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Staphylococcus hyicus exfoliative toxins selectively digest porcine desmoglein 1

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Abstract

Virulent strains of *Staphylococcus hyicus* can cause exudative epidermitis in pigs. The major symptom of this disease is exfoliation of the skin in the upper stratum spinosum. Exfoliation of the skin is strongly associated with exfoliative toxin including ExhA, ExhB, ExhC, ExhD, SHETA, and SHETB. Recently, genes for ExhA, ExhB, ExhC and ExhD were cloned. Exfoliative toxins produced by *S. aureus* have been shown to selectively cleave human or mouse desmoglein 1, a desmosomal adhesion molecule, that when inactivated results in blisters. In this study, we attempted to identify the molecular target of Exhs in porcine skin. Each of recombinant Exhs injected in the skin of pigs caused superficial epidermal blisters or crust formation. Cell surface staining of desmoglein 1, but not that of desmoglein 3, was abolished when cryosections of normal porcine skin were incubated with one of Exhs suggesting that Exh selectively degrade porcine desmoglein 1. In vitro incubation of the recombinant extracellular domains of desmoglein 1 and desmoglein 3 of human, mouse or canine origin demonstrated that only mouse desmogleins 1α and 1β were cleaved by ExhA and ExhC at high concentration. Furthermore, injection of ExhA and ExhC at high concentration caused superficial blisters in neonatal mice. These findings strongly suggest that Exhs cause blister formation of porcine skin by digesting porcine desmoglein 1 in a similar fashion to exfoliative toxins from *S. aureus*.

Keywords: *Staphylococcus hyicus*; Exfoliative toxin; Desmoglein; Exudative epidermitis

1. Introduction

Certain strains of *Staphylococcus hyicus* are able to cause a skin disease in pigs called exudative epidermitis (EE), which primarily occurs in piglets and has been known by its clinical signs for a long time [1]. EE occurs typically as an acute infection characterized by separation of the cells in the upper stratum spinosum of the epidermis, exfoliation of the skin, erythema and serious exudation, which may lead piglets to dehydration and subsequent death [2,3]. The most important virulence determinant of virulent *S. hyicus* is exfoliative toxin [4–7]. Exfoliative toxins responsible for the characteristic lesion of EE have been identified and purified from strains in Japan [8] and in Denmark [6]. The toxins isolated in Japan were designated SHETA and SHETB [8] and those isolated in Denmark were ExhA, ExhB, ExhC [9] and ExhD [10]. Recently, the genes encoding ExhA-D from four respective virulent strains were cloned [10]. The toxins from *S. hyicus* differ each other with regard to their antigenic properties [9,11], but overall amino acid sequences clearly demonstrated that they together with exfoliative toxins ETA, ETB and ETD from *S. aureus* constitute a homologous exfoliative toxin family of genus *Staphylococcus* [10].

In humans, some *S. aureus* strains cause complex skin infection with blister formation called bullous impetigo (BI) or Staphylococcal scalded skin syndrome (SSSS) [12,13]. Most of them produce either ETA or ETB [14]. These two classical exfoliative toxins cause intraepidermal cleavage through the granular layer and induce exfoliation or blister

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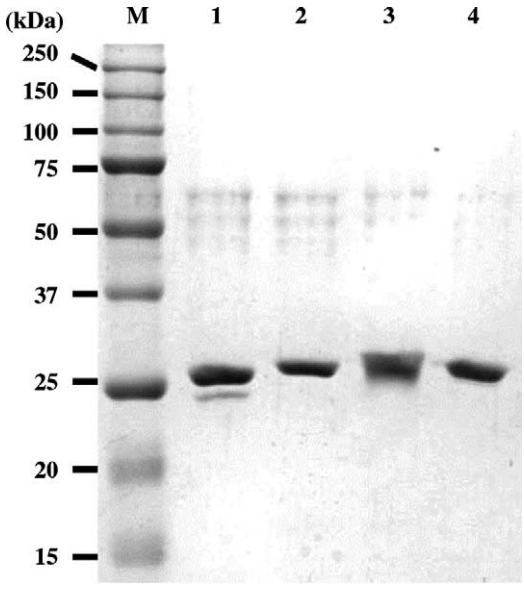

Fig. 1. Purification of recombinant Exh from *E. coli*. Purified ExhA, ExhB, ExhC and ExhD were resolved on an SDS-12% PAGE gel and stained with Coomassie brilliant blue. Lane M, Molecular marker; lane 1, rExhA; lane 2, rExhB; lane 3, rExhC; lane 4, rExhD.

formation in the diseased skin lesions. Recently, another serotype ETD was discovered in an *S. aureus* isolate from the pus of wounded skin [15]. Several lines of evidence have suggested that these ETs are serine proteases, and recently, the common target molecule was identified as desmosomal cadherin-like cell–cell adhesion molecule, desmoglein 1 (Dsg1) [15–17]. In spite of diverged amino acid sequences, *S. aureus* ETs share the same target molecule and the same cleavage site just after the Glu of human and mouse Dsg1 showing that they are acting as Glu-specific serine proteases [18,19]. In the human or mouse epidermis, isoforms of desmogleins, Dsg1 and Dsg3 are present, and these proteins possess overlapping functions in cell-to-cell adhesion [20,21]. Dsg1 is present throughout the epidermis, while Dsg3 is in the lower portion of epidermis. Consequently, blister formation through Dsg1 digestion by ET occurs in the superficial region, namely in the granular layer.

In this study, we hypothesized that exfoliative toxins from *S. hyicus* has a similar pathophysiological function as *S. aureus* ETs do in humans and demonstrate the direct effect of Exhs on porcine Dsg1 in epidermis of porcine skin.

2. Results

To investigate the molecular mechanism of the exfoliation or crust formation by Exh, we first examined the skin of pigs injected with recombinant Exh. A DNA fragment corresponding to the possible processed form of each *exh* was constructed by PCR and placed it in frame into a plasmid to express a His_6-tagged fusion protein. The His_6-tagged proteins were purified to homogeneity (Fig. 1) and intracutaneously injected into the skin of pigs. Macroscopically, all Exh tested, ExhA, ExhB, ExhC and ExhD induced either exfoliation or crust formation at the site of injection after 48 h (Fig. 2, upper). The appearance of the lesions and their development over time concurred well with previous results obtained with injection of concentrated culture supernatant of a virulent *S. hyicus* strain and these results indicated that the recombinant Exhs were as active as natural Exhs. Histological examination of the biopsies of pig skin taken at 48 h of post-injection of Exh showed the characteristic splitting at the granular layer (Fig. 2, lower). We then switched to use cryosections of normal pig skin to incubate with Exh in vitro. The cryosection was treated with either ExhA, ExhB, ExhC, ExhD or TBS-Ca as a control, and was examined by immunofluorescence with pemphigus foliaceus antisera (PFA) which presumably recognizes the extracellular domain of Dsg1 and with monoclonal antibody 5H10 which presumably recognizes the amino terminal extracellular domain of Dsg3. Examination of the cryosection of TBS-treated skin with PFA demonstrated the cell surface staining of epidermal keratinocytes throughout the epidermis layer whereas that with 5H10 preferentially stained the surface of keratinocytes in basal region (Fig. 3). The cell surface staining of Dsg1 was greatly

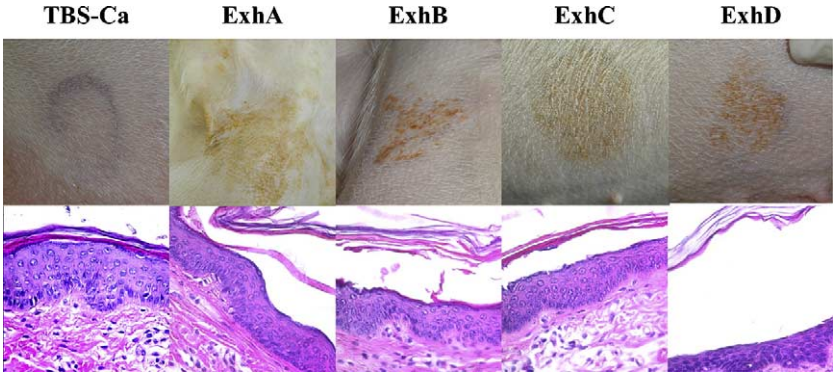

Fig. 2. Gross appearance and histological examination of pig skin injected with Exh.

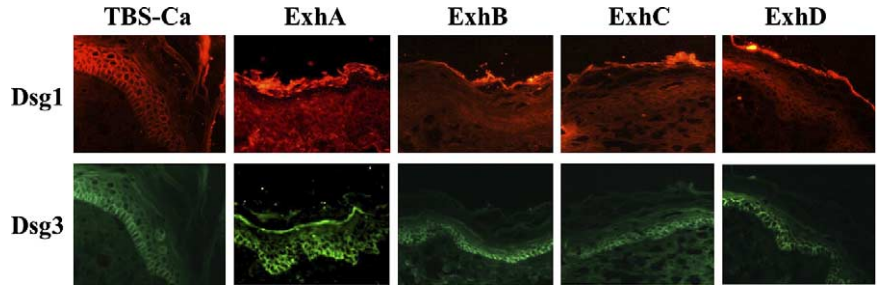

Fig. 3. Immunofluorescence of desmogleins in the epidermis of cryosection of pig skin incubated with Exh.

diminished in all Exh-treated samples, whereas that of Dsg3 by 5H10 was not affected at all. These effects by Exhs on Dsg1 and Dsg3 staining were very similar to those by ETs on that of human and mouse epidermis and indicated that Exhs selectively affect the extracellular domain of putative Dsg1 molecule in the absence of living cells, possibly by cleavage.

Since, the pig genes equivalent to human *dsg1* and *dsg3* are not available at present, we tried to see if Exhs are able to digest any of available Dsgs. We incubated a soluble recombinant form of the extracellular domain of human Dsg1-4, mouse Dsg1α-γ, 3, 4, or canine Dsg1, 3 with Exh, respectively, in vitro. As shown in Fig. 4, ExhA and ExhC were able to cleave the 80 kDa recombinant mouse Dsg1α and Dsg1β down to a 30 kDa peptide, otherwise Exhs did not cleave any of Dsg proteins at all. Knowing that ExhA and ExhC selectively cleave mouse Dsg1α and 1β, we subcutaneously injected each Exh into neonatal mice. As expected ExhA and ExhC induced gross blisters around the injection site. However, minimum doses to induce blisters within 6 h (20 μg for ExhA and 25 μg for ExhD) were relatively higher than that for ETA (0.25 μg). In case of ExhB and ExhD, even 800 μg of protein did not show any blisters in 16 h of post-injection (Fig. 5).

3. Discussion

Different patterns of cell surface staining of Dsg1 and Dsg3 have suggested a compensational function of these molecules in cell–cell adhesion in epidermis in human and mouse. This is also the case in porcine epidermis since 5H10 monoclonal antibody principally reacted with cell surface of the basal layer whereas pemphigus foliaceus sera recognized the cell surface of throughout the epidermis. Clear selective diminution of cell surface staining by pemphigus foliaceus sera in Exh-treated cryosection of pig epidermis indicated that all Exhs selectively target a Dsg1-equivalent cell–cell adhesion molecule in pig skin epidermis suggesting that Exhs may play similar pathophysiological function in onset of exfoliation of the skin in EE as ETs do in BI and SSSS.

ExhD is the latest member added to the family of Exh-proteins. Sequence analysis of *exhD* indicated that it has amino acid substitution at one of the conserved amino acids constituting catalytic triad for serine protease, asparagine to glutamine at 150 [10], which might raise a question whether ExhD is active or not. In this study, recombinant ExhD induced gross alteration of skin in vivo and selective diminution of presumable Dsg1 staining of cryosection.

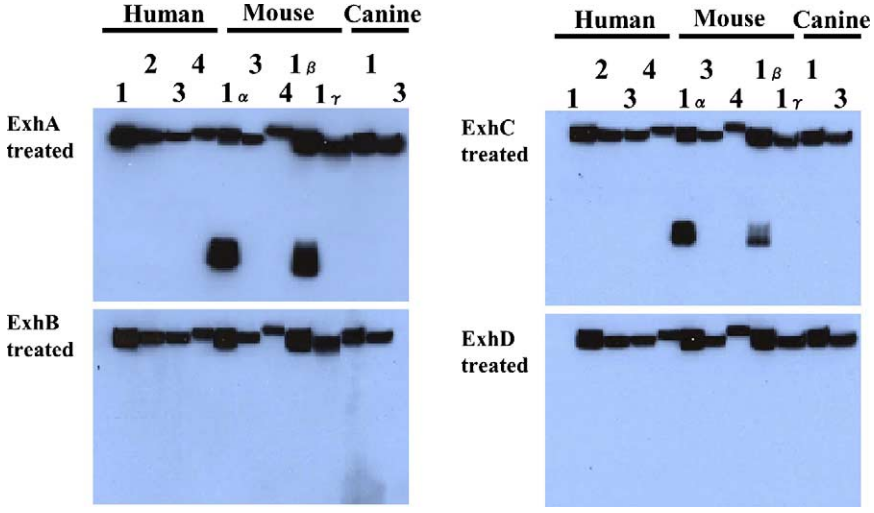

Fig. 4. In vitro treatment of baculovirus-expressed recombinant desmogleins by Exh. Extracellular domain of mouse, human or canine Dsgs produced by a baculovirus was incubated with Exh (10 μg/ml) [16]. The extracellular domain of mouse Dsg1α and 1β were cleaved by ExhA and ExhC at high concentration (10 μg/ml). Other Dsgs were resistant to digestion by all Exhs.

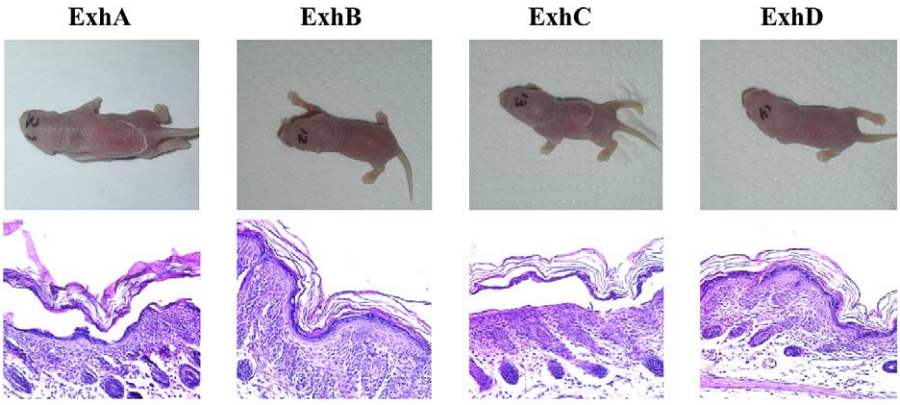

Fig. 5. Gross appearance of neonatal mouse skin injected with ExhA or ExhC. Neonatal mice injected with 100 μg of ExhA or ExhC show extensive blisters within 2 h after injection.

In addition, an investigation of the prevalence of toxigenic *S. hyicus* from cases of EE in Danish pig herds has shown that ExhD-producing *S. hyicus* frequently can be isolated from diseases animals as the disease-causing agent [22]. These observations suggest that ExhD is active as an exfoliative toxin though the relative potency of its activity compared to the other exfoliative toxins from *S. hyicus* is not known. In vitro digestion assay using extracellular domain of Dsg of human, mouse or dog as substrate indicated that Exhs have some species specificity in substrate. Human and canine Dsg1s are resistant to digestion by Exhs and mouse Dsg1 α/β are only susceptible to digestion by high concentration of ExhA or by ExhC. On the other hand, human and mouse Dsg1s are susceptible to ETA, ETB and ETD, but canine Dsg1 is not [18,19]. These results clearly indicate that Exhs and ETs possess species specificity. Ability of ExhA and ExhC to digest mouse Dsg1α/β cannot be simply attributed to their closer sequence similarity to ETA, ETB, and ETD. The molecular mechanism by which the Exhs and ETs sharply discriminate the substrates remains to be elucidated. These results also suggest that so far the only substrate for exfoliative toxins is Dsg1 but not Dsg3. ETA, ETB and ETD were shown to cleave just after the 381th glutamate in human and mouse Dsg1s indicating that they are acting as glutamate-specific serine proteases [18]. Given the similarity between those ETs and Exhs it was speculated that Exhs also possess similar specificity. However, final identification of Exhs as glutamate specific serine protease awaits identification and molecular characterization of the target molecule, porcine Dsg using Exhs.

4. Experimental/materials and methods

4.1. Bacterial strains

The *S. hyicus* strains used were NCTC10350 (ExhA producer), 1289D-88 (ExhB producer), 842A-88 (ExhC producer) and A2869C (ExhD producer). Primers used in this study are listed in Table 1. Other materials and chemicals were from commercial sources. *Staphylococcus* was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mitch) or Trypticase soy broth (TSB)(Becton Dickinson). *E. coil* was grown in Luria-Bertani broth. When necessary, ampicillin (100 μg/ml) was added for plasmid maintenance.

Table 1
Primers used in this study

Character	Name	PCR protocol	Oligonucleotide sequence (5′–3′)
ExhA	ExhA-1		GCATGCTTAGCGAATTATCAAAAGAA
	ExhA-2		AGATCTATTATTTATTTTTTGTTTTAATTC
exhB	ExhB-1		GCATGCTTAGCGAATTAGAACTTACT
	ExhB-2		AGATCTTTGAATTTCTTTTTTTAAGAA
exhC	ExhC-1		GCATGCTTGACGAAGAAAGTGACTTG
	ExhC-2		CGATCCTTTAATTAATTGTTTGAGATCTC
exhD	ExhD-1		GCATGCTTAGTGATTTATCAGAGCAA
	ExhD-2		AGATCTTTTATTCAATTTTTCTTTCAG
Mouse Dsgl-β	Primer1	RT-PCR	GCAACCATGGACTGGCACTCCTTCAGG
	Primer2		AGGCTCGAGGTGAACGTTGTCTTCTGTGATG
Mouse Dsgl-γ	Primer 4	RT-PCR	CTGGAAAATTAATTGAATCAAACACCTGC
	Primer 3		TGCCTCGAGGAAGTGAACATTGTCTACGTTTGGAGTG
Mouse Dsgl-γ	Primer1	Nested PCR	GCAACCATGGACTGGCACTCCTTCAGG
	Primer 3		TGCCTCGAGGAAGTGAACATTGTCTACGTTTGGAGTG

Table 2
Exfoliative activity of Exhs of ETA in neonatal mice

	Dose(μg)									
	800	25	20	10	5	2.5	1	0.5	0.25	0.1
ExhA			3/3	0/3						
ExhB	0/3[a]									
ExhC		3/3	1/3	0/3						
ExhD	0/3									
ETA									3/3	0/3

[a] A number of mice showing blister formation/a total number of mice.

4.2. Preparation of recombinant Exh

For purification of Exh with a His tag on the carboxyl-terminal end, primer sets were designed to amplify DNA fragments corresponding to the mature form of ExhA, ExhB, ExhC or ExhD according to the nucleotide sequences deposited in GenBank: *exhA*, AF515453; *exhB*, AF515454; *exhC*, AF515455; *exhD*, AF515456. Chromosomal DNA of each *S. hyicus* strain producing Exh was isolated as described previously [23]. PCR amplified DNAs were cloned into pQE70 (Qiagen, Valencia, Calif.) in order to express the His tag protein in *E. coli* DH10B. DNA sequences of the amplified DNAs were confirmed by an automated sequencer. His tag proteins were expressed in *E. coli* DH10B and recovered from the soluble fraction in lysis buffer (20 mM Tris–Cl [pH 8.0], 0.2 M NaCl, 0.2% Triton X-100, and protease inhibitor cocktail [Complete Mini, EDTA free; Roche Diagnostics, Mannheim, Germany]). Recombinant Exhs were purified by using TALON affinity resin (Clontech, Palo Alto, Calif.) (Table 2).

4.3. Pig skin assay

Pigs with the age of three weeks were housed individually in isolation units and fed antibiotic free feed and sow milk replacement ad libitum. The animal experiments were performed in accordance with a license from the Danish Animal Experiments Inspectorate. The pigs were intracutaneously injected with recombinant Exh (500 μg) dissolved in 100 μl of phosphate-buffered saline. Injections were placed proximal to both auxiliary and inguinal regions and on both sides of the abdomen between the second and the third nipple from the front of the pig. Samples were injected in duplicate, one on each of the two pigs, so that each sample was injected at two different positions. The sites of injection were examined and skin reactions recorded once every day for 5 days.

Neonatal mouse assay. Neonatal ICR mice (age, <24 h) were subcutaneously injected with recombinant Exh dissolved in 100 μl of phosphate-buffered saline, and the skin was examined by eye and microscopically at 1–6 h after injection. The mouse experiments were performed in accordance with the guidelines for animal experiments of the Research Facility for Laboratory Animal Science, Hiroshima University.

4.4. Immunofluorescence

Cryosections of nonfixed normal pig skin were incubated with 10 μg per ml of recombinant Exh in Tris–Cl with 1 mM $CaCl_2$ (TBS-Ca) or PBS-Ca alone for 1 h at room temperature. The sections were then stained with anti-Dsg1 sera obtained from patients with pemphigus foliaceus, anti-Dsg3 mouse monoclonal antibody, 5H10, which reacts with the extracellular domain [24].

4.5. In vitro digestion of recombinant Dsg

Recombinant Dsgs used were human Dsg1-4, mouse Dsg1α-γ, 3, 4, and canine Dsg1, 3. The entire extracellular domain of recombinant Dsg of a various origin with an E-tag on the carboxyl terminus was produced as a secreted protein by baculovirus expression. Extracellular domains of mouse Dsg1α, Dsg3 and Dsg4, human Dsg1, Dsg2, Dsg3 and Dsg4 as well as canine Dsg1 and Dsg3 with E tags and His tags on the carboxyl termini were expressed in a baculovirus and collected from culture supernatants as previously described [25–27]. To obtain cDNA for the entire extracellular domain of mouse Dsg1β (GenBank AY192158), total RNA was isolated from ICR mouse skin using RNeasy Mini Kit (Qiagen, Hilden, Germany), and used as a templates for RT-PCR amplification with Superscript TM One-Step RT-PCR for Long templates (Invitrogen, Carlsbad, CA) and appropriate primers (Table 1). cDNA for the entire extracellular domain of mouse Dsg1γ (GenBank AY192159) was also isolated by RT-PCR with total RNA from ICR mouse liver, and followed by nested PCR with primers (Table 1). These cDNAs were digested with *Nco*I and *Xho*I and ligated into the pQE-TriSystem vector (Qiagen Inc.) to generate pQE-mDsg1ß-His and pQE-mDsg1γ-His, respectively. The E-tag and His-tag were inserted in tandem at the C-terminal end of this protein. Nucleotide sequencing of the mouse Dsg1ß and γ constructs revealed no nucleotide and amino acid changes. pQE-Dsg1β-His and pQE-mDsg1γ-His were cotransfected with Sapphire Baculovirus DNA (Orbigen Inc.) into cultured Sf9 insect cells. Recombinant viruses produced in the culture supernatants were infected into High Five cells and cultured for 3 days. All of these supernatants were collected and directly used for in vitro digestion study as described [17]. Digested samples were assessed by immunoblot analysis with an anti-E tag mouse monoclonal antibody (Pharmacia Biotech, Uppsala, Sweden) for detection of the recombinant proteins.

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