High Expression of Collagen XVII Compensates for its Depletion Induced by Pemphigoid IgG in the Oral Mucosa

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The basement membrane zone consists of multiple components, including collagen XVII (COL17), which is the target of bullous pemphigoid. To our knowledge, no research has addressed the differences in basement membrane zone components between the skin and oral mucosa; therefore, we investigated the basement membrane zone proteins, with a focus on COL17. The mRNA and protein expression levels of COL17 were significantly higher in oral keratinocytes than in skin keratinocytes. Hemidesmosomal COL17 expression was markedly higher in oral keratinocytes than in skin keratinocytes, and its level was associated with adhesion strength. Oral keratinocytes adhered to the extracellular matrix more tightly than did skin keratinocytes in vitro. Based on these results, we attempt to explain the clinical diversity of bullous pemphigoid. COL17 depletion was more prominent in skin keratinocytes than in oral keratinocytes after treatment with COL17-NC16A mAbs, which have in vivo pathogenicity. COL17 C-terminus mAbs, which are not pathogenic, facilitated COL17 depletion in combination treatment with COL17-NC16A mAbs in both types of keratinocytes. In summary, the greater amount of COL17 in oral keratinocytes than in skin keratinocytes is associated with the higher strength of oral keratinocyte hemidesmosomal adhesion at the basement membrane zone. Our results may explain why bullous pemphigoid blistering tends to be more prevalent in the skin than in the oral mucosa.

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INTRODUCTION

Both skin and oral mucosa have several important functions, such as providing a barrier, thermoregulation, sensation, and immunity to maintain homeostasis of the body. However, distinct differences exist between the skin and the oral mucosa. Structurally, the skin has a keratinized epidermis and has hair follicles and sweat glands in the dermis. In contrast, most oral mucosa consists of nonkeratinized epithelia, with salivary glands in the lamina propria (Stephens and Genever, 2007). Although keratins 5 and 14 are expressed in both types of basal cells, keratins 4 and 13 are mainly expressed in the oral mucosa, and keratins 1 and 10 are mainly expressed in the skin (Shetty and Gokul, 2012). Secretory IgA plays an important role in mucosal immunity (Brandtzaeg, 2013). Isolated oral keratinocytes show accelerated migration and greater proliferation than skin keratinocytes (Drukała et al., 2005; Kim et al., 2013; Shetty and Gokul, 2012; Turabelidze et al., 2014). However, no research has focused on the differences between the skin and the oral mucosa in terms of their epithelial basement membrane zone (BMZ) components.

The BMZ can be differentiated by transmission electron microscopy into four major subregions (Borradori and Sonnenberg, 1999): (i) the plasma membrane of the basal keratinocytes, (ii) the lamina lucida, (iii) the lamina densa, and (iv) the sublamina densa. Within the BMZ, multiprotein complexes called hemidesmosomes anchor keratinocytes to the underlying basement membrane (Borradori and Sonnenberg, 1999; Green and Jones, 1996; Jones et al., 1998; Walko et al., 2015). Classic type I hemidesmosomes are found in the epidermis, and they consist of six major components: BP230, plectin, CD151, integrin α6, integrin β4, and collagen XVII (COL17, also called BP180) (Walko et al., 2015). Laminin 332 and collagen IV are extracellular matrix (ECM) proteins that are located in the lamina densa (Aumailley and Smyth, 1998; Sugawara et al., 2008). Collagen VII (COL7), which is a major component of anchoring fibrils, is found under the lamina densa (Keene et al., 1987; Woodley et al., 1984). In hemidesmosome assembly, transmembrane COL17 interacts extracellularly with integrin α6 (Hopkinson et al., 1998) and laminin 332 (Nishie et al., 2011; Tasanen et al., 2004) and intracellularly with BP230 (Hopkinson and Jones, 2000; Koster et al., 2003), plectin (Koster et al., 2003; Natsuga et al., 2017) and integrin β4 (Aho and Uitto, 1998; Hopkinson et al., 1998; Hopkinson and Jones, 2000). In the congenital skin blistering disease

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Abbreviations: BMZ, basement membrane zone; BP, bullous pemphigoid; COL17, collagen XVII; COL7, collagen VII; Dsg, desmoglein; ECM, extracellular matrix; MMP, mucous membrane pemphigoid; NC, noncollagenous; NHEK, normal human epidermal keratinocyte; NHOM, normal human oral mucosa; NHOMK, normal human oral mucosal keratinocyte; NHS, normal human skin

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epidermolysis bullosa, these BMZ proteins are defective (Fine et al., 2014). In addition, BMZ proteins are targeted by autoantibodies in several autoimmune subepidermal blistering diseases, such as COL17 for bullous pemphigoid (BP) and mucous membrane pemphigoid (MMP), COL7 for epidermolysis bullosa acquisita, and laminin 332 and integrin α6β4 for MMP (Amber et al., 2017; Bernard and Antonicelli, 2017; Murrell et al., 2015; Schmidt and Zillikens, 2013; Vorobyev et al., 2017). Among them, COL17 is targeted by both BP and MMP, each of which shows site-specific clinical phenotypes (Amber et al., 2017; Schmidt and Zillikens, 2013).

COL17 is a type II-oriented 1,497-amino acid transmembrane protein. It contains 15 collagenous domains and 16 noncollagenous (NC) domains (Giudice et al., 1992). COL17 presents in both the skin and the oral mucosa; nevertheless, no convincing evidence has explained why BP shows mainly skin manifestations. Recently, we reported that the reactivity of IgG from BP patients differs from that from MMP patients in normal human skin (NHS) and normal human oral mucosa (NHOM) (Kamaguchi et al., 2018). IgG from MMP patients preferentially reacted to the epithelial BMZ in NHOM. This difference may be caused by the targeting epitopes of autoantibodies in BP and MMP. In addition to diversity of antibodies, COL17 itself may differ in some properties between the skin and mucosa. This study explores the differences in BMZ components, which are known to be antigens targeted in autoimmune subepidermal blistering diseases. We obtained oral and skin keratinocytes from a single person; this is a great advantage for investigating differences. We show that the COL17 expression level is associated with the different hemidesmosomal adhesion strength.

RESULTS

COL17 stains more brightly in the BMZ of the oral mucosal epithelia than in the skin

First, we investigated the staining pattern of several BMZ proteins, including COL17, laminin 332, COL7, and integrin α6β4, which are targeted by autoantibodies of autoimmune subepidermal blistering diseases, using NHS and NHOM (n = 3). Histologically, the epithelium is thicker in the oral mucosa than in the skin, as previously reported (Glim et al., 2014) (Figure 1). COL17 is distributed at the bottom and the lateral-apical sides of basal keratinocytes in the skin (Hirako et al., 1998; Kitajima et al., 1992; Watanabe et al., 2017). COL17 was clearly detected at the bottom and the lateral-apical sides of oral mucosa cells, just as it was detected there in skin cells (Figure 1). However, COL17 stained brighter in the oral mucosa BMZ than in the skin BMZ (Figure 1). Integrin α6β4 stained slightly in the apical-lateral regions of the skin and the oral mucosa (Figure 1). Laminin 332 and COL7 stained only at the BMZ in both the skin and mucosa.

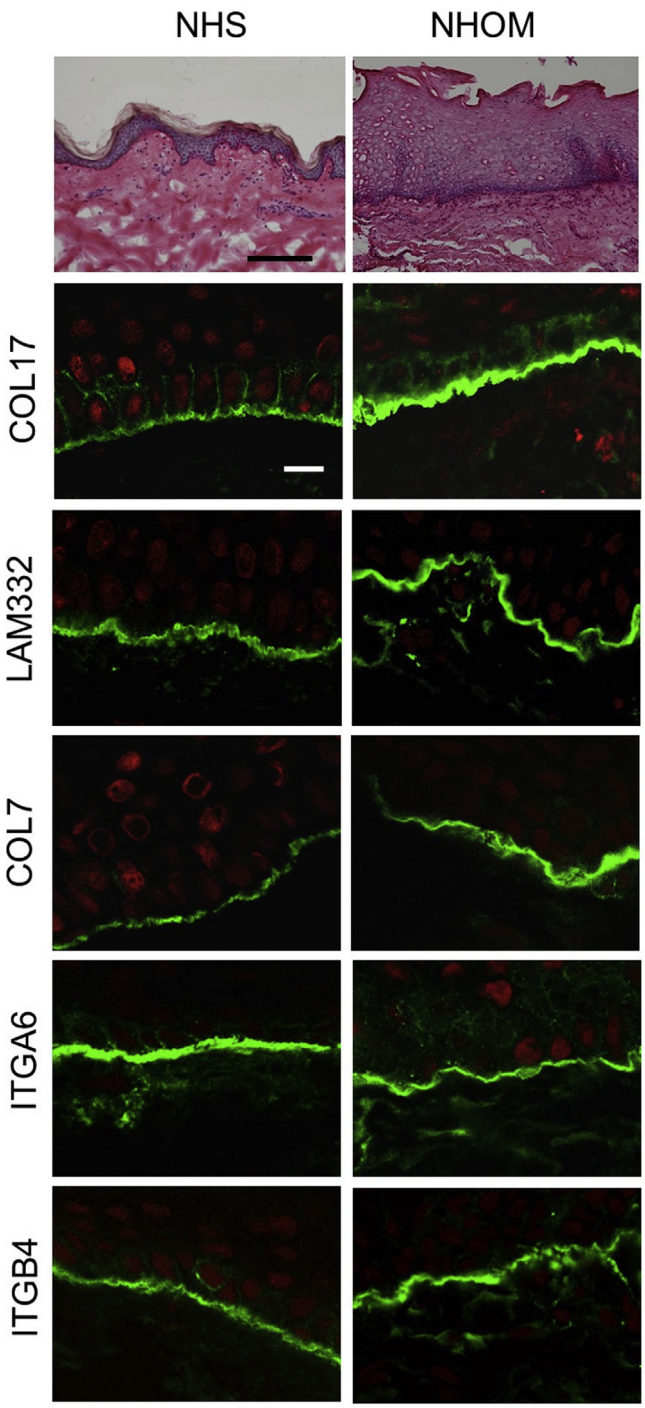

Figure 1. Immunofluorescence staining of BMZ proteins in the skin and oral mucosa. Hematoxylin and eosin staining was performed on NHS and NHOM. For immunofluorescence staining, NHS and NHOM were stained with anti-COL17, anti-laminin 332 (LAM332), anti-COL7, anti-integrin α6 (ITGA6), or anti-integrin β4 (ITGB4), followed by staining with FITC-conjugated anti-mouse, anti-rabbit, or anti-rat IgG. The nuclei were stained with propidium iodide. Images were taken by confocal laser scanning microscope (n = 3). Hematoxylin and eosin staining, scale bar = 200 μm. Immunofluorescence staining, scale bar = 10 μm. BMZ, basement membrane zone; NHOM, normal human oral mucosa; NHS, normal human skin.

Artificial splits in the oral mucosa were not along the lamina lucida

Indirect immunofluorescence using 1 mol/L NaCl-split skin is useful to differentiate autoimmune subepidermal blistering disease autoantibodies from lamina densa- and sublamina densa-targeted autoantibodies (Gammon et al., 1992). The separation in 1 mol/L NaCl-split skin runs along the intra-lamina lucida, which suggests a weakness in the lamina lucida (Masunaga et al., 1997). We introduced artificial splits

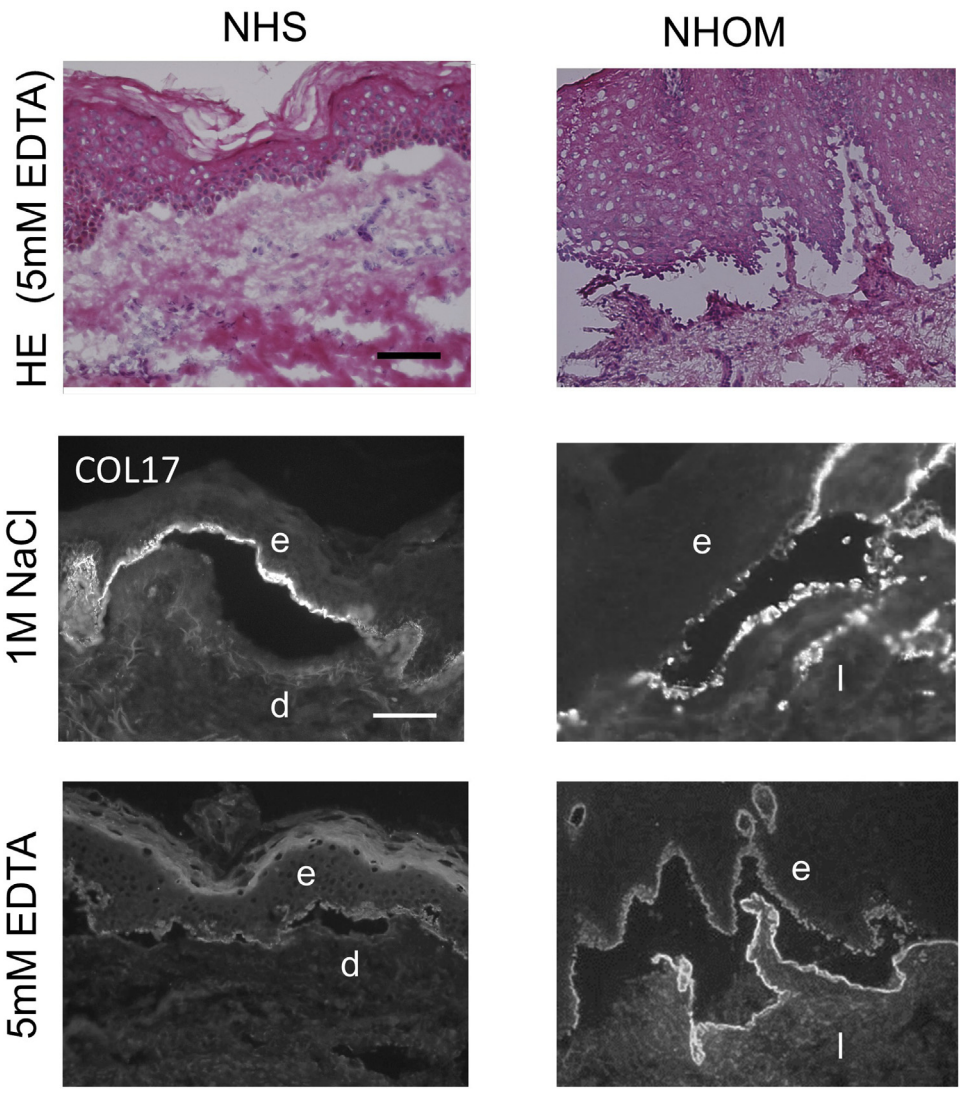

Figure 2. Artificial split formation treated with 1 mol/L NaCl or 5 mmol/L EDTA. Artificial dermal-epidermal separations on (left panels) NHS and (right panels) NHOM were created by incubation with 1 mol/L NaCl or 5 mmol/L EDTA. Hematoxylin and eosin staining and immunofluorescence staining of COL17 were performed on NHS and NHOM (n = 3). Scale bars = 100 μm. d, dermis (NHS); e, epidermis (NHS) or epithelium (NHOM); l, lamina propria (NHOM); M, mol/L; NHOM, normal human oral mucosa; NHS, normal human skin.

in NHS and NHOM treated with 1 mol/L NaCl or 5 mmol/L EDTA (n = 3). Histologically, the separation was less clear in NHOM than in NHS. Basal keratinocytes were detected on both sides of the split in NHOM (Figure 2, upper panel). COL17 stained on the epidermal side of the split in the NHS treated with 1 mol/L NaCl or 5 mmol/L EDTA (Figure 2, left panels), which is consistent with a previous report (Masunaga et al., 1997). Unexpectedly, COL17 stained on both the epithelial and the subepithelial sides of the split in NHOM treated with 1 mol/L NaCl or 5 mmol/L EDTA (Figure 2, right panels). Furthermore, it took longer for the separation to occur in NHOM than in NHS by treatment with EDTA. These results suggest that the basal keratinocytes in the oral mucosa show stronger adhesion to the basement membrane than the basal keratinocytes of the skin.

COL17A1 expression is significantly higher in NHOM keratinocytes (NHOMKs) than in normal human epidermal keratinocytes (NHEKs)

The ability of immunofluorescence to detect the precise protein levels existing in human tissues is limited. To prove the results of immunofluorescence staining, we compared the mRNA and protein expression levels using cultured keratinocytes or murine tissues. We generated normal human oral mucosal keratinocytes (NHOMKs) from healthy individuals. We first examined the expression of those keratins that are useful differentiation markers of the skin and oral mucosa (Shetty and Gokul, 2012). *KRT1* expression was higher in normal human epidermal keratinocytes (NHEKs) than in the NHOMKs, whereas *KRT13* expression was higher in the NHOMKs than in the NHEKs ($P < 0.0001$, $P = 0.0344$, respectively) (Figure 3a).

To explore the quantitative differences in the BMZ proteins (COL17, laminin 332, COL7, integrin α6β4), we compared the mRNA levels using keratinocytes (n = 3) (Figure 3a). Laminins are heterotrimer proteins that consist of α-, β-, and γ-chains. It is known that laminin 332, laminin 311, laminin 511, and possibly laminin 321 are present in the epidermal basement membrane (McMillan et al., 2006). Because γ2 is a unique chain to compose laminin 332, we selected laminin γ2 to analyze laminin 332. *COL17A1* expression was 50% higher in the NHOMKs than in the NHEKs ($P = 0.0033$). *LAMC2* and *COL7A1* expression levels were also higher in the NHOMKs than in the NHEKs (*LAMC2*: $P = 0.0258$,

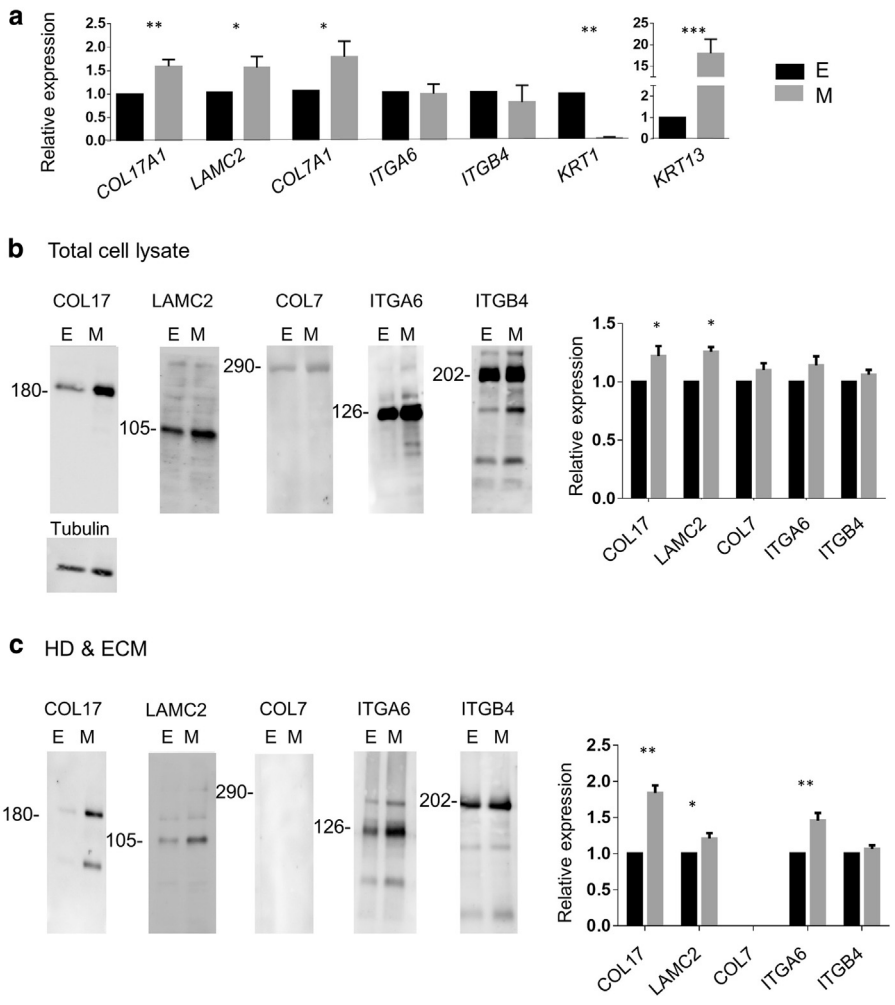

Figure 3. mRNA and protein expression of BMZ proteins in NHEKs and NHOMKs. (**a**) The gene expression levels of *COL17A1, LAMC2, COL7A1, ITGA6, ITGB4, KRT1,* and *KRT13* in NHOMKs are compared with those in NHEKs (n = 3). Immunoblotting using (**b**) total cell lysate and (**c**) hemidesmosome and ECM fraction (HD & ECM) of NHEKs and NHOMKs detecting COL17, laminin γ2 (LAMC2), COL7, integrin α6 (ITGA6), or integrin β4 (ITGB4) (n = 3). The data are expressed as the mean ± standard deviation. Student *t* test, *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$0.0001 < P < 0.001$. BMZ, basement membrane zone; E, NHEK (normal human epidermal keratinocyte); ECM, extracellular matrix; M, NHOMK (normal human oral mucosa keratinocyte).

COL7A1: $P = 0.0167$). There were no significant differences for *ITGA6* and *ITGB4*.

To exclude the possibility of the contribution of interindividual variation to the differences, we subsequently investigated the mRNA levels using keratinocytes obtained from a single individual (n = 1). Keratinocytes were established using both skin and oral mucosa samples from a healthy 33-year-old woman. Just as with the results for different persons, the *COL17A1* and *COL7A1* expression levels for a single individual were higher in NHOMKs than in NHEKs (*COL17A1,* $P = 0.0251$; *COL7A1,* $P = 0.0097$) (see Supplementary Figure S1a online). Although the *LAMC2* expression level was slightly higher in the NHOMKs in the interindividual data, there were no significant differences. The sample number of analysis using a single individual is a disadvantage in this study. It is difficult to obtain both skin and the oral mucosa samples from a single individual, so we attempted to analyze the murine tissues and keratinocytes.

mCol17a1 expression is higher in murine oral mucosa than in murine skin

The mRNA was extracted from murine tissues and keratinocytes (tail skin and buccal mucosa). mCol17a, mLamc2, and mCol7a1 have higher expression in the murine oral mucosa than in the murine skin (n = 5; mCol17a, $P < 0.0001$; mLamc2, $P = 0.0005$; mCol7a1, $P = 0.0021$) (see Supplementary Figure S2a online). Just as with the human results, mCol17a, mLamc2, and mCol7a1 expression levels were higher in the murine mucosal keratinocytes than in the murine skin keratinocytes (n = 4; mCol17a, $P < 0.0001$; mLamc2, $P < 0.0001$; mCol7a1, $P = 0.029$) (see Supplementary Figure S2b).

NHOMKs produced greater amounts of COL17 than NHEKs

Next, to evaluate the protein expression, we performed immunoblotting to detect the BMZ components (COL17, laminin 332, COL7, and integrin α6β4) using total cell lysate or hemidesmosome and ECM fraction. It is reported that the enriched hemidesmosomal proteins and ECM proteins remained after cells are removed by NH_4OH (Hirako et al., 2014). We compared these protein expressions between NHEKs and NHOMKs. Regarding total cell lysate, COL17 and laminin 332 had significantly higher expression in NHOMKs than in NHEKs (n = 3; COL17, $P = 0.0254$; laminin 332, $P = 0.0178$); calculated band is 105 kDa laminin γ2) (Figure 3b). In line with these results, the amounts of COL17 and laminin 332 were greater in NHOMKs than NHEKs from a single individual. (n = 1; COL17, $P = 0.0002$; laminin 332, $P = 0.0281$) (see Supplementary Figure S1b). With regard to hemidesmosome

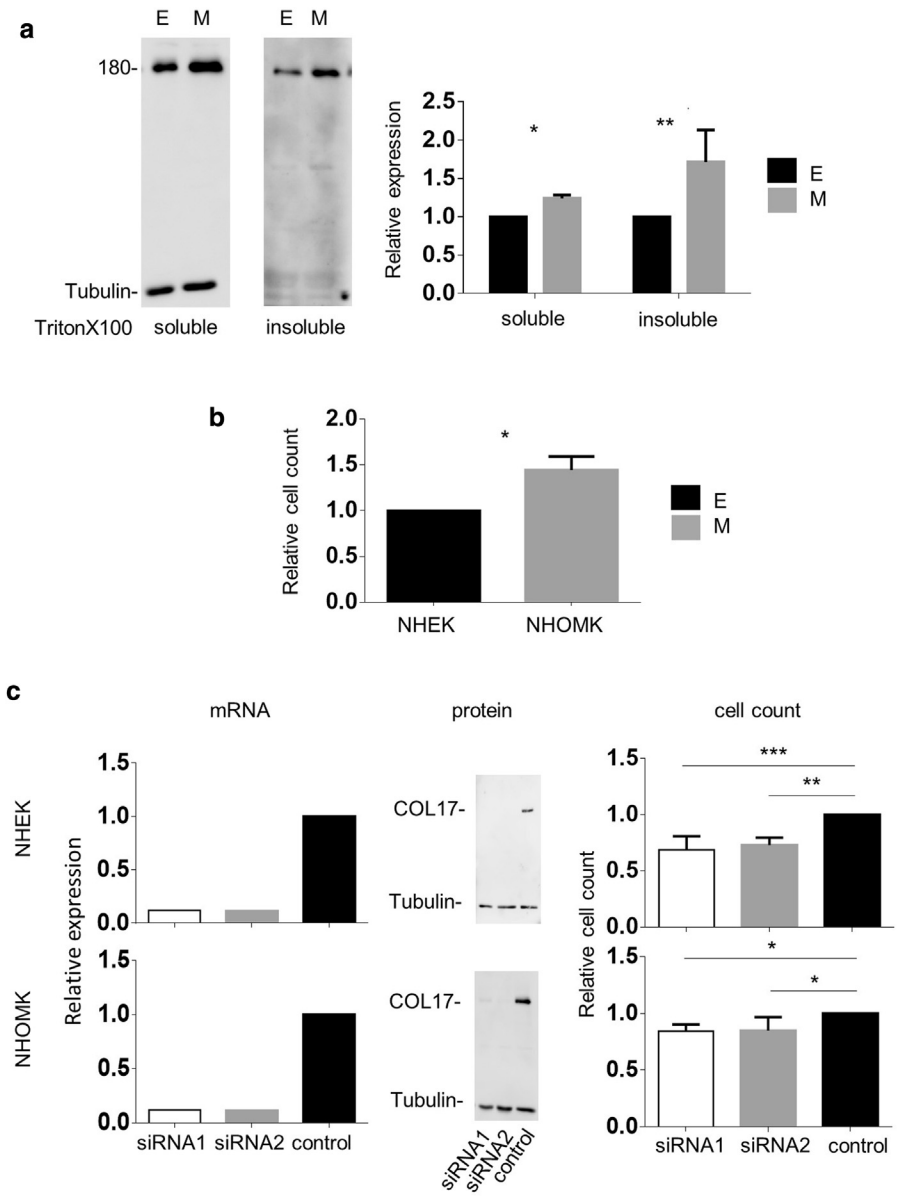

Figure 4. Immunoblotting of Triton X-100–soluble and –insoluble fractions and cell adhesion test. (**a**) Immunoblotting of COL17 using Triton X-100–soluble and –insoluble fractions from NHEKs and NHOMKs (n = 3). (**b**) Culture plates were placed on the vortex for 20 minutes, and the remaining cells were counted (n = 2). (**c**) Cell adhesion strength was tested using NHEKs and NHOMKs treated with COL17 knockdown (n = 3). The data are expressed as the mean ± standard deviation. Student *t* test, *0.01 < *P* < 0.05, **0.001 < *P* < 0.01, ***0.0001 < *P* < 0.001. E, NHEK (normal human epidermal keratinocyte); M, NHOMK (normal human oral mucosa keratinocyte); siRNA, small interfering RNA.

and extracellular matrix fraction, COL17, laminin 332, and integrin α6 had significantly higher expression in NHOMKs than in NHEKs (n = 3; COL17, $P = 0.0043$; laminin 332, $P = 0.0267$; integrin α6, $P = 0.0023$) (Figure 3c). The amounts of COL17, laminin 332, and integrin β4 were significantly greater in NHOMKs than NHEKs from a single individual (n = 1; COL17, $P = 0.0097$; laminin 332, $P = 0.0185$; integrin β4, $P = 0.0089$) (see Supplementary Figure S1c). The results of cell lysates and hemidesmosome and ECM fraction showed that amount of COL17 was considerably greater in NHOMKs than in NHEKs.

Cell adhesion strength is significantly higher for NHOMKs than for NHEKs

COL17 exists in both hemidesmosomes and non-hemidesmosomes in the skin (Hirako et al., 1998; Kitajima et al., 1992; Watanabe et al., 2017). Our immunofluorescence staining of COL17 clearly showed lateral-apical staining not only in the skin but also in the oral mucosa. To evaluate the amount of COL17 consisting in hemidesmosomes and in nonhemidesmosomes individually, we prepared Triton X-100–insoluble or –soluble fractions (Sigma Aldrich, St Louis, MO) of NHOMKs and NHEKs. Triton X-100–insoluble fraction represents hemidesmosomal COL17, whereas Triton X-100–soluble fraction represents nonhemidesmosomal COL17. For both fractions, the amount of COL17 was markedly greater in NHOMKs than in NHEKs (n = 3; soluble, $P = 0.0148$; insoluble, $P = 0.0033$) (Figure 4a). Because COL17 is apparently involved in cell adhesion, these results led us to test the cell adhesion strength by counting the cells remaining after vibration stress. The counts of remaining cells were significantly greater for NHOMKs than for NHEKs, which indicates that NHOMKs adhered to the culture plates more strongly than the NHEKs (n = 3, $P = 0.028$) (Figure 4b). Next, we assessed the correlation of cell adhesion strength with COL17 expression levels using COL17-knockdown keratinocytes. The number of cells remaining was much lower with COL17 knockdown

by small interfering RNAs than without such knockdown (n = 3) (Figure 4c). The cell adhesion and knockdown experiments were reproducible in the keratinocytes from a single individual (n = 1) (see Supplementary Figure S3 online). The knockdown efficiencies were measured by quantitative PCR and immunoblotting and were found to exceed 80% (Figure 4c). These results indicate that differential COL17 expression accounts for the differences in cell adhesion properties between NHEKs and NHOMKs.

Anti-COL17 antibodies induce significantly greater COL17 depletion in NHEKs than NHOMKs

Finally, to explain the clinical diversity of the predominant lesions in pemphigoid, we examined the COL17 depletion of NHEKs and NHOMKs treated with anti-COL17 IgG (Iwata et al., 2009). The C-terminus of COL17 is thought to be a major epitope of MMP, whereas the NC16A domain of COL17 is known as a pathogenic epitope for BP (Schmidt et al., 2001; Schmidt and Zillikens, 2013). We treated the keratinocytes with mAbs targeting the NC16A domain (TS39-3) (Ujiie et al., 2014) and/or the C-terminus (C17-C1) (Wada et al., 2016). Under treatment with mAb TS39-3, the amount of COL17 was reduced in NHEKs and NHOMKs in a dose-dependent manner (n = 3) (see Supplementary Figure S4a online); however, much more COL17 remained in the NHOMKs than in the NHEKs (n = 5) (Figure 5). This may be due to the great amounts of COL17 in NHOMKs under normal conditions (Figures 3 and 4a). No reduction was seen for either cell type under treatment with mAb C17-C1. When keratinocytes were treated with mAbs TS39-3 and C17-C1 in combination, COL17 depletion was remarkably enhanced. mAb C17-C1 caused additive shortages of COL17 in NHEKs and NHOMKs. We examined the cell adhesion strength of NHEKs and NHOMKs with mAbs TS39-3 and/or C17-C1. The cell adhesion strengths of both cells underwent a dose-dependent decrease with mAb TS39-3 alone or together with mAb C17-C1 (n = 3) (see Supplementary Figure S4b). These results indicate the possible pathogenicity of non-NC16A IgG.

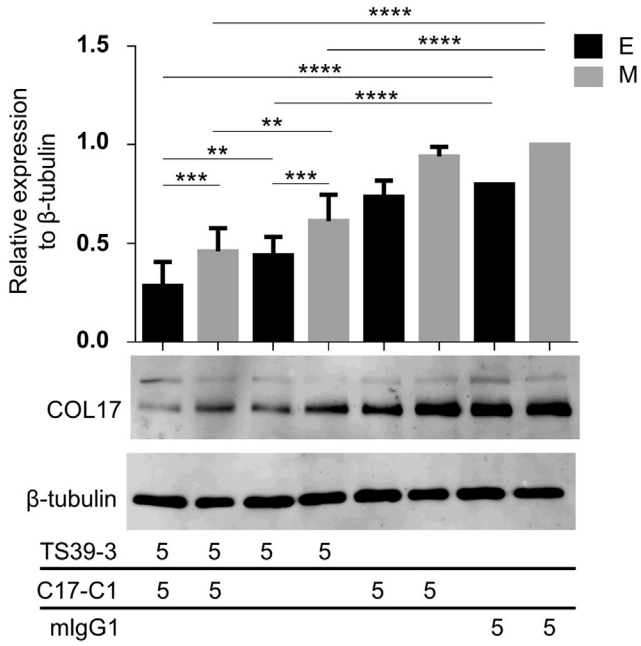

Figure 5. COL17 depletion in keratinocytes treated with mAbs against COL17. NHEKs and NHOMKs were treated with mAbs TS39-3, C17-C1, or mIgG1 and a combination of mAbs TS39-3 and C17-C1. The total concentration of each treatment was 2.5 μg/ml (n = 5). The graph shows the COL17 amount relative to the β-tubulin (upper panels). The representative immunoblotting is presented (lower panels). The data are expressed as the mean ± standard deviation. One-way analysis of variance, $^{**}0.001 < P < 0.01$, $^{***}0.0001 < P < 0.001$, $^{****}P < 0.0001$. E, NHEK (normal human epidermal keratinocyte); M, NHOMK (normal human oral mucosa keratinocyte).

DISCUSSION

This investigated the BMZ proteins, especially COL17, in the skin and oral mucosa. The immunofluorescence staining showed that COL17 staining was brighter in NHOM than in NHS. This was confirmed by quantitative PCR and immunoblotting using NHEKs and NHOMKs. The genetic shortage of COL17 is relevant to the pathogenesis of junctional epidermolysis bullosa, which shows blistering along the lamina lucida (Fine et al., 2014; McGrath et al., 1995). This indicates that the amount of hemidesmosomal COL17 is associated with the adhesion strength of basal cells anchoring to the ECM. Therefore, we studied the distribution of COL17 on the plasma membrane, such as hemidesmosomes and non-hemidesmosomes. COL17 stained not only on the bottom side but also on the lateral-apical sides of basal keratinocytes in the skin and the oral mucosa. Immunoblotting using Triton X-100−insoluble and −soluble fractions confirmed that greater amounts of COL17 were present in both the hemidesmosomes and nonhemidesmosomes of the NHOMKs than in those of the NHEKs. Given these results, we expected that the cell adhesion strength may differ between the skin and the oral mucosa. Indeed, NHOMKs showed stronger adhesion to culture plates than did NHEKs. COL17 depletion induced by BP patient IgG attenuates the cell adhesion strength (Iwata et al., 2009). In addition, COL17 knockdown induced a reduction in the number of cells adhering on the culture plate after vibration stress both in NHEKs and NHOMKs. Our results suggest that the amount of COL17 is associated with the basal cell attachment strength. We assume that the difficulty of inducing artificial separation in the oral mucosa may be attribute to the strong adhesion of the basal cells to the ECM.

The differences of BMZ proteins are expected to influence the clinical phenotype of BP. Although mAb targeting the NC16A domain depletes COL17 in both NHEKs (Wada et al., 2016) and NHOMKs (Figure 5), the clinical blisters in BP are mainly observed in the skin. The predominant skin blistering may be related to the amount of COL17 remaining after mAb binds to COL17; that is, the high expression of COL17 in the oral mucosa compensates for its BP-IgG−induced depletion. Pemphigus, another autoimmune blistering disease, is caused by autoantibodies against desmogleins (Dsgs). The autoantibodies targeting Dsg1 generate blisters only in the skin and not in the oral mucosa, because Dsg3 is expressed throughout the oral mucosa. Conversely, autoantibodies targeting Dsg3 induce oral mucosal lesions but not skin lesions, because Dsg1 counters the reduction of Dsg3 in the skin. This theory would mean that Dsg1 and Dsg3 compensate for each

other in pemphigus pathogenesis (Mahoney et al., 1999). Even if BP does not have exactly the same mechanism as pemphigus, the expression level of COL17 may determine the site of blister formation in BP. The blister mechanisms in BP do not have simple explanations but are associated with various factors such as complement activation and inflammatory cell infiltrates (Liu et al., 1995, 1997). We showed that the COL17 depletion and cell adhesion were correlated with mAb concentrations in vitro (see Supplementary Figure S4). The level of COL17 expression may not be solely associated with the blister formation in the oral mucosa, but the high autoantibody titer may involve the loss of attachment strength of basal cell to ECM not only in the skin but also in the oral mucosa. Furthermore, the higher expression of laminin 332 may be also related to the strong adhesion in the oral mucosa because of the interaction with COL17 and laminin 332 (Nishie et al., 2011). In addition to laminin 332, various molecules such as integrin α6β4 or BP230 interact with COL17. The integrins α6β4 were not depleted when the cells were treated by anti-COL17 antibodies (Iwata et al., 2009; Kamaguchi et al., 2017); therefore, they may not contribute to compensation of cell detachment induced by pemphigoid IgG. In addition, BP230 should not be associated with the cell adhesion in line with the depletion of COL17 because it is an intracellular molecule.

Additionally, we found that IgG against the C-terminus of COL17 may have pathogenicity. IgG against regions outside the NC16A domain that are considered nonpathogenic do not induce COL17 depletion (Imafuku et al., 2017; Wada et al., 2016). However, for the combination of IgG against the NC16A domain and the C-terminus, COL17 depletion was significantly enhanced (Figure 5). The enhancement of the pathogenicity by nonpathogenic IgG has been reported in pemphigus vulgaris (Kawasaki et al., 2006) and pemphigus foliaceus (Yoshida et al., 2017). The depletion activity of pathogenic mAbs against Dsg3 is known to be boosted when those mAbs act in combination with nonpathogenic mAbs (Yamamoto et al., 2007). These observations are similar to our results for mAbs to the C-terminus. BP patients with IgG targeting not only the NC16A domain but also the C-terminus may show blisters on the skin and the mucosa because of the enhanced effect of anti-C-terminus IgG on COL17 depletion. A previous study has also suggested the association with autoantibodies to the C-terminus and mucosal lesions in BP (Hofmann et al., 2002). Our results support the possible pathogenicity of autoantibodies targeting the C-terminus.

To summarize, we have shown that the greater amounts of COL17 in the oral mucosa than in the skin are associated with hemidesmosomal adhesion at the BMZ and that this compensates for the COL17 depletion induced by pemphigoid IgG. The differences in the expression levels of BMZ proteins, such as COL17 and laminin 332, may influence the clinical differences in pemphigoid.

MATERIALS AND METHODS

Artificially split skin and mucosa using human tissues

NHS and NHOM were obtained from uninvolved skin or mucosa of surgical specimens. To create artificial dermal-epidermal separations in skin and oral mucosa, the specimens were incubated with 1 mol/L NaCl for 24 hours at 4°C (Gammon et al., 1992), or 5 mmol/L EDTA for 6 hours in the skin or 8 hours in the oral mucosa at 4°C (Hybbinette et al., 1999). This study was approved by the ethical committee of Hokkaido University, and full written informed consent was obtained from all patients and healthy volunteers for the use of their materials. All studies using human materials were performed according to the principles of the Declaration of Helsinki.

Cell culture

Primary NHOMKs were generated from uninvolved skin or mucosa of surgical specimens or normal healthy volunteers. Murine skin and oral mucosal keratinocytes were generated from neonate C57BL/6J mice, mixed from more than five neonate mice. Each specimen was placed in dispase (1,000 Protease Units/ml; Wako, Osaka, Japan) overnight at 4°C, and the epidermis was separated from the dermis. After incubation in trypsin (0.05% weight/volume trypsin-0.53 mmol/L EDTA 4Na; Wako, Osaka, Japan) for 7 minutes at 37°C, the cell suspension was filtered through a 40-μm cell strainer (Thermo Fisher Scientific, Waltham, MA) followed by centrifuging at 270*g* for 3 minutes at 20°C. After the supernatant was aspirated, the pellet was resuspended in keratinocyte growth medium (KGM-Gold; Lonza, Basel, Switzerland) and cultured at 37°C under 5% CO_2. NHEKs were purchased for the control (lot number 0000399827; Lonza, Basel, Switzerland). For NHEKs and NHOMKs, no more than four passages were used in this study.

Quantitative reverse transcriptase–PCR

The expressions of hCOL17A1, hLAMC2, hCOL7A1, hITGA6, hITGB4, hKRT1, hKRT13, mCol17a1, mLamc2, mCol7a1, mItga6, mItgb4, mKrt1, and mKrt13 were measured by the cycle threshold ($\Delta\Delta CT$) method using each primer set (see Supplementary Table S1 online). Reverse transcriptase–PCR procedures are described in the Supplementary Materials.

Hematoxylin and eosin, immunofluorescence staining, and immunoblotting

Staining procedures and immunoblotting are described in the Supplementary Materials.

Treatment of cultured keratinocytes with mAbs against COL17

To investigate COL17 depletion, NHEKs and NHOMKs were treated with mAbs as previously reported, with some modifications (Iwata et al., 2009) (see Supplementary Materials).

Cell adhesion test

Cell adhesion strength to the culture bottom was investigated as previously reported, with minor modifications (Iwata et al., 2009) (see Supplementary Materials).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

MK performed the experiments. HI, HU, KN, WN, YK, and HS designed the experiments. MK and HI wrote the manuscript, and all the coauthors had final approval of the submission.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.03.002.

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