Effect of Wound Type on Smad 2 and 4 Translocation

Audrey E. K. Hutcbeon, Xiaqing Q. Guo, Mary Ann Stepp, Kenneth J. Simon, Paul H. Weinreb, Shelia M. Violette, and James D. Zieske

PURPOSE. In a prior study, it was reported that both TGF-β receptors type-I and -II are upregulated after wounding, suggesting that TGF-β signaling may play a role in corneal epithelial repair. The Smad proteins, which translocate into the nucleus after activation of the TGF-β receptors, are key factors in the major TGF-β signaling pathway. The present study was undertaken to examine whether Smads 2 and 4 translocate into the nucleus during wound repair and whether the wound type affects the extent of translocation.

METHODS. Either a 3-mm superficial keratectomy or epithelial debridement was performed on adult Sprague-Dawley rats. The eyes were allowed to heal from 4 hours to 2 weeks. Indirect immunofluorescence was performed with anti-Smads 2 and 4, anti-αβ6 integrin, which has been implicated in TGF-β activation. In addition, the effect of the p38MAPK inhibitor SB202190 on healing rates of debridement and keratectomy wounds was determined in organ culture.

RESULTS. In unwounded tissue, Smad 2 was cytoplasmic. By 4 hours after keratectomy, nuclear localization was visible in a few epithelial basal cells at the leading edge of the wound. The number of basal cells expressing nuclear Smad 2 in the wound area increased with time, peaking at 48 hours (95%). However, in the debridement model, Smad 2 localization remained primarily cytoplasmic. Smad 4 showed similar localization. In both wound models, p38MAPK inhibitor slowed epithelial migration, and αβ6 integrin appeared to be upregulated with localization primarily observed in the basal cells migrating over the wound area.

CONCLUSIONS. The presence of the basement membrane appears to have an effect on the extent and duration of translocation of the Smad 2 and 4 proteins during corneal epithelial wound repair. The Smad pathway does not appear to be essential for migration; rather, it may play a role in resynthesis of the basement membrane. (Invest Ophtalmol Vis Sci. 2005; 46:2362–2368) DOI:10.1167/iovs.04-0759

The cornea is an excellent model for studying wound healing. It has been found that within unwounded corneal epithelium, there are various degrees of cellular proliferational. The suprabasal cells do not proliferate and appear to have exited the cell cycle, and therefore are believed to be terminally differentiated. Only approximately 20% of the limbal basal cells are actively cycling, and these consist of a mix of slow-cycling stem cells and more rapidly proliferating transient amplifying cells. Finally, approximately 40% of corneal epithelial basal cells are proliferating at any given time. On wounding, the cells peripheral to the wound are stimulated to proliferate, increasing the number of actively cycling limbal and peripheral corneal basal cells to 90% to 100%. The proliferation rate of the cells migrating to cover the wound area, however, decreases significantly. A possible explanation for this decrease in proliferative activity in the migrating cells is that they are inhibited from proliferating by TGF-β signaling.

It has been observed that the cell cycle inhibitor p15INK4b is upregulated in these nonproliferative migratory cells, and because both inhibition of proliferation and p15INK4b synthesis are associated with TGF-β signaling, we localized the TGF-β receptors (TβR-I and -II) in rat corneal epithelium, to determine whether their spatial and temporal expression correlates with the inhibition of migration. We found that both TβR-I and -II were upregulated after wounding, and that TβR-II was specifically upregulated in the cells migrating to cover the wound. Because both receptors are necessary for TGF-β signaling, these data suggest that TGF-β signaling is present at higher levels in the cells migrating to cover the wound than in keratectomy wounds to test this idea, we examined the Smad family of transcription factors, which mediates the TGF-β signal.

Smad, which is derived from Mad (mothers against decapentaplegic) and sma, was discovered almost simultaneously by two different groups. On activation of the TGF-β signaling pathway, TβR-I binds to and phosphorylates TβR-II, which in turn phosphorylates either Smad 2 or 3. Subsequently, Smad 2 or 3 complexes with Smad 4, and the whole complex is translocated into the nucleus. Once inside the nucleus, the Smad complex, along with other transcription factors, regulates the transcription of the target gene. The expression of the target gene is variable depending on the cell type and the wound type.

METHODS

Cell Culture

Primary human corneal epithelial cells (HCECs) were isolated from limbal rims, as previously described. All limbal rims (gifts from Kenneth Kenyon and Peter Repoza of University Eye and Ear Consultants of Boston, Boston, MA) would have otherwise been discarded. Use of human tissue adhered to the tenets of the Declaration of Helsinki. HCECs were grown to 70% confluence in four-well chamber slides (VWR, West Chester, PA) and then serum starved overnight. Cells were either treated or not treated with 2 μg/mL of TGF-β1 or -β2 (R&D Systems, Minneapolis, MN) for 30 minutes in serum-free medium. They were then fixed and immunostained with anti-Smad 2 and 4, as described above.

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were then rinsed with PBS, fixed with 100% methanol for 10 minutes at -20°C, and stained for indirect immunofluorescence, as previously published. In brief, cells were incubated with primary antibody, anti-Smad 2 (BD Transduction Laboratories, San Diego, CA), anti-Smad 3 (Zymed, South San Francisco, CA), or anti-Smad 4 (B8; Santa Cruz Biotechnology, Santa Cruz, CA), for 1 hour at room temperature. Please note that the antibody against Smad 2 is reported by the manufacturer to show some cross-reactivity with Smad 3. However, in Western blot analysis (data not shown), the antibody reacted primarily with a band of the molecular mass corresponding to that of Smad 2 (58 kDa). The cells were then incubated for an additional hour with corresponding secondary antibody conjugated to fluorescein (Jackson Immunoresearch, West Grove, PA). The slides were coverslipped with mounting medium containing propidium iodide; a marker for all nuclei (Vectashield; Vector Laboratories, Burlingame, CA). Slides were viewed and documented by microscope (Eclipse E800; Nikon, Melville, NY) equipped with a digital camera (SPOT; Diagnostic Instruments, Sterling Heights, MI). Negative control reactions, in which the primary antibody was omitted, were performed with each experiment. Similar results were obtained in three separate experiments.

**Generation of the αβ6 Integrin Chimeric Antibody**

Chimeric antibody 2G2 containing a human constant domain was generated to detect αβ6 integrin in rodent tissue. Briefly, cDNAs were generated from 2G2 hybridoma (generated as described in Weinreb et al.); cell total RNA with constant region primers 5'-AGGTCTCATGAAYCTCCACACACAGGRRCGACATGAGAC-3' for the heavy chain and 5'-GGCTCTAAGCTTGGAGGTGGTTGGAGATGGA-3' for the light chain, using a cDNA synthesis kit (First Strand; Amersham Biosciences Corp., Piscataway, NJ). The heavy- and light-chain variable-region genes were amplified by polymerase chain reaction using the same 3' primers as were used for cDNA synthesis and pools of degenerate primers specific for most murine antibody gene signal sequences (sequences available on request). The cloned heavy- and light-chain variable regions were ligated into mammalian expression vectors along with human IgG1-constant regions generating mouse/human chimeric 2G2 (ch2G2). These constructs were transfected into the human embryonic kidney cell line 293 and after 3 days in culture, ch2G2 was harvested from the cell supernatant. The chimeric antibody was purified by affinity chromatography (Protein A Sepharose Fast Flow; Amersham Biosciences Corp.), as described elsewhere.

**Tissue**

All animal studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Adult male Sprague-Dawley rats were anesthetized with an intramuscular injection of a rodent anesthesia cocktail (21.5 mg/kg body weight of ketamine, 4.3 mg/kg body weight of xylazine, 0.7 mg/kg body weight of acepromazine). Once the animals were anesthetized, a drop of 0.5% proparacaine was applied to both eyes, and a 3-mm debridement or superficial keratectomy was made in both eyes. Eyes were enucleated and pinned to a paraffin post. Eyes were allowed to heal for either 16 or 20 hours, with or without 10 μM p38MAPK inhibitor SB202190 (Calbiochem, La Jolla, CA) in a completely defined medium. In the control samples, an equal concentration of dimethyl sulfoxide (DMSO; 0.33%) that was found in the p38MAPK inhibitor medium was added. After the appropriate time, eyes were stained with Richardson’s stain for 30 seconds and washed with PBS. The corneas were then documented with a microscope (model SMZ800; Nikon) equipped with a digital camera (SPOT; Diagnostic Instruments, Inc.). The remaining wound area was then measured with NIH ImageJ 1.32 software (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

**Results**

To determine whether TGF-β stimulates the translocation of the Smad 2(3)/4 complex into the nucleus of corneal epithelial cells, primary HCECs were grown to approximately 70% confluence and exposed to TGF-β1 and -β2. Both Smads 2 and 4 were present in the cytoplasm of primary HCECs (Figs. 1A, 1E). In contrast, Smad 3 was primarily nuclear, even in unstimulated cells (Fig. 1C). On treatment of the cells with TGF-β1 or -β2 for 30 minutes, both Smads 2 and 4 translocated from the cytoplasm to the nucleus (Figs. 1B, 1F). Little change in Smad 3 localization was noted (Fig. 1D). No detectable differences were observed in the translocation patterns stimulated by TGF-β1 and -β2. Cells immunostained with secondary antibody only were negative (Fig. 1C, 1E insets).

In previous experiments, it was found that both TGF-β1 and -4 were elevated in the nonproliferating corneal epithelial cells migrating to cover the wound area. To determine whether the Smad 2/4 pathway is involved in this wound-healing process, the localization patterns of Smads 2 and 4 were examined in 3-mm superficial keratectomies, which were allowed to heal in vivo for various times. The localization of Smad 2 is shown in Figure 2. In unwounded tissue (Fig. 2A, inset), there was a diffuse and somewhat cytoplasmic staining in the corneal epithelial cells. By 4 hours after wounding (Fig. 2A), Smad 2 appeared to localize in the nuclei of the basal corneal epithelial cells in the wound area (as indicated by the lack of laminin staining). This pattern continued through 8 and 16 hours (Figs. 2B–D). Over time, the nuclear staining became more distinct, and remained present, even after wound closure at 48 hours (Figs. 2E, 2F).

As seen in Figure 3, similar localization was present with Smad 4. A diffuse cytoplasmic localization was apparent in unwounded cornea (Fig. 3A), and nuclear staining was present in the basal corneal epithelial cells at early time points, such as 8 hours (Fig. 3B). As with Smad 2, the nuclear staining became more distinct and remained present, even after wound closure, 48 hours (Figs. 3C, 3D). Unlike Smad 2, however, there ap-
peared to be an occasional cell with nuclear staining just beyond the wound edge (as indicated by laminin staining). Because the nuclear localization patterns of Smads 2 and 4 were maintained at least through wound closure (48 hours), longer time points were examined to determine when the staining patterns returned to control levels. As seen in Figure 4, Smad 2 remained clearly nuclear at 4 days (Figs. 4A, 4B) and appeared to continue until approximately 6 days after wounding (data not shown). By 1 week (Figs. 4C, 4D), however, Smad 2 nuclear localization diminished considerably. The decrease in nuclear localization appeared to initiate at the edges of the wound (Fig. 4A, bracket). The loss of nuclear Smad 2 at 1 week corresponded with laminin localization appearing to be more linear and continuous (Figs. 4C, 4D). To semiquantify the levels of nuclear Smad 2, we counted the number of basal cells expressing nuclear Smad 2. The percentage of basal cells expressing nuclear Smad 2 increased rapidly after wounding (Fig. 5). Peak levels were seen at 48 hours when 95% ± 2.4% of the basal cells in the wound area expressed nuclear Smad 2. Levels of nuclear Smad 2 decreased gradually with time until 1 week. The standard deviations of the amounts increased with time, which was the result of the variability caused by sections at the wound edge having far fewer cells with nuclear Smad 2 than sections in the wound center.

Because Smad 2 and 4 translocation was seen primarily in the wound area lacking basement membrane, we went on to examine Smad localization in a debridement wound model in which the basement membrane was left intact. Both the extent and temporal pattern of Smad 2 translocation varied between keratectomy and debridement (Fig. 6). In contrast to a superficial keratectomy, Smad 2 maintained its cytoplasmic localization through 8 hours after debridement (Fig. 6A). Even by 16 hours (Fig. 6B), there was little if any nuclear Smad 2. By wound closure (Fig. 6C, 24 hours) and beyond (Fig. 6D, 48 hours), only small patches of nuclear localization were observed, with most of the Smad 2 being localized in the cytoplasm. Smad 4 localized in a pattern identical with that of Smad 2 (data not shown). Quantitation of the levels of nuclear Smad after debridement gave no meaningful results, presumably because of low cell counts and high variability.

In an attempt to determine the cause of the differential levels of Smad 2 and 4 translocation between keratectomy and debridement, we examined the effect of the p38MAPK inhibitor on migration rates. Saika et al.33 have shown that TGF-β activates the p38 pathway preferentially over the Smad pathway in debridement in mice. Examination of the effect of the inhibitor resulted in results similar to those reported by Saika et al.33 in our rat debridement model, with 43% and 38% slowing of healing seen at 16 and 20 hours, respectively. Furthermore, addition of the p38MAPK inhibitor slowed migration rates in the keratectomy model (Table 1).

Finally, we examined the localization of αvβ6 integrin in wounded and unwounded corneas in an attempt to discover whether this integrin may be involved in the differential Smad localization. The pattern of nuclear localization of αvβ6 integrin in wound edge (A, B, C, E) and in the wound area (D, F) in rat corneas, before and after a 3-mm superficial keratectomy. In unwounded corneas (inset), Smad 2 was primarily cytoplasmic. By 4 hours after wounding, nuclear Smad 2 was observed in the wound area (A). The number of cells expressing nuclear Smad 2 increased with time—8 hours (B) and 16 hours (C, D)—with the extent and intensity appearing to peak at 48 hours after wounding (E, F). This peak occurred 12 hours after wound closure. Lack of laminin staining (red) indicates the wound edge. Bar, 50 μm.
response to debridement and keratectomy. The αvβ6 integrin has been implicated in activation of TGF-β134 and -β3.35 The integrin was present at low levels in unwounded corneal epithelium (Fig. 7A), with levels appearing to increase in both wound types (debridement: Figs. 7B, 7C, 7E, 7G; keratectomy: Figs. 7D, 7F, 7H). The major difference observed in αvβ6 integrin localization in the two wound models was the duration of increased expression with the debridement, returning to normal appearance by 72 hours (Fig. 7G) and the keratectomy not returning to control levels until 1 week after wounding (data not shown). Of note, the localization of the αvβ6 integrin was most apparent in the wound area (Fig. 7).

**DISCUSSION**

We and others36,37 have demonstrated that the response to a corneal wound is compartmentalized—that is, the epithelium migrating to cover the wound has a varied response compared to the epithelium outside the original wound area. One of the intriguing differences is that cells outside the original wound area are stimulated to proliferate, presumably to repopulate the wound area, whereas cells migrating over the wound show a greatly decreased level of cell proliferation. These observations indicate that the two cell populations are receiving different signals or are interpreting similar signals differently. Several growth factor signaling pathways have been implicated in corneal wound repair. For example, we have demonstrated that the epidermal growth factor receptor is activated within minutes after wounding.38 In addition, we have demonstrated that both TGF-β receptors (R)-I and -II are upregulated after wounding and that TβR-II appears to be specifically upregulated in cells migrating over the original wound bed.13 Thus, in the current investigation, we sought to determine whether the TGF-β signaling pathway involving the Smad proteins was activated in corneal wound repair.

In our initial studies, we chose to use a superficial keratectomy wound model. This model has the advantage of being able to identify the wound area easily by localizing basement membrane components such as laminin. As seen in Figures 2 to 4, both Smads 2 and 4 translocate into the nucleus within 4 to 8 hours after wounding, indicating that TGF-β signaling pathways are activated during wound repair. This result is consistent with our previous findings that TGF-β receptors are upregulated after wounding.13 One of our most striking observations was that nuclear translocation of Smad 2 correlated closely with the wound edge, as indicated by the absence of laminin (Fig. 2). Smad 4 also exhibited this pattern to a lesser extent, with some nuclear localization extending beyond the wound edge (Fig. 3). The number of cells expressing nuclear Smads 2 and 4 increased with time, peaking at 48 hours after wounding (12 hours after epithelial wound closure), with virtually every basal cell in the wound area exhibiting nuclear Smads. The number of cells expressing nuclear Smad 2 gradually decreased (Fig. 5). This gradual decrease originated from the wound edge (Fig. 4), and the loss of nuclear Smad appeared to correlate with the reappearance of a continuous band of laminin in the basement membrane zone.

The results from the keratectomy suggested that the presence or absence of a basement membrane may play a role in the extent of Smad 2 and 4 translocation after wounding. This concept was supported by the findings of Saika et al.,33 who reported that Smads 3 and 4 do not translocate to any extent after a debridement in mice. We, therefore, examined a debridement in rats to inspect further the effect of basement membrane on TGF-β signaling through the Smad pathway. As seen in Figure 6, the wound type had a profound effect on

**FIGURE 3.** Immunolocalization of Smad 4 (green) in unwounded (A), at the wound edge (B, C), and in the wound area (D) in rat corneas, before and after a 3-mm superficial keratectomy. In unwounded cornea (A), Smad 4 was primarily cytoplasmic. After wounding (B, 8 hours), nuclear localization was observed in the wound area. As seen with Smad 2 (Fig. 2), the extent and intensity of nuclear Smad 4 localization also appeared to peak at 48 hours after wounding (C, D). Whereas Smad 4 nuclear localization was present primarily in the wound area, a few cells expressed nuclear Smad 4 in areas that exhibited continuous laminin (red) localization. Bar, 50 μm.

**FIGURE 4.** Immunolocalization of Smad 2 (green) at the wound edge (A, C) and in the wound area (B, D) of longer time points after keratectomy in rat corneas. Smad 2 nuclear localization decreased at 4 days (A, B). The decrease initiated at the wound edge (A, bracket) and appeared to correlate with restoration of laminin (red) deposition in the basement membrane zone. By 1 week after wounding (C, D), Smad 2 and laminin localization appeared to have returned to their unwounded condition. Bar, 50 μm.
Smad 2 and 4 translocation. Little, if any, translocation was observed during the period of active epithelial migration (wound closure occurred at 24 hours in this model). Also, in contrast to the keratectomy, only a few isolated cells showed nuclear Smads 2 or 4 at later time points. It is possible that the isolated cells with nuclear Smads correspond to small breaks in the basement membrane. This possibility is supported by Sta Iglesia and Stepp,39 who demonstrated that the basement membrane is degraded to variable amounts after debridement. Thus, our results in rats are in conceptual agreement with those of Saika et al.,33 who stated that TGF-β signaling through the Smad pathway does not appear to play a major role in corneal epithelial migration in wounds with the basement membrane intact. In their studies, they reported that Smad 3 was nuclear in unwounded corneal epithelium. We were not able to identify a Smad 3 antibody that gave consistent results in rat tissue; however, we did observe that Smad 3 had a nuclear localization in human corneal epithelial cells, with or without stimulation by TGF-β (Fig. 1). These data may suggest that Smad 3 translocates into the nucleus of corneal epithelial cells independent of TGF-β signaling.

TGF-β signaling has been implicated in a number of cellular processes, including regulation of proliferation, migration, and extracellular matrix deposition (for review see Ref. 24). Our data that Smads 2 and 4 do not translocate into the nucleus during migration over a debridement suggest that Smad signaling does not play a major role in regulation of migration. The finding that the extent of Smad 2 and 4 translocation peaks after epithelial wound closure in a keratectomy, also suggests that signaling through Smads 2 and 4 is not a major factor in regulation of migration. The findings in the debridement model suggest that signaling through Smads does not play a major role in the absence of cell proliferation in the migrating cells. Indeed, Saika et al.35 and Sharma et al.37 have reported that signaling involving p38, another downstream protein activated by TGF-β, plays a major role in the regulation of migration and proliferation. Our results using a p38MAPK inhibitor also indicate that activation of the p38 pathway is crucial to migration in rat corneas healing after debridement or keratectomy (Table 1). Whether Smads 2 and/or 4 are involved in the regulation of proliferation in a superficial keratectomy is less clear. It should also be noted that it is not clear what level of translocation is necessary to stimulate gene expression. For example, we have observed that the cell-cycle inhibitor p15INK4b (which has been shown to be regulated by Smads 2 and 3)40 is upregulated in the migrating corneal epithelial cells during debridement, even though little translocation of the Smads is observed.2 Last, our results are consistent with Smad translocation’s playing a role in stimulating regeneration of the basement membrane zone. There is a strong correlation between the presence of nuclear Smad 2 and the lack of basement membrane, as indicated by laminin localization. As the laminin returns, nuclear

**Figure 5.** Graphical representation of the average percentage of basal cells in the wound area exhibiting nuclear Smad 2- in 3-mm superficial keratectomies 8 hours to 2 weeks after wounding. Wound closure in a 3-mm superficial keratectomy performed on a rat cornea was approximately 36 hours, 12 hours before the average percentage of nuclear Smad 2 peaked in the wound area. Also note that at later time points, the SD increased, because of the loss of nuclear Smad 2 at the wound's edge.

**Figure 6.** Immunolocalization of Smad 2 (green) in rat corneas after an epithelial debridement. In contrast to the keratectomy, nuclear Smad 2 was not observed in the epithelium migrating to cover the wound at 8 hours (A). By 16 hours (B), little if any nuclear staining was present. Also in contrast to the keratectomy, only a few cells expressed nuclear Smad 2 at 24 (C) and 48 (D) hours. Laminin localization (red) was continuous across the wound area at all time points. Bar, 50 μm.
Smad 2 diminishes. This concurs with many findings from other studies that syntheses of basement membrane and extracellular matrix components are regulated by TGF-β signaling through the Smad pathway.24 One of the intriguing questions raised by these data is why Smad translocation was not observed to any great extent after debridement. The answer in part may lie in the fact that TGF-β signaling is transmitted through both Smad-dependent and independent pathways. It is not known how the cell "decides" which pathway should be used. One possibility is that TGF-β signaling pathways are influenced by other growth factor signaling pathways. For example, it has been demonstrated that epidermal growth factor (EGF) signaling can block Smad translocation by stimulating phosphorylation of the Smads at sites that prevent binding to Smad 4 and subsequent translocation into the nucleus.41 The pathway chosen may also be dependent on the level of active TGF-β present after wounding. Stramer et al.42 found that TGF-β2 is the major isoform present after wounding and the level of expression is greatly dependent on whether the wound penetrates the basement membrane. Thus, higher levels of TGF-β may be present after a keratectomy, which may swing the signaling toward the Smad pathway rather than toward other pathways, such as those involving p38. In addition, the relationship between the p38 and Smad pathways has been further complicated by the findings that p38 may phosphorylate Smad 2 and 3. Kamaraju and Roberts43 have demonstrated that this phosphorylation is required for Smad translocation into the nucleus. They also report that both p38 and Smad activation are essential for TGF-β mediated inhibition of growth.

Finally, in an attempt to understand further the differential translocation of Smad 2 and 4 in keratectomy versus debridement, we examined the localization of αvβ6 integrin. This integrin has been demonstrated to be involved in activation of TGF-β.28,34,35 We hypothesized that the extent of Smad translocation may be related to the extent of expression of this integrin, leading to more or less active TGF-β and, subsequently, more or less Smad translocation. However, we observed that αvβ6 integrin was upregulated in both models. Thus, its expression does not appear to explain the differential Smad translocation. We plan to pursue the role of αvβ6 integrin in corneal wound repair.

In summary, we have demonstrated that TGF-β signaling through the Smad pathway is activated in response to corneal wounds where the basement membrane is removed. This is particularly evident in the localization of Smad 2, which exhibits nuclear localization, specifically in the wound area. This pattern peaks after epithelial wound closure, suggesting that Smad signaling may be more important in the regenerative portion of wound healing than in the migratory process. Little translocation of Smads 2 and 4 was observed in wounds where the basement membrane was left intact. These findings suggest that the wound type may regulate whether TGF-β signaling involves Smad-dependent or Smad-independent pathways.

**Table 1.** Extent of Epithelial Wound Closure following Debridement or Keratectomy in the Presence or Absence of p38MAPK Inhibitor

<table>
<thead>
<tr>
<th>Sample</th>
<th>Remaining Wound Area ± SEM</th>
<th>% Inhibition</th>
</tr>
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<tbody>
<tr>
<td>16 hr Control — Debridement</td>
<td>0.95 ± 0.14</td>
<td>43.0</td>
</tr>
<tr>
<td>16 hr Inhibitor — Debridement</td>
<td>3.60 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>20 hr Control — Debridement</td>
<td>0.21 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>20 hr Inhibitor — Debridement</td>
<td>2.85 ± 0.54</td>
<td>38.4</td>
</tr>
<tr>
<td>16 hr Control — Keratectomy</td>
<td>5.11 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>16 hr Inhibitor — Keratectomy</td>
<td>5.59 ± 0.44</td>
<td>42.0</td>
</tr>
<tr>
<td>20 hr Control — Keratectomy</td>
<td>2.06 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>20 hr Inhibitor — Keratectomy</td>
<td>5.57 ± 0.81</td>
<td>50.6</td>
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Percent inhibition was determined by subtracting remaining wound area from original wound size. Values of 7.1 mm² and 9.0 mm² for debridement and keratectomy, respectively, were determined by measurement of corneas immediately after wounding.

**Figure 7.** Immunolocalization of αvβ6 integrin in rat corneas after either debridement (B, C, E, G) or keratectomy (D, F, H). αvβ6 integrin was present in the epithelium of unwounded control cornea (A) at low levels. By 16 hours after debridement (B), the αvβ6 integrin was present in the basal cells of the leading edge of migrating epithelium. The αvβ6 integrin continued to be upregulated, primarily in the basal cells of the wound area at 24 (C, D) and 48 (E, F) hours. Levels of αvβ6 integrin returned to control levels by 72 hours after debridement (G). However, they remained high at 72 hours after keratectomy (H). (F, H, arrows) Wound edge. Bar, 50 μm.
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