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Activated leukocyte cell adhesion molecule is a component of the endothelial junction involved in transendothelial monocyte migration

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Abstract Transendothelial leukocyte migration is a major aspect of the innate immune response. It is essential in repair and regeneration of damaged tissues and is regulated by multiple cell adhesion molecules (CAMs) including members of the immunoglobulin (Ig) superfamily. Activated leukocyte cell adhesion molecule (ALCAM/CD166) is an Ig CAM expressed by activated monocytes and endothelial cells. Hitherto, the functional relevance of ALCAM expression by endothelial cells and activated monocytes remained unknown. In this report, we demonstrate soluble recombinant human ALCAM significantly inhibited the rate of transendothelial migration of monocyte cell lines. Direct involvement of ALCAM in transendothelial migration was evident from the robust inhibition of this process by ALCAM blocking antibodies. However, soluble recombinant ALCAM had no impact on monocyte migration or adhesion to endothelium. Localization of ALCAM specifically at cell–cell junctions in endothelial cells supported its role in transendothelial migration. This study is the first to localize ALCAM to endothelial cell junctions and demonstrate a functional relevance for co-expression of ALCAM by activated monocytes and endothelial cells.

Keywords: ALCAM; Endothelium; Monocytes; Diapedesis

1. Introduction

Migration of monocytes from circulation to extravascular tissues is critical for generating tissue monocytes and macrophages, which are an integral feature of both innate and adaptive immune responses, and the repair process in many tissues. Monocyte recruitment is a complex process that involves multiple adhesive interactions with the endothelium [1–3]. Early stages in this process involve rolling of circulating monocytes in post capillary venules in the systemic circulation. A marginating pool of monocytes physically deform to transit pulmonary capillary vessels and are therefore routinely slowed-down in this vascular segment, which is the primary site for their retention in the lung [4,5].

The initial relatively weak adhesion characteristic of rolling is followed by markedly stronger adhesion between monocytes and endothelial cells mediated by leukocyte-specific β2 integrins and cognate ligands such as intercellular cell adhesion molecule family members expressed on endothelium. Adherent monocytes become activated and upregulate expression of several adhesion molecules, which promotes their migration through endothelial junctions, or through the body of endothelial cells in a process called diapedesis. Several adhesion molecules play important roles in this final stage of recruitment. Platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD31), is expressed on the surfaces of most leukocytes and at the borders of endothelial cell contacts, and is required for monocyte diapedesis [6]. Interestingly, inhibition of PECAM-1 function with blocking antibodies [7] or by genetic ablation [8] does not effect diapedesis in several vascular beds including the pulmonary microcirculation [7]. CD99 is expressed on subsets of leukocytes as well as at endothelial cell junctions, and has been shown to control the final step of monocyte diapedesis across human umbilical vein endothelial cell barriers [9]. More recently, the role of junctional adhesion molecules (JAM) in diapedesis have been documented [10–15]. There is robust recruitment of neutrophils by inflamed lungs in transgenic mice over-expressing endothelial JAM-C [16], although JAM-C over-expression has no significant impact on monocyte recruitment [16]. These findings are consistent with a model whereby diapedesis is tightly controlled at the local level by several factors including adhesion molecules tethered at endothelial junctions that exert a unique barrier phenotype in different vascular segments [17].

ALCAM (CD166) is an immunoglobulin (Ig) cell adhesion molecule consisting of five extracellular Ig domains, a single transmembrane domain and a short cytoplasmic tail. It is expressed by several cell types including endothelial cells, and given multiple names including BEN and HB2 [18–21]. ALCAM is not expressed in peripheral blood monocytes, however, it is upregulated in activated monocytes [22,23]. The functional relevance of this induction currently remains poorly understood. ALCAM has been implicated in invasion of endothelial cells into cartilage [24], implantation of blastocysts [25], neurite outgrowth [26] and invasion of melanoma cells [27]. While each of these studies implicates ALCAM in cell migration, a similar role for the molecule in activated monocytes has not previously been reported. In this study, we show that soluble recombinant AL-

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CAM (ALCAM-Fc) markedly inhibited transendothelial migration of THP1 and HL60 monocytes, but had no impact on migration or adhesion of monocytes to endothelium. Confocal analysis showed localization of ALCAM at endothelial cell junctions consistent with its specific role in diapedesis. Moreover, ALCAM-GFP was recruited to cell–cell contacts in live endothelial cells. These findings represent the first demonstration of ALCAM's subcellular localization in endothelial cells and its role in diapedesis.

2. Materials and methods

2.1. Human cell lines

Human monocyte cell lines THP1 (catalog no. TIB-202), HL-60 (catalog no. CCL-240) and U937 (catalog no. CRL-1593) were purchased from American Type Culture Collection (ATCC), Manassas, VA. The human K562 erythroleukemia cell line was a gift from Dr. Pace. THP1 cells were cultured in RPMI 1640 medium (ATCC), supplemented with 10% fetal bovine serum (FBS) and 0.05 mM 2-mercaptoethanol. HL60 cells were cultured in RPMI 1640 medium (ATCC) containing 10% FBS. HL60 and K562 cells were cultured in Iscove's modified Dubelcco's medium (IMDM) (ATCC) containing 10% FBS.

2.2. Isolation and culture of primary rat endothelial cells

Pulmonary microvascular endothelial cells (PMVECs) were isolated and cultured using a modification of a method we have previously described [28]. Male Sprague-Dawley rats (300–400 g) were euthanized by intraperitoneal injection of 50 mg of pentobarbital sodium (Nembutal, Abbott Laboratories). After sternotomy, the heart and lungs were removed en bloc and placed in a DMEM bath containing 90 μg/ml penicillin and streptomycin. Thin strips were removed from the lung periphery adjacent to the pleural surface, finely minced, and transferred with 2–3 ml DMEM to a 15-ml conical tube containing 3-ml digestion solution. [0.5 g BSA, 10 000 U type 2 collagenase (Worthington Biochemical Co, Lakewood, NJ), and cmf-PBS (Gibco BRL) to make 10 ml total volume]. The digestion mixture was allowed to incubate at 37 °C for 15 min before pouring through an 80-mesh sieve into a sterile 200-ml beaker. An additional 5 ml of normal medium [10% fetal bovine serum (FBS), Hyclone, Logan, UT) with 30 μg/ml penicillin and streptromycin in DMEM] was used to wash the sieve. The isolation mixture was transferred to a 15 ml conical tube and centrifuged at $300 \times g$ for 5 min, the medium aspirated, and the cells resuspended with 5 ml complete medium [1 part microvascular conditioned medium: three parts incomplete medium (80% RPMI 1640, 20% FBS, 12.3 U/ml Heparin (Elkins-Sinn, Cherry Hill, NJ), and 6.7 μg/ml Endogro (Vec Technologies, Rensselaer, NY) with 30 μg/ml penicillin and streptomycin]. Centrifugation/aspiration was repeated, the cells resuspended in 2–3 ml complete medium and allowed to incubate at 37 °C for 30 min before being placed drop wise onto 35-mm culture dishes. After 1 h at 37 °C with 5% CO_2, 3 ml of complete medium was added. The dishes were checked daily for contaminating cells that were removed by scraping and aspiration. Endothelial cell colonies were isolated with cloning rings, trypsinized, resuspended in 100 μl complete medium and placed as a drop in the center of a T-25 flask. The cells were allowed to attach (1 h at 37 °C with 5% CO_2) before the addition of 5 ml complete medium. Cultures were characterized using, uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled low-density lipoprotein (DiI-acetylated LDL), a lectin-binding panel and were routinely passaged by scraping [29].

2.3. Chimeric molecules and antibodies

Recombinant human ALCAM-Fc, recombinant mouse ALCAM-Fc and recombinant human VE-cadherin-Fc chimeric proteins were purchased from R&D Systems (Minneapolis, MN). Generation of rabbit anti-rat ALCAM antibody BRI-1 has previously been described as anti-HB2 [21,30]. BRI-1 antiserum was purified on a protein-A column (ImmunoPure Plus High Capacity, Pierce Biotechnology, Inc. Rockford, IL). Monoclonal anti-ALCAM antibodies used were anti-human clone MOG/07 (Novacastra, Newcastle, UK), anti-rat ALCAM clone 2117 (gift from Genetech) and purified and FITC-labeled anti-human ALCAM clone J4-81 (Antigenix America, Huntington Station, NY). Horse radish peroxidase (HRP) conjugated antibodies used included anti-mouse and -rabbit IgG (Santa Cruz Biotech and Jackson Laboratories, West Grove, PA). Alexafluor 488 and 594 were from Molecular Probes, (Eugene, OR). Fab fragments were made by incubating monoclonal antibodies with immobilized Papain beads (Pierce, Biotechnology, Rockford) for 3 h at 37 °C, according to the manufacturer's protocol; uncut IgG and Fc fragments were then removed by repeated passage over Protein G Sepharose. Purity and proper size of IgG and Fab fragments were confirmed by SDS–PAGE. Anti-human IgG (Fc-specific) Fab was purchased from Sigma (St Louis, MO) and FITC-labeled mouse IgG1 from Antigenix America.

2.4. Flow cytometry

THP1 and HL60 monocytes were washed once in IMDM with 5% FBS, then resuspended in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide (FACS buffer) with saturating amounts of human Fc receptor block (Miltenyi Biotech Inc. Auburn, CA). After five-minute incubation on ice, FITC-labeled anti-human ALCAM antibody clone J4-81 or an isotype-matched control (FITC-labeled mouse IgG1) was added to the cells for 30 min. After 30 min of incubation on ice, cells were washed three times and then resuspended in FACS buffer and analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). Additional control cell suspensions were incubated with secondary antibody alone.

2.5. Cell fractionation and Immunoblotting

Cell lysates prepared with ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing 1% triton X-100 (v/v) and supplemented with 1% protease inhibitor cocktail (Roche, Indianapolis, IN) was clarified by centrifugation at 13 000 rpm for 15 min at 4 °C, and soluble cell fractions harvested. Protein content in cell lysates was measured using a Lowry protein assay (Sigma). Lysates were combined with Laemmli buffer (Sigma), boiled for 2 min and resolved by electrophoresis on a 10% polyacrylamide gel. Samples were blotted unto nitrocellulose membranes, probed with antibodies, and protein bands identified by chemiluminescence (Fujifilm LAS-1000 imaging system, FujiFilm, Valhalla, NY).

2.6. Immunostaining

Endothelial cells seeded on glass cover slips were fixed with methanol and blocked with 2% normal goat serum for 10 min, followed by staining with affinity purified BRI-1 (1: 200 dilution), and Alexflour 594 (1/1000 dilution) (Molecular Probes). Cells were mounted with Dako Fluorescent mounting media (Dako, Carpinteria, CA). For negative control, the steps with primary antibody were omitted, and no specific immunoreactivity was detected in those slides. Stained cells were visualized using a laser confocal scanning microscope (Leica TCS SP2, Leica, Exton, PA) and by epifluorescence (Nikon TE2000, Nikon Instruments Inc., Melville, NY).

2.7. Cloning and expression of ALCAM-GFP

ALCAM cDNA was amplified from total RNA isolated from cultured rat PMVECs using gene-specific primers flanked with recognition and cleavage sites for Age I and SaI I. (Forward primer; 5′-TTGTCGGTGGCCTTCTAGGA-3′, reverse primer; 5′-GGCTTCTGTTTTGTGATTG-3′). PCR product was sub-cloned into the *Age*I/*Sal*I site in pRV-CMV-eGFP vector (Applied Vironomics, Fremount, CA). Multiple clones were isolated and verified by sequence analysis. For transfection, log-phase growing human K562 cells and semi-confluent rat PMVECs seeded on glass cover slips were transfected with ALCAM-GFP plasmid DNA (2.5 μg) using lipofectamine 2000 (Invitrogen). Stable lines of K562-ALCAM-GFP and ALCAM-GFP were selected with G418 (700 μg/ml) for 20 days. Expression of ALCAM-GFP was examined by epifluorescence (Nikon TE2000). MetaMorph premier software was used to obtain *z*-stacks of K562-ALCAM-GFP clusters.

2.8. Monocyte–endothelial cell adhesion

PMVECs were grown to confluence in 96-well plates. THP1 monocytes were labeled with 5 μg/ml calcein-AM (Molecular Probes) at 37 °C for 20 min, and washed twice with phenol-red free IMDM containing 10% FBS. Fluorescent-labeled THP1 monocytes (10^4/well) were added to PMVEC monolayers at 37 °C for 30 min in the presence