Cerebral Cell Adhesion Molecule: A Novel Leukocyte Adhesion Determinant on Blood-Brain Barrier Capillary Endothelium

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The blood-brain barrier (BBB) is comprised of cerebral endothelial cells joined by specialized tight junctions [1, 2]. The combination of intercellular tight junctions and sparse pinocytic activity effectively prevents the majority of bloodborne molecules from reaching the brain parenchyma. However, leukocytes readily traverse the BBB in response to inflammatory stimuli such as infection, suggesting that they can trigger a relaxation of the restrictions on movement across the BBB. The mechanics of trespassing this sealed barrier most certainly involve more complicated steps than leukocyte movement across fenestrated endothelia of the peripheral vasculature, but no specifics for this process have been described.

It is increasingly recognized that bacterial pathogens can traffic through the human body by pathways meant for normal cell targeting, a strategy that aids in evading immune surveillance [3–6]. An example of this phenomenon has been described for the causative agent of whooping cough, *Bordetella pertussis* [7, 8], which uses a two-part strategy to invade leukocytes. The first involves carbohydrate binding by pertussis toxin. Such binding is reminiscent of leukocyte interactions promoted by selectins [9, 10]. The second involves presentation by *B. pertussis* of ligands that bind the CD11b/CD18 integrin [11]. Together, these interactions afford the bacteria a mechanism of phagocytosis into macrophages that is not accompanied by a lethal respiratory burst [12]. The use of both mechanisms to target leukocytes allows *Bordetella* organisms to safely reach an intracellular environment in the pulmonary tract, where they can persist for extended periods. As a result of challenge of a host with bacteria-bearing molecules that mimic or interact with host receptors, antibodies can be generated that cross-react with normal host determinants. Many examples of such postinfectious autoimmune phenomena are known [13], including cross-reactivity with BBB determinants [14]. An understanding of the details of mimicry between host and pathogen can provide unique insights into host cell functions.

Filamentous hemagglutinin (FHA), a large adhesive protein of *B. pertussis* [15], can recognize and bind the CD11b/CD18 signal transduction complex of human leukocytes [1, 16]. For the pathogen, this binding results in entry into leukocytes without invoking an oxidative burst, thereby prolonging bacterial presence in the infected lung [12]. However, a side effect of FHA binding to CD11b/CD18 is disruption of integrin-mediated leukocyte adhesion to endothelial cells [17, 18]. In vitro, FHA or peptides derived from FHA block CD18-dependent leukocyte adherence to cultured endothelial cells. When used in animal models of inflammation, FHA and the peptides prevent migration of leukocytes into the cerebrospinal fluid during bacterial meningitis [18, 19] and reduce brain infarct volume and neutrophil infiltration into the brain during a rat model of cerebral ischemia [20]. This suggests that FHA can competitively interfere with CD18-dependent leukocyte migration to the central nervous system (CNS).

Competitive inhibition of CD18-dependent leukocyte adhesion could arise by a resemblance between FHA and any of several natural ligands of CD11b/CD18. This integrin binds coagulation factor X, C3bi, fibrinogen, intercellular adhesion molecules (ICAMs), and other unknown ligands on vascular endothelium [21–24]. Detailed analysis of domains in FHA has...
revealed regions that resemble and, therefore, competitively block recruitment of coagulation factor X and C3bi to the integrin on the leukocyte surface [17, 25]. However, the mechanism of interference by FHA peptides with CD18-dependent leukocyte adhesion to vascular endothelium remains unclear, since the effect seems to be independent of ICAM-1 [8]. This suggests that CD11b/CD18 might bind additional endothelial surface determinants, one of which resembles FHA.

Evidence in support of a possible resemblance between FHA and an endothelial surface determinant was gathered using a panel of monoclonal antibodies (MAbs) to FHA. Three anti-FHA MAbs bound to cerebral endothelial cells but not peripheral endothelia and resulted in opening of the tight junctions and enhanced BBB permeability [26]. Two of these antibodies, 12.2B11 and 12.6F8, cross-reacted in immune blots with two polypeptides (apparent molecular masses, 52 and 64 kDa) present in human cerebral microvessels but not found in human peripheral vascular endothelial cells [26]. Antibody 12.2B11 bound cultured rabbit cerebral endothelial cells but not aortic cells and, on intravenous administration, localized to cerebral microvessels and attenuated leukocyte recruitment to the CNS in a rabbit model of meningitis [26]. Antibody 12.6F8 also targeted brain microvessels on intravenous injection and reduced clinical symptoms in a rat model of autoimmune encephalomyelitis (O. Rott, J. George, R.M.S., unpublished data). These findings suggest that FHA mimics a ligand on cerebral endothelia and that antibodies to FHA may serve to identify this ligand. Thus, on the basis of the bioactivities of FHA, FHA peptides, and anti-FHA antibodies, the endothelial ligand may be involved in leukocyte trafficking to the brain.

Materials and Methods

Screen for clones reactive with anti-FHA antibodies. To clone cDNAs encoding proteins on human brain microvessels cross-reactive with anti-FHA antibodies, cerebral microvessels were isolated from fresh-frozen human brain (courtesy of Miklos Palkovits, Semmelweis University Medical School, Budapest) as described elsewhere [27, 28]. Poly A+ RNA was prepared (Fast Trak mRNA isolation kit; Invitrogen, San Diego) and was used to construct a λgt11 expression library [29]. The library was screened (picoblue immunoscreening kit; Stratagene, La Jolla, CA) with whole mouse polyclonal antisera that were raised against FHA. The chosen sera bound to cerebral microvessels but to no other cell types or structures on fresh-frozen slices of human brain (not shown). Seventeen positive clones were identified, of which 7 encoded cerebral cell adhesion molecule (CerCAM). Lysogens were made from all [30], and proteins were subjected to immunoblot analysis by transfer to Immobilon membrane (Millipore, Bedford, MA) and incubation at 24°C for 2 h [31] with mouse MAbs 12.6F8 or 12.2B11 (Danish Serum Institute, Copenhagen) or a control antibody of the same IgG1 isotype (Sigma, St. Louis) at 1 μg/mL in PBS with 0.1% Tween. Only the CerCAM clones reacted with the two antibodies. Subsequent screening of the human brain microvessel library was done with DNA probes [32].

Tissue distribution of CerCAM. Northern blots containing human RNA from 16 different tissues (Clontech, Palo Alto, CA) were probed according to the manufacturer’s directions with two 300-bp fragments corresponding to s and 3’ ends of clone 60.

Purification of native CerCAM. Brain capillaries (12 g) were extracted from three fresh-frozen human cortices over a dextran gradient as described elsewhere [27, 28]. The preparation was homogenized at 4°C in the presence of protease inhibitors and centrifuged at 100,000 g. The pellet was solubilized in 0.5% Nonidet with protease inhibitors for 1 h on ice and centrifuged at 100,000 g for 30 min at 4°C. The supernatant fluid was applied to a 12.6F8-bearing affinity column (10 mg of MAb 12.6F8 from Statens Serum Institute, Copenhagen, immobilized on a protein G Sepharose 4 FF MAbTrap G kit, Pharmacia, Stockholm). Protein bound to the column was eluted with 0.1 M glycine-HCl, pH 2.7, 0.5% Nonidet.

Expression of recombinant (r) CerCAM. rCerCAM was expressed in Escherichia coli DH5α by use of the pBLL21/PM11 expression vector bearing a polyhistidine tag. Primers derived from the s and 3’ coding sequences of CerCAM were used to amplify the full-length gene by polymerase chain reaction. The resulting 14-kb fragment was digested with BamHI and ligated into the vector pET14b (Novagen, Madison, WI). The vector was transformed into E. coli DH5α, and recombinant E. coli DH5αpM11 was confirmed by minipreparation and HindIII digestion. To induce CerCAM expression, E. coli DH5αpM11 was grown in the presence of isoprropyl-β-d-thiogalactopyranoside (1 mm) for 3 h. Cells were pelleted and lysed, and the homogenate was solubilized in 0.5% Triton X-100 for 15 min at room temperature. The mixture was centrifuged at 12,000 g, and inclusion bodies were solubilized with 8 M urea. Recombinant protein was purified over a nickel column (Novagen, Madison, WI) and concentrated with a Centricon-10 (Amicon, Beverly, MA) filter. Analysis by silver stain failed to detect any contaminating polypeptides. Polyclonal antibody to rCerCAM was raised in pathogen-free, Bordetella-free rabbits by HRP (Denver, PA) antiserum.

Full-length CerCAM and clone 60 were expressed in COS-7 cells. For transfection, the sequence immediately 5’ to the ATG start of CerCAM was altered to a perfect Kozak consensus sequence [33], and transfection was performed with the pcDNA I/Neo expression system (Invitrogen; 2 μg DNA) incubated with 50 μg of lipofectAMINE according to the manufacturer’s protocol (Life Technologies, Gaithersburg, MD) [34]. To confirm the expression of CerCAM, COS cell membranes were harvested at 72 h, solubilized in 0.1% SDS, and protein in the supernatant was immunoprecipitated by incubation under rotation with 10 μg of 12.6F8 antibody and 100 μL of protein G beads (Pierce Biochemicals, Rockford, IL) at 4°C for 1 h. Beads were washed in 0.5 M NaCl, and the material was analyzed by SDS-PAGE and immune blotting with antibodies 12.2B11 and 12.6F8 and rabbit polyclonal sera raised against rCerCAM.

Leukocyte adherence assay. Leukocyte adherence was assessed for resting and activated leukocytes adherent to Terasaki tissue culture wells coated overnight with rCerCAM, human serum albumin (0.5%), or fibrinogen (1 mg/mL). The albumin served as a negative control (neither resting nor activated cells would adhere well), and fibrinogen served as a positive control (resting cell ad-
Figure 1. Schematic representation of deduced amino acid (aa) sequence of cerebral cell adhesion molecule (CerCAM) with aa residues indicated. Shaded box, region similar to carbohydrate recognition domain of filamentous hemagglutinin (FHA); black box, region similar to bacterial transglycosylases; striped box, similar to far C-terminal region of FHA. Caenorhabditis elegans: region similar to uncharacterized open-reading frame from C. elegans. Vertical bars: predicted transmembrane domains spanning residues 168–184 and 383–401. Inset: top, schematic of 3 cDNA CerCAM clones (aa residues indicated); bottom, immunoblot analysis of clone 21 β-galactosidase fusion protein probed with anti-FHA antibody 12.6F8. Band of expected size of clone 21 fusion protein is seen; no band is seen with λgt11 negative control.

Results

Characterization of CerCAM cDNA. Initial screening of the human brain microvessel library yielded a group of clones sharing a common 3’ terminus. A lysogen of clone 21, the clone with the longest insert and having an in-frame stop codon, expressed a β-galactosidase fusion protein that cross-reacted on immune blots with antibodies 12.2B11 and 12.6F8 (figure 1). Subsequent screening of the human brain microvessel library with DNA probes identified two overlapping clones, 5.1 and 60, the latter of which includes the sequence of clone 21 (figure 1). Together, the two clones contain a 5′ 357-bp region followed by an open-reading frame (ORF) of 1554 bp encoding a predicted polypeptide of 517 amino acids (aa) with a molecular mass of 59,039 Da.

Ninety-nine percent of the CerCAM coding sequence has

herence would remain at baseline, but CD18-dependent adherence by activated cells would be increased [35]). After 3 washes, the wells were blocked with 5% human serum albumin for 1 h at room temperature. Human peripheral blood neutrophils (isolated by neutrophil isolation medium; Cardinals Associates, Santa Fe, NM) were adjusted in PBS without divalent cations to 10^6 cells/mL and were divided into two aliquots. One aliquot was kept on ice as resting cells; the other was activated with phorbol 12,13 dibutyrate (300 ng/mL) for 15 min at 37°C. Resting and activated cells (5 μL/well) were then allowed to adhere to the coated wells for 30 min at 37°C. Wells were washed and fixed with 2.5% glutaraldehyde, and adherent neutrophils were counted visually by phase microscopy (mean ± SD). For some experiments, leukocytes in PBS were preincubated with antibody (50 μg/mL) for 15 min at 37°C; anti-CD18 IB4 (Merck, Rahway, NJ), anti-CD18 MEM48 (BioDesign International, Kennebunk, ME), anti-CD11b D12 (Becton Dickinson, Franklin Lakes, NJ), anti-CD11b BMS104 (Bender MedSystems, Vienna), anti-CD11b 44 (BioDesign), or anti-CD11a (Dako, Carpinteria, CA).

Leukocyte adhesion was also determined to monolayers of COS cells transfected with full-length CerCAM cDNA. Transfected cells and untransfected control cells were incubated in Terasaki wells to confluence. Neutrophils were labeled with DiI according to the manufacturer’s directions (Molecular Probes, Eugene, OR) and activated with FMLP (1 μM) for 15 min at 37°C, and 10^6 cells were added to each well for 15 min at 37°C in 5% CO2. Wells were washed twice and fixed in 1% paraformaldehyde for 10 min in the dark, and adherent neutrophils were visualized by fluorescence microscopy (×20 magnification; Nikon, Osaka, Japan). For some experiments, leukocytes were incubated with anti-CD18 antibody IB4 (50 μg/mL; Merck) 15 min prior to incubation with COS cells.
been identified in the form of uncharacterized human expressed sequence tags (ESTs) from brain and placenta. One of these ESTs is located on chromosome 9 [36], indicating that this gene resides on this chromosome. The untranslated region contains a functional splice acceptor site [36], which raises the possibility that there are unidentified upstream coding exons. The predicted polypeptide lacks an N-terminal signal sequence and contains two strongly predicted transmembrane domains (figure 1). The region of the predicted polypeptide between aa 143 and aa 435 is 36% identical (62% similar) to an uncharacterized predicted polypeptide from Caenorhabditis elegans (cosmid D2045, ORF 9) [37] (figure 1). The region between 284 and 382 is 40% identical to several bacterial transglycosylases, and the contiguous residues 276–284 show 60% identity to a carbohydrate-recognition domain characterized in FHA [38] (figure 1).

Antibodies 12.2B11 and 12.6F8 recognize determinants in the C-terminal 20% of FHA [39]. Despite cross-reactivity of CerCAM and FHA with anti-FHA antibodies, the two predicted protein sequences show little similarity, with only a short sequence (residues 396–404) demonstrating 67% identity to the far C-terminal region of FHA (figure 1). CerCAM, unlike FHA, does not contain a predicted RGD sequence. There are no other stretches of significant homology between FHA and CerCAM.

Distribution of CerCAM. Northern blots containing human RNA from 16 different tissues (Clontech) were probed with two ~700-bp fragments corresponding to the 5′ and 3′ ends of clone 60. The two probes showed a highly expressed, brain-specific transcript of 6.2 kb (figure 2). A transcript of 2.7 kb was found in all tissues but was highly expressed in placenta and ovary tissue.

Functional analysis of CerCAM. Full-length CerCAM and clone 60 were expressed in COS-7 cells and analyzed by immunoprecipitation and immune blotting with antibodies 12.2B11 and 12.6F8 and rabbit polyclonal sera raised against rCerCAM. Transfection with cDNA corresponding to clone 60 yielded a polypeptide that cross-reacted with both 12.2B11 and 12.6F8 (not shown). Cell lysates from COS cells transfected with full-length CerCAM and probed with anti-CerCAM sera showed a single polypeptide at ~52 kDa (figure 3) with no cross-reactive band seen in the COS-conditioned medium or in immunoblots with preimmune serum (not shown). The 52-kDa species comigrated with the smaller of the two anti-FHA- and anti-CerCAM-reactive polypeptides present in cerebral microvessel preparations pooled from three human brains and purified by immunoaffinity chromatography over columns bearing antibody 12.2B11 or 12.6F8 (figure 3). The shared cross-reactivity of both anti-FHA and anti-CerCAM antibodies with two polypeptides from brain tissues suggests that CerCAM may be present in two related forms in cerebral capillaries.

Because the anti-FHA antibodies used to identify CerCAM block leukocyte migration to the CNS in vivo, the ability of CerCAM to support leukocyte adhesion was tested. Transient expression of CerCAM in COS-7 cells conferred the ability to support specific binding of neutrophils (figure 4). Neutrophils also bound in a dose-dependent manner directly to substrates coated with rCerCAM (figure 4). Because FHA can interact with the CD11b/CD18 signal transduction complex, the involvement of this integrin in leukocyte binding to CerCAM was investigated. Binding via CD11b/CD18 is augmented on activation of the leukocyte [40], a phenomenon that can be measured by leukocyte adhesion to the CD11b/CD18 ligand fibrinogen [35]. Resting neutrophils showed a reproducibly modest level of binding to rCerCAM and to COS cell transfectants that was not affected by antibodies to CD11b or CD18.
Figure 4. Neutrophil adherence to cerebral cell adhesion molecule (CerCAM). A, Wells were coated with recombinant (r) CerCAM at indicated concentrations. Resting (●) or activated (■) neutrophil binding was quantitated as cells/well. Adherence of resting and activated cells to albumin was <10 and <40 cells/well, respectively (negative control). Values are mean ± SD of 3 experiments. B, Adherence of resting (shaded bars) or activated (black bars) neutrophils to COS-7 cells (control) or cells transfected with CerCAM cDNA (+CerCAM). Neutrophils were also pre-incubated with anti-CD18 antibody IB4 (+IB4), and mixture was added to CerCAM-transfected cells. Values are mean ± SD of 5 experiments. C, Inhibition of binding of activated (black bars) or resting (shaded bars) neutrophils to rCerCAM-coated surfaces by antibodies to CD11b/CD18. Wells were coated with albumin (negative control), fibrinogen (positive control for increased adherence of activated cells), or rCerCAM (50 μg/mL). Neutrophils were pretreated with no antibody or with anti-CD11 or anti-CD18 antibodies; the mixture was applied to rCerCAM-coated wells. Mean (±SD) values were calculated from >3 experiments of 3 wells/experiment. mAb, monoclonal antibody.

However, there was significant augmentation of leukocyte binding to rCerCAM, to fibrinogen (control), and to CerCAM transfectants upon activation of the neutrophils. This binding was attenuated by anti-CD11b or anti-CD18 antibody (figure 4).

Discussion

Bacteria adapt to the human host to promote the course of infection. For B. pertussis, this process has targeted the CD11b/CD18 integrin as a mechanism to enter intrapulmonary leukocytes and avoid killing. The ligand on B. pertussis that binds this integrin complex is FHA. That binding arises through mimicry of a natural ligand for CD11b/CD18 on endothelia was suggested by cross-reactivity of anti-FHA antibodies with cerebral endothelial cells. This cross-reactivity was limited to cerebral vessels, suggesting that the endothelial ligand may relate to the leukocyte adhesion process for specialized endothelia characterized by tight junctions [26]. It would stand to reason that leukocyte migration across the BBB would require the opening of tight junctions to allow leukocytes to pass between endothelial cells. This opening may require molecules or events in addition to the single event of CD11b/CD18 binding to receptor on the endothelial surface [41]. The demonstrated ability of anti-FHA antibodies that cross-react with CerCAM to also transiently open the BBB to bloodborne markers is consistent with this notion [26]. Further, the ability of the same antibodies or cognate peptides to block leukocyte migration to the brain in vivo supports the relationship between CerCAM and leukocyte trafficking [18–20]. Thus, in the course of understanding the biologic activities of a bacterial adhesive protein, a significant amount of information was obtained on the physiology...
of a novel leukocyte adhesion determinant on human BBB microvessels.

Because CerCAM can support leukocyte adhesion in a manner inhibited by antibodies against CD11b and CD18, it may be involved in leukocyte extravasation in general and possibly in leukocyte interactions with an endothelium characterized by the presence of tight junctions. The predominant endothelial ligands for β2 integrins are ICAM-1 and ICAM-2, both of which are on cerebral and peripheral vessels [42]. A neuron-specific adhesion ligand for CD11a/CD18, telencephalon, has been described but is not present on endothelial cells [43]. The ICAMs and telencephalon are members of the immunoglobulin superfamily, a feature not shared by CerCAM. The presence of a brain-specific transcript for CerCAM suggests that there may be a form of CerCAM specific to brain endothelial cells, a conclusion consistent with the brain-specific in vivo binding of anti-FHA antibodies. A transcript of 2.7 kb was found in all tissues but was highly expressed in placenta and ovary, sites in the periphery also characterized by endothelia forming tight junctions. Antibody 12.6F8 recognizes a determinant on rat ovary microvessels (J. George, R.M.S., unpublished data). The two transcripts in the brain may reflect differential splicing to produce the two cross-reactive proteins of 52 and 64 kDa seen on immune blots of brain microvessels probed with antibodies 12.2B11 and 12.6F8. Alternatively, one or the other transcript may encode both proteins.

Given the potent functional relationship between FHA and CerCAM, it might be expected that the CerCAM sequence would resemble that of FHA. This was not found; however, it is possible that both FHA and CerCAM possess lectin-like activity. A carbohydrate recognition domain has been characterized in FHA [38], a feature that contributes to the ability of FHA to bridge the bacteria–human cell surface glycocompounds on cells not bearing β2 integrins, such as ciliated lung cells. This region of FHA is not represented in the CerCAM sequence (except for a 10-aa region of near identity). However, a more general similarity of a 100-aa region of CerCAM (region 284–382) to several bacterial transglycosylases suggests potential interactions between CerCAM and carbohydrates. The particularly high similarity to transglycosylases synthesizing lacto-N-neotetraose and the ability of FHA to recognize lactosamine [38] implicate lactose-containing moieties, common components of blood group determinants, as possible targets. A relationship between any potential lectin activity for CerCAM and its ability to bind CD11b/CD18 remains to be elucidated. However, recognition of more than one element of the β2 integrin complex is a precedent already described for many ligands, including FHA [16]. Alternatively, a lectin type of interaction may explain the ability of CerCAM to support some adherence of resting leukocytes in a non–CD11b/CD18–mediated interaction.

The biologic importance of a determinant that characterizes endothelia formed by tight junctions can be appreciated from several points of view. Such a determinant would serve as a marker for cerebral and related endothelia, perhaps allowing the differential targeting of leukocytes to brain or, conversely, restricting the trafficking of activated leukocytes to the periphery in non-CNS inflammatory states. The ability to pharmacologically modulate leukocyte trafficking to the CNS has improved the outcome of bacterial meningitis in humans and in animal models [44–46]. However, this approach has suffered from the lack of ability to narrow the antiinflammatory effects to the CNS, an advantage held by targeting CerCAM. The potential function of CerCAM in BBB permeability may be useful in designing delivery of brain-active pharmacologic agents to the CNS. The ability of anti-FHA antibodies to enhance the delivery of antibiotics and radiopaque imaging agents across the restrictive BBB is an example of this application [26].

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