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Original article

Streptococcus agalactiae invasion of human brain microvascular endothelial cells is promoted by the laminin-binding protein Lmb

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Abstract

Streptococcus agalactiae (*S. agalactiae*) can cause severe pneumonia, sepsis and meningitis in neonates and remains one of the most prevalent causes of invasive neonatal infections. During the course of infection, *S. agalactiae* colonizes and invades a number of host compartments, thereby interacting with different host tissues. Deletion of the *scpB-lmb* region, coding for the C5a peptidase and the laminin-binding protein Lmb, respectively, resulted in a 64% decreased invasion of *S. agalactiae* into human brain microvascular endothelial cells (HBMEC). Decreased invasion was also seen in *lmb* mutant strains *lmb*-k1 and *lmb*-k2 (74% and 69% reduction, respectively). Finally, host cell invasion was significantly reduced in competition experiments with either purified recombinant laminin-binding protein by 46% or a polyclonal antibody directed against the laminin-binding protein of *S. agalactiae* by 45%. The *S. agalactiae scpB-lmb* mutant induced an equal amount of the neutrophil chemoattractant interleukin (IL)-8 release in comparison to the wild-type. Taken together, our studies support the conclusion that Lmb promotes invasion of *S. agalactiae* into HBMEC but does not play a role in IL-8 release from HBMEC.

Keywords: Group B streptococci; HBMEC; Meningitis; Laminin-binding protein; IL-8

1. Introduction

Streptococcus agalactiae, also named group B streptococcus, is the leading cause of bacterial sepsis and meningitis in neonates in many industrialized countries. In addition, it causes substantial pregnancy-related morbidity and has emerged as an increasingly common cause of invasive disease in the elderly and in immunocompromised persons [1]. In order to cause meningitis, blood-borne pathogens must interact with cerebral microvascular endothelial cells which constitute the blood–brain barrier (BBB); subsequent bacterial replication within the CNS provokes host inflammatory response, resulting in meningitis.

While in vitro and in vivo models have been informative about the pathogenesis of bacterial meningitis and CNS factors that contribute to inflammation and brain injury, little is known about the contribution of specific virulence factors of the invading pathogens at the blood–brain barrier endothelium. Like many other pathogens, *S. agalactiae* can attach to host cell surfaces by binding to different host cell proteins. Surface proteins of *S. agalactiae* are likely to play important roles during different stages of an infection and also hold promise as vaccine components [2]. Moreover, *S. agalactiae* surface proteins are of interest for studying bacterial pathogenesis, such as adhesion to epithelial cells, interactions with human extracellular matrix or plasma proteins, and escape from host immunity.

Binding of *S. agalactiae* to human laminin is mediated by the lipoprotein Lmb (laminin-binding protein), which has been studied at the molecular level [3,4]. Lmb shows homology to members of the Lra1 family of proteins, which have been implicated in adhesion and metal transport in gram-positive bacteria [5]. The protein is expressed by most, if not all, human

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S. agalactiae strains and was first identified as a laminin-binding surface protein. It has been suggested to play a role in colonization and/or invasion of damaged epithelium [3]. The name Lmb was introduced for the *S. agalactiae* protein [3], but the virtually identical protein in *Streptococcus pyogenes* has been referred to as Lsp or Lbp [4,6]. In both *S. agalactiae* and *S. pyogenes*, the gene is located downstream of the C5a-peptidase gene *scpB* and nucleotides sequence identity between the two species is >98% in this region. However, adjacent sequences in the two genomes show no homology, indicating that the region has been horizontally transferred [4,7].

C5a peptidase (ScpB) from *S. agalactiae* has recently been identified as a mediator of bacterial binding to fibronectin [8,9]. Although *S. agalactiae* does not bind soluble fibronectin on its surface [10], adherence of the bacteria to immobilized fibronectin has been demonstrated [11]. In addition, soluble fibronectin has been shown to promote *S. agalactiae* invasion into host epithelial cells [9]. Fibrinogen-binding of *S. agalactiae* is mediated by the proteins FbsA and FbsB [12,13].

Human brain microvascular endothelial cells (HBMEC) have been used previously to study the pathogenesis of central nervous system infections by meningitis-causing pathogens such as *Escherichia coli*, *S. agalactiae* and *Streptococcus pneumoniae* [14–16]. Recently, we demonstrated that the fibrinogen-binding protein FbsA promotes the adherence to *S. agalactiae* to human brain microvascular endothelial cells [17]. Doran et al. [18] identified a role of lipoteichoic acid (LTA) anchoring in blood–brain barrier invasion by *S. agalactiae*. Besides BBB penetration this factor promotes lethality in a mouse model of hematogenous meningitis. Furthermore, proteins of *S. agalactiae* involved in pilus formation, pilA and pilB, have recently been shown to facilitate adhesion to and invasion of HBMEC [19]. In this study we investigated the role of laminin-binding protein Lmb in adherence to and invasion of HBMEC.

2. Material and methods

2.1. Bacteria and growth conditions

For all infection assays we used the *S. agalactiae* wild-type strain 090R (ATCC 12386, a serotype Ia derivative), and an isogenic *scpB-lmb* mutant of 090R, which comprises a deletion of the complete *scpB-lmb* composite transposon structure, as described by Franken et al. [4]. The laminin-binding protein mutants *lmb*-k1 and *lmb*-k2 were constructed by insertion duplication mutagenesis as described by Spellerberg et al. [3]. In strain *lmb*-k1 the pGhost5 vector was inserted at amino acid 165 and in *lmb*-k2 at amino acid 229 of the Lmb protein. *S. agalactiae* strains were cultivated at 37°C in Todd–Hewitt yeast broth containing 1% yeast extract. Mutant strains harboring chromosomally integrated pGhost5 vectors were maintained in medium containing 5 mg of erythromycin per liter at a temperature of 37°C. The *scpB-lmb*, *lmb*-k1 and *lmb*-k2 mutant grew equally well as wild-type in tissue culture media and showed no increased susceptibility to the antibiotics gentamicin and penicillin, respectively. They also revealed no differences in their sensitivity to sonication, and in respect to its buoyancy and chain length. For comparison experiments a *Staphylococcus aureus* (*S. aureus*) strain Cowan I (ATCC 12598) was grown in nutrient broth at 37°C.

2.2. Endothelial cell culture

The HBMEC used in this study were isolated from a brain biopsy of an adult female with epilepsy by the method previously described [20]. HBMEC were thawed and cultured in medium supplemented with 10% heat-inactivated fetal calf serum and 10% NuSerum (Becton Dickinson, Bedford, MA). Thereafter, cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum in culture flasks (Costar, Cambridge, MA) and split weekly in 1:10 ratios by using trypsin-EDTA (Gibco, Grand Island, NY). For adherence and invasion assays, HBMEC were transferred to 12-well tissue culture plates at seeding density of 5×10^5 cells per well and cultivated in RPMI tissue culture medium until confluence was reached. The medium was subsequently replaced with 2 ml of fresh RPMI medium each day.

2.3. Adhesion and invasion assay

Adherence of *S. agalactiae* to HBMEC and invasion into HBMEC were assayed essentially as described elsewhere [15,17]. In brief, for adherence assays, the HBMEC were infected with log-phase *S. agalactiae* and a multiplicity of infection (MOI) of 10 ($\sim 5 \times 10^6$ bacteria/5×10^5 cells per well) in RPMI tissue culture medium supplemented with 2% heat-inactivated fetal calf serum for 2 h at 37°C in 5% CO_2. The HBMEC were washed three times with PBS and were subsequently detached from the well by sonication. The number of cell-adherent bacteria was determined by plating appropriate dilutions of the lysate onto blood-agar plates. Due to the lysis of the eukaryotic cells in this process, the calculation of cell-adherent bacteria also included bacteria that had invaded into HBMEC. Therefore, the number of invaded bacteria was subtracted from the numbers of cell-adherent bacteria to calculate the actual number of adherent bacteria. For invasion assays, the HBMEC were also infected with log-phase *S. agalactiae* and an MOI of 10. Streptococci were incubated for 2 h at 37°C in 5% CO_2, and washed three times with PBS. Subsequently, the infected cells were incubated for 2 h in tissue culture medium supplemented with penicillin G (5 μg/ml) and gentamicin (100 μg/ml) to kill extracellular bacteria. After three washes with PBS, the HBMEC were detached by sonication. The number of invasive bacteria was quantified by plating serial dilutions of the lysate onto blood-agar plates. Each experiment was performed at least three times in triplicate.

2.4. Inhibition studies with recombinant Lmb protein and Lmb antibodies

To better understand the interaction of Lmb with the host cell surface at the molecular level, we used recombinant Lmb protein or polyclonal rabbit antibodies directed against