Wounding Alters Epidermal Connexin Expression and Gap Junction-mediated Intercellular Communication

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Submitted July 25, 1995; Accepted August 8, 1995
Monitoring Editor: Richard Hynes

We show that connexin expression and in vivo patterns of communication were dramatically altered in response to epidermal wounding. Six hours after injury, Cx26 was up-regulated in the differentiated cells proximal to the wound, but was down-regulated in cells located at the wound edge. In contrast, Cx31.1 and Cx43 were down-regulated in cells both peripheral to and at the wounded edge. These patterns of altered connexin expression were detectable as early as 2 h after wounding and were most pronounced in 24-h old wounds. Increased expression of Cx26 was still evident in the hyperproliferative epidermis of 6-day old wounds. In vivo dye transfer experiments with Lucifer yellow and neurobiotin confirmed that junctional communication patterns were altered in ways consistent with changes in connexin expression. The data thus suggest that intercellular communication is intimately involved in regulating epidermal wound repair.

INTRODUCTION

The epidermis rapidly regenerates in response to wounding to quickly re-establish the cutaneous permeability barrier. Epidermal cells located at the wound edge undergo several phenotypic alterations, called keratinocyte activation, to permit migration across the wound, including the disassembly of desmosomes, retraction of tonofilaments, expression and/or redistribution of several integrin receptors, and the formation of peripheral actin bundles (reviewed in Clark, 1985). Initial migration of activated keratinocytes does not depend on epidermal proliferation (Krawczyk, 1971). However, within 24–48 h after injury, cells residing at the original wound edge become hyperproliferative and generate additional migratory cells (Winter, 1972). Upon closure of the epithelium, keratinocytes underlying the wound stratify and differentiate while continuing to hyperproliferate to such an extent that the newly formed epidermis becomes notably thicker than normal (Hertle *et al.*, 1992). Subsequently, a uniform thickness is re-established.

Although mechanisms regulating wound repair are not known, it has been suggested that gap junction-mediated intercellular communication (GJIC) may

synchronize the migration and/or proliferation of the appropriate cell populations (Larson, 1990). Gap junctions are collections of intercellular channels that regulate the passage of ions, small metabolites, and second messenger molecules (<1 kDa) between the cytoplasm of adjoining cells. These channels gate open and closed in response to changes in voltage, pH, and phosphorylation (reviewed in Hall *et al.*, 1993). Gap junction channels are composed of connexins, a family of at least 12 highly related proteins that differ in their developmental and tissue-specific patterns of expression (White *et al.*, 1992, and references therein). Moreover, each connexin oligomerizes to form channels that may exhibit unique physiological properties (Bennett and Verselis, 1992).

We and others have previously shown that multiple connexins are present in developing and mature rat epidermis (Risek *et al.*, 1992; Goliger and Paul, 1994). These epidermal connexins are expressed in strata-specific patterns that often overlap but are clearly distinct for each connexin. For example, in newborn rat tail skin, Cx26 is found predominantly in the upper spinous and granular cell layers whereas Cx31.1 and Cx43 are confined to lower spinous and basal cell layers. Expression patterns of these connexins are consistent with ultrastructural studies detecting gap junctions in the basal, spinous, and granular cell layers of rat (Risek *et al.*, 1992) and human (Caputo and Pe-

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luchetti, 1977) epidermis, as well as with Lucifer yellow dye transfer experiments demonstrating GJIC in these three epidermal layers (Kam *et al.*, 1986; Salomon *et al.*, 1988).

Data suggesting that GJIC is involved in epidermal wound repair derive in part from ultrastructural investigations performed by Gabbiani *et al.* (1978). These studies demonstrated that epithelial cells at the periphery of a healing wound have nearly four times more surface area occupied by gap junctions than do normal epidermal cells. This increase in junctional surface area was concomitant with an increase in expression of the contractile proteins actin and myosin, suggesting that GJIC may be involved in synchronizing epidermal cell migration. Similar studies using wounded squamous epithelium from guinea pig palate found increased gap junction density in stratifying cells within the regenerating epithelium, suggesting a role for GJIC in cytodifferentiation (Andersen, 1980). The molecular mechanisms underlying these changes in gap junction density, and the connexins involved are not known. We have thus assayed for changes in connexin expression in wounded rat tail epidermis at different times after injury. We demonstrate that Cx26 expression is rapidly increased in differentiating cells proximal to the wound, but is decreased in cells at the wound edge proper. In contrast, Cx31.1 and Cx43 are rapidly decreased in cells located at both the wound edge and the wound periphery. We also show that wound-induced changes in connexin expression resulted in altered patterns of intercellular communication between keratinocytes in the newly regenerating epidermis.

MATERIALS AND METHODS

Tissue Preparation and Immunohistochemistry

Epidermal wounds ~0.5 mm deep and ~1 cm long were made by gouging with a microscalpel (Roboz Surgical Instruments, Rockville, MD) or an 18-gauge needle along the anterior/posterior axis and in a rostral portion of the tails of 10-day old Sprague-Dawley rat pups (Charles River, Wilmington, MA). At indicated times after injury, wounded tail sections were dissected, fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 1–2 h, and processed for paraffin embedding. Serial cross sections of tail through the wounds (~3 μ m thick) were mounted on slides, deparaffinized, and immunohistochemically stained for connexin expression as previously described (Goliger and Paul, 1994), with the following modifications: primary antibody dilutions were 1:5000 for Cx26 (Ab #9770), 1:100 for Cx31.1 (Ab 31.1/Gex), and 1:20,000 for Cx43 (Ab petunia); the ABC Elite solution used in the amplified staining procedure (Adams, 1992) was made according to manufacturer's instructions (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA), but then diluted 10-fold in PBS before use. In contrast to our previous studies (Goliger and Paul, 1994) in which amplification parameters were optimized for each connexin antiserum and for each stage of tissue development, all sections were treated identically in these experiments. Photomicrographs were taken with a Zeiss Axiophot microscope using Nomarski optics. Low magnification montages were constructed by scanning photographic slides of overlapping fields with a Microtek ScanMaker 1850S slide scan-

ner (Microtek Lab, Torrence, CA), fusing these images on a Macintosh computer using Adobe Photoshop (Adobe Systems, Mountain View, CA), and then scaling to size.

In Vivo Dye Transfer Experiments

Ten-day old rat pups were wounded as described above. Approximately 18–24 h after wounding, rat pups were killed and wounded tails were dissected apart from the animal body. Immediately following dissection, approximately 50 μ l of PBS containing 1% rhodamine-dextran (10,000 MW, lysine fixable; Molecular Probes, Eugene, OR) and either 2% lucifer yellow ($C_{13}H_9N_5O_9S_2Li_2$; Sigma, St. Louis, MO) or 2% neurobiotin tracer (N-2 [aminoethyl]-biotinamide hydrochloride; Vector Laboratories) was placed on top of the wound. Tails were then cut with a razor blade diagonally across the wound coagulum. Wounded portions of tail were dissected, deboned, incubated in PBS at room temperature for an additional 15–30 min, and then fixed in 4% paraformaldehyde and processed for paraffin embedding. Cross-sections of tail (~3 μ m thick) cut-loaded with Lucifer yellow were deparaffinized, cover-slipped with Permount mounting medium (Sigma) and photographed using epifluorescence. Tail sections cut-loaded with neurobiotin were deparaffinized, rehydrated, incubated with fluorescein-conjugated avidin at the manufacturer's recommended dilution (avidin-fluorescein isothiocyanate; Boehringer Mannheim, Indianapolis, IN), coverslipped with Vectashield mounting medium (Vector Laboratories), and photographed with epifluorescence. Figures were constructed using Adobe Photoshop software on a Macintosh computer as described above.

RESULTS

Wound Morphologies

Tail skin from 10-day old rats was wounded with a microscalpel or needle and then examined at selected times after injury. The morphologies of these incisional wounds are schematically illustrated in Figure 1. Six hours after injury, wounds appeared as discontinuities in the epidermis with wound edges at the indicated positions. Hematoxylin and eosin staining of the wounded tail skin revealed that the epithelial "tongue" beyond the wound edge was comprised of predominantly necrotic tissue. By 24 h after wounding, activated keratinocytes had migrated from opposite sides of the wound to form a continuous epithelium underlying the coagulum. Cells at the center of this epithelium had presumably stopped migrating and had commenced differentiating. In addition, by 24 h a "proliferative zone" of cells at the wound periphery had formed. The mitotically active cells in this zone resided in the basal cell layer and occasionally in the lowest spinous cell layer (Hell and Cruickshank, 1963; our unpublished observations), whereas cells in the upper layers were differentiating to restore the epidermal permeability barrier. Six days after injury the epidermis had almost healed. Wounds were occasionally identified by residual coagula but were more frequently identified by the abnormal thickness of the hyperproliferative epidermis at the original wound site (Hertle *et al.*, 1972). All wounds that we examined were essentially symmetric about the long axis of the wound with respect to morphology and connexin

Figure 1. Wound morphologies. Wound morphologies at indicated times after injury are schematically illustrated. Six-hour old wounds appeared as discontinuities in the epidermis, with epithelial "tongues" of predominantly necrotic tissue extending beyond the viable wound edge. Twenty-four hour old wounds contained proliferative zones at the wound periphery, and migrating and/or stratifying epithelia (M/S) underlying the center of the coagula (C). Six-day old wound sites were identified as regions of thicker, hyperproliferative epidermis with residual coagula (C) occasionally present.

staining. Thus, in Figures 2–10, we show only the left side of each wound.

Connexin Expression

Serial sections of wounded skin were immunohistochemically stained for the expression of three connexins, Cx26, Cx31.1, and Cx43. In epidermis far from the wound edge, Cx26 was detected primarily in the granular cell layers (Figure 2, A and B). However within 6 h after injury, increased amounts of Cx26 were detected in granular and upper spinous layers near, but not at, the wound edge (Figure 2, A and C, arrowhead indicates wound edge). In contrast, Cx26 decreased in cells within ~6 basal

cell diameters of the wound edge and in the necrotic epithelial tongue (Figure 2C, dot). Cx31.1 and Cx43 were predominantly expressed in the lower and middle spinous cell layers of normal epidermis (Figure 3, A and B; Figure 4, A and B). Within 6 h after wounding, these connexins were down-regulated in cells at the wound edge and in the adjacent epithelial tongue, as well as in a large number of cells peripheral to the wound (Figures 3 and 4). Similar but less pronounced changes in the expression patterns of all three connexins were observed at 2 h after wounding (our unpublished observations), indicating that these are relatively rapid responses to injury.

Altered expression patterns of Cx26, Cx31.1, and Cx43 were most obvious 24 h after wounding. Cx26 signal was more abundant than at 6 h and had now spread into the middle spinous as well as granular and upper spinous layers (Figure 5). Cx26 was also detectable near but not at the center (Figure 5, A and C, arrowhead at center) of the continuous epithelium underlying the coagulum. In addition, Cx26 was not readily detectable in the mitotically active basal cell layers of the proliferative zone and the regenerating epithelium (Figure 5, A and C). In contrast, Cx31.1 and Cx43 staining was nearly absent in the proliferative and regenerative areas and was clearly decreased many basal cell diameters from the edge of the wound coagulum (Figures 6 and 7). Although the extent of this decreased connexin expression varied with wound severity, regions closest to the original wound site displayed the maximal effect.

The latest stage of wound healing examined was 6 days after injury, at which time normal barrier functions of the epidermis had been restored. However, continued abundant expression of Cx26 was observed in the middle spinous, upper spinous, and granular layers of the hyperproliferative epidermis (Figure 8, A and C). This was in striking contrast to the restricted expression of Cx26 in the granular layers of normal 16-day old rat tail epidermis (Figure 8B). By 6 days after injury, Cx31.1 and Cx43 levels were nearly the same in the hyperproliferative epidermis as in unwounded areas with regard to both intensity and location of staining (Figures 9 and 10).

Dye Transfer Experiments

Results of the expression studies suggested that actively migrating keratinocytes at the leading edge of a discontinuous regenerating epithelium, or the corresponding keratinocytes at the center of a newly joined epithelium, would not communicate efficiently because they were devoid of all three connexins examined. Similarly, we expected that GJIC

Figure 2: Cx26 expression 6 hr. after wounding

Figure 3: Cx31.1 expression 6 hr. after wounding

Figure 4: Cx43 expression 6 hr. after wounding

Figures 2–4. Connexin expression 6 h after wounding. Serial sections of wounded rat tail epidermis were examined 6 h after injury for expression of Cx26 (Figure 2), Cx31.1 (Figure 3), and Cx43 (Figure 4). Each figure displays a low magnification montage of the left side of the wound (A), and high magnifications of regions of normal (B) and wounded (C) epidermis. Arrowheads in panels A and C correspond to the same location and indicate the dermal-epidermal junction at the wound edge. Black dots indicate the epithelial “tongue” adjacent to the wound edge. Scale bar in A, ~50 μm ; scale bars in B and C, ~30 μm .

within the mitotically active basal cell layers of the proliferative zone and regenerating epithelium would be decreased because these cells often ex-

pressed reduced levels of Cx31.1 and Cx43 and were devoid of Cx26. In addition, we anticipated that differentiated cells in the proliferative zone and

Figure 5: Cx26 expression 24 hr. after wounding

Figure 6: Cx31.1 expression 24 hr. after wounding

Figure 7: Cx43 expression 24 hr. after wounding

Figures 5–7. Connexin expression 24 h after wounding. Serial sections of wounded rat tail epidermis were examined 24 h after injury for expression of Cx26 (Figure 5), Cx31.1 (Figure 6), and Cx43 (Figure 7). Each figure displays a low magnification montage of the left side of the wound (A), and higher magnifications of regions of normal (B) and wounded (C) epidermis. Arrowheads in panels A and C correspond to the same location and indicate the dermal-epidermal junction at the approximate center of the newly forming epithelium underlying the coagulum. Scale bar in A, ~50 μ m; scale bars in B and C, ~30 μ m.

Figure 8: Cx26 expression 6 days after wounding

Figure 9: Cx31.1 expression 6 days after wounding

Figure 10: Cx43 expression 6 days after wounding

Figures 8–10. Connexin expression 6 days after wounding. Serial sections of wounded rat tail epidermis were examined 6 days after injury for expression of Cx26 (Figure 8), Cx31.1 (Figure 9), and Cx43 (Figure 10). Each figure displays a low magnification of the left side of the wound (A), and higher magnifications of normal (B) and wounded (C) epidermis. Arrowheads in panels A and C correspond to the same location and indicate the dermal-epidermal junction at the approximate center of the hyperproliferative epidermis. Scale bar in A, ~50 μm ; scale bars in B and C, ~30 μm .

parts of the regenerating epidermis might have altered GJIC properties associated with an up-regulation of Cx26 and a down-regulation of Cx31.1 and Cx43. To test these predictions, we examined patterns of dye transfer in control and wounded epidermis.

A "cut-loading" protocol was developed to simultaneously introduce gap junction-permeable (Lucifer yellow or neurobiotin) and -impermeable (rhodamine-dextran) dyes in rat tail skin 18–24 after wounding. The dye-loading "cut" always penetrated through the epidermis and usually the dermis as well. This resulted in dye uptake by both epidermal keratinocytes and dermal fibroblasts, as well as dye seepage into extracellular spaces (e.g. Figure 11A). We assumed that the dye transfer observed in epidermis was derived solely from cut-loaded keratinocytes because it has been well established that keratinocytes do not communicate with dermal fibroblasts (Kam *et al.*, 1986; Salomon *et al.*, 1988). Artifactual staining of the cornified layers and coagulum, as evidenced by coincident staining with both dyes, was frequently observed (Figures 11, B and C).

Control experiments with unwounded skin demonstrated that Lucifer yellow was transferred to a large

number of cells distal to the cut edge (Figure 11A, black arrow indicates cut edge) even when only a few keratinocytes were directly loaded (Figure 11A, white arrowheads). In addition, Lucifer yellow was detected in the lateral diffusion channel of the stratum lucidum (Figure 11A white arrow; Mansbridge and Knapp, 1993). As expected, dye transfer occurred among cells residing in all metabolically active layers of the epidermis (basal, spinous, and granular but not cornified; Figure 11A, dotted outline). Lucifer yellow was detected in >100 cells in unwounded skin although the extent of dye transfer was variable between experiments. Moreover, the fluorescent intensity of Lucifer yellow decreased gradually with distance from the cut edge. These results apparently contrasted with earlier observations of restricted and compartmentalized epidermal dye transfer (Kam *et al.*, 1986; Salomon *et al.*, 1988; Mansbridge and Knapp, 1993).

Experiments with wounded tail skin demonstrated that wound-induced changes in connexin expression affected Lucifer yellow transfer. A comparison of dye transfer patterns among all wounded tails ($n = 12$) revealed common features that are illustrated by two experiments depicted in Figure 11. In the first example (Figure 11B) the dye-loading

Figure 11. Lucifer yellow transfer was less efficient in the regenerating wound epithelium than in normal skin. Nomarski and fluorescent images show sections of an unwounded (A) and two wounded (B and C) tails cut-loaded with rhodamine-dextran and Lucifer yellow. Basal, spinous, and granular cell layers of epidermis are located between the dotted outlines. Cells located along the cut edge (black arrows) that were directly loaded with dyes are indicated (white arrowheads). In addition, Lucifer yellow diffused into a lateral diffusion channel in the stratum lucidum (white arrow). The presence of Lucifer yellow in cells not containing rhodamine-dextran indicated gap junction-mediated dye transfer. Scale bars, 50 μ m.

cut (black arrow) occurred within the proliferative zone of a 24-h old wound that had already formed a continuous epithelium (dotted outline). At least one, and possibly a few cells within the proliferative zone, were directly loaded with dye (Figure 11B, white arrowheads). Lucifer yellow was transferred to most of the cells in the proliferative zone and part of the adjoining regenerating epithelium, including cells within the basal and lowest spinous cell layers. This was unexpected because basal and lowest spinous cells in the proliferative zone frequently expressed reduced amounts of Cx31.1 and Cx43 and did not express Cx26. The overall extent of transfer in wounded epidermis was significantly less than in controls (Figure 11, compare panels A and B), with no transfer occurring to the more central regions of the newly formed epidermis. This is consistent with our finding that neither Cx26, Cx31.1, nor Cx43 were strongly expressed in this region.

The second example (Figure 11C) shows a 20-h old wound that had not yet formed a continuous epithelium (dotted outline). The dye-loading cut (indicated by the black arrow) occurred through the proliferative zone and the underlying dermis, but shear force most likely tore dermis away from epidermis. This resulted in direct loading of both dyes into a number of basal cells in the regenerating epithelium (Figure 11C, white arrowheads). We observed that Lucifer yellow efficiently transferred to about 20–25 cells located primarily in the basal and spinous layers of the regenerating epidermis. However, Lucifer yellow did not transfer to a group of cells located at the leading edge of the regenerating epithelium, consistent with the lack of connexin expression in this area (Figures 5–7). In addition, Lucifer yellow was not strongly detected in the more differentiated cells immediately underlying the coagulum. This is likely to reflect predominant expression of Cx26 in these upper layers because Lucifer yellow transfers poorly through channels comprised of this connexin (Elfgang *et al.*, 1995; Cao, Willecke, and Nicholson, personal communication). Although patterns of dye transfer in wounds shown in Figure 11, B and C, were somewhat dissimilar due to differences in the location of dye-loaded cells, the results were comparable; dye transferred between cells in the basal and lower spinous layers of the proliferative zone and lateral regions of the regenerating epidermis but transferred poorly into the remaining portions of this newly forming epithelium.

A second gap junction-permeable tracer, neurobiotin, was also used to investigate wound-induced alterations in GJIC. Neurobiotin differs from Lucifer yellow in both size (LY = 443 Da; Nb = 287 Da) and charge (LY = -2 ; Nb = $+1$) and is generally thought to be a more sensitive tracer for intercellular communication (Elfgang *et al.*, 1995). Control studies with normal epidermis showed that neurobiotin trans-

ferred significantly greater distances from the cut-loaded edge than Lucifer yellow (compare Figures 11A and 12A). However, similar to Lucifer yellow, neurobiotin transfer occurred only in the metabolically active layers, and the concentration of neurobiotin gradually decreased with distance from the cut edge.

In experiments with wounded skin, neurobiotin transferred to nearly all keratinocytes within the regenerating epithelium (Figure 12, B–D), which is in striking contrast to the limited transfer of Lucifer yellow between these keratinocytes. For the more differentiated cell layers, efficient transfer of neurobiotin but not Lucifer yellow is consistent with predominant expression of Cx26 in these cells because neurobiotin permeates Cx26 channels more efficiently than Lucifer yellow (Cao, Willecke, and Nicholson, personal communication; Elfgang *et al.*, 1995). For the less differentiated basal and lowest spinous cell layers, transfer of both dyes is inconsistent with the apparent lack of connexin expression in these cells. Most cells within the newly forming epidermis were apparently well coupled to each other. However, we frequently observed that neurobiotin did not transfer efficiently to a few cells at the leading edge of the regenerating epithelium (Figure 12C, arrows; Figure 12D). These cells may be the actual migrating keratinocytes suggesting that keratinocyte activation requires changes in GJIC.

DISCUSSION

We have shown that connexin expression and in vivo patterns of communication were dramatically altered in response to epidermal wounding. Cx31.1 and Cx43 were down-regulated in the regenerating epithelium and often in the adjacent proliferative zone and wound periphery. In contrast, Cx26 was strongly up-regulated in the differentiated cells within the proliferative zone and in the lateral regions of the regenerating epidermis, but down-regulated at the leading edges of the regenerating epithelium or corresponding central portions of a newly joined epidermis. In vivo dye transfer experiments with Lucifer yellow and neurobiotin showed that intercellular communication was a highly regulated aspect of wound healing, which correlated to a certain extent with changes in connexin expression.

Our finding of extensive Lucifer yellow transfer in normal unwounded epidermis contrasts with previous experiments showing restricted dye transfer. Mansbridge and Knapp (1993) introduced Lucifer yellow into the epidermis by a cut-loading procedure somewhat similar to ours but reported little or no dye transfer. This was likely due to a delay between cutting the tissue and incubating with dye, which did not occur in our experiments. Other groups have micro-injected individual epidermal keratinocytes with Lu-

Figure 12. Wounding uncouples presumptive migrating keratinocytes. Nomarski and fluorescent images show sections of an unwounded (A) and two wounded (B–D) tails cut loaded with rhodamine-dextran and neurobiotin. Several cells (white arrowheads) located along the cut edge (black arrows, panels A, B, and D) were directly loaded with dyes, although no such cells were detected in the plane of section shown in panel D. Panel A shows epidermis at a low magnification to illustrate the full extent of neurobiotin transfer in unwounded tissue (basal, spinous, and granular cell layers are located between the dotted outlines). Panel C shows higher magnification of the leading edge of the regenerating epithelium depicted in panel B. Panel D shows a second example of a newly forming epithelium also at high magnification. Whereas most cells in these regenerating epithelia were well coupled, neurobiotin transferred poorly to several presumptive migrating keratinocytes located at their leading edges (C, black and white arrows; D). Scale bar in A, 100 μ M; scale bars in B, C, and D, 25 μ m.

cifer yellow and observed compartmentalized transfer to only 20–40 neighboring cells (Kam *et al.*, 1986; Salomon *et al.*, 1988). We have no clear explanation for the differences between our results and theirs. Nevertheless, our findings are more consistent with patterns of connexin expression in the epidermis (Risek *et al.*, 1992; Kamibayashi *et al.*, 1993; Butterweck *et al.*, 1994; Goliger and Paul, 1994; Risek *et al.*, 1994; Salomon, *et al.*, 1994). Furthermore, our results with Lucifer yellow are comparable, albeit less extensive, to those we obtained with neurobiotin. Together, experiments with both dyes clearly indicate that epidermal keratinocytes are well coupled over large areas and do not appear to form restricted dye transfer compartments.

There were two properties of intercellular communication in wounded epidermis that appeared to be inconsistent with connexin expression patterns. The first was that basal and lowest spinous cells in the proliferative zones and regenerating epithelium transferred dye efficiently but apparently lacked Cx26, Cx31.1, and Cx43. The simplest explanation for this behavior is that additional connexins might be expressed in these layers. Several other connexins including Cx30.3, Cx31, and Cx37 are found in normal rodent skin (Risek *et al.*, 1992; Goliger and Paul, 1994; Butterweck *et al.*, 1994) but their expression patterns in wounded tissue are not known. In addition, other connexins not typically found in the epidermis could

be up-regulated in response to wounding. The second inconsistency is that Lucifer yellow transferred efficiently to the more differentiated cell layers in the proliferative zone but not to the more differentiated layers within the regenerating epithelium. Both of these regions predominantly expressed Cx26. Transfer to the proliferative zone was thus unexpected because Lucifer yellow diffuses poorly through Cx26 channels (Elfgang *et al.*, 1995; Cao, Willecke, and Nicholson, personal communication). However, the proliferative zone usually expressed Cx26 more abundantly than the regenerating epithelium. Moreover, amounts of Cx31.1 and Cx43 were always markedly decreased in the regenerative epidermis but varied in the proliferative zone depending on wound severity. Thus, efficient transfer of Lucifer yellow in the proliferative zone may have been facilitated by high levels of Cx26, and/or low levels of Cx31.1 and Cx43.

Changes in GJIC may be important in regulating two aspects of wound healing. The first involves the regulation of keratinocyte activation. Based on the observation that presumptive migratory cells became uncoupled, we postulate that keratinocyte activation may be partly initiated by the loss of connexin expression and a concomitant decrease in GJIC. The molecular mechanisms by which GJIC affect activation are not known although the modulation of cell-cell adhesion subsequent to the inhibition of GJIC could be involved (Meyer *et al.*, 1992; Paul *et al.*, 1995). The second aspect of wound repair that correlated with altered GJIC is the restoration of normal epidermal growth. Keratinocyte differentiation was associated with a dramatic increase in the expression of Cx26 throughout middle and upper spinous cell layers and granular cell layers. This increased expression could theoretically lead to changes in local Ca^{2+} concentrations within the differentiated cell strata. It has been previously demonstrated that epidermal wounding results in the loss of a Ca^{2+} gradient normally found between keratinocyte layers, and that premature regeneration of this gradient impedes permeability barrier repair (Menon *et al.*, 1992). High levels of Cx26-mediated GJIC could help prevent premature regeneration of the normal Ca^{2+} gradient and thus facilitate repair. Eventual restriction of Cx26 to the granular cell layers upon re-epithelialization would permit subsequent restoration of the epidermal Ca^{2+} gradient.

Results of our studies demonstrate that wounding the epidermis induced dramatic changes in the expression of three connexins, causing detectable alterations in the communication properties of the regenerating tissue. Epidermal wound healing thus provides an attractive model system in which to address the biological significance of multiple connexin expression, as well as the physiological importance of GJIC in a non-excitable tissue.

ACKNOWLEDGMENTS

We thank Dr. John Gibbins for help with the histology, and members of the Goodenough and Paul laboratories for critically reading and discussing the manuscript. This work was supported by a National Institutes of Health grant GM-37751 to D.L.P. J.G. is the recipient of an award from The Medical Foundation.

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