〈Brief Note〉

Conformation-dependent fibronectin binding to *Clostridium perfringens*

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Summary Fibronectin (Fn), an extracellular matrix protein that is found in plasma and on the cell surface, is involved in wound healing, cell migration, and tissue reconstitution. Fn consists of 12 type I, two type II, and 15 to 17 type III modules, and has various conformations; *in vitro*, it has a compact or extended conformation in solutions with a low or high ionic strength, respectively. Previously, we reported that *Clostridium perfringens* cells bind to gelatin via Fn, and recognize Fn type $III_{9\text{-}10}$ ($III_{9\text{-}10}$). In the present study, we investigated the interaction between *C. perfringens* cells and Fn in solutions with a low (l-Fn) or high (h-Fn) ionic strength. l-Fn more bound to dry-fixed cell than h-Fn. Furthermore, Fn-prebound gelatin was prepared by reacting l-Fn or h-Fn with coated gelatin (l-Fn gelatin or h-Fn gelatin, respectively), and a substantial amount of an anti-$III_{9\text{-}10}$ monoclonal antibody (HB39) and a substantial number of *C. perfringens* cells bound to both the l-Fn gelatin and h-Fn gelatin. However, both HB39 and *C. perfringens* cells showed a predilection for binding to l-Fn gelatin when compared to h-Fn gelatin. These results indicated that the Fn $III_{9\text{-}10}$ modules that are recognized by *C. perfringens* cells are more accessible in l-Fn than in h-Fn, although *C. perfringens* cells can recognize both l-Fn gelatin and h-Fn gelatin. These results imply that regardless of the conformation of the reacting Fn, *C. perfringens* cells can efficiently adhere to collagen-binding tissues as long as Fn is bound to collagen. This may confer a major advantage for *C. perfringens* infection.

Key words: Fibronectin, *Clostridium perfringens*, Fn Type $III_{9\text{-}10}$, Gelatin, Fn compact conformation

1. Introduction

Fibronectin (Fn), an abundant glycoprotein found in plasma, is an extracellular matrix protein. Fn is involved in many cellular processes, including wound healing, tissue structure formation, and cell migration[1]. Fn is composed of two very similar subunits that are linked by two asymmetric disulfide bonds located near the carboxyl terminal to form a dimeric structure. The monomer polypeptide of Fn consists of 12 type I, two type II, and 15 to 17 type III modules (Fig. 1)[2]. Fn binds to collagen or gelatin through the $I_6II_{1\text{-}2}I_{7\text{-}9}$ site of Fn[3,4]. Although Fn is known to have a compact conformation (compact Fn) under normal physiological conditions, it can change to the extended conformation (extended Fn) under other conditions, *e.g.*, changes in the pH or the

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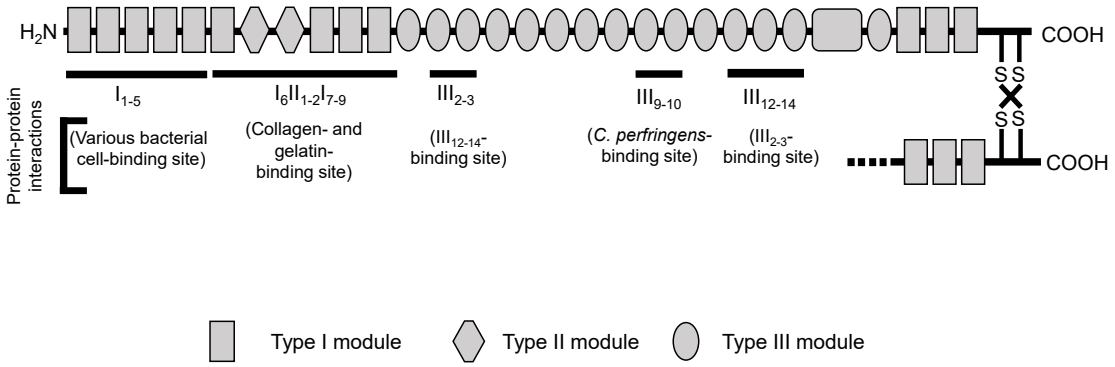

Fig. 1. Diagram of an Fn monomer showing selected binding sites. Fn exists as a dimer comprised of two nearly identical monomers linked at the C-terminus by disulfide bonds. The rectangles, hexagons, and ellipses represent Type I, Type II, and Type III modules, respectively. The regions I_{1-5} (various bacterial cell-binding site), $I_6II_{1-2}I_{7-9}$ (collagen- and gelatin-binding site), III_{2-3} (III_{12-14}-binding site), III_{9-10} (*C. perfringens* cell-binding site), and III_{12-14} (III_{2-3}-binding site) are indicated by bold lines.

presence of glycerol[5–7]. The conversion from compact Fn to extended Fn can also be induced *in vitro* by dissolving Fn in a solution with a high salt concentration[7–9]. Fn conformation changes have been detected by various methods, including circular dichroism[8,10], transmission electron microscopy[7], size exclusion chromatography[11,12], and light scattering[13–15].

Clostridium perfringens is a Gram-positive, spore-forming, obligate anaerobe that causes gas gangrene, a serious wound-associated infection[16]. We previously reported that *C. perfringens* cells adhered to type II collagen, type III collagen, and gelatin when they were prebound with Fn, but not to collagens and gelatin themselves[17]. Although a few *C. perfringens* cells may adhere to type I collagen, many more cells adhere to Fn-prebound type I collagen[17]. In the early phase of the wound-healing process, Fn binds to collagen in the wound tissue, which may facilitate the adhesion and infiltration of *C. perfringens* cells into the wounded tissue. In a previous study, it was shown that an anti-Fn III_{9-10} monoclonal antibody, HB39, inhibited *C. perfringens* cell binding to immobilized Fn[18]. Furthermore, *C. perfringens* cells bound to immobilized recombinant Fn Type III_{9-10} (III_{9-10}) as well as immobilized Fn[19]. These results suggested that *C. perfringens* cells recognize III_{9-10}. However, the number of *C. perfringens* cells that bound to either recombinant Fn Type III_9 (III_9) or recombinant Fn Type III_{10} (III_{10}) was marginal[18], suggesting the importance of the combination of the III_9 and III_{10} modules for the effective binding of *C. perfringens* cells to Fn.

Erickson *et al*. showed by transmission electron microscopy that extended Fn could be seen as an extended thin strand with a few bends, whereas compact Fn was more substantially bent, and folded upon itself[7]. A pronounced hinge point at the III_{9-10} interface was also observed as a bend in compact Fn[9]. Bending at the hinge region of III_{9-10} could be accounted for by the folding of Fn into the compact conformation by structural analysis[20]. To date, it remains unclear which Fn form is preferably recognized by *C. perfringens* cells. Thus, the present study investigated the binding of various forms of Fn to *C. perfringens* cells, and the interactions of *C. perfringens* cells and HB39 with the various forms of Fn prebound to gelatin.

2. Materials and Methods

Bacterial strains and growth conditions

C. perfringens HN13 (strain 13 Δ*galK* Δ*galT*)[21] was derived from the wild-type strain 13, which was isolated from soil. HN13 was cultured anaerobically in Gifu anaerobic medium (Nissui Co., Tokyo, Japan) using the Anaero Pack system (Mitsubishi Gas Chemical, Tokyo, Japan).

Preparation of Fn and anti-Fn monoclonal antibody HB39

Fn was purified from pooled human serum using a gelatin-sepharose column as described previously[22]. The purified Fn was dissolved in 10 mmol/L veronal buffer, pH 7.4 (VB), containing 50 mmol/L NaCl (50 mmol/L NaCl VB), 140 mmol/L NaCl (140 mmol/L NaCl VB), 250 mmol/L NaCl (250 mmol/L NaCl VB), or 750 mmol/L NaCl (750 mmol/L NaCl VB). Gelatin was purchased from Thermo Fisher Scientific (Waltham, MA, USA). A hybridoma cell line producing HB39 (immunoglobulin (Ig) G) was purchased from the American Type Culture Collection (Manassas, VA, USA). HB39 was purified from the hybridoma cell supernatant using a Protein G affinity column (Cytiva, Tokyo, Japan).

Biotinylation of *C. perfringens* cells and Fn

HN13 cells were suspended in 50 mmol/L NaCl VB to an optical density at a wavelength of 600 nm (OD_{600}) of 0.1, then reacted with 0.1 mmol/L biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester (Dojindo, Kumamoto, Japan) for 1 h at room temperature. After washing with 50 mmol/L NaCl VB, the biotinylated HN13 cells were resuspended in 50, 140, or 250 mmol/L NaCl VB to an OD_{600} value of 0.1. Biotinylation of Fn was performed as previously described[22].

Enzyme-linked avidin-biotin complex system

All plate-binding assays were carried out in enzyme immunoassay (EIA) plates (Corning Inc., Corning, NY, USA).

Dry-fixed HN13 cells on an EIA plate were prepared as described previously[18]. After blocking with BSA, the wells were reacted with 1 μg of biotinylated Fn in 50, 140, or 250 mmol/L NaCl VB (Fig. 2). The wells were washed three times with 20 mmol/L phosphate-buffered saline (pH 7.4) containing 0.02% (v/v) Tween 20 (PBST).

The wells of EIA plates were individually coated with gelatin. For coating, 50 μL of gelatin solution at a concentration of 20 μg/mL in 10 mmol/L borate buffer (pH 8.5) was added to each well, then incubated for 30 min at room temperature.

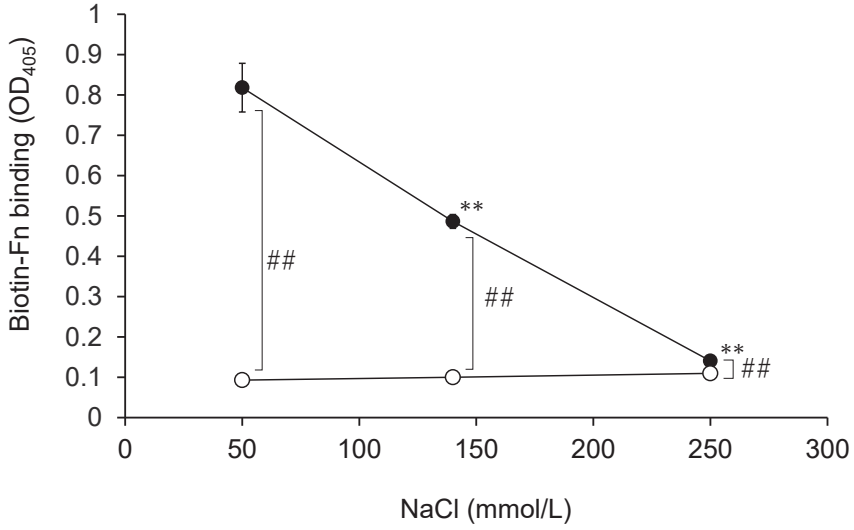

Fig. 2. The binding of l-Fn or h-Fn to dry-fixed HN13 cells. The dry-fixed HN13 cells were reacted with biotinylated Fn (1 μg/well) in 50, 140, or 250 mmol/L NaCl VB. After several washes, HRP-streptavidin was added, and binding was detected by a colorimetric assay. BSA was used as a blocking reagent. Closed circles indicate the binding of biotinylated Fn to dry-fixed HN13 cells. Open circles indicate the binding of biotinylated Fn to BSA. Data are shown as the mean ± SD (n = 3). **, $p < 0.01$ vs. the binding of biotinylated Fn in 50 mmol/L NaCl VB to dry-fixed HN13 cells (Dunnett's multiple comparison tests). ##, $p < 0.01$ (Student's *t*-test).

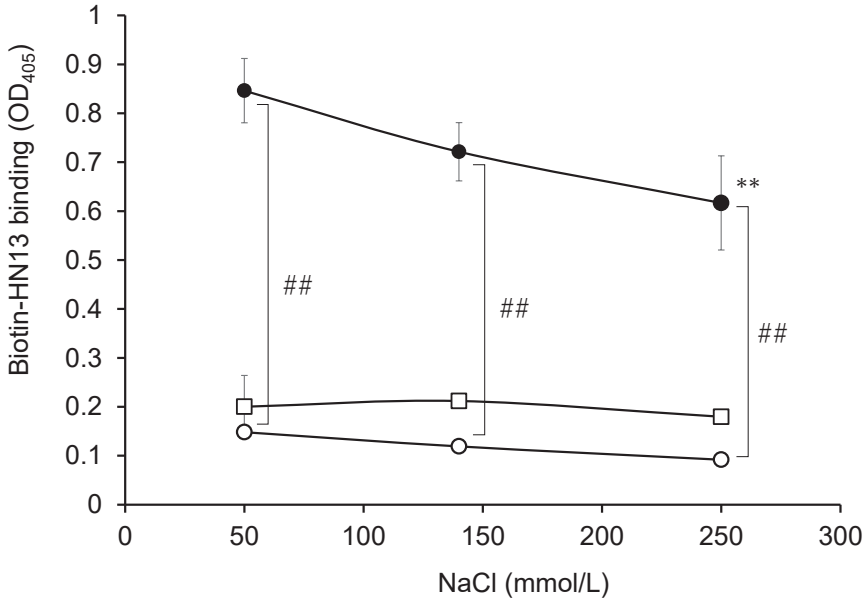

Fig. 3. The binding of biotinylated HN13 cells to l-Fn gelatin or h-Fn gelatin. Coated gelatin (1 μg/well) was reacted with or without Fn (1 μg/well) in 50, 140, or 250 mmol/L NaCl VB. Then, 0.1 mL of a biotinylated HN13 cell suspension (OD_{600} = 0.1) was added. After several washes, HRP-streptavidin was added, and binding was detected by a colorimetric assay. BSA was used as a blocking reagent. Closed circles indicate the binding of biotinylated HN13 cells to Fn-prebound gelatin. Open circles indicate the binding of biotinylated HN13 cells to gelatin. Open squares indicate the binding of biotinylated HN13 cells to BSA. Data are shown as the mean ± SD (n = 3). **, $p < 0.01$ vs. the binding of biotinylated HN13 cells to l-Fn-prebound gelatin (Dunnett's multiple comparison tests). ##, $p < 0.01$ (Student's *t*-test).

After blocking with bovine serum albumin (BSA), the wells were reacted with or without 1 μg of Fn in 50, 140, or 250 mmol/L NaCl VB (Figs. 3 and 4). After three washes with PBST, the wells were reacted with biotinylated HN13 (Fig. 3) or biotinylated HB39 for 1 h at room temperature (Fig. 4).

Bound proteins and HN13 cells were detected as follows. After washing with PBST, the wells were reacted with 100 μL of horseradish peroxidase (HRP)-streptavidin (Vector Laboratories, Burlingame, CA, USA) or secondary antibody (HRP-conjugated goat anti-mouse IgG antibody; SeraCare Life Sciences Inc., Milford, MA, USA) in 10 mmol/L Tris-buffered saline (pH 7.4), then incubated for 30 min at room temperature. After washing with PBST, the color was developed by adding 0.1 mL of 0.91 mmol/L 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) in 0.1 M citrate buffer (pH 4.1) containing 0.03% H_2O_2. The reaction was stopped by adding 0.1 mL of 0.1 M citric acid containing 0.01% NaN_3. The absorbance of each well at 405 nm was then measured using a microplate reader.

Size exclusion chromatography

For size exclusion chromatography, 100 μL of Fn solution (0.5 mg/mL) in 50, 140, 250, or 750 mmol/L NaCl VB was applied to a Superose 6 Increase 10/300 GL column (Cytiva) on an Äkta FPLC system (Cytiva) equilibrated with VB containing 50, 140, 250, or 750 mmol/L NaCl, respectively, in the isocratic mode at a flow rate of 0.5 mL/min.

Statistical analysis

Data are presented as the means ± standard deviation (SD). Statistical comparisons were made using one-way analysis of variance followed by a

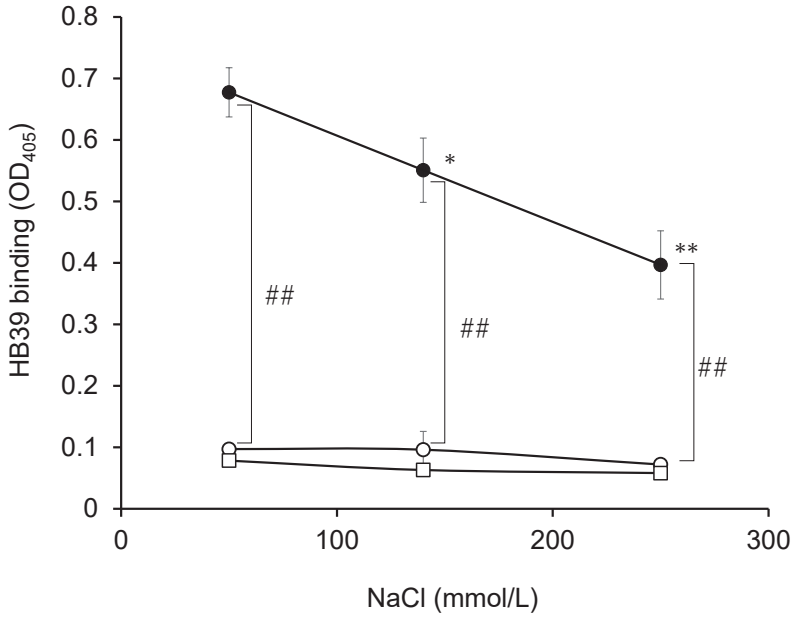

Fig. 4. The binding of HB39 to l-Fn gelatin or h-Fn gelatin. Coated gelatin (1 μg/well) was reacted with or without Fn (1 μg/well) in 50, 140, or 250 mmol/L NaCl VB. Then, 0.1 mL of HB39 was added. After several washes, HRP-conjugated anti-mouse-IgG antibody was added, and binding was detected by a colorimetric assay. BSA was used as a blocking reagent. Closed circles indicate the binding of HB39 to Fn-prebound gelatin. Open circles indicate the binding of HB39 to gelatin. Open squares indicate the binding of HB39 to BSA. Data are shown as the mean ± SD (n = 3). *, $p < 0.05$ and **, $p < 0.01$ vs. the binding of HB39 to l-Fn-prebound gelatin (Dunnett's multiple comparison tests). ##, $p < 0.01$ (Student's *t*-test).

Student's *t*-test[23] or Dunnett's multiple comparison test[24]. A *p*-value of <0.05 was considered to be indicative of statistical significance.

3. Results

Fn binding to dry-fixed HN13 cells under various ionic strengths

We reported that the biotinylated Fn in 140 mmol/L NaCl VB binds to dry-fixed strain13 cells[29]. In this study, the binding of biotinylated Fn to dry-fixed HN13 cells under various concentrations of NaCl was measured. Fn in 50 mmol/L NaCl VB (l-Fn), Fn in 140 mmol/L NaCl VB, and Fn in 250 mmol/L NaCl VB (h-Fn) bound significantly to dry-fixed HN13 cells than coated BSA. l-Fn bound significantly more to dry-fixed HN13 cells than Fn in 140 mmol/L NaCl VB and h-Fn. The binding of Fn in 250 mmol/L NaCl VB (h-Fn) to the cells was marginal. The colorimetric value of h-Fn was only approximately 4.3% of that of l-Fn (Fig. 2).

HN13 cell binding to Fn-prebound gelatin under various ionic strengths

As reported in a previous study, *C. perfringens* cells adhere to Fn-prebound gelatin, but not to gelatin itself[17]. Thus, the binding of HN13 cells was examined by using Fn-prebound gelatin, which was prepared by reacting gelatin with Fn under various NaCl concentrations. A substantial number of HN13 cells bound significantly to the Fn-prebound gelatin prepared with 50 mmol/L NaCl VB (l-Fn gelatin), 140 mmol/L NaCl VB and 250 mmol/L NaCl VB (h-Fn gelatin), compared with to gelatin itself and to coated BSA, although the cell binding to h-Fn gelatin was 32.3% lower than that to l-Fn gelatin

(Fig. 3). Almost equal amounts of biotinylated Fn bound to all of the Fn-prebound gelatin prepared with Fn in 50, 140, and 250 mmol/L NaCl VB (data not shown).

Binding of HB39 to Fn-prebound gelatin under various concentrations of NaCl

We previously found that HB39 recognizing $III_{9\text{-}10}$ inhibits the binding of *C. perfringens* cells to immobilized Fn[18]. Furthermore, *C. perfringens* cells bound equally to both immobilized $III_{9\text{-}10}$ and immobilized Fn[19]. The results shown in Fig. 3 therefore prompted us to investigate HB39 binding to gelatin, which was prepared by reacting gelatin with Fn under various concentrations of NaCl. HB39 binding to Fn-prebound gelatin significantly decreased as the NaCl concentration increased (Fig. 4). However, substantial amounts of HB39 also significantly bound to h-Fn gelatin, compared with to gelatin itself and to coated BSA. The colorimetric value of HB39 binding to h-Fn gelatin was approximately 56.5% of that binding to l-Fn gelatin (Fig. 4).

Size exclusion analysis of Fn under various ionic strengths

Mariotti *et al*. demonstrated by size exclusion chromatography that the retention time of Fn in phosphate buffer containing 750 mmol/L NaCl was shorter than that in phosphate buffer containing 150 mmol/L NaCl[12]. Therefore, we investigated the retention times in Fn in 50, 140, 250, and 750 mmol/L NaCl VB using size exclusion chromatography under conditions similar to theirs. Contrary to expectation, in the size exclusion chromatography experiment, the retention times of Fn in 50, 140, and 250 mmol/L NaCl VB were 25.1, 26.5, and 27.1 min, respectively (Fig. 5). However, the retention time of Fn in 750 mmol/L NaCl VB was 23.2 min, and this result was similar to previous study[12] (data not shown).

4. Discussion

Previously, we found that *C. perfringens* cells adhered to collagen and gelatin through the $III_{9\text{-}10}$

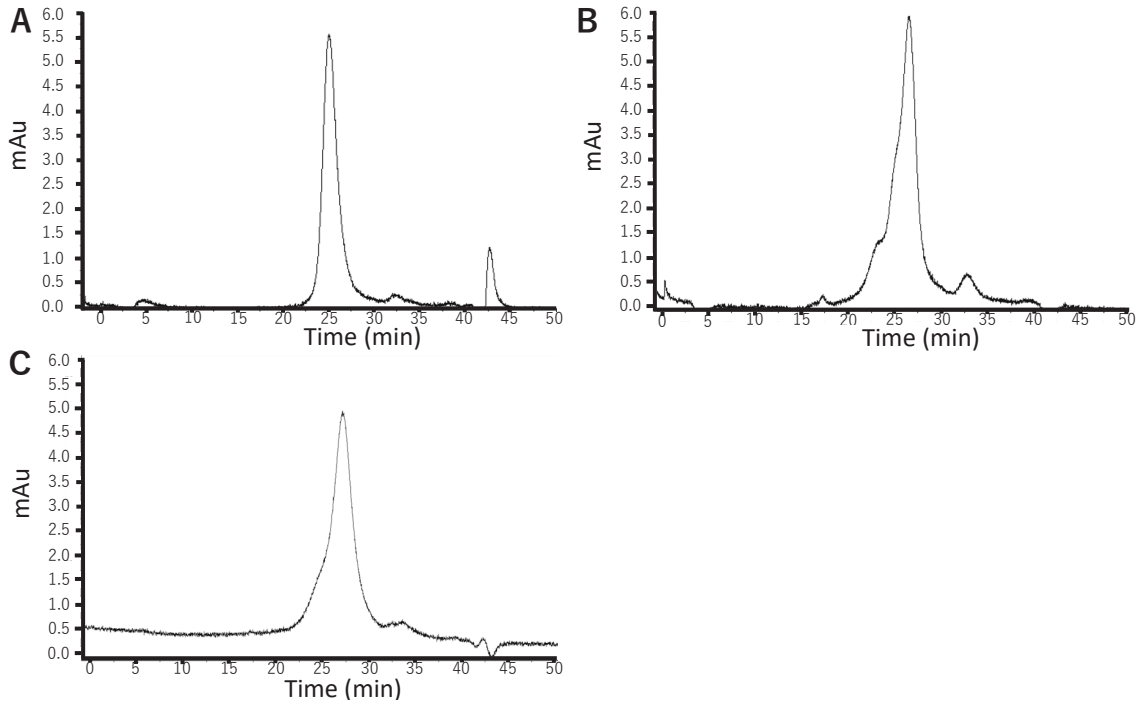

Fig. 5. Size exclusion chromatography of Fn in 50, 140, and 250 mmol/L NaCl VB. Fn (50 μg) in 50 (A), 140 (B), or 250 mmol/L NaCl VB (C) was separated by a Superose 6 Increase 10/300 GL column on an Äkta FPLC system equilibrated with 50 (A), 140 (B), or 250 mmol/L NaCl VB (C) in the isocratic mode at 0.5 mL/min. mAu: milli absorbance units.

moiety of Fn[17–19]. The bending of the $III_{9\text{-}10}$ site depends on the conformation of the Fn molecule[9,20]. Fn is known to have a compact or extended conformation at a low or high ionic strength, respectively[7,8,11–14]. In this study, it is noteworthy that although the soluble Fn in the high ionic strength (250 mmol/L NaCl) solution almost completely lost its binding activity to dry-fixed HN13 cells (Fig. 2), both a substantial number of HN13 cells and a substantial amount of HB39 bound to Fn-prebound gelatin that was prepared with 250 mmol/L NaCl (Figs. 3 and 4). This may be explained by possible differences in the conformation between soluble Fn and Fn prebound to gelatin.

The conformation of Fn changes depending on various factors, such as the pH, oxidative stress and ionic strength, and the presence of urea, glycerol and proteins. We expected that the apparent size of compact Fn is smaller than extended Fn. Thus, we attempted to determine the difference of Fn conformation using by size exclusion chromatography. However, contrary to our expectation, the retention time of Fn in 50 to 250 mmol/L NaCl VB decreased with increasing NaCl concentration (Fig. 5 A-C). The profiles of Fn in 140 and 250 mmol/L NaCl VB showed an asymmetric fronting peak by same analytical technique in the previous study[12], resulting in a delay of the retention time (Fig. 5 B and C).

In a previous study, it was suggested that the site between III_9 and III_{10} is a hinge region in compact Fn[9]. The bending of the hinge region of $III_{9\text{-}10}$ is required for the compact conformation of Fn[20]. In the present study, more l-Fn bound to dry-fixed HN13 cells than h-Fn (Fig. 2). Therefore, the bending between III_9 and III_{10} may be important for the increased Fn binding to *C. perfringens* cells.

In the early phase of the wound-healing process, Fn released from disrupted blood vessels binds to collagen. Upon injury, ion leakage (such as Na^+, Cl^-, K^+, and Ca^{2+}) occurs across wounded cells or cell layers. This establishes a voltage gradient that is laterally orientated at wounds, pointing toward the wound center[26]. Furthermore, the pH in the wounded area increases[27], promoting the extended conformation of Fn[8]. Nonetheless, *C. perfringens* cells are able to bind to wound tissue efficiently even when the Fn bound to collagen is in the extended form.

In this study, we demonstrated that the site on Fn for the binding of dry-fixed HN13 cells is more highly recognized on l-Fn than on h-Fn, and that HN13 cells can recognize not only l-Fn gelatin, but also h-Fn gelatin. In previous studies, we identified some Fn-binding proteins (Fbps) in *C. perfringens*, including FbpC, FbpD, and glyceraldehyde-3-phosphate dehydrogenase[28,29]; however, the interrelationships between these Fbps and the Fn conformations remain unknown. We consider that the ability of *C. perfringens* cells to bind to Fn, despite the broad spectrum of Fn conformations, provides a great advantage to the bacterium for infecting efficiently.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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