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Anaplasma Phagocytophilum Major Surface Protein (Msp)-2 Directly Binds to Platelet Selectin Glycoprotein Ligand-1 (CD162) Prior to Cell Entry and Infection

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Abstract : *Anaplasma phagocytophilum* major surface protein-2 (Msp2 or p44) is the immunodominant outer membrane protein of the bacterium. Recently, we disclosed that Msp2 was an *A. phagocytophilum* adhesin for binding to host neutrophils and HL-60 cells, probably mediated by attachment to platelet selectin glycoprotein ligand-1 (PSGL-1). In this study, we further elucidated that Msp2 bound to PSGL-1/FucT IV-transfected BJAB but not nontransfected BJAB cells. Binding of recombinant Msp2 or cell free bacteria to the surface of PSGL-1/FucT IV-transfected BJAB cells was significantly higher than to nontransfected BJAB cells ($p<0.01$ and $p<0.01$). Also, Msp2 monoclonal antibody and soluble recombinant Msp2 as antagonist led to concentration-dependent reductions in *A. phagocytophilum* adhesin ($p<0.05$ and $p<0.01$) to transfected BJAB cells. Thus, we conclude that Msp2 of *A. phagocytophilum* acts as an adhesin by which the bacterium binds to PSGL-1 on host neutrophils and myeloid cells.

Key words **:** *A. phagocytophilum* Msp2, PSGL-1, CD162, adhesin.

Introduction

Anaplasma (*Ehrlichia*) *phagocytophilum* is a tick-borne obligate intracellular bacterium that causes granulocytic ehrlichiosis in humans (HGE), horses, dogs, ruminants, and other animals (3,5,11,14,17). The bacterium infects, resides, and propagates within vacuoles of myeloid cells, chiefly blood neutrophils in mammalian hosts (3,5,6,18). It was previously reported that *A. phagocytophilum* binding to neutrophils was blocked by some monoclonal antibodies that recognized sCD15 (sialylated Lewis X), a frequent modification on the surfaces of leukocytes (9). Subsequent studies indicated that *A. phagocytophilum* could bind to neutrophils via a surface exposed glycoprotein, platelet selectin glycoprotein ligand-1 (PSGL-1 or CD162), which had been specifically modified by the action of α-(1,3) fucosyltransferase (FucT IV) (7).

Major surface protein-2 (Msp2) is the quantitatively and immunologically dominant protein on the surface of *A. phagocytophilum* (1,2,4,8,13,19,20). We recently proposed that Msp2 was at least one component of an *A. phagocytophilum* adhesin that mediated binding to host myeloid cell surfaces, presumably by binding directly to PSGL-1 (submitted for publication). In this study, we suggest that *A. phagocytophilum* Msp2 binds directly to PSGL-1 by the assay of PSGL-1/FucT IV-transfected BJAB cells.

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Materials and Methods

BJAB and PSGL-1/FucT IV-transfected BJAB cells

BJAB and PSGL-1/FucT IV-transfected BJAB cells were kindly provided by Dr. Snapp, Northwestern University Medical School. BJAB and transfected cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37°C in 5% CO_2 (15).

Monoclonal antibodies

The monoclonal antibody 20B4 (IgG2aκ) used in this study reacts with Msp2 of at least 7 different laboratory strains of *A. phagocytophilum* collected from California, Minnesota, Wisconsin, and New York (1). Isotype-matched control IgG2aκ monoclonal antibody purified from mouse ascites was used as control (Sigma Chemical Co., St. Louis, MO, USA). KPL-1 (IgG1aκ) monoclonal antibody that reacts with an extracellular domain of PSGL-1 (CD162) was purchased commercially (BD Pharmingen, San Diego, CA, USA).

Western blot analysis of PSGL-1

To confirm that the transfected BJAB cells express PSGL-1, transfected and nontransfected cells were harvested and washed with PBS, then lysed with the M-PER Kit (Pierce, Rockford, IL, USA). Cell lysates were separated on an 8% SDS-PAGE gel under reducing conditions, and the separated proteins were transferred to a nitrocellulose membrane by standard methods. Western blot analysis was performed using anti-PSGL-1 monoclonal antibody KPL-1 (IgG1aκ). Bound

PSGL-1 antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA). The blots were developed by BCIP/NBT (Bio-Rad) in alkaline phosphate buffer.

Cell free *A. phagocytophilum*

A. phagocytophilum Webster strain was propagated in HL-60 cell line in RPMI 1640 medium supplemented with 1% fetal bovine serum and 2 mM L-glutamine (1,10). Cell free *A. phagocytophilum* was prepared from heavily infected HL-60 cells that were disrupted by 3 to 5 passages through a 26-gauge syringe needle. Cellular debris was removed by centrifugation at $750 \times g$ for 10 min, and cell free bacteria were harvested from the supernatant by centrifugation ($2,500 \times g$ for 10 min). The cell free bacteria were labeled with the lipophilic fluorescent compound, PKH-67 (Sigma Chemical Co., St. Louis MO, USA), and checked using the fluorescence microscopy.

Recombinant Msp2

A recombinant Msp2 was expressed using pCAL-n-EK vector system and tested by protein immunoblotting with Msp2 monoclonal antibody 20B4 and polyclonal rabbit anti-*A. phagocytophilum*. After induction for 4 h with 1 mM IPTG to induce expression of recombinant Msp2 as a fusion with calmodulin-binding protein, the transformed *E. coli* was harvested, lysed by sonication or with the B-PER Kit (Pierce, Rockford, IL, USA), and purified on calmodulin affinity resin columns (Stratagene, LaJolla, CA, USA). The purified recombinant protein retained reactivity with both Msp2 monoclonal antibody 20B4 and rabbit polyclonal anti-*A. phagocytophilum* in protein immunoblots.

Binding assay of rMsp2 and cell free *A. phagocytophilum*

To determine whether Msp2 binds to PSGL-1 in PSGL-1/FucT IV-transfected BJAB cells, cell-free *A. phagocytophilum* or rMsp2 was incubated with 2×10^5 cells for 1 h at 37°C in 5% CO_2. The cells were then harvested by centrifugation and washed two times with RPMI 1640 tissue culture medium to remove unbound bacteria. The cells were then cytocentrifuged onto glass slides and fixed in cold acetone. Binding of bacteria or recombinant Msp2 to BJAB and PSGL-1/FucT IV-transfected cells was assessed by staining with Msp2 monoclonal antibody 20B4 and fluorescein-labeled anti-mouse Ig (Kierkegaard and Perry Laboratories, Gaithersburg, MD, USA) for 1 h at room temperature, followed by counterstaining with Evans blue; slides were mounted with PBS:glycerol solution under glass cover-slips. Fluorescence was initially evaluated by microscopy and quantitated using a Vistra Systems Fluorimager IS (Sunnyvale, CA, USA) and analyzed using Molecular Dynamics Image QuaNT software (version 4.2a). Relative fluorescence intensity was compared among groups and replicates to determine differences in binding.

Inhibition assay of *A. phagocytophilum* binding to PSGL-1

To determine whether the binding of *A. phagocytophilum* to PSGL-1 in PSGL-1/FucT IV-transfected BJAB cells is inhibited by anti-Msp2 monoclonal antibody, cell-free bacteria labeled with PKH-67 (Sigma Chemical Co., St. Louis MO, USA) were first reacted with varying concentrations of Msp2 monoclonal antibody or isotype-matched monoclonal antibody for 1 h at 37°C in 5% CO_2. Cells were then incubated with 2×10^5 BJAB and PSGL-1/FucT IV-transfected BJAB cells. The cells were washed two times, resuspended in 100 μl of tissue culture medium, and cytocentrifuged onto glass slides. Slides were examined microscopically and fluorescence was measured as above.

Alternately, BJAB and PSGL-1/FucT IV-transfected BJAB cells were mixed with 5 or 1 μg/ml of recombinant Msp2, bovine serum albumin (BSA) as control protein, or medium only for 1 h at 37°C in 5% CO_2, and then washed as above prior to treatment with fluorescence-labeled cell-free *A. phagocytophilum* for 1 h. The cells were washed again, prepared on slides, and analyzed as above.

Results

Detection of PSGL-1 in PSGL-1/FucT IV-transfected BJAB cells

PSGL-1 was confirmed to be expressed by PSGL-1/FucT IV-transfected BJAB cells by immunoblot under reducing conditions. Reaction of transfected cells with KPL-1 monoclonal antibody showed a 220 kDa band that corresponded to the extracellular domain of PSGL-1 in PSGL-1/FucT IV-transfected BJAB cells. PSGL-1 was not detected in non-transfected BJAB cells. Moreover, transfected but not non-transfected cells could be infected with cell free *A. phagocytophila* in culture (data not shown).

Cell free *A. phagocytophilum* and rMsp2 binding to PSGL-1/FucT IV-transfected BJAB cells

To confirm whether *A. phagocytophilum* binds to host cells via PSGL-1 and specifically by Msp2, binding of cell free *A. phagocytophilum* to nontransfected BJAB and PSGL-1/FucT IV-transfected BJAB cells were initially compared. After only one hour of *in vitro* incubation, cell free bacteria bound to the surfaces of PSGL-1/FucT IV-transfected BJAB cells significantly more ($p<0.01$) than to nontransfected BJAB cells (Fig. 1). To determine whether Msp2 could mediate PSGL-1 binding directly, recombinant Msp2 was incubated with BJAB and PSGL-1/FucT IV-transfected BJAB cells. Similar to the results with cell free bacteria, binding of recombinant Msp2 to the surfaces of PSGL-1/FucT IV-transfected BJAB cells was dose dependent and significantly greater than binding to nontransfected BJAB cells incubated with control protein ($p<0.01$) (Fig. 2).

Inhibition of *A. phagocytophilum* binding by Msp2 monoclonal antibodies

An antibody concentration-dependent reduction in *A. phagocytophilum* binding was observed in replicated experiments

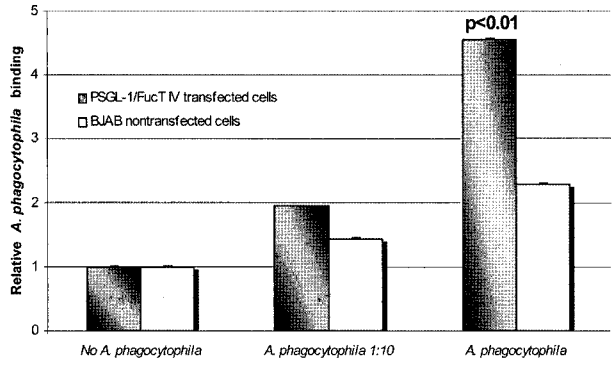

Fig. 1. Binding of cell free *A. phagocytophilum* to PSGL-1/FucT IV-transfected and nontransfected BJAB cells. Fluorescence of cells without *A. phagocytophilum* is defined as a unit. Cell free bacterial binding is measured and expressed relative to the fluorescence measured on cells in the absence of any bacteria.

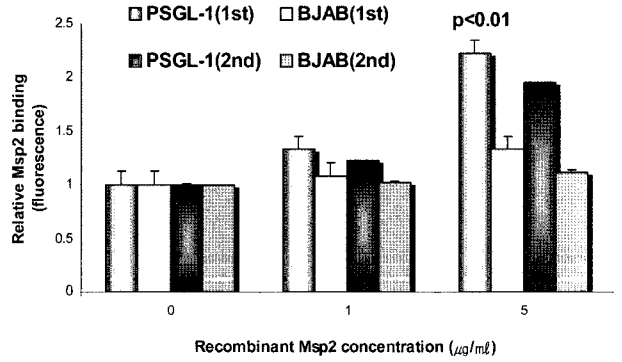

Fig. 2. Binding of recombinant Msp2 to PSGL-1/FucT IV-transfected and nontransfected BJAB cells. Fluorescence of cells without Msp2 is defined as a unit. Recombinant Msp2 binding is measured and expressed relative to the fluorescence measured on cells in the absence of any Msp2. (*p<0.01). Results of 2 separate experiments are shown (1st and 2nd).

using PSGL-1/FucT IV-transfected BJAB cells. Cell free bacteria supplemented with 10 μg/ml of monoclonal antibody 20B4 show a significant reduction of binding to PSGL-1/FucT IV-transfected BJAB cells as compared to cells supplemented with isotype-matched control monoclonal antibody ($p<0.05$) (Fig. 3). In cultures supplemented with 1 μg/ml of monoclonal antibody 20B4, a slight reduction of *A. phagocytophilum* binding was also noted.

Inhibition of *A. phagocytophilum* binding by recombinant Msp2

An rMsp2 dose-dependent antagonism of *A. phagocytophilum* binding to PSGL-1/FucT IV-transfected BJAB cells was observed, similar to the results of experiments conducted with blocking monoclonal antibody. BSA was used as a nonspecific protein control. Overall, significant reductions in *A. phagocytophilum* binding to PSGL-1/FucT IV-transfected BJAB cells

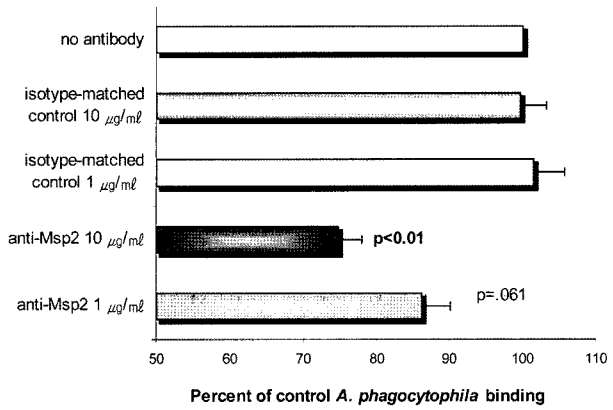

Fig. 3. Msp2 monoclonal antibody pre-incubation with fluorescence-labeled *A. phagocytophilum* blocks adhesion to PSGL-1/FucT IV-transfected BJAB cells in a dose dependent manner. Results are derived from 2 separate experiments and are shown as the mean percentage of fluorescence in Msp2-monoclonal antibody- or isotype-matched control treated *A. phagocytophilum* cultures vs. cultures with no added antibody (defined as 100% adhesion).

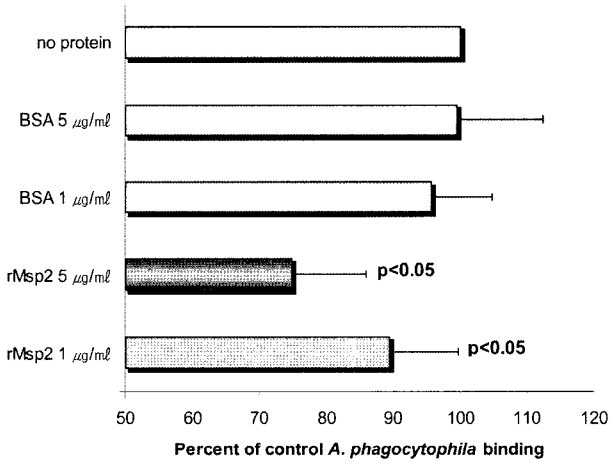

Fig. 4. Pre-incubation of PSGL-1/FucT IV-transfected BJAB cells for 1 h with recombinant Msp2 blocks adhesion of fluorescently labeled *A. phagocytophilum*. Results are fluorescence intensity as a percentage of cultures not incubated with recombinant Msp2 shown and are the means of 2 separate experiments.

were noted in the cultures pre-incubated with 5 μg/ml of rMsp2 ($p<0.05$) and 1 μg/ml of rMsp2 ($p<0.05$) when compared to the same concentrations of control protein (Fig. 4).

Discussion

Recently, it was demonstrated that PSGL-1 exposed on HL-60 cells, and presumably on neutrophil surfaces, act as host myeloid cell ligands for binding of *A. phagocytophilum* (7,9). Our recent investigations showed that antibody to Msp2 or

antagonistic blocking of Msp2 binding to PSGL-1 expressed on host myeloid cells inhibited adhesion and propagation of *A. phagocytophilum*. However, Goodman *et al* (7) noted that a specific derivative of HL-60 cells that is deficient in FucT-VII could also become infected, albeit at a significantly reduced rate. Thus, despite the data that strongly suggests a role for Msp2 as adhesin for *A. phagocytophilum*, the interaction between the specific adhesin and the proposed PSGL-1 ligand still needs more study. Here, we demonstrate that PSGL-1 as modified by FucT IV, binds directly to Msp2 and is the major ligand that mediates binding of intact *A. phagocytophilum* to neutrophils prior to endosomal entry. The data to support this role includes direct binding of cell free *A. phagocytophilum* to transfected but not nontransfected BJAB cells, the direct binding of recombinant Msp2 to transfected but not nontransfected BJAB cells, and the dose-dependent inhibition or antagonism of cell free *A. phagocytophilum* binding when assayed in the presence of Msp2 monoclonal antibodies or recombinant Msp2, respectively.

A. phagocytophilum Msp2, like that of the closely related bovine erythrocyte pathogen *A. marginale*, is involved in adhesion of the bacterium to the surface of its host cell. Like *A. phagocytophilum*, *A. marginale* also binds to a surface glycosylated protein, but the identity of erythrocytes has not yet determined (1,2). A number of unanswered questions still exist, including the presence and identity of other potential membrane components that could be part of an adhesin complex (1,6), whether the natural diversity of Msp2 that is presumed to be related to antigenic variation can contribute to continued host cell binding even with an aggressive anti-Msp2 immune response, and whether any of this information may help to design appropriate strategies to improve control of HGE.

Acknowledgments

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숙주세포 침입을 위한 *Anaplasma phagocytophilum*의 주요 표면단백질 (Msp)-2과 PSGL-1 (CD162)과의 반응

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요 약 : *Anaplasma phagocytophilum*의 주요 표면단백질인 Msp2 (p44)는 세균의 표면에 발현되는 주요한 항원 단백질이다. 본 실험에서는 *A. phagocytophilum*이 주요 숙주세포인 호중구나 HL-60 세포에 침입하는 데 있어, 세균의 주요 표면 단백질인 Msp2와 숙주세포의 표면에 발현되는 PSGL-1과의 반응 여부를 알아보았다. 그 결과, 재조합 단백질인 Msp2나 순수하게 분리된 *A. phagocytophilum*이 PSGL-1/FucT IV 유전자가 형질전환되어 PSGL-1이 발현되는 BJAB 세포와는 결합하지만, 순수한 BJAB 세포와는 전현 반응하지 않는 것을 관찰할 수 있었다($p<0.01$ & $p<0.01$). 또한, 순수하게 분리된 *A. phagocytophilum*과 형질전환된(PSGL-1/FucT IV) BJAB 세포와의 결합이 Msp2의 단-클론항체나 Msp2 재조합 단백질의 농도에 따라 억제됨을 관찰할 수 있었다($p<0.05$ & $p<0.01$). 따라서 *A. phagocytophilum*의 Msp2가 숙주세포인 호중구의 표면에 발현되는 PSGL-1과 직접적으로 결합하는 부착물질임을 알 수 있었다.

주요어 : *A. phagocytophilum*, 주요 표면단백질, 호중구, PSGL-1, 부착물질