SHORT COMMUNICATION

Fibronectin-Binding Proteins of Host-Associated Spirochetes

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Introduction

Fibronectin, one of the extracellular matrix components, is found on the surface of eucaryotic cells including fibroblasts and hepatocytes¹⁻³⁾, and can mediate cytoadherence of certain gram-positive and gram-negative bacteria⁴⁻¹¹⁾. Spirochetes which cause or are associated with many diseases such as syphilis, Lyme disease, relapsing fever, leptospirosis and swine dysentery have an affinity for this surface-associated protein¹²⁻¹⁵⁾. We also reported that a human oral spirochete, *Treponema denticola*, a predominant species in diseased periodontal pockets, possesses fibronectin-binding outer envelope proteins with molecular sizes of 53 kDa and 72 kDa, suggesting that these fibronectin-binding surface proteins may play a role in their colonization of the oral cavity¹⁶⁾. In this study we conducted a detailed examination of the fibronectin-binding proteins of further host-associated spirochetes including *T. socranskii* and a human oral intermediate-sized isolate.

Materials and Methods

Seven strains of host-associated spirochetes, *Treponema denticola* ATCC 33520 and ATCC 35404, *T. socranskii* subsp. *buccale* ATCC 35534, a human oral intermediate-sized isolate strain G7201, *T. pallidum* ATCC 27087 and *T. phagedenis* biotypes Reiter and

Kazan 8 were anaerobically grown in TYGVS medium, Treponema medium, or Nos medium at 37°C for 4 to 7 days and collected by centrifugation, washed twice in 0.01 M phosphate-buffered saline (PBS ; pH 7.2), and resuspended again in PBS as whole cell samples¹⁷⁾. The polypeptides from each bacterial strain were separated on a 1-mm thick, 10 % polyacrylamide gel by discontinuous SDS-PAGE as reported by Laemmli¹⁸⁾. Mixtures of an aliquot of each whole cell sample (10 µg of protein) and 5 µl of the sample buffer were heated at 100°C for 5 min, and were then loaded in wells of the stacking gels¹⁶⁾. The separated polypeptides from each strain were stained by Coomassie brilliant blue dye for specification and determination of molecular sizes of individual protein bands.

In order to examine the reactivity of the polypeptide of whole cells with human plasma fibronectin (1.3 µg/ml ; Sigma Chemical Co., St. Louis, Mo., U. S. A.), an immunoblot analysis was conducted on the basis of the method of Towbin et al¹⁹⁾. Separated polypeptides on SDS-polyacrylamide gels were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Ca., U. S. A.)¹⁶⁾. Fibronectin-binding of the separated polypeptides was examined by using rabbit anti-human fibronectin serum (1 : 400 dilution ; Organon Teknika Corp., West Chester, Pa., U. S. A.) and horseradish peroxidase-conjugated affinity-purified anti-rabbit immunoglobulin G [H+L] (1 : 1,000 dilution ; Bio-Rad). The nitrocellulose

membranes were incubated for 30 min in 30 ml of Tris-buffered saline (TBS; pH 7.5) containing 1 % of bovine serum albumin (Fraction V, Nakalai Tesque Inc., Kyoto, Japan), and each reaction proceeded at room temperature for more than 2 hrs. The membranes were washed three times (20 min each) with 20 ml of TBS containing 0.005 % Tween 20 after incubation of nitrocellulose membranes with fibronectin solution, anti-human fibronectin serum, or anti-rabbit immunoglobulin G.

Immunoelectron microscopy was done as previously reported¹⁷⁾. Briefly, 10 μ l of each bacterial suspension (*T. phagedenis* biotype Reiter, *T. denticola* ATCC 35404, or *T. socranskii* subsp. *buccale* ATCC 35534) was mounted on collodion membrane-coated nickel grids. The grids were dried, placed on the surface of a 100 μ l sample of each polyclonal rabbit antiserum at a dilution of 1 : 30 for two hrs at room temperature, and washed five times with PBS to remove unbound antiserum. The spirochetal cells on the grids were reacted with a protien A-gold complex (gold particle size, 15 nm; E. Y. Laboratories, Inc., San Mateo, Ca., U. S. A.) at a dilution of 1 : 30 for 60 min at room temperature. The microorganisms were washed again five times in distilled water, negatively stained with 2 % phosphotungstic acid (pH 7), and observed under an electron microscope.

Results and Discussion

The Coomassie brilliant blue-stained SDS-polyacrylamide gel profiles of the seven treponemal strains showed polypeptide bands ranging from 10 kDa to 170 kDa (Fig. 1, upper lanes). Immunoblot analysis revealed that human plasma fibronectin bound to several polypeptides separated by SDS-PAGE (Fig. 1, lower lanes). They included major and minor polypeptides such as the 40-kDa, 44-kDa, 66-kDa, 97-kDa and approximately 150-kDa polypeptides from *T. phagedenis* biotype Reiter, the 40-kDa, 46-kDa, 66-kDa, 83-kDa and 97-kDa polypeptides from *T. phagedenis* biotype Kazan 8, the 40-kDa, 46-kDa, 66-kDa, 83-kDa and 97-kDa polypeptides from *T. pallidum* ATCC 27087, the 38-kDa, 52-kDa, 66-kDa, 72-kDa and approximately 150-kDa polypeptides from *T.*

Fig. 1 Polypeptide patterns of seven strains of treponemes and their immunoblot analysis with fibronectin. The Coomassie brilliant blue-stained polypeptides (10 μ g of protein per lane) (upper lanes) of *T. phagedenis* biotypes Reiter (lane 1) and Kazan 8 (lane 2), *T. pallidum* ATCC 27087 (lane 3), *T. denticola* ATCC 35404 (lane 4), ATCC 33520 (lane 5), *T. socranskii* subsp. *buccale* ATCC 35534 (lane 6), and a human clinical isolate strain G7201 (lane 7) were reacted with human plasma fibronectin (1.3 μ g/ml) (lower lanes). Seven bands with molecular sizes in kilodaltons (arrows) show typical fibronectin-binding polypeptides with particular interests for their localizations in bacterial cells. Molecular size standards (in kilodaltons) are shown on the upper right.

denticola ATCC 35404, the 38-kDa, 53-kDa, 66-kDa, 72-kDa, 83-kDa, 97-kDa, and approximately 140-kDa polypeptides from *T. denticola* ATCC 33520, the 45-kDa, 53-kDa, 66-kDa, 83-kDa, 97-kDa and approximately 150-kDa polypeptides from *T. socranskii* subsp. *buccale* ATCC 35534, and the 53-kDa, 68-kDa, 83-kDa, 97-kDa and approximately 150-kDa polypeptides from a human isolate strain G7201. Im-

Fig. 2 Electron micrographs showing negatively stained treponemes after incubation with polyclonal antisera, followed by incubation with protein A-gold conjugate. All figures show the typical images of each reacted sample. (A) *T. phagedenis* biotype Reiter reacted with anti-66-kDa protein antigen serum, (B) *T. denticola* ATCC 35404 reacted with anti-72-kDa protein antigen serum, (C) *T. denticola* ATCC 35404 reacted with anti-52-kDa protein antigen serum, (D) *T. socranskii* subsp. *buccale* ATCC 35534 reacted with anti-83-kDa protein antigen serum, (E) *T. denticola* ATCC 35404 reacted with anti-38-kDa protein antigen serum, (F) *T. socranskii* subsp. *buccale* ATCC 35534 reacted with anti-45-kDa protein antigen serum, and (G) *T. denticola* ATCC 33520 reacted with anti-53-kDa protein antigen serum. Bars, 100 nm.

munoelectron microscopy using protein A-gold particles and the individual polyclonal rabbit antiserum against each pooled protein from SDS-polyacrylamide gels demonstrated that five polypeptides with molecular sizes of 66 kDa from *T. phagedenis*

biotype Reiter, 52 kDa and 72 kDa from *T. denticola* ATCC 35404, 45 kDa and 83 kDa from *T. socranskii* subsp. *buccale* ATCC 35534, originated from outer envelopes or cell wall-membrane complexes (Fig. 2). The 38-kDa polypeptide of *T. denticola* ATCC 35404

was from its axial flagella (Fig. 2).

Immunoblotting using rabbit antisera against the human plasma fibronectin and anti-rabbit IgG-horse-radish peroxidase conjugate demonstrated that the five species, *T. pallidum*, *T. socranskii*, *T. phagedenis*, *T. denticola* and the unidentified intermediate-sized human isolate strain G7201, which is morphologically and biochemically different from any other cited treponemes of human oral origins, possessed several fibronectin-binding proteins. Of the fibronectin-binding proteins detected here by immunoblot analysis, the 45-kDa, 52-kDa, 66-kDa, 72-kDa and 83-kDa polypeptides originated from the outer envelopes or wall-membrane complexes. The 38-kDa flagellar protein and the 53-kDa surface protein from *T. denticola* ATCC 33520 are also fibronectin-binding outer envelope proteins¹⁶⁾. While 62-kDa, 66-kDa and 84-kDa polypeptides were commonly detected in all seven strains, the 38-kDa, 48-kDa, 52-kDa and 72-kDa polypeptides were detected in two ATCC strains of *T. denticola*¹⁷⁾. The outer envelope fractions from three serovars of *T. denticola* comprise 15 to 22 Coomassie brilliant blue-stained polypeptides with molecular sizes of 14 kDa to 97 kDa²⁰⁾. Although further detailed studies are necessary to determine whether or not the other fibronectin-binding polypeptides seen in Figure 1 will be surface outer envelope proteins, some fibronectin-binding polypeptides may be from the surface outer envelopes of the treponemes.

When seen in an electron microscope, axial flagella wrap around a helical cytoplasmic cylinder, comprising the cell wall-membrane complex and the cytoplasmic region, and are situated in the periplasmic space below the outer envelope. One end of each axial flagellum is inserted at a subterminal end of the cylinder, whereas the other end is free. The outer envelopes covering the axial flagella are fragile enough to be broken down by mechanical treatment²¹⁾. Interestingly, *T. denticola* and *T. phagedenis* have been shown to expose their axial flagella outward under physiologically normal culture conditions²²⁾. Electron microscopical specimens of host-associated treponemes which were prepared using a gentle care procedure also showed that some axial flagella at the subterminal ends of the cytoplasmic cylinder protrud-

ed out of the outer envelope^{23,24)}. Fibronectin, laminin, and collagen are referred to as adhesins which have diverse biological activities including promoting adherence, migration and activation of lymphocytes^{25,26)}. In the host-associated spirochetes the fibronectin-binding polypeptides on the surface of the outer envelopes, the 66-kDa polypeptide of *T. phagedenis*, the 52-kDa polypeptide of *T. denticola* ATCC 35404, and the 38-kDa polypeptide on projecting axial flagella may permit the spirochetes to adhere to periodontal tissue cells the fibronectin molecules through their receptor or receptor sites. This specific fibronectin-surface protein interaction facilitates their colonization on specific surfaces in periodontal pockets.

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