb or normal mouse IgG (Figure 5c).

Anti-A2ML1 pAb increased plasmin activity in NHK culture medium

Although we tried to detect protease activity using colorimetric protease assay kit, the sensitivity is too low to detect the protease activity (data not shown). Then, we examined protease activity in culture medium by casein gel zymography. Increased serine protease activity, corresponding to plasmin, was detected in culture medium of NHK incubated with anti-A2ML1 pAb, but not with anti-PPL mAb, normal mouse IgG, or no antibody addition (Figure 5d). No activity of other proteases was detected.

DISCUSSION

In this study, double-staining IF of COS7 cells transiently transfected with human A2ML1 cDNA confirmed the specific reactivity of PNP sera with A2ML1. By this IF, 30 (51.7%) of 58 PNP sera reacted with A2ML1-expressing COS7 cells. By IP–IB using lysates of A2ML1-expressing COS7 cells, A2ML1 was detected by 35 (60.3%) of 58 PNP sera, but not by any disease and normal control sera. Precisely, the IF showed negative results in 10 IP–IB–positive sera, and conversely positive results in 5 IP–IB–negative sera. Thus, 40 (69.0%) of 58 PNP sera recognized A2ML1, when the results in IP–IB and IF studies of A2ML1-expressing COS7 cells were combined.

This frequency of anti-A2ML1 antibodies is higher than the positive reactivity in 10 (50%) of 20 PNP sera in our previous IP–IB study (Schepens et al., 2010). The detection rate of about 70% is similar to those obtained by conventional IP with radiolabeled-cultured NHK extracts in previous case reports and smaller population systematic studies (Nishibori et al., 1995; Izaki et al., 1996; Mahler et al., 1998; Williams et al., 2000; Inaoki et al., 2001; Fujimoto et al., 2002; Mimouni et al., 2002; Martinez De Pablo et al., 2005; Marzano et al., 2005; Santi et al., 2005; Lee et al., 2006).

Thus, by using a larger series of unselected PNP sera, this study unequivocally and independently confirms that A2ML1 is one of the major PNP antigens. Although the sensitivity of IF of A2ML1-expressing COS7 cells was slightly lower than that of IP–IB, the easier IF may become a useful method to detect anti-A2ML1 autoantibodies after adequate technical improvements.