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Responses of brain and non-brain endothelial cells to meningitis-causing *Escherichia coli* K1

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Abstract

Bacterial interaction with specific host tissue may contribute to its propensity to cause an infection in a particular site. In this study, we examined whether meningitis-causing *Escherichia coli* K1 interaction with human brain microvascular endothelial cells, which constitute the blood–brain barrier, differed from its interaction with non-brain endothelial cells derived from skin and umbilical cord. We showed that *E. coli* K1 association was significantly greater with human brain microvascular endothelial cells than with non-brain endothelial cells. In addition, human brain microvascular endothelial cells maintained their morphology and intercellular junctional resistance in response to *E. coli* K1. In contrast, non-brain endothelial cells exhibited decreased transendothelial electrical resistance and detachment from the matrix upon exposure to *E. coli* K1. These different responses of brain and non-brain endothelial cells to *E. coli* K1 may form the basis of *E. coli* K1's propensity to cause meningitis.

Keywords: Tissue tropism; Endothelial cells; Meningitis; HBMEC; HUVEC; HMEC; HDMEC; TEER; *Escherichia coli*

Adherence is considered the initial interaction of a pathogenic microorganism with its host, providing a route to cellular invasion and/or injury [4,23]. Pathogenic microorganisms preferentially interact with certain cells or tissues, a phenomenon referred to as cellular or tissue tropism, which determines specific microbial–host relationships. The host in turn exhibits its response depending on the organism and its site of infection. For example, during urinary tract infection, the innate host defenses are involved in the early stages of the infection, clearing up to 99% of the infecting bacteria by exfoliation [30]. Similarly, apoptosis is another defense mechanism to a variety of stimuli including microbes [3].

The endothelium is specialized at different anatomic sites and plays a role in host defense when exposed to various microorganisms during bacteremia. Different capillary beds display widely disparate phenotypes such as differing tightness ranging between the highly tight junctions of brain microvessels (2000 Ω/cm^2) and the open spaces between the sinusoidal endothelial cells of spleen [6,8–11,13]. Such differences among the endothelial lining in different vascular segments and in different tissues may contribute to different host responses.

Our focus is on blood–brain barrier (BBB) dysfunction during bacterial meningitis [19–22], where barrier permeability is maintained during bacterial translocation, but associated inflammatory responses to bacterial entry into the CNS cause a breakdown of the barrier [17]. *Escherichia coli* (*E. coli*) K1 strain RS218 is a prototype strain for neonatal meningitis [1]. Previous investigations in our group, using in vitro and in vivo models of the BBB, have indicated that successful traversal of the blood–brain barrier by circulating *E. coli* K1 requires a high level of bacteremia

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as well as invasion of human brain microvascular endothelial cells (HBMEC). Since the route of entry of *E. coli* K1 into the host is implicated to be via the gut [7] and *E. coli* subsequently disseminates to the bloodstream and the brain, we investigated if different endothelial cells exhibit different responses to *E. coli* K1. Published literature for *E. coli* K1 strains interaction with different epithelial cells from bladder (T24), kidney (MDCK and OK), intestine (Caco-2 and T84), respiratory tract (A549), and larynx (Hep-2) showed that bacterial invasion occurred only in the T24, A549, and T84 epithelial cells, not in Caco-2 cells [7,24]. *E. coli* K1 strain IHE3034 exhibited a lower invasion rate in the human umbilical vein endothelial cells (HUVEC) Ea-hy926 compared to human bladder epithelial T24 cells [24], but there is no information comparing the interactions of *E. coli* K1 strain RS218 with human endothelial cells of brain and non-brain origin. The main objective of this study was to investigate if *E. coli* RS218 exhibited any tropism towards the brain microvascular endothelial cells lining the BBB. We used the in vitro model of endothelial cells of human origin isolated from three different sites-brain (HBMEC), skin (HDMEC), and umbilical cord (HUVEC).

Materials and methods

Endothelial cells. All the endothelial cells used were of human origin—from brain (HBMEC) [31–33], umbilical vein (HUVEC from Cambrex), and skin (HDMEC from CDC [HMEC-1]). HBMEC were propagated in HBMEC medium composed of RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 10% NuSerum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM non-essential amino acids, 1% vitamins, 100 U/ml penicillin, and 100 μg/ml streptomycin. HUVEC were propagated as per supplier's instructions (Cambrex, Walkersville, MD) in endothelial cell growth medium EGM-2 supplemented with bullet kit containing hydrocortisone, heparin, recombinant human (rh) fibroblast growth factor-B, rh-long-R insulin like growth factor-1, rh-epidermal growth factor, rh-vascular endothelial growth factor, ascorbic acid, gentamicin sulfate, amphotericin-B, and 2% FBS. HDMEC were propagated in MCDB131 containing 100 U/mL penicillin and 100 μg/mL streptomycin, with 10% fetal bovine serum and 2 mM L-glutamine at 37 °C in 5% CO_2. Prior to the experiments, all the endothelial cells were acclimatized and propagated in HBMEC medium followed by experimental medium (XM) composed of equal volumes of M199 and Ham's F12, both containing 2 mM L-glutamine, supplemented with 5% FBS.

Bacterial strains. Escherichia coli RS218^{STR} (O18ac:K1:H7) is a spontaneous streptomycin resistant mutant of *E. coli* RS218. Strain HB101, a laboratory *E. coli* K-12 strain [5], was used as a negative control and grown on LB agar. The *cnf1* deletion mutant (Δ*cnf1*) that does not express cytotoxic necrotizing factor-1 [18] and the *fimH* deletion mutant (Δ*fimH*) that does not express type 1 fimbriae [34] have been previously described. Bacterial strains were grown on LB agar containing streptomycin. During experiments, an MOI 10 was used to infect the cells.

Association assay. Association assays were performed as described earlier [29]. Briefly, all the three types of endothelial cells were grown to confluence in a 24-well tissue culture plate in HBMEC medium. The cells were washed and incubated for 1 h in XM and then with *E. coli* RS218 and HB101 at an MOI of 10. The associated bacteria were counted after 60 min of infection since the monolayer was eroded when incubated for 90 min. Similarly, *E. coli* Δ*cnf1* and Δ*fimH* were also tested to determine the responses of endothelial cells to CNF1 and FimH.

Phase-contrast microscopy. Phase-contrast microscopy of the wells containing cells before and after infection was done under the Olympus microscopy system (Olympus America, Melville, NY) equipped with an inverted Olympus microscope IX-70, a CCD camera OlymPix (model TE3/A/S; AstroCam), a 40×, 1.3-numerical aperture oil-immersion objective, supported by UltraView software.

Measurement of transendothelial electrical resistance. To study the real-time endothelial barrier dysfunction, the transendothelial electrical resistance (TEER) was measured with the ECIS (electric cell-substrate impedance sensing) system (Applied Biophysics, Troy, NY) which measures characteristics of cell growth, attachment/spreading, and barrier function of confluent cell layers [35]. HBMEC, HDMEC, and HUVEC were directly seeded on a collagen-coated eight-well gold electrode array in 400 μl HBMEC medium without antibiotics. Each well had one active electrode (250-μm diameter) and a large counter electrode. Once the resistance reached a constant maximum value [28], the cells were washed and incubated 1 h in 300 μl XM. One hundred microliters of bacterial inoculum in XM was quickly added to reach an MOI of 10 and incubated at 37 °C. TEER was measured until all the three types of endothelial cells dropped in their resistance.

Results and discussion

While most bacteria are believed to have a specific interaction with certain tissues [14], many host cells prevent their prolonged interaction by exfoliating [26,30] or undergoing apoptosis in the presence of bacteria [3]. *E. coli* K1 strain RS218 is a prototype *E. coli* associated with meningitis. The initial route of entry for this bacterium is via the gut [7] and *E. coli* travels through the bloodstream and infects the brain. In contrast to non-brain endothelial cells, the endothelial cells lining the BBB have a characteristically higher TEER (>2000 $\Omega\,cm^2$) [16,28,32]. The fact that only a limited number of pathogens are able to traverse this barrier suggests that the BBB endothelial cells may posses attributes that allow specific interactions with meningitis-causing microbes [2,36]. We examined and compared the human endothelial cells from non-brain sites such as skin and umbilical cord with HBMEC for their interactions with *E. coli* K1.

Only HBMEC sustained association with E. coli K1 strain RS218 for 60 min

We examined the interaction of *E. coli* K1 with human endothelial cells by performing the association assays [29] and bacterial counts were taken at 60 min of infection. The association rate more than 60 min of infection could not be analyzed for non-brain endothelial cells since the monolayers had eroded and the bacteria adhered to the matrix substrate. The association rates of *E. coli* RS218 on HBMEC at 60 min were comparable with the previously published data (Fig. 1). To examine the responses of endothelial cells to *E. coli* K1, phase-contrast microscopy was done. On infecting HBMEC with *E. coli* RS218 for 60 min, the monolayer remained intact (Fig. 2A), while a similar infection of the non-brain endothelial HDMEC (Fig. 2B) and HUVEC (Fig. 2C) resulted in rounding-up of the endothelial cells and detachment from the wells. However, such responses were not observed in HDMEC and HUVEC when infected with *E. coli* HB101. These

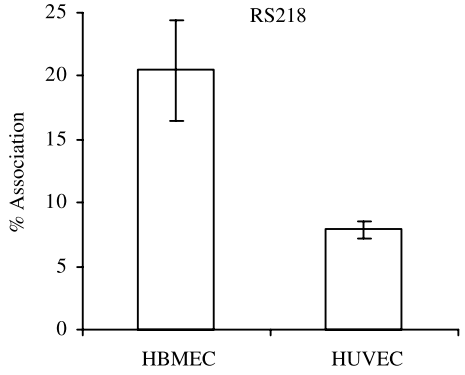

Fig. 1. Comparison of association rates of *E. coli* RS218 between HBMEC and HUVEC at 60 min of incubation at 37 °C.

findings suggest that the non-brain endothelial cells cannot sustain intercellular junctional resistance upon exposure to *E. coli* K1, resulting in dislodgement from their basement membrane. The morphology of brain endothelial cells, in contrast, remained intact throughout the infection process.

Maintenance of brain endothelial cell monolayer is due to high junctional resistance in HBMEC

To study the effect of bacterial infection on the integrity of monolayer in all the three cell lines, an ECIS based real-time measurement of transendothelial resistance was done. After reaching confluence the cell lines were incubated for 1 h in XM, and the TEER measurements were started. At the end of 1 h, all the three cell lines were infected with *E. coli* K1 strain RS218 at an MOI 10 and the TEER measurements continued post-infection. As shown in Figs. 3A–C, all the cells grew normally even in the low serum-containing XM. On addition of bacteria, within 45 min the resistance of non-brain endothelial cells started to drop, but surprisingly the resistance of HBMEC increased until nearly $2\frac{1}{2}$ h. By the time the HBMEC resistance reached

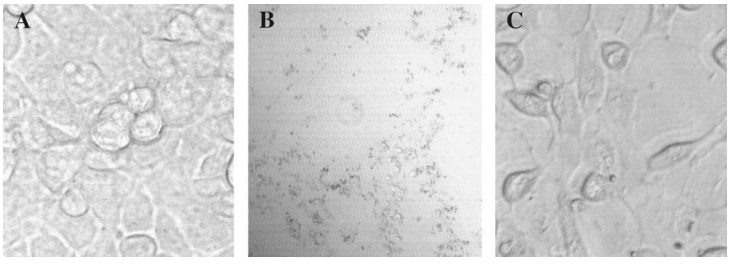

Fig. 2. Microscopic images showing the HBMEC (A), HDMEC (B), and HUVEC (C) infected with *E. coli* RS218 for 60 min.

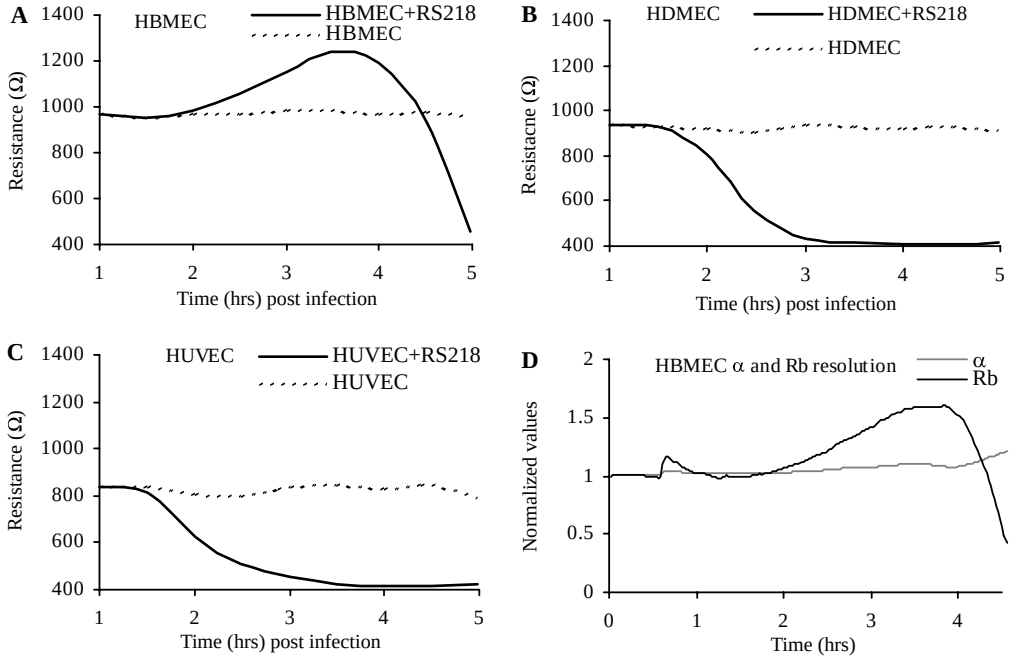

Fig. 3. Real-time measurements of transendothelial electrical resistance (A, B, and C) for the three cell types after 1 h infection with *E. coli* K1 strain RS218 (solid lines) and uninfected cells (dotted lines). HBMEC (A) exhibited increased resistance between 2 and 4.5 h followed by a decrease, while resistance in HDMEC (B), and HUVEC (C), started to fall within 2 h. (D) The resolution of resistance components into resistance to current flow between adjacent cells, Rb (dark line) and resistance to current flow beneath the cells (gray line).

its peak, the non-brain endothelial cells had lost their resistance completely. The resistances of all types of uninfected cells were unchanged. To further analyze whether the increased resistance is due to increased intercellular tight junctions, or due to increased adherence to the substrate, we applied the analytical model of Giaver and Keese [15]. This model resolves the data into components reflecting resistance to current flow between the adjacent cells (Rb), which represents the true barrier function, and beneath the cell layer (α), which depends on the characteristics of cell adhesion to the substrate due to shape and size of the endothelial cells. As shown in Fig. 3D, Rb increased gradually until the cells started to disrupt while α was not affected. This indicates that the tightness exhibited by the HBMEC was due to tighter barrier function rather than the increased attachment of the cells to the substrate. Such an analysis could not be applied for the non-brain endothelial cells since their resistance dropped very early post-infection. Microscopic examinations of these non-brain endothelial cells post-infection suggest that the resistance drop is due to the intercellular junctional breach since they had all rounded up and then detached from the basement of the cell culture wells.

Disruptions of non-brain endothelial cell monolayer are not CNF1- or FimH-dependent

CNF1 of UPEC was shown to intoxicate and kill cultured human uroepithelial cells via apoptosis induction [25]. In addition, presence of the FimH adhesin was also demonstrated to be necessary for the UPEC strain NU14 to induce apoptotic exfoliation and subsequent invasion of the disrupted bladder surface [27]. Based on this information, we investigated if during *E. coli* K1 infection, the disruption of non-brain endothelial cell monolayer is CNF1- or FimH-dependent. Subsequently, Δ*cnf1* and Δ*fimH* mutants of *E. coli* RS218 were examined for their association rates and abilities of disruption of non-brain endothelial cell monolayer by association assays and phase-contrast microscopy, respectively. We showed that the non-brain endothelial cell monolayers were disrupted with both these mutants, similar to the wild type, indicating that non-brain endothelial disruption is not due to CNF1 and type 1 fimbriae. The association rates with HBMEC after 60 min incubation with all the strains were comparable with the previously published data (Fig. 4).

In summary, *E. coli* association assays and phase-contrast microscopy examinations revealed that the monolayer of non-brain endothelial cells was disrupted during an infection with *E. coli* K1 strain RS218 at an MOI 10, while HBMEC monolayer remained intact upon exposure to *E. coli* K1. Our real-time measurements of the cell integrity of the three human endothelial cells using the ECIS revealed that the cells of non-brain origin lost their transendothelial electrical resistance upon exposure to *E. coli* K1 while resistance was increased in HBMEC. Since CNF1 and FimH have been shown to be involved in such effects in other epithelial cells [25,27], we investigated their roles in the different responses of brain and non-brain endothelial cells to *E. coli* K1. Microscopic examinations of the endothelial cells infected with either of the type 1 fimbriae and CNF1 deletion mutants still showed disruption of non-brain endothelial cell monolayer. These findings indicate that *E. coli* K1-mediated disruption of non-brain endothelial cells is not due to type 1 fimbriae and CNF1.

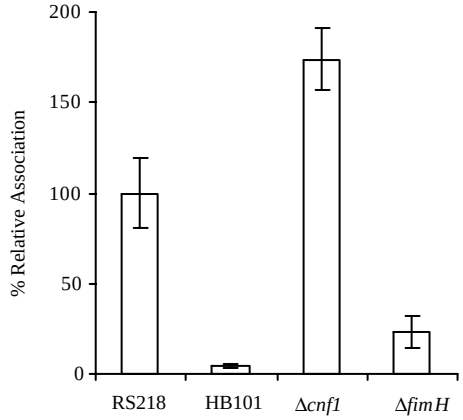

Fig. 4. Relative association rates (60 min incubation) of *E. coli* RS218 Δ*cnf1* and Δ*fimH* mutants compared to the wild-type RS218^{STR}. HB101 is the negative control.

In conclusion, the divergent behavior of different endothelial cells of human origin in response to meningitis-causing *E. coli* K1 may contribute to tissue tropism. While the non-brain endothelial cells shed, on being infected with *E. coli* RS218, the brain endothelial cells exhibit an opposite reaction. The primary function of the HBMECs being to provide a barrier, they become tighter in their intercellular junctions during stress. A similar phenomenon was recently reported by us, when zinc was depleted the intercellular resistance of HBMEC increased, while that of non-brain endothelial cells was decreased [12]. The increased resistance of HBMEC during *E. coli* K1 infection, which may be to prevent entry of the bacteria, in turn may provide a platform for *E. coli* to use its virulence factors to attach, invade and transcytose across the BBB. Further studies are required to elucidate the mechanisms involved in divergent responses of brain and non-brain endothelial cells to *E. coli* K1.

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