PURPOSE. Adhesion molecules play a critical role in leukocyte emigration to wound sites, but differences are evident in different vascular beds. In this study, the contributions of P-selectin to neutrophil emigration into the cornea after central epithelial abrasion were investigated.

METHODS. Re-epithelialization, neutrophil influx, and platelet accumulation were assessed in C57BL/6 mice after removal of a 2-mm diameter area of central corneal epithelium that did not directly injure the limbal vessels or the avascular stroma of the cornea. Comparisons were made between wild-type (WT) mice and mice with targeted deletions of genes for P-selectin, CD18, or CD54, or mice with antibody-induced neutropenia or thrombocytopenia.

RESULTS. After central corneal epithelial abrasion, platelets localized in the limbal vessels and neutrophils emigrated from the limbal vessels to the region of the epithelial wound. There was temporal correspondence of platelet and neutrophil localization, peaking within 12 hours of wounding. Platelet accumulation, neutrophil emigration and corneal epithelial healing as measured by wound closure, basal epithelial cell density, and epithelial cell division were significantly reduced in P-selectin-deficient mice (P-sel−/−). Anti-GP1bα antibody-induced thrombocytopenia in WT mice significantly reduced platelet and neutrophil accumulation and wound healing. Passive transfer of wild-type platelets into P-sel−/− mice significantly restored platelet localization in limbal vessels, neutrophil emigration, epithelial cell division, and epithelial cell migration into the abraded region of the cornea.

CONCLUSIONS. Platelet localization in the limbus of abraded corneas contributes to re-epithelialization, and P-selectin provides a necessary step in this process. (Invest Ophthalmol Vis Sci. 2006;47:4794 – 4802) DOI:10.1167/iovs.06-0381

Neutrophil emigration at sites of inflammation depends on a cascade of adhesive and activating events. The common hypothetical model describes neutrophils interacting with endothelial cells under conditions of shear where adhesive interactions of the selectin family of adhesion molecules (expressed on leukocytes and endothelial cells) lead to rolling, and activation of integrin affinity/avidity by chemokines leads to stationary adhesion to the apical surface of the endothelial cells. Recent evidence indicates that, under some circumstances, platelets contribute significantly to the localization of leukocytes in acute inflammation. For example, Nishijima et al. found that ischemia/reperfusion-induced leukocyte localization in venules of the retina was reduced greater than 70% by depletion of circulating platelets. Conversely, leukocytes appear to contribute to platelet localization under some circumstances. Cooper et al. demonstrated that anti-neutrophil antiserum diminished the localization of platelets by approximately 50% in intestinal venules after ischemia and reperfusion. Similarly, anti-neutrophil antibodies have been shown to inhibit endotoxin-induced platelet localization in intestinal6 and cremasteric venules. Numerous publications document the contributions of platelets to inflammation and tissue injury, and inhibition of the platelet’s role in inflammation is now of considerable therapeutic interest.

We have been investigating the contributions of neutrophils and platelets in the inflammatory response to corneal epithelial abrasion. Superficial wounds in corneal epithelium stimulate leukocyte infiltration from limbal vessels at the periphery of the cornea into the avascular connective tissue stroma,13,14 and the extravasated leukocytes migrate through the stroma to the region of the wound.15 Under normal circumstances, re-epithelialization is rapid, and infiltrating leukocytes return to very low baseline levels, presumably as a result of apoptosis.16 In earlier studies, we found that corneal epithelial abrasion (a lesion that did not directly damage the underlying connective tissue stroma or the blood vessels of the limbus) in C57BL/6 mice induced two waves of neutrophil infiltration. The first peaked at 12 to 18 hours and the second at 30 to 36 hours after injury.18 Closure of a central corneal epithelial wound in C57BL/6 wild-type mice was complete within 24 hours, a time before the second wave of neutrophils. The adhesion molecules required for these two waves of infiltration differed. Mice deficient in both P- and E-selectin (P/E−/−) exhibited little leukocyte infiltration throughout the entire observation time, while mice deficient in CD18 (CD18−/−) exhibited a single wave of emigration corresponding temporally to the second of wild-type mice. In both CD18−/− and P/E−/− mice epithelial wound closure was delayed by 12 to 24 hours. Given these observations and our results that neutropenic mice also exhibited delayed wound healing, it appeared that early inflammation evidenced by neutrophil infiltration facilitated corneal re-epithelialization.

The current report continues the analysis of neutrophil adhesion and emigration and their relationship to re-epithelialization in abraded corneas by analyzing these events in mice with targeted deletions of P-selectin, CD18, or ICAM-1. P-selectin-deficient mice were studied in an effort to determine whether this selectin alone was important to neutrophil emigration and epithelial wound-healing, in light of our earlier study with P/E−/− mice.18 An unexpected finding was that P-selectin deficiency not only resulted in reduced neutrophil

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Platelet Response to Corneal Abrasion Is Necessary for Acute Inflammation and Efficient Re-epithelialization

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4794
Localization but also in significantly delayed platelet accumulation in the region of the limbal vessels and in significantly delayed epithelial wound closure. These observations raise questions about the possible link between platelet localization in the limbal vessels shortly after epithelial injury and the efficiency of wound healing. The data suggest a role for platelets in the inflammatory and epithelial response to corneal injury.

**MATERIALS AND METHODS**

**Animals**

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice with targeted deletions of P-selectin (P-sel−/−),19 CD18 (CD18−/−),20 and CD54 (ICAM-1−/−)21 were backcrossed at least 10 generations with C57BL/6 mice. All mice used in this study were 6 to 8 weeks old, weighed 18 to 20 g, and were handled according to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine.

**Mouse Model for Corneal Epithelial Wound Healing**

The central corneal wound was made as previously described.18,22 Briefly, the central cornea was marked by a trephine 2 mm in diameter and the epithelium was debrided with a diamond blade for refractive surgery (Accutome, Malvern, PA) under a dissecting microscope. After wounding, the extent of corneal wound closure was examined by fluorescein staining every 6 hours and photographed with a digital camera. The size of the epithelial defect was expressed as a percentage of the original wound area.

**In Vivo Platelet Depletion and In Vivo Neutrophil Depletion**

Mice were injected intraperitoneally with rat monoclonal antibodies directed against mouse GPIbα with 0.2 mg in 200 μL PBS 24 hours before corneal wounding. To confirm anti-GPIbα treatment depleted platelets in vivo, the absolute number of circulating platelets was measured with an automated cell counter. The specificity of the anti-GPIbα treatment was assessed by determining the blood leukocyte counts with an automated cell counter. As we have reported,18 a rat anti-mouse Gr-1 monoclonal antibody was used to deplete neutrophils.

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**FIGURE 1.** Morphometric analysis of the response to central corneal epithelial abrasion in C57BL/6 mice. (A) Schematic representation of a wholemount, excised cornea showing microscopic fields (40× objective) across perpendicular diameters of the cornea from limbus to limbus. The diameter of each microscopic field was approximately 0.53 mm. The parameter reflecting epithelial cell division is shown in two photomicrographs of the same field of view stained with DAPI to reveal chromatin (B) and FITC-tagged anti-tubulin (C) and to show the spindle apparatus of mitotic cells at the focal plane of the basal epithelial cells. Scale bar, 20 μm.

**FIGURE 2.** Epithelial wound closure after central corneal epithelial abrasion in C57BL/6 mice. (A) Percentage of the original wound area is plotted over time and was calculated (n = 6, *P < 0.01) from the area of fluorescein staining at each time point (mean ± SEM). (B) Sum of dividing epithelial cells at 18 hours in the microscopic fields of limbus, peripheral, and parawound regions; average of four quadrants sampled per cornea from four mice (*P < 0.01, mean ± SEM). (C) Kinetics of cell accumulation in corneas of wild-type and P-sel−/− mice. Data are the sum of neutrophils (Gr-1+ cells with polymorphic nuclei visible in the stroma of wholemount preparations of corneas) in five fields of view from the limbus to the center (pattern of analysis illustrated in Fig. 1A; n = 4, mean ± SEM). Data for platelets are the sum in eight microscopic fields of view in the limbus region of the wholemount preparations of each cornea (n = 4, mean ± SEM).
antibody (BD-PharMingen, San Diego, CA) was used to deplete peripheral neutrophils. The antibody (0.25 mg) was administered intraperitoneally (IP) 1 day before corneal epithelial wounding was initiated. Treatment with this dose of the antibody induced severe neutropenia for up to 5 days, as assessed by counting more than 200 leukocytes on blood smears, similar to that reported.18,25 Control mice received an equivalent amount of a nonbinding monoclonal antibody (SFDR5).24

Immunohistology

Morphometric analysis of the corneal response to injury used a defined pattern described in our previous publications18 (shown in Fig. 1A). Excised wounded corneas including the limbus were fixed and stained with anti-Gr-1-FITC (to identify neutrophils), anti-CD31-PE, or anti-CD41-PE (to identify platelets) using a previously described protocol.18 The antibodies were obtained from PharMingen. Radial cuts were made in the cornea so that wholemounts could be flattened by a coverslip in mounting medium (Airvol; Air Products and Chemicals, Allentown, PA) containing 1 μM 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO), to assess nuclear morphology and cell division. Figure 1B shows DAPI-stained microscopic images in which cells dividing basal epithelial cells were counted and confirms the presence of associated spindle tubulin (labeled with FITC-conjugated monoclonal anti-tubulin; Sigma-Aldrich), as reported by Song et al.25 The pattern of microscopic analysis illustrated in Figure 1A included counting parameters of inflammation (e.g., leukocytes) or healing (basal cell density or dividing epithelial cells) within nine microscopic fields of view (40× objective, field of view diameter of 0.53 mm) across the cornea from limbus to limbus. Digital images were captured and saved for analysis (DeltaVision; Applied Precision, Issaquah, WA). To compare the relative level of neutrophils in the different areas from the limbus to the central cornea, we counted each cornea separately. To compare the relative level of platelet accumulation in the limbal areas, we counted eight random fields from each cornea separately. At least four corneas were examined for immunohistology, and each cornea was analyzed to obtain the average number per field. The limbus was defined as the intervening zone between the cornea and sclera as the most peripheral field.

Electron Microscopy

Excised corneas were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (1 hour) followed by 1% tannic acid (5 minutes). Postfixation in 1% osmium tetroxide (1 hour) was followed by en bloc fixation in aqueous uranyl acetate. The samples were subsequently dehydrated in a graded ethanol series, embedded in resin (LX 112; Polysciences, Warrington, PA), and ultrathin transverse sections (80 nm) were obtained with an ultramicrotome (RMC 7000; Venana Medical Systems, Tucson, AZ) equipped with a diamond knife. Sections were stained with uranyl acetate and lead citrate before viewing with an electron microscope (200CX; JEOL, Tokyo, Japan).

Isolation of Platelets for Passive Transfer Experiments

Platelets were isolated as described previously.7 Briefly, ~0.9 mL of blood was withdrawn via cardiac puncture from a mouse mixed with 0.1 mL of acid-citrato-dextrose buffer (Sigma-Aldrich), and placed in a polypropylene tube. The blood was centrifuged at 260g for 8 minutes, and then platelet-rich plasma (PRP) was collected and subjected to repeat centrifugation at 260g for 3 minutes. Platelets were pelleted from the PRP by centrifugation at 740g for 10 minutes. The platelet pellet was resuspended gently in 500 μL phosphate-buffered saline (PBS; Sigma-Aldrich) and allowed to rest for 30 minutes, during which platelets in a 20-μL sample of the solution were stained with cresyl blue and counted on a hemocytometer. Thereafter, an aliquot of the solution containing 200 × 10⁶ platelets was centrifuged at 740g for 10 minutes, and the pellet was resuspended gently in 200 μL PBS and injected intravenously into the recipient mouse. This method of platelet isolation has been shown by others5 and us7 to result in less than ~0.01% leukocytes in the platelet suspensions.

Statistical Analysis

Data analysis was performed by using ANOVA and pair-wise multiple comparisons with the Tukey test. P < 0.05 was considered significant. Data are expressed as the mean ± SEM.

Results

A central corneal epithelial abrasion sufficient to remove a 2-mm area of epithelium healed rapidly in the wild-type mice.
wild-type mice (Fig. 3B) but not in P-selectin–deficient mice. (Wistar) IgG1 bound limbal vessels in uninjured corneas in the interpretation that P-selectin was absent, Wug.E9/Rat RB40.34 clone failed to bind limbal vessels, but in contrast to P-selectin. Consistent with the results of Zhu and Dana, the we used two different monoclonal antibodies against murine observations that P-selectin was not detectible in limbal vessels, vessels, the site of leukocyte emigration. To confirm the ob-
P-selectin was not detected by immunostaining in the limbal
mice, P-sel
hours after injury.18 In contrast to the response of the wild-
evident within 2 hours, and peak emigration was evident by 12
recovery of neutrophils within the first 24 hours of injury (Fig. 2C). To determine the possible contribution of platelets in the response to corneal injury, we analyzed platelet accumulation within the
limbal vessels. Platelet accumulation appeared to parallel that of neutrophils, both in the wild-type mice and in the P-sel
mice (Fig. 2C). As we have shown in an earlier publication, neutrophil emigration progresses through the avascular stroma of the cornea extending to the wound margin within 6 hours. Platelet accumulation appeared to be restricted to the region of the limbal vessels (Figs. 3C–G).

**Neutrophil and Platelet Accumulation in P-selectin–Deficient Mice**

Analysis of the inflammatory response to the epithelial injury revealed that neutrophil emigration in wild-type mice was evident within 2 hours, and peak emigration was evident by 12 hours after injury.18 In contrast to the response of the wild-type mice, P-sel
mice had a markedly blunted accumulation of neutrophils within the first 24 hours of injury (Fig. 2C). To determine a possible contribution of platelets in the response to corneal injury, we analyzed platelet accumulation within the

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<th>Neutrophils</th>
<th>Lymphocytes</th>
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<td>Anti-GP1bα‡</td>
<td>64 ± 18.9</td>
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<td>0.3–2.0</td>
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Data were recorded when the corneas were collected for analysis of response to central epithelial abrasion.

* All data are expressed as ×10³/μL of blood.
† Mice underwent central corneal epithelial abrasion.
‡ Wild-type mice treated with anti-GP1bα 24 hours before corneal abrasion.
§ Data were recorded in mice in the Center for Comparative Medicine, Baylor College of Medicine.

(Fig. 2A). Mice deficient in P-selectin (P-sel
) exhibited a significant delay in re-epithelialization (Figs. 2A, 3A) and a significant reduction in epithelial cell division in the regions that were not directly injured (Fig. 2B). The contribution of P-selectin was puzzling, since Zhu and Dana15 reported that P-selectin was not detected by immunostaining in the limbal vessels, the site of leukocyte emigration. To confirm the observations that P-selectin was not detectible in limbal vessels, we used two different monoclonal antibodies against murine P-selectin. Consistent with the results of Zhu and Dana, the RB40.34 clone failed to bind limbal vessels, but in contrast to P-selectin.

**Effect of Thrombocytopenia on Neutrophil Accumulation and Epithelial Healing**

Because accumulation of platelets and neutrophils was markedly reduced in the abraded corneas of P-sel
mice (Figs. 2C, 3E, 3F), we attempted to determine whether platelet localization is necessary for neutrophil accumulation in response to the epithelial injury. The experimental condition used was the induction of thrombocytopenia in wild-type mice by adminis-
tration of an anti-GP1bα monoclonal antibody. In preliminary experiments we found that the protocol described in the Methods section resulted in ~85% reduction in circulating platelets within 1 hour after intravenous administration. Similarly, Nieswandt et al.26 reported that this protocol resulted in comparable thrombocytopenia in mice within 1 hour, and a further reduction to less than 3% of control values at 24 hours that remained constant for 3 to 4 days. The authors found that the selectivity of this treatment was indicated by the finding

**Figure 4.** Effects of systemic administration of anti-GP1bα monoclonal antibody in wild-type mice on neutrophil accumulation in corneas of C57BL/6 mice after central corneal epithelial abrasion. (A) Corneas were abraded and collected either 18 or 30 hours later for wholemount preparations to be stained with FITC-labeled anti-Gr-1 and PE-labeled anti-CD41. Neutrophils (Gr-1
cells with polymorphic nuclei visible in the stroma) were counted in five fields of view from the limbus to the center (pattern of analysis illustrated in Fig. 1A).

Platelets were counted in eight randomly selected fields in the limbal region of the cornea (n = 4, mean ± SEM; *P < 0.01). (B, C) The distribution of neutrophils over regions of the cornea at 18 (B) and 30 (C) hours after central corneal epithelial abrasion (n = 4, mean ± SEM).
that neutrophils, lymphocytes, and monocytes were not significantly different from levels in mice matched for age and strain that received a nonbinding antibody. Thus, over the time span of the acute inflammatory response and re-epithelialization after corneal abrasion, mice receiving anti-GP1bα monoclonal antibody exhibited a normal number of blood neutrophils and marked depletion of blood platelets. Two time points after injury were chosen for the evaluation of neutrophil accumulation and wound healing. These were 18 hours, a time when neutrophils are known to be abundant throughout the stroma of the abraded corneas and epithelial cell division peaks, and 30 hours, a time when a second wave of neutrophil infiltration is peaking and epithelial cell division is sustained. Animals receiving anti-GP1bα monoclonal antibody (Table 1 for blood platelet and leukocyte counts) exhibited significant reductions in platelet accumulation (Fig. 4A) and extravasated neutrophils throughout the corneal stroma at both 18 and 30 hours after wounding (Figs. 4B, 4C). Re-epithelialization, as measured by fluorescein staining of the corneal surface, was significantly delayed (Fig. 5A), and epithelial cell division was significantly reduced as well at both 18 and 30 hours after wounding (Figs. 5B, 5C).

**Figure 5.** Effects of platelet depletion in wild-type mice on parameters of wound healing in corneas of C57BL/6 mice after central corneal epithelial abrasion. (A) Wound closure determined by fluorescein staining of the cornea (technique illustrated in Fig. 3A; n = 5, mean area ± SEM; *P < 0.01). (B, C) Dividing basal epithelial cells in each region of the cornea (pattern given in Fig. 1A) at 18 and 30 (respectively) hours after epithelial abrasion. L, L', opposite limbal regions; PL, PL', opposite paralimbal regions; PW, PW', opposite parawound regions; PC, PC', opposite paracentral regions; and C, center. The PC and C regions encompassed the area of corneal epithelial abrasion (n = 4, mean ± SEM; *P < 0.01).

**Figure 6.** Response to central corneal epithelial abrasion of three adhesion molecule in knockout mice and neutropenic wild-type mice. (A) Twelve or 18 hours after corneal abrasion, corneas were processed as wholemounts and stained for platelets (anti-CD41), neutrophils (anti-Gr-1), and nuclei (DAPI). Data are the sum of platelets in eight microscopic fields of view in the limbal region of the wholemount preparations of each cornea at 12 hours after wounding (n = 4, mean ± SEM). Data below the bar graph are percentage reduction in platelets in the knockout mice at 12 hours, reduction in neutrophils (sum of neutrophil counts from the limbus to the center of the cornea at 12 hours) and basal epithelial cell division (sum of dividing cells from the limbus to the center of the cornea at 18 hours). All values given are significantly different from the wild-type mice (P < 0.01, n = 4, only the mean is given for ease of presentation). (B) Neutrophil and platelet accumulation in the ICAM-1−/− mice are plotted over time after central corneal epithelial abrasion, demonstrating corresponding kinetics in response to injury (n = 4, mean ± SEM). (C) Neutropenic wild-type mouse corneas were evaluated for platelet localization in the limbal vessels and for basal epithelial cell division at 18 hours after central corneal epithelial abrasion. Values for platelets given here are the sum of platelets in eight microscopic fields of view in the limbal region of the wholemount preparations of each cornea (n = 4), and the values for epithelial division are the sum of dividing cells in limbal, paralimbal, and central regions per quadrant of the cornea (n = 4). Anti-PMN indicates mice treated with the monoclonal antibody Gr-1; Control: mice treated with the nonbinding monoclonal antibody SFDR5.
Dependence of Platelet Accumulation on Neutrophils

In an effort to determine whether platelet accumulation in the limbus after central corneal epithelial abrasion was dependent on neutrophil adhesion, mice with targeted CD18 deficiency were evaluated. We have shown that these mice exhibit little neutrophil infiltration within the first 24 hours after corneal wounding. These mice also exhibited marked reduction in platelet accumulation at 12 hours after epithelial injury (Fig. 6A), a time when platelet accumulation was peaking in wild-type mice (Fig. 2C). In addition, evaluation of epithelial cell division was significantly lower at 18 hours after injury than levels in wild-type mice (Fig. 6A). ICAM-1-deficient mice also exhibited a similar pattern of reduced platelet accumulation, neutrophil emigration and epithelial cell division (Fig. 6A). The time courses of platelet and neutrophil accumulation in the injured corneas in ICAM-1-deficient mice were quite similar (Fig. 6B), and in this regard consistent with the studies in both wild-type and P-sel−/− mice. To provide additional data on the dependence of platelet accumulation on neutrophils, platelet localization was assessed at 18 hours after corneal epithelial wounding in neutropenic WT mice (Fig. 6C). Platelet accumulation and epithelial cell division were significantly reduced in neutropenic WT mice (Fig. 6C). These results strengthen the interpretation that neutrophils are necessary for platelet localization and in addition extend our earlier observation that wound healing in neutropenic mice is significantly delayed.

Effect of Wild-type Platelets in Neutrophil Accumulation and Epithelial Healing

To determine whether passive transfer of wild-type (WT) platelets into P-sel−/− mice could restore neutrophil extravasation and epithelial healing, platelets isolated from wild-type mice were transfused (200 × 10⁶ platelets) into P-sel−/− mice immediately before central corneal epithelial abrasion. To add a second precise parameter of wound healing to this experiment, we analyzed the density of basal epithelial cells in the region shown in Figure 1B as paracentral. This area has proven to be a sensitive indicator of re-epithelialization as a region shown in Figure 1B as paracentral. This area has proven to be a sensitive indicator of re-epithelialization.18,22 As shown in Figure 5B, epithelial cell division was evident only in the epithelium that was not directly injured at 18 hours after wounding, because as shown in Figure 5B, epithelial cell division was evident only in the epithelium that was not directly injured at 18 hours (and at 24 hours, not shown). As shown, epithelial migration in the P-sel−/− mice was reduced (n = 4, P < 0.01 at 12, 18, and 24 hours).

Response characterized by platelet localization in the limbal vessels and neutrophil emigration from the limbal vessels to the region of the epithelial wound. (2) The kinetics of platelet and neutrophil localization corresponded, peaking within 12 hours of wounding. (3) P-selectin-deficient mice exhibited significantly reduced platelet accumulation in limbal vessels, neutrophil emigration within the first 24 hours after the central corneal abrasion, and corneal epithelial healing as measured by wound closure, epithelial cell density, and epithelial cell division. (4) Antibody-dependent depletion of circulating platelets also resulted in significant reductions in platelet and neutrophil accumulation and significant reductions in measures of wound healing. (5) Passive transfer of freshly isolated platelets from wild-type mice into mice deficient in P-selectin significantly restored platelet localization in limbal vessels, neutrophil emigration, epithelial cell division, and epithelial cell migration into the abraded region of the cornea.

DISCUSSION

The results in this study support the conclusion that platelets localize in the limbus after central epithelial abrasion and that induced expression of P-selectin provides a necessary step in this process. In addition, platelet localization appears to be necessary for efficient early emigration of neutrophils and efficient re-epithelialization of the cornea. The data supporting these conclusions are as follows: (1) Central corneal epithelial abrasion that did not directly injure the limbal vessels or the avascular stroma of the cornea induced an inflammatory re-
In addition to the apparent contribution of P-selectin to these events, CD18 and ICAM-1 appear to be involved in platelet localization in limbal vessels after corneal abrasion (Fig. 6). One interpretation of these observations is that leukocyte adhesion is necessary for platelet accumulation, since CD18 integrins and ICAM-1 can serve as critical adhesion molecules for neutrophil emigration in acute inflammation.39,40 and platelets have been shown to attach readily to adherent neutrophils.41 Additional support for the interpretation that neutrophil localization is critical to platelet localization is evident in neutropenic mice where platelet accumulation was markedly reduced at 18 hours after wounding (Fig. 6C), a time of significantly elevated platelets in limbal vessels of normal mice. Cooper et al.42 found that depletion of neutrophils significantly reduced platelet accumulation in intestinal venules subjected to ischemia and reperfusion. However, given our observation that platelet depletion markedly reduces neutrophil accumulation (Fig. 4) and published evidence that platelets may have surface CD18,43 the initiating sequence in the coordinated localization of platelets and neutrophils in the limbal vessels remains unresolved.

There is little published evidence regarding the early accumulation of endogenous platelets and their effect on re-epithelialization of a wound. The linking of platelets to wound healing (including the cornea) has largely been in the preparation of platelet-derived topical therapeutics (lysatc or releasates),30–34 rationalized by their rich content of growth factors.30–34 Thrombocytopenia in mice failed to alter the extent of neutrophil accumulation or the rate of skin wound re-epithelialization.36,37 Healing of gastric ulcers in rats was found to be significantly retarded by platelet depletion,38,39 though the primary contribution of platelets in this setting was thought to be their proangiogenic activity, a consequence of platelet accumulation observable in experimental models such as neovascularization of the cornea and subcutaneous implants (Matrigel; BD Biosciences, San Diego, CA).44 or in the context of tumor vascularization.41,42 The role of platelets in hemostasis is well-established,12,43 but most evidence linking platelets to inflammation focuses on the pathogenic aspects of inflammation and the protective or ameliorating influence of reducing platelet activation or aggregation.12,43,44 The results presented in the current paper indicating a potential role for endogenous platelets in the re-epithelialization of the cornea may represent a specialized case in a unique anatomic setting.

In normal conditions, the corneal epithelium is continuously renewed by division in the basal cell layer or by stem cells within the region of the limbus.30–34 The stem cells in the limbus appear to be sensitive to injury by entry into the cell cycle, providing the expansion of basal cells necessary for the coverage of wounded area. Various growth factors are expressed in the renewal process and have been shown to induce proliferation of corneal epithelial cells by autocrine or paracrine mechanisms.14,45 Of particular interest are transforming growth factor (TGFβ), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, IGF-2, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF).49–51 One possible mechanism by which the accumulating leukocytes and platelets contribute to re-epithelialization is the delivery of growth factors to the limbus, the site of stem cells thought to be essential to healing. Platelets are known to contain several growth factors such as PDGF,53 TGFβ,54 and IGF-1,55 all of which are available for release with activation.

Grenier et al.50 reported that HGF is stored in the secretory and secondary granules of the neutrophils and is released in an active form on neutrophil degranulation. Exocytosis of these classes of neutrophil granules occurs rapidly after activation by chemokines59 shown to be present in wounded corneas within the time frame of leukocyte accumulation.60 Another factor found in neutrophil granules of potential importance is CAP37, shown by Pereira et al.61 to augment corneal epithelial cell migration and proliferation. Thus, it is likely that the contribution of platelets and leukocytes is multifactorial.

That re-epithelialization is less efficient in P-selectin–deficient mice is most likely explained by the roles P-selectin plays in the interactions of leukocytes and platelets with endothelium and the interactions between leukocytes and platelets. Since P-selectin appears to be expressed by endothelium in limbal vessels, it could participate in the tethering of both leukocytes and platelets, a phenomenon evident in other vascular beds.62–64 P-selectin in platelets clearly participates in the
adhesion of platelets to leukocytes and in the localization of leukocytes in tissues. An argument that limbal vessel endothelial P-selectin contributes to corneal inflammation is supported by the observation that limbal vessel was labeled with anti-P-selectin antibody WugE9 (Fig. 3B) and by the observed delay in the accumulation of passively transferred wild-type platelets in P-selectin–deficient mice (Fig. 8A). This delay could reflect the absence of endothelial P-selectin in the knockout mice. Platelet P-selectin, however, substantially restores the early phases of corneal epithelial healing and neutrophil localization, as evidenced by the results of passive transfer of wild-type platelets to P-selectin–deficient mice. Thus, the observations in the current study indicate for the first time that P-selectin is a significant determinant in the events after corneal epithelial abrasion that contribute to wound healing of the cornea.

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