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Short Communication: Expression and Regulation of Activated Leukocyte Cell Adhesion Molecule in Human Retinal Vascular Endothelial Cells

Justine Ruth Smith, MBBS, PhD^{a,b}, Timothy Chipps, MAT^a, Hoda Ilias, BS^a, Yuzhen Pan, MB^a, and Binoy Appukuttan, PhD^a

^aCasey Eye Institute, Oregon Health & Science University

^bDepartment of Cell & Developmental Biology, Oregon Health & Science University

Abstract

Activated leukocyte cell adhesion molecule (ALCAM; CD166) is an immunoglobulin superfamily member that has been described in several non-ocular endothelial populations, but not in relation to endothelium within the eye. Studies in extraocular systems have implicated ALCAM in angiogenesis and leukocyte transendothelial migration, which are key processes in retinal vascular diseases. We investigated the expression of ALCAM in human retinal endothelium, and studied the regulation of expression by established angiogenic and inflammatory stimuli. Retinal endothelial expression of ALCAM was detected in primary retinal endothelial cultures isolated from human cadavers by RT-PCR (n = 4 donors) and Western blot (n = 4 donors), and in intact human retina by immunohistochemistry (n = 3 donors). In the 4 donors studied by RT-PCR, transcript encoding the truncated soluble isoform, sALCAM, was also detected. Quantitative real-time RT-PCR demonstrated significant up-regulation of ALCAM and sALCAM in response to stimulation with master cytokine, tumor necrosis factor (TNF)- α . However, general inflammatory stimulus, lipopolysaccharide (LPS), and the prototype Th1, Th2 and Th17 cytokines, interferon (IFN)- γ , interleukin (IL)-4 and IL-17A, respectively, did not impact ALCAM or sALCAM expression. In contrast, expression of ALCAM was significantly up-regulated by vascular endothelial growth factor (VEGF)₁₆₅. Up-regulation in the presence of VEGF and TNF- α , but not LPS, IFN- γ , IL-4 and IL-17A, suggests a potential role for ALCAM in human retinal angiogenesis in some settings.

Keywords

activated leukocyte cell-adhesion molecule; retina; endothelial cell

Activated leukocyte cell adhesion molecule (ALCAM; CD166) belongs to the immunoglobulin (Ig) superfamily, and thus is a transmembrane protein with five extracellular Ig domains and a short cytoplasmic segment (Swart, 2002). Since the first description of ALCAM – or BEN – in the chick embryo (Pourquie et al., 1990), the protein

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Corresponding author: Justine R. Smith, MBBS, PhD, Address: Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239, United States, Telephone: 1-503-494-5023, Facsimile: 1-503-494-6875, smithjus@ohsu.edu.

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has been identified in many species, including humans (Swart, 2002). A wide variety of stem cells and differentiated cells express ALCAM; these include neuronal cells, epithelial cells, different mesenchymal cells, endothelial cells and leukocytes (Swart, 2002). The primary receptor for ALCAM is CD6 (Bowen et al., 1995), but ALCAM also functions as a ligand for galectin-8 (Delgado et al., 2011) and demonstrates low affinity homophilic interactions (van Kempen et al., 2001). A soluble endothelial isoform, sALCAM, which consists of the amino-terminal variable-type Ig domain (Ikeda and Quertermous, 2004), is also described.

To date much research has focused on the role of ALCAM in neural development. Indeed, although never studied in the human eye, the ability of ALCAM to guide retinal axons is well documented in *Drosophila*, goldfish and rodents (Petrausch et al., 2000; Ramos et al., 1993; Weiner et al., 2004). Multiple other functions for ALCAM are reported, however. Several groups have independently demonstrated the ability of endothelial ALCAM to promote the growth of new blood vessels in extraocular systems, using endothelial precursor cells and adult microvascular endothelial cells in cluster and/or tube formation assays (Arai et al., 2002; Delgado et al., 2011; Ikeda and Quertermous, 2004; Ohneda et al., 2001). Other studies point to involvement of endothelial ALCAM in the transmigration of various leukocyte subpopulations; ALCAM blockade prevents lymphocyte and/or monocyte diapedesis across simulated pulmonary, brain and pancreatic tumor endothelium (Cayrol et al., 2008; Masedunskas et al., 2006; Nummer et al., 2007).

Ischemic retinal vasculopathies, diabetic retinopathy and retinopathy of prematurity, and posterior uveitis represent important causes of vision loss in the United States and worldwide (Congdon et al., 2003; Suttrop-Schulten and Rothova, 1996; Wheatley et al., 2002). Angiogenesis and leukocyte transendothelial migration are key mechanisms in the pathogenesis of ischemic retinal vasculopathy and posterior uveitis, respectively (Cheung et al., 2010; Crane and Liversidge, 2008; Sapieha et al., 2010b), and as discussed above, studies conducted outside the eye suggest the possibility that ALCAM might participate in both processes. Expression of ALCAM within the human eye has not been previously described, and there are no publications reporting expression of ALCAM by retinal endothelium in any species. Endothelial cells show considerable molecular heterogeneity depending on vascular location (Chi et al., 2003), and expression of other Ig superfamily members, in particular, varies in different endothelial subpopulations (Kanda et al., 1998). In this study, we evaluated retinal endothelial expression of ALCAM, using primary human retinal endothelial cells and intact human retinal tissue, and subsequently investigated regulation of expression by common angiogenic and inflammatory stimuli.

Retinal endothelial cells were isolated from paired globes of human cadavers without history of retinal vascular disease (Lions Eye Bank of Oregon, Portland, OR), following a method that is well established in our laboratory and published (Smith et al., 2007). For RNA studies, cells were treated with RLT lysis buffer (Qiagen, Valencia, CA), and RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA), including the optional on-column DNase treatment. To extract protein, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (i.e., 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM ethylene diamine tetra-acetic acid, 50 M sodium fluoride, 0.15 M sodium chloride and 0.01 M sodium phosphate (pH 7.2), with Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN)).

For studies on the regulation of ALCAM expression, which required large numbers of endothelial cells, the cells were transformed by transduction with the murine recombinant amphotropic retrovirus, LXS_N16E6E7, as we have previously reported (Furtado et al., 2012). The immortalized cells retain the endothelial phenotype as evidenced by: cobblestone

morphology; expression of CD31 and von Willebrand factor; and capillary-like tube formation on provisional extracellular matrix. Retinal endothelial cells were grown to confluence in 6-well plates in MCDB-131 medium with 10% fetal bovine serum (FBS) and EGM-2 SingleQuots supplement (Clonetics-Lonza, Walkersville, MD), subsequently rested for 24 hours in MCDB-131 medium with 2% FBS, and finally treated in quadruplicate with one of the following proteins or medium alone for 4 hours: (1) lipopolysaccharide (LPS, 10 µg/ml, Sigma-Aldrich, St. Louis, MO); (2) tumor necrosis factor alpha (TNF-α, 10 ng/ml, R&D Systems, Minneapolis, MN); (3) interferon-gamma (IFN-γ, 10 ng/ml, R&D Systems); (4) interleukin (IL)-4 (10 ng/ml, R&D Systems); (5) IL-17A (100 ng/ml, R&D Systems); and (6) vascular endothelial growth factor A, isoform 165 (VEGF₁₆₅, 20 ng/ml, Millipore, Temecula, CA).

Standard RT-PCR was used to evaluate the presence of ALCAM and sALCAM in primary retinal endothelial cell isolates from 4 human donors, and quantitative real-time RT-PCR was employed to evaluate the response of immortalized human retinal endothelial cells to stimulation with one of the six molecules described above. cDNA was synthesized from total RNA of the retinal endothelial cells using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Sequences of the primers were as follows: ALCAM (5'-TCTGCAGGAGGTTGAAGGAC-3' and 5'-GCCTCATCGTGTCTGGAAT-3'); sALCAM (5'-ATACCTTGCCGACTTGACGTACCT-3' and 5'-AAAGAACATGGTCTGGTACTGGCC-3'); intercellular adhesion molecule (ICAM)-1 (5'-TAAGCCAAGAGGAAGGAGCA-3' and 5'-CATATCATCAAGGGTTGGGG-3'); β-actin (5'-GAGAAGATGACCCAGATCATG-3' and 5'-ATCTCCTGCTTGCTGATCCACAT-3'); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-AGCTGAACGGGAAGCTCACTGG-3' and 5'-GGAGTGGGTGTCGCTGTGAAGTC-3'). Standard PCR, with initial “touch down” was performed on the GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). Products were electrophoresed on 1.5% agarose gel for visualization on the Eagle Eye II (Stratagene, Santa Clara, CA). For quantitative real-time PCR, relative expression of gene products, normalized to GAPDH, was determined on a thermocycler (Chromo4; Bio-Rad Laboratories, Hercules, CA) with SYBR Green nucleic acid dye (iQ SYBR Green Supermix; Bio-Rad Laboratories). Known concentrations of purified control product, simultaneously amplified, were used to generate a standard curve from which the relative expression of experimental samples could be read. Levels of expression of transcript by control and stimulated cells were compared by two-tailed Student t-test. A p-value less than 0.05 was taken to indicate a statistically significant difference. All comparisons of stimulated versus control cells were repeated, and if results from two experiments were contradictory, a third study was performed.

Expression of ALCAM protein in the human retina was studied by Western blot of protein extract from cultured retinal endothelial cells from 4 human donors and immunostaining of intact retinal tissue. For the Western blot, boiled protein samples were loaded to 10% polyacrylamide gel in the amount of 10 µg/well. After gel electrophoresis, proteins were transferred to nitrocellulose membrane, which was blocked with 10% milk and probed with polyclonal goat immunoglobulin G (IgG) anti-human ALCAM antibody (R&D Systems), diluted 1:200, and monoclonal mouse IgG1 anti-human β-actin antibody (clone AC-15; Sigma-Aldrich). Antigen-antibody complexes were detected with IRDye 800CW-conjugated donkey anti-goat IgG antibody, diluted 1:5000, and IRDye 680-conjugated donkey anti-mouse IgG antibody, diluted 1:5000 (both obtained from LI-COR Biosciences, Lincoln, NE), and imaged on the Odyssey Infrared Imager (LI-COR Biosciences). For immunohistochemical analysis, human retina was dissected from eyes of 3 human cadavers with no history of retinal vascular disease (Lions Eye Bank of Oregon), fixed in 70% ethanol for up to 24 hours, and embedded in paraffin. ALCAM was visualized in 5 µm

tissue sections, according to our published indirect immunostaining method (Smith et al., 2003), with omission of the antigen retrieval step, using mouse monoclonal anti-human ALCAM antibody (Clone: MOG/07, IgG2a κ , Novocastro-Leica Microsystems, Newcastle Upon Tyne, United Kingdom), at 1:10 dilution (i.e., 14 μ g/ml), or as a negative control, mouse IgG2a directed against irrelevant antigen (R&D Systems), at an equivalent dilution.

Studies conducted in extra-ocular systems (Arai et al., 2002; Cayrol et al., 2008; Delgado et al., 2011; Ikeda and Quertermous, 2004; Masedunskas et al., 2006; Nummer et al., 2007) imply that retinal endothelial ALCAM has the potential to participate in new vessel growth in retinal ischemic vasculopathy and leukocyte migration in posterior uveitis. However, expression of ALCAM by human retinal endothelium has not been previously reported. We examined the expression of ALCAM by cultured primary human retinal endothelial cells at both transcript and protein level. The molecule was detected in isolates from 7 human cadavers (Figure 1A and 1B). Cells may change as a result of culture in isolation from their microenvironment. To address this possibility, we immunostained sections of intact retina dissected from eyes of 3 additional human cadavers. For the 3 human eyes, positive staining of vascular endothelium was seen in sectioned retina stained with anti-human ALCAM antibody in comparison to tissue stained with isotype-matched control antibody directed against an irrelevant antigen (Figure 1C). Diffuse staining in the inner plexiform layer, ganglion cell layer and nerve fiber layer was also noted, as expected from results of studies of retinal neural development in the mouse (Weiner et al., 2004).

The soluble form of ALCAM, sALCAM, is described in non-ocular endothelial cells (Ikeda and Quertermous, 2004). This is a truncated isoform of the protein that modulates ALCAM activity in capillary-like tube formation assays. There is no commercially available antibody to detect the specific Ig domain that constitutes sALCAM. However, expression may be readily studied at transcript level using RT-PCR with primers corresponding to sequence in one exon unique to sALCAM. In addition to identifying ALCAM transcript in retinal endothelial isolates from 4 human cadavers, we also identified sALCAM transcript by RT-PCR (Figure 1A).

After establishing that human retinal endothelial cells expressed ALCAM and sALCAM, we used real-time quantitative PCR to evaluate the regulation of expression in response to five inflammatory molecular mediators, i.e., TNF- α , LPS, IFN- γ , IL-4 and IL-17A (Figure 2). For comparison and to provide a positive control for the stimulation procedure, we also studied expression of ICAM-1. Intercellular cell adhesion molecule 1, also an Ig superfamily member, is the best characterized leukocyte adhesion molecule on vascular endothelium (Long, 2011), and has been strongly implicated in leukocyte migration into the retina in posterior uveitis (Liversidge et al., 1990; Uchio et al., 1994; Whitcup et al., 1992; Whitcup et al., 1993; Xu et al., 2003; Zaman et al., 1994). Tumor necrosis factor alpha is a “master cytokine”, having multiple activities that promote intraocular inflammation (Khera et al., 2010). Exposure of human retinal endothelial cells to TNF- α significantly increased cellular expression of both ALCAM and sALCAM, as well as ICAM-1. Interestingly, and in contrast, LPS, which is a general inflammatory stimulus and strongly up-regulated ICAM-1, did not impact expression of ALCAM or sALCAM. Expression of ALCAM and sALCAM was also not altered in the presence of prototype Th1, Th2 and Th17 inflammatory cytokines, IFN- γ , IL-4 and IL-17A, respectively, although ICAM-1 expression was increased in response to IFN- γ .

Vascular endothelial growth factor was the first angiogenic factor to be identified in the retina (Aiello et al., 1994), and VEGF165 has since been shown to play a central role in the pathogenesis of retinal neovascularization (Cheung et al., 2010; Sapieha et al., 2010a). We observed a significant increase in expression of ALCAM in human retinal endothelial cells

after exposure to VEGF₁₆₅; although an increase in sALCAM was not statistically significantly different following exposure, there was trend to up-regulation of the soluble isoform also. Expression of ICAM-1 by the cells was not affected by the 4-hour stimulation with VEGF, although an increase would have been expected had the exposure to VEGF been longer, per previous report (Chen et al., 2005). Interestingly, TNF- α , which did impact levels of ALCAM and sALCAM, is also implicated in the development of retinal new vessels in murine oxygen-induced retinopathy, which is a standard animal model of retinal ischemic vasculopathy (Higuchi et al., 2009; Kociok et al., 2006; Majka et al., 2002).

In summary, using RT-PCR and western blot of primary retinal endothelial total RNA and protein lysate, respectively, and immunostaining of intact retina, from multiple human cadaver donors, we demonstrate retinal endothelial expression of ALCAM and soluble isoform, sALCAM. Studies of regulation show induction by VEGF and TNF- α , but not LPS, IFN- γ , IL-4 and IL-17A. These findings suggest the possibility that ALCAM might participate in the process of angiogenesis in the human retina in certain settings, but are not strongly supportive of a role in leukocyte migration.

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Figure 1.

(A) Agarose gel demonstrating RT-PCR products from retinal endothelial cells isolated from 4 human cadaver donors, plus no template control. Expected product sizes: ALCAM: 349 bp; sALCAM: 348 bp; β -actin: 735 bp. Lanes: L = ladder; D = donor; / = no cDNA. (B) Western blot showing expression of ALCAM protein in human retinal endothelial cells from 4 human cadaver donors. Expected protein sizes: ALCAM: 107 kDa; β -actin: 42 kDa. L = protein ladder; D = donor. (C) Photomicrographs of immunostained human retina: (a) diffuse expression of ALCAM within inner plexiform layer, ganglion cell layer and nerve fiber layer of human retina, but (b) no staining in negative control (Original Magnification: 400X); and (c) expression of ALCAM by retinal vascular endothelium, but (d) no staining in negative control (Original Magnification: 1000X). Fast Red with hematoxylin counterstain. PR = photoreceptors; ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL = ganglion cell layer; NFL = nerve fiber layer.

Figure 2.

Results of quantitative real-time RT-PCR analysis showing ALCAM, sALCAM (pink) and ICAM-1 (blue) expression by human retinal endothelial cells stimulated for 4 hours with LPS, TNF- α , VEGF, IFN- γ , IL-4 and IL-17 or medium alone. Conditions were tested in quadruplicate. Bars indicate mean. Error bars indicate standard error of mean. * = $p < 0.05$. Each graph is representative of two independent experiments.