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Letter to the Editor

N-linked glycosylation on laminin γ1 influences recognition of anti-laminin γ1 pemphigoid autoantibodies

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Anti-laminin γ1 pemphigoid; Autoantibody; Laminin γ1; N-linked glycosylation; Epitope

After the two original cases of anti-p200 pemphigoid [1,2], many similar cases were reported [3]. After many failures in autoantigen identification [4], mass-spectrometry identified laminin (LM) γ1, proposing the name of anti-LMγ1 pemphigoid [5,6]. However, subsequent studies failed to show pathogenic role [7], or suggested other pathogenic antibodies [8].

Detection of LMγ1 by immunoblotting (IB) of normal human dermal extract (NHDE) is diagnostic hallmark [5,6]. However, patient sera reacted with epidermal basement membrane zone, but not blood vessel walls, and some sera failed to react with LMγ1 recombinant protein (RP) in LM111 and LM211/221 trimers [6]. This unique recognition of anti-LMγ1 autoantibodies may be caused by epitopes specific in epidermal basement membrane zone, which are produced by different glycosylation or conformation.

In this study, to further characterize anti-LMγ1 autoantibodies, we examined reactivity of both anti-LMγ1-specfic antibodies and patient sera with LMγ1 in various antigen sources with or without deglycosylation. All information for the materials and methods are described in supplementary data. We collected 24 patient sera, all of which reacted with LMγ1 in IB of NHDE (Fig. 1a and Table S1).

We first characterized LMγ1 produced by four different keratinocyte cell lines (HeLa cells, HaCaT cells, DJM-1 cells and normal human keratinocytes) and one lung cancer cell line (A549). Immunofluorescence (IF) using rabbit polyclonal antibody (pAb) raised against mouse LMγ1 N-terminal domain RP showed positive reactivity in all cell lines (Fig. 1b).

IB of the 5 cell lines using anti-LMγ1 monoclonal antibody (mAb) detected LMγ1 molecules with various molecular weights (MWs) in cell lysates, and those with similar and higher MWs in culture supernatants (Fig. 1c). The mAb also reacted with LMγ1 in both LM521 and LM411 trimer RPs, which showed identical MW to LMγ1 in NHDE (Fig. 1d).

Because LMγ1 is an N-linked glycosylation protein [4,9], we next examined glycosylation condition of LMγ1 in various antigen sources. After deglycosylation with PNGase F, all LMγ1 molecules in both cell lysates and culture supernatants became smaller to molecules with almost identical MWs (Fig. 1e). LMγ1 molecules in deglycosylated LM521 and LM411 RPs also became smaller to identical MWs (Fig. 1f). These results indicated that N-linked glycosylation was the main cause for different MWs of LMγ1 among various antigen sources.

We then examined the reactivity of patient sera by the same methods. Unexpectedly, no patient sera showed positive reactivity in IF of 5 cell lines (Fig. 1b, representative data). In addition, in IB of 5 cell lines, selected 11 and 17 patient sera did not react with LMγ1 either in cell lysates or culture supernatants (Fig. S1a, b and Table S1). In contrast, 20 (83%) and 18 (75%) of 24 patient sera reacted with LMγ1 in LM521 and LM411 RPs, respectively (Fig. S1c and Table S1).

To examine whether N-linked glycosylation influences LMγ1 recognition by patient sera, we examined patient sera by IB of various antigen sources with or without deglycosylation. None of 11 patient sera reacted with deglycosylated LMγ1 in either cell lysates or culture supernatants of any cell lines (Fig. S1d, e and Table S1).

In contrast, most patient sera reacted with both non-deglycosylated and deglycosylated LMγ1 molecules in LM521 RP (Fig. 2a and Table S1). Intensity of patient sera with deglycosylated LMγ1 was significantly stronger than non-deglycosylated LMγ1 in LM521 RP ($p = 0.005$) (Fig. 2b). The ratios of intensity (deglycosylated/non-deglycosylated) are also shown (Fig. 2c).

IB of non-deglycosylated and deglycosylated LM411 RPs was also performed using serum dilution of 1:400. Two patient sera examined also showed stronger reactivity to deglycosylated LMγ1 (Fig. S2 and Table S1). These results suggested that N-linked glycosylation inhibited recognition of patient sera to LMγ1 in LM521 and LM411 RPs. Intriguingly, LMγ1 pAb reacted more strongly with non-deglycosylated LMγ1 in both LM521 and 411 RPs (Figs. 2a, c and S2a, c).

We also performed IB of NHDE with or without deglycosylation. Most patient sera reacted with both non-deglycosylated and deglycosylated LMγ1 molecules in NHDE (Fig. 2d and Table S1). Intensity of patient sera to deglycosylated LMγ1 was significantly weaker than non-deglycosylated LMγ1 in NHDE ($p < 0.001$) (Fig. 2e). The ratios of intensity (deglycosylated/non-deglycosylated) are also shown (Fig. 2f). These results indicated that patient sera reacted more strongly with non-deglycosylated LMγ1 in NHDE. The same reactivity was also shown by the rabbit pAb (Fig. 2d and f).

To elucidate further the epitopes on molecular basis, we prepare a novel RP of C-terminal domain of LM511 with mutations on the three N-linked glycosylation sites (LM511E8/γΔNG), because most patient sera recognize C-terminal domain of LMγ1 [5]. By IB, we compare the reactivity of patient sera between LM511E8/γΔNG and wild type RP (LM511E8/wild) (Fig. 2g and

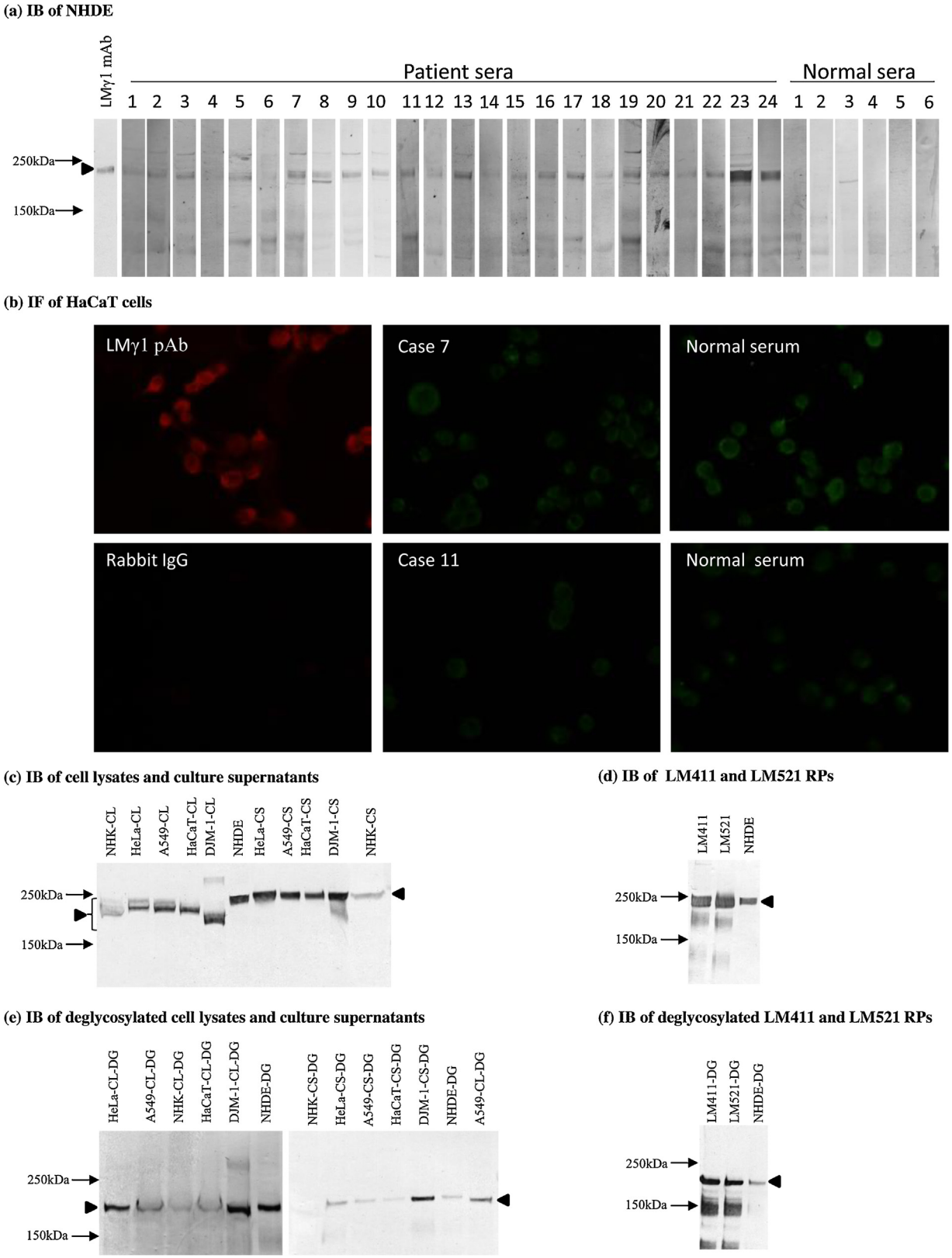

Fig. 1. Detection of anti-LMγ1 antibodies in patient sera and the studies of LMγ1 in various antigen sources. (a) Immunoblotting (IB) of normal human dermal extract (NHDE) for all patient sera. (b) Immunofluorescence (IF) of HaCaT cells for patient sera. Normal rabbit IgG was used as negative control. (c) IB of cell lysates (CL) and culture supernatants (CS) of various cell lines. (d) IB of LM521 and LM411 trimer RPs. (e) IB of cell lysates (CL) and culture supernatants (CS) of various cell lines before and after deglycosylation. (f) IB of LM521 and LM411 trimer RPs before and after deglycosylation. MWs are shown in the left. Closed arrowheads indicate the position of LMγ1 protein bands. NHK, normal human keratinocyte; DG, deglycosylated.