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Original article

Streptococcus pyogenes CAMP factor promotes bacterial adhesion and invasion in pharyngeal epithelial cells without serum via PI3K/Akt signaling pathway

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Abstract

Streptococcus pyogenes is a bacterium that causes systemic diseases, such as pharyngitis and toxic shock syndrome, via oral- or nasal-cavity infection. *S. pyogenes* produces various molecules known to function with serum components that lead to bacterial adhesion and invasion in human tissues. In this study, we identified a novel *S. pyogenes* adhesin/invasin. Our results revealed that CAMP factor promoted streptococcal adhesion and invasion in pharyngeal epithelial Detroit562 cells without serum. Recombinant CAMP factor initially localized on the membranes of cells and then became internalized in the cytosol following *S. pyogenes* infection. Additionally, CAMP factor phosphorylated phosphoinositide 3-kinase and serine−threonine kinase in the cells. ELISA results demonstrate that CAMP factor affected the amount of phosphorylated phosphoinositide 3-kinase and serine−threonine kinase in Detroit562 cells. Furthermore, CAMP factor did not reverse the effect of phosphoinositide 3-kinase knockdown by small interfering RNA in reducing the level of adhesion and invasion of *S. pyogenes* isogenic *cfa*-deficient mutant. These results suggested that *S. pyogenes* CAMP factor activated the phosphoinositide 3-kinase/serine−threonine kinase signaling pathway, promoting *S. pyogenes* invasion of Detroit562 cells without serum. Our findings suggested that CAMP factor played an important role on adhesion and invasion in pharyngeal epithelial cells.

Keywords: *Streptococcus pyogenes*; CAMP factor; Invasion; PI3K/Akt signaling pathway; Endocytosis

1. Introduction

Streptococcus pyogenes causes not only superficial diseases, such as streptococcal pharyngitis, but also invasive infections, including streptococcal toxic shock syndrome and necrotizing fasciitis [1]. Most diseases manifest through infection of the pharynx, nasal cavity, or skin. In Japan, approximately 400,000 individuals have contracted streptococcal pharyngitis per year.[1] Initial stage of infection requires the adhesion of *S. pyogenes* to epithelial cells, followed by production of various pathogenic proteins that promote

Abbreviations: rCAMP factor, recombinant CAMP factor; GTBS, Tris-buffered saline containing 0.25% gelatin; LDH, lactate dehydrogenase; PI, propidium iodide; pAkt, phosphorylated Akt.

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epithelial-cell adhesion and invasion. Most of these include bacterial-cell-wall-localized proteins that bind to several proteins present in the human epithelial cell membrane, including fibronectin, laminin, plasmin, collagen, and immunoglobulins. Reported examples of these interactions include those associated with the M1 protein [2−4], fibronectin-binding protein (Fba) [2,5−7], protein H [8], and M49 plasminogen [9]. These previous studies of *S. pyogenes* adhesins/invasins supposed infection from the wound site; therefore, these were assumed to occur in the presence of serum. However, serum is scant in the pharynx and oral cavity. Consequently, in this study, we examined mechanisms that involved in *S. pyogenes* adhesion and invasion in the pharynx or skin without serum components.

Christie Atkins Munch-Petersen (CAMP) factor was identified as a co-hemolyzed protein that enhances hemolysis caused by *Staphylococcus aureus* [10] and *Clostridium perfringens* [11]. Several bacteria, including *S. pyogenes* [12], *Streptococcus agalactiae* [13], and *Propionibacterium acnes* [14], possess CAMP factor, with all exhibiting high levels of sequence homology. It is reported that *P. acnes* CAMP factor contributes to bacterial invasion in skin keratinocytes [15,16]. In *S. pyogenes*, Kurosawa et al. demonstrated that CAMP factor forms vacuoles on macrophages and attenuates phagocytic activity [17]; however, little is known about the mechanism of streptococcal invasion by CAMP factor. Therefore, we demonstrate here that CAMP factor of *S. pyogenes* is a serum-independent adhesin/invasin of pharyngeal epithelial cells.

2. Materials and methods

2.1. Bacteria

Invasive *S. pyogenes* clinical strain SSI-9 (serotype M1) was isolated from wound site of patients with streptococcal toxic shock syndrome [18]. *S. pyogenes* SSI-9 wild-type, isogenic *cfa*-deficient (Δ*cfa*) mutant and isogenic *fba*-deficient (Δ*fbaA*) mutant strain was grown in Todd Hewitt broth (Becton Dickinson, MD, USA) supplemented with 0.2% yeast extract at 37 °C. *cfa* and *fba*A are gene of CAMP factor and Fba, respectively.

2.2. Cells

Human pharyngeal carcinoma Detroit562 cells were grown in modified Eagle medium α (Wako, Osaka, Japan) supplemented with 10% heat-treated fetal bovine serum (FBS; Japan Bio Serum Co. Ltd., Hiroshima, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Wako) at 37 °C in aerobic incubator supplemented with 5% CO_2. Human lung adenocarcinoma epithelial A549 cells and human keratinocyte HaCaT cells were grown in Dulbecco's modified Eagle medium (Wako) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in aerobic incubator supplemented with 5% CO_2. This study was carried out in accordance with the recommendations of Niigata University. The protocol was approved by Niigata University.

2.3. Materials

A rabbit antibody against *S. pyogenes* CAMP factor was generated by Eurofins Genomics (Tokyo, Japan). A rabbit antibody against phosphoinositide 3-kinase (PI3K), Akt, phospho-Akt (Ser473) and β-actin were purchased from Cell Signaling Technology, Inc (MA, USA). Horseradish peroxidase-labeled goat anti-rabbit IgG and an enhanced chemiluminescence western blot kit were obtained from GE Healthcare (Little Chalfont, UK). A carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescent intracellular labeling kit was purchased from Dojindo (Kumamoto, Japan). LY294002, LY303511 and Wortmannin were obtained from Cell Signaling Technology, and MK-2206 was from Santa Cruz Biotechnology (TX, USA).

2.4. Purification of recombinant CAMP factor and integration mutagenesis using targeted plasmid

Recombinant CAMP factor (rCAMP factor) was prepared as described previously [17]. rCAMP factor expression plasmid was constructed using a pGEX-6P-1 vector (GE Healthcare). The pGEX-6P-1 vector containing the *cfa* gene was transformed into *Escherichia coli* strain Able-K (Stratagene, CA, USA) by heat shock method. The Able-K transformants were grown in Luria−Bertani broth (Nacalai Tesque, Kyoto, Japan) supplemented with 100 μg/ml ampicillin (Meiji Seika, Tokyo, Japan) to select for the pGEX-6P-1 vector, allowing its expression. Then, the rCAMP factor protein was purified by glutathione-sepharose 4B (Stratagene), and the GST tag was cleaved by prescission protease (GE Healthcare). The purified rCAMP factor protein was dialyzed against Tris-buffered saline containing 0.25% gelatin (GTBS). The amount of lipopolysaccharide in 1 μg of purified rCAMP factor protein was determined to be less than 2 pg using lipopolysaccharide detection kit (Genscript Corp., NJ, USA).

S. pyogenes SSI-9 isogenic Δ*cfa* mutant [17] and isogenic Δ*fbaA* mutant strain [18] was prepared as described previously. In briefly, PCR product of the internal portion of *cfa* and *fbaA* genes were amplified by PCR, and ligated into a suicide vector pSF151. The resultant plasmids were transformed into wild type strain SSI-9 by electroporation and the inactivated mutant strains were selected on kanamycin (Meiji Seika)-containing agar plate. The GenBank accession numbers of investigated isolates of *S. pyogenes* determined in this work are NP_269402 for *cfa* gene and BAB62098 for *fbaA* gene, respectively.

2.5. Cytotoxicity assay

Cells (3×10^5 in 300 μl) were seeded into a 48-well plate (Becton Dickinson) and stimulated with 3 different concentrations of rCAMP factor (0.2, 1, and 5 μg/ml) or 0.1% Triton X-100 at 37 °C for 24 h or 30 min, respectively. Cytotoxicity was determined as the amount of lactate dehydrogenase (LDH) using a LDH measurement kit (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's

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