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ICAM-1 mediates surface contact between neutrophils and keratocytes following corneal epithelial abrasion in the mouse

Debjani Gagen [a,b,*], Sara Laubinger [b], Zhijie Li [b,c], Matei S. Petrescu [d], Evelyn S. Brown [a], C. Wayne Smith [b], Alan R. Burns [a,b]

[a] College of Optometry, University of Houston, Houston, TX, USA
[b] Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA
[c] Jinan University, Guangzhou, China
[d] Department of Pediatrics, Tulane School of Medicine, New Orleans, LA, USA

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ABSTRACT

Corneal epithelial abrasion elicits an inflammatory response involving neutrophil (PMN) recruitment from the limbal vessels into the corneal stroma. These migrating PMNs make surface contact with collagen and stromal keratocytes. Using mice deficient in PMN integrin CD18, we previously showed that PMN contact with stromal keratocytes is CD18-dependent, while contact with collagen is CD18-independent. In the present study, we wished to extend these observations and determine if ICAM-1, a known ligand for CD18, mediates PMN contact with keratocytes during corneal wound healing. Uninjured and injured right corneas from C57Bl/6 wild type (WT) mice and ICAM-$1^{-/-}$ mice were processed for transmission electron microscopy and imaged for morphometric analysis. PMN migration, stromal thickness, and ICAM-1 staining were evaluated using light microscopy. Twelve hours after epithelial abrasion, PMN surface contact with paralimbal keratocytes in ICAM-$1^{-/-}$ corneas was reduced to ~50% of that observed in WT corneas; PMN surface contact with collagen was not affected. Stromal thickness (edema), keratocyte network surface area and keratocyte shape were similar in ICAM-$1^{-/-}$ and WT corneas. WT keratocyte ICAM-1 expression was detected at baseline and ICAM-1 staining intensity increased following injury. Since ICAM-1 is readily detected on mouse keratocytes and PMN-keratocyte surface contact in ICAM-$1^{-/-}$ mice is markedly reduced, the data suggest PMN adhesive interactions with keratocyte-stromal networks is in part regulated by keratocyte ICAM-1 expression.

1. Introduction

Corneal epithelial abrasion, resulting from physical damage or refractive surgery, elicits an inflammatory response (Belmonte et al., 2004; Burns et al., 2005; Chinnery et al., 2009; Hamrah et al., 2003; Li et al., 2006a, 2006b; O'Brien et al., 1998; Pearlman et al., 2008; Wilson et al., 2001) characterized by an acute recruitment of neutrophils (PMNs) into the stroma (Burns et al., 2005; Li et al., 2006a, 2006b; Wilson et al., 2001). Li and colleagues showed PMN emigration from limbal vessels is facilitated by leukocyte-specific Beta (β)-2 integrin, CD18. Furthermore, they showed that the total absence of CD18, or absence of specific CD18 family members (CD11a/CD18-LFA-1 or CD11b/CD18-Mac-1), not only delayed entry of PMNs into the stroma but also significantly delayed wound closure (Li et al., 2006a, 2006b).

PMN transendothelial migration in the systemic circulation is well known to be regulated by CD18 integrins (Carlos and Harlan, 1994; Jaeschke and Smith, 1997; Ley, 2001; Phillipson et al., 2006). Most recently, in response to central corneal epithelial abrasion, we identified a novel extravascular role for CD18 during PMN migration through the corneal interstitium (Petrescu et al., 2007). We observed that PMNs preferentially infiltrate the anterior stroma and migrate within the interlamellar spaces where they make close surface contacts with the surrounding collagen and paralimbal keratocytes. Close contact with keratocytes requires CD18 while contact with collagen does not. It is well known that keratocytes form a cellular network and we suggested keratocytes function as a "cellular highway" for CD18-dependent PMN migration within the stroma. The identity of the CD18 ligand expressed on the keratocyte was not determined.

* Corresponding author. University of Houston, College of Optometry, 505 J Davis Armistead Bldg, Houston, TX 77204-2020, USA. Tel.: +1 713 743 0740; fax: +1 713 734 2053.
E-mail address: dgagen.2010@alumni.opt.uh.edu (D. Gagen).

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It is well established that endothelial intercellular adhesion molecule-1 (ICAM-1) plays a key role during inflammation, serving as a ligand for CD18 integrins and thereby facilitating PMN recruitment (Brake et al., 2006; Burns et al., 1994; Ley, 1996; Moreland et al., 2002; Oberyszyn et al., 1998). ICAM-1 is a five-domain transmembrane glycoprotein that binds to PMN integrin CD11a/CD18 at domain one and CD11b/CD18 at domain three (Diamond et al., 1993; Huang and Springer, 1995; Lynam et al., 1998, Roebuck and Finnegan, 1999; Smith et al., 1989). *In vitro* and *in vivo* studies of the cornea show that ICAM-1 is expressed on epithelial cells (Byeseda et al., 2009; Hobden et al., 1995; Kumagai et al., 2003; Li et al., 2007, Liang et al., 2007; Yannariello-brown et al., 1998), keratocytes (Hobden et al., 1995; Kumagai et al., 2003; Liang et al., 2007; Pavilack et al., 1992; Seo et al., 2001), and endothelial cells (Elner et al., 1991; Hobden et al., 1995; Pavilack et al., 1992). We and others have observed increased ICAM-1 staining on mouse corneal epithelial cells following epithelial abrasion or *Pseudomonas* infection (Byeseda et al., 2009; Hobden et al., 1995; Li et al., 2007, 2006a). With respect to the corneal keratocyte, *in vitro* studies of human corneal explants show increased levels of ICAM-1 staining after cytokine treatment (Pavilack et al., 1992). In the mouse, baseline immunostaining for keratocyte ICAM-1 reportedly increases *in vivo* after *Pseudomonas* infection (Hobden et al., 1995) but whether it increases after simple epithelial abrasion is unknown. Furthermore, it remains to be determined if ICAM-1 expression on mouse keratocytes mediates PMN close surface contact with keratocytes.

The purpose of this study is to evaluate the relative contribution of ICAM-1 to PMN stromal migration by determining if close surface contact between migrating PMNs and stromal keratocytes is ICAM-1-dependent.

2. Methods

2.1. Animals

Male C57Bl/6 wild type mice (WT) were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at Baylor College of Medicine animal housing facilities. ICAM-$1^{-/-}$ mice were backcrossed at least 10 generations with C57Bl/6 mice. Twenty-eight mice ($n = 14$ of each strain), ages 6–10 weeks, were used in this study (Byeseda et al., 2009). All animals were treated according to the guidelines described in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Baylor College of Medicine Animal Care and Use Committee policy.

2.2. Wound protocol

Pentobarbital (Nembutal; Ovation Pharmaceuticals, Deerfield, IL) was administered intraperitoneally (50 mg/kg body weight) to anesthetize the mice. A 2 mm diameter trephine was used to demarcate the central epithelial region of the right eye and the epithelium within the demarcated region was mechanically removed using an Algerbrush II (Alger Equipment Co., Inc., Lago Vista, TX) under a dissecting microscope.

2.3. Immunohistochemistry

For histologic studies, WT and ICAM-$1^{-/-}$ mice were humanely euthanized (1-chloro-2,2,2-trifluoroethyldifluoromethyl ether-Isofluorane inhalation followed by cervical dislocation) and the eyes were enucleated. Corneas were excised from ICAM-$1^{-/-}$ and WT mice and incubated at 37 °C for 30 min. Epithelial sheets were removed and corneas were fixed in 2% paraformaldehyde (Tousimus Research Corporation, Rockville, MD) in 0.1 M phosphate buffered saline (PBS, pH 7.2) at 4 °C for 60 min, blocked with PBS with 2% bovine serum albumin (BSA), and permeabilized with 0.1% Triton-X. Radial cuts were made from the peripheral edge to the paracentral region. Uninjured and 12 h injured corneas (a time point when PMN stromal infiltration is underway, (Li et al., 2006c)) were incubated with unconjugated rabbit anti-ALDH3A1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C. All corneas were washed three times with PBS/2% BSA and incubated overnight with goat-anti-rabbit Cy5 conjugated secondary IgG (Abcam, San Francisco, CA) to identify ALDH3A1-positive keratocytes, PE conjugated anti-ICAM-1 antibody (clone YN-1, Abcam, San Francisco, CA) to evaluate ICAM-1 expression on keratocytes, FITC conjugated Ly6-G antibody to detect PMNs (BD Bioscience, Pharmingen, San Jose, CA), and DAPI (4′,6-diamidino-2-phenylindole, Sigma, St. Louis, MO) to detect nuclei. Separate 12 h injured corneas were stained with a PE conjugated antibody against Thy1.2 (Ishihara et al., 1987; Pei et al., 2004) (BD Bioscience, Pharmingen, San Jose, CA), a fibroblast marker, and FITC conjugated antibody against alpha-smooth muscle actin, a myofibroblast marker (Jester et al., 1995; Yoshida et al., 2005) (Sigma, St. Louis, MO). A third set of uninjured and injured corneas served as control for non-specific antibody staining and was incubated with the appropriate isotype matched non-immune IgG antibodies. All corneas were mounted in AIRVOL mounting media (Celanese, Dallas, TX). Images through the full thickness of the paralimbal corneal region, immediately central and adjacent to the limbus, were obtained using a DeltaVision Core inverted microscope (Applied Precision, Issaquah, WA) and processed using SoftWorx software.

2.4. Electron microscopy

2.4.1. Tissue processing

Whole uninjured and injured mouse eyes were fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 2 h at room temperature. Fixed eyes were rinsed in 0.1 M sodium cacodylate buffer and stored at 4 °C until further processing. Corneas, with the limbus intact, were carefully excised from the whole eye and cut into four equal-sized quadrants. These quadrants were post-fixed in 1% tannic acid for 5 min and transferred to 1% osmium tetroxide. Specimens were then dehydrated through an acetone series and embedded in resin (EMbed-812, Electron Microscopy Sciences, Hatsfield, PA). Thin plastic sections (80–100 nm) were cut and imaged on a JEOL 200CX (Tokyo, Japan) electron microscope or a Tecnai G^2 Spirit BioTWIN (FEI Company, Hillsboro, OR) electron microscope.

2.4.2. Morphometric analysis

Morphometric analyses using stereological techniques were performed, as previously described (Petrescu et al., 2007), to quantify the percentage of PMN membrane in close contact with stromal structures (keratocytes, other PMNs and collagen) and determine keratocyte network size and surface area. Stereology, a well-established technique, is used for analyzing two-dimensional images (e.g., tissue sections) to obtain unbiased and accurate estimates of geometrical features including feature number, length, surface area, and volume. It has been used extensively over the past 40 years in the study of biological structures (Anderson et al., 1994; Gibbons et al., 2009; Howard and Reed, 1998; Knust et al., 2009; Mahon et al., 2004; Michel and Cruz-Orive, 1988; Petrescu et al., 2007; Schmitz and Hof, 2005; Weibel, 1981), including the cornea (Petrescu et al., 2007) and retina (Anderson et al., 1994; Mahon et al., 2004). Briefly, electron micrographs were recorded from the anterior and posterior paralimbal regions. To avoid observer sampling bias, systematic uniform random sampling (SURS) (Howard and Reed, 1998; Petrescu et al., 2007) of PMNs, keratocytes, and collagen lamellae were obtained by imaging the