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Dual Role of Melanoma Cell Adhesion Molecule (MCAM)/CD146 in Lymphocyte Endothelium Interaction: MCAM/CD146 Promotes Rolling via Microvilli Induction in Lymphocyte and Is an Endothelial Adhesion Receptor

Borhane Guezguez,* Pascale Vigneron,* Nathalie Lamerant,† Claudine Kieda,† Thierry Jaffredo,* and Dominique Dunon3*

The melanoma cell adhesion molecule (MCAM)/CD146 is expressed as two isoforms differing by their cytoplasmic domain (MCAM long (MCAM-l) and MCAM short (MCAM-s)). MCAM being expressed by endothelial cells and activated T cells, we analyzed its involvement in lymphocyte trafficking. The NK cell line NKL1 was transfected by MCAM isoforms and submitted to adhesion on both the endothelial cell monolayer and recombinant molecules under shear stress. MCAM-I transfection reduced rolling velocity and increased NK1 adhesion on the endothelial cell monolayer and VCAM-1. Scanning electron microscopy revealed that MCAM-I induced microvilli formation and extension. In contrast, MCAM short or mock transfection had no effect on adhesion of NKL1 cells and microvilli formation. As shown by mutagenesis, serine 32 of the MCAM-I cytoplasmic tail, belonging to a putative protein kinase C phosphorylation site, was necessary for MCAM-I-actin cytoskeleton interaction and microvilli induction. Accordingly, chelerythrine chloride, a protein kinase C inhibitor, abolished MCAM-I-induced microvilli and rolling of MCAM-I-transfected NKL1 cells. Inhibition of adhesion under shear stress by anti-MCAM Abs suggested that both lymphoid MCAM-I and endothelial MCAM were also directly involved in lymphocyte endothelium interaction. MCAM-I-transfected NKL1 and activated CD4 T cells adhered to rMCAM under shear stress whereas anti-MCAM Ab treatment inhibited this process. Taken together, these data establish that MCAM is involved in the initial steps of lymphocyte endothelium interaction. By promoting the rolling on the inflammation marker VCAM-1 via microvilli induction and displaying adhesion receptor activity involving possible homophilic MCAM-I-MCAM-I interactions, MCAM might be involved in the recruitment of activated T cells to inflammation sites. The Journal of Immunology, 2007, 179: 6673–6685.
promotes microvilli induction in NKL1-transfected cells which re-
results in the slowing of leukocytes in the bloodstream and their
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mediated by selectins, P-selectin (37–40). The initial steps engage lymphocyte microvilli at
the firm adhesion and transendothelial migration of lympho-
spond to the first steps of the extravasation process, followed by
NKL1). Collectively, these studies suggest a significant role of
the laminin family (30–33). Moreover, MCAM is able to trans-
duce signals and induces phosphorylation of cellular proteins
such as Pyk2, focal adhesion kinase, CAS, and paxillin (34–36).
In addition, mammalian and avian MCAM are expressed as
two isoforms differing by the cytoplasmic region. These
isoforms are named MCAM long (MCAM-l) and MCAM
short (MCAM-s) for long and short cytoplasmic tail, respec-
tively, but both isoforms present similar adhesion properties (4,
12, 22, 23).
Lymphocyte tethering and rolling on endothelial cells corre-
spond to the first steps of the extravasation process, followed by
the firm adhesion and transendothelial migration of lymphocytes
(37–40). The initial steps engage lymphocyte microvilli at
the endothelial contact zone and are largely mediated by select-
tins and their ligands (41). Tethering corresponds to the trans-
ient contact between leucocytes and endothelium (42–44) and
results in the slowing of leucocytes in the bloodstream and their
rolling on the surface of the endothelium. Selectins, P-selectin
glycoprotein ligand 1, and α4 integrin, another molecule in-
volved in rolling, are clustered at the tip of microvilli and this
localization is essential for the initial steps of homing (41, 45–
47). Collectively, these studies suggest a significant role of
MCAM isoforms in lymphocyte-endothelium interaction. In
this report, we show that MCAM is involved in the rolling and
adhesion of lymphocytes. We established that MCAM-l pro-
motes microvilli induction in NKL1-transfected cells which re-
distributes rolling receptors, including MCAM-l itself. In a pro-
cess distinct from VCAM-1-dependent adhesion, the role of
MCAM-l in lymphocytes was confirmed with an activated CD4+ T cell line expressing endogenous MCAM-l. Moreover, we showed that endothelial MCAM acts as an adhesion receptor.

Materials and Methods

cDNA and plasmid constructs

To obtain the MCAM-GFP construct, the chicken MCAM-l (long iso-
form) and MCAM-s (short isoform) were amplified by PCR using
specific primers as described previously (4). The cDNAs were inserted in a pcDNA3-GFP vector (BD Clontech), placing the MCAM sequence in frame with sequence encoding GFP at the C terminus. Recombinant sequences encoding mutated cytoplasmic and extracellular domain of
MCAM-1 were obtained by PCR using specific primers (see Table I).
The PCR products were subcloned in the pcRII-Topo (Invitrogen Life Technologies). The cDNAs were digested with HindIII/BamHI and in-
serted in a pcDNA3-GFP vector, as described above. In MCAM-deleted mutants, numbers indicate the first amino acid deleted of the cytoplasmic tail (see Fig. 1). The MCAM-1 mutant in which a serine (residue 594) was substituted by alanine residue hereafter termed MCAM-1(594S/A), was constructed by in vitro mutagenesis using the Quick-
change XL site-directed mutagenesis kit, according to supplier’s in-
structions (Stratagene). Briefly, complementary primers (22 nM) and 50 ng of MCAM-1-GFP cDNA template were used in PCR under the fol-
lowing conditions: 18 cycles of denaturation for 30s at 95°C and primer
annealing and extension at 68°C for 15 min. The primers used (mutated
nucleotides are underlined) were shown in Table I.

| Table I. Primers used for mutagenesis and RT-PCR experimentsa |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| cDNA            | Expt. | Amplicon Size (bp) | Primer Sequences                      |
| MCAM-l          | PCR   | 1878             | (F) 5′-CCCCAAGCTTGCGCAAGCAGATGGCTG-3′ |
|                 |       | 1752             | (R) 5′-CCCCAAGCTTGCGCAAGCAGATGGCTG-3′ |
|                 |       | 1806             | (F) 5′-CCCACAACTCGAGATGGCTGCTG-3′    |
|                 |       | 1773             | (R) 5′-CCCACAACTCGAGATGGCTGCTG-3′    |
|                 |       | 1686             | (F) 5′-CCCACAACTCGAGATGGCTGCTG-3′    |
|                 |       | 1878             | (R) 5′-CCCACAACTCGAGATGGCTGCTG-3′    |
| MCAM-s          | PCR   | 1250             | (F) 5′-ATGCTAGCTGAGAAATGGCGCTG-3′    |
|                 |       | 1080             | (R) 5′-ATGCTAGCTGAGAAATGGCGCTG-3′    |
| MCAM-l Δext(12) | PCR   | 600 (MCAM-l)    | (F) 5′-CTATCGCTAGCTACCTTGCGGCAATC-3′ |
|                 |       | 480 (MCAM-s)    | (R) 5′-ATGCTAGCTGAGAAATGGCGCTG-3′    |

a Primer design was based on previous studies (4, 12). (F), Forward; (R), reverse.

| Table II. Low magnification (×2000) SEM characterization of lymphocytes |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell Type       | Sparsed Shortb  | Numerous Shortb | Sparsed Longc   | Numerous Longd |
|                 | Microvilli %    | Microvilli %    | Microvilli %    | Microvilli %    |
| NK-GFP (control)| 98              | 0               | 2               | 0               |
| NK-MCAM long    | 0               | 8               | 31              | 61              |
| NK-MCAM short   | 47              | 41              | 12              | 0               |
| CEMT4           | 48              | 48              | 0               | 52              |

b Density from 1 to 3 microvilli/μm².
c Density from 4 to 6 microvilli/μm².
d Median length from 0.2 to 0.4 μm.
e Interestingly, CEMT4 cells which roll on endothelial cells exhibit similar phenotype than NK-MCAM-l cells.
Cells and culture conditions

The NKL1 human NK cells were provided by S. Chouaib (Unité 487, Institut National de la Santé et de la Recherche Médicale, Institut Gustave Roussy, Villejuif, France). They were established from peripheral blood of a patient with large granular lymphocyte leukemia, as described previously (48). CEMT4 are human leukemic CD4⁺/H11001 T cells, provided by P. Olivier (Institut Pasteur, Paris, France). Both cell lines were cultured in OptiMEM-1 with glutamax-I, 2% FBS, and antibiotics (Invitrogen Life Technologies). All organ-specific endothelial cell lines (OSECs), respectively, human appendix endothelial cells (HAPEC), human skin microvascular endothelial cells (HSkMEC), and HPLNEC.B3 (endothelial cells from human peripheral lymph node of a patient with Hodgkin’s lymphoma) were established in the laboratory of C. Kieda (49), Centre National de la Recherche Scientifique patent 99-16169). OSECs were maintained in culture with OptiMEM-1 supplemented with glutamax-I, 3% FBS, and antibiotics (Invitrogen Life Technologies). The mouse myogenic C2 cell line and L929 mouse fibroblast cells (American Type Culture Collection) were grown in DMEM plus glutamax supplemented with 10% heat-inactivated FCS and antibiotics (Invitrogen Life Technologies). All cultures were performed in a 5% CO₂ humidified atmosphere at 37°C. Before cell culture, glass coverslips 22 × 22 mm (CML) were placed in 6-well plates (TPP), UV sterilized, 100% ethanol washed, and air dried in a laminar flow of tissue-culture cabinet for 2 h.

RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from human OSECs and NKL1 and CEMT4 cell lines (49) using the RNeasy Mini kit (Qiagen). To remove any trace of DNA, RNA was treated with DNase I using the Message Clean kit from GenHunter. Semiquantitative RT-PCR was performed with the Quantum RNA Actin Internal Standards kit (Ambion), according to the manufacturer’s instructions. To amplify the control target (actin) at a level roughly similar to our gene of interest (MCAM), the ratio of actin primers to competitors used was 2:8. An oligo(dT) (15) primer was used for the reverse transcription reaction and the primers used to amplify MCAM are shown in Table II giving two PCR products of 600 and 480 bp, respectively, corresponding to MCAM-1 and MCAM-s. Twenty-five amplification cycles were performed. PCR products were resolved on a 1% agarose gel and quantified using the ImageQuant 5.1 program (Molecular Dynamics/Amersham Biosciences).
FIGURE 2. MCAM-I decreases rolling velocity and increases firm adhesion of NKL1 cells to endothelial cells in flow conditions. A, Expression of MCAM isoforms by RT-PCR. Templates were cDNA from human endothelial cell lines. Controls correspond to PCR experiments performed in the absence of template. After 30 cycles of amplification, PCR products were electrophoresed, stained with ethidium bromide, and photographed. Letters on the right indicate the different spliced isoforms. Actin expression was used to standardize cDNA amounts and to allow semiquantitative PCR analysis. Leukocytes correspond to: human NK cell line for NKL1 and human CD4-activated T cells for CEMT4. Human endothelial cell corresponds to cell lines of different origins (49): umbilical cord for HUVEC, lung microvasculature for HLMEC, spleen microvasculature for HSpMEC, intestine mucosa for HIMEC, brain microvasculature for HBrMEC, peripheral lymph node for HPLNEC.B3, mesenteric lymph node-HMLNEC, skin microvasculature for HSkMEC, appendix for HAPEC.1. Endothelial cell lines express higher amounts of the MCAM-I than the MCAM-s isoform. In contrast, lymphocytes (NKL1 and CEMT4) expressed exclusively the MCAM-I isoforms and NKL1 expressed a very low level of MCAM-I in comparison to the other cell lines. B, Microscopic analysis NKL1 cell adhesion to the HPLNEC.B3 line in flow condition. MCAM-I-GFP-, MCAM-s-GFP-, and GFP-transfected NKL1 cells (1 × 10⁶ cells/ml) were pumped over the HPLNEC.B3 line (50 ml/min for 10 min, shear stress 1.3 dyn/cm²). After the next 5 min of washing with medium to eliminate nonadherent NK cells, pictures were taken using a Zeiss Axiovert 200 inverted fluorescence microscope. C, Quantitative analysis of rolling and firm adhesion of NKL1 cells. Videomicroscopy of migrating NKL1 cells under flow conditions allowed to measure rolling velocity using the Metamorph software. After the next 5 min of washing with medium to eliminate nonadherent NK cells, the results were plotted as the number of NKL1 cells per a microscopy field. Results, expressed as the mean and SD, were calculated from quintuplicate determinations in one representative experiment of five performed.
Abs, chemicals, and immunocytochemistry

PE-conjugated anti-chicken MCAM (clone 264) (4) was purchased from Southern Biotechnology Associates and was used for flow cytometry analyses. The polyclonal Ab anti-chicken MCAM (4), the polyclonal anti-rat MCAM (1/200 dilution serum, provided by Prof. E. Taira, Department of Pharmacology, Iwate Medical School, Iwate, Japan) and soluble recombinant molecules were used for adhesion assay under shear stress. Soluble recombinant human VCAM-1 and P-selectin were purchased from R&D Systems. Soluble recombinant human MCAM was purchased from Biocytex. Soluble recombinant avian MCAM generated and produced as previously described (4) was a gift of Prof. B. Imhof (Centre Médical Universitaire, Geneva, Switzerland). An antiserum against the murine C/H9260 fusion protein of soluble recombinant avian MCAM was generated by immunizing rabbit (4). Anti-avian MCAM polyclonal Ab was affinity purified on an affinity column of cyanogen bromide-coupled soluble avian MCAM. Preimmune Ig were purified on a protein A column. Specificity of these Abs were further controlled by FACS analyses on L cells and NKL1 cell lines before and after transfection by MCAM. Data were identical with those obtained with an anti-MCAM mAb (clone 264). Antiserum against GST-fusion protein of rat MCAM Ig-like loop I-III (GST-I-III) and loop I-V (GST-I-V) were generated by immunizing rabbits (24). Anti-rat MCAM polyclonal Ab was further purified on a protein A column and checked by FACS on human cell lines.

Chelerythrine chloride, a protein kinase C (PKC) inhibitor, was purchased from Sigma-Aldrich and used in a scanning electron microscopy and adhesion assay under shear stress. For immunocytochemistry, transfected GFP cells were cultured on glass coverslips in 6-well tissue-culture plates (Tpit) and fixed with prewarmed 4% (w/v) paraformaldehyde solution in PBS for 15 min. Thereafter, cells were treated with 0.1% (w/v) Triton X-100 in PBS (Sigma-Aldrich) for 10 min and soaked in coating solution (PBS containing 5% BSA) for 30 min. Coverslips were incubated with primary mAbs in 1% PBS-BSA for 1 h, washed, and incubated with goat TRITC or Alexa Fluor 555-conjugated anti-mouse Abs (1/250 dilution; Molecular Probes). For cytoskeleton staining, TRITC-conjugated phalloidin (1/1500 dilution; Sigma-Aldrich) was used to visualize F-actin. All procedures were performed at room temperature. Samples were mounted in Immuno-mount (Thermo Shandon) and analyzed with a TCS-SP confocal microscope (Leica) set on sequential mode. Alternatively, qualitative and quantitative analysis of the lengths and the widths of microvilli were measured using Metamorph software (Roper Scientific).

Cell transfection and electroporation

Stable transfected L929 and NK1-1 cells expressing wild-type and mutated MCAM-GFP were obtained after transfection of the relevant expression vectors using Lipofectamine 2000, according to the manufacturer’s instructions (Invitrogen Life Technologies). Transfected cells were maintained in culture medium supplemented with 1 mg/ml neomycin (G418). Positive clones were detected by sorting GFP-positive populations and controlled using PE-conjugated anti-chicken MCAM (clone 264) by flow cytometry (Coulter Epics flow cytometer cell sorter; Beckman Coulter) and Western blotting of postnuclear lysates. Cells were then used either for immunofluorescence or confocal imaging.
FIGURE 4. MCAM-l increased the number and the extension of microvilli of human NK NKL1 cells and is associated with the actin cytoskeleton. Surface morphologies of human NKL1 cells. A. Scanning electron microscopy of GFP-, MCAM-s-GFP-, and MCAM-l-GFP-transfected NKL1 cells. MCAM-l-GFP expression increased microvilli whereas GFP transfection or MCAM-s-GFP did not modify NKL1 cell morphology. Bar, 2 μm. B. Confocal microscopy of MCAM-l-GFP-, MCAM-s-GFP-, and GFP- (control) transfected NKL1 cells. Cells were fixed with 4% paraformaldehyde and labeled with TRITC-phalloidin to detect actin cytoskeleton. Overlays revealed that MCAM-l-GFP was associated with the actin cytoskeleton whereas MCAM-s-GFP did not colocalize. Bar, 10 μm.

Scanning electron microscopy

Cell suspensions (150 μl) were fixed by addition of 1.35 ml of room temperature fixative (3.5% glutaraldehyde in 100 mM Na PO₄ (pH 7.4)) and rapidly mixed by inversion. After 2 h, cells were centrifuged in at 300 × g for 5 min. The cell pellet was washed twice in 1.5 ml of Na PO₄, then resuspended in 20 μl of this buffer. This drop of cells was placed on top of a 5-mm round acid-washed glass coverslip (CML), previously coated with hexamethyldisilazane (Sigma-Aldrich), desiccated overnight under vacuum. Surface morphologies of human NK NKL1 cells.

Flow chamber and laminar flow adhesion assay

The flow chamber (Immunetics) used has been previously described elsewhere (51, 52). The chamber was mounted on an Axiovert 200 epifluorescence inverted microscope (Zeiss) for direct real time visualization of dynamic cell adhesion process using a ×20 objective. The microscope was coupled to an AxioCam high-resolution numeric camera (Zeiss) to record 10 random fields per coverslip. The system was directly linked to a Pentium 3 computer equipped with the acquisition software Axiosvision (Zeiss). Before the experiments, endothelial cells were seeded 48 h at concentration onto polystyrene tissue-culture slides (Nagle Nunc International). Alternatively, chamber slides were coated overnight at +4°C with soluble recombinant VCAM-1, P-selectin, MCAM, or total IgG at 2.5 μg/ml and washed twice in PBS. Unbound protein-binding sites were blocked with BSA for 1 h at room temperature (53). Then, 1 × 10⁶/ml MCAM-GFP-transfected NKL-1 or CEMT4 cells in PBS were then perfused through the chamber on a monolayer of endothelial cells or on a soluble recombinant molecule using a withdrawal syringe pump (Harvard Apparatus) under constant shear stress at 1.3 dyn/cm². In experiments involving Ab treatment, transfected NKL1, CEMT4 cells, and endothelial cells were preincubated before perfusion, respectively, with polyclonal Ab anti-chicken MCAM (1.7 μg/ml) and polyclonal anti-rat MCAM (1 μg/ml) in PBS 1% BSA (w/v) solution for 30 min on ice or 10 min at room temperature. Alternatively, the cells were preincubated with chelerythrine chloride (5 μM) for 10 min at 37°C in PBS. Cells were washed and immediately subjected to an adhesion assay under shear stress. Endothelial cells were preincubated before perfusion with the polyclonal Ab anti-rat MCAM. Each image was subjected to computer-assisted analysis with Metamorph software (Roper Scientific) and used to count the number of arrested cells (bound cells) for separate fields of view (n = 10). Firmly adherent lymphocytes were only included in the analysis after a 5-min washing perfusion period with medium. Each flow experiment is as done in quintuplicate and the mean value ± SD of specific adhesion were calculated per field. Recognition, rolling, and adhesion of the lymphocytes to the endothelial cells were allowed at a fixed flow rate (50 μl/min) for 5 min. For this period of time, the experiment sequence was recorded at 1 frame/s during 5 min, on the time-lapse setting of the Axiosvision (Zeiss). Rolling velocity was measured for each condition (with or without Abs) by tracking 30 cells, frame by frame, in the flow direction for 15 s to yield a 300 s total observation time. Lymphocyte rolling on the endothelial cell monolayer was considered for the analysis when the interaction time was >2.0 s and the distance traveled by lymphocytes during 15 s was more than one cell diameter. Data are expressed as mean ± SD of rolling velocity (micrometer per second) per quintuplicate replicate experiments. The number of rolling cells was analyzed during 10 min following the instant when cells first appeared in the observation window. All cells that rolled across a line perpendicular to the capillary axis in the center of the field were counted. Typically, the rolling flux increased steadily during the first 6 min of perfusion and reached a plateau thereafter. These kinetics were independent of
the wall shear rate (at least within the range applied) reflecting the period necessary for the system to reach equilibrium.

Statistical analysis
Results from flow experiments of at least five independent experiments were pooled and expressed as means ± SD. Statistical comparison of means was performed using the two-tailed unpaired Student t test. The null hypothesis was rejected at p < 0.05.

Results
Lymphoid MCAM-l supports the rolling and arrest of lymphocytes on endothelial monolayers
The MCAM molecule is expressed by activated lymphocytes and vascular endothelium (4, 8, 12, 13). To test whether MCAM isoforms (Fig. 1) are involved in lymphocyte trafficking, we analyzed the in vitro rolling and adhesion of a NK cell line (NKL1) on vascular endothelial cells under flow (HPLNEC.B3 and data not shown for HSKMEC and HAPEC1 lines (51)). NKL1 cells were selected for their endogenous expression of MCAM at a very low level and their previous use in adhesion assays with endothelium (Fig. 2A) (51). Compared with mock-transfected cells, transfected NKL1 with MCAM-l exhibited a 20-fold increase in adhesion to endothelial cells under shear stress (Fig. 2B). In contrast, transfection of MCAM-s did not increase NKL1 cell adhesion although the MCAM-l and -s expression levels were identical (Fig. 2B). Video-microscopy analysis revealed that MCAM-l increased the number of rolling cells and reduced the rolling velocity of NKL1 cells (Fig. 2C). Transfection of MCAM-s has a mild effect on rolling. Treatment of NKL1 cells expressing MCAM-l by anti-MCAM polyclonal Ab inhibited rolling and adhesion while Ig purified from preimmune serum had no effect (Fig. 3A). These Ab-blocking data were confirmed with a human activated CD4+ T cell line expressing endogenous MCAM-l (CEMT4, Figs. 2A and 3B (51)).

MCAM-l induces microvilli in NKL1 cells and promotes rolling and adhesion to VCAM-1
Lymphocyte rolling is mediated by adhesion molecules located at the tip of microvilli (54). Therefore, the effect of MCAM transfection on the shape of microvilli was studied by scanning electron microscopy on NKL1 cells. MCAM-s and mock-transfected NKL1 cells both showed a smooth cell surface with rare microvilli (Fig. 4 and Table I). In contrast, transfection of MCAM-l led to the formation of numerous and long microvilli, while MCAM-s formed only few and short protrusions. Confocal microscopy showed that MCAM-l was located on microvilli whereas MCAM-s distribution was diffuse over the cell surface. MCAM-l induction of microvilli appeared to be a general phenomenon because similar microvilli extension was found in myogenic C2 and L929 cells transfected with MCAM-l (data not shown and Fig. 5).

Microvilli formation depends on the actin cytoskeleton. We indeed found that MCAM-l colocalized with polymerized actin, as verified by phalloidin costaining in NKL1 and L929 cells (Figs. 4B and 5). MCAM-l and MCAM-s cytoplasmic tails differ by 50 aa. Transfection of MCAM-l cytoplasmic deletion mutants into L929 cells showed that the motif involved in microvilli induction and actin colocalization was located between amino acids 30 and 41 of the cytoplasmic tail. This sequence, VKSDKLSEEAG, contains a putative PKC phosphorylation site (KSDK) and the replacement of the corresponding serine 32 by alanine abolished the induction of microvilli extension (Fig. 5). Inhibition of MCAM-l-dependent microvilli extension by chelerythrine chloride, an inhibitor of the PKC pathway (55), confirmed that the PKC pathway was involved in the control of microvilli extension (Fig. 6). Chelerythrine chloride also inhibited rolling and adhesion of MCAM-l-transfected NKL1 cells.

FIGURE 6. PKC pathway is required in MCAM-l-induced microvilli extension and lymphocyte rolling. A, Scanning electron microscopy of untreated and treated MCAM-l-GFP-transfected NKL1 cells with chelerythrine chloride (C.C, 5 mM), a PKC inhibitor. MCAM-l microvilli extension depends on the PKC pathway. Bar, 2 mm. B, Microscopic analysis of PKC pathway inhibition in MCAM-l-GFP-transfected NKL1 cell adhesion to HPLNEC.B3 line in flow condition. MCAM-l-GFP lymphocytes were incubated with chelerythrine chloride (5 mM) 10 min at 37°C. Cells (1 × 10⁶ cells/ml) were then washed three times and pumped over the HPLNEC.B3 line (50 μl/min for 10 min, shear stress 1.3 dyn/cm²). After the next 5 min of washing with medium to eliminate nonadherent NK cells, pictures were taken using a Zeiss Axiovert 200 inverted fluorescence microscope. C, Quantitative analysis of rolling and firm adhesion of chelerythrine chloride treatment (as described in Fig. 2). Inhibition of the PKC pathway led to a decrease of rolling and adherents cells.

To test the role of microvilli induction in the rolling process, MCAM-l-transfected NKL1 cells were submitted to adhesion on endothelial receptors under shear stress (Fig. 7). MCAM-l increased NKL1 rolling and firm adhesion to rVCAM-1 but had no effect on NKL1 interaction with P-selectin or total IgG as a control (Fig. 7A). Interestingly, the anti-MCAM polyclonal Ab did not
affect rolling of MCAM-l-transfected NKL1 cells to VCAM-1 (Fig. 7B) showing that MCAM-l is not directly involved in the interaction with VCAM-1 during this step.

Endothelial MCAM is a rolling receptor

The fact that anti-MCAM Abs inhibited rolling of MCAM-l-transfected cells to endothelial cells but not on VCAM-1 suggested that MCAM plays another role in lymphocyte rolling, in addition to microvilli induction. Because both lymphocytes and endothelial cells express MCAM, we analyzed whether endothelial MCAM isoforms were involved in lymphocyte adhesion (Fig. 8). Pretreatment of endothelial cells by anti-MCAM polyclonal Ab inhibited rolling and adhesion of NKL1 cells, similar to the blocking of MCAM on lymphocytes (Fig. 8A). These experiments were confirmed with the human CD4+ T cell line (data not shown) because endothelial cells do express VCAM-1; the adhesion and inhibition involves a mechanism other than VCAM-1 interactions that requires MCAM on both lymphocytes and endothelial cells. Adhesion of NKL1 cells to rMCAM under shear stress was analyzed to determine whether endothelial MCAM was a rolling receptor (Fig. 8, A and C). MCAM-l transfection of NKL1 cells induced rolling and dramatically increased adhesion to rMCAM. In contrast, MCAM-s-GFP transfection increased only the rolling step. Treatment of MCAM-l-expressing NKL1 cells by polyclonal anti-MCAM Ab abolished rolling and adhesion to rMCAM while preimmune purified Ig had no effect (Fig. 8D). Similar data were obtained with polyclonal anti-MCAM Ab treatment of the human activated CD4+ T cell line (Fig. 8D). Thus, MCAM-mediated rolling appears to require MCAM expression by both cell types, vascular endothelial cells and lymphocytes, and thus might occur via homophilic interactions.

Firm adhesion of MCAM-l-positive leukocytes requires endothelial MCAM-l expression

Adhesion experiments of NKL1 cells to rMCAM under flow did not allow for determination of the precise function of either endothelial MCAM isoform in lymphocyte-endothelium interactions. For this purpose, MCAM-l-expressing NKL1 cells were submitted to adhesion on L929 cell transfected either by MCAM-l or MCAM-s under shear stress (Fig. 9). MCAM-l or MCAM-s expression by L929 cells supported both rolling of MCAM-l-positive NKL1 cells, whereas firm adhesion was only supported by MCAM-l-transfected L929 cells.

Discussion

In this report, we showed that MCAM is involved in lymphocyte recruitment by endothelium. MCAM-l induces microvilli in lymphocytes and increases the rolling and adhesion of lymphocytes on endothelial cells notably via the endothelial receptors VCAM-1 and MCAM. Microvilli formation depends on the interaction of MCAM-l with the actin cytoskeleton and requires a PKC phosphorylation site in the cytoplasmic tail. Rolling of MCAM-l+ lymphocytes is supported by both the MCAM-s- and MCAM-l-expressing endothelial cell monolayer, but firm adhesion requires only MCAM-l.

Induction of MCAM-l-dependent microvilli

Interaction of MCAM-l with the actin cytoskeleton leads to the formation of microvilli. It was previously reported that MCAM
associates with actin microfilaments (13) and is localized in microvilli (17). However, no function was proposed for this association. We show here that microvilli extension requires serine 32 of the MCAM-l cytoplasmic domain. Serine 32 is part of a PKC phosphorylation site conserved from zebrafish to human (Fig. 10). An additional domain corresponding to the membrane proximal sequence KKGK is conserved in both MCAM-s and MCAM-l. This domain has recently been involved in the binding of L-selectin to ezrin-radixin-moesin (ERM) proteins (56). Interestingly, both avian MCAM-l and MCAM-s isoforms have been shown to bind ERM proteins in pull-down experiments at low ionic concentration (17). However, at high ionic concentration MCAM-s failed to interact with ERM proteins. Under these latter conditions, interaction with ERM needed the cytoplasmic sequence 25–39 present only in MCAM-l. Thus, minimal binding of MCAM to ERM protein may be ensured via the KKGK sequence, whereas firm binding may require additional sequence encompassing serine 32, which is responsible for microvilli extension.

Lymphocyte MCAM-l is involved in lymphocyte-endothelium interaction

Lymphocyte microvilli, specifically induced by the MCAM-l isoform, are involved in rolling and firm adhesion. Indeed, these microvilli are essential in this phenomenon because MCAM-s, which does not promote microvilli formation, had a weak effect on rolling and did not induce adhesion of NK1 cells. Lymphocyte MCAM-l is potentially involved in lymphocyte trafficking at different levels (Fig. 10 B). First, MCAM-l would participate indirectly in this process by controlling microvilli formation and redistribution of rolling actors at the tip of microvilli. Second, inhibition of rolling and adhesion of MCAM-l+/H11545 leukocytes by anti-MCAM Abs and similar properties shared by MCAM-l and selectins strongly support the hypothesis that MCAM-l is a new tethering and rolling receptor of leukocytes. Third, similarly to VCAM-1 and mucosal addressin cell adhesion molecule-1 (39), MCAM-l would also be involved in firm adhesion. MCAM-l, like selectins, CD44, and P-selectin glycoprotein ligand 1, is linked to the actin cytoskeleton via ERM proteins (17). In addition, rapid shedding of L-selectin upon cell activation via PKC during rolling facilitates further progression to firm adhesion and transendothelial migration (57–59). Interestingly, a PKC site is involved in the control of MCAM-l/actin interaction and a soluble form of MCAM, ~10 kDa smaller than the cell-associated MCAM (110 and 120 kDa for MCAM-s and -l, respectively) is also found in plasma (60, 61). This soluble form could be generated by a similar shedding process from leukocyte as well as endothelial cell surfaces. Finally,
Whereas MCAM-I and MCAM-s exhibit similar adhesion properties and ligands in static conditions (4, 12, 23), we showed that these two isoforms exhibit different properties under dynamic flow. Both MCAM-s and MCAM-I expressed by a monolayer of L929 cells supported rolling, but only MCAM-I was able to promote firm adhesion. Both MCAM-I and MCAM-s have similar extracellular domains (as well as the soluble rMCAM) suggesting that different adhesion properties are accounted for by their cytoplasmic tail. Notably, firm adhesion would require the strong interaction of MCAM-I with the actin cytoskeleton which is missing for MCAM-s (Figs. 4 and 5) (17). MCAM isoforms are differentially targeted in polarized cells, MCAM-s to apical membranes and MCAM-I to basolateral membranes (62). MCAM-I+ lymphocyte-endothelium interaction could depend on the endothelium state. MCAM-I+ lymphocytes would roll on apical MCAM-s of resting polarized endothelium but would require the recruitment of additional players to induce firm adhesion. In contrast, MCAM-I+ lymphocytes would roll and adhere to MCAM-I presented by the incompletely polarized endothelium, for instance, at inflammation sites. Indeed, treatment of HUVEC by proinflammatory cytokines TNF-α and IFN-γ leads to redistribution of adhesion molecules such as JAM-A and PECAM-1 away from intercellular junctions (63–65).

Rolling of MCAM-I-expressing lymphocytes to endothelial MCAM-I may lead to different signal transduction in endothelial cells and lymphocytes. MCAM clustering by a mAb induces an outside-in signaling pathway recruiting p95Tyk2 and leading to tyrosine phosphorylation of focal adhesion kinase, CAS, and paxillin (35). Activation of these molecules is known to promote cell spreading (66). This transduction pathway might be involved in rolling and transendothelial migration of activated T cells.

**MCAM/CD146 would promote activated lymphocyte migration to inflammation sites**

MCAM-I is expressed in all CD146+ lymphocytes and is the only isoform detected in PCR experiments. Our data establish that MCAM expression in activated lymphocytes (8, 67) modifies their migration properties in comparison to resting lymphocytes. Notably, MCAM-I expression could favor the targeting of activated T cells to inflammasome sites. Detection of MCAM+ activated lymphocytes at inflammation sites supports this hypothesis. In vivo expression of MCAM was detected in activated T cells infiltrating hypersensitivity skin lesions and synovial fluid T cells of rheumatoid patients, whereas lymphocytes of healthy donors did not show significant MCAM expression (8). Reciprocally, regulation of MCAM expression in inflamed endothelium is correlated with the potential role of endothelial MCAM in the targeting of activated T cell to inflammation sites. Recently, increased expression of CD146 was detected in endothelial cells in active inflammatory bowel disease (68).

Repertoires of adhesion molecules expressed in activated T cells and inflamed endothelium strengthen the role of MCAM in lymphocyte targeting to inflamed endothelium. First, migration at inflammation sites depends mainly on expression of VCAM-1, ICAM-1, P-selectin, and E-selectin adhesion molecules by endothelial cells (39, 40, 69). Second, T cell activation via TCR engagement, in addition to inducing MCAM expression, leads to the rapid shedding of L-selectin, preventing the re-entry of activated T cells into peripheral lymph nodes (70). Thus, in the presence of low levels of L-selectin, MCAM would play a major role in the control of activated T cell rolling. In these cells, MCAM-I induces microvilli and would promote rolling via redistribution of \( \alpha_\beta_i \) integrin, which interacts with VCAM-1 specifically expressed in the inflamed endothelium. In addition, endothelial MCAM is very

Endothelial MCAM isoforms play different roles in lymphocyte MCAM-I-induced rolling and adhesion

Our adhesion experiments using soluble rMCAM as a substrate demonstrated that endothelial MCAM is a receptor involved in lymphocyte rolling and adhesion. More precisely, we showed that rMCAM specifically supported MCAM-I-induced rolling of NKL1 cells. Using anti-MCAM-blocking Abs, we showed that lymphocyte MCAM-I is involved in rolling on rMCAM. Because MCAM homophilic interaction has been shown to support cell-cell adhesion (4, 22, 24, 25), it is very likely that lymphocyte MCAM-I mediates rolling via interaction with vascular MCAM isoforms (Fig. 10B). However, because MCAM-I expression in lymphocytes is related to several mechanisms such as microvilli induction or signal transduction (34, 35), we cannot exclude that endothelial MCAM might interact with a heterophilic ligand.

similarly to selectins (47), MCAM-I might also contribute to integrin activation via signal transduction (34, 35).
FIGURE 10. MCAM functional model. A, Functional motifs of MCAM-l and MCAM-s cytoplasmic domains. Alignment of amino acid sequences of MCAM cytoplasmic regions of chicken (cMCAM), murine (mMCAM), human (hMCAM), rat (rMCAM-s), zebrafish (zMCAM-l), and bovine (bMCAM-l) (4, 12, 16). MCAM-s sequence presents three conserved motifs, a KKGK motif (pink triangle) that would allow binding to ERM molecules, a PKC site (blue triangle) and its C terminus that might interact with a PDZ domain. In addition to KKGK sites and the PKC site encountered in the MCAM-s cytoplasmic domain, MCAM-l exhibits a second PKC site (blue triangle). Serine 32 (red) of this site is involved in MCAM-l induction of microvilli and of their extension in lymphocytes and fibroblasts. A dileucine motif (orange) is required for MCAM-l basolateral targeting of polarized epithelial cells (62). A putative endocytosis motif YXXL is also encountered in the MCAM-l cytoplasmic tail. Note that these different motifs are conserved in vertebrates. B, Potential roles of MCAM in lymphocyte endothelium interaction (1). Lymphoid MCAM-l promotes microvilli extension known to favor rolling via rolling receptor redistribution such as VCAM-1 (2). Endothelial MCAM-s and MCAM-l are rolling receptors that interact likely with lymphoid MCAM-l but heterophilic interaction (X) cannot be excluded (3). Endothelial MCAM-l is involved in firm adhesion of MCAM-l⁺ lymphocytes. In contrast to MCAM-s, MCAM-l is strongly associated to actin cytoskeleton and ligand binding may induce signal transduction.
likely involved in this process. First, similar to VCAM-1, MCAM isoforms are up-regulated in inflammatory diseases (68) and following our data act as rolling receptors. Second, restriction of endothelial MCAM-1 luminal expression to activated endothelium and specific arrest of MCAM-1+ activated lymphocytes on the MCAM-1-expressing cell monolayer suggest an additional role for the endothelial MCAM-1 isoform in the targeting to inflammation sites. Taken together, our data establish that MCAM plays a major role in the control of microvilli formation and regulates the rolling velocity of activated T cells.

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Disclosures

The authors have no financial conflict of interest.

References


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