Deregulation of keratinocyte differentiation and activation:
a hallmark of venous ulcers

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Abstract

Epidermal morphology of chronic wounds differs from that of normal epidermis. Biopsies of non-healing edges obtained from patients with venous ulcers show thick and hyperproliferative epidermis with mitosis present in suprabasal layers. This epidermis is also hyperkeratotic and parakeratotic. This suggests incomplete activation and differentiation of keratinocytes. To identify molecular changes that lead to pathogenic alterations in keratinocyte activation and differentiation pathways we isolated mRNA from non-healing edges deriving from venous ulcers patients and determined transcriptional profiles using Affymetrix chips. Obtained transcriptional profiles were compared to those from healthy, unwounded skin. As previously indicated by histology, we found deregulation of differentiation and activation markers. We also found differential regulation of signalling molecules that regulate these two processes. Early differentiation markers, keratins K1/K10 and a subset of small proline-rich proteins, along with the late differentiation marker filaggrin were suppressed, whereas late differentiation markers involucrin, transglutaminase 1 and another subset of small proline-rich proteins were induced in ulcers when compared to healthy skin. Surprisingly, desmosomal and tight junction components were also deregulated. Keratinocyte activation markers keratins K6/K16/K17 were induced. We conclude that keratinocytes at the non-healing edges of venous ulcers do not execute either activation or differentiation pathway, resulting in thick callus-like formation at the edge of a venous ulcer.

Key words: venous ulcers • chronic wounds • keratinocyte differentiation • keratinocyte activation • desmosomes • wound healing • transcriptional profiling • microarrays

Introduction

Chronic wounds, such as venous ulcers, affect hundreds of thousands of patients each year. They represent a serious healthcare problem around the world. Venous ulcers develop as a consequence of venous valvular incompetence causing venous reflux, obstruction and subsequently venous hypertension. Their chronicity, frequent relapses and associated complications heavily impact patient’s quality of life and increase healthcare costs.

Venous ulcers are responsible for more than half of lower extremity ulcerations, with prevalence ranging from 0.06% to 2% [1]. A rough estimate shows that the prevalence of unhealed venous ulcers is about 0.3%, that is about 1 in 350 adults [2], or approximately 400,000–600,000 cases in the United States [3]. Treatment of these ulcers sometimes requires numerous hospitalizations and treatment modalities incorporating topical dressings, antibiotics, debridement, compression therapy and cellular therapy. After wound healing with non-operative methods the incidence rate of recurrent ulcerations after 3 and 5 years was reported to be 37% and 48%, respectively [3]. The annual cost of

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Healthy epidermis provides a protective barrier that undergoes constant renewal. In this multi-layered, highly cellular tissue cell-to-cell connection occurs via desmosomes, adherens junctions, gap and tight junctions (TJs). TJs control paracellular permeability and maintain cell polarity, thus maintaining barrier function [7]. Basal keratinocytes divide and as they start differentiating and leaving the basal compartment they undergo changes in gene expression and commit to terminal differentiation resulting in formation of enucleated corneocytes. Once the barrier is broken, keratinocytes become activated in response to epidermal injury and start to proliferate, migrate and express a specific subset of keratin proteins suprabasally, keratins 6, 16 and 17 [8].

We have shown previously that skin deriving from the non-healing edge of chronic ulcers (e.g. venous, pressure and diabetic foot ulcers) exhibits distinct morphology. The epidermis becomes hyperproliferative, hyper- and parakeratotic with the presence of mitotically active cells in suprabasal layers [9, 10]. We have also shown that activation of c-myc and nuclearization of β-catenin in the epidermis of patients with chronic wound play a role in inhibition of keratinocyte migration and contribute to impairment of healing in chronic wounds [9]. These changes suggest inadequate execution of either of the two major processes important for epidermal maintenance and homeostasis: activation and differentiation.

To test if indeed these two processes are impaired in venous ulcers, we utilized large-scale microarray analyses and biopsies of non-healing edges of three patients suffering from venous ulcers. Microarray technology has brought the ability to simultaneously analyse the expression patterns of tens of thousands of genes and thereby identify groups of differentially regulated genes involved in pathogenesis of many different diseases. It has been used successfully in gene expression analyses of various tumours [11, 12] and wound healing of different tissues [13–18]. To the best of our knowledge a focused, large-scale microarray analysis has not been performed for patients with chronic wounds. In this study, we compared expression profiles of patients' biopsies from non-healing edges of venous ulcers to profiles obtained from biopsies of healthy skin. Among 1557 genes that are differentially regulated in a statistically significant manner (P < 0.05), we particularly focused on groups of genes that characterize regulation of main biological processes in keratinocytes: activation and differentiation. We found keratinocyte activation markers to be induced. Proliferation is a component of keratinocyte activation. Cell cycle related genes, both cell cycle activators and repressors, were differentially regulated suggesting the loss of cell cycle control. Furthermore, we found deregulation of early and late differentiation markers, as well as regulators of keratinocyte differentiation, suggesting improper execution of either the early or late phase of differentiation. Microarray data were evaluated and confirmed using quantitative real-time PCR and immunohistochemistry.

We conclude that keratinocytes at the non-healing edges of venous ulcers are caught in a loop of trying to execute either of two processes, proliferation and differentiation, thus resulting in a thick hyperproliferative, hyper- and parakeratotic epidermis.

### Materials and methods

#### Skin specimens used in study

Institutional review board approval was obtained and skin biopsies deriving from non-healing edges of venous ulcers were collected from normally discarded tissue after surgical debridement procedures on three consented patients with venous reflux ulcers in accordance with the Declaration of Helsinki Principles. Three healthy skin specimens were obtained as discarded tissue from reduction mammoplasty. As previously shown, three biological replicates are sufficient to generate valuable microarray analyses [19]. A portion of biopsies were embedded in tissue-freezing media (Triangle Biomedical Sciences, NC, USA) and frozen on dry ice, whereas the rest of the samples were stored in RNA later (Ambion, Foster City, CA, USA) for the immediate RNA isolation. Prior to RNA isolation from skin specimens, tissue morphology was evaluated histologically and mixed cell population was addressed as previously described [10].

#### Debridement techniques of venous ulcers

Venous ulcers biopsies were obtained from normally discarded tissue from patients. All patients were debrided in the operating room under monitored anaesthesia care or general anaesthesia and in all cases local lidocaine injection was used for local anaesthesia. After the wound was prepped in betadine, a No. 20 sterile surgical scalpel was used to debide the flat wound edges. No undermining was encountered. The non-healing edges used in this study were clinically identified by a single surgeon (Dr. Brem) as the edge of the most proximal skin edge to the ulcer bed, often times indicated by the presence of callus. Multiple biopsies were taken of each patient within 30 min. of the injection of local anaesthetic. Once the samples were brought to the laboratory, routine histopathological analysis with hematoxylin & eosin were performed to verify typical morphology of the non-healing edge as described by Stojadinovic et al. [9]. Briefly, acanthotic epidermis with hyperkeratosis and parakeratosis are indicative of a non-healing edge of the wound, in addition to presence of fibrosis. Furthermore, immunohistochemistry staining for markers of chronic wounds (c-myc and β-catenin) was performed and all samples stained positive. Only biopsies that fulfilled these two assays were used in the study.

#### Preparation and hybridization of probes

Samples were homogenized and total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. Approximately 5 μg of total RNA was reverse transcribed, amplified and labelled as described [20]. Labelled cRNA was hybridized to HG-U133A-set Gene Chip probe arrays that contain probe sets representing approximately
22,000 genes (Affymetrix, Santa Clara, CA, USA). The arrays were washed and stained with avidin-biotin streptavidin-phycocerthyrin labelled antibody using Affymetrix fluidics station and then scanned using the Agilent GeneArray Scanner system (Hewlett-Packard, Palo Alto, CA, USA) as described by Affymetrix.

Gene array data analysis

Microarray Suite 5.0 (Affymetrix) was used for data extraction and for further analysis, data mining tool 3.0 (Affymetrix, Santa Clara, CA, USA) and GeneSpring™ software 7.3.1 (Silicon Genetics, Redwood City, CA, USA) were used for normalization to the median and filtration on the Volcano plot for degree of change and P-value calculations. Samples were normalized per chip: to the 50 th percentile and per gene to a median. Statistical comparisons of expression level between each condition were performed using ANOVA test. Only genes with a P-value less than 0.05 were considered to be statistically significant. Differential expressions of transcripts were determined by calculating the degree of change. Genes were considered regulated if the expression levels differed more than 2-fold relative to healthy skin. Clustering was performed based on individual gene expression profiles. We developed an extensive gene annotation table describing the molecular function and biological category of the genes present on the chip as previously described [10]. The genes were annotated according to this table.

Immunohistochemistry

Slides containing frozen five micrometer thick skin sections were fixed in acetone (–20°C) for 1 min. Sections were blocked in 5% bovine serum albumin (BSA) and following primary antibodies diluted in 5% BSA/Phosphate Buffered Saline (PBS) were used: antibody against Filaggrin [21] and K10 (gift from Dr. H Sun, New York University), Involucrin (Neomarkers, Fremont, CA, USA) and K17 (Gift from Dr. P. Coulombe, Johns Hopkins University ) [22]. For staining with Dsg3, Dsc2 and DP, sections were permeabilized with 1% Triton-X-100 in PBS for 1 min., washed and blocked in 1% BSA/0.1% Triton X-100 in PBS for 1 hr at room temperature (RT). Sections were incubated with antibodies against desmocollin 2 (7G6), desmoglein 3 (5H10) and desmoplakin at room temperature (RT). Sections were incubated with antibodies against albumin (BSA) and following primary antibodies diluted in 5%

Quantitative real-time PCR analysis

For real-time PCR, 0.5 μg of total RNA from healthy skin and venous ulcer was reverse transcribed using a Omniscript Reverse Transcription kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed in triplicate using the iCycler IQ thermal cycler and detection system and an IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Relative expression was normalized for levels of HPRT1. The primer sequences used were: HPRT1, fw (5'-AGGGAAAAGACTGGATGACA-3'); KLK6, fw (5'-CATGGCCGAGCCCTTGGAAGAC-3') and rev primer (5'-TGGATCACAGGCCGGAGAGACAGGAGC-3'); MMP11fw (5'-AGATCTACTCTTTCCGAGGC) and rev primer (5'-TTCGAGACCTCTACCTCA-3'); CCL27 fw (5'-TCTGAGGCCAGCCGCTCAC-3') and rev primer (5'-CATTTCCACTGGATGACCT-3'); APOD fw (5'-AACATGAAAGTGAAGACGCA) and rev primer (5'-ACAGGAGCATGCTTCTCAGT-3'); S100A7fw (5'-GGAGAACTTCCCAAATTC-3') and rev primer (5'-ACATCGGCAGGTATTTGT-3'); BMP2 fw (5'-TCAAGCCAACAGACACG-3') and rev primer (5'-GGTGCATGAAAAGGCTGAT-3'); BMP7 fw (5'-AGGCTGTGAAGACGAG-3') and rev primer (5'-GTTGCGGTTCATGTGGGAGT-3'); K17 fw (5'-GGTGGGTTGAGATCACATGT-3') and rev (5'-CAGGTTGCCATTTCTGTC). Statistical comparisons of expression levels were performed using Student's t-test.

Results

To determine genes that play a role in pathogenesis of venous ulcers we performed microarray analysis, utilizing biopsies from non-healing edges of venous ulcers from three patients with venous stasis ulcers and three healthy skin specimens. All three patients presented with an ulcer on a lower left leg, longer than an year in duration (Fig. 1). Proper diagnosis was made following previously published guidelines and protocols [3, 23]. Evaluation for ischaemia was performed either by non-invasive flow exams (ankle-brachial index of <0.9) or by arteriogram. All patients did not have evidence of ischaemia. In addition, all patients had a venous duplex exam and confirmed the presence of venous valvar incompetence and venous reflux. None of the patients had diabetes. All patients had haemosiderosis upon clinical exam.

To compare gene expression profiles from obtained skin specimens, we performed cluster analyses using GeneSpring™ 7.3.1 software. We generated a gene tree employing all genes present on the chip and visualized the expression profile of each sample (Fig. 2). As expected, skin specimens obtained from healthy, control skin were clustered together separate from cluster of skin specimens obtained from venous biopsies, thus confirming differential gene expression profiles derived from venous ulcers versus healthy skin.

Overall microarray data analyses

When the gene expression profile of skin derived from edges of non-healing wounds was compared to healthy skin, a differential expression of transcripts was determined by calculating the degree of change. Genes were considered regulated if the expression levels differed more than 2-fold relative to healthy skin. Duplicates were eliminated and genes of unknown function were excluded. Of approximately 22,000 genes presented on the chip, we found 1557 genes to be differentially regulated between non-healing edges of venous ulcers and healthy skin. Out of 1557 genes, 55% of genes were down-regulated and...
45% were up-regulated. We sorted regulated genes according to their biological functions, i.e. process in which they participate (Fig. 3). Furthermore, we selected the top 50 up-regulated and the top 50 down-regulated genes based on the degree of change and P-values ($P < 0.05$) and grouped them by cellular functions and biological processes (Fig. 4). The most regulated genes fell into following biological processes: contact and motility, tissue remodelling, inflammation and immunity, proliferation, differentiation, cell death control, metabolism and signal transduction and transcription. To better understand molecular changes in non-healing edges of venous ulcers we focused on specific cellular functions and processes affected by venous ulcer environment.

### Keratinocyte activation markers

Activation of keratinocytes is marked by the induction of a specific set of keratins: keratins 6, 16 and 17. We have shown previously induction of K6 at the non-healing edges of chronic wounds [9]. Microarray data confirmed induction of keratin 6 (K6). In addition, induction of keratin 16 (K16) was also found in venous ulcers. Interestingly, we could not detect keratin 17 (K17), another keratinocyte activation marker, among genes regulated more than 2-folds. We checked generated gene list of statistically regulated genes before we made the 2-folds cut-off, and found K17 among up-regulated genes. K17 expression was induced 1.86-fold. To confirm changes in K17 expression we performed real-time PCR and found K17 induced in non-healing edges of venous ulcers as compared to normal skin (Fig. 5A). Furthermore, we used K17-specific antibody to stain healthy skin and skin biopsies harvested form venous ulcers. As expected, healthy epidermis does not show K17 staining, whereas epidermis from non-healing edge of venous ulcers shows robust signal (Fig. 5B). Therefore, keratinocytes at the non-healing edge of venous ulcers express early activation markers.

### Keratinocyte activation: proliferation

Keratinocyte proliferation is one of the cellular processes defining keratinocyte activation. Hyperproliferation of keratinocytes is characteristic of the epidermis of the non-healing edge. Interestingly, our microarray analyses show deregulation of cell cycle associated genes. In mammalian cells, a crucial checkpoint control for proliferation is provided by the pocket proteins of the retinoblastoma (Rb) family [24, 25]. All three pocket proteins of the Rb family, Rb, p107 and p130 were found down-regulated in
our microarray data, suggesting loss of cell cycle control. Furthermore, cyclin B1, cyclin D2, cyclin A2, cyclin F and cyclin M4 were up-regulated along with cell division cycle 2 (CDC2) suggesting an increase of CDC2/cyclin B1 and CDC2/cyclin A2 complexes, promoting both cell cycle G1/S and G2/M transitions. In contrast, cyclin D1 and eukaryotic translation initiation factor 4E (EIF4E) which promotes the nuclear export of cyclin D1, were found down-regulated in non-healing edges. Microarray analyses further show a loss of cell cycle checkpoint regulation. Checkpoint suppressor 1 (CHES1) and Wee1-like protein kinase (WEE1) were down-regulated. WEE1 catalyses the inhibitory tyrosine phosphorylation of CDC2/cyclinB kinase and appears to coordinate the transition between DNA replication and mitosis by protecting the nucleus from cytoplasmically activated CDC2 kinase. Since both CDC2 and cyclin B1 were up-regulated coupled with loss of inhibitory phosphorylation by WEE1, we propose that this might account for the hyperproliferative phenotype in non-healing edges of venous ulcers. Two cyclin-dependent kinase inhibitors,
| FC  | P-value | SYMBOL | Unigene Comment | Comment |
|-----|---------|--------|-----------------|---------|
| 8.74 | 0.00 | TP53I3 | tumor protein p53 inducible protein 3 | Pro-apoptotic |
| 6.81 | 0.03 | BCL11A | Bcl2/adenovirus E1B 19KDa interacting protein 3 | |
| 6.32 | 0.01 | BIRC5 | baculoviral IAP repeat-containing 5 (survivin) | Anti-apoptotic |
| -22.62 | 0.02 | TNFAIP3 | tumor necrosis factor, alpha-induced protein 3 | |
| -59.88 | 0.00 | SCGB1D2 | secretoglobin, family 1D, member 2 | |
| -23.53 | 0.00 | FABP7 | fatty acid binding protein 7 | |
| -8.12 | 0.00 | FAP4 | fatty acid binding protein 4, adipocyte | Lipid |
| -59.88 | 0.00 | SCGB2A2 | secretoglobin, family 2A, member 2 | |
| -14.39 | 0.05 | CLU | clusterin | |
| -8.20 | 0.00 | PGRMC2 | progestosterone receptor membrane component 2 | |
| -8.00 | 0.01 | CYP3A5 | cytochrome P450, family 3, subfamily A, polypeptide 5 | Steroid |
| 8.67 | 0.00 | RYNO | protein kinase (L-lysine), hydroxylase | Carbohydrate |
| 6.09 | 0.04 | TDO2 | tryptophan 2,3-dioxygenase | Amino acid |
| 18.27 | 0.00 | LOC91316 | immunoglobulin lambda-like polypeptide 1, pre-B-cell specific | |
| 9.82 | 0.01 | DIO2 | deiodinase, iodothyronine, type II | Other |
| 7.19 | 0.04 | D2S448 | melanoma associated gene | Other |
| 6.09 | 0.01 | GPX7 | glutathione peroxidase 7 | |
| 6.69 | 0.02 | FM03 | flavin containing monoxygenase 3 | Energy |
| 8.54 | 0.02 | GALNT6 | UDP-N-acetylgalactosamine 2-0-sulfotransferase 6 | Other |
| 73.56 | 0.00 | TCN1 | transcobalamin I | Other |
| -8.13 | 0.00 | GOLGA4 | golgi autoantigen, golgin subfamily a, member 4 | |
| -8.70 | 0.00 | SFPG | splicing factor proline/glutamine rich, RNA metabolism | Other |
| -10.83 | 0.03 | SLC2A3 | solute carrier family 2, member 3 | Other |
| -3.33 | 0.01 | TFCR | transferrin receptor (p90, CD71) | Transport |
| 6.35 | 0.00 | SLC36A1 | solute carrier family 36 (proton/amino acid symporter, member 1) | |
| 13.78 | 0.00 | NELL2 | NELL-like 2 | Other |
| -63.69 | 0.04 | ADH1B | alcohol dehydrogenase family 1B, class I, beta polypeptide | Other |
| 9.99 | 0.00 | ALDH4A1 | aldehyde dehydrogenase 4 family, member A1 | Other |
| 9.03 | 0.00 | AKR1B10 | aldo-keto reductase family 1, member B10 (aldose reductase) | Other |
| -3.13 | 0.01 | GATA3 | GATA binding protein 3 | Other |
| -62.64 | 0.01 | FOSS | F6U murine osteosarcoma viral oncogene homolog B | Other |
| -70.42 | 0.01 | ID4 | inhibitor of DNA binding 4 | Other |
| -47.50 | 0.01 | ZFP36L1 | zinc finger protein 36, C3H type-like 1 | Other |
| -19.57 | 0.02 | MAF | v-maf musculoaponeurotic fibrosarcoma oncogene homolog F | Other |
| -14.79 | 0.03 | KLF2 | Kruppel-like factor 2 (lung) | Other |
| -13.02 | 0.02 | TIEG | TGFβ inducible early growth response | Other |
| -13.05 | 0.02 | SCA7 | spinocerebellar ataxia 7 | Other |
| -12.72 | 0.00 | KLF4 | Kruppel-like factor 4 | Other |
| -12.59 | 0.00 | EGFR | early growth response 3 | Other |
| -11.73 | 0.00 | KLF5 | Kruppel-like factor 5 | Other |
| -10.58 | 0.00 | JUN | v-jun sarcoma virus 17 oncogene homolog | Other |
| -10.13 | 0.01 | EGR1 | early growth response 1 | Other |
| -9.62 | 0.02 | PER2 | period homolog 2 | Other |
| -9.43 | 0.01 | JUND | jun D proto-oncogene | Other |
| -9.26 | 0.00 | SP3 | Sp3 transcription factor | Other |
| -8.77 | 0.04 | BAZ1A | bromodomain adjacent to zinc finger domain, 1A | Other |
| -8.70 | 0.00 | NFIB | nuclear factor I/B | Other |
| -41.14 | 0.01 | CD44 | CD44 antigen | Other |
| -15.85 | 0.03 | SF1 | splicing factor 1 | Other |
| -12.05 | 0.02 | ZFP36 | zinc finger protein 36, C3H type, homolog | Other |
| -9.51 | 0.00 | ZNF451 | zinc finger protein 451 | Other |
| -42.19 | 0.04 | ATF3 | activating transcription factor 3 | Other |
| -17.53 | 0.00 | TCF8 | transcription factor 8 | Other |
| -6.27 | 0.01 | PITX1 | paired-like homeodomain transcription factor 1 | Other |
| -9.43 | 0.03 | OSR2 | odd-skipped-related 2A protein | Other |
| -10.06 | 0.00 | EIP1A | eukaryotic initiation factor 1A | Other |
| -57.14 | 0.03 | NR4A2 | nuclear receptor subfamily 4, group A, member 2 | Other |
| -38.76 | 0.03 | NR4A1 | nuclear receptor subfamily 4, group A, member 1 | Other |
| -7.81 | 0.01 | PNR1 | proline-rich nuclear receptor coactivator 1 | Other |
| -8.93 | 0.00 | TGFB3 | transforming growth factor, beta receptor III | Other |
| -16.64 | 0.02 | LEPR | leptin receptor | Other |
| -8.08 | 0.02 | EBB2ZP2 | erb2 interacting protein | Other |
| -8.80 | 0.03 | RB1 | retinol binding protein 1, cellular | Other |
| -10.47 | 0.01 | FZRL1 | coagulation factor II (thrombin) receptor-like 1 | Other |
| -9.43 | 0.00 | ADRB2 | adrenergic, beta-2, receptor, surface | Other |
| -6.83 | 0.01 | AGTR1 | angiotensin II receptor-like 1 | Other |
| -8.56 | 0.02 | RHEB | Ras homolog enriched in brain | Other |
| -2.39 | 0.02 | FZD7 | frizzled homolog 7 (Drosophila) | Other |
| -7.69 | 0.04 | NCKAP1 | NCK-associated protein 1 | Other |
| -8.65 | 0.01 | FKBPA | nuclear factor of kappa light polypeptide gene enhancer alpha | Other |
| -15.70 | 0.01 | PTGIS | prostaglandin I2 (prostacyclin) synthase | Other |
| -18.85 | 0.00 | PTGD2 | prostaglandin D2 synthase | Other |
| -15.13 | 0.02 | TMEM1 | transmembrane protein 1 | Other |
| -9.59 | 0.04 | HBB | hemoglobin, beta | Other |

**Fig. 4** A hundred top regulated genes. Fold changes are shown in left columns. P-values, gene symbols and unigene comments are given next. The genes are marked with brackets and grouped according to cellular functions and biological processes.
| FC | P-value SYMBOL | Unigene Comment |
|----|---------------|-----------------|
| -11.24 | 0.00 HAS1 | hyaluronan synthase 1 |
| -6.48 | 0.02 EVA1 | epithelial V-like antigen 1 |
| 6.59 | 0.04 TNC | tenascin C (hexabrachion) |
| 17.19 | 0.01 DISC2 | desmocollin 2 |
| 10.40 | 0.00 MCAM | melanoma cell adhesion molecule |
| -11.30 | 0.03 PDZK3 | PDZ domain containing 3 |
| -8.06 | 0.03 RABGGTB | Rab geranylgeranyltransferase, beta subunit |
| -9.71 | 0.00 DMN | desmuslin |
| -10.64 | 0.01 ADD3 | adducin 3 (gamma) |
| -24.78 | 0.00 KRT6A | keratin 6A |
| -37.66 | 0.01 KRT6B | keratin 6B |
| 46.29 | 0.04 KRT16 | keratin 16 |
| 6.47 | 0.00 GMFG | glia maturation factor, gamma |
| -29.94 | 0.04 TNA | tetranectin |
| -21.32 | 0.02 TNXB | tenascin X B |
| -11.20 | 0.01 MAGP2 | microfibril-associated glycoprotein-2 |
| 6.88 | 0.00 COL5A3 | collagen, type V, alpha 3 |
| 7.53 | 0.00 COL4A2 | collagen, type IV, alpha 2 |
| 8.48 | 0.00 COL5A1 | collagen, type V, alpha 1 |
| 19.28 | 0.02 COL4A1 | collagen, type IV, alpha 1 |
| 38.57 | 0.01 COL11A1 | collagen, type XI, alpha 1 |
| -9.17 | 0.03 AFP | amyloid beta (A4) precursor protein |
| 7.98 | 0.05 WFDC1 | WAP four-disulfide core domain 1 |
| 18.41 | 0.02 KLLK13 | kalreikin 13 |
| 25.63 | 0.02 MPP11 | matrix metalloproteinase 11 (stromelysin 3) |
| 30.60 | 0.01 KLK6 | kalreikin 6 |
| 32.08 | 0.01 HA1 | airway trypsin-like protease |
| 7.95 | 0.00 UBE2C | ubiquitin-conjugating enzyme E2C |
| 6.51 | 0.03 CSTB | cystatin B (stefin B) |
| 22.98 | 0.03 SERPINB13 | serine (cysteine) proteinase inhibitor, member 13* |
| 94.96 | 0.00 SERPINB4 | serine (cysteine) proteinase inhibitor, member 4 |
| 143.80 | 0.00 SERPINB3 | serine (cysteine) proteinase inhibitor, member 3 |
| 146.60 | 0.00 P3D | protease inhibitor 3, skin-derived (SKALP) |
| 12.84 | 0.01 THY1 | Thy-1 cell surface antigen |
| 6.36 | 0.00 TRB | T cell receptor beta locus |
| 9.46 | 0.00 TRA | T cell receptor alpha locus |
| -48.78 | 0.00 DAF | decay accelerating factor for complement |
| -8.77 | 0.00 CD3 | CD3 antigen |
| 6.39 | 0.01 IGFL3-10 | immunoglobulin lambda variable 3-10 |
| 13.67 | 0.02 IGKV10R2-108 | immunoglobulin kappa variable 1/2R-108 |
| 21.88 | 0.00 IGKV10-13 | immunoglobulin kappa variable 1D-13 |
| 37.21 | 0.00 IGKC | immunoglobulin kappa constant |
| 67.16 | 0.01 MGC27165 | hypothetical protein MGC27165 |
| 91.18 | 0.00 IGLJ3 | immunoglobulin lambda joining 3 |
| 96.96 | 0.00 IGGL | immunoglobulin lambda locus |
| 126.70 | 0.00 IGHG3 | immunoglobulin heavy constant gamma 3 |
| -12.06 | 0.04 IGL8 | interleukin 6 |
| 63.08 | 0.05 DEF8B4 | defensin, beta 4* |
| 95.11 | 0.02 S100A8 | S100 calcium binding protein A8 (calgranulin A)* |
| 22.78 | 0.00 S100A12 | S100 calcium binding protein A12 (calgranulin C)* |
| -9.71 | 0.00 RAD21 | RAD21 homolog |
| -7.87 | 0.01 STAG2 | stromal antigen 2 |
| 7.48 | 0.02 UPP1 | uridine phosphorylase 1 |
| 8.79 | 0.01 CDA | cytidine deaminase |
| 13.66 | 0.00 CHN1 | chimerin (chiamaerin) 1 |
| 14.99 | 0.00 RR2M2 | ribonucleotide reductase M2 polypeptide |
| -10.60 | 0.01 WEE1 | WEE1 homolog |
| -8.77 | 0.01 INSIG1 | insulin induced gene 1 |
| 6.50 | 0.00 CDC2 | CDC20 cell division cycle 20 homolog |
| 10.33 | 0.01 EGFL6 | EGF-like-domain, multiple 6 |
| 7.07 | 0.02 NDRG4 | NDRG family member 4 |
| -124.38 | 0.00 GFBP5 | insulin-like growth factor binding protein 5* |
| -30.96 | 0.05 APOD | apolipoprotein D |
| -25.77 | 0.00 CCL27 | chemokine (C-C motif) ligand 27 |
| -14.31 | 0.04 CXCL2 | chemokine (C-X-C motif) ligand 2 |
| 6.38 | 0.00 BMP1 | bone morphogenetic protein 1 |
| -22.83 | 0.00 THH | trichohyalin |
| -8.70 | 0.03 PLG | filaggrin |
| 6.18 | 0.00 IL1 | interleukin 1 |
| 15.72 | 0.02 SPRR1B | small proline-rich protein 1B (cornifin) |
| 13.70 | 0.01 SPRR3 | small proline-rich protein 3 |
| 33.93 | 0.00 SPRR1A | small proline-rich protein 1A |
| 118.00 | 0.00 S100A9 | S100 calcium binding protein A9 (calgranulin B)* |
| 165.80 | 0.01 S100A7 | S100 calcium binding protein A7 (psoriasin 1)* |
| 10.96 | 0.02 CALML3 | calmodulin-like 3 |

**Fig. 4 Continued.**

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cyclin-dependent kinase inhibitor 2B (CDKN2B) and cyclin-dependent kinase inhibitor 3 (CDKN3) were up-regulated. Deregulation of cell cycle associated genes suggest a loss of cell cycle control, which may account for the hyperproliferative potential of the keratinocytes present in the epidermis of venous ulcers.

**Keratinocyte activation: regulators**

Among secreted molecules that participate in keratinocyte activation, insulin-like growth factor binding protein 5 (IGFBP5) was among top 50 down-regulated genes. Furthermore, bone morpho-morphocamental proteins (BMP) were differentially regulated. The expression of BMP-2 and BMP-7 was down-regulated (Fig. 6A), whereas the expression of BMP1 was up-regulated. Furthermore, we found that the leptin receptor was down-regulated; however, there was no change in leptin expression. Angiogenesis factors, vascular endothelial growth factor (VEGF), epiregulin (EREG) and angiopoietin-like 6 (ANGPTL6) were markedly down-regulated. Previous data have shown that ANGPTL6 promotes epidermal proliferation, remodelling and regeneration [26]. Conversely, we detected induction of additional pro-angiogenic growth factors and receptors, such as platelet-derived endothelial cell growth factor (ECGF1), receptor neuropilin (NRP1) and stromal cell-derived factor 1-α (CXCL12/SDF-1α). We have found profound down-regulation of apolipoprotein D (APOD) and up-regulation of defensin B4 (DEFB4) (Fig. 6B). Up-regulation of DEFB4 has been shown to be associated with benign hyperplasia in skin [27].

Chemokines which mediate T cell chemotaxis were found suppressed. The expression of cutaneous T-cell attracting chemokine (CCL27), was almost abolished (Fig. 6C), along with IL-7, which is essential for T-cell memory generation, while the expression of I17R was induced. Surprisingly, the expression of platelet-derived growth factors α and β (PDGFA and PDGFB) was increased, but the expression of transforming growth factor-β2 (TGFβ2), transforming growth factor β receptor III (TGF-βR3), fibroblast growth factor 13 (FGF13) and interleukin 6 (IL-6) was decreased. Expression of the stromelysin-3 gene (MMP-11) was up-regulated (Fig. 6C), consistent with previously published data [28].

In summary, microarray data show changes consistent with morphology, suggesting deregulation and incomplete activation of keratinocytes at the non-healing edge in epidermis of venous ulcers.

**Keratinocyte differentiation: markers, cornified envelope and barrier formation**

In intact skin, the major role of keratinocyte terminal differentiation is to form a physical barrier that acts as permeability barrier against water loss, foreign microbes and toxins. The cornified cell envelope and TJs [7] are two important components of skin barrier function.

Microarray data obtained from this study confirm epidermal morphology of non-healing edges. Early markers of differentiating keratinocytes, K1 and K10, were down-regulated as well as the late markers, filaggrin (FLG) and trichohyalin (THH), which are molecules that associate with the keratin cytoskeleton during terminal differentiation (Fig. 7). Interestingly, additional components of the cornified envelope, including involucrin (IVL) and small proline-rich proteins (SPRR1A, SPRR1B, SPRR2B, SPRR3) were up-regulated, whereas transglutaminase 1 (TGM1), an enzyme responsible for cross-linking SPRR’s and IVL into the cornified envelope, was up-regulated. To confirm microarray data at the protein level, we used immunohistochemistry and stained patients’ biopsy specimens with K10, IVL and FLG-specific antibodies. We found robust increase of IVL, whereas K10 and FLG were barely detected in specimens derived from venous ulcers.
Among newly identified potential markers of keratinocyte terminal differentiation [29], we found skin-derived protease inhibitor 3 (SKALP/PI3), oxysterol-binding protein-like 8 (OSBPL8), adducin 3 (ADD3), early growth response 3 (EGR3), inhibitor of DNA binding 4 (ID4), occluding (OCLN) and decay-accelerating factor for complement (DAF) down-regulated, whereas septin 8 (SEPT8), serine/threonine kinase 10 (STK10) and serine/cysteine proteinase inhibitor, clade B, member 3 (SERPINC3) were up-regulated (Fig. 7). In addition, several other molecules that participate in epidermal differentiation were found to be regulated. S100A7, a member of human epidermal differentiation complex (EDC), S100A8, S100A9, S100A12 and kalikrein 6 (KLK6) were among the top up-regulated genes. We used real-time PCR to confirm these findings (Fig. 6D). TJ in skin are complex structures localized in granular layer. These junctions are composed of transmembrane (claudin 1–20, occludin) and plaque (symplekin (SYMPK) and ZO 1–3) proteins [30, 31]. We found that different structural components of TJs, such as TJ protein 3 (ZO3, TJP3), spectrin 1 (SPTBN1), InaD-like protein (MUPP1, INADL), occludin (OCLN), claudin 5 (CLDN5), claudin 8 (CLDN8) down-regulated in the non-healing edge of venous ulcers. Interestingly, only SYMPK was found up-regulated. Formation of TJs in epidermis, during differentiation, is a precisely regulated spatiotemporal process. An important component of this regulation is polarity complex Par3, Par6, atypical PKC-iota (PARD) and CDC42 [32]. We found that different components of polarity

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complex, PARD and CDC42 are down-regulated in chronic wounds when compared to healthy skin.

Taken together, data obtained from microarray analysis indicate deregulation of early and late differentiation markers, aberrant cornified envelope assembly and deregulation of TJ molecules. This suggests a possible loss of barrier function in the epidermis of venous ulcers.

**Keratinocyte differentiation: regulators**

Microarray analyses revealed down-regulation of kuppel-like factor (KLF4), transcription factor, important for the establishment of skin barrier function as well as cross-linking of cornified envelope proteins [7, 33]. Furthermore, up-regulation of phospholipase D2 (PLD2) but not phospholipase D1 (PLD1) was found. The activation of these regulators has been implicated in late keratinocyte differentiation [34]. The Notch signalling pathway has been shown to play a role in defining different steps of keratinocyte differentiation [35, 36]. We have found Manic Fringe (MFNG) to be up-regulated and NOTCH2 to be down-regulated. Taken together, these data demonstrate deregulation of molecules that govern both early and terminal stages of differentiation.

**Adhesion molecules of differentiating keratinocytes**

In addition to changes in TJ expression, we observed a deregulation of several genes coding for proteins of the desmosomal complex in venous ulcers. Among desmosomal cadherins, desmocollin 2 (DSC2) and desmoglein 3 (DSG3) were up-regulated whereas desmocollin 3 (DSC3) and desmoglein 2 (DSG2) were down-regulated. Two other desmosomal molecules, desmoplakin (DP) and plakophilin 2 (PKP2) were also down-regulated in venous ulcers. To confirm the microarray data we stained healthy skin and skin specimens derived from venous ulcers with K10, involucrin and filaggrin-specific antibodies showed increased involucrin expression while K10 and filaggrin are barely detected. Scale bar 50 μm.
increased signal throughout the epidermis of venous ulcers while signal of DP was decreased thus confirming deregulation of desmosomal proteins (Fig. 9).

**Discussion**

In this paper we present comprehensive analyses of the pathogenesis of non-healing edges of venous ulcers using transcriptional profiling, real-time PCR and immunohistochemistry. We found that hyperproliferation of keratinocytes in the epidermis of non-healing edges is, in part, due to a loss of a cell cycle control and incomplete activation. Furthermore, we found deregulation of early and late differentiation markers, as well as regulators of this processes, coupled with aberrant cornified envelope assembly and deregulation of TJ and desmosomal molecules. Taken together, data obtained from this study suggest that keratinocytes at the non-healing edges of venous ulcers are not capable of accomplishing either of two main processes in skin homeostasis: proliferation and differentiation.

In healthy skin, basal epidermal keratinocytes proliferate and, as they exit the basal cell compartment, they commit to a differentiation program. Keratinocyte differentiation requires DNA degradation, nuclear destruction and substantial proteolytic activity and leads to cell death resulting in the formation of a cornified layer and subsequently desquamation of dead cells. Our previous findings show that epidermis of chronic wounds is hyperproliferative with mitotically active cells present in suprabasal cell layers. These findings, coupled with the presence of the nuclei in a very thick cornified layer, suggest aberrant activation and incomplete differentiation of keratinocytes in chronic wounds.

When a wound occurs, keratinocytes at the wound edge become activated and start to express activation markers, keratins...
differentiation [52], and that perhaps this process demarcates FLG into FLG they undergo specific changes that mark terminal 

lated. It has been shown that once keratinocytes process pro-
cates improper cornified envelope assembly [51]. FLG, one of 
well as S100A7 and SPRR1A, SPRR1B, SPRR2B, SPRR3, indi-
major early cross-linked component of cornified envelope as 
linking during cornification process along with IVL, which is a 

Up-regulation of TGM1, an enzyme involved in the initial cross 

findings and further validates our microarray analyses [50]. Interestingly, we found SDF1/H9251 expression may be asso-

SDF-1/H9251 up-regulated in venous ulcers. This may indicate that changes in SDF-1/H9251 expression may be asso-
ciated specifically with diabetes rather than with impaired healing 
in general.

Keratinocyte early differentiation markers K1/K10 were down-
regulated in our experiment, which is consistent with previous 
findings and further validates our microarray analyses [50]. Up-regulation of TGM1, an enzyme involved in the initial cross 
linking during cornification process along with IVL, which is a 

a major early cross-linked component of cornified envelope as 
well as S100A7 and SPRR1A, SPRR1B, SPRR2B, SPRR3, indi-
cates improper cornified envelope assembly [51]. FLG, one of 
the key proteins that facilitate terminal differentiation of the epi-
dermis and formation of the skin barrier, was found down-regu-

lated. It has been shown that once keratinocytes process pro-
FLG into FLG they undergo specific changes that mark terminal differentiation [52], and that perhaps this process demarcates 

the 'line of regenerative potency' among the population of differenti-
ating keratinocytes [53]. Down-regulation of FLG in venous 
ulcer coupled with deregulation of other keratinocyte differentia-
markers suggests that keratinocytes at the edge of non-
healing venous ulcers start to differentiate but are not capable of 
completing the process. In support of this finding, we also 
detected down-regulation of PLD1 in venous ulcers. It has been 
shown that during keratinocyte differentiation PLD1 mRNA 
levels increase [54]. Furthermore, the highest levels of PLD1 
expression were found in the more differentiated layers of the 
epidermis, with negligible expression in basal keratinocytes 
[55]. Down-regulation of PLD1 expression in venous ulcers may 
suggest some form of de-differentiation and supports the notion 
that these keratinocytes do not execute the process to its com-
pletion. Similarly, we also found APOD (recently associated with 
differentiating keratinocytes) to be down-regulated in our venous 
ulcers [29].

Similar to findings of deregulation of differentiation markers 
and molecules that participate in this process and consistent with 
morphology of epidermis in venous ulcers, we also found dereg-
ulation of signalling molecules that control the process of differ-
entiation. The overall findings are that the regulatory signals are 
sending 'mixed messages' to the cells leading to deregulation of 
the process. In differentiating epidermal keratinocytes, BMPs 
stimulate expression of K1, IVL and distal-less homeobox 3 (Dlx3) 
transcription factor, suggesting that these molecules may be tar-
gets for BMP signalling [56, 57]. It's been shown that BMP2 

inhibits keratinocyte proliferation and promotes their terminal dif-
ferentiation [58]. Thus, down-regulation of BMP2 in venous ulcers 
may account for keratinocyte hyperproliferation and deregulation of 
terminal differentiation. In addition, down-regulation of KLF4, a 
transcription factor highly expressed in differentiating layers of 
epidermis important for the establishment of skin barrier function 
and expression/cross-linking of cornified envelope proteins, fur-
ther confirms improper regulation of differentiation process in 
venous ulcers [7, 33].

Another interesting observation is the down-regulation of \( \alpha \text{B} \)-deficient mice are highly proliferative with deregulated epider-
mal differentiation [59], thus exhibiting phenotype similar to that 
of a non-healing epidermis of venous ulcers. Changes in expression 
of genes participating in regulation of keratinocyte differentiation program contribute to abnormal keratinocyte differentiation. 

Adhesion molecules exert a dynamic role in epidermal differen-
tiation stratification. Down-regulation of TJ proteins, particularly 
claudins suggest a loss of permeability barrier function of the epi-
dermis in our venous ulcer samples. This is corroborated by the 
inability of claudins to prevent TJ formation in KO mice, as well as 
a complete alteration in their function [60, 61]. Recent papers sug-
gest that activity of complex Par3, Par6, atypical PKC-iota and 
CDC42 in granular layer of epidermis is necessary for TJ forma-
tion and keratinocyte differentiation [62]. Furthermore during cal-
cium-induced differentiation of keratinocytes, atypical PARD was 

necessary for establishment of barrier formation. This complex
had characteristic re-distribution during wound healing and it may also be an endogenous regulator of asymmetric cell division of basal keratinocytes. Asymmetric cell division, on other hand, promotes stratification and wound healing in the skin by keeping balance between proliferation and differentiation [30, 63]. Down-regulation of atypical PARD and CDC42 suggests loss of cell polarity further suggesting a loss of balance between keratinocyte proliferation and differentiation and deregulation of TJ formation. Further investigation is necessary to analyse the exact localization of the polarity complex as well as TJ in hyperproliferative epidermis of venous ulcers.

We also observed deregulation of desmosomal components in the non–healing edges of venous ulcers. In the normal epidermis, DSC2 and DSG2 are found highly expressed in the proliferative basal layers of the epidermis. We recently showed that DSG2 plays a role in keratinocyte cell proliferation and survival [64]. Therefore a suppression of DSG2 expression may alter keratinocyte proliferation in venous ulcers. DSG3 expression, on the other hand, extends from the basal to spinous layers with intensity decreasing with differentiation. In venous ulcers, DSG3 was up-regulated and expressed throughout hyperproliferative epidermis, which is in agreement with previously publish data obtained from murine models, suggesting that differential expression of desmosomal proteins within the epidermis participate in the regulation of the tissue proliferation and differentiation [64–68]. When DSG3 was overexpressed under the control of K1 promoter in the suprabasal epidermis of transgenic mice, histological analysis of the skin revealed a hyperproliferative epidermis with hyper- and parakeratosis, along with abnormal epidermal differentiation [65]. Interestingly, despite the high expression of DSG3 and DSC2 in venous ulcers, expression of their associated plaque protein DP was greatly suppressed. This apparent lack of association between DSG3 and DP has been shown in DP RNAi-treated cells [69]. Similar to that observed here in venous ulcers, reduction of DP expression in a mild form of striate palmoplantar keratoderma also results in hyperproliferation and hyperkeratosis [70] further supporting a role for DP in regulating keratinocyte proliferation and differentiation. These data suggest that deregulation and atypical expression of desmosomal components may play a role in altered keratinocyte differentiation.

In the future, several different comparative genomics studies would prove very useful in delineating molecular pathogenesis of chronic wounds. For example, comparing transcriptional profiles of non-healing edges of chronic to acute wounds in different stages would identify specific wound-healing genes which contribute to non-healing phenotype. In addition, comparing transcriptional profiles of non-healing edges from venous to diabetic foot ulcers or pressure ulcers will identify common pathways that lead to non-healing phenotype in majority of chronic wounds. Proposed experiments underscore technical challenges in this field. Specifically, selection of controls is fundamental to the outcome of the data. Our laboratory has worked over the years to establish how variable human samples actually show consistent expression profiles [37] and, further, how to standardize the wound biopsy from patient to patient [10]. To address the issue of mixed cell populations in our skin samples, mRNA levels of abundantly expressed genes specific for two major cell types: perlecan and vimentin (fibroblasts) and stratifin and junctional plakoglobin (keratinocytes) were compared using microarrays. The signal intensity levels correlate to the hybridization signal intensity of vimentin, perlecan, stratifin and junctional plakoglobin and are a direct measure of the mRNA levels in each biopsy. All biopsies show consistent levels of expression for perlecan, stratifin and junctional plakoglobin suggesting that both the keratinocytes and fibroblasts are present at the consistent numbers in our venous ulcer biopsies [10]. Evaluations of microarray findings were performed on biopsies from venous ulcers of subsequent set of patients, which further documents the validity of the data obtained. In addition, technical aspects of harvesting the tissue, such as lidocaine injection, presence of infection, could be perceived as factors that may influence the data outcome. There is evidence in literature that lidocaine decreases proliferation of cultured human keratinocytes [71]. However, samples for this study were harvested within 30 min. after the lidocaine injection and immediately processed for RNA purification. Therefore, the lidocaine did not affect the outcome. In addition, we have established that specimens harvested using this procedure exhibit marked hyperproliferation [9, 10] which was further confirmed by microarrays presented in this manuscript.

Our data indicate that keratinocytes in venous ulcers initiate differentiation, carry out atypical cornified envelope assembly, and proceed towards terminal differentiation, but fail to completely execute the terminal process of differentiation and barrier formation. Most of the current understanding of the pathophysiology of venous ulcers comes from the tissue level [72], but our data expands on previous work to examine why a venous wound might not heal on a cellular level. For a physician, the implications of this research provide a rational biologic basis for debridement of venous ulcers to remove the skin cells that do not have the ability to migrate properly, leaving those that do.

Taken together, the data collected from this microarray study suggest that, on the one hand, keratinocytes at the non-healing edges of venous ulcers express activation markers, show deregulation of a cell cycle associated genes, and loss of cell cycle control. On the other hand, they show deregulation of early and late differentiation markers, TJ and desmosomal proteins as well as regulators of differentiation and perform aberrant cornified envelope assembly. Thus, keratinocytes at the non-healing edges of venous ulcers do not successfully complete either of the two possible pathways in a proper manner: proliferation or differentiation. Instead, keratinocytes are constantly trying, without success, to address either of the two processes.

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