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Integrin-dependent neutrophil migration in the injured mouse cornea

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ABSTRACT

As an early responder to an inflammatory stimulus, neutrophils (PMNs) must exit the vasculature and migrate through the extravascular tissue to the site of insult, which is often remote from the point of extravasation. Following a central epithelial corneal abrasion, PMNs recruited from the peripheral limbal vasculature migrate into the avascular corneal stroma. *In vitro* studies suggest PMN locomotion over 2-D surfaces is dependent on integrin binding while migration within 3-D matrices can be integrin-independent. Electron micrographs of injured mouse corneas show migrating PMNs make extensive surface contact not only with collagen fibrils in the extracellular matrix (ECM), but also keratocytes. Evidence supporting involvement of integrins in corneal inflammation has prompted research and development of integrin blocking agents for use as anti-inflammatory therapies. However, the role of integrin binding (cell–cell; cell–ECM) during stromal migration in the inflamed cornea has previously not been clearly defined. In this study *in vivo* time lapse imaging sequences provided the means to quantify cell motility while observing PMN interactions with keratocytes and other stromal components in the living eye. The relative contribution of β1, β2 and β3 integrins to PMN locomotion in the inflamed mouse cornea was investigated using blocking antibodies against the respective integrins. Of the 3 integrin families (β1, β2 and β3) investigated for their potential role in PMN migration, only β1 antibody blockade produced a significant, but partial, reduction in PMN motility. The preferential migration of PMNs along the keratocyte network was not affected by integrin blockade. Hence, the dominant mechanism for PMN motility within the corneal stroma appears to be integrin-independent as does the restriction of PMN migration paths to the keratocyte network.

1. Introduction

Corneal insult, such as a central corneal epithelial abrasion, evokes prompt and extensive inflammation with neutrophils (PMNs) providing the major early cellular response (reviewed in Silva, 2010). PMN infiltration into the injured cornea is necessary for efficient epithelial wound healing (Li et al., 2006). Upon extravasation from the limbal vessels, PMNs must make their way through the compact stroma to reach a remote site of injury. During the course of this journey they encounter an extracellular matrix (ECM) of tightly-spaced sheets (lamellae) of predominantly parallel fibrils of type I collagen laid down in criss-crossed fashion with keratocytes interspersed between lamellae. Keratocytes form an intricate network of inter-connected flattened cells with long cytoplasmic processes arranged in layers parallel with the corneal surface (i.e., "keratocyte network") (Assouline et al., 1992; Nishida et al., 1988; Poole et al., 1993). As such, the corneal stroma presents a unique challenge for infiltrating cells migrating from the limbal vasculature.

Electron micrographs of injured mouse corneas show migrating PMNs make extensive surface contact not only with collagen fibrils, but also keratocytes. Mice deficient in leukocyte β2 (CD18) integrin, or its ligand ICAM-1 (CD54), show reduced contact between PMNs and keratocytes with no change in PMN contact with collagen (Burns et al., 2005; Gagen et al., 2010; Petrescu et al., 2007). Based on these studies, it was suggested that PMNs migrate preferentially upon the keratocyte network, mediated, at least partially, by β2 integrin on the PMN and ICAM-1 on the keratocyte. However, β1 (CD29) and β3 (CD61) integrin families are also expressed on extravascular migrating PMNs and may be involved in interstitial migration (Carter, 2009; Gonzalez et al., 2007; Lindbom and Werr, 2002). *In vitro* studies suggest PMN locomotion over 2-D surfaces is dependent on integrin binding while migration within 3-D matrices can be integrin-independent (Friedl and Brocker, 2000; Friedl et al., 1998; Khandoga et al., 2009; Koenderman et al.,

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2010; Lammermann et al., 2008; Lindbom and Werr, 2002; Mandeville et al., 1997). Interest in the involvement of integrins in corneal inflammation has prompted research and development of integrin blocking agents for use as anti-inflammatory therapies (Chen et al., 2007; Dietrich et al., 2007; Ecoiffier et al., 2008). However, the role of integrin binding during *in vivo* leukocyte migration within the corneal stroma has yet to be clearly defined.

The purpose of the present study was to investigate the role of integrin binding in facilitating PMN motility within the corneal interstitium using *in vivo* confocal microscopy. Time lapse image sequences obtained using the Heidelberg Retina Tomographer III with Rostock Corneal Module (HRT-RCM) provided the means to quantify cell motility while observing PMN interaction with keratocytes and other stromal components in the living eye. The relative contribution of β1, β2 and β3 integrins to PMN locomotion in the inflamed mouse cornea was investigated using blocking antibodies against the respective integrins.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice between the ages of 8–16 weeks were bred and housed at the University of Houston, College of Optometry (UHCO) and were handled according to the guidelines described in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Vision and Ophthalmic Research and UHCO animal handling guidelines.

2.2. Corneal inflammation induced by epithelial debridement

Animals were anesthetized with an intraperitoneal (IP) injection of ketamine (75 mg/kg body weight) and xylazine (7.5 mg/kg body weight). With the aid of a stereo dissecting microscope, eyelashes were trimmed to prevent interference with later imaging. The corneal epithelium was removed in a single vertical stripe approximately 0.5 mm wide and extending to within 0.5 mm of the inferior and superior vascular limbus using an AlgerbrushII with a 0.5 mm burr (Alger Equipment Co., Inc., Lago Vista, TX) held tangentially to the corneal surface so that the direction of burr rotation was downward at the advancing edge. The wound was initiated in the upper cornea (superior or inferior, depending on the orientation of the mouse) moving toward the lower limbus. The mouse was then rotated 180° and the Algerbrush again applied moving from upper to lower cornea. This method provided the most consistent results with well-defined wound edges. The vertical stripe injury elicited an acute inflammatory response initiated at the peripheral vascular limbus. Within the wound area, keratocyte death was observed. However, the wound was small enough that ample parawound area was preserved for imaging PMN migration in the uninjured portion of the stroma where keratocytes remained viable. Mice were kept on an isothermal heating pad while under anesthesia and then placed in an isolation cage for the duration of the 8 h prior to imaging using the Heidelberg Retinal Tomographer III with Rostock Cornea module (400 μm size lens) (HRT-RCM).

2.3. Antibodies

Blocking antibodies were used to assess the contribution of integrins to PMN migration within the stroma. Specific antibody clones were selected based on previously published reports documenting their ability to selectively block β1, β2 or β3 integrin-mediated adhesion. The following antibodies were diluted in normal saline and applied topically immediately post wounding (25 μg/ml or 250 μg/ml working concentrations): anti-β1 (anti-CD29; BioLegend, clone HMβ$_1$-1); anti-β2 (anti-CD18; BD Pharmingen, clone GAME-46); anti-β3 (anti-CD61; BioLegend, clone HMβ$_3$-1). The specificity of these antibodies for their specific integrin targets is well described in the literature [anti-β1 (clone HMβ$_1$-1) (Sangaletti et al., 2008; Werr et al., 1998); anti-β2 (clone GAME-46) (Bowden et al., 2002; Ridger et al., 2001); anti-β3 (clone HMβ$_3$-1) (Piali et al., 1995)].

A combination of all three antibodies (same individual concentrations) was used to test for additive or compensatory effects. Isotype-matched non-immune IgG antibodies were used to control for non-specific antibody effects. Topically applied antibodies (5 μl) were allowed to penetrate the wounded cornea for 5 min. The excess solution was then wicked off the cornea along with any cellular debris and a second application of the antibody solution was applied and allowed to penetrate until the animal recovered from anesthesia (typically 20–30 min without blinking). Left undisturbed, the droplet of solution could still be seen after 30 min and covered the entire cornea (Fig. 1).

2.4. HRT imaging

In vivo leukocyte cell motility was evaluated 8 h after epithelial abrasion. At that time, each mouse was anesthetized with a slightly higher dose of IP ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) to ensure adequate anesthesia during the imaging session. For imaging, the mouse was placed in a heated holding device consisting of a 50 ml centrifuge tube (VWR, Houston TX) with the bottom cut out to allow the mouse head to protrude for imaging and a triangular piece remaining to support the head. The tube was wrapped with a rheostat-controlled heating cable (Zoo Med, Inc., San Luis Obispo, CA) and insulating foam. Body temperature was monitored by rectal probe and maintained between 36.0 and 37.0 °C (Microtherma 2, ETI Ltd. UK). As we observed in pilot experiments, and as others have reported (Miller et al., 2002), cell migration speed is influenced by body temperature and declines as body temperature falls below normal. Corneal temperature was initially measured before and after imaging using an infrared thermistor (Vario-Therm 6000L, Everest Interscience,

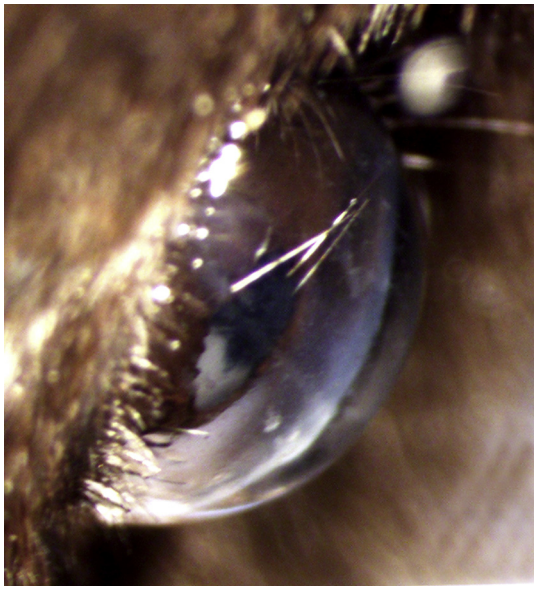

Fig. 1. A 5 μl drop of anti-integrin antibody solution covered the entire cornea and remained intact during the anesthesia recovery time (30–40 min).