Meningitic *Escherichia coli* K1 IbeA is an Outer Membrane Protein Involved in Inducing Apoptosis of Human Brain Endothelial Cells

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E. coli K1 is the most common gram negative pathogen causing neonatal sepsis and meningitis. During *E. coli* K1 infection accompanied with bacterial invasion and hematogenous spreading, brain microvascular endothelial cells (BMEC) are directly targeted by *E. coli* K1 and their virulence factors. Among a number of known virulence factors contributing to *E. coli* K1 invasion of BMEC, IbeA is the major invasin which is unique to pathogenic *E. coli* K1 but not present in nonpathogenic *E. coli* K12. Therefore, we tested the hypothesis that IbeA, an outer membrane protein, induced human BMEC apoptosis. IbeA was shown to be an outer membrane protein, which shares significant sequence homology to another *E. coli* K1 virulence factor CglE. We investigated the potential of recombinant His-tagged IbeA to induce human BMEC (HBMEC) apoptosis. The TUNEL and caspase assays were used to measure apoptosis. The results showed that IbeA was able to induce apoptosis in HBMEC. Furthermore, caspase-8 was found to be activated by IbeA and IbeA+ *E. coli* K1. However, the *ibeA* mutant 10A-23 and another *E. coli* K1 invasion protein IbeB were unable to stimulate caspase 8. Hence, caspase-8-dependence of IbeA-induced endothelial apoptosis may bear relevance to novel insight into the pathogenesis of *E. coli* K1-induced sepsis and meningitis.

Key words: Apoptosis; BMEC; *E. coli*; IbeA; meningitis; outer membrane protein.

Abbreviations: BBB (blood-brain barrier), BMEC (Brain Microvascular Endothelial Cells), HBMEC (Humen BMEC), CNS (Central Nervous System), OMP (Outer Membrane Protein).

Despite the availability of effective bactericidal antibiotics over the last sixty years, bacterial meningitis continues to be the most common serious infection of the central nervous system (CNS) with high morbidity and mortality[1-5]. This high morbidity and mortality is due to inadequate knowledge of the pathogenesis of bacterial meningitis. *E. coli* is the most common gram-negative bacteria causing neonatal sepsis and meningitis[1-5]. Most cases of bacterial meningitis in newborns develop as a result of hematogenous spread. Strains causing meningitis possess traits that distinguish them from commensal strains of *E. coli* and other pathogenic strains such as those

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causing diarrhea and urinary tract infection. Characteristically, meningitic strains of *E. coli* are predominately carrying K1 capsule (over 84%), produce S fimbriae, and express *ibeA*[6]. The presence of these features implies that meningitic strains possess a defined set of virulence determinants that allow the bacterium to penetrate the blood-brain barrier (BBB) and enter the CNS. In order to dissect the molecular and cellular mechanisms responsible for *E. coli* K1 entry into the CNS, an *in vitro* HBMEC culture model with characteristics of the BBB has been established[1-3]. In combination of this cell culture model with animal models, several virulence factors including IbeA, IbeB IbeC, AslA, OmpA, FimH, and F-plasmid encoded TraJ are identified as invasion determinants which are involved in *E. coli* crossing of the BBB (1-7). Among these known invasion determinants, IbeA is a major invasion protein that is unique to pathogenic *E. coli* K1 but absent in nonpathogenic *E. coli* K12[1-2, 4, 6].

IbeA was the first gene of the GimA island to be isolated. The work that led to its discovery was aimed at identifying *E. coli* structures that contributed to the invasion of the BBB. Bioinformatic analysis suggests that IbeA is a putative outer membrane protein[6,8]. Vimentin and PTB-associated splicing factor (PSF) have been identified as IbeA-binding proteins[9, 10]. The distribution of *ibeA* has been studied in a number of *E. coli* strains, most of them being responsible for extraintestinal infections in humans[6]. The prevalence of *ibeA* is highly dependent on the origin of the strain and on the subgroup it belongs to (A, B1, B2, and D)[6]. In all the studies where the presence of *ibeA* has been analyzed, *ibeA* was found to be restricted to the B2 subgroup, a subgroup that includes strains with the highest virulence in mice and the highest level of virulence déterminants[6]. Most of these strains belong to the B2 subgroup[6, 11]. A recent study demonstrated that only the *ibeA* gene was significantly more prevalent in *E. coli* strains causing early infection in neonates (0-3 days after birth)[12]. O18:K1:H7 *E. coli* strains are mainly responsible for neonatal sepsis and meningitis in humans and belong to a limited number of closely related clones[14]. The same serotype is also frequently isolated from avian pathogenic *E. coli* (APEC) strains. We have shown that IbeA is an invasion determinant in avian

strains, indicating a closer relationship between avian and meningitic strains[13]. These studies suggest that poultry may be a vehicle for human *E. coli* pathogens and that avian strains may potentially serve as a reservoir of virulence genes for meningitic strains. It has been demonstrated that APEC strains were able to induce apoptosis in macrophages[14-15]. However, the apoptotic factors were not identified in these studies. As shown in our previous studies, IbeA-dependent *E. coli* K1 invasion was observed in both intestinal epithelial and brain endothelial cells [1-4, 16]. One of the most intriguing findings is that transmission electron microscopy studies demonstrated that a high density of mitochondria was seen in the area of engulfment during the invasion of intestinal epithelial cells (Caco-2) by *E. coli K1* strain E44, suggesting mitochondrial involvement in the invasion process (16). Since mitochondria play a critical role in apoptosis, we proposed that apoptosis might be associated with *E. coli* K1 invasion of brain endothelial cells.

There is increasing evidence that apoptosis plays an important role in the complex balance among invading bacteria, the immune system, and host cells leading to inflammation and tissue damage in infections[17]. A number of studies suggest that neuronal apoptosis is a characteristic feature of bacterial meningitis in human disease and in several animal models of meningitis[17-23]. Disruption of the BBB is a key event in meningitis and in sepsis-associated encephalopathy. The integrity of the BBB affects invasion of bacteria, recruitment of leukocytes, and barrier functions. A recent report reveals the impressive ability of *S. pneumoniae* to induce apoptosis in brain-derived endothelial cells[17]. Currently, it is unclear whether *E. coli* K1 is able to induce apoptosis in BMEC. Identification of specific factors from meningitic *E. coli* K1 responsible for inducing apoptosis will provide insight into pathogenesis and may suggest novel strategies for disease prevention or treatment.

In the present study we demonstrated that IbeA is an outer membrane protein inducing apoptosis in HBMEC. Based upon results of two independent measurements for apoptosis, namely the TUNEL assay and caspase assay, we present evidence that IbeA does act to induce apoptosis in endothelial cells of the human brain microvasculature.

MATERIAL AND METHODS

Bacterial strains, plasmids and reagents

Bacterial strains, plasmids, and their relevant characteristics are described in Table 1. *E. coli* E44 is a rifampin-resistant strain of a clinic isolate *E. coli* RS218 (O18:K1:H7) from the cerebrospinal fluid (CSF) of a newborn infant with meningitis [24]. *E. coli* DH5a was used as the host strain for subcloning and protein expression. BL21(DE3), which carries the T7 RNA polymerase gene, was the host strain for protein expression of IbeA and IbeB[4, 25]. All strains were grown at 37 ºC in LB broth (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter) supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml) and/ or rifampin (100 µg/ml) if required. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Strains containing plasmids were grown at 37°C in Luria broth (LB; 10 g of tryptone, 0.5 g of NaCl, and 5 g of yeast extract/L) with ampicillin (100 µg/mL), kanamycin (50 µg/mL), and rifampin (100 µg/mL) for the positive selection of plasmids or bacterial strains (Table 1). Bacteria were cultured in LB and were stored at -70°C in LB plus 20% glycerol. Recombinant bactericidal/permeability-increasing protein (BPI) was obtained from XOMA Corporation. Recombinant proteins IbeA, IbeB and BPI were pre-treated with polymyxin B. The anti-IbeA antibody was prepared by immunizing rabbits with recombinant IbeA protein with an N terminal His6 tag that was purified by Ni-Sepharose column[4].

Extraction and manipulation of plasmids and subcloning

All genetic manipulations were done by using standard methods, as described elsewhere[26]. Plasmid DNA was extracted by using a plasmid mini kit (Qiagen). DNA fragments were purified and were extracted from agarose gel slices, using Geneclean (Bio 101). Competent *E. coli* cells were made in 10% glycerol and were transformed with electroporation, as described elsewhere[4,5].

Recombinant protein expression and purification

BL21 (DE3)/pET28a expression system was used to produce recombinant IbeA and IbeB proteins. The expression constructs pET17A and pET14B were made as described previously[4, 25]. Small-scale expression and purification of the recombinant protein was carried out before a large scale isolation according to the manufacturer's instructions (Novagen). The results indicated that IbeA and IbeB predominately resided in the insoluble fraction. Insoluble IbeA and IbeB proteins with a histidine tag were purified by binding to Ni-NTA resin in 6 M guinidine-HCl according to the manufacturer's instructions. The eluted proteins containing 8 M urea were refolded

Table 1. *E. coli* strains and plasmids used in this study

Strains and plasmids	Genotype / characteristics	Reference/source
E. coli strains		
RS218	O18:K1:H7 (causing 32% of *E. coli* meningitis)	(24)
E44	Rif[r] derivative of RS218	(1-4)
10A-23	E44 *ibeA*::TnphoA	(4)
DH5α	FΦ80d*lacZ*ΔM15Δ(*lacZYA-arg*F)U169 *rec*A1 *end*A1	
	*hsd*R17 *phoA sup*E44 l⁻ *thi*-1 *gyr*A96 *rel*A1	Invitrogen
BL21 (DE3)	F⁻*ompT hsdS*$_B$ (r$_B$⁻ m$_B$⁻) *gal dcm* D*ompT*	Novagen
Plasmids		
pUC13	Amp[r], lacZ	(4)
pUC1030	pUC13 carrying ibeA locus (18-kb)	(4)
pET28a(+)	Kan[r], F1 origin, His • Tag	Novagen
pET17A	pET28a(+) carrying *ibeA* gene (1.7 kb)	(4)
pET14B	pET28a(+) carrying *ibeB* ORF (1.4 kb)	(25)

as described previously[4]. Purity of the final products were assessed by subjecting the indicated amount of protein to sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE). Protein was assayed by using the Bio-Rad protein assay reagents according to the manufacturer's instruction.

Localization of IbeA in the outer membrane

To analyze the cellular location of IbeA, the outer membrane proteins were isolated as described previously[27]. Briefly, bacteria were sonicated in 0.5 ml PBS, followed by centrifugation at 6,000 x g to remove debris. The supernatant was subjected to ultracentrifugation at 120,000 x g for 1 h at 4ºC. The upper half of the supernatant was used as the soluble fraction, which contained cytoplasmic proteins and periplasmic proteins. The pellet was used as the mixed membrane fraction. The pellet was further washed with 0.6 ml of buffer (10 mM Tris-HCl, pH7.5, 5 mM EDTA, 0.3% lithium dodecyl sulfate) and centrifuged again at 100,000 x g for 1 h. The precipitate was used as the outer membrane protein. Isolated proteins were separated on a polyacrylamide gel and analyzed by Western blot.

SDS-PAGE and Western blot

Protein samples were resolved on SDS-12.5% polyacryamide Phastgels (Amersham) at 16ºC. For Western blot the resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The PVDF membrane was blocked with 5% non-fat milk powder in PBS plus 0.05% Tween 20 (PBS-T) at room temperature for 1 h. Then the membrane was incubated in the primary antibody (1:3000 dilution in PBS-T) for 1.5 h at room temperature, and washed in PBS-T. The horseradish peroxidase (HRP) conjugated secondary antibody (1:5000 dilution in PBS-T) was incubated with the membrane for 1.5 h at room temperature. After washing the proteins were detected using Lumi-light western blotting substrate kit (Roche).

Cell cultures

HBMECs were routinely cultured in RPMI 1640 medium (Mediatech, Herndon, VA) containing 10% heat inactivated fetal bovine serum, 10% Nu-serum, 2 mM glutamine, 1mM sodium pyruvate, essential amino acids, vitamins, penicillin G (50 mg/ml) and streptomycin (100 mg/ml) at 37 ºC in 5% CO_2.

TUNEL (ApopTag) staining of human BMEC

Human BMEC were grown on collagen-coated eight-well chamber slides. At confluence, recombinant proteins IbeA, IbeB and BPI (5 µg/ml) were added to human BMEC and the mixture was incubated for up to 48 h at 37°C. Subsequently, human BMEC were washed three times with experimental medium and fixed with 1% paraformaldehyde in PBS at 4°C (28). Apoptotic cells were detected by ApopTag *in situ* apoptosis detection kit (Intergen, Purchase, NY), according to the manufacturer's instructions. Briefly, the 3'-OH DNA ends that were generated by DNA fragmentation become substrates for terminal deoxynucleotidyl transferase (TdT). Then digoxigenin nucleotides were catalytically added to the apoptotically produced DNA ends. These nucleotides were detected by antidigoxigenin antibody carrying a conjugated peroxidase. Diaminobenzidine was then reacted with peroxidase to produce insoluble brownish-colored products in apoptotic bodies where DNA fragmentations were present. Human BMEC were counterstained with methyl green, mounted, and viewed and photographed in Nikon Diaphot microscope[28].

Measurement of Caspase-8 Activity

Activation of caspase-8 was determined by detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate IETD-pNA (ApoAlert Caspase-8 Colorimetric Assay, Clontech)[29]. Briefly, HBMEC were infected with *E. coli* K1 strains (E44 or its mutant 10A-23) for 2 h. After bacteria removed, the incubation continued for 3 h. For testing the effects of IbeA on caspase-8 activation, HBMEC were treated with Ibe proteins (5 µg/ml) for 4h. After incubation, HBMEC were resuspended in ice-cold lysis buffer, and incubated on ice for 10 minutes. Cell lysate supernatants were prepared after centrifugation. Caspase-8 activity of the supernatants was measured according to manufacturer's instruction.

Bioinformatics approaches

The IbeA protein sequences were used to search the protein databases at NCBI/NIH by using BLAST and PSI-BLAST[30]. Sequences were compared to existing sequences at the protein level to identify homologous proteins. ClustalW[31] and Boxshade[32] programs were used for multiple alignment of protein sequences.

Statistical analysis

Data were analyzed as described previously[33]. A paired t test was used to determine the statistical significance between the control and treatment groups. *P*<0.05 was considered to be significant.

RESULTS AND DISCUSSION

Outer membrane localization of IbeA. As suggested in our previous report (8), IbeA is a putative outer membrane protein (OMP)

To assay the possible outer membrane localization of the target protein IbeA, we isolated crude OMPs and soluble proteins (SP) from the *ibeA* mutant 10A-23, complemented strain and E44 (the parent strain). An anti-IbeA antibody was used for immunoblotting detection of IbeA. Analysis of the protein profiles of the SP fractions (left lane in Panels A-C of Fig.1) in SDS/PAGE/Western blot showed that no IbeA was detected in the wild-type, *ibeA* mutant and complemented bacteria, excluding the possibility of cytoplasmic and periplasmic location of IbeA. The same Western blot revealed that a 50-kDa protein band was detected in the OMP fractions from the wildtype strain E44 and pUC1030-complemented mutant strain (10A-23/30). As expected, IbeA was not present in the OMP fraction isolated from the mutant (10A-23/13). The mutant 10A-23 is phenotypically noninvasive *in vitro* and *in vivo* (4, 8, 16, 34). The noninvasive phenotype of 10A-23 can be restored by pUC1030 carrying the *ibeA* locus[8]. The protein content of IbeA in this mutant was not examined in the previous studies[8,16, 34]. Here we demonstrated that the noninvasive phenotype of this mutant was due to the absence of the IbeA protein (Panel C in Fig.1). The *ibeA* locus is able to complement this deficiency (Panel B in Fig.1). Many new *E. coli* OMPs have recently been identified by proteomics techniques[35]. However, poorly expressed proteins like IbeA may escape detection when wild-type cells are grown under standard conditions. In order to efficiently determine the outer membrane location of underexpressed proteins, there is an increasing use of a combination of bioinformatics with experimental approaches[35]. IbeA was predicted as a putative OMP in our previous bioinformatics studies[8]. The current work has experimentally verified the predicted outer membrane localization of IbeA.

IbeA is homologous to CglE, a putative dihydrolipoamide dehydrogenase LDH) which may be involved with apoptosis. In order to get more insight into the function and structure of IbeA, the protein sequence of IbeA was used to search the protein databases at NCBI/NIH by using BLAST and PSI-BLAST (30). Our bioinformatic studies showed that CglE and IbeA are present in GimA as a pair of homologous proteins that are encoded by two different operons, *cglDTEC* and *ibeRAT*, at two different locations. The functionality of both genes was initially defined by comparing the deduced their open reading frames (ORFs) to existing sequences with known functions at the protein level, and the results showed that a similar pair of proteins (IB-SP1 and IB-SP2) is also present in *Silicibacter sp* which belongs to the most abundant and ecologically relevant marine bacterial groups. BLAST analysis indicates that IbeA shows significant protein sequence homology/similarity to CglE (50%/70%), IB-SP1 (48%/64%) and SP2 (46%/65%). Fig.2 shows multiple sequence alignment of these four proteins. Boxshaded areas represent homology (heavy) or similarity (light). Bioinformatics analysis suggests that CglE is a putative DLDH since it shares sequence homology to DLDH of various species from bacteria to humans (6). DLDH (E3 component of pyruvate dehydrogenase complex, and á-ketoglutarate dehydrogenase) are mitochondrial matrix enzymes involved in the the tricarboxylic acid *cycle (TCA cycle)*. Mitochondrial dysfunction mediates increased oxidative stress and apoptosis cascades as well as impaired energy metabolism[36]. A proteomic study suggests that DLDH may be involved in neuronal apoptosis[36]. Our previous studies showed that vimentin was the common receptor for IbeA and CglE[9,37], suggesting that these two virulence factors may have common pathogenic functions including the induction of apoptosis.

IbeA induces HBMEC apoptosis and caspase activation

In order to determine whether IbeA + *E. coli* K1 induces HBMEC apoptosis, we tested apoptotic activity of recombinant proteins (IbeA, IbeB and BPI), IbeA+ strain (E44) and IbeA-deficient *E. coli* K1 (10A-23) by two different approaches [TUNEL (terminal deoxynucleotidyl
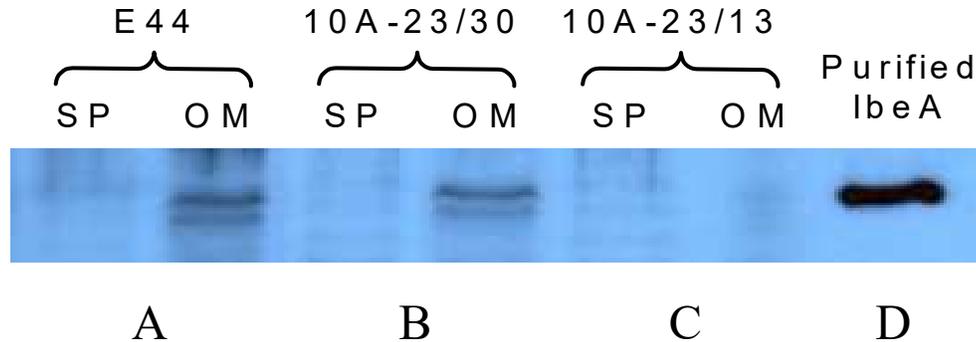
Fig. 1. SDS-PAGE and Western blot analysis of outer membrane proteins (OM), soluble proteins from supernatants (SP), and purified IbeA protein. OM and SP proteins were prepared from strains E44 (wildtype), complemented IbeA mutant with pUC1030 carrying *ibeA* locus (10A-23/30) and the mutant with the empty vector pUC13 (10A-23/13) (8). Five micrograms of OM, SP protein or 500 ng of recombinant IbeA protein was loaded in each lane of an SDS-10% polyacrylamide gel. After electrotransfer, IbeA proteins were detected with affinity-purified anti-IbeA antibody

Fig. 2. Multiple alignment of IbeA protein sequence (Accession no. AAF98391) with other homolgous proteins from *E. coli* K1 (CglE/Accession no. AAL61901) and *Silicibacter pomeroyi* DSS-3(Ib-SP1/Accession no. YP_166890 and Ib-SP2/Accession no. YP_165962)

transferase-mediated dUTP nick-end-labeling) and caspase assays]. IbeB, an OMP virulence factor[5], was used as a negative control for IbeA. BPI is an apoptotic inhibitor and bactericidal protein against gram negative bacteria[38,39]. After HBMEC treated with Ibe proteins or IbeA plus BPI, apoptotic cells were detected by TUNEL assays. As shown in Fig. 3B, extensive apoptosis was induced by IbeA. Recombinant IbeA used in this study was expressed in *E. coli* as a His6-tagged fusion protein. To control for nonspecific effects, His6-tagged-IbeB was used in parallel. Fig. 3A showed that IbeB, a

nonapoptotic invasion protein, was not capable of inducing apoptosis in HBMEC. BPI treatment alone did not induce apoptosis (Fig. 3C). A combination of IbeA with BPI led to significantly reduced apoptosis (Fig. 3D), suggesting that IbeA-induced apoptotic cellular effects were efficiently blocked by BPI. This blockage may be due to direct interaction between BPI and IbeA since BPI is a very basic protein (pI=10.62), which easily interacts with the acidic protein IbeA (pI=4.68). BPI has been shown to induce apoptosis in HUVEC at high doses (14-100 µg/ml)(40). This
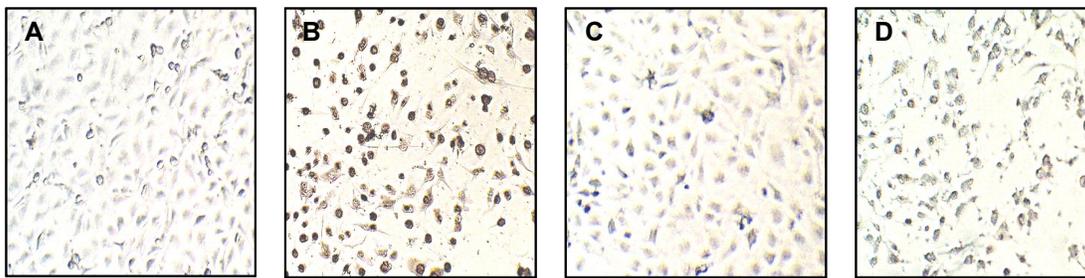
Fig. 3. IbeA-induced apoptosis in human BMEC. IbeA and other proteins were pre-incubated on ice for 1h and then incubated with BMEC monolayers for 4h. Apoptosis assays were performed according to the manufacturer's instruction. A: IbeB (5 µg/ml) (Control); B: IbeA (5 µg/ml); C: BPI (5 µg/ml); D: IbeA + BPI. IbeA and IbeB are recombinant proteins expressed in pET28a and purified with Ni-NTA chromatography

discrepancy may be due to (a) the size of dosage and (b) different types of endothelial cells.

To further define the role of IbeA in *E. coli* K1-induced apoptosis in HBMEC, we tested whether IbeA was able to activate caspase-8, an initiator casapase [41]. Various apoptotic pathways lead to the proteolytic activation of this protease from its proenzyme form[42]. It has been shown that caspase-8 can be activated during the infection of host cells by *M. tuberculosis. H. pyroli* and *S. staphylococcus*[41-43]. Confluent monolayers of human BMEC cultured on dishes were infected with E44 and the *ibeA* mutant 10A-23 or treated with IbeA and IbeB proteins. Caspase-8 activity was detected by a commercial kit from ClonTech. As shown in Fig.4, IbeA and E44 were able to significantly enhance caspase-8 activity comparing to IbeB and the *ibeA* mutant 10A-23. This study demonstrated that caspase-8 contributes to IbeA+ *E. coli* K1-induced apoptosis in HBMEC.
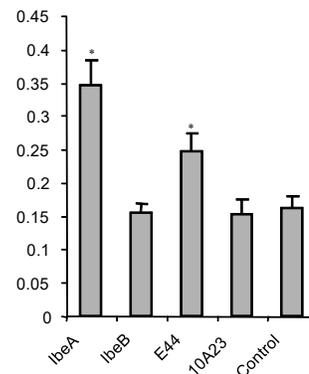
Fig. 4. Caspase 8 activities in human BMEC infected with *E. coli* K1 E44 (parent strain) and the *ibeA* mutant 10A-23 and treated with IbeA and IbeB proteins. BMECs were infected with *E. coli* for 2 h. After bacteria removed, the incubation continued for 3 h. BMECs were treated with proteins for 4h. Error bars represent the standard errors of triplicate samples. A paired t test was done with significance set at $P<0.05$.

Apoptosis is a tightly regulated suicide program leading to cell death and plays a central role in maintaining the integrity and homeostasis of multicellular organisms[42]. Recently, there has been increasing interest in understanding how microbial invasion may affect apoptosis of eukaryotic cells and whether this contributes to microbial pathogenesis. For example, uropathogenic and other microbial pathogens have been reported to induce apoptosis of host cells[41-44]. Apoptosis involves the proteolytic action of caspases, a family of cysteine proteases that cleave a large group of cellular protein substrates on the C-terminal side of aspartic acid. Caspase-8 involves in one of the major pathways of caspase activation leading to apoptosis. Using recombinant His6-tagged IbeA, IbeA+ *E. coli* K1 and the IbeA deficient mutant, our results show that IbeA is an OMP contributing to the induction of caspase-8-initiated apoptosis in HBMEC. We and others have demonstrated that IbeA is an important invasion determinant that is required for *E. coli* K1 entry into the CNS. Here our data demonstrate that IbeA induces HBMEC apoptosis. Due to its dual pathogenic functions in the pathogenesis of *E. coli* K1 meningitis, IbeA might be regarded as a 'bridge' protein that can link invasion processes and apoptotic pathways, which may play a crucial role in the development of *E. coli* K1 meningitis. Thus, it is tempting to speculate that both IbeA-induced *E. coli* K1 invasion and BMEC apoptosis are coordinately regulated. Further work is needed to examine how IbeA contributes to signal transduction and subsequently *E. coli* K1 entry into the host cells.

ACKNOWLEDGMENTS

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