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Abnormal connexin expression in human chronic wounds

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Summary

Background Regulated alteration of connexin expression has been shown to be integral to acute wound repair. Downregulation of the gap-junction protein connexin 43 at the wound edge has been correlated with keratinocyte and fibroblast migration, while abnormal overexpression of connexin 43 significantly perturbs healing, as shown in the streptozotocin diabetic rodent impaired healing model.
Objectives To examine the protein expression levels of connexin 43, in addition to connexins 26 and 30, in a variety of human chronic wounds.
Methods Wound-edge punch biopsies and a matched control from the arm were taken from a cohort of patients with venous leg, diabetic foot or pressure ulcers. Wound connexin expression in each patient was compared with that in a matched, nonwounded arm punch. Tissue was sectioned, stained and imaged by confocal microscopy using identical parameters per patient to permit quantification.
Results Epidermal connexin 43, connexin 26 and connexin 30, and dermal connexin 43 were discovered to be strikingly upregulated in every ulcer from all three wound types, pointing to connexin upregulation as a common feature between chronic wounds.
Conclusions This result supports efforts to target connexin 43 to promote cell migration and wound healing in chronic ulcers.

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Conflicts of interest
J.E.S.S is supported at UCL by a BBSRC CASE studentship with CoDa Therapeutics Inc. D.L.B. is a founding scientist of CoDa Therapeutics Inc. and holds shares in the company. S.O.N., R.H., A.M.W. and A.R.J.P. are or were employed by CoDa Therapeutics Inc. T.E.S obtained the human tissue for use by CoDa Therapeutics Inc.

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What's already known about this topic?

- Connexin 43 (Cx43) protein levels are reduced at the wound edge, enabling migration of keratinocytes and fibroblasts to aid wound healing.
- In wounded diabetic rats, Cx43 is elevated in the wound edge and healing is delayed.
- In human diabetic epidermal wound edge, Cx43, Cx26 and Cx30 are detectable.

What does this study add?

- We quantify the wound-edge expression of Cx43, Cx26 and Cx30 in chronic venous, diabetic foot and pressure ulcers and show their tissue distribution.
- Cx43 is massively upregulated in the epidermis and dermis.

Chronic wounds are a global problem[1] that places immense strain on healthcare resources while often dramatically reducing the quality of life of patients.[2] The most prevalent chronic wounds are diabetic foot ulcers (DFUs), venous leg ulcers (VLUs) and pressure ulcers (PRUs).[3] The incidence of all three wound types is predicted to increase dramatically over the coming decades.[1] This is partially accounted for by an increasingly ageing population in combination with the rising global prevalence of diabetes and obesity.[4,5]

It is well understood that a chronic wound does not pass through the normal phases of acute cutaneous wound repair, resulting in a long-standing deficit in tissue integrity. Healing is abnormally slowed or stalled, as shown by persistent inflammation and limited re-epithelialization, leaving the wound susceptible to infection and physical damage that may further attenuate healing.[6] The lack of effective and specific treatment options reveals that our understanding of chronic wound pathobiology is still incomplete.

One emerging factor in the pathogenesis of chronic wounds is the role of the gap-junction protein connexin 43 (Cx43). Connexin proteins are integral components of gap junctions, which are membrane channels that facilitate direct cell-to-cell communication and permit the intercellular movement of molecules less than ~1 kDa in size. Gap junctions are essential to homeostasis within skin, as witnessed by the detrimental effects of naturally occurring mutations that lead to a variety of skin abnormalities.[7] In addition to Cx43, nine other connexin proteins are reported to be expressed in the human epidermis (connexins 26, 30, 30·3, 31, 31·1, 32, 37, 40 and 45),[8] with Cx43 also identified in dermal tissue.[9]

Connexins are closely involved in normal wound repair, and show dynamic changes in expression after wounding. In the first 24 h, Cx43 is naturally downregulated in wound-edge keratinocytes and fibroblasts as they become migratory, while Cx26 and Cx30 are upregulated in the epidermal leading edge.[10–13] Manipulation of this process in rodent models via the application of a Cx43 antisense oligodeoxynucleotide to the acute wound accelerates downregulation and the healing process, while also dampening the inflammatory response.[14,15] Consistent with this finding, reduced levels of Cx43 in the Cx43-inducible knockout mouse model were reported to double the rate of skin wound repair.[16]

Other approaches to interfere with connexins for therapeutic benefit are being explored in a variety of tissues and models of injury. There is a range of mimetic peptide approaches. Danegaptide (or GAP-134) is reported to activate gap-junction channels.[17] Another peptide, ACT-1, reportedly interrupts the binding partner interaction with the PDZ2 domain of Cx43.[18,19] Peptides that mimic the extracellular loops of connexins have been reported to influence both gap junctions and hemichannel function,[20–22] although the precise mode of action is still to be defined.

Misregulation of Cx43 impairs healing, as observed in the streptozotocin diabetic rat, where, in this model of human chronic disease, Cx43 is not downregulated in the cells of the wound edge but is instead abnormally increased.[12,13,23] Application of Cx43 antisense oligodeoxynucleotides to diabetic rat wounds prevents the abnormal upregulation of Cx43 at the wound edge and restores normal healing rates.[12,13,23] The connexin status of the cells of the dermis may also be very important. Recently it has been reported that Cx43 expression in fibroblasts changes their cell-to-cell adhesion and cytoskeletal response during wound healing, with Cx43 upregulation retarding their rate of migration.[13] In biopsies from patients with mixed ulcers and DFUs, Cx43, Cx26 and Cx30 were detected at epidermal wound margins, as well as in cells at some distance from the epidermal wound edge.[24] However, the involvement of connexin regulation within the epidermis in chronic wound persistence has not been thoroughly investigated. Documenting the levels of connexin expression in a variety of chronic wounds is an important step in our understanding of the link between connexin expression and impaired healing.

Materials and methods

Biopsy acquisition, preservation and cryosectioning

Ethical approval for the tissue collection was obtained from the Western Institutional Review Board (Olympia, WA, U.S.A.), and all patients gave informed consent. All approvals and procedures were carried out in accordance with the Declaration of Helsinki. Details of the patients are provided in Table 1. All biopsies were obtained in Warren, PA, U.S.A., while all laboratory work yielding the results shown was carried out at University College London, London, U.K.

Patients were eligible for study inclusion if they were aged > 18 years and had an uninfected chronic wound present for at least 4 weeks, irrespective of current or previous treatments. Wound-edge biopsies of chronic wound tissue (VLU, $n = 19$; DFU, $n = 11$; PRU, $n = 6$) were obtained by a single operator (T.E.S.) via a 4-mm full-thickness punch biopsy taken from the visible wound edge, along with a matched biopsy of arm skin.

All biopsies were immediately immersed in 4% paraformaldehyde for 24 h and then transferred into 20% sucrose in phosphate-buffered saline (PBS). Tissue blocks were embedded in optimal cutting temperature compound (BDH, Poole, U.K.) and stored at −80 °C. Frozen sections, 14 μm thick, were obtained using a Leica CM1900 UV cryostat (Leica, Wetzlar, Germany). Some sections were stained with haematoxylin and eosin using standard methods. The large skin sections were imaged as montages on a Leica DMI6000 wide-field microscope with a 10 × objective lens, followed by 20 × images of selected regions.

Immunofluorescence

The tissue sections were permeabilized for 5 min in acetone and blocked using PBS-L-lysine (0·1 mol L^{-1}) for 30 min. Primary antibodies were prepared in PBS-lysine: Cx43 (1 : 4000; rabbit polyclonal; Sigma, Poole, U.K.), Cx26 (1 : 200;

Table 1 Clinical characteristics

	Venous leg ulcers (n = 19)[a]	Diabetic foot ulcers (n = 11)[b]	Pressure ulcers (n = 6)[c]
Age (years), median (range)	59 (31–79)	59 (48–82)	62 (34–88)
Male, %	63	64	83
Wound location, %			
Gaiter/lower leg	79	0	0
Ankle	16	0	0
Foot	5	0	0
Dorsal foot	0	46	0
Plantar foot	0	36	0
Toe	0	9	0
Ankle	0	9	0
Sacral	0	0	67
Malleolus	0	0	17
Heel	0	0	17
Wound age (months)			
Median (range)	6 (1·5–108) (n = 17)	4 (1–26)	21 (4–48)
≤ 3	35%	46%	0%
3–6	24%	27%	17%
6–12	12%	18%	17%
> 12	29%	9%	67%
Wound size (cm^2), median (range)	9·9 (2–113)	6·6 (0·56–22·2)	7·0 (3·4–40·5)
Diabetes present	58% (n = 17)	100%[d]	60% (n = 5)
Body mass index ($kg\ m^{-2}$), median (range)	46 (28–70) (n = 12)	34 (24–44)	27 (25–29)

[a]Two subjects with venous leg ulcers were eligible and enrolled twice during the study period presenting with different ulcers. [b]One subject with pressure ulcers was eligible and enrolled twice during the study period. [c]Minimum duration 4 weeks, but in two venous leg ulcers the precise wound age beyond this was not recorded. [d]Seven patients with diabetic foot ulcers were on insulin, two patients had a confirmed diagnosis but with no diabetes treatment specified, and two patients were on oral hypoglycaemics only.

Gap28H rabbit polyclonal)[25] and Cx30 (1 : 200; rabbit polyclonal; Invitrogen, Paisley, U.K.). The tissue was incubated with the primary antibody for 1 h. For negative controls the primary antibody was omitted. The tissue was washed with PBS-lysine 3 × 5 min followed by the secondary antibody for 1 h (Alexa Fluor 488 goat antirabbit, 1 : 400; or Alexa Fluor 568 goat antimouse, 1 : 400; Invitrogen). Nuclei were stained using Hoechst (1 : 50 000 in PBS; Sigma) for 5 min followed by 2 × 10 min PBS washes. Coverslips were mounted using Citifluor (glycerol/PBS solution; Citifluor Ltd, London, U.K.).

Confocal microscopy

An Olympus FV-1000 confocal microscope (Olympus, Center Valley, PA, U.S.A.) was used to obtain 10 and 20× qualitative montage images of whole-tissue sections and 40× quantitative images of the epidermis and dermis. The 4-mm biopsies were imaged (epidermis and dermis) across their diameter at three locations: at the wound edge, 1 mm from the wound edge, and at the far edge. Hoechst was excited by a 405-nm laser, Alexa Fluor 488 by a 488-nm laser and Alexa Fluor 568 by a 565-nm-wavelength laser. A minimum of two sections were qualitatively analysed per connexin for each patient. This ensured that the staining pattern observed truly represented the distribution of the protein being investigated. The imaging and image analysis parameters for each antibody were kept constant between a patient's arm and wound biopsy.

One of these sections was quantitatively analysed per connexin, with images taken at the wound edge, 1 mm from the wound edge and at the far edge of each biopsy, within both the epidermal and dermal compartments. Due to the multiple sampling from each tissue section, in combination with the control group being patient matched, a modified two-way ANOVA was used. This allowed the influence of these factors to be accounted for and acknowledged.

Each cut section was numbered to allow orientation within the biopsy. Structural comparisons were made between stained samples, irrespective of the connexin being probed for, with increasing depth to ensure consistency. Due to the orientation in which the biopsies were taken and subsequently mounted, every sample contained an entire cross-section of each biopsy, from the wound edge to a maximum of 4 mm from the wound edge. Minimal changes were observed. These variations were limited primarily to the positioning of blood vessels and the precise shape, for example undulations and rete peg depth within the epidermal compartment. No major differences in cellularity were identified within the epidermis or dermis between sections, serial or nonserial. The use of sections from varying points within each biopsy further supports

our finding that connexin upregulation is a universal feature of chronic wounds.

Image quantification and statistical analysis

Connexin quantification was carried out using ImageJ (http://imagej.nih.gov/ij/). Epidermal and dermal thresholds were kept constant between all images, being set at 80 and 100–255, respectively, with a recognized pixel threshold size of 2–infinity used for all images.[23] In the epidermis connexin expression was related to the cell number as pixels per cell, and in the dermis as pixels μm^{-2}.

The data from the connexin measurements are presented as the 'absolute connexin expression level', which was used for the statistical analysis and is presented in the graphs. The corresponding fold-change data are presented in the tables as (i) 'fold difference of the group means', this being the fold difference between the forearm biopsy group mean and the various wound-location group means (wound edge, 1 mm, far edge); and (ii) the 'mean of the individual fold changes'. This was based on calculating each individual's unique fold difference by first normalizing their wound biopsy connexin expression to their matched forearm connexin level. Then a mean individual fold difference was calculated for each study group (this was the mean of the individually normalized connexin fold changes). This dataset gives an indication of how much the individual connexin fold differences varied between patients.

Statistical analysis

The connexin expression data were analysed using a two-way ANOVA, the two factors/variables being location (arm, wound edge, 1 mm from the wound edge, and far edge) and patient. The residuals were tested for normality using the Kolmogorov–Smirnoff test, with a parametric distribution being assumed in all cases with $P > 0{\cdot}05$. Normality was not reached in three groups: VLU Cx30, DFU Cx30 and DFU Cx43 epidermal values. These specific datasets were independently transformed using the natural log before analysis. A Dunnett's *post hoc* test compared all three wound measurements back to the reference group (i.e. arm values). Significance was taken at $P < 0{\cdot}05$.

Results

Features of chronic wound biopsies

The histology of chronic wound biopsies varied, but consistent features were identified that distinguished them from healthy tissue (Fig. 1). These include increased depth to the epidermal rete pegs, a greater number of blood vessels, and a large abundance of neutrophils both within dead tissue at the wound edge and throughout the dermis.

In acute wounds the early hallmark of active healing is the formation of a thin keratinocyte tongue at the wound edge, indicating the start of re-epithelialization. These cells have a migratory phenotype and crawl forward across the wound bed. None of the DFU biopsies presented with a thinning of the epidermal wound edge. However, a thinning tongue of wound-edge keratinocytes was identified in some VLUs (six of 19 biopsies), which may represent the beginning of healing or attempts to heal in some wounds. In the PRU cohort, two of the six wounds examined had this feature.

Biopsies from venous leg ulcers

Biopsies from VLUs revealed several consistent features (Fig. 1). The epidermis of the 4-mm biopsies was typically hyperthickened, increasing in depth with distance from the wound edge. However, in some samples, the epidermis consistently thinned towards the wound edge and had an appearance consistent with a migratory phenotype, as noted above. The epidermal expression of Cx43 and Cx30 was increasingly elevated along the length of the biopsy as the epidermis became increasingly thickened upon moving away from the wound edge, whereas Cx26 was uniformly elevated in the epidermis along the biopsy (Fig. 2). The levels of Cx43 at the epidermal wound edge of these biopsies, while having a fourfold higher absolute group mean than that seen in the normal unwounded arm tissue, were not significantly different. However, 1 mm from the wound edge the absolute group mean elevation in Cx43 was eightfold higher than in the reference arm tissue, and highly statistically significant ($P < 0{\cdot}01$), while on the far edge of the biopsy the increase was on average 14-fold and very highly statistically significant ($P < 0{\cdot}001$).

Cx26 and Cx30 are normally expressed at relatively low levels in the intact skin in comparison with Cx43, but were reported to be increased in hyperproliferative human keratinocytes.[26,27] These two proteins had a many-fold greater elevation than that observed for Cx43 in the chronic wound tissues examined. For example, epidermal wound-edge Cx30 was significantly elevated by an average of 213-fold rising to 226-fold at the thicker, far edge location ($P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively). Cx26 was also significantly elevated, 73-fold, at the wound-edge epidermis, rising to 123-fold at the far edge of the biopsy when compared with the matched, intact reference tissue ($P < 0{\cdot}001$).

A common feature within the dermis of VLUs was an increased number of blood vessels along with a loss of the autofluorescent extracellular matrix in the upper third of the dermis (Fig. 2c). Dermal fibroblasts do not express Cx26 or Cx30 but do express Cx43, and this was significantly elevated across the dermis, increasing by 20-fold at the wound edge and 32-fold at the far edge when compared with matched, unwounded tissue ($P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively). The corresponding means of the individual normalized fold changes for each connexin are shown in Fig. 2d.

Biopsies from diabetic foot ulcers

Biopsies from DFUs also had common features (Figs 1 and 3). The epidermis was hyperthickened but, unlike VLUs, this was

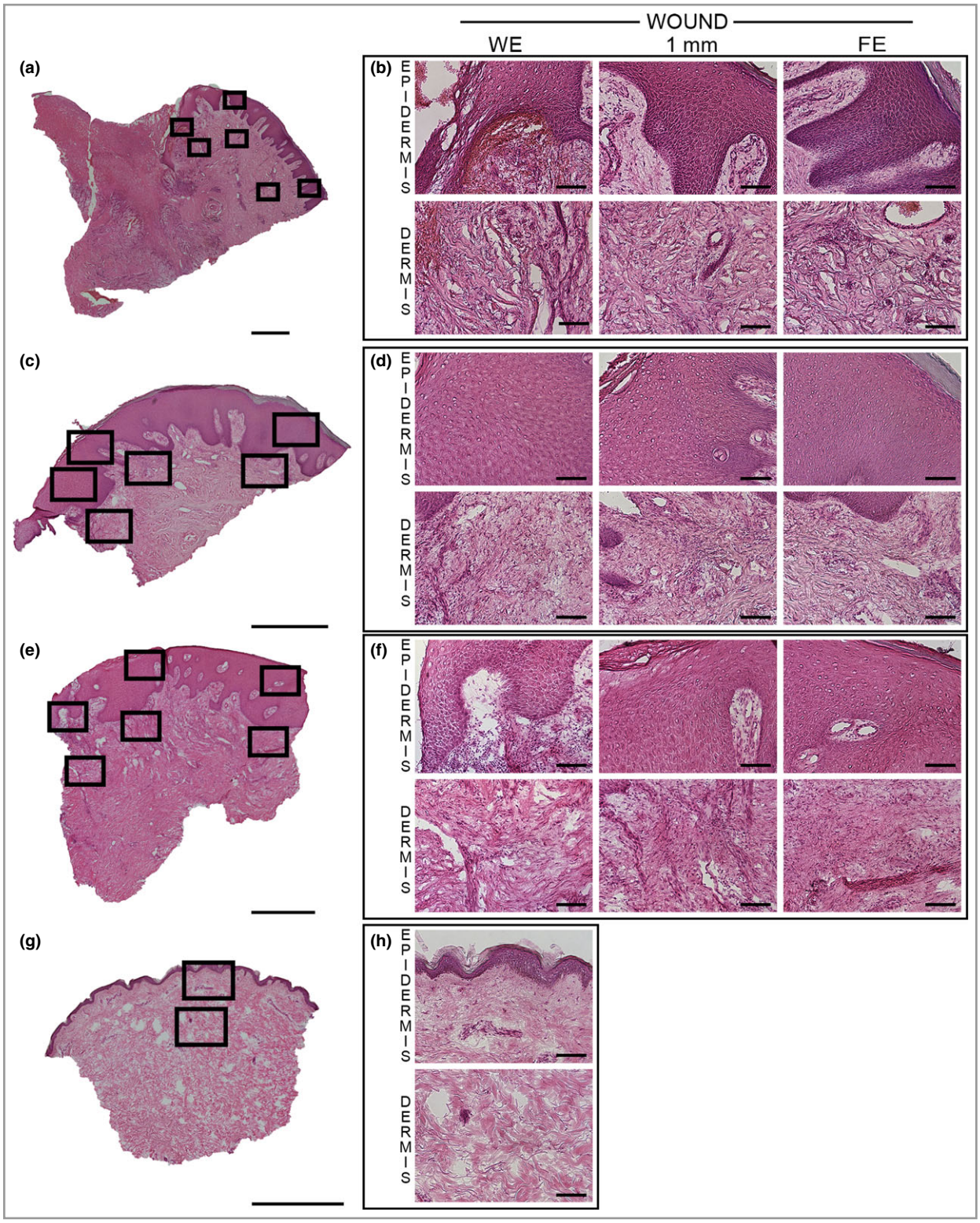
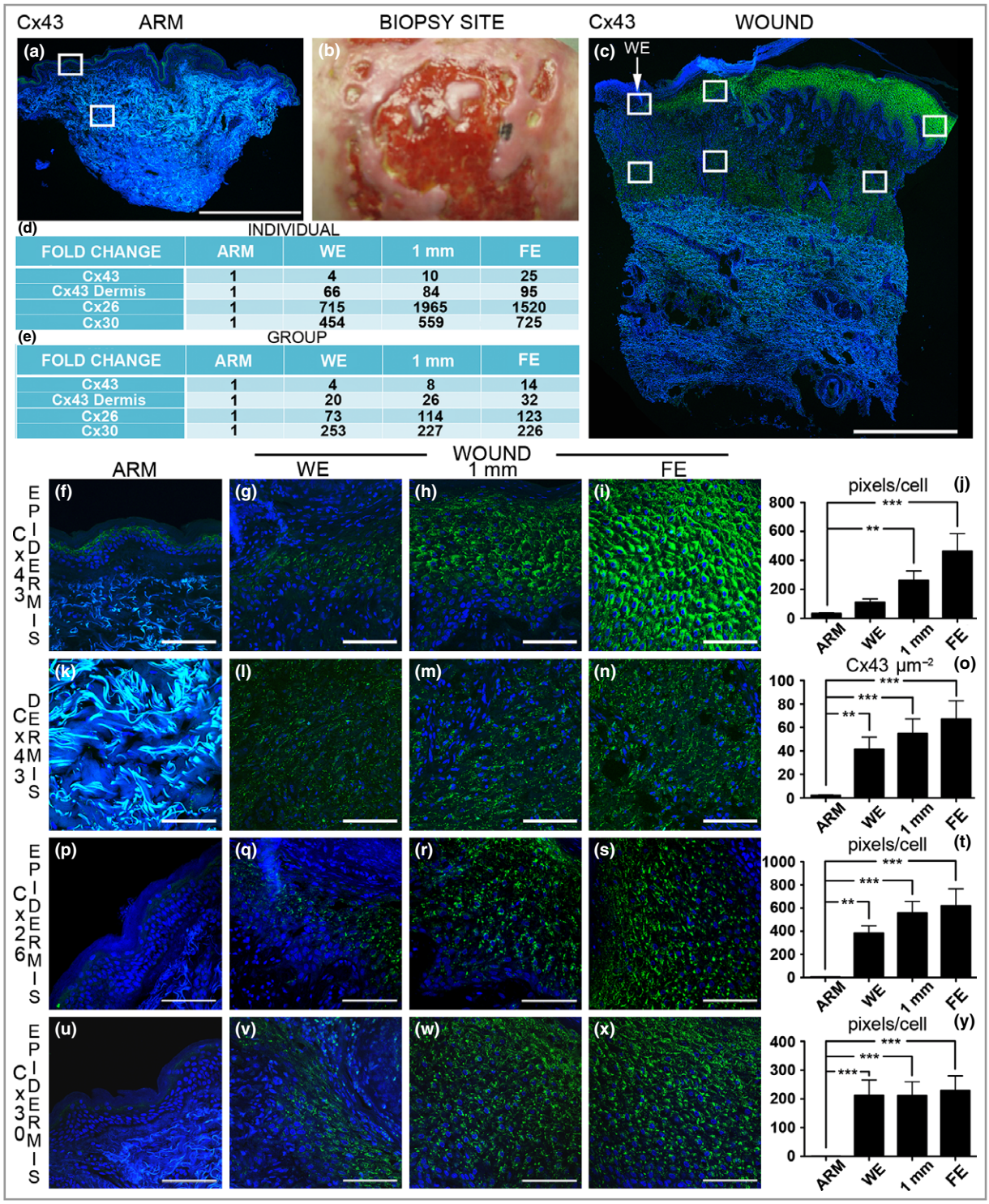


Fig 1. Haematoxylin and eosin staining of biopsies shown as montages of the whole tissue (a, c, e, g), and higher-power images of the demarcated regions: wound edge (WE), 1 mm from the WE and at the far edge (FE) (b, d, f, h). (a, b) Venous leg ulcers, (c, d) diabetic foot ulcers, (e, f) pressure ulcers and (g, h) control skin from the arm. Scale bars: montages 1 mm, higher power 100 μm.

more uniform in DFU samples. None of the biopsies showed any signs of thinning towards the wound edge and had no appearance of healing (Figs 1 and 3c). Like VLUs, DFUs also had elevated levels of connexin expression, but this was fairly consistent across the length of the biopsy. The Cx43 absolute group mean was elevated ninefold at the wound edge and

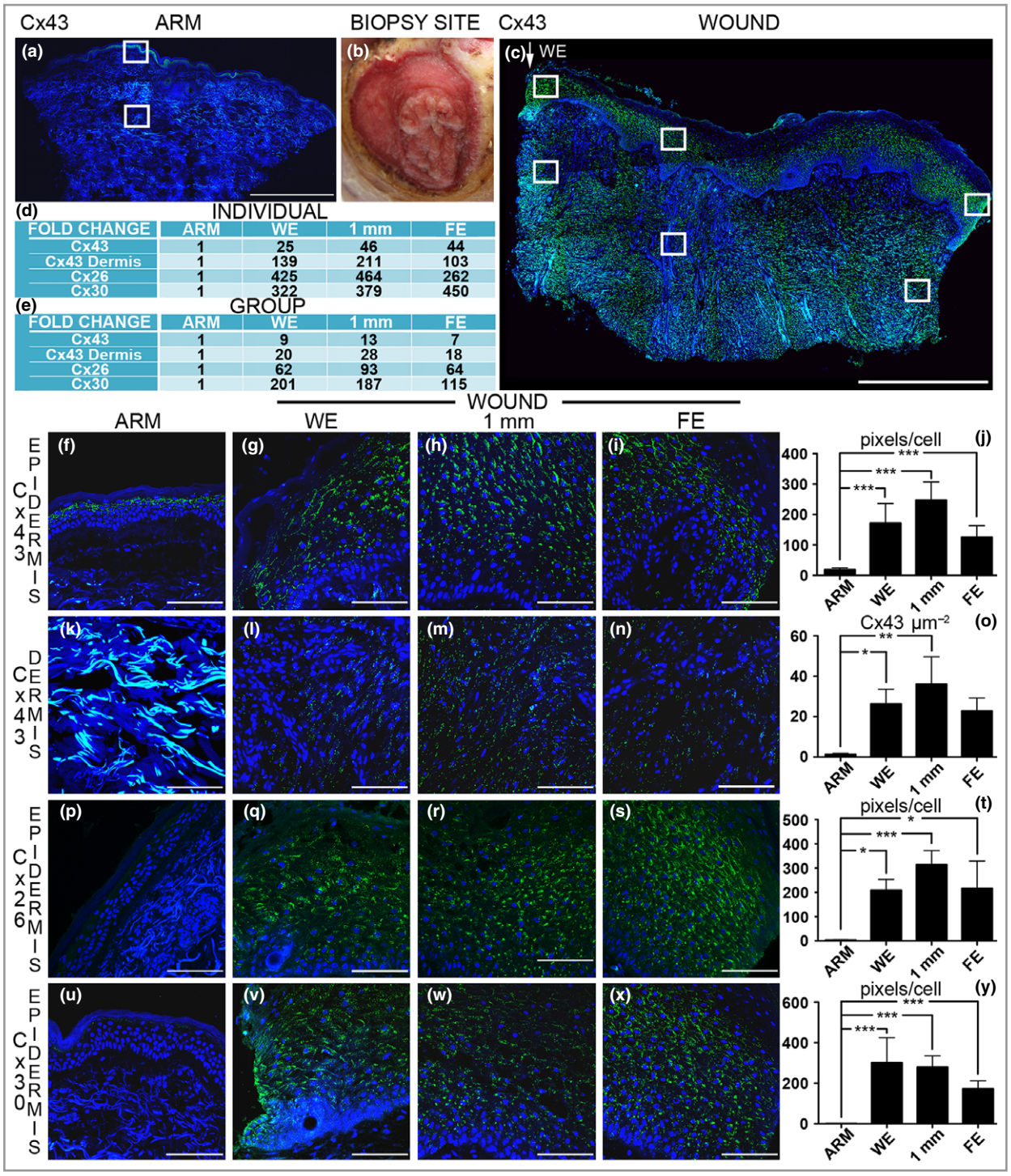


FOLD CHANGE	ARM	WE	1 mm	FE
Cx43	1	4	10	25
Cx43 Dermis	1	66	84	95
Cx26	1	715	1965	1520
Cx30	1	454	559	725

FOLD CHANGE	ARM	WE	1 mm	FE
Cx43	1	4	8	14
Cx43 Dermis	1	20	26	32
Cx26	1	73	114	123
Cx30	1	253	227	226

Fig 2. Connexin (Cx)43, Cx26 and Cx30 expression in venous leg ulcers (VLUs). (a, c) Location of quantification sites. (b) Representative VLU. (d, e) Mean of the individual and group connexin fold changes compared directly with the reference values from the arm. (f–y) Cx43, Cx26 and Cx30 expression with associated summary graphs for the wound edge (WE), 1 mm from the WE and at the far edge (FE). Scale bars: 10 × montages, 1000 µm; 40× images, 100 µm. Green: Cx43, Cx26 and Cx30; blue: nuclei. **P < 0·01; ***P < 0·001. Error bars show the mean ± SEM. Epidermis, n = 19 (except FE, n = 14); dermis, n = 17 (except WE, n = 15; FE, n = 13).

sevenfold at the far edge compared with the unwounded forearm (both P < 0·001). Cx26 and Cx30 were also significantly increased, by 62-fold (P < 0·05) and 201-fold (P < 0·001), respectively, at the wound edge and 64-fold (P < 0·05) and 115-fold (P < 0·001) at the far edge of the biopsy.



(d) INDIVIDUAL FOLD CHANGE	ARM	WE	1 mm	FE
Cx43	1	25	46	44
Cx43 Dermis	1	139	211	103
Cx26	1	425	464	262
Cx30	1	322	379	450

(e) GROUP FOLD CHANGE	ARM	WE	1 mm	FE
Cx43	1	9	13	7
Cx43 Dermis	1	20	28	18
Cx26	1	62	93	64
Cx30	1	201	187	115

Fig 3. Connexin (Cx)43, Cx26 and Cx30 expression in diabetic foot ulcers (DFUs). (a, c) Location of Cx43 quantification sites. (b) Representative DFU. (d, e) Mean of the individual and group connexin fold changes compared directly with the reference values from the arm. (f–y) Cx43, Cx26 and Cx30 expression and associated summary graphs for the wound edge (WE), 1 mm from the WE and at the far edge (FE). Scale bars: 10 × montages, 1000 µm; 40× images, 100 µm. Green: Cx43, Cx26 and Cx30; blue: nuclei. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$. Error bars show the mean ± SEM. Epidermis, $n = 11$ (except FE, $n = 8$); dermis, $n = 6$.

The dermis of the DFUs was distinctly different from that of the VLUs, as in many cases it lacked any signs of autofluorescent signal from the fibres of the dermal extracellular matrix, or, if autofluorescence remained, the organizational pattern was absent. This suggested that a large proportion of the native collagen and elastin had either been degraded or was no longer being arranged into mature fibrils. The DFU dermis featured significantly increased levels of Cx43, by an

average of 20-fold ($P < 0{\cdot}05$) at the wound edge and 18-fold at the far edge of the biopsy. These data and the means of the individual normalized fold changes for each connexin are shown in Fig. 3d.

Biopsies from pressure ulcers

Biopsies from PRUs were variable in their appearance. The epidermis was typically thickened along the length of the biopsy with the formation of deep rete pegs (Figs 1e and 4). The degree of healing was variable, manifesting in some instances as a thinning tongue of wound-edge epidermis and reminiscent of the VLUs. Again, connexin expression was elevated in the epidermis but unevenly so at the wound edge. The Cx43 absolute group mean was significantly increased at 1 mm by 10-fold ($P < 0{\cdot}01$), while Cx26 was elevated 90-fold and Cx30 by 471-fold when compared with the low baseline levels found in intact arm skin ($P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively).

The dermis of the PRUs could be distinguished from that of VLUs and DFUs by the consistent presence of an autofluorescent signal from the extracellular matrix. Cx43 expression in the PRU dermis was significantly increased, on average 57-fold at the wound edge ($P < 0{\cdot}05$) and 37-fold on the far edge of the wound. These data and the means of the individual normalized fold changes for each connexin are shown in Fig. 4d.

Distribution of epidermal connexin overexpression

The distribution of connexins within the epidermis varied along the length of the biopsies and with depth corresponding to the varying layers of the epidermis. The deep rete pegs were characterized by a dominance of Cx26 and Cx30. In some regions, a large proportion of the cell membrane appeared to be taken up by connexin, giving the staining a 'fish scale' appearance.

Discussion

Here we show a statistically significant, substantial upregulation of three connexin gap-junction proteins in VLUs, DFUs and PRUs: epidermal Cx43, Cx26 and Cx30 and dermal Cx43. Precise spatial and temporal control of connexin proteins has been shown to be integral to the regular wound reparatory process, where downregulation of Cx43 at the wound edge is correlated to keratinocyte and fibroblast migration. The connexin misregulation we have identified here may serve to slow healing and/or prolong ulceration.[23]

In this study, human chronic wounds of all three major aetiologies were found to have abnormally high connexin expression at the wound edge. By way of an example, Cx43 wound-edge expression was nine times greater in the DFU cohort than in basal, unwounded skin. When this is considered in the context of the substantial preclinical data that link delayed healing with elevated Cx43 expression,[12,13,23] it strongly indicates that the upregulation of this protein is a common feature of chronic wound pathology.

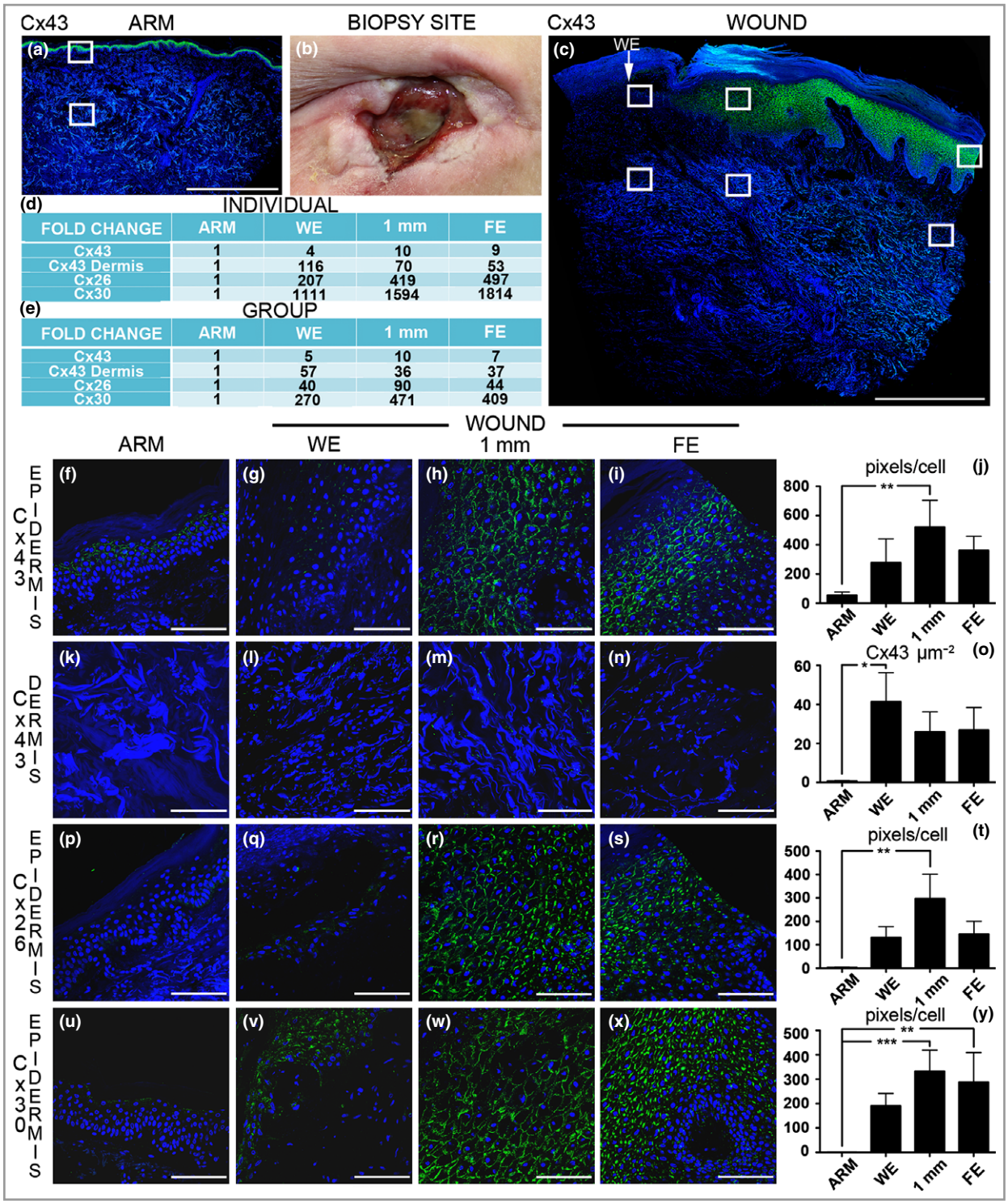
Incisional and excisional wounding studies in rodents and humans report that re-epithelialization is characterized by an initial Cx43 downregulation at the wound edge with no expression detected within the cells of the leading edge prior to migration.[10,11,24,28] The presence of very high levels of the Cx43 protein within the epidermal and dermal wound edges of VLUs, DFUs and PRUs could show it to be a specific primary inhibitor of keratinocyte and fibroblast migration, and may underlie the stalled nature of chronic wounds as it does in streptozotocin diabetic rats,[23] where targeting Cx43 with Cx43 antisense oligodeoxynucleotides reverses a slower healing rate.[14,15,23]

It is now also known that the downregulation of Cx43 in 3T3 fibroblasts via Cx43 antisense oligodeoxynucleotide application or short hairpin RNA transduction results in significantly faster rates of migration in scratch-wound assays.[13] Attenuated Cx43 expression leads to diminished cell adhesion via a reduction in N-cadherin expression, enhanced activation of the small GTPases Rac1 and RhoA and subsequent changes in the actin and tyrosinated tubulin cytoskeletal dynamics, resulting in significantly longer migratory lamellipodial extensions.[12]

Conversely, overexpression of Cx43 is associated with the opposite effect. Increasing Cx43 expression by pharmacological or molecular means results in repressed production of lamellipodia and retarded migration.[12,13] In these studies, only a onefold increase in Cx43 expression within cultured fibroblasts was required to halve their migration rate. This increase is minimal when compared with the striking multiple-fold increases we found in the dermal wound-edge Cx43 levels in both VLUs and DFUs.

The significantly elevated Cx43 levels observed in all chronic wounds examined in this study are likely to reduce the migratory ability of fibroblasts and keratinocytes, compromising healing in both key tissue compartments of the skin. Retarded fibroblast migration may also contribute to the lack of granulation tissue formation reported in some ulcers.[3] Furthermore, the negative effects of Cx43 overexpression on migration may be due not solely to gap-junction intercellular communication but also to nonjunction-mediated effects. Cx43 is known to act at the centre of a nexus interacting with adhesion molecules, tight junctions and cytoskeletal components via interactive domains on its cytoplasmic C-terminal tail.[12,29] Cx43 upregulation may also prolong and facilitate the inflammatory state.[15,30–32]

To date most research on connexin dynamics throughout wound repair has focused on understanding the role of Cx43. Cx26 and Cx30 are usually detected only at very low levels within the intact interfollicular epidermis, but are significantly upregulated after wounding within the migratory epidermal leading edge.[11] Examination of these proteins within chronic wound tissue showed them both to be significantly overexpressed across the entirety of the epidermis, which correlates with a variety of skin proliferative conditions. For example, upregulation of Cx26 and/or Cx30 has previously been reported in psoriasis,[33,34] warts[33] and a variety of genetically inherited conditions that lead to skin abnormalities, such as



FOLD CHANGE	ARM	WE	1 mm	FE
Cx43	1	4	10	9
Cx43 Dermis	1	116	70	53
Cx26	1	207	419	497
Cx30	1	1111	1594	1814

FOLD CHANGE	ARM	WE	1 mm	FE
Cx43	1	5	10	7
Cx43 Dermis	1	57	36	37
Cx26	1	40	90	44
Cx30	1	270	471	409

Fig 4. Connexin (Cx)43, Cx26 and Cx30 expression in pressure ulcers (PRUs). (a, c) Location of Cx43 quantification sites. (b) Representative PRU. (d, e) Mean of the individual and group connexin fold changes compared directly with the reference values from the arm. (f–y) Cx43, Cx26 and Cx30 expression and associated summary graphs for the wound edge (WE), 1 mm from the WE and at the far edge (FE). Scale bars: 10 × montages, 1000 µm; 40× images, 100 µm. Green: Cx43, Cx26 and Cx30; blue: nuclei. *P < 0·05; **P < 0·01; ***P < 0·001. Error bars show the mean ± SEM. Epidermis and dermis, n = 6 (except FE, n = 5).

porokeratosis of Mibelli[35] and Clouston syndrome.[34] A common phenotypic factor between these syndromes and chronic wounds is keratinocyte hyperproliferation.

Keratinocyte proliferation and differentiation are misregulated in DFUs and VLUs.[36,37] In VLUs there is a loss of cell-cycle control, along with the misexpression of activation and

differentiation pathways.[36] In DFUs, keratinocytes at the wound edge are hyperproliferative, independent of ulcer-edge thickness. Interestingly this extends into the nonulcerated region, with tissue of a histologically 'normal' phenotype staining strongly for the cell proliferation marker Ki67.[37] The overexpression of Cx26 and Cx30, as detected along the entire length of the 4-mm punch biopsies independently of ulcer type, could reflect a direct involvement in the epidermal thickening.

Studies of acute incisional wound healing in transgenic mice, where Cx26 is ectopically expressed within keratinocytes, showed delayed wound healing and a hyperproliferative epidermal state.[38] After 21 days postwounding, only 42% of the heterozygous mice had healed, while full epidermal barrier restoration was seen in all wild-type animals by day 14.[38] Alternatively, Cx26 and Cx30 may merely be markers of hyperproliferation, with their expression predominantly influencing cellular differentiation. Overall, enhanced connexin expression may also in part be required to maintain homeostasis within the avascular epidermis via gap-junctional intercellular communication and paracrine hemichannel ATP release.[38]

The precise mechanisms that result in the observed persistent connexin upregulation in chronic wounds remain unclear, and may be dependent upon a group of events. Two influential and potentially important physiological factors may be hypoxia and hyperglycaemia.

There are two potential limitations within this study. The first was that the inclusion criteria were intentionally broad. Thus, the biopsies in each category were taken from a wide spectrum of patients rather than from a medically homogeneous set of individuals. Consequently there is an expected level of underlying variation within the dataset that is reflective of the range in patient ages, ulcer severity, ulcer duration and the possible impacts of previous treatments. However, this inclusive approach has highlighted that connexin misregulation appears to be a unifying feature of chronic wounds from a wide range of common clinical settings. Secondly, connexin expression in ulcers was compared with that of the patient's matched nonwounded arm skin. This reference sample was used because it offered a consistent background against which to make our assessments, while avoiding the risk of further biopsy injury to the already ulcerated regions of the leg or foot.

In summary, this investigation has found that the overexpression of Cx43, Cx26 and Cx30 in the epidermis and that of Cx43 in the dermis of ulcer biopsies is a signature feature of chronic wounds, identified in all patients irrespective of ulcer type, either VLU, DFU or PRU. The full biological implications of these consistent findings are unknown and are worthy of further intensive functional investigation.

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