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Intestinal Restitution: Progression of Actin Cytoskeleton Rearrangements and Integrin Function in a Model of Epithelial Wound Healing

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Superficial injury involving the mucosa of the gastrointestinal tract heals by a process termed restitution that involves epithelial sheet movement into the damaged area. The forces that drive epithelial sheet movement are only partially understood, although it is known to involve changes in the morphology of cells bordering the damage, such as the formation of large, flat, cytoplasmic extensions termed lamellae. We investigated the mechanism of epithelial sheet movement by following the response of the actin cytoskeleton and specific integrins ($\alpha6\beta4$, $\alpha6\beta1$, and $\alpha3\beta1$) to wounding. To model this event *in vitro,* monolayers of T84 cells, well-differentiated colon carcinoma cells, were damaged by aspiration and the ensuing response was analyzed by a combination of time-lapse video microscopy, fluorescence confocal microscopy and antibody inhibition assays. We show that wound healing begins with retraction of the monolayer. $\alpha6\beta4$ integrin is localized on the basal surface in structures referred to as type II hemidesmosomes that persist throughout this early stage. We hypothesize that these structures adhere to the substrate and function to retard retraction. Once retraction ceases, the wound is contracted initially by actin purse strings and then lamellae. Purse strings and lamellae produce a pulling force on surrounding cells, inducing them to flatten into the wound. In the case of lamellae, we detected actin suspension cables that appear to transduce this pulling force. As marginal cells produce lamellae, their basal type II hemidesmosomes disappear and the $\alpha6$ integrins appear evenly distributed over lamellae surfaces. Antibodies directed against the $\alpha6$ subunit inhibit lamellae formation, indicating that redistribution of the $\alpha6$ integrins may contribute to the protrusion of these structures. Antibodies directed against the $\alpha3\beta1$ integrin also reduce the size and number of lamellae. This integrin's contribution to lamellae extension is most likely related to its localization at the leading edge of emerging protrusions. In summary, wounds in epithelial sheets initially retract, and then are contracted by first an actin purse string and then lamellae, both of which serve to pull the surrounding cells into the denuded area. The $\alpha6$ integrins, particularly $\alpha6\beta4$, help contain retraction and both the $\alpha6$ integrins and $\alpha3\beta1$ integrin contribute to lamellae formation. *(Am J Pathol 2000, 156:985–996)*

Disruptions in the mucosal lining of the gastrointestinal tract reseal by a process termed restitution.[1,2] Restitution is an important component of the barrier function of the gut lining because it prevents luminal contents from seeping into the underlying intestinal tissue during normal wear and tear of the epithelium. Initial light microscopic studies of fixed tissue discerned that gut epithelial cells respond to injury by altering their morphology. Further work showed that mucosal injuries are resealed by the concerted movement of the surrounding cells, not by cell division or by contributions from blood clot formation.[3,4] These points illustrate that restitution is a very specific example of wound healing in epithelial monolayers.

Epithelial sheets respond to injury by mobilizing their actin cytoskeleton. Two different types of responses have been noted.[5,6] One involves lamellae formation, a key feature of restitution both *in vivo* and *in vitro*.[4,7] Lamellae are large, flat, cytoplasmic protrusions that are extended by the marginal cells into the denuded area. Much of what is known about lamellae is derived from the study of solitary migrating cells such as fibroblasts.[8] During fibroblast locomotion the actin cytoskeleton within the lamella associates with integrins on the surface. The traction to pull the cell forward is provided by the adhesion between the integrins and their specific extracellular matrix ligands. Eventually the lamella contracts, detaching the rearward part of the cell and allowing it to translocate. Although this process seems relevant to epithelial wound healing, many normal epithelia reseal defects without

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loss of cell contact.[9,10] In the case of restitution, marginal cells surrounding gut injuries are thought to use lamellae to migrate into the wound, but cells are not observed to detach from their neighbors and translocate into the injury.[1,2,4,7,11] This observation raises the question of how lamellae participate in the healing process. Other epithelial wounds heal by a process called purse string contraction in which cells marginal to the damage arrange their actin in a belt that tightens to close the injury.[5,6] It is thought that small wounds (<0.008 mm^2) heal by purse string contraction, whereas larger wounds use lamellae.[6] However, recent studies of corneal abrasions suggest that the two processes may not be mutually exclusive.[12] It is unclear from previous findings whether actin purse strings form during gut epithelial wound healing or whether lamellae that form during restitution preclude these structures.

We were interested in understanding the underlying molecular processes that drive restitution. To model the processes *in vitro* we used T84 cells, well-differentiated human intestinal carcinoma cells that are polarized along their apical to basal axis and express well-developed intercellular junctions.[13] Previously we found that the laminin family of extracellular matrix proteins and the integrins that bind these proteins are instrumental in restitution.[14] Our findings targeted the laminin-binding integrins, $\alpha6\beta1$, $\alpha6\beta4$, and $\alpha3\beta1$, as part of the molecular machinery responsible for epithelial wound healing, but the point at which these integrins participate in the healing process is unknown. It would be informative to correlate the changes in cell morphology that occur on wounding with changes in the function and distribution of laminin-binding integrins.

Here, we examine the mechanism of epithelial cell movement during wound closure by following the mobilization of the actin cytoskeleton and laminin-binding integrins in response to injury of T84 monolayers. We show that lamellae formation is a later step in a progression of cytoskeletal rearrangements that begins with actin purse strings. The $\alpha6$ integrins and $\alpha3\beta1$ integrin, which we found to be instrumental in lamellae formation, redistribute to distinctive locales on the lamellae during wound resealing. We conclude that the purpose of both the actin purse string and lamellae is to allow the usually columnar T84 cell to flatten greatly, providing as much cytoplasmic coverage of the denuded area as possible.

Materials and Methods

Culture of T84 Cells

T84 colon carcinoma cells were cultured as described previously.[13,14] Briefly, cells were grown in DME-low glucose/Ham's F12 (GIBCO, Grand Island, NY) supplemented with 15 mmol/L Hepes, 6% normal calf serum, 2 mmol/L L-glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin. Cells were grown for 2 to 3 days after reaching confluency before being used in assays.

Antibodies

The following monoclonal antibodies specific for integrin subunits were used in the present study: mouse antibody UM-A9 (integrin $\beta4$ subunit), provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI); rat antibody GoH3 (integrin $\alpha6$ subunit) and mouse antibody against CD29 (integrin $\beta1$ subunit), purchased from Immunotech (Marseille, France); mouse antibody PIB5 (integrin $\alpha3$ subunit), purchased from Becton Dickinson (San Jose, CA). Mouse and rat IgG was purchased from Sigma (St. Louis, MO). Fluorescein-conjugated goat anti-rat and goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Immunofluorescence Microscopy

T84 cells were dissociated with trypsin-EDTA and, except where mentioned in the figure legends, plated onto glass coverslips. The cells were grown in a 37°C, 5% CO_2 atmosphere for 10 to 14 days. Subsequently, the confluent monolayers were wounded by aspiration through a micropipet tip. These were made by pulling a heated glass Pasteur pipet so that a very narrow bore resulted.

At various times after wounding, cells were fixed for 10 minutes with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) with 7% sucrose followed by 0.4% Triton X-100 in Tris-buffered saline (TBS, pH 7.4) for 2 minutes. In some cases, noted in the figure legend, cells were permeabilized before fixation for 30 seconds on ice in F1 buffer (10 mmol/L Pipes, pH 6.8, 0.5% Triton-X 100, 300 mmol/L sucrose, 100 mmol/L potassium chloride, 3 mmol/L magnesium chloride, 10 mmol/L ethyleneglycoltetraacetic acid, and 2 mmol/L phenylmethylsulfonyl fluoride). After fixation, the coverslips were incubated in a blocking solution (3% bovine serum albumin and 1% normal donkey serum in TBS) for 1 hour at room temperature. After incubation with either primary antibody or nonspecific IgG, the coverslips were washed in TBS (3 times, 10 minutes each) and incubated for 1 hour at room temperature in the appropriate fluorescein-conjugated secondary antibody.[1:100] In most experiments the secondary antibody was accompanied by rhodamine-phalloidin (1 μg/ml, Sigma) or rhodamine-phalloidin and Hoechst stain (0.5 μg/ml, Sigma). All antibodies and fluorescent compounds were diluted in blocking solution. After staining, the coverslips were washed in TBS and mounted in a mixture (8:2) of glycerol and PBS, pH 8.5, containing 1% propylgallate. Slides were examined by confocal imaging using a Zeiss LSM410 Laserscan Microscope equipped with a peripheral Argon-UV laser for exciting Hoechst fluorescence (Carl Zeiss, Thornwood, NY).

Video Microscopy and Function Blocking Experiments

T84 cells were grown for 10 to 14 days on 35-mm tissue culture dishes. Circular wounds were made using micropipet tips. The healing process was examined by

time-lapse video-microscopy using a Nikon Diaphot 300 inverted microscope with phase contrast optics, equipped with a stage warmer. Images were captured using a CCD camera (Dage-MTI, Michigan City, IN), a frame-grabber (Scion) and a 7600 Power Macintosh computer. Films of wounds healing at 37°C, 5% CO_2 were made by capturing images every 2 minutes for up to 12 hours. Some wounds were filmed for various amounts of time and then fixed and stained for immunofluorescence. For function-blocking experiments, T84 cell monolayers were wounded, fresh medium containing function-blocking integrin antibodies was added, and then images of the wounds were captured so that their initial diameters could be measured. After incubation for 2 hours in a 37°C, 5% CO_2 incubator, images were captured again for a final wound diameter measurement. Alternatively, images of wounds were captured for measurements of initial diameters and then wound healing was allowed to progress until the first appearance of lamellae. At this point, function-blocking antibodies were added. After two hours, images were captured again for measurement of final wound diameters. Wound diameters were measured using IP lab spectrum software (Scanalytics, Fairfax, VA). The initial and final diameters for each wound were used to determine effects of the monoclonal antibodies on wound closure.

Results

Wound Closure in T84 Monolayers Progresses via Retraction, Actin Purse String Formation, and Lamellae Extension

To identify the sequence of events involved in the closure of T84 wounds (in this case, 0.018 mm^2, approximately 400 cells), we used time-lapse video microscopy (Figure 1). Initially after wounding, the wound edge actually retracted for 10 minutes and the surface area of the wound increased by ~14%. At this time, a refractile ring that surrounded the wound edge was evident. This ring was similar in appearance to actin purse strings that have been shown to contract smaller wounds.[5,6] Subsequent to the appearance of the refractile ring, the cells surrounding the wound edge elongated and increased their surface area approximately twofold, a process that resulted in the initiation of wound closure. Although the surface area of the wound was reduced by 30% at 40 minutes relative to its largest size at 10 minutes, surface protrusions such as lamellae were not evident in cells at the wound edge. In fact, distinct lamellae extending from a few cells abutting the damage were not seen until 60 minutes after wounding. Lamellae protrusion was not synchronous, but gradually most of the marginal cells produced these extensions, while the refractile ring became less evident. At 120 minutes after wounding, the denuded area was covered entirely by lamellae. Based on this video analysis, we conclude that the closure of T84 wounds involves a sequence of morphologically-defined events that involve an initial retraction of the wound edge, subsequent refractile ring formation accompanied by cell elongation, and, finally, lamellae extension.

The possibility that the refractile ring we observed during the closure of a 0.018 mm^2 wound is an actin purse string is intriguing because actin purse strings have been associated with smaller wounds (<0.008 mm^2).[6] This possibility was examined by staining monolayers 30 minutes after wounding with rhodamine-phalloidin to visualize F-actin (Figure 2). Indeed, an intense band of F-actin, characteristic of an actin purse string, was seen around the circumference of such wounds (Figure 2A). In fact, even larger wounds exhibited actin purse string formation within 30 minutes after wounding (Figure 2B). Interestingly, cells that were damaged as a result of wounding were excluded and appeared in the denuded area outside the actin purse string. Cross-sectional images revealed that these damaged cells were detached from the substrate (Figure 2C).

Coincident with actin purse string formation, cells surrounding the denuded area elongated with their long axis at right angles to the wound edge. Image analysis indicated that cells as far as 10 cell diameters away from the injury elongated in response to wounding and increased their basal surface area by approximately twofold (Figure 1). Moreover, rhodamine-phalloidin staining revealed that cells surrounding the wound rearranged their stress fibers orthogonally to the wound edge such that these stress fibers stretched toward the actin purse string (Figure 3A). Cross-sectional images highlighted the impression of an intact monolayer tapered down to the actin purse string (Figure 3B). Collectively, these images demonstrate that cells distant from the wound contribute to closure by elongating, and that actin purse strings, which generate a contractive force, are associated with this elongation.

The actin rearrangements involved in lamellae formation were also examined by rhodamine-phalloidin staining. *En face* views of wounds surrounded by lamellae demonstrated the presence of F-actin within the lamellae protruding into the denuded area (Figure 4). However, we observed regions in the monolayer adjacent to the lamellae that contained no detectable F-actin (Figure 4A). Cross-sectional analysis revealed that these F-actin-deficient regions corresponded to the bodies of the marginal cells that produced the lamellae and actually contained fine F-actin cables that were suspended above the basal plane (Figure 4B). For this reason, images taken at this basal plane gave the appearance of F-actin holes in the monolayer. These F-actin suspension cables extended from the marginal cell's rearward apicolateral cell surface to the dense actin network at the base of the lamella. This unique arrangement of actin suggested that the cables were under tension (Figure 4B). A cross-section taken in a neighboring region revealed shorter actin suspension cables (Figure 4C). These cross-sectional views imply that the cable's suspension from the rearward apicolateral cell surface down to the lamella base is related to the extent to which the cell containing it elongates. Together, the images presented in Figure 4 demonstrate that lamellae serve to stretch the marginal cells that produce them and result in the formation of

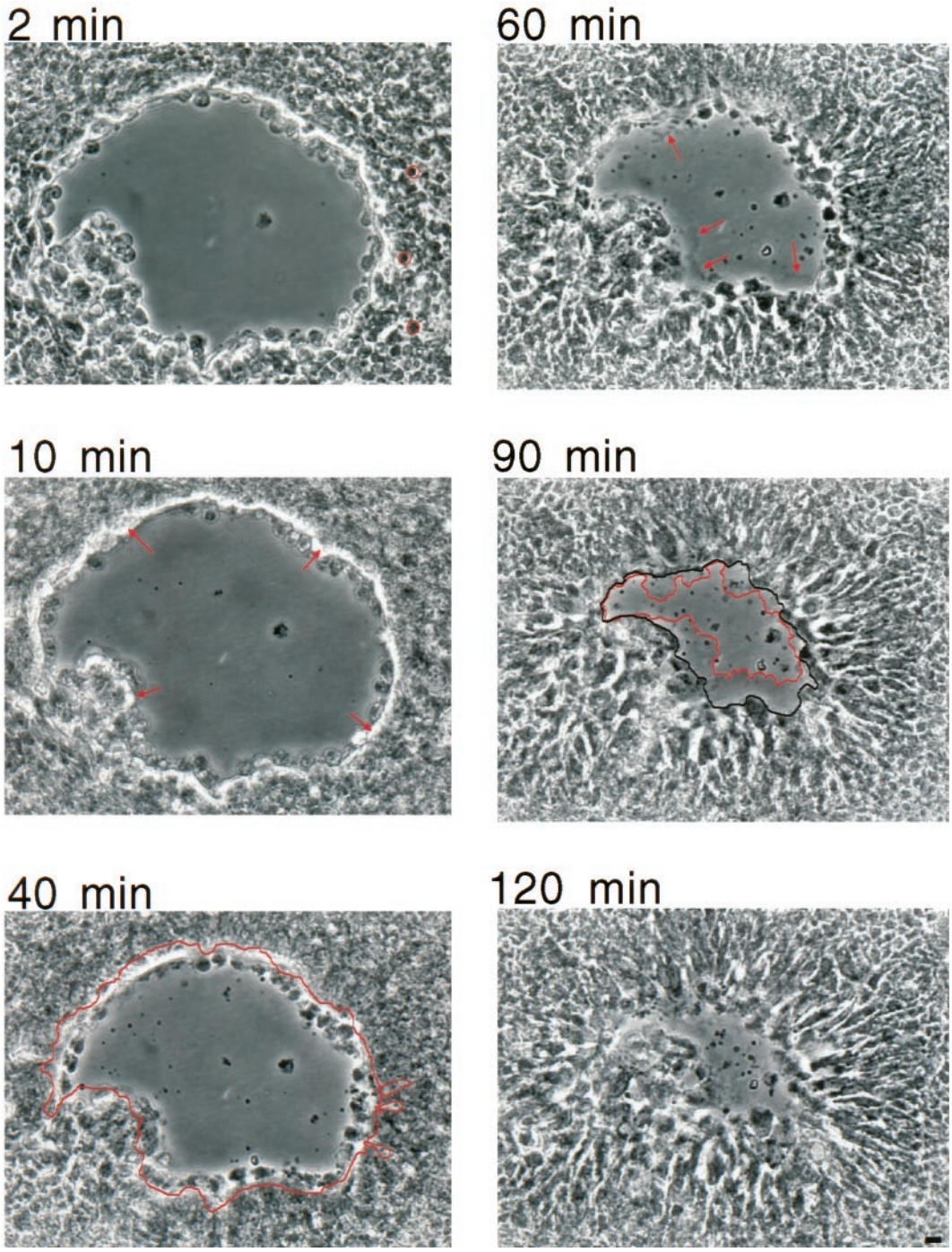


Figure 1. Time-lapse video microscopy reveals the sequence of events during wound healing of a monolayer of T84 cells. Times above the panels refer to the number of minutes elapsed after wounding. The first image of the wound, approximately 0.018 mm^2, was taken at 2 minutes. Cell bodies were randomly chosen on the right side of the denuded area and outlined in red. At 10 minutes postwound, retraction reached its fullest extent and the denuded area was now approximately 0.021 mm^2. The refractile ring, greatly resembling an actin purse string, is indicated by **arrows**. By 40 minutes, the wound closed to 0.015 mm^2, a 30% reduction from its largest size at 10 minutes. The outline of the injury at 10 minutes is transposed onto the image taken at 40 minutes. Cell bodies, also outlined, were randomly chosen on the right side of the denuded area for comparison with those at 2 minutes. Similar comparisons of 10 randomly chosen cells, followed from 16 minutes to 40 minutes, showed that during this period of wound contraction, cell surface area increased on the average twofold (from 54 ± 3 μm^2 to 116 ± 7 μm^2, $P < 0.01$). By 60 minutes, lamellae, indicated by **arrows**, were obvious. At 90 minutes, many of the cells adjacent to the wound protruded lamellae. Lamellae are highlighted in red, and the margin of the cell bodies is outlined in black. The area between these two outlines is considered to be the amount of lamella surface area contributing to wound sealing up to this point in time, a measurement used in Figure 5. By 120 minutes, the denuded area was nearly completely covered by lamellae. Scale bar, 10 μm.

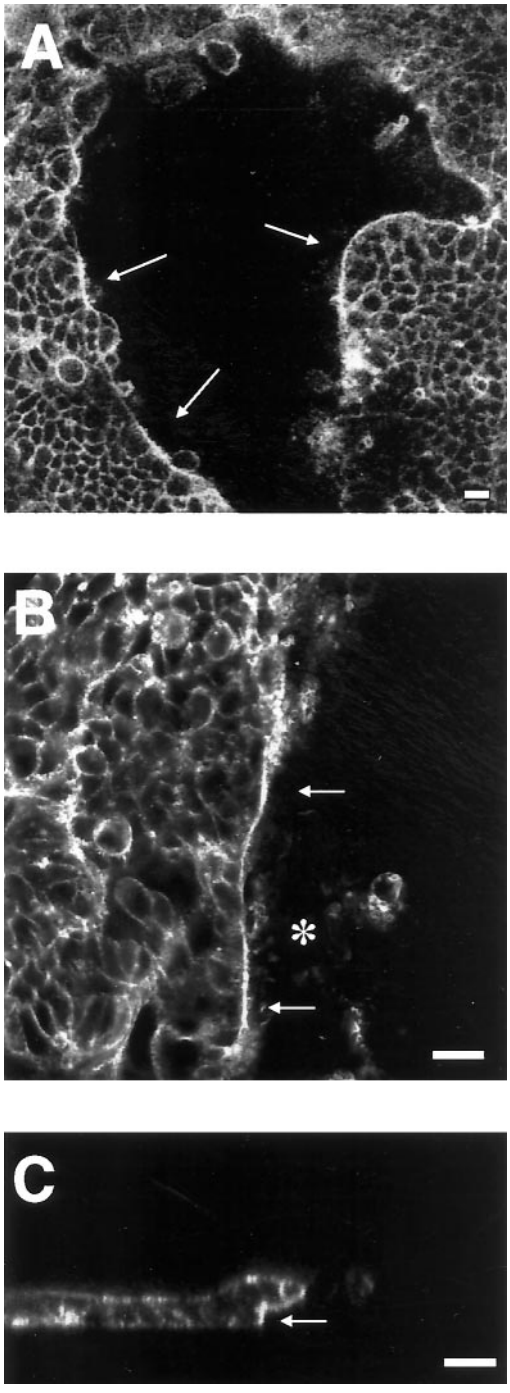


Figure 2. Rhodamine-phalloidin fluorescent staining of recently wounded T84 monolayers reveals actin purse string formation. T84 monolayers were wounded by pipet aspiration and fixed 30 minutes later. **A:** Monolayers grown on porous supports were wounded. This *en face* view of the injury shows a continuous belt of actin or purse string, indicated by **arrows**, encircling the denuded area. The purse string is not visible in the upper half of the injury because the porous support at this point declines away from the plane of focus. **B:** An *en face* view of a larger injury reveals that it is not yet fully surrounded by an actin belt. The actin purse string (**arrows**) is in the process of forming. The **asterisk** marks cells, not included in the purse string, that were damaged and have lifted off the substrate. **C:** An optical cross-section taken at the point of the **upper arrow** in **B** reveals the actin purse string (**arrow**) forming in the basolateral compartment of the attached cells marginal to the wound. Cells not included in the purse string are detached from the substrate. Scale bars, 10 μm.

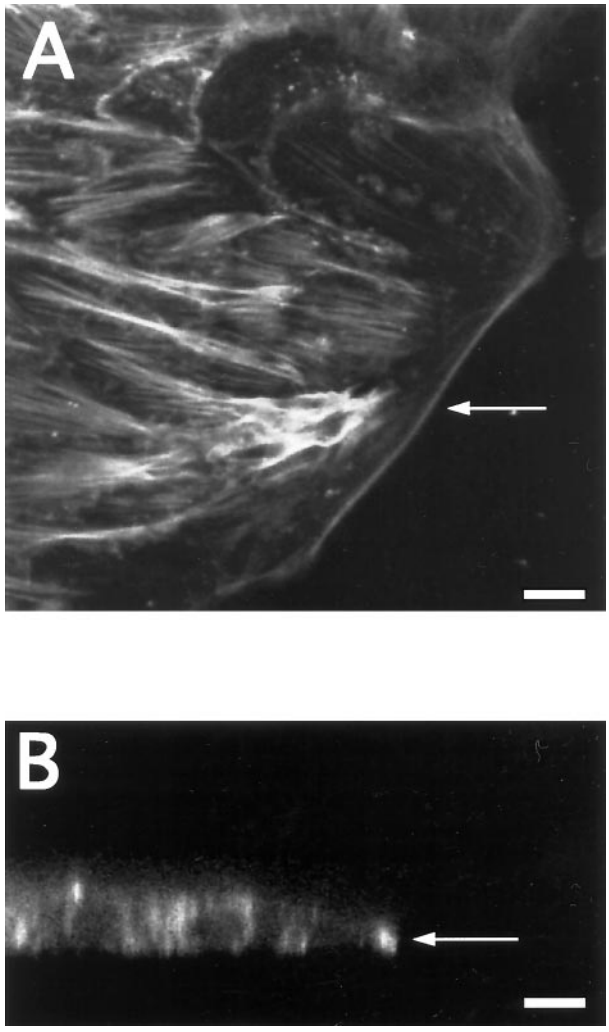
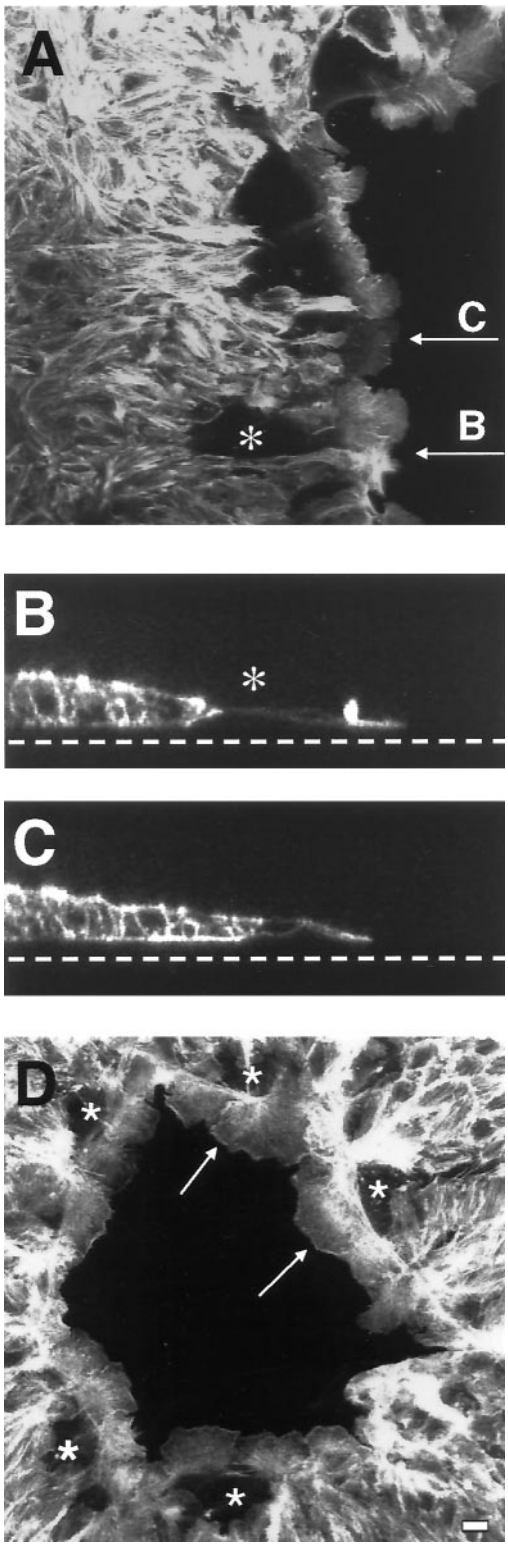


Figure 3. Rhodamine-phalloidin staining demonstrates that cell elongation is accompanied by actin stress fiber rearrangement. **A:** T84 monolayers were fixed 3 hours after wounding. In this *en face* view taken at the basal surface, the **arrow** points to the actin purse string. Cells rearward of those producing the purse string exhibit stress fibers arrayed at right angles to the injury. **B:** An optical cross-section of an area similar to that shown in **A** shows how the monolayer flattens as it reaches the actin purse string, highlighted by an **arrow**. Scale bars, 10 μm.

actin suspension cables that bridge the rearward, lateral cell surface with the lamella base.

Correlating Function and Localization of the α6 Integrins and α3β1 Integrin during Wound Healing with Changes in Cell Morphology Induced by Wounding

Previously we demonstrated that α3β1 and the α6 integrins were involved in wound resealing of T84 cell monolayers. In the current study, we determined at which stage (retraction, actin purse string formation or lamellae extension) these integrins contributed to the healing process. Wounds were allowed to reseal for 2 hours in the presence of nonspecific antibody or antibodies against the α3 or α6 integrin subunit (Figure 5a). All of the wounds, regardless of treatment, exhibited refractile ac-



tin purse strings, suggesting that neither the $\alpha 3\beta 1$ integrin nor the $\alpha 6$ integrins were involved in the formation of this structure. Wounds treated with nonspecific antibody closed 35%, whereas those treated with $\alpha 6$ subunit antibody were 11% larger compared with their initial sizes. These data demonstrate that cells treated with the $\alpha 6$ subunit antibody were unable to halt retraction within 2 hours of wounding, suggesting that the $\alpha 6$ integrins promote the cessation of retraction. In contrast, wounds treated with $\alpha 3$ subunit antibody were 12% smaller. Thus, injured monolayers treated with $\alpha 3$ subunit antibody were able to halt retraction within the 2-hour time span, but resealed more slowly than those treated with nonspecific antibody.

We next assessed the effects of the integrin-specific antibodies on lamellae protrusion. To determine the contribution of $\alpha 3\beta 1$ integrin and the $\alpha 6$ integrins to this process, inhibitory antibodies were added just as lamellae were beginning to emerge, rather than immediately after wounding (Figure 5b). This strategy allowed wound resealing to proceed past the retraction and purse string stages before the inhibitory antibodies were added. Wounds were analyzed 2 hours later for the contribution of lamellae to wound closure. In the presence of an $\alpha 3$-specific antibody, lamellae contributed only 10% of the surface area to wound healing as compared to 25% in the presence of nonspecific antibody. When antibodies against the $\alpha 6$ integrin subunit were added at this later time point, lamellae formation was also reduced by 50% relative to that seen with nonspecific antibody. These findings demonstrate that both the $\alpha 6$ integrins and $\alpha 3\beta 1$ integrin participate in lamellae formation.

We examined the localization of the $\alpha 3$, $\alpha 6$, and $\beta 4$ subunits in cells at distinct stages of wound closure to obtain additional insights into their contribution to wound healing. We focused on the $\alpha 6\beta 4$ integrin because a significant fraction of the $\alpha 6$ signal colocalizes with the $\beta 4$ subunit on the basal surface of intact T84 monolayers in plaque-like structures.[14] The basal surface was examined by immunofluorescence microscopy soon after wounding, as retraction ceased and purse strings formed (Figure 6A). Superimposing a relatively apical section containing the forming actin purse string (in blue) on a basal section containing actin stress fibers (in red) and $\alpha 6\beta 4$ integrin (in green) demonstrated that intact plaques remained on the basal surface of marginal cells during retraction. Higher magnification revealed that basal plaques occupied almost the entire basal surface of T84 cells and contained striations through which actin stress fibers ran (Figure 6B). These actin-striated plaques are most likely Type II hemidesmosomes, described in several mammary and colon carcinoma cell lines.[15,16] Be-

Figure 4. Rhodamine-phalloidin staining of lamellae formation. Monolayers were wounded and then fixed 7 hours later. **A:** An *en face* view shows lamellae protruding into the denuded area. The **arrows**, pointing to lamellae, indicate areas through which optical cross-sections were taken (**B** and **C**). Rearward of many of these lamellae lies a relatively actin-deficient area. One such area is denoted by an **asterisk**. **B:** A cross-section taken through the monolayer at the point indicated by the **lower arrow** in **A** shows fine actin suspension cables (**asterisk**) reaching down from the rearward, apical-lateral cell surface to the dense actin array at the base of the lamella. The **dashed line**, level with the substratum, is shown to help visualize the angle at which these cables descend. **C:** An optical cross-section taken through the monolayer at the point indicated by the **upper arrow** in **A**. In this area, where filamentous actin is visible rearward of the lamellae, actin cables are present but not as elongated as those in **B**. **D:** A wound surrounded by lamellae. These lamellae exhibit a dense actin array at their base and actin-rich lamellopodia at their leading edges, to which the **arrows** point. Areas behind the lamellae that appear actin-deficient are denoted by **asterisks**. Based on cross-sections in **B** and **C**, these areas correspond to cell bodies that contain fine actin suspension cables extending down to the bases of the lamellae. Scale bar, 10 μm.

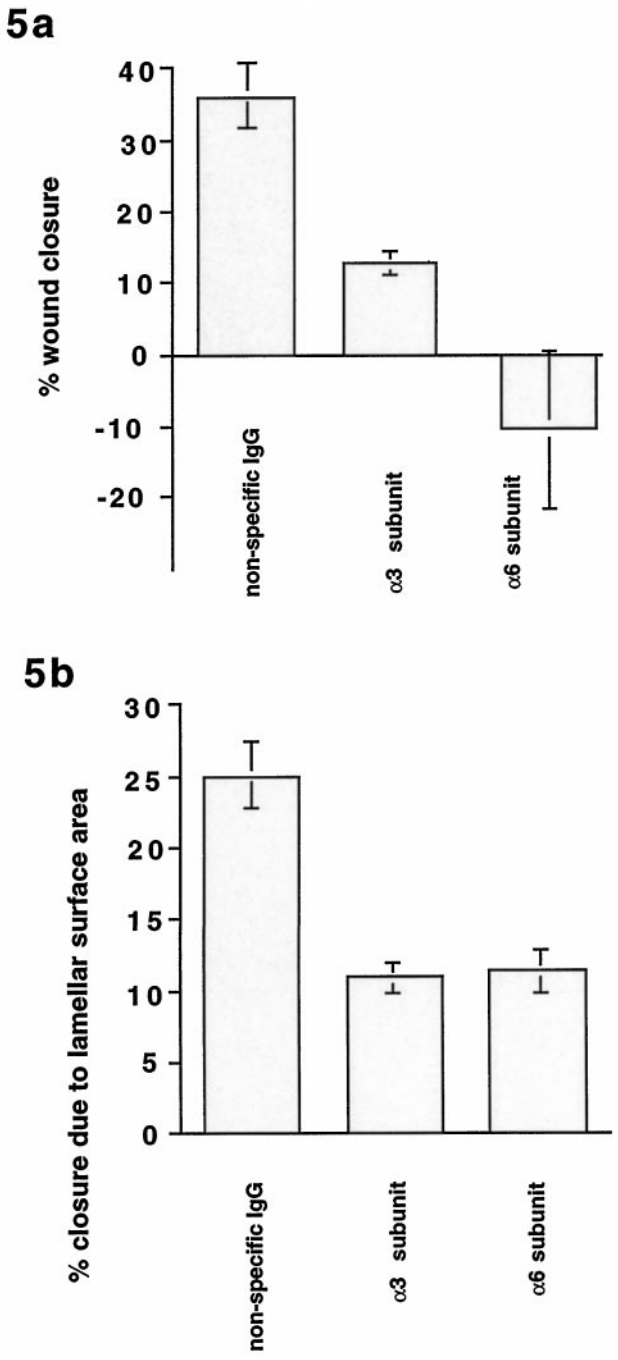


Figure 5. Antibody inhibition of T84 monolayer wound healing. **a:** Wounds were made in the presence of 20 μg/ml of nonspecific IgG or monoclonal antibodies against the α3 or α6 integrin subunit. The area of the denuded region measured immediately after wounding was compared to that value measured 2 hours later to determine the percentage of total closure. The mean percent closure of wounds treated with α3 and α6 integrin-specific antibodies was statistically significantly different from those treated with nonspecific IgG ($P < 0.01$). **b:** Wounds were allowed to close until lamellae appeared and then 20 μg/ml nonspecific IgG, antibody against the α3 subunit or antibody against the α6 integrin subunit was added. After 2 hours, the percent closure due to lamella surface area was determined by subtracting the area of the denuded region from the area bounded by the cell bodies of the marginal cells. (See the 90 minute panel in Figure 1, where the red line encircles the denuded region and the black line traces the area bounded by the cell bodies of the marginal cells). The mean percent closure due to lamellar surface area of wounds treated with α3 and α6 integrin-specific antibodies was statistically significantly different from those treated with nonspecific IgG ($P < 0.01$).

sides resembling type II hemidesmosomes in appearance, they contained the protein HD-1, also known as plectin, but lacked the proteins BP-1 and BP-2 (data not shown). These findings indicate that $\alpha6\beta4$ integrin is found in type II hemidesmosomes, basal structures that survive injury and thus are present during retraction.

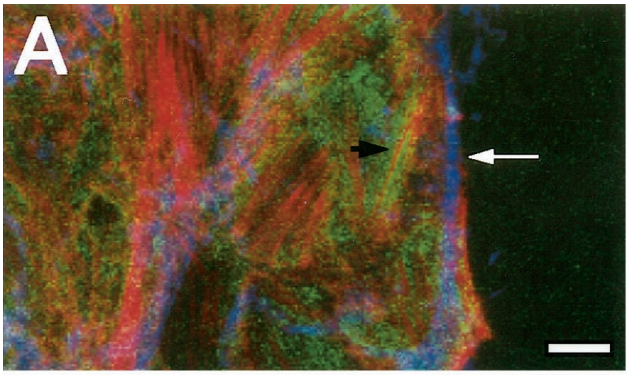
We next analyzed the distribution of the α6 integrins on lamellae to determine how they participate in the formation of these processes. Examination of marginal cells revealed that $\alpha6\beta4$ integrin does not reside in plaques on those producing lamellae. Rather, $\alpha6\beta4$ integrin was dispersed on the marginal cell surface and was not found in the characteristic plaque pattern, in which it alternates with actin. This was seen in a wound fully encircled by lamellae and stained for nuclei (Figure 6C, blue), actin (red), and $\alpha6\beta4$ integrin (green). Marginal cells exhibited a uniform distribution of the β4 subunit, although cells located rearward to the marginal cells displayed this integrin in basal plaques stretched toward the injury (Figure 6D). Close inspection of a lamella revealed the β4 subunit distributed between the fine actin filaments radiating out from the lamella base (Figure 6E). To detect $\alpha6\beta1$ integrin on lamellae, double-staining experiments were performed with antibodies to the α6 and β1 integrin subunit. Points of colocalization, indicating the presence of $\alpha6\beta1$ integrin, were dispersed over these extensions (Figure 6F). These images demonstrate that the α6 integrins respond to injury by distributing themselves over lamellae.

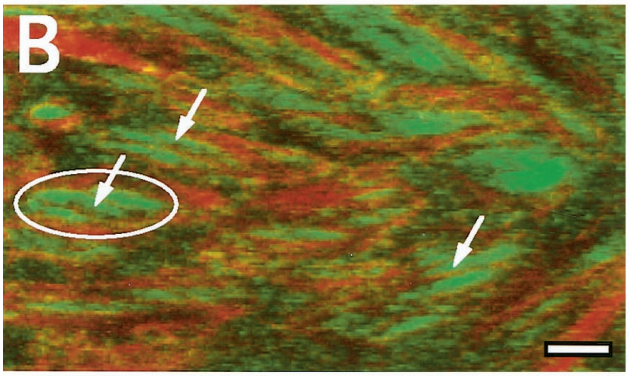
The localization of $\alpha3\beta1$ integrin during lamellae formation was also analyzed. Before the appearance of large, adherent lamellae, small cytoplasmic protrusions were often found emerging into the denuded region in areas where the actin purse string was no longer intact (Figure 7). A distinctive feature of these small protrusions was that they were observed when wounds were stained for the α3 integrin subunit, but not when they were stained for the α6 or β4 integrin subunit. They also stained weakly for actin. Approximately 1 out of 5 of these emerging lamellae exhibited more intense α3 integrin subunit staining at their leading edge than on their bodies. Optical cross-sectioning revealed that this intense band corresponded to a concentration of α3 integrin subunit at the outermost edge into which thin actin filaments ran (Figure 7D). The α3 subunit on more mature lamellae was evenly distributed across these larger extensions and was not found as concentrated at the edge of larger lamellae as it was on small emerging ones (Figure 7E). The localization of $\alpha3\beta1$ integrin on the leading edge of small protrusions and the antibody inhibition data suggest that this integrin plays a distinct role in lamellae formation.

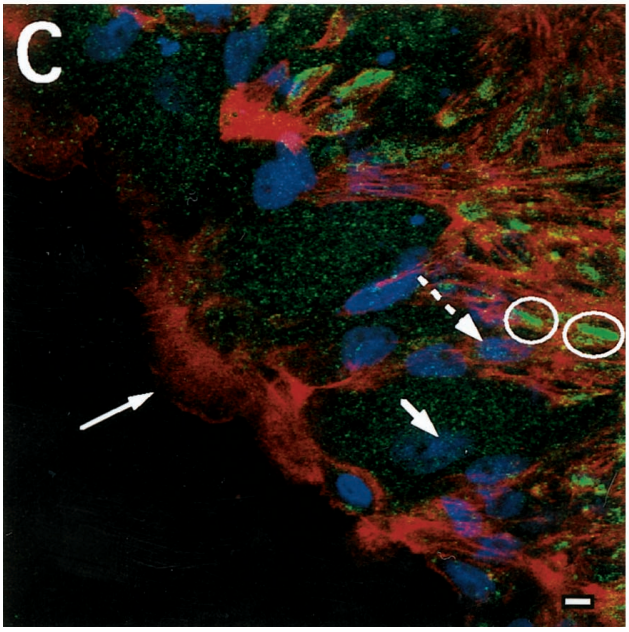
Discussion

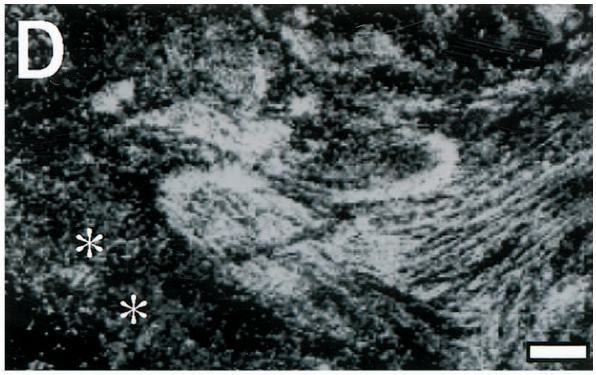
In this paper, we document the progression of events leading to wound closure of an epithelial sheet. These findings yield new insight into gastrointestinal restitution, a specific example of epithelial sheet wound healing. Previous studies on restitution focused primarily on the

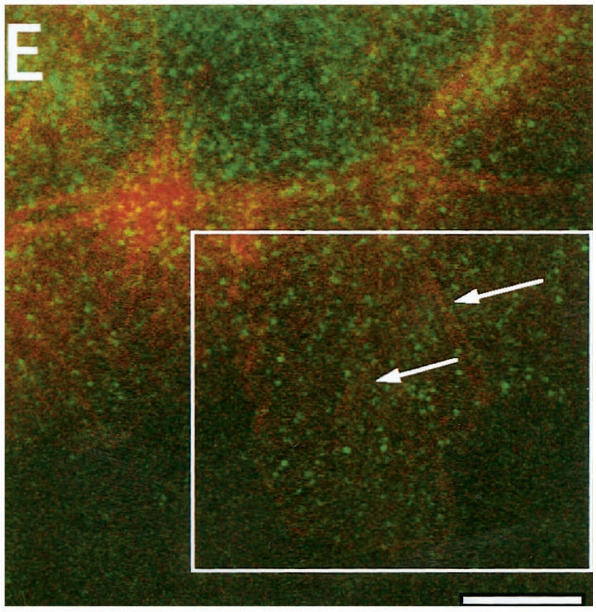
morphology of cells involved in wound closure. Our study extends this work by describing not only the morphology but also changes in cytoskeleton organization and integrin localization in the context of a progression that leads to wound closure. More specifically, we examined filamentous actin and the laminin-binding integrins $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 3\beta 1$ because they have been implicated in epithelial sheet healing.[14] We found that after an initial retraction, wound closure progressed by a series of actin rearrangements that generated a contractive or pulling force on the cells surrounding the defect, inducing them to flatten into the denuded area, thereby sealing it. The laminin-binding integrins participated at different points during the healing process. The $\alpha 6$ integrins, particularly $\alpha 6\beta 4$, helped to contain retraction, and both the $\alpha 6$ integrins and $\alpha 3\beta 1$ integrin contributed to lamellae formation.

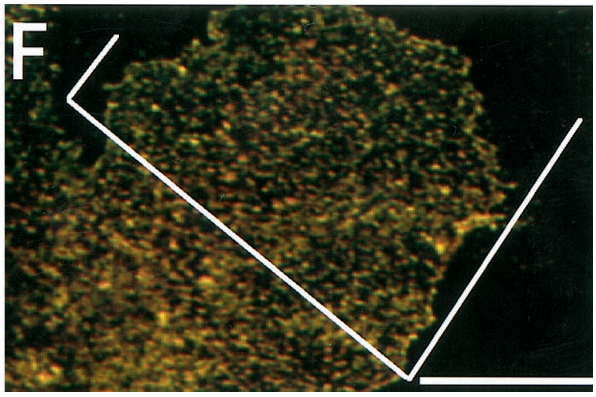












Actin purse strings were first described in studies that demonstrated the ability of small epithelial wounds to heal without any contribution from lamellae.[5,6] These studies show that actin purse strings mechanically pull on marginal cells, drawing them together. When purse string formation is inhibited by the inactivation of endogenous Rho, small, embryonic skin wounds fail to heal.[17] In this study we show that purse strings also participate in the resealing of larger wounds that later produce lamellae. Our results demonstrate that closure begins after purse strings form and before lamellae appear. In the case of larger wounds, the force generated by the purse string appears to be transduced to more remote cells, probably via cell-to-cell contacts. The result is that remote cells stretch toward the injury, increasing their surface area on the average twofold as their basal stress fibers become aligned at right angles to the purse string. These findings suggest that actin purse strings contribute to the resealing of larger wounds by providing a pulling force that induces surrounding cells to flatten, thus increasing coverage of the denuded area.

Lamellae, a key feature of restitution, appear after the purse string completely encircles the wound. We suggest that the wounds made in this study (with surface area of >50 cells), cannot be closed solely by actin purse contraction but require further efforts provided by lamellae. The signal to extend lamellae may be related to the down-regulation of adherens junctions that link the filamentous actin components together to form the purse string.[12] It has been inferred from studies of migrating, isolated cells that the primary function of lamellae during epithelial wound healing is that of a motile apparatus. In contrast, the images presented here indicate that the primary function of lamellae is to generate a pulling force on distal cells, much like purse strings. In fibroblasts, the pulling force generated by lamellae results in the detachment of the cell tail and translocation of the cell.[18,19] However, in intestinal epithelium, the marginal cells do not detach from their rearward neighbors.[2,4,7,11] Rather, the force generated by lamellae is transferred rearward, inducing cells that are distant from the wound to flatten as they stretch toward the injury. This force can be detected by the fact that $\alpha6\beta4$-containing basal plaques become aligned orthogonally to the wound edge in cells that are rearward to those abutting the damage. Cell flattening serves to cover the denuded area in addition to surface area provided by the lamellae themselves.

A novel finding in our study is that marginal cells rearrange their actin cytoskeleton to produce an actin suspension cable. There is no equivalent of this structure in solitary migrating cells, and to our knowledge it has not been described previously. We propose that this actin suspension cable is used to pull the rearward cells toward the lamellae at the wound margin. Actin cables course down from the rearward, apicolateral surface of the marginal cell into the dense actin array at the base of the lamellae. This array in the lamellae of solitary migrating fish keratocytes consists of actin and myosin arranged to produce the contractive force required for detachment of the trailing edge.[20] Our data suggest that this contractive force in T84 cell monolayers and other epithelial sheets is transduced along actin suspension cables to cell-cell contacts at the surface distal from the lamellae. In this way the contractive force is communicated to surrounding cells, inducing them to flatten.

Past work by our group pertaining to extracellular matrix interactions during wound healing demonstrated that laminins secreted by T84 cells, particularly laminin-5, and the laminin-binding integrins $\alpha6\beta1$ and $\alpha6\beta4$ contribute to closure.[14] However, these studies did not pinpoint the stages during resealing at which these integrins act. Here we show that the $\alpha6$ integrins act during retraction and lamellae formation. We hypothesize that $\alpha6\beta4$ integrin within type II hemidesmosome-basal plaques is responsible for generating substratum adhesion that helps contain retraction. This is suggested by the observation that Type II hemidesmosomes share important features with classical hemidesmosomes, complex structures that mediate very tight adhesion of the epidermis to the underlying basal lamina of the skin.[21–26] Both types of hemidesmosome link the keratin cytoskeleton to the cell substratum via $\alpha6\beta4$ integrin.[16,27] Our hypothesis that the $\alpha6\beta4$ integrin is responsible for containing retraction is supported by the sharp distinction seen during retraction between nonadherent cells lying in the damaged area and the adherent cells that express basal plaques. It is

Figure 6. The $\alpha6\beta4$ integrin resides in basal plaques that are disassembled during lamellae formation. T84 monolayers, either intact or injured, were fixed and then stained with rhodamine-phalloidin and by immunofluorescence for the $\beta4$ integrin subunit (**A–C** and **E**) or for the $\beta4$ integrin subunit alone (**D**) or by immunofluorescence for the $\beta1$ and $\alpha6$ integrin subunit (**F**). **A:** An injured T84 monolayer was fixed during wound retraction and stained with rhodamine-phalloidin (red and blue) and for the $\beta4$ integrin subunit (green). An *en face* view of an actin purse string (blue, denoted by the **white arrow**) forming in a relatively apical plane is superimposed over the corresponding view of the basal compartment showing actin stress fibers (red, denoted by **black arrow**) running through $\alpha6\beta4$ integrin (green). This image demonstrates that $\alpha6\beta4$-containing plaques remain intact after wounding, including marginal cells that form the purse string during retraction. Scale bar, 5 μm. **B:** An intact T84 monolayer was fixed and permeabilized in F1 buffer. Treatment with F1 buffer allowed antibody access to the basal surface but left the actin cytoskeleton intact. This *en face* view of the basal plane was stained with rhodamine-phalloidin (red) and for $\beta4$ integrin subunit (green). The $\alpha6\beta4$ integrin is found in ovoid plaques that are interrupted by actin stress fibers, three of which are indicated by **arrows**. The outline of a single cell demonstrates that almost the entire basal surface expresses $\alpha6\beta4$ integrin. Scale bar, 5 μm. **C:** An *en face* view of the basal surface of an injured T84 monolayer fixed 6 hours after wounding and stained with Hoechst stain for nuclei (blue) as well as rhodamine-phalloidin (red) and for the $\beta4$ integrin subunit (green). (Not every cell in this image exhibits a nucleus because many nuclei lie above the basal plane.) Many of the cells adjacent to the wound extend lamellae (**long arrow**). The relatively actin-deficient areas that contain thin actin cables as described in Figure 4 are here seen to contain nuclei, one of which is denoted by the **short arrow**. This image demonstrates that the actin-deficient areas, which contain nuclei and therefore are the bodies of marginal cells, do not exhibit plaques. Rearward of the marginal cells lies a second tier of cells that surround the wound but are not directly adjacent to it. One of the nuclei of these cells is indicated by the **dashed arrow**. Cells in the second tier do exhibit plaques, some of which are circled. Scale bar, 10 μm. **D:** An enlargement of the basal surface of cells in the second tier stained for the $\beta4$ integrin subunit. The wound is out of view to the left of the image and the **asterisks** are placed in F-actin-deficient areas. This image shows plaques stretched toward the wound edge. Scale bar, 10 μm. **E:** Higher magnification reveals that the $\beta4$ subunit (green) is scattered over the surface of a lamella (contained within the box) but does not colocalize with actin filaments (red, **arrows**) radiating out from the base. Scale bar, 10 μm. **F:** Cells were double-stained for the $\beta1$ (green) and $\alpha6$ (red) integrin subunits to detect $\alpha6\beta1$ integrin (yellow). Yellow points of colocalization are dispersed across the lamella, contained within the lines. Scale bar, 10 μm.

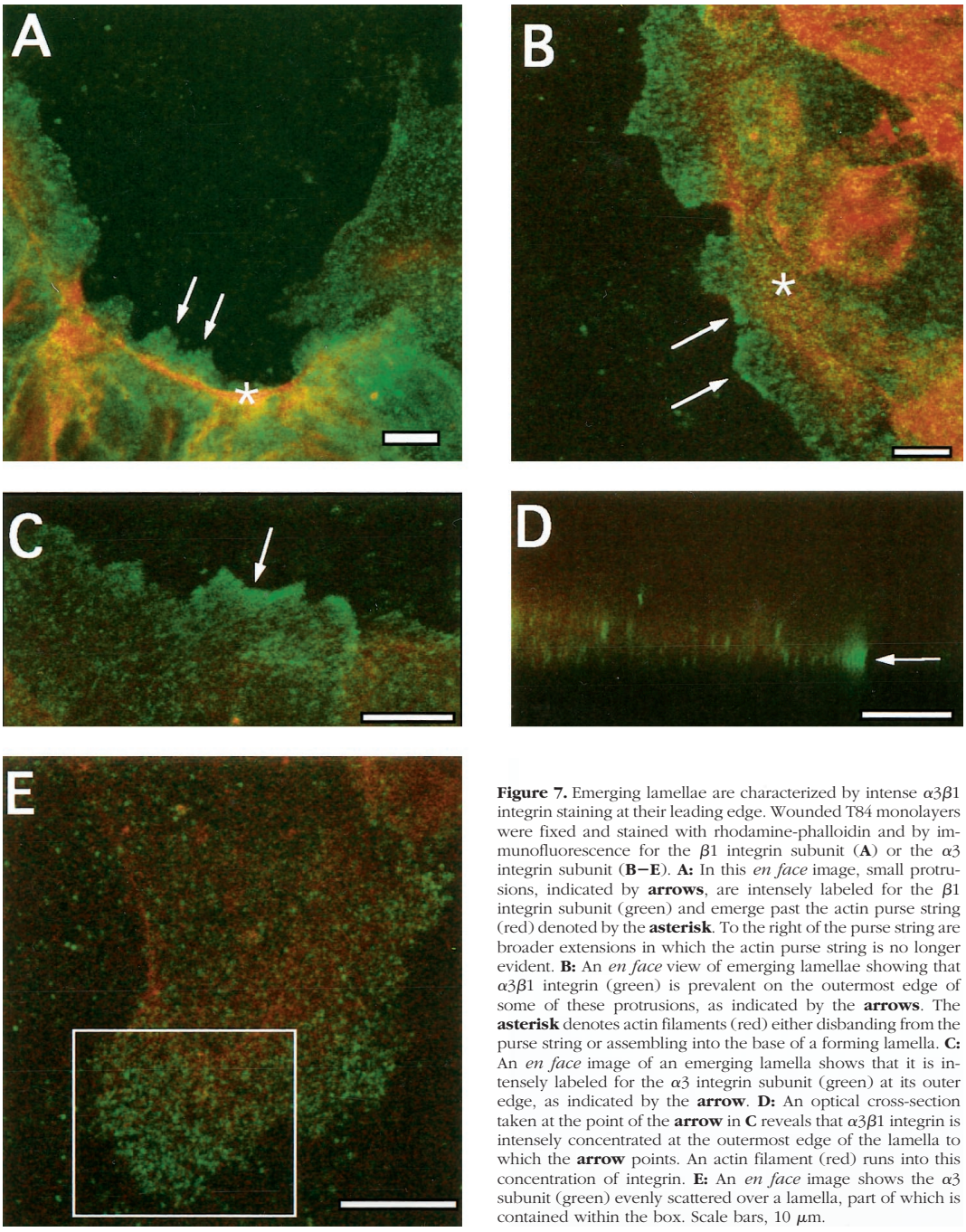


Figure 7. Emerging lamellae are characterized by intense $\alpha3\beta1$ integrin staining at their leading edge. Wounded T84 monolayers were fixed and stained with rhodamine-phalloidin and by immunofluorescence for the $\beta1$ integrin subunit (**A**) or the $\alpha3$ integrin subunit (**B–E**). **A:** In this *en face* image, small protrusions, indicated by **arrows**, are intensely labeled for the $\beta1$ integrin subunit (green) and emerge past the actin purse string (red) denoted by the **asterisk**. To the right of the purse string are broader extensions in which the actin purse string is no longer evident. **B:** An *en face* view of emerging lamellae showing that $\alpha3\beta1$ integrin (green) is prevalent on the outermost edge of some of these protrusions, as indicated by the **arrows**. The **asterisk** denotes actin filaments (red) either disbanding from the purse string or assembling into the base of a forming lamella. **C:** An *en face* image of an emerging lamella shows that it is intensely labeled for the $\alpha3$ integrin subunit (green) at its outer edge, as indicated by the **arrow**. **D:** An optical cross-section taken at the point of the **arrow** in **C** reveals that $\alpha3\beta1$ integrin is intensely concentrated at the outermost edge of the lamella to which the **arrow** points. An actin filament (red) runs into this concentration of integrin. **E:** An *en face* image shows the $\alpha3$ subunit (green) evenly scattered over a lamella, part of which is contained within the box. Scale bars, 10 μm.

likely that $\alpha6\beta4$ integrin within the type II hemidesmosome attaches to laminin-5, one of its ligands that is secreted as a basal sheet by T84 cells.[14,28] Our previous finding that antibodies to laminin-5 added at the time of injury, like those against $\alpha6$ integrin subunit, allow the monolayer to retract unimpeded lend credence to the idea that $\alpha6\beta4$ integrin within the basal plaques mediates attachment to laminin-5, helping to halt retraction.[14]

Antibody inhibition data also demonstrated that the $\alpha6$ subunit-containing integrins participate in lamellae protrusion during T84 wound resealing. Localization studies revealed that on extending lamellae, marginal cells no longer exhibit alternating actin and $\alpha6\beta4$ integrin characteristic of type II hemidesmosomes, nor the lateral cell surface expression of $\alpha6\beta1$ integrin seen in intact monolayers.[14] Instead, $\alpha6\beta4$ and $\alpha6\beta1$ integrin are found dispersed over the lamellae, indicating that both integrins redistribute as lamellae extend. A similar observation has been made during corneal wound healing. In this case, $\alpha6\beta4$ integrin relocates over the cell surface and classical hemidesmosomes disassemble on corneal epithelial cells bordering the injury.[10,24,29] We suggest that redistribution of $\alpha6\beta4$ and $\alpha6\beta1$ integrin is important for lamellae formation and that the basis for $\alpha6$-subunit antibody inhibition is in-

terference with the rearrangement of either one or both of these integrins. In agreement with this conclusion, $\alpha6\beta4$ integrin has been shown to promote the formation and stabilization of lamellae in carcinoma cells by stimulating cyclic AMP-related and phosphoinositide 3-OH kinase pathways.[30–32] It is intriguing to speculate that the surface relocation and release of $\alpha6\beta4$ integrin from hemidesmosome-like structures coincides with its signaling to these pathways during epithelial wound healing.

Antibody inhibition assays also demonstrate that $\alpha3\beta1$ integrin participates in lamellae formation. Images presented here suggest that $\alpha3\beta1$ integrin plays a prominent role in lamellae formation because they show this integrin concentrated at the leading edge of small protrusions where actin filaments terminate. Similarly, $\alpha3\beta1$ integrin associates with actin in keratinocytes and in doing so participates in cell spreading as opposed to cell anchoring.[23] Its role in lamellae formation may also help explain the finding that $\alpha3\beta1$ integrin is important for pancreatic carcinoma cell migration.[33] The observed concentration of $\alpha3\beta1$ integrin at the leading edge of small lamellae suggests that it moves over the cell surface during T84 wound resealing similarly to $\beta1$ integrins during fibroblast migration.[34] In fibroblast migration, integrins are preferentially transported to the cell's leading edge where they bind to their extracellular matrix ligand and then associate with the actin cytoskeleton. The actin cytoskeleton exerts a rearward force on the adherent integrin resulting in the traction required to move the cell forward. Our data indicate that $\alpha3\beta1$ integrin on T84 cells preferentially moves toward the leading edge of emerging lamellae. We hypothesize that it then binds to laminin-5, one of its ligands, helping to generate the traction or a pulling force required for lamella growth.[35] Eventually, this force is transmitted rearward and serves to pull on surrounding cells.

Our findings on T84 epithelial sheet wound healing can be summarized in the following model. On injury, the monolayer first retracts. Retraction ceases, at least in part, due to the adhesion mediated by $\alpha6\beta4$ integrin in type II hemidesmosomes on the basal surface of the cells. Once retraction ceases, the actin purse string exerts a pulling force on the monolayer. The result is that cells flatten into the denuded area and the wound begins to contract. Type II hemidesmosomes remain intact during this process even though the cells stretch to cover 2× more surface area. Some wounds do not close entirely by the efforts of actin purse strings and, in these cases, lamellae are extended by marginal cells. During lamellae extension, type II hemidesmosomes disassemble and actin suspension cables are formed. Disassembly of type II hemidesmosomes is part of lamellae formation and is accompanied by redistribution of the $\alpha6$ integrins. $\alpha3\beta1$ integrin contributes to the initial phase of lamellae protrusion by helping to create the adhesive traction required to form actin suspension cables. The actin suspension cables pull on the rearward cells and flatten them, similarly to the actin purse string, creating more cytoplasmic coverage of the denuded area.

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