Simultaneous immunolocalization of desmoglein 3 and IgG4 in oral pemphigus vulgaris: IgG4 predominant autoantibodies in its pathogenesis

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BACKGROUND: Oral pemphigus vulgaris (PV), an autoimmune blistering disease, is mainly mediated by autoantibodies against desmoglein (Dsg) 3. However, no attention has been paid to IgG subclasses of the autoantibodies against Dsg3 in the diagnostic procedure for PV. Thus, our aim in this study was to investigate whether Dsg3 and any of IgG subclasses are immunohistochemically colocalized in tissue sections of PV oral mucosa.

MATERIALS AND METHODS: Serial sections cut from formalin-fixed paraffin blocks of biopsy specimens of 9 PV cases and those of normal buccal mucosa surgically removed for fibro-epithelial polyps were comparatively examined for immunohistochemical localizations for Dsg3, IgG4, and IgG.

RESULTS: Dsg3 was demonstrated in a dot-like pattern on the cell border and in the cytoplasm of the whole epithelial layer in both normal and PV specimens, while its staining was irregular among floating epithelial sheets of PV. IgG4 was also demonstrated in a punctuated fashion on the cell border among floating epithelial sheets, which was nearly identical to the immunohistochemical profile of Dsg3. In addition to being detected in the epithelial part, IgG4 signals were prominently localized in plasma cells scattered in the granulation tissue, where ratios of IgG4-positive (+) plasma cells to IgG+ cells were extraordinarily higher (mean 28%) than those in normal mucosa.

DISCUSSION: These findings confirmed for the first time that autoantibodies against Dsg3 are mainly composed of IgG4 in oral PV and that the combined immunohistochemistry for Dsg3 and IgG4 can be a valuable aid in confirming a histopathological diagnosis of PV.

Keywords: desmoglein 3; IgG4; immunohistochemistry; oral mucosa; pemphigus vulgaris

Introduction

Pemphigus vulgaris (PV) is an autoimmune blistering mucocutaneous disease mediated by autoantibodies against desmoglein (Dsg) 3 (1). Dsg family members consist of the transmembrane core of desmosomes together with desmocollins, and both of these groups of homophilic cell adhesion molecules belong to the cadherin superfamily (2). Among the Dsg isoforms, Dsg3 is characteristically expressed in the lower half of the epidermis and in wider zones of the oral mucosal epithelium, from basal to prickle cell layers (3), while Dsg1 is expressed throughout the epidermis and oral mucosa, and Dsg2 is widely detected in simple epithelia as well as in the lower layers of the epidermis (4), while Dsg4 is represented in the hair follicle (5). Thus, autoantibodies against Dsg3 tend to cause PV more frequently in the oral mucosa than in the skin by inhibiting the Dsg3 homophilic adherence between lower prickle cells, especially parabasal cells (6). The diagnosis of PV is confirmed by four findings: (i) mucocutaneous bulla formation, (ii) histopathological demonstration of intra-epithelial acantholysis, (iii) the presence of IgG autoantibodies bound to squamous epithelial cells, and (iv) the presence of circulating autoantibodies (7). However, no special attention has been paid to which IgG subclasses the autoantibodies against Dsg3 are composed of.

In the past two decades, since the entity of IgG4-related disease (IgG4RD) was established in pancreatitis, the clinical significance of IgG4 in the pathogenesis of various autoimmune diseases has attracted considerable attention (8). IgG4RD has been documented in various organs and tissues, such as the biliary tract, salivary gland, thyroid, breast, prostate, kidney, lung, skin, meninges, lymph nodes, aorta, and pericardium (9). However, the phenomenon of
serum IgG4 level elevation, which has been considered as one of the pivotal criteria of IgG4RD, does not always seem to be specific to IgG4RDs (10).

In addition to IgG1, IgG4 has been shown to be one of the two dominant IgG subclasses against Dsg3 in PV patients’ sera by means of enzyme-linked immunosorbent assays (ELISA) (11, 12) as well as immunofluorescence and immunoblottings (13, 14), although IgG4 and Dsg3 colocalization was not described distinctively in these studies. There has been only one article reporting that IgG4 was immunolocalized on the cell border of epidermal cells facing intra-epithelial blisters in paraffin sections obtained from the skin of patients with PV and pemphigus foliaceus (PF), although no immunohistochemical localization of Dsg3 was evaluated in them (15). Therefore, no actual immunohistochemical relationship between Dsg3 and IgG4 in PV has so far been documented in the literature (15).

Thus, the aim of this article is to demonstrate immunohistochemically the relationship between Dsg3 and IgG4 in oral biopsy specimens from patients with oral PV to confirm whether these two molecules are colocalized and whether this combined immunohistochemistry could be an aid for the histopathological diagnosis of PV.

Materials and methods

Materials
Nine cases of PV with oral mucosal bullae were collected from the surgical pathology files of the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences. Our diagnostic criteria for PV were as follows: (i) clinical finding, blister formation on oral mucosa or skin; (ii) histopathological finding, intra-epithelial, and suprabasal cleft due to acantholysis; and (iii) immunofluorescent or serological confirmation of autoantibodies with sera from the individual patients (7). However, serum levels for anti-Dsgs autoantibodies in their sera were not examined except for in the most recent cases. This was because we started the serum tests after 2012 in our clinic. Before that, the tests were only carried out by dermatologists beginning in 2003, when the Governmental health insurance started to cover the tests. Thus, serum data for the autoantibodies were not available to us before 2012, although we confirmed that the patients were treated under the final diagnoses of PV by dermatologists. No serum data were obtained for patients diagnosed before 2002. Clinical data including age, sex, lesion sites, serum data, and date of diagnoses have been summarized in Table 1. The mucocutaneous findings were reassessed by pictures as well as inspection and palpation records. In addition, 20 surgical specimens of fibro-epithelial polyp, which contained normal buccal mucosa, were also collected for control immunohistochemical experiments. The experimental protocol for analyzing surgical materials was reviewed and approved by the Ethical Board of the Niigata University Graduate School of Medical and Dental Sciences (Oral Life Science).

Histopathology
Tissue specimens were fixed routinely in 10% formalin and embedded in paraffin. Serial 5-μm sections were cut from paraffin blocks, and one set each of the sections was stained with hematoxylin and eosin (HE) and was used for re-evaluation of the histological diagnosis.

Antibodies
A mouse monoclonal antibody against human Dsg 3 (clone 3G133, IgG1, diluted at 1:40) was obtained from Abcam plc (Cambridge, UK). A mouse monoclonal antibody against human IgG4 (HP6025, IgG1, 1:500) was purchased from Zymed, Life Technologies Corp. (Carlsbad, CA, USA). Rabbit polyclonal antibodies against human IgG (Cat. No. 760-2653, pre-diluted) were obtained from F. Hoffman-La Roche (Basel, Switzerland).

Immunohistochemistry
Immunohistochemistry was performed using the ChemMate Envision™ system (Dako, Glostrup, Denmark). For Dsg3, sections were treated with 0.4% pepsin (Sigma Chemical Co., St. Louis, MO, USA) in 0.01 N HCl at 37°C for 30 min. For IgG4, sections were incubated with 0.2% trypsin (Sigma) at 37°C for 30 min. For IgG, sections were autoclaved in citric acid buffer (pH 6.0) at 121°C for 10 min. After pre-treatment, the sections were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 0.5% milk protein (Megmilk Snow Brand Co. Ltd., Tokyo, Japan) and 0.05% TritonX-100 (T-PBS) and treated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) to visualize reaction products.

For control studies on antibodies, the primary antibodies (anti-rabbit or anti-mouse immunoglobulins) conjugated with peroxidase-labeled dextran polymers for 1 h at room temperature to block endogenous peroxidase activities. After rinsing in T-PBS, sections were incubated with 0.02% 3,3′-diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide to visualize the reaction products. Finally, the sections were counterstained with hematoxylin.

Results
Clinical findings
Nine biopsy samples of the oral mucosa obtained from patients with PV were collected from the surgical pathology file during a 31-year period from 1983 to 2013. Their clinical and histological data were summarized in Table 1. The patients were composed of 3 males and 6 females with a
Table 1  Clinicopathological findings of pemphigus vulgaris cases

<table>
<thead>
<tr>
<th>Case #</th>
<th>Age (year) and sex</th>
<th>Site</th>
<th>Intraoral findings</th>
<th>Nikolsky phenomenon</th>
<th>Intraepithelial clefts</th>
<th>Tzanck cells</th>
<th>Epithelial cell borders</th>
<th>Cytoplasm</th>
<th>Dsg3</th>
<th>IgG4</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>46 M</td>
<td>Soft palate</td>
<td>Erosion</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Dot</td>
<td>Yes</td>
<td>Unclear</td>
<td>N.E.</td>
</tr>
<tr>
<td>2</td>
<td>29 F</td>
<td>Gingiva</td>
<td>Erosion</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Dot</td>
<td>Yes</td>
<td>Unclear</td>
<td>N.E.</td>
</tr>
<tr>
<td>3</td>
<td>69 M</td>
<td>Lip</td>
<td>Ulcer</td>
<td>–</td>
<td>Yes</td>
<td>Yes</td>
<td>Linear</td>
<td>No</td>
<td>Dot</td>
<td>42 ± 5.5</td>
</tr>
<tr>
<td>4</td>
<td>67 F</td>
<td>Buccal mucosa</td>
<td>Erosion</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Dot</td>
<td>Yes</td>
<td>Diffuse</td>
<td>N.E.</td>
</tr>
<tr>
<td>5</td>
<td>69 F</td>
<td>Oropharynx</td>
<td>Ulcer</td>
<td>–</td>
<td>Yes</td>
<td>Yes</td>
<td>Dot</td>
<td>Yes</td>
<td>Unclear</td>
<td>N.E.</td>
</tr>
<tr>
<td>6</td>
<td>73 F</td>
<td>Oropharynx</td>
<td>Erosion + white lesion</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Linear</td>
<td>No</td>
<td>Dot</td>
<td>63 ± 15</td>
</tr>
<tr>
<td>7</td>
<td>53 M</td>
<td>Buccal mucosa, soft palate</td>
<td>Erosion + white lesion</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Linear</td>
<td>No</td>
<td>Diffuse</td>
<td>48 ± 6.7</td>
</tr>
<tr>
<td>8</td>
<td>57 F</td>
<td>Buccal mucosa, gingiva</td>
<td>Erosion + white lesion</td>
<td>–</td>
<td>Yes</td>
<td>Yes</td>
<td>Dot/linear</td>
<td>Yes</td>
<td>Diffuse</td>
<td>30 ± 9.3</td>
</tr>
<tr>
<td>9</td>
<td>59 F</td>
<td>Gingiva</td>
<td>Erosion + white lesion</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Dot/linear</td>
<td>Yes</td>
<td>Dot/diffuse</td>
<td></td>
</tr>
</tbody>
</table>

M, male; F, female; –, no record available; Dsg, desmoglein; ELISA, enzyme-linked immunosorbent assay; +, positive titer but no exact value available; N.E., no tissue samples available for examination.
Figure 1  Histopathological and immunohistochemical findings of pemphigus vulgaris. (A, B) hematoxylin and eosin stains; immunoperoxidase stains for desmoglein (Dsg) 3 (C, F) immunoglobulin (Ig) G4 (D, G, I), and IgG (E, H, J), hematoxylin counter stain. (A) × 100; (C–E, I, J) × 400; (B, F–H) × 600, bars (A) 200 μm; (B, F) 50 μm; (C, I) 100 μm. Most of the gingival epithelial compartment, which was hyperplastic, was detached from the basal area, resulting in the formation of an intra-epithelial cleft. There was a dense infiltration of chronic inflammatory cells in subepithelial granulation tissues (A). Densely, eosinophilic prickle cells in singular or aggregated forms were floating within the cleft space. Basal cells remained in the lamina propria connective tissue side through the basement membrane (B). In the upper part of the prickle cell layer where no acantholytic change occurred, Dsg3 was demonstrated in a linear pattern on the cell border as if the signals were tracing intercellular bridges (C). IgG4 was only faintly and occasionally localized in the intercellular space (D). IgG was linearly and more definitely positive on the cell border (E). In the lower half layer affected with acantholysis, Dsg3 was demonstrated in a dot-like pattern on the cell border as well as within the cytoplasm (arrows) of epithelial cells (F). IgG4 also in dot-like signals was shown on the cell border (arrows) in a similar manner to Dsg3 ones (G). IgG was localized on the cell border, while it was not obviously seen in the basal to parabasal zone (H). There were definitely large numbers of IgG4-positive (+) plasma cells within the subepithelial granulation tissue (I), while IgG+ ones were more frequently seen (J). However, the proportion of IgG4+ to IgG+ was extraordinarily higher.
mean of age of 58 years, ranging from 29 to 73 years. The biopsy samples were obtained from the gingiva (three cases), buccal mucosa (3), palate to oropharynx (4), and lip (1). Their major clinical symptoms were ulcer or erosion, while Nikolsky phenomena were disclosed only in three cases. Unfortunately, serum IgG4 levels were not examined in any of the cases.

**Histopathology**
In biopsy specimens, the mucosal epithelia were basically hyperplastic, and they were detached from the lower prickle cell layer, leaving the basal zones with the basement membrane in the subepithelial connective tissue. The lower prickle cells tended to be detached from each other, and singular-isolated cells which had deeply stained, eosinophilic cytoplasm (so-called Tzanck cells) or those in aggregated forms were floating in a cleft-like space formed within the epithelial layer. As basal cells remained along the basement membrane, the intra-epithelial cleft looked to be located in the suprabasal to lower prickle cell zone and was at least lined by the first basal cells along the basement membrane (Fig. 1A). There was a dense infiltration of chronic inflammatory cells, mainly composed of plasma cells, in the subepithelial granulation tissues (Fig. 1B). These histological findings were shared by all the cases summarized in Table 1, and their histopathological diagnoses of PV were confirmed.

**Immunohistochemistry**
Immunohistochemically, Dsg3 was clearly localized in a linear fashion on the cell border of squamous epithelial cells in the whole epithelial layer except for the surface keratinized layer in normal mucosa of fibroepithelial polyp samples (Fig. 2A). However, in PV samples, its immunolocalization profiles were obviously different between the upper and lower epithelial layers. In the upper layer, Dsg3 was localized in a linear fashion on the cell border of prickle cells as seen in normal samples (Fig. 1C). In contrast, it was demonstrated in a dot-like pattern on the cell border of the basal cells to lower prickle cells, which were acantholytic (Fig. 1F). The dot-like Dsg3 staining became weaker, less frequent, and more irregular in detached epithelial sheets floating in intra-epithelial clefts, while its granular signals were enhanced within the cytoplasm (Fig. 1F). IgG4 was also localized in a dot-like pattern on the cell border of lower prickle cells, which was similar to Dsg3 signals, although it was not so obvious in the basal zone. Its dot-like signals decreased in number and intensity in detached epithelial sheets (Fig. 1G). However, in the upper prickle cell layer, IgG4 was only faintly demonstrated in a linear fashion (Fig. 1D). IgG was also linearly localized on the cell border in the upper prickle cell layer with some enhancement toward the surface (Fig. 1E), and the same linear staining was obtained in the lower prickle cells, although it was not obviously seen in the basal to parabasal zone. In the detached epithelial sheets, its linear staining became interrupted and partially in irregular dot-like fashions (Fig. 1H). In normal epithelia, there was no apparent immunolocalization for IgG4 (Fig. 2B), while faint interrupted ones for IgG were linearly localized along the cell border of the whole epithelial layer except for the surface keratinized layer (Fig. 2C). Thus, the staining patterns for Dsg3 and IgG4 were nearly the same but different from the IgG staining profile in PV samples. The IgG4 deposition on the epithelial cell membrane was confirmed in six (66.7%) of the nine cases examined, while in the other three cases, we could not precisely evaluate immunohistochemical profiles because of extensive hemorrhage (indicated as ‘unclear’ in the column for epithelial cell border in Table 1).

In the subepithelial granulation tissue (Fig. 1A), there were definitely large numbers of IgG4-positive (+) plasma cells (Fig. 1I), more than 10 in a × 400 magnification field, which fulfilled the diagnostic criteria of IgG4RD (10), while IgG+ ones were of course more frequently seen (Fig. 1J). The ratios of IgG4+/IgG+ plasma cells ranged from 20% to 36% with a mean of 28% in the subepithelial granulation tissue part (Table 1). Those IgG4+/IgG+ ratios were extraordinarily higher than those in normal buccal mucosa in fibroepithelial polyp samples (< 1%).

**Discussion**
We have shown for the first time in the present study that Dsg3 and IgG4 were colocalized in the oral mucosa of patients with oral PV and that the combined immunohisto-

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**Figure 2** Immunohistochemical findings in normal squamous epithelium of the buccal mucosa. Immunoperoxidase stains for Dsg3 (A), IgG4 (B), and IgG (C), hematoxylin counter stain. × 400, bar 100 μm. Dsg3 was regularly localized in a linear fashion on the cell border of squamous epithelial cells in the whole epithelial layer except for in the surface keratinized layer (A). No IgG4 signals were observed in the epithelial layer (B), while IgG was demonstrated in the intercellular space of the prickle cells and in lympho-plasma cells infiltrating into the epithelial layer as well as in the subepithelial granulation tissue of fibroepithelial polyp (C).
chemistry for Dsg3 and IgG4 was helpful in confirming the histopathological diagnosis of PV arising in the oral mucosa, especially when autoantibody serum data were not available. The histopathological diagnosis of PV might have been performed solely on HE by confirming such classic histological features as intra-epithelial (suprabasal) clefts and floating prickle cells due to acantholysis and immunofluorescence (2). However, as its pathogenesis due to autoimmune reaction to Dsg molecules has already been clarified (16), the immunohistochemistry for Dsg should provide important objective evidence for the final diagnosis, especially when bullous signs are restricted to the oral mucosa, because similar blistering lesions, which need differential diagnoses from PV, occur in the oral mucosa. The Dsg3 dot-like signals on the cell membrane as well as in the cytoplasm, which we demonstrated in the present study, may indicate that the cell membrane of prickle cells was disrupted due to autoantibodies as well as that Dsg3-immune complexes were endocytosed. These characteristic findings must be helpful in confirming the diagnosis of PV. Interestingly, the Dsg3 dot-like signals along the cell membrane resembled those of IgG4 on the cell membrane, which was never observed for IgG. Based on the present results, we consider that autoantibodies against Dsg3 were primarily composed from IgG4, although unfortunately, no exact serum data for these patients were available except for one case.

IgG4-related autoimmune pathogenesis was firstly reported in pancreatitis by Hamano et al. (17). As then, it has been well known that IgG4 is involved in various autoimmune diseases arising in the salivary gland, lacrimal gland, and many other organs (8, 9), which nowadays have constituted a specific clinical entity as IgG4RD. According to the diagnostic criteria of IgG4RD proposed by Japanese Ministry of Health, Labor and Welfare study groups in 2012 (10), IgG4RD is diagnosed by combinations of such clinical and histopathological findings as characteristic swellings of single/multiple organs, high serum IgG4, prominent fibrosis of organs, and histological infiltration of IgG4+ plasma cells, although so far PV has not been regarded as IgG4RD.

Pemphigus vulgaris is recognized as an autoimmune disease mediated by autoantibodies binding to Dsg3 (18). This pathogenetic mechanism has been confirmed by in vivo and in vitro studies (6, 19, 20). Among the Dsg family, Dsg3 is expressed in wider ranges from the basal to upper layers of the oral epithelium, although in the skin epidermis, it is restricted to the basal zone (21). This has been considered to be a reason for the fact that PV caused by anti-Dsg3 predominantly occurs in the oral mucosa (6). Recently, IgG4 has been found to be a dominant IgG subclass in sera of patients with both PV and PF, although no detailed information was available for lesional locations (skin or oral) of their cases (11). Calkins et al. (22) demonstrated data that autoantibodies against Dsg3 might be internalized by endocytosis and degraded upon their binding to antigens. Based on these lines of evidence, it is reasonable to consider that IgG4 and Dsg3 should be colocalized in the oral tissue samples of PV. In the present study, we have confirmed this hypothesis and shown successully that simultaneous immunohistochemical demonstrations of IgG4 and Dsg3 could be a helpful aid for the diagnosis of oral manifestation of PV even though in case serum data are not available and no clinical symptoms other than oral mucosal ones are detected. As far as we have been able to determine from the literature, this is the first report for the immunohistochemical colocalization of IgG4 and Dsg3 in PV tissue specimens.

As mentioned above, the demonstration of enhanced IgG4 levels in sera and tissue specimens should be essential for the IgG4RD diagnosis. In particular, the presence of IgG4+ plasma cells has been considered as important histological evidence. In our series of oral PV samples investigated in the present study, we demonstrated increased numbers of strongly-IgG4+ plasma cells with their increased IgG4+/IgG+ ratios in the subepithelial connective tissue, although no clinical data for serum IgG4 levels were available. In addition to autoimmune diseases, serum IgG4 levels are also known to be elevated in such diseases as asthma (23), allergic atopic dermatitis (24), parasitic infections (25), and pancreatic adenocarcinoma (26), which were not included in the entity of IgG4RD. IgG4+ plasma cells may infiltrate in any kind of inflammatory situation (27). Thus, the biosynthesis and secretion of IgG4 by plasma cells may not always be specific to PV and should also be inferred in any type of disease. However, it is necessary to elucidate general and specific functions of IgG4 in the pathogenesis of not only IgG4RD but also the other autoimmune diseases before we fully understand the actual molecular mechanism underlying PV.

References


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**Conflict of interest**

We declare that we have no conflict of interest.