Production of recombinant extracellular domains of canine desmoglein 1 (Dsg1) by baculovirus expression

Koji Nishifuji\textsuperscript{a,b,*}, Masayuki Amagai\textsuperscript{b}, Takeji Nishikawa\textsuperscript{b}, Toshiroh Iwasaki\textsuperscript{a}

\textsuperscript{a}Department of Veterinary Internal Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan
\textsuperscript{b}Department of Dermatology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan

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Abstract

The aim of this study was to generate a recombinant protein to represent the entire extracellular domain of canine desmoglein 1 (Dsg1), a desmosomal cell–cell adhesion molecule, by the baculovirus expression system. Cotransfection of a baculovirus transfer vector containing the cDNA for the entire extracellular domain of canine Dsg1 with baculovirus DNA into insect cells resulted in the secretion of soluble canine Dsg1 into insect culture supernatants. Immunoreactivity of 11 human pemphigus foliaceus (PF) sera against the cell surface of canine keratinocytes was completely removed when the sera were preincubated with the canine Dsg1 baculoprotein. This recombinant canine Dsg1 produced by baculovirus shares the major epitopes of the authentic canine Dsg1 recognized by human PF sera, and will be useful in studying the molecular pathophysiological mechanisms in PF and impetigo in canine patients.

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1. Introduction

Desmoglein 1 (Dsg1) is a cadherin-type cell–cell adhesion molecule found in desmosomes (Koch and Franke, 1994). The expression of Dsg1 is predominantly restricted to stratified squamous epithelia in certain mammals (Schafer et al., 1994; Aoki et al., 2000). It has been proposed that in human patients with pemphigus foliaceus (PF), serum autoAb interfere the adhesive function of Dsg1 and that blisters occur in the superficial epidermis (Stanley, 1993; Amagai, 1995). Our recent studies demonstrated that the target of exfoliative toxins (ET) produced by \textit{Staphylococcus aureus} is also Dsg1 (Amagai et al., 2000a, 2002; Yamaguchi et al., 2002). Inactivation of Dsg1 by ET results in a histology of epidermal blisters typical in human patients with bullous impetigo (BI), and its generalized form, staphylococcal scalded-skin syndrome (SSSS).

Our understanding of the pathophysiology of blisters in human PF, BI and SSSS has been advanced by the generation of the recombinant proteins to represent the entire extracellular domain of human Dsg1 by baculovirus expression (Amagai et al., 1995; Ishii...
et al., 1997). Immunoreactivity of autoAb in human PF sera against the cell surface of keratinocytes was completely absorbed by preincubation with the human Dsg1 baculoprotein, indicating that the baculoproteins have the most, if not all, of the conformational epitopes of authentic human Dsg1 recognized by human PF sera. In addition, we have demonstrated that ETA, ETB and ETD, three serotypes of ET isolated from human patients, specifically and directly cleave the recombinant extracellular domains of human Dsg1 (Amagai et al., 2000a, 2002; Yamaguchi et al., 2002).

Besides affecting humans, PF and impetigo are also recognized in canine patients (Werner et al., 1983; Scott et al., 1987, 1980). Clinical and histopathological features of blisters in these canine patients are similar to those in corresponding human diseases, except for the numerous infiltration of neutrophils in the blister cavity of canine PF (Scott et al., 1980). Immunoblotting studies have revealed that serum IgG in canine PF recognized a canine keratinocyte protein that may correspond to Dsg1 (Suter et al., 1993; Iwasaki et al., 1997); however, the exact molecular pathophysiological mechanisms of PF and impetigo in canine patients have not been well documented. In this study, we aimed to generate a recombinant protein to represent the entire extracellular domain of canine Dsg1 with the proper conformation by the baculovirus expression system.

2. Materials and methods

2.1. Human sera

Human sera were obtained from 11 patients with human PF and 5 patients with mucosal-dominant human pemphigus vulgaris (PV). All human PF sera tested were positive for anti-human Dsg1 IgG and negative for anti-human Dsg3 IgG, whereas all human PV sera tested were negative for anti-human Dsg1 IgG but positive for anti-human Dsg3 IgG, as determined by ELISA against recombinant human Dsg1 and Dsg3 produced by baculovirus expression (Ishii et al., 1997; Amagai et al., 1999).

2.2. Cell culture of keratinocytes

A canine acanthomatous epulis cell line, MCA-B1, was a gift from Dr. S. Tateyama (Miyazaki University, Miyazaki, Japan) (Priosoeryanto et al., 1995). These cells were maintained in Dulbecco’s Modified Eagle’s and Ham’s Nutrient Mixture F-12 medium (DME/F-12; Sigma Biosciences, St. Louis, MO) supplemented with 10% fetal calf serum (GIBCO BRL, Grand Island, NY) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; GIBCO BRL).

2.3. Immunofluorescence

Indirect immunofluorescence (IIF) using normal canine lip, and living keratinocyte staining using MCA-B1 cells were performed as described previously (Ishii et al., 1997; Amagai et al., 2000b).

2.4. Construction of recombinant canine Dsg1 protein

cDNA encoding the entire extracellular domain of canine Dsg1 (GenBank AF005360) (Muller et al., 2000) was isolated using reverse transcription-polymerase chain reaction (RT-PCR) technique. Briefly, poly (A)^+ RNA extracted from MCA-B1 cells using a Quickprep Micro mRNA Purification Kit (Amersham Biosciences Corp., Piscataway, NJ) was transcribed into the first-stranded cDNA with a sequence-specific RT primer, 5’-GAGTAGTCCGATGCCAGC-3’, and MMLV reverse transcriptase (ReverTra Ace; TOYOBO, Osaka, Japan). Then the cDNA fragment encoding the entire extracellular domain of canine Dsg1 was amplified by PCR with 5’ primer, 5’-ccgatcttaaatATGAACTGGCACTTTTAAG-3’, and 3’ primer, 5’-cggctcgagATGGACATTATCTGACACACTTTTAAG-3’. A baculovirus transfer vector, pEVmod-hDsg3-His, which contains the entire extracellular domain of human Dsg3 fused with E tag (a tag recognized by anti-E tag mAb from Amersham Biosciences Corp.) and His tag, has been described previously (Ishii et al., 1997). The cDNA for human Dsg3 in pEVmod-hDsg3-His was then replaced with the cDNA for canine Dsg1 (pEVmod-cDsg1-His), and the final construct was further subjected to nucleotide sequencing. Recombinant baculovirus was obtained by cotransfection of pEVmod-cDsg1-His and BaculoGold baculovirus DNA (Pharmingen, San Diego, CA) into cultured insect Sf9 cells. High Five insect cells (Invitrogen, San Diego, CA) cultured in serum-free EX
cells 405 medium (JRH Biosciences, Lenexa, KS) were infected with recombinant virus and incubated for three days at 27 °C. Production of recombinant canine Dsg1 (cDsg1-His) in culture supernatants was confirmed by immunoblotting analysis as previously described (Ishii et al., 1997). Culture supernatants were stored at −70 °C after removal of cell debris by centrifugation.

2.5. Immunoadsorption assay

Immunoadsorption assays were performed as follows: Human PF and PV sera were diluted at 1:20 and 1:80 with the insect culture supernatants containing hDsg1-His (a recombinant baculoprotein of the entire extracellular domain of human Dsg1 fused with E tag and His tag) (Ishii et al., 1997), cDsg1-His or control supernatant. After incubation at 4 °C overnight, the diluted sera were used for IIF using normal canine lip or living keratinocyte staining using MCA-B1 cells.

3. Results and discussion

3.1. Anti-Dsg1 IgG in human PF sera are able to crossreact with canine Dsg1 expressed on MCA-B1 cells

We first assessed whether anti-Dsg1 IgG in human PF sera are able to recognize canine Dsg1. IIF of cryosectioned-normal canine lip with five human PF sera showed the deposition of IgG between keratinocytes throughout the canine lip epithelium in all human PF sera tested. When these sera were incubated with insect culture supernatants containing hDsg1-His, the immunoreactivity of these sera was completely removed, while preincubation with control supernatant did not alter their immunoreactivity (Fig. 1).

We next wanted to determine if there are some canine keratinocyte cell lines expressing the canine counterpart of human Dsg1. We, therefore, performed living keratinocyte staining with five human PF sera using a canine keratinocyte cell line, MCA-B1. Fluorescent deposition of IgG on the surface of MCA-B1 cells was detected in all human PF sera tested, and their immunoreactivity was completely removed by preincubation with hDsg1-His baculoprotein (Fig. 1).

These findings taken together indicate that anti-Dsg1 IgG in human PF sera are able to crossreact with a canine counterpart of human Dsg1 expressed on the surface of MCA-B1 cells.

3.2. Production of a secreted form of canine Dsg1 by baculovirus expression

Using poly (A)+ RNA extracted from MCA-B1 cells as a template, a cDNA encoding the entire extracellular domain of canine Dsg1 was amplified by RT-PCR and ligated in the frame with the cDNA encoding E tag and His tag in a baculovirus transfer vector, pEVmod. Comparison of nucleotide sequences of pEVmod-cDsg1-His with a sequence previously reported (GenBank AF005360) (Muller et al., 2000) revealed that one nucleotide at position

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**Fig. 1.** Immunoreactivity of anti-Dsg1 IgG in human PF sera against the cell surface of canine keratinocytes. Human PF sera were incubated with cryosectioned-normal canine lip (A) or a canine keratinocyte cell line, MCA-B1 (C). Human PF sera were incubated with High Five supernatant containing hDsg1-His and were subsequently incubated with normal canine lip (B) or MCA-B1 cells (D). Immunoreactivity of IgG in human PF sera to the cell-cell contact sites of canine keratinocytes was completely removed by preincubation with hDsg1-His. Bar, 20 μm.
560 of the GenBank data was A instead of G, resulting in one amino acid change at position 183 from Gly to Asp in our clone. This aspartic acid residue at position 183 was conserved in bovine, human and mouse Dsg1 as previously reported (Koch et al., 1990; Nilles et al., 1991; Wheeler et al., 1991; Mahoney et al., 2002); however, the precise function of such residues in maintaining the conformation of the molecule remains to be further studied.

Expression of this vector in insect High Five cells resulted in the secretion of a soluble form of canine Dsg1 baculoprotein, cDsg1-His. The region derived from the entire extracellular domain of canine Dsg1 consisted of the signal peptide (S), the prosequence (P), and the extracellular domains (EC1–5) which were fused with E tag (E) and His tag (H) at the C terminal end. (B) The secreted form of cDsg1-His (lane 1) was detected in insect culture supernatant by immunoblot analysis as a doublet of 95.6 and 86.7 kDa bands. Lane 2 shows bands for hDsg1-His. M: molecular mass standards.

3.3. Absorption of antibodies against the cell surface of canine keratinocytes in human PF sera by cDsg1-His

Previous studies demonstrated that serum autoAb in human PF mainly recognize the conformational epitopes expressed on the extracellular domains of Dsg1. To further assess whether cDsg1-His shares the most of the conformational epitopes with authentic canine Dsg1 specifically recognized by human PF sera, we performed immunoadsorption assay with cDsg1-His as well as hDsg1-His combined with living keratinocyte staining with MCA-B1 cells. We incubated 11 human sera obtained from patients with human PF or five human sera obtained from patients with mucosal-dominant human PV with insect culture supernatant containing cDsg1-His, hDsg1-His or control supernatant. The immunoreactivity against the surface of MCA-B1 cells in all 11 of the human PF sera tested was completely removed after incubation with the cDsg1-His or hDsg1-His baculoprotein, whereas the control supernatant did not alter their immunoreactivity. The immunoreactivity of all five of the human PV sera tested was not affected by preincubation with cDsg1-His nor hDsg1-His baculoprotein (Fig. 3, Table 1).

There are several lines of evidence to suggest that loss of adhesive function of Dsg1 may be involved in the pathogenesis of PF and impetigo in canine patients. Histopathological distribution of blisters in canine PF and impetigo is restricted only to the superficial epidermis, where Dsg1 is expressed without concomitant Dsg3 (Aoki et al., 2000). In addition,

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<th>Human sera</th>
<th>Number positive/number tested (immunoadsorption with)</th>
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<td></td>
<td>Control</td>
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<td>PF</td>
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<td>PV</td>
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* 11 human PF sera or five human pemphigus vulgaris (PV) sera were incubated with High Five supernatant containing cDsg1-His, hDsg1-His or control supernatant and then applied to living keratinocyte staining with MCA-B1 cells.
immunoblotting studies have revealed that serum Ab in patients with canine PF recognized a canine keratinocyte protein that showed identical migration to the protein recognized by human PF sera (Suter et al., 1993; Iwasaki et al., 1997). Our results of immunoadsorption assay suggest that cDsg1-His represents not only the amino acid sequence but also the conformation of the extracellular regions of authentic canine Dsg1. It is our hope that future studies using this recombinant molecule will provide us explanations whether Dsg1 is involved in the pathogenesis of these canine skin diseases.

In summary, we have successfully generated a recombinant protein to represent the entire extracellular domain of canine Dsg1 by the baculovirus expression system. Immunoadsorption studies revealed that the canine Dsg1 baculoprotein shares the major epitopes of authentic canine Dsg1 specifically recognized by anti-Dsg1 Ab in human PF sera. Availability of the canine Dsg1 baculoprotein will further allow us to investigate the molecular pathophysiological mechanisms of blister formation of PF and impetigo in canine patients.

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