Cytotoxic Necrotizing Factor-1 Contributes to Escherichia coli K1 Invasion of the Central Nervous System*

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Naveed Ahmed Khan‡, Ying Wang§, Kee Jun Kim‡, Jin Woong Chung‡, Carol Ann Wass§, and Kwang Sik Kim‡§¶

From the §Division of Infectious Diseases, Childrens Hospital Los Angeles, University of Southern California School of Medicine, Los Angeles, California 90027 and the ¶Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287

Escherichia coli K1 invasion of brain microvascular endothelial cells (BMECs) is a prerequisite for penetration into the central nervous system and requires actin cytoskeletal rearrangements. Here, we demonstrate that E. coli K1 invasion of BMECs requires RhoA activation. In addition, we show that cytotoxic necrotizing factor-1 (CNF1) contributes to E. coli K1 invasion of brain endothelial cells in vitro and traversal of the blood-brain barrier in the experimental hematogenous meningitis animal model. These in vitro and in vivo effects of CNF1 were dependent upon RhoA activation as shown by (a) decreased invasion and RhoA activation with the Δcnf1 mutant of E. coli K1 and (b) restoration of invasion frequency of the Δcnf1 mutant to the level of the parent E. coli K1 strain in BMECs with constitutively active RhoA. In addition, CNF1-enhanced E. coli invasion of brain endothelial cells and stress fiber formation were independent of focal adhesion kinase and phosphatidylinositol 3-kinase activation. This is the first demonstration that CNF1 contributes to E. coli K1 invasion of BMECs.

Inadequate knowledge of the pathogenesis associated with bacterial entry into the central nervous system contributes to considerable mortality and morbidity associated with bacterial meningitis. For example, most cases of bacterial meningitis occur as a result of hematogenous spread, but it is unclear how circulating bacteria cross the blood-brain barrier (1). Escherichia coli is the most common Gram-negative microorganism causing meningitis in the neonatal period. We have previously shown that E. coli K1 crossing of the blood-brain barrier in vivo requires a threshold level of bacteremia and invasion of brain microvascular endothelial cells (BMECs)† and identified several E. coli determinants (OmpA, Ibe proteins, AsLA, and TraD) contributing to BMEC invasion in vitro and in vivo (2–7). We have also demonstrated that host cell actin cytoskeletal rearrangements are required for E. coli K1 invasion of BMECs, as shown by invasive E. coli K1-associated F-actin condensation and blockade of E. coli K1 invasion of BMECs by the microfilament-disrupting agents cytochalasin D and latrunculin A (8); but the specific host cell signaling pathways involved in E. coli K1 invasion and actin cytoskeletal rearrangements remain incompletely understood.

Our recent studies have shown that tyrosine phosphorylation of several host cell signaling molecules is involved in E. coli K1 invasion of BMECs, as treatment of BMECs with genistein, a protein-tyrosine kinase inhibitor, blocks E. coli K1 invasion of BMECs (9). In addition, focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K) are crucial signaling pathways contributing to E. coli K1 invasion of BMECs (9, 10), but the basis of E. coli K1 activation of FAK and PI3K has yet to be defined. It is also unclear which microbial factors contribute to actin cytoskeletal rearrangements in BMECs.

Cytotoxic necrotizing factor-1 (CNF1) is a dermonecrotic protein toxin produced by human and animal isolates of E. coli. CNF1 consists of 1014 amino acids with a molecular mass of 113.7 kDa. CNF1 is frequently associated with E. coli strains that cause extraintestinal infections in humans (11, 12). However, the exact role of CNF1 in the pathogenesis of extraintestinal E. coli infections is not fully understood. Previous studies have indicated that CNF1 induces phagocytosis in epithelial cells (13–15). CNF1 has been shown to activate Rho GTPases by deamidation of glutamine 63, converting it into glutamic acid, thereby inhibiting GTP-hydrolyzing activity and constitutive activation of Rho, resulting in polymerization of F-actin and increased formation of stress fibers (16–18). In this study, we examined the role of CNF1 in E. coli K1 invasion of BMECs in vitro and traversal of the blood-brain barrier in the experimental hematogenous meningitis animal model and the mechanisms associated with CNF1 in E. coli K1 invasion of BMECs.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—E. coli K1 strain E44 is a spontaneous rifampin-resistant mutant of strain RS218 (O18;K1;H7), a cerebrospinal fluid isolate from a neonate with meningitis (4). E. coli K12 strain JM101 (New England Biolabs Inc., Beverly, MA) was used as the host for plasmids, and SM10 (pRK2013) was used as the host for suicide plasmids. E. coli strains were routinely grown at 37 °C in Luria broth. Where appropriate, the medium was supplemented with ampicillin (100 µg/ml), kanamycin (40 µg/ml), chloramphenicol (25 µg/ml), or rifampin (50 µg/ml).

Construction of the cnf1 Deletion Mutant—Oligonucleotide primers were designed based on the published sequence of the cnf1 gene (20). A 1-kb DNA fragment was obtained using primers 5′-GGGAGCCATTTCGAATTTGG-3′ and 5′-TTCCGCTTGTAATGATTAC-3′ and cloned into pBluescript KS (Stratagene, La Jolla, CA). A 0.9-kb DNA fragment upstream of cnf1 was produced using primers 5′-ACCATCTCCGGCGCACAGGAAGCT-3′, incorporating a BamHI site (underlined), and 5′-AAATTCCTTAATGTAATCCTGCT-3′, incorporating an EcoRI site (underlined), and cloned into the above-mentioned plasmid; and then a kanamycin resistance cassette from pUC4K (Amersham Biosciences)

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† To whom correspondence should be addressed: Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine, 600 N. Wolfe St., Park 256, Baltimore, MD 21287. Tel.: 410-614-3917; Fax: 410-614-1491; E-mail: kwangkim@jhmi.edu.
‡ The abbreviations used are: BMECs, brain microvascular endothelial cells; FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase; CNF1, cytotoxic necrotizing factor-1; TRITC, tetramethylrhodamine isothiocyanate.
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§§ To whom correspondence should be addressed: Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine, 600 N. Wolfe St., Park 256, Baltimore, MD 21287. Tel.: 410-614-3917; Fax: 410-614-1491; E-mail: kwangkim@jhmi.edu.
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was inserted at the EcoRI site. The recombinant DNA was moved to suicide plasmid pET185.2 and then transferred to strain E44 by conjugation. The cnf1 deletion mutant was selected on kanamycin and verified by PCR.

**Purification of CNF1**—The cnf1 gene was amplified from E. coli K1 strain E44 using oligonucleotide primers 5′-ACGGATCCGGGACGC-GAAGCT-3′, incorporating a BamHI site (underlined), and 5′-AC- GAATTCTTCATGGTGGTGCCT-3′, incorporating an EcoRI site (underlined), and cloned into pBluescript KS at the same sites to produce pBl-cnf1. CNF1 was expressed in E. coli JM101 and purified using published protocols (14).

**Endothelial Cell Cultures and Transfections**—Human BMECs were isolated and cultured as previously described (21). BMEC cultures were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 10% NuSerum, 2 mM glutamine, 1 mM pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, essential amino acids, and vitamins. PI3K mutants pAp110 (a kinase-negative catalytic subunit of PI3K) and pAp65 (defective in interaction with p110) were prepared as previously described (10). A FAK mutant lacking the autophosphorylation site (Phe397FAK) was prepared as described previously (9). Dominant-negative RhoA (N19RhoA, constructed by replacing threonine 19 with asparagine) and constitutively active RhoA (V14RhoA, constructed by replacing glycine 14 with valine using oligonucleotide-directed mutagenesis) constructs were used as previously described (22, 23). All constructs were cloned into the pCMV-based vector pCMV Invitrogen) and transfected into human BMECs using LipofectAMINE (Invitrogen) as previously described (10). Briefly, a DNA-LipofectAMINE complex in RPMI 1640 medium was added to 50% confluent human BMEC monolayers. After 6 h of incubation, cells were washed and grown in complete medium for 72 h, followed by selection with G418 (400 µg/ml). Antibiotic-resistant colonies were pooled and confirmed by Western blotting.

**In Vitro Human BMEC Invasion Assays**—Invasion assays were performed as previously described (4, 9). Cultured confluent human BMECs (grown in 24-well plates) were incubated with 10⁷ E. coli cells (multiplicity of infection of 100) in experimental medium (Medium 199/Ham’s F-12 medium (1:1) containing 5% heat-inactivated fetal bovine serum, 2 mM glutamine, and 1 mM pyruvate). Plates were incubated at 37°C in a 5% CO₂ incubator for 90 min. Monolayers were washed with RPMI 1640 medium and incubated in experimental medium containing gentamicin (100 µg/ml) for 1 h to kill extracellular bacteria. The monolayers were washed again and lysed in 0.5% Triton X-100. The released intracellular bacteria were enumerated by plating on sheep blood agar plates. To examine the role of RhoA in E. coli invasion of human BMECs, cells were treated with the Rho kinase inhibitor, and invasion assays were performed.

**RESULTS**

**The cnf1 Deletion Mutant of E. coli K1 Is Less Efficient in Invasion of Human BMECs in Vitro and Penetration of the Blood-Brain Barrier in the Hematogenous Meningitis Model in Vivo**—To determine whether CNF1 plays a role in E. coli K1 invasion of BMECs, an isogenic E44 mutant (Δcnf1) was generated (Fig. 1). The deletion of cnf1 in the mutant strain was verified by PCR (data not shown) as well as by Western blotting (Fig. 2). The growth characteristics of strain E44 and the Δcnf1 mutant were identical on Luria broth and blood agar. In addition, the total protein profiles as assessed by SDS-PAGE were identical for strain E44 and the Δcnf1 mutant (data not shown). Both strain E44 and the Δcnf1 mutant were used for invasion assays. We observed that the cnf1 deletion mutant was significantly less invasive in BMECs compared with the parent strain (Fig. 3). To examine whether the cnf1 deletion mutant is indeed less invasive in vivo, the cnf1 mutant and the parent strain were administered to 5-day-old rats. As shown in Table I, subcutaneous injection of 10⁷ colony-forming units of strain E44 or its Δcnf1 mutant resulted in bacteremia in 100% of the animals, and the magnitude of the bacteremia was similar between the two groups. A total of 10 of 26 animals (38%) infected with strain E44 were found to have meningitis. In contrast, only 3 of 28 animals (11%) developed meningitis, and this rate of meningitis was significantly less (p = 0.026) than the rate observed with the cnf1 mutant. These findings indicate that CNF1 is a critical determinant for E. coli K1 to invade human BMECs in vitro and to cross the blood-brain barrier in vivo. It is also important to recognize that the frequency of in vitro BMEC invasion (90%) corresponds to the enhanced bacterial penetration of the blood-brain barrier in vivo (Fig. 3 and Table I) (2–4, 7).

**Requirement of RhoA in E. coli K1 Invasion of Human BMECs**—To determine the role of RhoA in E. coli K1 invasion of human BMECs, monolayers were treated with Y-27632 (a Rho kinase inhibitor), and invasion assays were performed. Y-27632 exhibited a dose-dependent inhibition of E. coli K1
invasion of human BMECs (Fig. 4), suggesting that RhoA plays a role in E. coli K1 invasion of human BMECs. To further confirm these findings, invasion assays were performed using BMECs expressing constitutively active RhoA (ΔRhoA) or BMECs with constitutively active RhoA (RhoA). E. coli K1 invasion was decreased by >50% in human BMECs expressing constitutively active RhoA (Fig. 5 and Table II), whereas BMECs with constitutively active RhoA exhibited a marked increase in E. coli K1 invasion of human BMECs (Fig. 5). Taken together, these findings indicate that RhoA activation is required for efficient E. coli K1 invasion of human BMECs.

The cnf1 Deletion Mutant Is Less Efficient in RhoA Activation—Rho GTPases, shown to affect actin cytoskeletal rearrangements, are the target of CNF1 (13, 16, 17). To assess whether the cnf1 deletion mutant lacks the ability of RhoA activation, human BMECs were incubated with E. coli K1 strain E44 and its cnf1 deletion mutant, and total lysates were compared for the activated GTP form of RhoA. The cnf1 deletion mutant was significantly less efficient in activation of RhoA GTPase compared with parent strain E44 (p < 0.05) (Fig. 6, a and b). Of interest, the cnf1 deletion mutant of E44 exhibited increased RhoA activation compared with the negative control (laboratory E. coli strain HB101) (Fig. 6, a and b). We have previously shown that several E. coli determinants such as OmpA, Ibe proteins, AsLA, and TraJ contribute to BMEC

Table I

| Strain       | No. of animals (mean ± S.D.) | No. of animals with positive CSF cultures for E. coli |
|--------------|------------------------------|-----------------------------------------------------|
| E44          | 26                           | 0.60 ± 1.49                                       |
| Δcnf1        | 20                           | 0.60 ± 1.20                                       |

* a CSF, cerebrospinal fluid; cfu, colony-forming units.  
  b p = 0.006 in two-sided Fisher exact test.

Table II

| Human BMECs | Invasion* |
|-------------|-----------|
| Control     | +CNF1     |
| pcDNA       | 0.121 ± 0.009 | 4.15 ± 0.86 |
| Y-27632 (50 μM) | 0.045 ± 0.002 | 0.13 ± 0.03 |
| ΔRhoA       | 0.047 ± 0.003 | 0.09 ± 0.004 |
| ΔFAK        | 0.045 ± 0.008 | 3.35 ± 0.74 |
| ΔPI3K       | 0.048 ± 0.008 | 3.43 ± 0.65 |

* Expressed as mean ± S.D.
invasion (2–7), and it may be possible that one or more of these microbial determinants also contribute to RhoA activation. Overall, these findings suggest that CNF1 contributes to *E. coli* K1 invasion of BMECs most likely via RhoA activation. This concept was further supported by the demonstration that the invasive ability of thecnf1 deletion mutant was restored to the level of the parent *E. coli* K1 strain in BMECs with constitutively active RhoA (Fig. 5).

**CNF1 Treatment Dramatically Increases *E. coli* K1 Invasion of Human BMECs**—Next, to determine the effects of CNF1 on *E. coli* K1 invasion, we incubated human BMECs with purified CNF1 for 2 h prior to invasion assays. A >40-fold increase in *E. coli* K1 invasion of BMECs was observed in CNF1-treated cells (Table II). We previously reported that tyrosine phosphorylations of FAK and PI3K are essential for *E. coli* K1 invasion of human BMECs, as shown by significantly decreased invasion of *E. coli* K1 in dominant-negative FAK- and PI3K-expressing BMECs (Table II) (9, 10). However, CNF1-mediated *E. coli* K1 invasion of human BMECs was independent of FAK and PI3K. This was shown by the demonstration that CNF1 enhanced *E. coli* K1 invasion of pcDNA-transfected BMECs as well as of both dominant-negative FAK- and PI3K-expressing BMECs (Table II).

However, prior treatment of dominant-negative RhoA-expressing BMECs with CNF1 resulted in a minimal increase in *E. coli* K1 invasion, documenting that RhoA is the major target in CNF1-mediated invasion of *E. coli* K1 in human BMECs. In addition, human BMECs were treated with Y-27632 and then with CNF1, followed by the invasion assays. We observed that CNF1 did not increase invasion substantially in cells that were pretreated with Y-27632 (Table II), thus further confirming that CNF1-mediated increased *E. coli* K1 invasion of human BMECs requires RhoA activation.

**CNF1 Induces Stress Fiber Formation in Human BMECs**—We have demonstrated that host cell actin cytoskeletal rearrangements are required for *E. coli* K1 invasion of BMECs (8). CNF1 has been shown to activate Rho GTPases, resulting in polymerization of F-actin and increased formation of stress fibers in human umbilical vein endothelial cells (18). To determine whether CNF1 induces stress fiber formation in human BMECs, cells were incubated with CNF1 for 2 h, and immunofluorescence assays were performed. We observed increased stress fiber formation in CNF1-treated pcDNA-transfected BMECs (Fig. 7). Consistent with the above invasion data, CNF1-induced stress fiber formation was also observed in both dominant-negative FAK- and PI3K-expressing BMECs (Fig. 7). In contrast, CNF1 did not induce stress fiber formation in dominant-negative RhoA-expressing BMECs (Fig. 7) and in Y-27632-pretreated BMECs (data not shown).

**CNF1-Mediated Invasion and Cytoskeletal Rearrangements Are Independent of FAK and PI3K, but Dependent on RhoA**—To verify the activation of RhoA following CNF1 treatment, human BMECs were stimulated with CNF1 for 30 min, and GTP-RhoA was collected as described under "Experimental Procedures." We observed increased levels of GTP-RhoA in response to CNF1 in pcDNA-transfected BMECs as well as in both dominant-negative FAK- and PI3K-expressing BMECs (Fig. 8, a and b). CNF1 treatment of dominant-negative RhoA-expressing BMECs did not result in RhoA activation (Fig. 8, a and b). Taken together, these findings indicate that CNF1-mediated *E. coli* K1 invasion of BMECs and cytoskeletal rearrangements are independent of FAK and PI3K, but dependent on RhoA.

**DISCUSSION**

To our knowledge, this is the first report describing the role of CNF1 in the pathogenesis of meningitis due to *E. coli* K1. The cnf1 deletion mutant was significantly less invasive in
human BMECs in vitro as well as significantly less efficient in penetration of the blood-brain barrier in the experimental hematogenous E. coli meningitis animal model in vivo compared with the parent E. coli K1 strain. We have previously shown that a high degree of bacteremia is a primary determinant for meningeval invasion by E. coli K1 (27); however, the magnitude of bacteremia was similar between the two groups of animals infected with the cnf1 deletion mutant or the parent strain (Table I). These findings indicate that CNF1 is indeed a critical determinant for E. coli K1 penetration of the central nervous system in vivo. This finding is in contrast to previous reports describing the inconsistent contribution of CNF1 to uropathogenesis in vivo (28–30).

We have previously shown that FAK and PI3K are essential for E. coli K1 invasion of human BMECs (Table II) (9, 10). In addition, we have shown that P3K interacts with FAK in human BMECs and that its interaction is increased in human BMECs stimulated with E. coli K1 (9,10), suggesting that FAK recruits PI3K to the sites of bacterial entry. PI3K activation is abolished in dominant-negative FAK mutants (10), indicating that FAK is upstream of PI3K in E. coli K1 invasion of human BMECs. CNF1 has been shown to enhance tyrosine phosphorylation of FAK and formation of stress fibers in Swiss 3T3 fibroblasts (31). In contrast, as shown in this study, increased stress fiber formation and E. coli K1 invasion in response to CNF1 in human BMECs were independent of FAK and PI3K. These findings suggest that the requirement of FAK and PI3K for E. coli K1 invasion of human BMECs was overcome by CNF1 treatment of human BMECs. We have previously shown that several determinants of E. coli K1 such as OmpA and Ibe proteins contribute to BMEC invasion (2–7), and it is tempting to speculate that the contribution of OmpA and Ibe proteins to E. coli K1 invasion of BMECs may involve FAK and PI3K. Additional studies are needed to determine microbial factors contributing to E. coli K1 invasion of BMECs and also requiring FAK and PI3K activation.

One of the novel findings in this study is that E. coli K1 invasion of human BMECs was dependent on Rho GTPase. This was shown using dominant-negative RhoA-expressing BMECs as well as the Rho kinase inhibitor Y-27632, both of which showed significant decreases in E. coli K1 invasion of human BMECs. Of interest, the cnf1 deletion mutant was significantly less efficient in invasion of human BMECs as well as in RhoA activation compared with the parent E. coli K1 strain, suggesting that CNF1 contributes to E. coli K1 invasion of BMECs via RhoA activation. This concept was further supported by the demonstration that the decreased invasion observed with the cnf1 deletion mutant was restored to the level of the parent E. coli K1 strain using BMECs with constitutively active RhoA. Also, similar to the enhancing effects of CNF1 on E. coli K1 invasion of BMECs, invasion assays using BMECs with constitutively active RhoA resulted in significantly increased invasion, suggesting that RhoA activation can substitute for CNF1 in enhancing E. coli K1 invasion of BMECs. Taken together, these findings suggest that CNF1 mediates E. coli K1 invasion of BMECs via RhoA activation. Of interest, the enhancing effects of constitutively active RhoA on E. coli K1 invasion were not as high as those achieved using CNF1 (~8-fold versus 40-fold increases, respectively). One explanation may be the use of different methods of activating RhoA, i.e., transfection versus CNF1 treatment. An alternative explanation may be related to the concept that CNF1 may activate RhoA and other Rho GTPases, resulting in greater E. coli K1 invasion of BMECs.

In their active GTP-bound form, Rho GTPases interact with many effectors, including serine/threonine kinases, lipid kinases, and several adaptor proteins (32–34), affecting the host cell actin cytoskeleton and leading to increased formation of stress fibers (14, 15, 18, 31). Several lines of evidence suggest that PI3K plays an important role in host cell cytoskeletal remodeling and trafficking (35). For example, PI3K controls Rho-mediated changes in the actin cytoskeleton in fibroblasts (36, 37). We found that CNF1 treatment increased stress fiber formation in dominant-negative PI3K-expressing BMECs, whereas this effect was minimal in dominant-negative RhoA-expressing BMECs and in Y-27632-pretreated BMECs. In addition, RhoA activation (as shown by GTP-RhoA) was shown to occur in response to CNF1 in dominant-negative PI3K-expressing BMECs. Overall, these data indicate that E. coli K1-induced stress fiber formation of BMECs is dependent upon RhoA, FAK, and PI3K, but that CNF1 treatment is able to overcome the requirement of FAK and PI3K, suggesting different mechanisms for E. coli K1 and CNF1 for their abilities to induce stress fiber formation and also to induce bacterial entry into human BMECs.

We have previously documented that other meningitis-causing bacteria such as group B Streptococcus (38) and Citrobacter (39) are able to invade human BMECs by inducing host cell actin cytoskeletal rearrangements, but the basis of actin cytoskeletal rearrangements by these meningitis-causing bacteria is unclear. In this study, we demonstrated that CNF1 contributes to E. coli K1 invasion of BMECs by modulating actin cytoskeletal rearrangements through activation of RhoA. More importantly, we identified CNF1 as a critical determinant for E. coli K1 penetration of the central nervous system in vivo. Our findings with CNF1-producing E. coli K1 illustrate that CNF1 in a whole bacterium is able to contribute to BMEC invasion in vivo and penetration into the central nervous system in vivo. In contrast, previous reports describing CNF1-mediated enhancement of bacterial invasion of host cells have shown with addition of only exogenous CNF1 (14). Of interest, CNF1 is considered to be a cytosolic protein and not to be secreted from the whole bacterium. It remains unclear how
CNF1 in the whole bacterium is able to provide the BMEC invasion phenotype and RhoA activation, and studies are in progress to address this issue.

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Naveed Ahmed Khan, Ying Wang, Kee Jun Kim, Jin Woong Chung, Carol Ann Wass and Kwang Sik Kim

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