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Intracellular Domain of Brain Endothelial Intercellular Adhesion Molecule-1 Is Essential for T Lymphocyte-Mediated Signaling and Migration

John Greenwood,* Claire L. Amos,* Claire E. Walters,* Pierre-Olivier Couraud,† Ruth Lyck,‡ Britta Engelhardt,§§ and Peter Adamson²*

To examine the role of the ICAM-1 C-terminal domain in transendothelial T lymphocyte migration and ICAM-1-mediated signal transduction, mutant human (h)ICAM-1 molecules were expressed in rat brain microvascular endothelial cells. The expression of wild-type hICAM-1 resulted in a significant increase over basal levels in both adhesion and transendothelial migration of T lymphocytes. Endothelial cells (EC) expressing ICAM-1 in which the tyrosine residue at codon 512 was substituted with phenylalanine (hICAM-1Y512F) also exhibited increased lymphocyte migration, albeit less than that with wild-type hICAM-1. Conversely, the expression of truncated hICAM-1 proteins, in which either the intracellular domain was deleted (hICAM-1ΔC) or both the intracellular and transmembrane domains were deleted through construction of a GPI anchor (GPI-hICAM-1), did not result in an increase in lymphocyte adhesion, and their ability to increase transendothelial migration was attenuated. Truncated hICAM-1 proteins were also unable to induce ICAM-1-mediated Rho GTPase activation. EC treated with cell-permeant penetratin-ICAM-1 peptides comprising human or rat ICAM-1 intracellular domain sequences inhibited transendothelial lymphocyte migration, but not adhesion. Peptides containing a phosphotyrosine residue were equipotent in inhibiting lymphocyte migration. These data demonstrate that the intracellular domain of ICAM-1 is essential for transendothelial migration of lymphocytes, and that peptidomimetics of the ICAM-1 intracellular domain can also inhibit this process. Such competitive inhibition of transendothelial lymphocyte migration in the absence of an effect on adhesion further implicates ICAM-1-mediated signaling events in the facilitation of T lymphocyte migration across brain EC. Thus, agents that mimic the ICAM-1 intracellular domain may be attractive targets for novel anti-inflammatory therapeutics. The Journal of Immunology, 2003, 171: 2099–2108.

E ndothelial cells (EC)⁰ of the blood-CNS barriers display characteristics that differentiate them from endothelium of other organs. In particular, these cells express well-developed intercellular tight junctions and are responsible for exceptionally low paracellular permeability. The migration of leukocytes across the specialized vascular barriers of the CNS also differs from that observed at other vascular beds and is dependent on a variety of controlling factors (1–3). One of these factors is provided by the endothelium, which facilitates the passage of migratory cells across the vessel wall through the expression of cell adhesion molecules. However, these molecules do not appear to be simply points of attachment, but are involved in transducing leukocyte adhesion-mediated signaling responses to EC (4–6).

The activation state of a circulating T lymphocyte is important in determining its ability to migrate out of the circulation (3, 7–10). Nevertheless, using functional assays we have shown that brain EC are intimately and actively involved in facilitating lymphocyte diapedesis (5). It has previously been demonstrated that the expression of the adhesion molecule ICAM-1 on EC is pivotal in supporting lymphocyte migration across vascular endothelium in both peripheral tissue (8, 9) and the CNS (2, 3, 6, 11). We have shown that following ICAM-1 cross-linking or coculture with T lymphocytes, brain EC ICAM-1 is capable of evoking signal transduction events, suggesting that EC actively respond to leukocyte adhesion (4, 5, 12). Mimicking leukocyte attachment through Ab cross-linking of EC ICAM-1 molecules in vitro, we have reported the induction of EC actin stress fibers (5) and an enhanced tyrosine phosphorylation of cortactin (12), focal adhesion kinase (FAK), paxillin, and p130Cas (4). Both ICAM-1 cross-linking and coculture of EC with T lymphocytes result in increased levels of GDP-loaded EC Rho proteins, indicating that EC Rho proteins are also involved in ICAM-1-mediated signaling events (5).

Transendothelial lymphocyte migration is also inhibited following inactivation of EC Rho protein function (5, 13). How ICAM-1 elicits these signals remains unclear. ICAM-1 has no catalytic activity associated with its short cytoplasmic domain, but despite this, it is known to bind a variety of molecules such as α-actinin (14), β-tubulin, GAPDH (15), and ezrin (16, 17). These proteins all have the potential to perform signaling functions. In addition, it has been described that ICAM-1 can interact with filamentous actin (F-actin) (18), and that the avidity of this interaction increases following ICAM-1 cross-linking (19).
The cytoplasmic domain of ICAM-1 consists of 27 aa in rat and 28 aa in human. A striking feature of this sequence is the presence of a high number of positively charged amino acid residues that appear to be important for ICAM-1 to bind ezrin (20), a property shared with other ezrin-binding adhesion molecules such as CD44 (20). It is also interesting to note that the tyrosine residue within the cytoplasmic domain at position 512 in hICAM-1 is conserved in both rat and mouse ICAM-1 and is also conserved in the shorter cytoplasmic domain of VCAM-1. It has been demonstrated that ICAM-1 can be phosphorylated on the cytoplasmic tyrosine residue in response to the binding of immobilized fibrogen to ICAM-1 (21) and that this results in the recruitment of the tyrosine phosphatase Src homology protein tyrosine phosphatase-2.

To define the role of the ICAM-1 cytoplasmic domain in EC signaling and control of transendothelial lymphocyte migration within the CNS, we have expressed mutant hICAM-1 molecules in rat brain EC and used competitive intracellular peptides mimicking the ICAM-1 cytoplasmic domain. We report here that the presence of the cytoplasmic domain of ICAM-1 is essential for transendothelial migration of T lymphocytes as well as ICAM-1-mediated activation of Rho. Thus, deletion of the cytoplasmic domain of ICAM-1 results in inhibition of lymphocyte adhesion and migration, whereas treatment with competitive peptides resulted only in a reduction in migration.

Materials and Methods

Materials

Mouse mAbs to rICAM-1 (1A29 and 3H8) were generated by hybridomas provided by Dr. M. Miyasaka (Osaka University, Osaka, Japan) and Dr. W. Hickey (Dartmouth Medical School, Hanover, NH). Mouse mAbs to hICAM-1 (clone BAA4) were obtained from R&D Systems (Oxford, U.K.). Rabbit anti-mouse Abs (RAM) were obtained from Sigma-Aldrich (Poole, U.K.). Mouse anti-phosphotyrosine mAb clone 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY), and clone PY20 was obtained from Autogenbioclear (Calne, U.K.). Mouse anti-rat FAK was obtained from Dr. J. Pearson, Kings College, London, U.K.). RNA was extracted from brain EC (1A29) by guanidinium thiocyanate and rendered using CellQuest software. A secondary isotype-matched IgG control sample for each cell line was also acquired.

Endothelial cells

The immortalized Lewis rat brain microvascular EC line GP8/3.9 (22) was maintained in Ham’s F-10 medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. GP8/3.9 cells transfected with RSVpuro hICAM-1 constructs were maintained in GP8/3.9 medium containing puromycin (20 µg/ml).

cDNA constructs

Wild-type (WT) hICAM-1 and mutant hICAM-1 molecules were generated using RT-PCR from CHO cells overexpressing hICAM-1 (a gift from Dr. J. Pearson, Kings College, London, U.K.). RNA was extracted from ICAM-1-expressing CHO cells using an RNasea kit (Qiagen, West Sussex, U.K.) according to the manufacturer’s instructions. Reverse transcriptase was used to generate cDNA from oligo(dT)15-18 primed RNA, which was used as a template for PCR. PCR reactions were performed using the following forward primer 5'-ggaggcaataagttgctggaacccg-3' and 5'-ggaggcaataagttgctggaacccg-3', and 5'-ggaggcaataagttgctggaacccg-3', to generate WT, C-terminal truncation at aa 504, and Tyr121-Phe112 point mutations, respectively. HindIII and SalI sites, which were engineered into the forward and reverse PCR primers, respectively, were used to clone the PCR fragment into a HindIII/XhoI-restricted pcDNA3/RSVpuro plasmid. The identity of the PCR-generated ICAM-1 inserts was confirmed by DNA sequencing. A GP-anchored hICAM-1 construct (pcDM8 GP1-ICAM-1) in which both the C-terminal domain and the transmembrane domain are deleted by truncating ICAM-1 at codon 480 and fusing this sequence to the GPI anchor sequence of human LFA3 (14) was a gift from T. Springer (Center for Blood Research, Harvard Medical School, Boston, MA). The GPI-ICAM-1 sequence was excised from pcDM8 using HindIII and XbaI and was directionally cloned into HindIII/XbaI-digested RSVpuro.

Penetratin-ICAM-1 peptides

Penetratin peptides were N-terminally biotinylated and consisted of 16 residues of the penetratin sequence (RQIKIWFQNRRMKWKK). The 13 C-terminal amino acids of human (h)- or rICAM-1 were synthesized distal to the terminal penetratin sequence. Peptides were HPLC-purified before use. The sequence used were hICAM-1 (QRKKYKXSLQ-YP-RFLQQQAQ), rICAM-1 (QRKKYQKLKQAQ-YP-rICAM-1 (QRKKIRYP)-KLQKAQ), and an irrelevant sequence from the soluble part of rat rod opsin (CYPKSNFRGGENH). Bold type indicates phosphorylated residue. Penetratin peptides were localized in cells using streptavidinFITC (1/50; Jackson Immunoresearch Laboratories) following fixation in 3.7% paraformaldehyde and permeabilization with 0.2% Triton X-100.

Flow cytometric analysis of ICAM-1 transfectants

Puromycin-resistant GP8/3.9 brain EC clones were transfected and assessed by flow cytometric analysis using a human-specific anti-ICAM-1 Ab (clone BBA4) to demonstrate the presence of hICAM-1 expression. After detachment with collagenase (1 mg/ml) for 20 min, cells were washed and incubated with the BBA4 mAb (10 µg/ml) for 1 h on ice. After washing, cell pellets were resuspended in 100 µl of FITC-conjugated goat anti-mouse IgG (1/100 dilution) and incubated for a further 30 min before standard paraformaldehyde fixation and flow cytometric analysis. Data were quantified and rendered using CellQuest software. A secondary isotype-matched IgG control sample for each cell line was also acquired.

Metabolic labeling

GP8/3.9 brain EC (5 × 10^5) were incubated in phosphate-free DMEM overnight in the presence of 0.2 mCi/ml of [32P]orthophosphate. Cells were washed in HBSS and were subsequently cocultured with Con A-stimulated lymphocytes (10^5) or were cross-linked with anti-ICAM-1 mAb in complete DMEM.

Immunoprecipitation of ICAM-1

Brain EC (1 × 10^6) were washed with ice-cold HBSS containing 1 mM Na3VO4 before lysis in buffer containing 20 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 3 mM MgCl2, 100 mM NaCl, 1 mM EGTA, 50 mM β-glycerophosphate, 1 mM Na3Vo4, 0.5 mM PMSF, 1 mM NaF, 10 µg/ml leupeptin, and 10 µg/ml aprotonin for 30 min at 4°C. Cell nuclei were pelleted by centrifugation and discarded. Lysates (0.1–0.5 mg of protein) were incubated with specific Abs (1/100) for 2 h at 4°C with end-over-end rotation. Immune complexes were captured by incubation with protein G-agarose for an additional 2 h at 4°C before extensive washing in lysis buffer. The washed immune complexes were eluted with SDS sample buffer (100 µl of 1×SDS-HESP (pH 6.8), 4% SDS, 20% glycerol, 1 mM orthovonadate, and 100 mM DTT with bromophenol blue) and heated to 95°C for 5 min, and proteins were resolved on SDS-PAGE.

Western blotting

Immunoprecipitated samples or cell lysates were heated in SDS sample buffer and subjected to SDS-PAGE before transfer to nitrocellulose membranes (Schleicher & Schuell, London, U.K.) using a semidry blotter (BioRad, Hernel Hempstead, U.K.) at 10 V (3.53 mACm²) for 30 min. The membranes were blocked in 5% fat-free milk in PBS for 2 h before incubation overnight at 4°C with 0.1% dilution of 388 hybridoma supernatant, 1 µg/ml of 1A29, or BBA4 at an appropriate dilution in PBS containing 5% BSA. Membranes were washed several times with PBS/0.1% Tween 20 before a 1-h incubation with an anti-mouse HRP-conjugated IgG at a dilution of 1/15,000 (Pierce). After several washes in PBS/0.1% Tween 20, blots were developed using the ECL system (Amersham International) according to the manufacturer’s instructions and were exposed to x-ray film.
Dot-blot analysis of penetratin-hICAM-peptides

Biotinylated peptides (100 μg/ml) were incubated with GP8/3.9 cells expressing WT-hICAM-1 for 2 h, and cells were subsequently lysed in buffer containing 20 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 3 mM MgCl₂, 100 mM NaCl, 1 mM EGTA, 50 mM β-glycerophosphate, 1 mM Na₂VO₃, 0.5 mM PMSF, 1 mM NaF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin for 30 min at 4°C. Incubated with 20 μl of streptavidin-Sepharose, and washed three times in the same buffer. Samples were subsequently boiled for 5 min and dot-blotted on nitrocellulose membranes before immunoblotted with anti-phyosphoryrosine (4G10) mAb.

Adhesion of peripheral lymph node lymphocytes to EC and T lymphocyte transendothelial migration

Adhesion assays and transendothelial migration assays were conducted as previously described using cells harvested from Lewis rat peripheral lymph nodes and myelin basic protein (MBP) Ag-specific T lymphocyte lines (2, 3, 10). The results are expressed as the mean ± SEM, and significant differences between groups were determined by Student’s t test.

Fluorescence microscopic analysis of biotinylated ICAM-1 peptides and F-actin localization

Untransfected brain EC were treated with 100 U/ml rat rIFN-γ for 48 h to up-regulate endogenous ICAM-1 expression. Cells were fixed with 3% paraformaldehyde for 20 min at room temperature, washed, and permeabilized with 0.25% Triton X-100, followed by blocking with 10% FCS in PBS. Streptavidin-Texas Red (1/100; Jackson Immunoresearch Laboratories) was used to localize C-terminal ICAM-1-biotinylated peptides or 0.1 μg/ml Oregon Green phalloidin (Molecular Probes, Eugene, OR) used to visualize F-actin. Cells were viewed on a laser scanning confocal microscope (Leica, Rockleigh, NJ) or Zeiss (New York, NY).

Rho activation

GST-rhotekin was expressed from pGEX-2T-rhotekin in Escherichia coli for 5 h using 1 mM isopropyl-β-D-galactopyranoside (Life Technologies/BRL, Paisley, U.K.) and was used to precipitate activated Rho as previously described (23, 24). Rho proteins were labeled by ADP-ribosylation with BRL, Paisley, U.K.) and was used to precipitate activated Rho as previously described (23, 24). Rho proteins were labeled by ADP-ribosylation with BRL, Paisley, U.K.) and was used to precipitate activated Rho as previously described (23, 24). Rho proteins were labeled by ADP-ribosylation with BRL, Paisley, U.K.) and was used to precipitate activated Rho as previously described (23, 24). Rho proteins were labeled by ADP-ribosylation with BRL, Paisley, U.K.) and was used to precipitate activated Rho as previously described (23, 24). Rho proteins were labeled by ADP-ribosylation with BRL, Paisley, U.K.) and was used to precipitate activated Rho as previously described (23, 24).

Analysis of ICAM-1 expression on GP8/3.9 brain EC

To evaluate whether manipulation of the EC lines resulted in alteration of ICAM expression, the surface expression of ICAM-1 and ICAM-2 was evaluated using ELISA as previously described (22).

Results

Coculture of T lymphocytes with brain EC does not result in phosphorylation of ICAM-1

The rat brain microvascular EC line, GP8/3.9, was prelabeled with [32P]phosphate and cocultured with Con A-stimulated peripheral lymph node lymphocytes (A) or cross-linked with anti-rICAM-1, followed by RAM (B). EC were washed, and ICAM-1 or FAK was immunoprecipitated from EC lysates using anti-rICAM-1 (1A29) or anti-FAK. ICAM-1 immunoprecipitates from 32P-labeled cells were resolved on 10% SDS-PAGE and exposed to autoradiographic film or immunoblotted with anti-phosphoryrosine mAb (4G10). Membranes were subsequently stripped and reprobed for rICAM-1 using 1A29 mAb. FAK immunoprecipitates (C) were immunoblotted with anti-phosphoryrosine mAb (4G10).

Expression of hICAM-1 in rat brain EC

To determine whether the intracellular domain, membrane-spanning domain, or C-terminal tyrosine residue is important in mediating EC signaling events induced by ICAM-1 and in supporting transendothelial migration of T lymphocytes, a series of hICAM-1 molecules was constructed. Human ICAM-1 molecules were generated that 1) carried a C-terminal truncation in which the intracellular domain was removed (hICAM-1ΔC), 2) consisted of a C-terminal truncation at the membrane-spanning domain, which was fused to the GPI anchor of LFA3 (GPI-hICAM-1), 3) a C-terminal domain carrying a single phenylalanine amino acid substitution at the conserved tyrosine residue codon 512 (hICAM-1Y512F), and 4) WT hICAM-1 (WT-hICAM-1; Fig. 2). Each cDNA was cloned within the pRSVpuro expression vector and was used for the transfection of GP8/3.9 rat brain EC.

Transfected brain EC colonies were selected following growth in medium containing 5 μg/ml of puromycin. Clonal cell lines were generated and evaluated for the expression of hICAM-1 by flow cytometry. Cell clones were selected expressing each of the human mutant ICAM-1 molecules at approximately equivalent levels similar or exceeding endogenous rICAM-1 expressed on GP8/3.9 cells following treatment with IFN-γ for 48 h (Fig. 3), apart from the GPI-

FIGURE 1. Endogenous endothelial rICAM-1 is not post-phosphorylated following coculture with T lymphocytes or cross-linking of endothelial ICAM-1. Unlabeled or 32P-labeled rat brain EC were either cocultured for various times with Con A-stimulated peripheral lymph node lymphocytes (A) or cross-linked with anti-rICAM-1, followed by RAM (B). EC were washed, and ICAM-1 or FAK was immunoprecipitated from EC lysates using anti-rICAM-1 (1A29) or anti-FAK. ICAM-1 immunoprecipitates from 32P-labeled cells were resolved on 10% SDS-PAGE and exposed to autoradiographic film or immunoblotted with anti-phosphoryrosine mAb (4G10). Membranes were subsequently stripped and reprobed for rICAM-1 using 1A29 mAb. FAK immunoprecipitates (C) were immunoblotted with anti-phosphoryrosine mAb (4G10).
Transendothelial migration of T lymphocytes was significantly augmented by hICAM-1. This observation was due to the transendothelial migration of T lymphocytes through hICAM-1, which resulted in a significant decrease in the basal adhesion of lymphocytes to the EC. Furthermore, the expression of hICAM-1 resulted in a decrease in the basal adhesion of lymphocytes to the EC, when compared to that observed in control EC or EC expressing RSV-puro control (p < 0.001 for both). Indeed, both mutant ICAM-1 proteins resulted in a decrease in the basal adhesion of lymphocytes to EC.
lymphocytes to rat brain EC expressing RSVpuro alone (85.0/11006 4.3% (p/11021 0.001; n/11005 32) and 73.8/11006 6.8% (p/11021 0.001; n/11005 32), respectively). Thus, hICAM-1 proteins lacking the intracellular domain are unable to mediate efficient adhesion of lymphocytes or their subsequent transendothelial migration. Although the tyrosine substitution mutant reduced the ability to mediate these events, the effects are not as striking as those carrying C-terminal truncations.

Cell-permeant peptides comprising the C-terminal domain of ICAM-1 attenuate transendothelial migration of lymphocytes, but not adhesion

Next, we determined whether ICAM-1-mediated support of transendothelial lymphocyte migration could be inhibited with cell-permeant peptide mimicking the intracellular domain of ICAM-1. Peptides comprising the membrane-proximal part of the rat or hICAM-1 sequence within the intracellular domain were used to antagonize the binding potential of ICAM-1-binding/signaling partners. Such ICAM-1 C-terminal sequences were fused to the GPI linker of LFA3 (GPI-hICAM-1). Data are expressed as the mean ± SEM percentage of the control for a minimum of three independent experiments (n = 6/experiment). Significant differences were determined by Student’s t test. Compared with control (RSVpuro vector): *, p < 0.01; **, p < 0.001. Compared with WT-hICAM-1: †, p < 0.02; ††, p < 0.001 (by Student’s t test).
the phosphopeptide (YP-rICAM-1) was also effective in significantly inhibiting transendothelial migration of T lymphocytes to 50 ± 4.6% of the control value \((p < 0.0001; n = 29; \text{Fig. 6A})\).

Significantly, both the phosphopeptide and the nonphosphorylated peptide were equally effective. However, treatment of untransfected brain EC with these peptides did not reduce lymphocyte adhesion, which was contrary to our findings with EC expressing hICAM-1 lacking the intracellular domain. Thus, treatment with either the rICAM-1 peptide or the YP-rICAM-1 peptide resulted in adhesion values 96.1 ± 1.8% \((n = 32)\) and 100.3 ± 2.2% \((n = 32)\) of control values, respectively \((\text{Fig. 6B})\). None of the rat penetratin-ICAM-1 peptides affected either the basal or IFN-γ-induced cell surface expression of ICAM-1, as determined by ELISA (data not shown).

When identical experiments were conducted in untransfected rat brain EC using penetratin ICAM-1 peptides comprising an identical region of the hICAM-1 molecule, both phosphorylated and nonphosphorylated human peptides were also effective in inhibiting transendothelial lymphocyte migration. Incubation of EC with hICAM-1 (QRKIKK(Y/P)RLQQAQ) or YP-hICAM-1 (QRKIKK(Y/P)RLQQAQ) peptides caused a reduction of transendothelial migration to 79.9 ± 3.8% \((p < 0.01; n = 27)\) and 53.9 ± 4.2% \((p < 0.001; n = 28)\) of control values respectively \((\text{Fig. 6C})\). In a similar fashion to the penetratin rICAM-1 peptides, hICAM-1 peptides were without effect on lymphocyte adhesion \((109.0 ± 1.1\% \ (n = 32)\) and 99.5 ± 2.4\% \((n = 32)\) for hICAM-1 and YP-hICAM-1, respectively \((\text{Fig. 6D})\). An irrelevant control peptide sequence corresponding to the soluble part of rat rod opsin (CKPMSNRFGENH) had no significant effect on either lymphocyte adhesion or transendothelial migration. N-terminal biotinylation of penetratin peptides was employed to evaluate the entry of penetratin peptides into rat brain EC. Following fixation and visualization of biotin peptides with streptavidin-TRITC, it was confirmed that in all cases there was efficient uptake of peptide. Thus, treatment with intracellular peptides comprising a sequence from the intracellular domain of either rat or human ICAM-1 appears to be effective in inhibiting transendothelial lymphocyte migration through rat brain EC without affecting lymphocyte adhesion.

**Human ICAM-1 intracellular domain peptides abolish the enhanced transendothelial migration of lymphocytes mediated through ectopic expression of hICAM-1 in rat brain EC without affecting lymphocyte adhesion**

When penetratin peptides containing the hICAM-1 sequence were used to treat rat brain EC expressing WT-hICAM-1, both nonphosphorylated and tyrosyl-phosphopeptides were able to abolish the increase in transendothelial lymphocyte migration associated with the ectopic expression of WT-hICAM-1. Lymphocyte migration was reduced to 56.3 ± 5.7\% \((p < 0.001\text{ vs cells expressing WT-hICAM-1; } n = 22\) and 57.5 ± 8.2\% \((p < 0.001\text{ vs cells expressing WT-hICAM-1; } n = 32\) following treatment with hICAM-1 C-terminal peptide and YP-hICAM-1 peptide, respectively \((\text{Fig. 7A})\). It is noteworthy that both peptides were effective in reducing migration to values significantly below that observed with control rat brain EC \((p < 0.001\text{ vs control brain EC})\), which is consistent with the effects of these peptides on EC expressing only endogenous rICAM-1 \((\text{Fig. 6C})\). Rat brain EC expressing WT-hICAM-1 treated with penetratin peptides containing the hICAM-1 sequence were unable to significantly reduce

**FIGURE 6.** Lymphocyte adhesion to and migration through rat brain EC monolayers in the presence of human and rat penetratin-ICAM-1 peptides. Migration of Ag-specific (MBP) T cells through \((A\text{ and } C)\) or adhesion of \(^{3}H\)-labeled activated rat peripheral lymph node lymphocytes to \((B\text{ and } D)\) control rat brain EC monolayers or EC pretreated with rat \((A\text{ and } B)\) or human \((C\text{ and } D)\) penetratin peptides. Penetratin peptide containing the C-terminal 16 aa of rICAM-1, penetratin peptide containing the C-terminal 16 aa of rICAM-1 in which the tyrosine residue is phosphorylated (YP-rICAM-1), penetratin peptide containing the C-terminal 16 aa of hICAM-1, penetratin peptide containing the C-terminal 16 aa of hICAM-1 in which the tyrosine residue is phosphorylated (YP-hICAM-1), and penetratin peptide containing 16 aa or rat opsin (irrelevant) are shown. Observations are from a minimum of three independent experiments \((n = 6/experiment)\). Data are expressed as the mean ± SEM percentage of the control of a minimum of three independent experiments \((n > 6/experiment)\). Significant differences compared with control were determined by Student’s \(t\) test. * \(p < 0.01; ** \(p < 0.001; *** \(p < 0.0001\).
lymphocyte adhesion, which again is consistent with those experiments conducted on rat brain EC expressing only endogenous ICAM-1 (Fig. 7B). Thus, adhesion of lymphocytes to brain EC expressing WT-hICAM-1 was 299.0 ± 12.4%, and following treatment of EC with hICAM-1 and YP-hICAM-1 peptides it was 278.0 ± 11.5% (n = 32; p = NS) and 251.3 ± 14.9% (n = 32; p = NS), respectively. To determine that penetratin-hICAM-1 peptides did not undergo phosphorylation or dephosphorylation, both penetratin-hICAM-1 and the corresponding phosphopeptide were incubated with GP8/3.9 cells expressing WT-hICAM-1. Isolation of penetratin peptides from cell lysates, RAM-treated cells, or cells that had been cross-linked with ICAM-1 mAb, followed by anti-phosphotyrosine immunoblotting, demonstrated that these peptides did not show altered phosphorylation status (Fig. 7C).

**The intracellular domain of ICAM-1 is essential for ICAM-1-mediated signal transduction**

Cross-linking of WT-hICAM-1 expressed in rat brain EC with a specific anti-hICAM-1 mAb resulted in activation of endothelial Rho proteins as previously described for endogenous ICAM-1 (5). The expression of hICAM-1 carrying the tyrosine to phenylalanine substitution at codon 512 (hICAM-1, Y512F) was also capable of inducing Rho activation. However, cross-linking of either hICAM-1ΔC or GPI-hICAM-1 failed to induce Rho activation (Fig. 8A). In contrast to this, preincubation of brain EC expressing WT-hICAM-1 with penetratin-hICAM-1 peptides did not result in the inhibition of hICAM-1-stimulated Rho activation (Fig. 8B). These findings were supported by the observation that treatment of cells with these peptides also failed to suppress ICAM-1-stimulated stress fiber formation (Fig. 8C, a–c and d–f). It was noted, however, that the hICAM-1 C-terminal domain peptides showed significant colocalization with F-actin stress fibers, which was not the case for the irrelevant penetratin peptide (Fig. 8C, g–i). When the concentration of penetratin hICAM-1 peptides was increased following cytosolic microinjection of cells, both the hICAM-1 peptide (Fig. 8D, d and e) and the corresponding phosphopeptide (Fig. 8D, a–c), but not an irrelevant sequence (Fig. 8D, f), were able to inhibit ICAM-1-stimulated stress fiber formation.

**Expression hICAM-1 in rat brain EC leads to formation of human-rat heterodimers**

Brain EC monolayers were treated with various concentrations of the reduction sensitive chemical cross-linker DTSSP, and lysates from EC expressing WT-hICAM-1 or hICAM-1ΔC were analyzed by Western blot analysis following resolution of proteins on non-reducing SDS-PAGE. As the concentration of DTSSP was increased, proteins of larger molecular mass were detected following immunoblotting with anti-hICAM-1. These higher molecular mass immunoreactive proteins suggest the presence of ICAM-1 dimers, given the apparent molecular mass of ~180 kDa (Fig. 9A). Higher molecular mass bands were not present when proteins were resolved by reducing SDS-PAGE (data not shown). Endogenous rICAM-1 immunoprecipitated from rat brain EC cells expressing WT-hICAM-1 with the specific anti-rICAM-1 mAb 1A29 was subsequently immunoblotted with anti-hICAM-1 mAb (BBA4). Positive bands demonstrated that hICAM-1 was effectively co-immunoprecipitated with endogenous rICAM-1 (Fig. 9B), since anti-ICAM-1 mAb is species specific (Fig. 9D). Indeed, small differences in molecular mass in the hICAM-1 molecules coimmunoprecipitated with rICAM-1 were observed in cells expressing WT-hICAM-1 or hICAM-1ΔC. This demonstrates that human and...
ICAM-1 can exist as heterodimers. In keeping with this suggestion, immunoprecipitation of hICAM-1 from WT-hICAM-1-expressing cells was found to coprecipitate endogenous ICAM-1 (Fig. 9C).

Discussion

It has previously been shown that ICAM-1 is required for efficient adhesion and migration of lymphocytes across monolayers of both peripheral (8, 9) and CNS EC (1–3, 11). Studies from our laboratories and those of others have shown that ICAM-1 plays a pivotal role in transducing signals in EC following adhesion of lymphocytes, an essential step in the EC signal cascade responsible for facilitating lymphocyte transendothelial migration. The precise mechanism by which this EC ICAM-1-mediated signaling occurs has yet to be fully defined, but is known to be dependent on both an intact endothelial actin cytoskeleton and functional endothelial and F-actin and biotinylated peptides were detected by direct immunofluorescence. a, Penetratin-hICAM-1; b, F-actin; c, merge of a and b; d, penetratin-YP-hICAM-1; e, F-actin; f, merge of a and e; g, penetratin-opsin; h, F-actin; i, merge of g and h. Scale bars = 15 μm. D, Brain EC lysate immunoblotted with anti-hICAM-1 mAb (BBA4) demonstrating no cross-reactivity with rICAM-1.
Rho proteins (4, 5, 13). ICAM-1 is found to associate with F-actin (18), possibly through an intermediate actin-binding protein such as α-actinin (14), and shows increased avidity following cross-linking of ICAM-1 or coculture of EC with T lymphocytes (19). Although it is clear that both the endothelial actin cytoskeleton and activation of Rho proteins are important, it is not known how the ICAM-1 molecule itself mediates intracellular signaling responses in EC, since the intracellular domain of ICAM-1 has no obvious catalytic activity.

Ectopic expression of human WT ICAM-1 in the rat brain EC line GAP3/3.9 mediated an increase in both the adhesion and subsequent migration of rat lymphocytes across the endothelial monolayer. The inability of either C-terminally truncated hICAM-1 or a glycoporpholin-anchored hICAM-1 protein to induce a similar enhancement of lymphocyte adhesion or transendothelial migration strongly implies that the intracellular domain is a vital upstream component of the signaling cascade initiated through the adhesion of lymphocytes, which subsequently controls lymphocyte transendothelial migration. In keeping with this suggestion, ICAM-1 proteins lacking the intracellular domain are incapable of activating Rho proteins following ICAM-1 cross-linking. These observations further support previous findings that efficient transendothelial migration of lymphocytes is dependent on ICAM-1-mediated Rho signaling in EC (5). These data are consistent with a report showing that transmigration of polymorphonuclear cells across monolayers of ICAM-1-transfected CHO cells was also abolished following deletion of the ICAM-1 intracellular domain (27). However, contrary to our data, leukocyte adhesion was not affected by cells expressing the truncated ICAM-1 protein (27). Studies in mouse ICAM-1-deficient EC that have been retransfected with WT mouse ICAM-1 or C-terminally truncated ICAM-1 also show that both these molecules are able to mediate the adhesion of lymphocytes to EC, but truncated ICAM-1 is unable to mediate transendothelial migration (unpublished observations). Why hICAM-1 constructs lacking the intracellular domain, when expressed in rat brain EC, are capable of reducing adhesion of lymphocytes to levels below those observed in untransfected EC is currently unknown. This may reflect the fact that these mutant ICAM-1 proteins may no longer be able to associate with the actin cytoskeleton. Indeed, treatment of EC with cytochalasin D, which inhibits lymphocyte transendothelial migration, does not result in reduced lymphocyte adhesion (5). It is interesting to note that under these conditions, ICAM-1 is still able to associate with polymerized actin (28). Alternatively, ICAM-1 molecules are known to exist as dimers (29), and the formation of cross-species ICAM-1 heterodimers in which one ICAM-1 molecule is C-terminally truncated may prevent efficient clustering of ICAM-1 or may result in a change in the conformation of ICAM-1 dimers, thus reducing lymphocyte adhesion. Such heterodimers would not have occurred in other studies employing ICAM-1-transfected CHO cells or ICAM-1-deficient mice EC retransfected with mouse ICAM-1.

Expression of ICAM-1 containing a tyrosine to phenylalanine substitution at codon 512, which is a highly conserved residue in ICAM-1 molecules from different species, also resulted in a significant enhancement of lymphocyte adhesion and migration, albeit without quite reaching the values achieved in the WT-hICAM-1-expressing EC. ICAM-1-deficient mouse brain EC transfected with mouse ICAM-1 carrying an identical mutation does not result in an inhibition of transendothelial lymphocyte migration compared with that in WT ICAM-1 controls (unpublished observations). Together, these observations suggest that with respect to EC-mediated lymphocyte adhesion and migration, this amino acid is not a critical residue within the ICAM-1 intracellular domain and are consistent with the observation that ICAM-1 is not tyrosine phosphorylated following ICAM-1 cross-linking or coculture with T lymphocytes. Other studies have suggested that ICAM-1 can be tyrosine-phosphorylated following adhesion of cells to immobilized fibrinogen; however, it was notable that in these studies an ICAM-1 mutant lacking the conserved intracellular tyrosine residue was still able to bind Src homology protein tyrosine phosphatase-2, and that both this ICAM-1 protein and a full C-terminally truncated version of ICAM-1 was capable of being tyrosine-phosphorylated in response to binding of fibrinogen (21). It is therefore interesting to note that in our exhaustive studies we have been unable to show phosphorylation of either endogenous or overexpressed ICAM-1 in response to either ICAM-1 cross-linking or coculture of EC with T lymphocytes. These studies were performed under conditions that induced ICAM-1-dependent signaling responses, as assessed by the hyperphosphorylation of FAK.

Cell-permeant ICAM-1 C-terminal peptides mimicking the ICAM-1 intracellular domain were shown to be effective in inhibiting transendothelial migration of lymphocytes. In contrast to studies with truncated ICAM-1 proteins, these peptides were unable to moderate lymphocyte adhesion to EC. Furthermore, inclusion of a phosphorylated tyrosine residue at a position in the peptide corresponding to codon 512 of ICAM-1 was no more potent an inhibitor of transendothelial migration than the nonphosphorylated peptide. This supports the view that phosphorylation of the conserved tyrosine residue is not required for ICAM-1-mediated signal transduction, enabling transendothelial migration of T cells. It is interesting to note that although the amino acid sequences of human and rat ICAM-1 are different, they are similar in the juxta-membrane region to which the peptides were targeted. Thus, peptides comprising the hICAM-1 were effective in inhibiting transendothelial migration of lymphocytes across monolayers of rat brain EC expressing only endogenous ICAM-1 and were almost as effective as rat-specific sequences. This suggests that common effector molecules bind both rat and human ICAM-1 C-terminal sequences. Such studies further implicate the intracellular domain of ICAM-1 in T cell-mediated signaling functions and the subsequent EC support of lymphocyte migration. However, treatment of EC with penetratin ICAM-1 peptides in the culture medium at levels that attenuated lymphocyte migration did not result in an inhibition of ICAM-1-mediated Rho activation or subsequent stress fiber formation, although it was apparent the ICAM-1 peptides showed a significant colocalization with F-actin stress fibers, suggesting that the ICAM-1 C-terminal domain interacts with the actin cytoskeleton as previously suggested (18, 19, 28). Conversely, when peptides were directly introduced into cells, stress fiber formation was ablated (Fig. 8D). This suggests that higher concentrations of peptides are required to block Rho-dependent signaling, but that other ICAM-1-mediated events, which are essential for support of transendothelial migration, are inhibited at lower levels. Similar studies using mouse ICAM-1 peptides also lead to attenuation of transendothelial migration of lymphocytes without affecting lymphocyte adhesion to mouse brain EC (our unpublished observations).

Overall, these data support our previous findings that transendothelial migration of T lymphocytes across monolayers of CNS EC can be inhibited by interfering with EC ICAM-1-mediated intracellular signaling responses without altering lymphocyte adhesion (5, 13, 30). Although ICAM-1 has been reported to bind a number of intracellular proteins, such as α-actinin (14), β-tubulin, GAPDH (15), and ezrin (16, 17), it is still not clear which, if any, of these molecules is responsible for transducing signals from the intracellular domain of ICAM-1. Ezrin is known to link ICAM-1 to the actin cytoskeleton, and it is clear that the actin cytoskeleton is important for both ICAM-1-mediated signaling and transendothelial lymphocyte migration. Coupled with the fact that proteins

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of the ezrin/radixin/moesin family are also effectors of Rho signaling pathways (31), ezrin is perhaps the leading candidate. It has recently been shown that ERM proteins may play a role in T lymphocyte adhesion to Ig superfamily molecules (32). However, we have to date been unable to show direct interaction between ICAM-1 and ezrin in vitro under conditions where ICAM-2/ezrin interactions can be demonstrated (28).

Finally, our data raise the interesting possibility that agents based on peptide sequences within the C-terminal domain of ICAM-1 may provide the basis for a novel class of anti-inflammatory agent that can antagonize ICAM-1-mediated intracellular signal transduction and hence subsequent transendothelial lymphocyte migration.

References


