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RhoA and Rac1 contribute to type III group B streptococcal invasion of human brain microvascular endothelial cells

Sooan Shin, Kwang Sik Kim *

Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

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Abstract

Type III group B streptococcus (GBS) has been shown to invade human brain microvascular endothelial cells (HBMEC), which constitute the blood–brain barrier, but the underlying mechanisms remain incompletely understood. In the present study, we showed that the geranylgeranyl transferase I inhibitor, GGTI-298, not the farnesyltransferase inhibitor, FTI-277 inhibited type III GBS invasion of HBMEC. The substrates for GGTI-298 include Rho family GTPases, and we showed that RhoA and Rac1 are involved in type III GBS invasion of HBMEC. This was shown by the demonstration that infection with type III GBS strain K79 increased the levels of activated RhoA and Rac1 and GBS invasion was inhibited in HBMEC expressing dominant-negative RhoA and Rac1. Of interest, the level of activated Rac1 in response to type III GBS was decreased in HBMEC expressing dominant-negative RhoA, while the level of activated RhoA was not affected by dominant-negative Rac1. These findings indicate for the first time that activation of geranylgeranylated proteins including RhoA and Rac1 is involved in type III GBS invasion of HBMEC and RhoA is upstream of Rac1 in GBS invasion of HBMEC.

Keywords: Group B streptococcus; Brain microvascular endothelial cell; Invasion; Actin rearrangement; RhoA; Rac1

Group B streptococcus (GBS) is the most common cause of neonatal meningitis and neonatal GBS meningitis is associated with high mortality and morbidity [1,2]. A major contributing factor to this high mortality and morbidity is our incomplete understanding of the pathogenesis of this disease. For example, several lines of evidence from experimental hematogenous meningitis and neonates with GBS meningitis indicate that GBS invasion into the brain follows a high-degree of bacteremia and cerebral capillaries are the portal of GBS entry into the brain, but it is incompletely understood how GBS traverses the blood–brain barrier [3,4].

We have established the in vitro blood–brain barrier model with human brain microvascular endothelial cells (HBMEC). Upon cultivation on collagen-coated Transwell inserts, these HBMEC exhibit morphological and functional properties of tight junction formation and polar monolayer [5,6]. Using this in vitro model, we have shown that serotype III strains of GBS which account for the majority of cerebrospinal fluid isolates have been shown to invade and traverse HBMEC [7]. We also showed that type III GBS invasion of HBMEC requires rearrangements of host cell actin cytoskeleton [7], but the basis of microbial-host interactions that are involved in HBMEC actin cytoskeleton rearrangements is incompletely understood. Several signal transduction pathways have been shown with meningitis-causing microbes for their invasion of HBMEC and associated HBMEC cytoskeleton rearrangements such as tyrosine kinases, phosphatidylinositol 3-kinase (PI-3 kinase), and Rho GTPases [8–13]. In the present study, we showed for the first time the involvement of RhoA and Rac1 in type III GBS invasion of HBMEC and RhoA is upstream of Rac1 in GBS invasion of HBMEC.

* Corresponding author. Fax: +1 410 614 1491.
E-mail address: kwangkim@jhmi.edu (K.S. Kim).

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Materials and methods

HBMEC and group B streptococcus. HBMEC were isolated and grown as described previously [6]. RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 10% Nu-Serum (BD BioSciences, Palo Alto, CA), 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), essential amino acids, and vitamins was used as the growing medium. For bacterial infection, the experimental medium (XM, M199-Ham F-12 [1:1] containing 5% heat-inactivated fetal bovine serum and 2 mM glutamine) was used. Type III GBS, strain K79 which was isolated from the cerebrospinal fluid of a neonate with meningitis [14] was previously shown to efficiently invade HBMEC [7] and grown in Todd-Hewitt broth (Difco laboratories, Detroit, MI). For HBMEC infection, GBS colonies grown on trypticase soy agar with 5% sheep blood (BD Biosciences) were cultured in XM for overnight at 37 °C.

Reagents and antibodies. GGTI-298 (a geranylgeranyl transferase inhibitor), FTI-277 (a farnesyltransferase inhibitor), and Y27632 (Rho kinase inhibitor) were purchased from EMD Biosciences, Inc. (La Jolla, CA). Mouse monoclonal anti-RhoA (26C4) and Rac1 (23A8) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Upstate Biotechnology (Charlottesville, VA), respectively. Mouse monoclonal anti-cMyc antibody was purchased from BD Biosciences. Rho Assay Reagent and Rac/Cdc42 Assay Reagent were purchased from Upstate Biotechnology. Adenoviruses encoding N19RhoA, N17Cdc42, and N17Rac1 were kindly provided by Dr. A.J. Ridley [15] and amplified as described previously [16]. GFP expressed adenovirus from pShuttle-IRES-hrGFP-1 vector (Stratagene, La Jolla, CA) alone was kindly provided by Dr. Lew Romer at Johns Hopkins University.

HBMEC invasion assays. Confluent HBMEC monolayers in collagen-coated 24-well plates were washed with XM and preincubated for 30 min at 37 °C in 5% CO_2 incubator, and then infected with 10^7 GBS (multiplicity of infection of 100) grown overnight in XM. After 2 h incubation at 37 °C in 5% CO_2 incubator, monolayers were washed with warmed PBS three times and further incubated with XM containing gentamicin (100 μg/ml) and penicillin G (5 μg/ml) for 2 h to kill extracellular GBS as previously described [7]. The monolayers were washed with PBS three times and lysed with 0.025% Triton X-100 in distilled water. The released intracellular GBS were enumerated by plating on trypticase soy agar with 5% sheep blood. Results were calculated as a percent of the initial inoculum and expressed as percent relative invasion compared to percent invasion of K79 without inhibitors. The number of total cell-associated bacteria was determined as described above except that the gentamicin-penicillin G step was omitted in duplicated experiments. For testing inhibitors, DMSO alone (no treatment, NT) and DMSO solubilized FTI-277 (a farnesyltransferase inhibitor) and GGTI-298 (a geranylgeranyl transferase inhibitor) were preincubated with HBMEC for 15 min before GBS infection. In case of Y27632, 30 min pretreatment was done. The inhibitors were also examined for their effect on bacterial viability by determining colony-forming units and for their effect on the integrity of HBMEC monolayers as described previously [9]. For adenovirus infection, 50% subconfluent HBMEC in 24-well plates were infected with dialyzed adenoviral particles (multiplicity of infection of 100). After 20 h of incubation, medium was completely removed and the cells washed, and incubated for additional 24 h for invasion assays.

Preparation of cell lysates and assays for Rho and Rac1 activation. For infection with bacteria, 2×10^4 HBMEC were seeded on 6-well plates and incubated at 37 °C for 3 days. The cells were washed with HBSS and incubated at 37 °C for one more day in XM without FBS. Type III GBS strain K79 grown overnight at 37 °C in XM without serum was used for infection of HBMEC. For adenovirus infection, 1×10^4 HBMEC on 6-well plates were cultivated at 37 °C for 2 days to reach approximately 50% confluency. HBMEC were infected with dialyzed adenoviral particles (multiplicity of infection of 100) and incubated for two more days. After incubation with type III GBS strain K79 (1×10^7 bacteria/ml) at 37 °C for the indicated periods of time, HBMEC were washed with cold PBS containing 1 mM sodium orthovanadate and lysed in modified RIPA buffer containing inhibitors for proteases and phosphatases at 4 °C [17]. The cell lysates were sonicated and centrifugated at 20,000*g* for 10 min at 4 °C. The supernatants were collected, and the protein concentrations were determined by Protein Assay Reagent (Bio-Rad). For RhoA and Rac1 activation assays, 500 μg of protein was incubated with 30 μg of Rhotekin-RBD (Rho assay reagent from Upstate) and 10 μg of PAK-1 PBD (Rac/Cdc42 assay reagent, Upstate) bound to glutathione agarose beads, respectively, for 45 min at 4 °C. The glutathione agarose pull-downed protein complexes were washed with the lysis buffer and subjected to 16% Novex Tris-glycine (TG) gel (Invitrogen) for SDS–PAGE. For visualizing total RhoA and Rac1 proteins, 25 μg of cell lysates were loaded in each lane of TG gel.

Western blotting. The separated proteins were transferred to PVDF membrane (Immobilon-P, Millipore), and the membranes were blocked with 5% bovine serum albumin in Tris-buffered saline (20 mM Tris [pH 7.5], 150 mM NaCl) containing 0.1% Tween 20 for 1 h at room temperature and incubated with appropriate primary antibody overnight at 4 °C. The blots were washed and incubated with HRP-conjugated anti-mouse IgG antibody (1:5000 dilution, Cell Signaling Technology) in 5% skim milk for 1 h at room temperature. Proteins were visualized with film development using ECL Western detection reagent (Amersham Biosciences, Pistcataway, NJ). In some cases films were scanned and subsequent densitometric analysis of protein bands was preformed using ImageJ software, version 1.34 (Research Services Branch of National Institute of Mental Health, NIH).

Results and discussion

The role of geranylgeranylated proteins in type III GBS invasion of HBMEC

We have previously shown that type III GBS invasion of HBMEC involves the host cell actin cytoskeleton rearrangements, as shown by transmission electron microscopic demonstration of microvillus-like protrusions around internalizing GBS on the surface of HBMEC and inhibition of GBS invasion of HBMEC by cytochalasin D [7]. However, the signaling mechanisms that are involved in GBS invasion of HBMEC and associated cytoskeleton rearrangements remain unclear. We first used two inhibitors for their effects on type III GBS invasion of HBMEC. When HBMEC were pretreated with GGTI-298, which inhibits protein geranylgeranylation, type III GBS invasion of HBMEC was inhibited in a dose-dependent manner (Fig. 1). In contrast, inhibition of protein farnesylation by FTI-277 did not significantly affect type III GBS invasion of HBMEC. It should be noted that GGTI-298 and FTI-277 did not affect the viability of GBS as well as the integrity of HBMEC during the experimental procedure. Of interest, the total numbers of HBMEC-associated GBS were not affected by pretreatment with GGTI-298 and FTI-277 (data not shown). These findings suggest that geranylgeranylated proteins are involved in type III GBS invasion of HBMEC.

The role of Rho GTPases in type III GBS invasion of HBMEC

The most well known substrates for geranylgeranyl transferase I include the Rho family of small GTPases such as Rho, Rac, and Cdc42 and the γ-subunits of trimeric

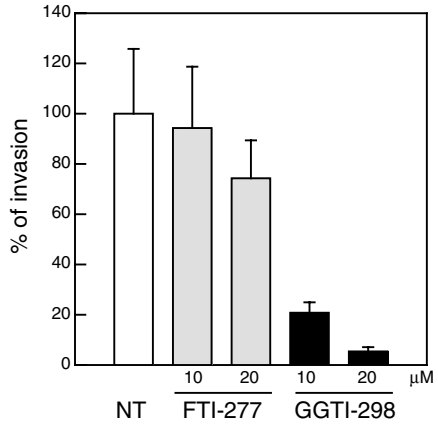

Fig. 1. Effects of GGTI-298 and FTI-277 on type III GBS strain K79 invasion of HBMEC. HBMEC were pretreated with DMSO alone (no treatment, NT) and indicated concentrations of DMSO-solubilized GGTI-298, and FTI-277 for 15 min. Each condition contained the same concentration of DMSO in the medium. Pretreated HBMEC were infected with GBS for invasion assays as described in Materials and methods. The intracellular CFUs were counted, and the results are expressed as percent relative invasion compared to percent invasion of K79 without inhibitors (NT), means ± SD of three independent experiments, each performed in triplicate.

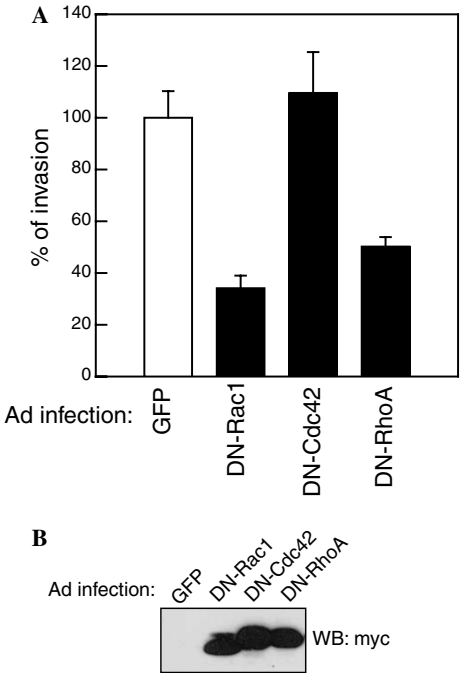

Fig. 2. Effect of dominant-negative Rac1, Cdc42, and RhoA expression on the type III GBS strain K79 invasion of HBMEC. (A) Subconfluent HBMEC in 24-well plates were infected with adenovirus constructs at a MOI of 100 in growing medium and were incubated for additional 48 h and processed for invasion assays. GFP expressed adenovirus from pShuttle-IRES-hrGFP-1 vector alone was used for adenovirus control. The results were expressed as percent relative invasion compared to percent invasion of K79 with GFP alone, means ± SD of three independent experiments, each performed in triplicate. (B) Confirmation of dominant-negative Rac1, Cdc42, and RhoA expression. Cell lysates were prepared from duplicated sets of HBMEC infected with adenoviruses and Western blottings were performed to detect Myc tag, which was fused with dominant-negative Rac1, Cdc42, and RhoA proteins.

G-proteins [18]. We have previously shown that Rho GTPases are key regulators of the actin cytoskeleton in response to meningitis-causing microbes in HBMEC [10–13]. We, therefore, examined the role of Rho GTPases in type III GBS invasion of HBMEC by using adenovirus constructs containing dominant-negative RhoA (N19RhoA), Rac1 (N17Rac1), and Cdc42 (N17Cdc42). Transfection of HBMEC with adenoviral constructs was verified by detection of myc tag, which was fused with dominant-negative constructs (Fig. 2). As shown in Fig. 2, GBS invasion was significantly decreased in HBMEC expressing dominant-negative RhoA and Rac1. In contrast, type III GBS invasion was not affected in HBMEC with dominant-negative Cdc42. These findings indicate that RhoA and Rac1 are most likely to be involved in type III GBS invasion of HBMEC.

The role of RhoA and Rac1 in type III GBS invasion of HBMEC

To further examine the roles of RhoA and Rac1 in type III GBS invasion of HBMEC, we next examined whether RhoA and Rac1 were activated in response to type III GBS strain K79 in HBMEC. We measured the levels of activated RhoA and Rac1 by pull-down assays with rhotekin-RBD and PBD of protein A kinase (PAK) fused to GST, which bind to the activated forms of RhoA and Rac1 (i.e., RhoA-GTP and Rac1-GTP, respectively). As shown in Fig. 3, type III GBS strain K79 infection of HBMEC increased the levels of GTP-bound RhoA (A) and Rac1 (B) in a time-dependent manner. RhoA activation was detectable after 15 min of infection and reached a maximum after 30 min. In contrast, Rac1 activation was detectable after 30 min of infection with type III GBS strain K79 and reached a maximum after 60 min. It is important to note that the total amounts of RhoA and Rac1 were similar between samples obtained at different times of infection, indicating that time-dependent activations of RhoA and Rac1 occur in response to type III GBS in HBMEC.

We have previously shown the involvement of RhoA in invasion of HBMEC by another meningitis-causing microbe, *Escherichia coli* K1 [10–12]. *E. coli* K1 invasion of HBMEC was significantly decreased in HBMEC exhibiting dominant-negative RhoA, and Y-29632 (an inhibitor of Rho kinase) inhibited *E. coli* K1 invasion of HBMEC in a dose-dependent manner [11]. We next examined whether or not similar RhoA activation pathways are involved in type III GBS invasion of HBMEC. As shown in Fig. 3C, Y-27632 did not affect GBS invasion of HBMEC. These findings suggest that the involvement of RhoA in GBS invasion of HBMEC is not through Rho kinase. We have also shown that host cell actin cytoskeleton rearrangements are required for invasion of HBMEC by meningitis-causing microbes such as *E. coli* K1, type III GBS, and *Listeria monocytogenes* [5,7,19]. However, signaling mechanisms

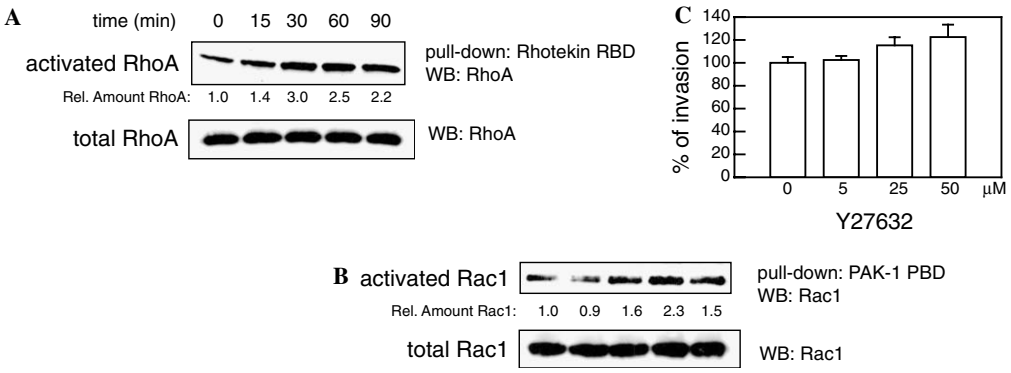

Fig. 3. Activation of RhoA and Rac1 in type III GBS infected HBMEC. Confluent HBMEC in 6-well plates were further incubated overnight without serum. HBMEC were infected with type III GBS strain K79 for indicated periods and the cell lysates were prepared as described in Materials and methods. RhoA and Rac1 activations in response to GBS infection are shown in upper panels of (A) and (B), using pull-down assays with GST fused Rhotekin RBD and PAK-1 PBD, respectively. Total RhoA and Rac1 amounts at each time point were detected by using RhoA and Rac1 Western blotting, shown in bottom panels. (C) To further test the involvement of Rho kinase, the downstream of RhoA activation, Y-27632 (a Rho kinase inhibitor), was examined for its effect on GBS invasion of HBMEC. The results were expressed as percent relative invasion compared to percent invasion of K79 without Y-27632, means ± SD of three independent experiments, each performed in triplicate. Amount of DMSO (a solvent of Y-27632) was adjusted to be equal for different concentrations of Y27632.

that are involved in actin cytoskeleton rearrangements differ between meningitis-causing microbes. For example, *E. coli* invasion of HBMEC involves activations of focal adhesion kinase, Src kinase, and PI-3 kinase [5,8,9], while GBS invasion of HBMEC was independent of Src kinase (Shin and Kim, preparation for publication) and *L. monocytogenes* invasion of HBMEC was independent of focal adhesion kinase [5,8]. As shown in the present study, both *E. coli* and GBS activate RhoA for their invasion of HBMEC, but the downstream signaling pathways of RhoA activation differ between *E. coli* and GBS. Studies are in progress to determine whether mDia, another effector of Rho activation is involved in type III GBS invasion of HBMEC.

Inhibition of Rac1 activation by RhoA dominant-negative construct

Type III GBS invasion is inhibited in HBMEC expressing dominant-negative RhoA and Rac1, and activations of RhoA and Rac1 occurred in response to type III GBS in HBMEC with different kinetics (Fig. 3A and B). We next examined whether activations of RhoA and Rac1 are related to each other by examining activations of RhoA and Rac1 in response to type III GBS in the presence of dominant-negative Rac1 and RhoA, respectively. HBMEC transfected with adenoviral constructs expressing dominant-negative Rac1 and RhoA were incubated with type III GBS strain K79 for 60 min. As shown in Fig. 4B, successful transfection with dominant-negative RhoA and Rac1 was documented by detecting myc tag. Activated RhoA and Rac1 were pulled down from cell lysates by rhotekin RBD and PAK-1 PBD, respectively, and pull-down precipitates were immunoblotted with anti-RhoA and Rac1 antibody, respectively. As shown in Fig. 4A, the level of activated RhoA in response to type III GBS

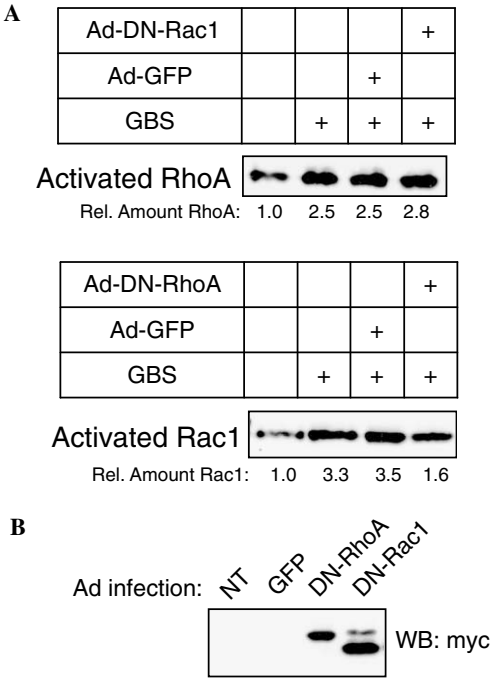

Fig. 4. Interaction of RhoA and Rac1 signaling in response to type III GBS in HBMEC. The activations of RhoA and Rac1 in response to type III GBS were examined in HBMEC expressing dominant-negative Rac1 and RhoA, respectively (A) Subconfluent HBMEC (approximately 70%) in 6-well plates were infected with adenovirus constructs at a multiplicity of infection of 100 in growing medium and incubated for 24 h, followed by additional incubation overnight at 37 °C without serum. Cell lysates were prepared after 60 min infection with GBS, and RhoA and Rac1 activations were measured by using pull-down assays with GST fused rhotekin RBD and PAK-1 PBD, respectively. (B) The detection of myc tag in HBMEC lysates for confirming the expression of dominant-negative RhoA and Rac1.

was not affected by dominant-negative Rac1 compared to the control GFP vector-transfected HBMEC and non-transfected HBMEC, while Rac1 activation was almost

abolished in HBMEC transfected with dominant-negative RhoA compared to the control vector-transfected HBMEC and non-transfected HBMEC. These findings suggest that RhoA may be upstream of Rac1 in type III GBS invasion of HBMEC. We also showed that RhoA activation occurred before Rac1 activation in response to type III GBS strain K79 (Fig. 3), e.g., RhoA activation was noted at 15 min of infection with a maximum at 30 min, while Rac1 activation occurred after 30 min of infection with a maximum at 60 min. Taken together, these findings indicate that RhoA and Rac1 contribute to type III GBS invasion of HBMEC and activations of RhoA and Rac1 occur in response to type III GBS in HBMEC, but RhoA is upstream of Rac1 in GBS invasion of HBMEC.

In summary, we showed that the geranylgeranylated proteins such as RhoA and Rac1 are activated in response to type III GBS and involved in GBS invasion of HBMEC. Additional studies are needed to understand how Rho GTPases contribute to type III GBS invasion of HBMEC.

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