The surface protease OmpT serves as *Escherichia coli* K1 adhesion in binding to human brain micro vascular endothelial cells

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Abstract: *Escherichia coli* (*E. coli*) K1 is the most common bacteria that cause meningitis in the neonatal period. But it's not entirely clear about how *E. coli* crosses the blood-brain barrier. The features of the OmpT deletion in meningitic *E. coli* infection were texted *in vitro*. In comparison with the parent strain, the isogenic OmpT deletion mutant was significantly less adhesive to human brain microvascular endothelial cells (HBMEC). The adhesion-deficient phenotype of the mutant was restored to the level of the wild-type by complementing with low-level OmpT expression plasmid. Interestingly, the adhesion was enhanced by point mutation at the OmpT proposed catalytic residue D85. Compared with the poor adhesive activity of bovine serum albumin-coated fluorescent beads, recombinant OmpT or catalytically inactive variant of OmpT-coated beads bound to HBMEC monolayer effectively. Our study suggests that OmpT is important for bacterial adhesion while entering into central nervous system, and the adhesion does not involve in the proteolytic activity of OmpT.

Keywords: OmpT, adhesion, meningitis, brain microvascular endothelial cells, *E. coli* K1.

INTRODUCTION

Escherichia coli is the most common bacteria of neonatal bacterial meningitis (NBM), however the pathogenesis of this disease, especially how bacteria cross the blood-brain barrier (BBB) is not entirely defined (Huang *et al*., 2001). Many bacterial surface determinants are involved in specific interaction with the host cell during the invasion process. The presence of S fimbriae is important in the binding of *E. coli* to human brain microvascular endothelial cells (HBMEC), which might be an important early step in the penetration of *E. coli* across the BBB (Stins *et al*., 1994). *E. coli* associated with HBMEC leads to up-regulation of the *fim* genes, and type 1 fimbria locked-off mutant and the *fimH* deletion mutant were significantly less adhesive and invasive than the parent strain (Teng *et al*., 2005). OmpA can enhance *E. coli* invasion by interaction with a 95-kDa HBMEC glycoprotein (Prasadarao *et al*., 2003). In addition, some outer membrane virulence factors (IbeA, IbeB, IbeC and IbeT et. al) are identified to participate in the *E. coli* crossing of the BBB (Huang *et al*., 2001 and Huang *et al*., 2001 and Zou *et al*., 2008).

E. coli outer membrane protease T (OmpT) belongs to the OmpTin family, comprises some enterobacterial surface proteases, most of them are multifunctional (Kukkonen *et al*., 2004). Compared with other OmpTin members, OmpT appears most likely to be a housekeeping protein, has been considered to play role in the hydrolysis of anti-microbial peptides (Stumpe *et al*., 1998), activation of human plasminogen and degradation of recombinant heterologous proteins (Kukkonen *et al*., 2001). Although crystal structure and substrate specificity of OmpT has been clarified (Vandeputte *et al*., 2001), the physiological function of the surface protease remains unclear. OmpT is a putative urinary tract infection virulence factor, which has not been demonstrated experimentally (Wang *et al*., 2009). Interestingly, *OmpT* significantly predominated (96%) over all traditional meningitis-associated traits in seventy *E. coli* isolates (Johnson *et al*., 2002). Previous studies showed many *E. coli* genes including *OmpT* were significantly regulated in HBMEC-bound *E. coli*, and OmpT was believed to mainly depend on proteolytic activity to facilitate the invasion (Xie *et al*., 2008). In our work, we studied the role of OmpT in the pathogenicity of *E. coli* K1 meningitis by using isogenic $OmpT^-$ and $OmpT^+$ strains.

MATERIALS AND METHODS

Bacterial strains, plasmids and reagents

E. coli strain RS218 (018:K1:H7) is a clinical isolate from the CSF of a neonate with meningitis and E44 is a spontaneous rifampin-resistant mutant of RS218 (Huang *et al*., 1995). The isogenic mutant E44: Δ*OmpT* was constructed as described previously (Hui *et al*., 2010). DH5α was used as the host strain in the subcloning and preparation of plasmids for DNA sequence determination. Competent cells of *E. coli* were made in 10% glycerol and transformed with electroporation. Strains containing plasmids were all grown at 37°C in LB Lennox broth, which supplemented with appropriate antibiotics for the positive selection of plasmids (table 1). Restriction endonucleases and T4 DNA ligase were both purchased from TaKaRa (Dalian, China). All chemicals used were

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purchased from Sigma (St. Louis, MO). All genetic manipulations were performed by the standard methods.

Cell culture and E. coli adhesion and invasion assays

HBMEC were cultured and used for assays as previously described (Zhang *et al*., 2009). Bacteria (10^7 CFU/well) were added to confluent monolayers of HBMEC in 24-well plates (about 10^5 cells/well) at a multiplicity of infection of 100. After 2h of incubation at 37ºC, one set of triplicate wells were washed four times with PRMI 1640 medium, then lysed by the addition of 20µl of 5% Triton X-100. Bacterial CFU were enumerated to give to the adherent bacteria. Adherence frequencies were calculated as a percentage of the total added bacteria or relative adhesion (percent adhesion as compared to the adhesion of wild-type strain).

To determine invasion frequencies, the HBMEC confluent monolayers were incubated with the same amount of bacteria (10^7) for 1.5h to permit invasion to occur. One set of triplicate wells was treated with 100µg/ml gentamicin for 1h to kill all extracellular bacteria. After washing with PRMI 1640 medium, the intracellular bacteria were enumerated. Invasion frequencies were computed as the number of intracellular bacteria divided by the number of bacteria associated with HBMEC. The transformants were subjected to the adhesion and the invasion assays as described previously.

Complementation of E44: ΔOmpT with a cloned OmpT gene

The 1.5 kb chromosome fragment containing the *OmpT* gene was cloned into pGEM-T vector, after digestion of the resulting plasmid pGET with *Bam*H I and *Eco*R I , the fragment was subcloned into high-copy-number vector pUC19 (resulting in plasmid pUT) or low copy expression vector pSTV29 (resulting in plasmid pST). Point mutation D85A was introduced into plasmid pUT and pST using two complementary primers with the sequences 5'-TCAGG**C**CTGGATGGATTCC-3' and 5'-GGAATCCATCCAGGCCTGA-3' (mutated bases are in bold). The E44: Δ*OmpT* strain was transformed by either pUT or pST for the expression of OmpT, E44: Δ*OmpT* (pUT) expressed OmpT at a level much higher than E44: Δ*OmpT* (pST) and the wild-type strain.

Lactate dehydrogenase (LDH) release test

The *E. coli* K1 stains were grown statically in brain heart infusion broth at 37°C for 18-24h. LDH release assay were done with approximately 10^7 bacteria added to confluent monolayers of HBMEC, and the monolayers were incubated for 4 and 6 h at 37°C. The supernatant was used for the LDH assay with a LD-L50 kit (Sigma). Releasing of LDH into the medium is proportional to the disruption of the cellular membrane (Nagai *et al*., 2005). The results were shown as follows: subtract background value from experimental LDH activity / total LDH activity × 100%.

Recombinant protein purified and in vitro folding

The complete ORF of *OmpT* without its signal sequence was amplified by PCR with primers 5'-ACAACCATGGGCCATCATCATCATCATCATTCTA CCGAGACTTTATCG-3' and 5'-CCGAATTCTTAAAATGTGTACTTAAGAC-3', cloned into the *Nco* I and *Eco*R I sites of pET28a. The construct was designated pET-OmpT expressing OmpT as an N-terminal His_6-tagged fusion protein under control of the T7 promoter. In addition, site mutation D85A was also introduced and resulted in pET-OmpT85. All expression plasmids were transformed into *E. coli* BL21 (DE3). After the induction of isopropyl-beta-D-thiogalactoside (IPTG), Recombinant OmpT fusion protein was isolated as inclusion bodies and purified by affinity chromatography on Chelating Sepharose Fast Flow (GE Healthcare, USA) under denaturing conditions. The refolding protocols of OmpTin protease were described previously (Kramer *et al*., 2000 and Lobo, 2006). In brief, the purified OmpT was refolded by diluting 5-fold into refolding buffer (10mM Tris-HCl, 25mM $DodMe_2NPrSO_3$, pH 8.0). After overnight incubation at 4°C, Lipopolysaccharide (LPS) was added in equimolar concentration, and finally dialyzed against the adhesion assay buffer. Recombinant His_6-OmpT was given 10% SDS-PAGE for purity analysis and quantified by bicinchoninic acid (BCA) assay (Pierce, Germany).

OmpT-coated beads association assay

For the association assays, Latex fluorescent beads (Invitrogen, USA) with a diameter of 1µm were coated with refolded OmpT and its variant D85A OmpT as described previously (Lobo, 2006 and Braun *et al*., 1998). Similar amount of bovine serum albumin (BSA) was coupled to beads as controls. Coated beads were added to HBMEC monolayers at a ration of 100 beads/cell. The monolayers were incubated for 1h at 37°C under gentle agitation. Unbound beads were washed three times with PRMI 1640 medium and fixed with 4% (w/v) paraformaldehyde for 20 minutes. The fixed monolayers were mounted and viewed with fluorescence microscopy (Olympus).

RESULTS

OmpT deletion in E. coli K1 results in decreased HBMEC adhesion rate

OmpT has been cloned and then sequenced from *E. coli* K1 strain E44. OmpT from *E. coli* K1 strain was found to be 96% identical to the paralogue in *E. coli* K12 strain (P09169) (data not shown). During the development of *E. coli* meningitis, the initial step is the adhesion of *E. coli* to HBMEC, which constitutes the BBB (Stins *et al*., 1994). In order to conform the role of OmpT in the pathogenesis of *E. coli* meningitis, the ability of both the parent strain and the isogenic *OmpT*-deletion mutant to bind HBMEC were firstly evaluated *in vitro*. The same amount of E44

and E44: ΔOmpT were incubated with the HBMEC confluent monolayers for 1, 2 and 3h and quantitative determination of bound was performed after extensive washing (fig. 1). Compared to the adhesion rates of the wild-type E44, 0.63% (1 h), 1.2% (2 h) and 9.13% (3 h), the adhesion rates of E44: Δ*OmpT* were 0.123, 0.63, 4.83% for 1, 2 and 3 h, respectively. Although both the adhesion rates of two strains increased in a time-dependent way, the degree of difference between them was significant. The *OmpT* mutant demonstrated reduced binding, indicating that OmpT may work in *E. coli* K1 binding to HBMEC.

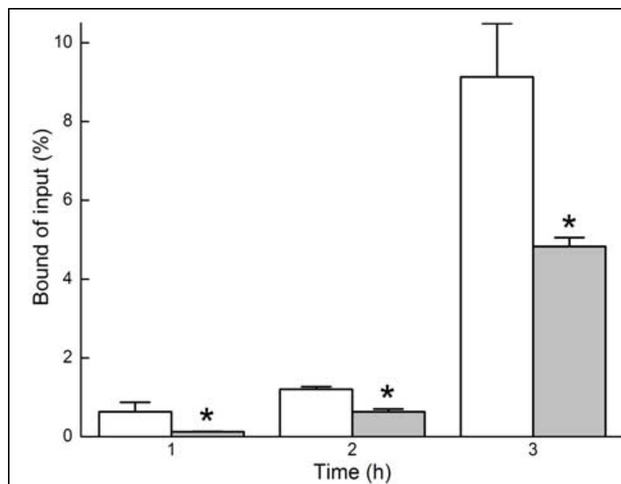

Fig. 1: Mutation in *OmpT* confers decrease in binding to cultured HBMEC. Equivalent amount of the wild-type E44 (*gray bar*) and isogenic mutant E44: Δ*OmpT* (*white bar*) were incubated with HBMEC monolayer for 1, 2 and 3h and bacteria bound (as a proportion of input bacteria) was quantified. The values are means of three independent assays with standard deviations. The asterisks indicate significant difference compared to the wild-type (Two-tailed *t* test, *P<0.01).

The proteolytic activity of OmpT is not involved in E. coli K1 adhesion to HBMEC

The defect in binding could not be complemented with the OmpT overproducer pUT, a derivative of high-copy-number vector pUC19. *E. coli* E44 harboring the empty vector pUC19, E44: Δ*OmpT* carring either pUC19 or pUT were incubated with the HBMEC confluent monolayers for 2 h, the relative adhesion rates were calculated after extensive washing (fig. 2a). The relative adhesion activity of E44: Δ*OmpT* (pUC19) (65%) was significantly less than that of the parent strain E44 (pUC19) (100%). However, the overexpression of OmpT impaired the adhesion ability of E44: Δ*OmpT* (pUT) (36%). In addition, the adhesion rates of E44: Δ*OmpT* (pUT) for 1, 3 and 4 h were also much lower than E44: Δ*OmpT* (pUC19) (data not shown). Substitution mutation D85A abolishes the enzymatic activity of OmpT (Vandeputte *et al*., 2001). We measured adhesion of E44: Δ*OmpT* (pUT85) to find out whether the enzymatic activity is involved in adhesion. Inactivated OmpT imparted E44: Δ*OmpT* high frequency of adhesion (108%). It is the uncontrolled enzymatic activity of OmpT that has an adverse infulence on bacterial adherence. Cytotoxicity induced by *E. coli* K1 was assessed by LDH leakage into the culture medium. At different times after infection, the amounts of LDH released from the damaged HBMEC cytoplasm were determined by using a commercially available Kit. As shown in fig. 2b, Upon infection of HBMEC with E44: Δ*OmpT* (pUT), the overexpressed OmpT from a multicopy plasmid pUT mediated a higher cytotoxicity than infected with wild-type E44 harboring the empty plasmid pUC19. It seemed like the amount of OmpT was positively correlated with the LDH release. Further information could be learned from mutated OmpT devoid of proteolytic activity, E44: Δ*OmpT*(pUT85) infection of HBMEC showed cytotoxicity close to that infected with E44: Δ*OmpT*. These data suggested there was a significant correlation between the proteolytic activity of OmpT and the cytotoxicity.

To deeply demonstrate the effect of OmpT on *E. coli* adhesion, another type rescue plasmid was used. The *OmpT* gene was cloned from E44 genome into the low-level expression vector pSTV29, and designated pST. Transformed with pST partly restored the adhesion ability of E44: Δ*OmpT* to the level of the parent strain (fig. 3a) (80%), and adhesion rate of E44: Δ*OmpT* (pST85) was similar to that of bacteria expressing wild-type OmpT. This means the enzymatic activity of OmpT is not required for adhesion. The adhesion of *E. coli* to the HBMEC is a prerequisite for cell internalization (Stins *et al*., 1994). Invasiveness of the isogenic *OmpT* deletion mutant, the wild-type strain, E44:Δ*OmpT* expressing OmpT or protease-negative mutant OmpT was assessed using the classic gentamicin protection assay. About 3-4% of bound bacteria were internalized in all experimental groups (fig. 3b). OmpT-mediated adhesion seems not to be followed by cell internalization. A multifactorial interaction is involved in *E. coli* K1 adhesion and invasion, many auxiliary virulence factors such as OmpT may contribute to the process synergistically with each other.

Association of purified OmpT or its variant-coated beads with HBMEC

From the results above, optimal expression of OmpT was associated with increased bacterial adhesion to HBMEC. However, uncontrolled enzymatic activity was harmful for adhesion process. Whether OmpT that mediates bacterial adherence directly as an adhesion molecule or not is still unknown. So we expressed OmpT and D85A OmpT as His_6-tagged proteins, which were purified by affinity chromatography under denaturing conditions. SDS-PAGE analysis of the recombinant proteins resulted in single bands with a calculated molecular mass of about 3.5 kDa (fig. 4a lane 1, 3). Without thermal denaturation,

the folded, compact OmpT and its variant migrates fast on SDS-PAGE, and all run at an apparent molecular mass of about 29 kDa (fig. 4a lane 2, 4). *In vitro* refolding conditions successfully induced the formation of the specific β-barrel structure of the OmpTins family, and then LPS was added, which was necessary for the enzymatic activity of OmpT (Kramer *et al*., 2000).

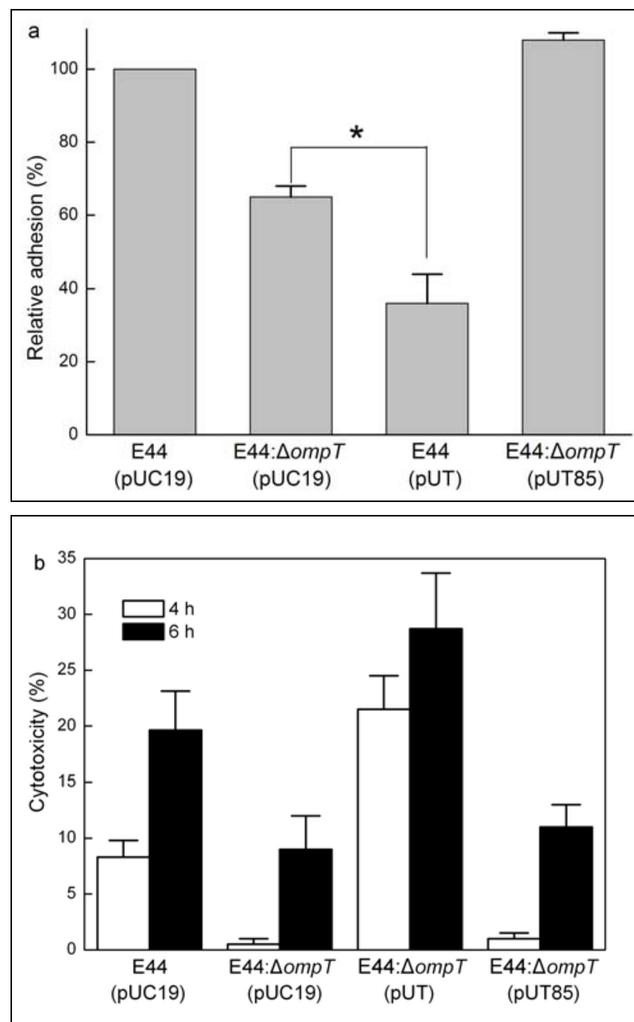

Fig. 2: Severe cell membrane damage and decreased adherene resulted from the uncontrolled protease activity of OmpT. **a** Adhesion to HBMEC by E44 and the *OmpT* isogenic mutant E44:Δ*OmpT* expressing OmpT (pUT), or site mutated OmpT devoid of proteolytic activity (pU85). After 2 h incubation, adherence frequency was calculated. The results are expressed as relative adhesion (%) of the wild-type E44, which is defined as 100%. (Student's *t* test, **P* < 0.01) **b** Cytotoxicity of *E. coli* K1 to HBMEC. Following infected with various *E. coli* K1 strains, cytotoxicity to HBMEC was assayed by determining the amount of LDH released from the damaged cells into the medium at 2, 4, and 6-h postinfection, and calculated as described in "Materials and methods". Each value represents the mean of triplicate independent measurements, and presented as means ± SD.

The refolded proteins and control protein BSA were incubated with Latex beads firstly, and then uncoated reactive sites on beads were blocked by excess BSA. Previously study showed the treatment slightly reduced the enzymatic activity of Pla, another OmpTin protease (Lobo, 2006). BSA-beads were seldom found to be associated with HBMEC after extensive washing. In contrast with the control, OmpT or D85A OmpT-beads bound to HBMEC and were variably aggregated (fig. 4b). The similar *in vitro* adhesive property of Pla was also reported before. This makes it difficult to calculate the precise data of the binding rate. Extensive binding of

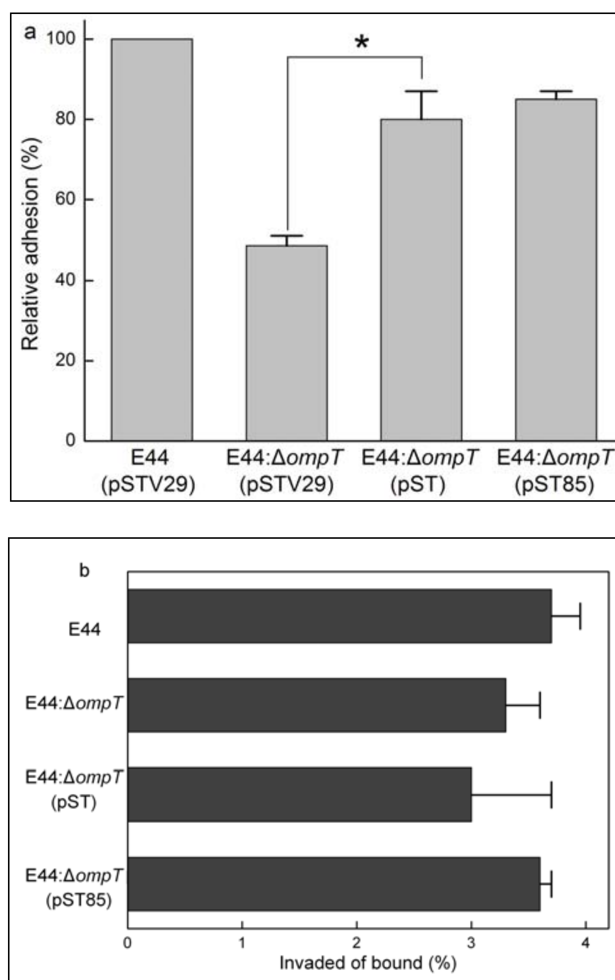

Fig. 3: The adhesion does not involve the proteolytic activity of OmpT. **a** Relative adhesion of E44 and E44:Δ*OmpT* containing low-copy vector pSTV29, E44:Δ*OmpT* complemented with pST or pST85. (Student's *t* test, **P* < 0.01) **b** *E.coli* K1 OmpT is not involved in the ivasion of HBMEC. Monolayers of cultured HBMEC were infected with E44, E44:Δ*OmpT* and E44:Δ*OmpT* complemented with pST or pST85. After 1.5 h incubation, Invasion was quantified as the number of intracellular *E. coli* number divided by the bound bacteria. Experiments were repeated at least three times with similar results.

Table 1: Bacterial strains and plasmids were used in this study

Strains	Genotype/characteristics	Reference/source
E44	Rif^r derivative of RS218 O18:K1:H7	14
E44: Δ*OmpT*	Δ*OmpT* derivative of E44 via allelic exchange	15
DH5α	F^- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1*hsd*R17 *phoA* *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Invitrogen
BL21 (DE3)	F^-*ompT hsdS*$_B$(r^-_B m^-_B) *gal dcm* (DE3)	Novagen
Plasmids		
pGEM-T	Amp^r	Promega
pGET	pGEM-T carrying the *ompT* gene under its native promoter	This study
pUC19	Amp^r, ori(ColE1), high copy	Amersham
pUT	pUC19 carrying in-frame *ompT* ORF	This study
pUT85	pUT with Asp85Ala	This study
pSTV29	pACYC184 derivative, Cm^r, low copy	Takara
pST	pSTV29 carrying in-frame *OmpT* ORF	This study
pST85	pST with Asp85Ala	This study
pET28a (+)	Kan^r, f1 origin	Novagen
pET-OmpT	pET28a(+) carrying *OmpT* gene under T7 promoter	This study
pET-OmpT85	pET-OmpT with Asp85Ala	This study

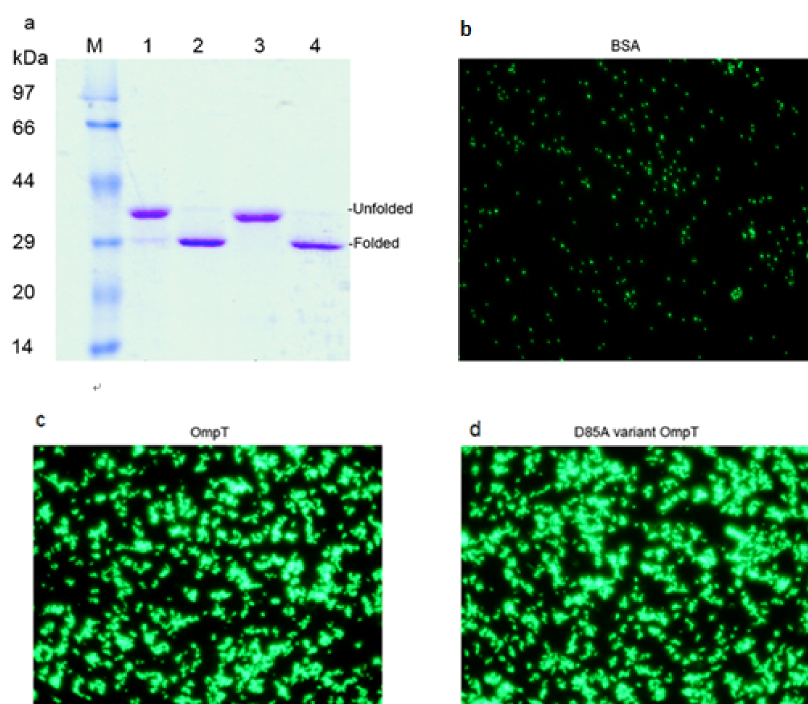

Fig. 4: Adhesion of Latex beads coated with OmpT and its variant to HBMEC monolayers. **a** *in vitro* folding of OmpT and D85A variant OmpT. Isolated inclusion bodies were purified by affinity chromatography under denaturing conditions, and then refolding was initiated by dilution in the presence of detergent. All samples were incubated for 5 min in loading buffer at room temperature before SDS-PAGE analysis. Lane M, molecular mass marker. Lane 1, purified recombinant OmpT. Lane 2, *in vitro* folded OmpT. Lane 3, purified D85A variant OmpT. Lane 4, *in vitro* folded D85A variant OmpT. **b** The group incubated with BSA-beads was selected as control. C, d Fluorescent micrographs of OmpT- or D85A variant OmpT-beads associated with cultured HBMEC

OmpT or D85A OmpT-beads were observed in randomly selected fields under fluorescence microscopy. The adhesive property of purified OmpT protein was demonstrated directly, and the adhesive function didn't involve the enzymatic activity of OmpT.

DISCUSSION

Neonatal bacterial meningitis represents a significant cause of mortality and morbidity and is most frequently caused by gram-negative bacteria. Most bacterial meningitis in the neonatal period develop by the way of hematogenous spread (Huang *et al*., 1995 and Teng *et al*., 2006). More and more evidence shows adhesion and invasion are separate steps for microbial penetration into CNS (Zou *et al*., 2008). The adherence of *E. coli* to HBMEC is the initial step towards the development of meningitis (Stins *et al*., 1994). There are a considerable number of specific microbial structures related to adherence traits of *E. coli* were characterized. *ibeT* located immediately downstream of invasion gene *ibeA* in the GimA pathogenic island of *E. coli* K1, *ibeT* isogenic mutant was deficient in adhesion to intestinal epithelia and HBMEC compared with the parent strain (Zou *et al*., 2008). Additionally there are many other genetic determinants, such as OmpA and CNF1, are considered to contribute to *E. coli* K1 entry into HBMEC (Prasadarao *et al*., 1996 and Khan *et al*., 2002. S fimbria mediated binding of *E. coli* K1 to HBMEC by a lectin-like activity of SfaS adhesin, but was not accompanied by *E. coli* crossing BBB (Stins *et al*., 1994). Type 1 fimbriae were also demonstrated to be involved in *E. coli* K1 binding to HBMEC (Teng *et al*., 2006). Both S and type 1 fimbria are all common bacterial structures exist in *E. coli*. In the present study, OmpT, a housekeeping surface protease of gram-negative bacteria, was investigated during the pathogenesis of meningitis.

Prior to this report, Lahteenmaki *et al*. reported that recombinant *E. coli* XL1 expressing OmpT adhered weakly to human umbilical vein endothelial cells (HUVEC), however, the expression of the OmpTin homolog from *Yersinia pestis* promoted intimate adhension of *E. coli* XL1 to HUVEC (Lahteenmaki *et al*., 2001). Y xie and colleagues found that several genes including *OmpT* contributed to bacterial adhesion to HBMEC and speculated that OmpT might degrade host proteins to facilitate invasion (Xie *et al*., 2008). Antimicrobial peptide protamine is the optimal substrate of OmpT (Stumpe *et al*., 1998). By comparing the protamine susceptibility of E44 and E44: Δ*OmpT*, we found that the expression level of OmpT in E44 was indeed very low (data not shown), but deletion of *OmpT* led to deficient adhesion to HBMEC. High copy plasmid pUT greatly improved the OmpT expression amount of E44: Δ*OmpT* (pUT), however the overexpression of OmpT impaired the adhesion ability of E44: Δ*OmpT* (pUT). Complemented with low-level expression plasmid pST, the nonadhesive phenotype was partly restored. It seems like the relevant amount of OmpT endow the mutant with better adhesion ability. OmpT is different from other reported adhesins with common mechanism that stronger binding can be got with increased adhesin/acceptor expression, so the different conclusion of Lahteenmaki and colleagues may be explained.

Interestingly, OmpT-mediated adhesion was not dependent on the proteolytic activity of OmpT. Complemented with a catalytically deficient active site variant of OmpT, stronger binding can be got with increased inactivitated OmpT expression. In addition, recombinant OmpT and inactivated D85A OmpT are able to adhere to HBMEC monolayer effectively *in vitro*. The surface protease OmpT appears to be an adhesion. HBMEC surface may be exactly modified by OmpT, an efficient extra cytoplasmic protease. Some OmpT potential substrates/acceptors on the membrane of endothelial cells may be involved in the adhesion process, which were yet to be proved. Our data showed that uncontrolled OmpT proteolytic activity adversely affected bacterial adhesion process. The reason will be elicited when potential substrate and the specific mechanism was clarified.

OmpT is a prototype of a unique family of bacterial endopeptidases known as the OmpTins. Most of the OmpTin proteins have been implicated in bacterial pathogenesis (Kukkonen *et al*., 2004). Although OmpT has been purified and well biochemical characterized (Sugimura *et al*., 1988 and McCarter *et al*., 2004), little is known about its *in vivo* function. Many pathogenic bacterial species intervene with the mammalian proteolytic plasminogen-plasmin system, which aggravates damage of extracellular matrix and microbial spread and organ invasion during infection (Lahteenmaki *et al*., 2005). OmpT has been demonstrated to be a weak plasminogen activator (Kukkonen *et al*., 2001). Added plasmin could promote *Candida* to cross-cultured HBMEC monolayers (Jong *et al*., 2003). OmpT has been believed to be involved in the resistance to human defensin anti-microbial peptides (Hui *et al*., 2010). Although OmpT showed most likely *E. coli* adhesin during interaction with HBMEC, the proteolytic activity of OmpT is important in bacterial resistance to innate immune defenses. Multi-functional adhesin/ protease OmpT should play several roles *in vivo* to facilitate bacterial infection.

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