RESEARCH

Dysregulated healing responses in diabetic wounds occur in the early stages postinjury

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Abstract

Chronic wounds are a serious and debilitating complication of diabetes. A better understanding of the dysregulated healing responses following injury will provide insight into the optimal time frame for therapeutic intervention. In this study, a direct comparison was done between the healing dynamics and the proteome of acute and obese diabetic wounds on days 2 and 7 following injury. Full thickness excisional wounds were induced on obese diabetic (B6.Cg-lepob/J, ob/ob, $n = 14$) (blood glucose 423.25 ± 127.92 mg/dL) and WT control (C57BL/6J, $n = 14$) (blood glucose 186.67 ± 24.5 mg/dL) mice. Histological analysis showed no signs of healing in obese DM wounds whereas complete wound closure/re-epithelisation, the formation of granulation tissue and signs of re-vascularisation, was evident in acute wounds on day 7. In obese DM wounds, substance P deficiency and increased MMP-9 activity on day 2 coincided with increased cytokine/chemokine levels within wound fluid. LC-MS/MS identified 906 proteins, of which 23 (Actn3, Itga6, Ebp41, Sncg, Nefm, Rsp18, Rsp19, Rpl22, Macroh2a1, Rpn1, Ppib, Snrnp70, Ddx5, Eif3g, Tpt1, FABP5, Cavin1, Stfa1, Stfa3, Cybs, Tkt, Mb, Chmp2a) were differentially expressed in wounded tissue on day 2 ($P < 0.05$; more than two-fold) and 6 (Cfd, Ptms, Hp, Hmga1, Cbx3, Syap1) ($P < 0.05$; more than two-fold) on day 7. A large number of dysregulated proteins on day 2 was associated with an inability to progress into the proliferative stage of healing and suggest that early intervention might be pivotal for effective healing outcomes. The proteomic approach highlighted the complexity of obese DM wounds in which the dysregulation involves multiple regulatory pathways and biological processes.

Key Words

- ▶ wound healing
- ▶ diabetes
- ▶ inflammation
- ▶ substance P

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Introduction

Depending on the underlying cause, chronic wounds are subdivided into three main classes that is, leg ulcers (associated with venous disease), pressure ulcers/pressure injuries (associated with prolonged bed rest) and diabetic foot ulcers (DFUs) (associated with uncontrolled

hyperglycaemia). Of these, DFUs are the most common and very difficult to treat, with standard of care involving debridement, offloading and infection control with amputation often being necessitated. It, therefore, places a socio-economic burden on the patients and enormous

financial strain on health care systems worldwide (Alexiadou & Doupis 2012, Frykberg & Banks 2015, Jones *et al.* 2019). In 2017, approximately 8.8% of the world's population was affected by diabetes mellitus (DM) and this percentage is projected to increase to 9.9% by the year 2045 (Cho *et al.* 2018). The prevalence of DFUs in DM patients is 4–10%, with a lifetime risk of 25% and due to the lack of effective treatment strategies, DFUs have a 20–80% recurrence rate (Mulder *et al.* 2012, Cho *et al.* 2018). The mortality rate amongst DFU patients with amputated limbs can be as high as 44% after 1 year (Fortington *et al.* 2013). The molecular characterisation associated with impaired/dysfunctional healing responses in pathological conditions has however not yet been fully elucidated (Ganesh & Ramkumar 2020).

The wound healing process which normally consists of four major phases (haemostasis, inflammation, proliferation and remodelling) (Ozgok Kangal & Regan 2020) is hindered in DM due to its deviant pathology. The pathogenesis of uncontrolled DM involves persistent hyperglycaemia resulting in the accumulation of advanced glycation end-products (AGEs) (glycosylated proteins and lipids) (Singh *et al.* 2014). Consequently, oxidative stress (reactive oxygen species, ROS) becomes prominent and a chronic self-perpetuating pro-inflammatory state develops (Goh & Cooper 2008, Zhong *et al.* 2017). Together, this toxic microenvironment leads to tissue damage and cellular dysfunction. In DM skin, chronic inflammation together with pathogenesis-induced ischaemia and neuropathy are the leading causes for the development of DFUs (Pradhan *et al.* 2009, da Silva *et al.* 2010). DM neuropathy is characterised by insufficient production of neuropeptides such as substance P and the overproduction of endopeptidases such as Neutral endopeptidase (also known as Neprilysin, NEP) and matrix metalloprotease-9 (MMP-9) (Olerud *et al.* 1999, Antezana *et al.* 2002, da Silva *et al.* 2010, Jones *et al.* 2019). Substance P is secreted by sensory nerves and inflammatory cells (macrophages and eosinophils) upon injury (O'Connor *et al.* 2004). It functions via its receptor, neurokinin-1 receptor (NKR-1), and has been shown to promote the wound healing process in both DM and non-DM skin wounds by initiating the acute inflammatory phase of healing (Spenny *et al.* 2002, Leal *et al.* 2015). This is thought to occur within the first 3 days postwounding (Leal *et al.* 2015). Substance P has also been shown to promote the transition into the proliferative stage of healing by promoting an M2 (pro-regenerative) phenotype in macrophages (Leal *et al.* 2015). During the proliferative stage of healing, substance P mediates angiogenesis, promotes keratinocyte

proliferation and fibrogenesis (Scott *et al.* 2007, Steyaert *et al.* 2010, Um *et al.* 2016). Excessive substance P activity, on the other hand, is associated with hypertrophic scar formation (Scott *et al.* 2005). In DM skin with underlying neuropathy there is a reduction in the bioavailability of substance P and an overproduction of the cell surface enzyme, NEP, that degrades substance P (Trengeve *et al.* 1999, Antezana *et al.* 2002, Spenny *et al.* 2002, Leal *et al.* 2015). Although these and other characteristics of chronic wounds are well defined a better understanding of the complex interaction associated with dysregulation that occurs following injury is crucial for the development of more effective treatments. Using fine-tuned animal wound models to identify potential therapeutic targets can bridge the translational gap between laboratory-based and clinical studies (Sabino *et al.* 2018).

This study compared the healing dynamics as well as the proteome of acute wounds with that of non-healing diabetic wounds during the initial and late stages following injury. This will provide significant insight into the optimal time frame for therapeutic intervention and aid in identifying critical role players at the different stages postinjury involved in the dysfunctional healing responses.

Materials and methods

Ethics statement

This study was approved by the animal research ethics committee (SU-ACUD17-000016) at Stellenbosch University and complied with the South African Animal Protection Act (Act no. 71, 1962). In accordance with the South African National Standards 10386:2008 and the Veterinary and Para-Veterinary Professions Act, 1982, all researchers involved in handling of the animals were authorised by the South African Veterinary Council (SAVC).

Induction of full-thickness excisional wounds and sample collection

Obese DM mice (B6.Cg-lep^{ob}/J, ob/ob, $n = 14$) (10–12 weeks; 46.1 ± 4.2 g) (blood glucose 23.49 ± 7.1 mmol/L or 423.25 ± 127.92 mg/dL) and healthy WT control mice (C57BL/6J, $n = 14$) (10–12 weeks; 27.3 ± 3.1 g) (blood glucose 10.36 ± 1.36 mmol/L or 186.67 ± 24.5 mg/dL) were anaesthetised using Isoflurane gas (Safeline Pharmaceuticals, Cape Town, Western Cape, South Africa). Dorsal hair was shaved, and the skin cleaned with povidone-iodine (Mundipharma, Cape Town, Western

Cape South Africa) prior to making two identical bilateral full-thickness skin excisions. The procedure included the removal of the underlying *panniculus carnosus* layer (Chen *et al.* 2013). Wounds (5 mm diameter) were located 1 cm below the base of the skull and on either side of the midline. To induce a non-healing obese DM wound, neutral endopeptidase (NEP) (1.02 mg/ μ L; 150 pmol/h/ μ g) (SRP6450, Neprilysin/CD10; Sigma-Aldrich) was injected subcutaneously around the wound edges in the ob/ob animals immediately postwounding. The expression of NEP is much greater in the ulcer margins of DFU patients (Antezana *et al.* 2002) than what is observed in the skin of diabetic animals (delayed healing) and healthy controls (Antezana *et al.* 2002, Spenny *et al.* 2002). Exogenous NEP administration into the wound edges of a commonly used diabetic animal model of delayed healing in this study was therefore used to induce a chronic wound that more closely mimic DFUs. Acute wounds were induced on WT control mice (saline injection) as parallel control. Additional control groups included WT control mice with NEP injection ($n = 3$) and streptozotocin-induced hyperglycaemic mice (intraperitoneal injection of 50 mg/kg body weight STZ, 3 days before wounding; blood glucose following STZ-injection 12.1 ± 0.6 mmol/L or 217.9 ± 10.8 mg/dL) with NEP injection ($n = 3$ per group). Pain management included the injection of a local anaesthetic, 7 mg/kg lignocaine (2%) (Bodene, Port Elizabeth, Eastern Cape, South Africa) immediately postwounding as well as administering 300 mg/kg acetaminophen (GSK, Cape Town, Western Cape, South Africa) in the drinking water for a period of 3 days.

Wounds were covered by a vapour-permeable PU film (Hydro-film, Paul Hartmann AG, Heidenheim, Baden-Württemberg, Germany) and the accumulated exudate (wound fluid) harvested by needle puncture (Tregrove *et al.* 1999, Schmohl *et al.* 2012) on day 2 postwounding. After recovery from the anaesthesia, mice were housed individually under standard conditions (12 h light:12h darkness cycle at a controlled temperature of 21°C) with *ad libitum* access to drinking water and chow (Rat and Mouse Breeder Feed, Animal Specialties, Pty, Ltd., Klapmuts, SA) and their appearance and behaviour closely monitored for signs of distress. Following cervical dislocation, wound tissue including a 5 mm margin was excised. One half of each tissue sample was snap-frozen in liquid nitrogen and stored at -80°C for protein extraction. The other half of each tissue sample was fixed in 10% buffered formalin solution and embedded in paraffin wax for histological analysis (hematoxylin & eosin; Masson's Trichrome staining) using standardised techniques.

Assessment of macroscopic wound closure and wound fluid analysis

Wound images were taken on days 0, 2 and 7 using a Canon digital camera (EOS 600D, Canon Inc, Taiwan). The surface area (cm^2) for each wound and the percentage wound closure was determined using Image J software (version 1.46, NIH.gov, USA). Wound fluid collected on day 2 was analysed using a Bio-Plex Pro Mouse Cytokine 23-plex assay (M60009RDPD, Bio-Rad) according to the manufacturer's instructions. The cytokines assessed included Eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma ($\text{IFN}\gamma$), interleukin (IL)-1 α , IL1 β , IL2, IL3, IL4, IL5, IL6, IL9, IL10, IL12(p40), IL12(p70), IL13, IL17A, keratinocyte chemoattractant (KC), macrophage chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , RANTES and TNF alpha ($\text{TNF}\alpha$). Note: due to the small volume of wound fluid collected from each animal it was necessary to pool the wound fluid from all the animals within each group prior to analysis ($n = 4$ per group).

MMP-9 expression in wounded tissue

The expression of MMP-9 within the wounded tissue was assessed using a sandwich ELISA kit (AB100732, Abcam) according to the manufacturer's instructions. Snap frozen tissue samples (100 mg) were homogenised in liquid nitrogen. Assay buffer (400 μ L) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (1083709100, Sigma-Aldrich) and 10 μ g/mL Aprotinin (A1153-5MG, Sigma-Aldrich) was added to each sample, incubated on ice (10 min) and centrifuged at 4°C for 10 min at 12,000 $\times$. The protein concentration within the supernatant was determined using a NanoDropTM 2000/2000c (Thermo Fisher Scientific) (260/280 nm) and samples stored at -20°C . For each sample 6 μ g of protein was used in the ELISA and the absorbance read at 450 nm using a micro-plate reader (Multiskan GO 1.00.40, Thermo-Scientific Group).

Immunohistochemistry substance P

Standard immunohistochemistry techniques were used to assess the expression of substance P within the wounded area using a primary anti-substance P antibody (ab10353, Abcam) and an HRP enzyme-conjugated secondary antibody (ab6789, Abcam). Images were taken at 5 $\times$ magnification using the Zeiss Axio Vision microscope (Zeiss Axio Observer 7 Inverted microscope, Zeiss).

The expression of substance P (% surface area) was determined using Image J software (version 1.46, NIH.gov, USA).

Histology quantification of healing dynamics

Tile scan images of H&E and Masson's Trichrome stained sections were taken at 10× magnification using the Zeiss Axio Vision microscope (Zeiss Axio Observer 7 Inverted microscope, Zeiss). Assessment of the histological parameters such as granulation tissue thickness (µm), epithelial thickness (µm), cellularity (nuclei within granulation tissue) (% surface area) and the number of blood vessels formed within granulation tissue was performed using Image J software (version 1.46, NIH.gov, USA). The histology index was calculated for each wound as indicated in Table 1.

Protein extraction for proteomics

Homogenised tissue samples (100 mg) were incubated in 89% ethanol containing 50 mM Tris(2-carboxyethyl) phosphine (TCEP; Fluka, Sigma-Aldrich), 1% acetic acid (Sigma-Aldrich) and 1 mg/mL caffeine (Pierce) for 1 h at 4°C. The supernatant was sonicated in methanol (Sigma-Aldrich) for 15 min at 4°C and incubated with 600 µL of methyl tert-butylether (MTBE; Sigma-Aldrich) for 1 h at 4°C. Phase separation was induced by adding 100 mM triethylammonium bicarbonate (TEAB; Sigma-Aldrich) to each sample and centrifugation (14 min at 12,000 g). Four volumes of methanol (Sigma-Aldrich) was added to the lower phase and centrifuged at 12,000 g for 10 min. The supernatant was removed, and the pellet was sonicated (10 min at 4°C) with 200 µL 50 mM TEAB pH 7.8 containing 8 M urea (Sigma-Aldrich), 0.5% SDS and a protease inhibitor cocktail (Sigma-Aldrich). Four volumes of methanol were added, and samples centrifuged at 12,000 g for 10 min. The supernatant was removed, and protein pellet was retained for proteomic analysis. The protein samples underwent an on-bead digestion step followed by a peptide quantification assay (Bradford assay). Samples were resuspended in 50 mM triethyl ammonium bicarbonate (TEAB; Sigma-Aldrich) before reduction with 5 mM triscarboxyethyl phosphine

(TCEP; Fluka, Sigma-Aldrich) 50 mM TEAB for 1 h at 60°C. Cystein residues were thiomethylated with 20 mM S-methyl methanethiosulfonate (Sigma-Aldrich) in 50 mM TEAB (Sigma-Aldrich) for 30 min at room temperature. After thiomethylation the samples were diluted two-fold with binding buffer (100 mM sodium acetate, 30% acetonitrile, pH 4.5). The protein solution was added to MagResyn (Resyn Biosciences, Pretoria, Gauteng, South Africa) HILIC magnetic particles prepared according to manufacturer's instructions and incubated overnight at 4°C. After binding the supernatant was removed and the magnetic particles washed twice with washing buffer (100 mM sodium acetate, 15% acetonitrile, pH 4.5). After washing the magnetic particles were suspended in 50 mM TEAB containing trypsin (New England Biolabs, Ipswich, MA, USA) to a final ratio of 1:10. After a 6 h incubation at 37°C the peptides were extracted once with 50 µL water and once with 50% acetonitrile (Romil). The samples were dried down and resuspended in 30 µL 2% acetonitrile:water; 0.1% FA.

Residual digest reagents were removed using an in-house manufactured C18 stage tip (Empore Octadecyl C18 extraction discs; Supelco). The samples were loaded onto the stage tip after activating the C18 membrane with 30 µL methanol (Sigma-Aldrich) and equilibration with 30 µL 2% acetonitrile:water; 0.05% TFA. The bound sample was washed with 30 µL 2% acetonitrile:water; 0.1% TFA before elution with 30 µL 50% acetonitrile:water; 0.05% TFA. The eluate was evaporated to dryness. The dried peptides were dissolved in 2% acetonitrile:water; 0.1% FA for LC-MS analysis. Half the contents of each vial of isobaric Tandem Mass Tag (TMT) (Thermo Fischer Scientific) were added to 14 µg of each sample. The use of 10-plex isobaric labels allowed the relative quantification of proteins by examining all the biological samples from one time point in a single run.

Proteomics liquid chromatography and mass spectrometry

Liquid chromatography (Dionex nano-RSLC) was performed on a Thermo Scientific Ultimate 3000 RSLC equipped with a 5 mm × 300 µm C₁₈ trap column (Thermo

Table 1 Histology index.

Score	Description
1	Little epidermal and dermal organisation (epithelial thickness < 30 µm); <10 capillary vessels; no granulation tissue formation.
2	Moderate epidermal and dermal organisation (epithelial thickness <100 µm), few newly formed capillary vessels evident, cellular infiltration; granulation tissue thickness (>100 µm).
3	Complete remodelling of the epidermis and dermis, well-formed capillary vessels.

Scoring parameters were adapted from Galeano *et al.* (2001).

Scientific) and a nanoEase M/Z Peptide CSH 25 cm $\times$ 75 μ m 1.7 μ m particle size C₁₈ column (Waters, Milford, Massachusetts, USA) analytical column. Chromatography was performed at 45°C and the outflow delivered to the mass spectrometer (Thermo Scientific Fusion, Nanospray Flex ionisation source) through a stainless-steel nano-bore emitter. MS1 scans were performed using the orbitrap detector set at 120,000 resolution over the scan range 350–1500 with AGC target at 4E5 and maximum injection time of 50 ms. MS2 acquisitions were performed using monoisotopic precursor selection for ion with charges +2–+7 with error tolerance set to ± 10 ppm. Fragment ions were detected in the orbitrap mass analyser set to 50,000 resolution with the AGC target set to 5E4 and the maximum injection time to 80 ms.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8.2.0). The normal distribution of data was determined using the Shapiro–Wilk and Kolmogorov–Smirnov test. Data are presented as mean $\pm$ s.d. Two-way ANOVAs with Tukey *post hoc* tests were performed to determine the effect of time,

group and group $\times$ time. Welch's T-test with two-tailed *P* value was used for comparison of the healing dynamics and proteome between the acute and DM wounds. Level of significance was accepted at $P < 0.05$. *P* values were multiple testing corrected via the Benjamini–Hochberg FDR procedure to obtain Q-values. Functional pathway analysis and protein interactions were mapped for the differential proteins of interest ($P < 0.05$) (identified through biostatistical analysis of the LC-MS/MS data) using STRING (version 11.0, string-db.org).

Results

Animal wellness

All animals tolerated the procedure well with a 100% survival rate observed for a period of 7 days postwounding. Refer to Fig. 1A for representative images illustrating macroscopic wound size on days 0 and 7 in the respective groups. For both the WT saline (body weight 27.3 ± 3.1 g) and obese DM (body weight 46.1 ± 4.2 g) wounded animals, an initial drop in body weight of approximately 3 g (WT 12% decrease ($P = 0.0011$); obese DM 4–6% decrease), were observed during the first 2 days postwounding. The animals did, however, recovered from this initial weight loss

Figure 1

Animal wellness. (A) Representative images of wounds on days 0 and 7 postwounding induced in the following animal models: WT mice + saline injection (WT saline wounds); WT mice + NEP injection (WT wounds); streptozotocin-induced hyperglycaemia in WT mice + NEP injection (lean DM wounds); ob/ob mice + NEP injection (obese DM wounds). (B) Animal weight (g) over time postwounding. (C) Animal wellness behaviour score over time postwounding with 1 = alert, curious, active, normal movement; 2 = less active, less responsive, abnormal movement; 3 = inactive, poorly responsive. (D) Comparison of wound size (cm²) on day 0 immediately postwounding. (E) Percentage wound closure (%) in acute DM wounds on days 2 and 7 postwounding. *Statistical analysis:* Two-way ANOVA with Tukey *post hoc* test. * $P < 0.05$ indicates a significant difference from day 0 within the same group and ### $P < 0.001$ indicates significant difference between groups (B, C, D and E). * $P < 0.05$ and *** $P < 0.001$ indicate significant difference between groups (D and E). A full colour version of this figure is available at <https://doi.org/10.1530/JME-20-0256>.

(weight day 7; WT 25.2 ± 0.9 g, obese DM 45.6 ± 0.9 g) (Fig. 1B). The body weight for the WT+NEP (29.9 ± 2.4 g) and lean DM+NEP (32.2 ± 0.7 g) groups remained stable throughout the study (Fig. 1B). There were no differences evident in the wellness scores (behaviour) of animals (Fig. 1C).

Wound healing dynamics

To identify the most appropriate model representative of acute and non-healing diabetic wounds, macroscopic wound closure was compared between treatment groups. At 7 days postwounding there was no significant difference in wound closure evident between the WT saline ($73.5 \pm 9.7\%$), WT+NEP ($74.9 \pm 13.2\%$) and STZ-injected WT+NEP ($74.2 \pm 16.3\%$) groups, whereas healing was significantly delayed in obese DM wounds ($43.5 \pm 21.9\%$)

(Fig. 1A and E). For all subsequent analysis, the WT saline wounds were chosen to represent acute wounds and the obese DM wounds were chosen to represent non-healing diabetic wounds. Immediately postwounding on day 0, there was no difference in wound size (WT 0.359 ± 0.021 cm², obese DM 0.424 ± 0.017 cm²) between the acute and obese DM groups (Fig. 1D). In the acute wounds, significant wound closure and re-epithelisation was evident (day 2: $29.9 \pm 6.7\%$; day 7: $73.5 \pm 3.1\%$) postwounding, whereas obese DM wounds remained open (day 2: $11.7 \pm 3.7\%$; day 7: $43.5 \pm 6.9\%$) with no epithelisation evident ($P = 0.008$) (Fig. 1A and E). This was confirmed using histological assessment of healing dynamics (Fig. 2A and B). On day 7 postwounding, significant granulation tissue formation (thickness 1015 ± 177 μ m) was evident in acute wounds whereas none had formed in the obese DM wounds ($P < 0.01$) (Fig. 2A and B). Within the granulation

Figure 2

Histological comparison of acute and obese diabetic (DM) wounds. (A) Masson's Trichrome staining. Representative images illustrating the wound area and wound edges, the formation of granulation tissue and the structural organisation within the wounded area on day 7 postwounding. Dotted line (---) indicates granulation tissue thickness. Arrows indicate capillaries. (B) Hematoxylin & eosin staining. Representative images illustrating the wound area and wound edges, the presence of cell nuclei (blue/purple) within granulation tissue and the epithelial layer. Dotted line (---) indicates the thickness of the newly formed epithelium. A full colour version of this figure is available at <https://doi.org/10.1530/JME-20-0256>.

tissue, cellularity was evident with nuclei representing $11.4 \pm 1.1\%$ of the surface area and newly formed capillaries were clearly visible in the acutely wounded areas (Fig. 2A and B). Quantification of the histological parameters indicated that acute wounds had an overall histological score of 2 ± 1 whereas obese DM wounds had a score of 1 ± 0 on day 7 postwounding with no signs of healing (Table 1).

Substance P and MMP-9

A significant difference was evident between groups with regards to the expression of both MMP-9 and substance P within the wounded area (Fig. 3). Despite variability in the expression of MMP-9 within the obese DM wounds, on day 2 postwounding, MMP-9 levels were four- to eight-fold higher in obese DM compared to acute wounds ($P = 0.0297$) and remained slightly elevated on day 7 ($P = 0.05$) (Fig. 3A). Immunohistochemistry illustrated a deficiency in the expression of substance P in the obese DM wounds immediately postwounding (acute $8.7 \pm 3.3\%$; DM $1.5 \pm 2.3\%$) ($P < 0.05$) as well as on day 2 (acute $19.3 \pm 8.8\%$; DM $4.8 \pm 2.6\%$) ($P < 0.05$) (Fig. 3B and C). Refer to Fig. 3C for representative images illustrating

substance P expression within the wounded areas at each of the respective time points.

Cytokine profile in wound fluid

On day 2 postwounding, the level of pro-inflammatory cytokines, chemokines and anti-inflammatory cytokines within the pooled wound fluid samples were overall higher in the obese DM compared to acute wounds as illustrated in Fig. 4. All of the chemokines assessed were overexpressed with GCSF (acute 516.65 pg/mL; DM 5199.68 pg/mL), KC (acute 89.75 pg/mL; DM 1258.26 pg/mL), MCP1 (acute 166.46 pg/mL; DM 5857.79 pg/mL), MIP1 α (acute 82.12 pg/mL; DM 435.79 pg/mL) and MIP1 β (acute 139.19 pg/mL; DM 554.89 pg/mL) being the most prominent in obese DM wound fluid. Similarly, the pro-inflammatory cytokines IL1 α (acute 28.19 pg/mL; DM 219.04 pg/mL), IL6 (acute 95.54 pg/mL; DM 873.31 pg/mL), IL12(p40) (acute 18.92 pg/mL; DM 44.71 pg/mL), IL12(p70) (acute 10.53 pg/mL; DM 96.87 pg/mL), RANTES (acute 0 pg/mL; DM 72.73 pg/mL) and TNF α (acute 8.87 pg/mL; DM 56.04 pg/mL) were prominent in obese DM wound fluid. In comparison, the expression levels of anti-inflammatory cytokines were relatively low in the obese DM wounds Fig. 4.

Figure 3

MMP-9 and substance P expression within acute and obese diabetic (DM) wounds. (A) MMP-9 expression (absorbance) in acute and DM wounds on days 0, 2 and 7 postwounding. (B) Substance P (% surface area) expression levels in acute and DM wounds on days 0, 2 and 7 postwounding. (C) Representative images illustrating substance P expression. Dotted line (---) indicates the wounded area. *Statistical Analysis:* Two-way ANOVA with Tukey *post hoc* test. * $P < 0.05$ indicates the significant difference between groups at the same time point. A full colour version of this figure is available at <https://doi.org/10.1530/JME-20-0256>.

Figure 4

Cytokine profile within wound fluid derived from either acute or obese diabetic (DM) wounds. Heat map illustration the levels (pg/mL) of pro-inflammatory cytokines, chemokines and anti-inflammatory cytokines in acute and DM wounds. Note: due to the small volume of wound fluid collected from each animal it was necessary to pool the wound fluid from all the animals within each group prior to analysis ($n = 4$ per group). A full colour version of this figure is available at <https://doi.org/10.1530/JME-20-0256>.

Proteome

A total of 906 proteins with each more than three unique peptides were identified (Fig. 5). On day 2 postwounding, 135 differentially expressed proteins ($P < 0.05$) were observed between acute and chronic wounds (Fig. 5A and B) with a prominent cluster of ribosomal protein-interactions evident. Of these 135 proteins, 20 were upregulated more than two-fold and 3 were downregulated ($>$ two-fold) in chronic wounds (Fig. 5C and Table 2). Functional grouping indicated that the dysregulated proteins of interest on day 2 were involved in either structural integrity (Actn3, Itga6, Epb41), the neurofilament network (Snca, Nefm), ribosomal function/transcriptional translation or protein modification

(Rsp18, Rsp19, Rpl22, Macroh2a1, Rpn1, Ppib, Snrnp70, Ddx5, Eif3g), cellular growth (Tpt1), metabolic regulation/lipid rafts (FABP5, Cavin1), co-factors/catalases/protease inhibitors (Stfa1, Stfa3, Cycs, Tkt), oxygen transport (Mb) or vestibular bodies (Chmp2a) (Table 2). On day 7 postwounding, 35 proteins were differentially expressed ($P < 0.05$) between acute and chronic wounds with only two upregulated and four downregulated more than two-fold (Fig. 5D, E and F). The functional grouping indicated that the dysregulated proteins of interest on day 7 were involved in either immune modulation (Cfd, Ptms, Hp), transcriptional regulation (Hmga1, Cbx3) or neural synapses (syap1). The relative expression levels, as well as the P -values for the identified proteins that were either upregulated or downregulated more than two-fold in the obese DM compared to acute wounds at the respective time points, are presented in Table 2.

Discussion

Wound repair is highly complex, comprising a series of coordinated and overlapping processes. Impaired healing is one of the most serious and debilitating complications of DM, yet current treatments are far from optimal (Sen *et al.* 2009). A better understanding of the complex nature of chronic DM wounds and the dysregulation that occurs at specific stages postwounding is crucial for the development of more effective treatments. A direct comparison of acute and obese DM wounds in the current study clearly illustrates that extensive dysregulation in the healing responses occur within the first 2 days postinjury suggesting that early intervention might be pivotal for effective healing outcomes and to potentially limit the reoccurrence of wounds. Taken together, the data from the current study is consistent with previous observations indicating that the chronic DM wounds have an inability to progress through the normal phases of healing into the proliferative stage (Landén *et al.* 2016). This was evident in both macroscopic and protein level. Histological analysis showed no signs of healing within the obese DM wounds even after 7 days, whereas complete wound closure/re-epithelisation, the formation of granulation tissue and signs of re-vascularisation was evident in acute wounds. During the early stage postinjury, deficiency in substance P expression and increased protease activity (MMP-9) within the wounded tissues coincided with excessive levels of chemokines and pro-inflammatory cytokines, known to attract phagocytic immune cells, in the wound

Figure 5

Proteome comparison of acute ($n=4$) and diabetic (DM) ($n=4$) wounds. (A and D) Heatmap indicating the maximum intensity projection of the 906 proteins detected at the respective time points. (B and E) Volcano plots indicating the differentially expressed proteins of interest ($P < 0.05$) as well as the number of proteins that were either up-regulated (green) or downregulated (red) more than two-fold in the DM vs acute wounds on day 2 (B) and day 7 (E), respectively. (C and F) Relative expression levels of the differentially expressed proteins of interest ($P < 0.05$; more than two-fold) identified on day 2 (B) and day 7 (D) postwounding. A full colour version of this figure is available at <https://doi.org/10.1530/JME-20-0256>.

fluid derived from DM wounds. This is consistent with observations from (Fadini *et al.* 2016) indicating that phagocytic neutrophil associated NETosis is involved in delayed DFU healing. A substantially larger number of dysregulated proteins were furthermore identified in the early stage (day 2) compared to the later stage of healing (day 7) confirming the importance of the initial response in predicting outcome. The proteomic comparison at different stages postwounding furthermore enabled the novel identification of proteins potentially involved in the dysregulated healing responses. These proteins and their known roles in either wound healing-associated processes or diabetes will be discussed based on functional grouping at either the initial (2 days) or late stages (7 days) postwounding.

Initial stages structural proteins (Itga6, Epb41, Actn3)

Integrins have an important function in cell–cell and cell–matrix adhesions and have been shown to play a crucial role in angiogenesis, matrix remodelling and epithelial/fibroblast/keratinocyte migration (Chen *et al.* 2001, Mercurio *et al.* 2001, Grose *et al.* 2002, Desai *et al.* 2013). The specific $\alpha 6$ subunit has been identified as a candidate gene in high-fat diet-induced DM (Kobayashi *et al.* 2014) and its expression is altered in various DM comorbidities such as retinopathy (Robbins *et al.* 1994), neuropathy (Guo *et al.* 2019) and nephropathy (Jin *et al.* 1996). Of specific relevance to wound healing is the role of integrin $\alpha 6$ in angiogenesis and fibroblast senescence (Jun & Lau 2010, 2017).

Table 2 Dysregulated proteins of interest in chronic wounds.

Gene	Protein	Accession #	Distinct peptides identified	Acute wounds (mean $\pm$ s.d.)	Chronic wounds (mean $\pm$ s.d.)	Log2 fold change	P-value
Day 2 postwounding							
Actn3	Alpha-actinin-3	sp O88990 ACTN3_MOUSE	68	1.73 $\pm$ 0.21	0.84 $\pm$ 0.11	-1.05	0.001
Cavin1	Caveolae-associated protein 1	sp O54724 CAVN1_MOUSE	46	0.40 $\pm$ 0.15	1.08 $\pm$ 0.36	1.42	0.026
Chmp2a	Charged multivesicular body protein 2a	sp Q9DB34 CHM2A_MOUSE	5	0.28 $\pm$ 0.03	0.97 $\pm$ 0.42	1.79	0.045
Cyts	Cytochrome c, somatic	sp P62897 CYC_MOUSE	15	2.23 $\pm$ 0.61	1.07 $\pm$ 0.23	-1.06	0.025
Ddx5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	sp Q61656 DDX5_MOUSE, tr Q8BTS0 Q8BTS0_MOUSE	6	0.37 $\pm$ 0.05	1.10 $\pm$ 0.39	1.57	0.032
Eif3g	Eukaryotic translation initiation factor 3 subunit G	sp Q9Z1D1 EIF3G_MOUSE	7	0.49 $\pm$ 0.20	1.06 $\pm$ 0.18	1.11	0.006
Epb41	Protein 4.1	sp P48193-2 41_MOUSE, sp P48193 41_MOUSE, tr A0A571BEG4 A0A571BEG4_MOUSE, tr A2A838 A2A838_MOUSE, tr A2A841 A2A841_MOUSE, tr A2A842 A2A842_MOUSE, tr F6S4K9 F6S4K9_MOUSE	7	0.46 $\pm$ 0.04	1.05 $\pm$ 0.32	1.17	0.036
Fabp5	Fatty acid-binding protein 5	sp Q05816 FABP5_MOUSE	9	0.46 $\pm$ 0.09	1.08 $\pm$ 0.17	1.23	0.002
Irga6	Integrin alpha-6	sp Q61739-2 ITA6_MOUSE, sp Q61739 ITA6_MOUSE, tr A0A5F8MPB5 A0A5F8MPB5_MOUSE	5	0.61 $\pm$ 0.24	1.32 $\pm$ 0.20	1.09	0.004
Macroh2a1	Core histone macro-H2A.1	sp Q9QZQ8-2 H2AY_MOUSE, sp Q9QZQ8 H2AY_MOUSE	10	0.53 $\pm$ 0.14	1.10 $\pm$ 0.19	1.05	0.004
Mb	Myoglobin	sp P04247 MYG_MOUSE	9	2.65 $\pm$ 0.81	1.23 $\pm$ 0.48	-1.11	0.029
Nefm	Neurofilament medium polypeptide	tr A0A0R4J036 A0A0R4J036_MOUSE	20	0.42 $\pm$ 0.10	1.05 $\pm$ 0.29	1.36	0.017
Pplb	Peptidyl-prolyl cis-trans isomerase B	sp P24369 PPIB_MOUSE	12	0.43 $\pm$ 0.07	0.99 $\pm$ 0.32	1.19	0.039
Rps18	40S ribosomal protein S18	sp P62270 RS18_MOUSE, tr A0A1Y7VKY1_MOUSE, tr F6VWP7 F6VWP7_MOUSE, tr S4R1N6 S4R1N6_MOUSE	10	0.43 $\pm$ 0.05	0.96 $\pm$ 0.28	1.14	0.031
Rps19	40S ribosomal protein S19	sp Q9CZX8 RS19_MOUSE	12	0.43 $\pm$ 0.08	1.01 $\pm$ 0.22	1.21	0.009
Rpl22	60S ribosomal protein L22	sp P67984 RL22_MOUSE	9	0.39 $\pm$ 0.19	1.01 $\pm$ 0.19	1.38	0.032
Rpn1	Dolichyl-diphospho-oligosaccharide-protein glycosyltransferase subunit 1	sp Q91YQ5 RPN1_MOUSE	14	0.48 $\pm$ 0.08	1.10 $\pm$ 0.19	1.19	0.004
Sncg	Gamma-synuclein	sp Q9Z0F7 SYUG_MOUSE	34	0.21 $\pm$ 0.02	1.07 $\pm$ 0.27	2.34	0.008
Snrrp70	U1 small nuclear ribonucleoprotein 70 kDa	sp Q62376 RUI7_MOUSE	9	0.46 $\pm$ 0.09	0.95 $\pm$ 0.29	1.06	0.036
Stfa1	Stefin-1	sp P35175 CYT1_MOUSE, tr A0A384DV91_MOUSE	10	0.36 $\pm$ 0.07	1.03 $\pm$ 0.36	1.52	0.031
Stfa3	Stefin-3	sp P35173 CYT3_MOUSE	6	0.42 $\pm$ 0.11	1.14 $\pm$ 0.43	1.43	0.041
Tpt1	Translationally-controlled tumor protein	sp P63028 TCTP_MOUSE, tr D3YU75 D3YU75_MOUSE	7	0.43 $\pm$ 0.07	1.04 $\pm$ 0.27	1.26	0.017
Tkt	Transketolase	sp P40142 TKT_MOUSE	37	0.50 $\pm$ 0.29	1.06 $\pm$ 0.19	1.09	0.021
Day 7 postwounding							
Cbx3	Chromobox protein homolog 3	sp P23198 CBX3_MOUSE, tr Q9DCC5_Q9DCC5_MOUSE, tr D3Z3T3 D3Z3T3_MOUSE	5	1.32 $\pm$ 0.34	0.62 $\pm$ 0.16	-1.08	0.019
Cfd	Complement factor D (Adipsin)	sp P03953-2 CFAD_MOUSE, sp P03953 CFAD_MOUSE	4	1.42 $\pm$ 0.35	0.46 $\pm$ 0.19	-1.63	0.006
Hmg1	High mobility group protein HMG-I/HMG-Y	tr A0A338P6F6 A0A338P6F6_MOUSE	4	1.66 $\pm$ 0.63	0.53 $\pm$ 0.15	-1.66	0.034
Hp	Haptoglobin	sp Q61646 HPT_MOUSE	16	0.63 $\pm$ 0.36	1.54 $\pm$ 0.48	1>29	0.025
Ptms	Parathymosin	sp Q9D0J8 PTMS_MOUSE	7	1.74 $\pm$ 0.39	0.81 $\pm$ 0.43	-1.09	0.019
Syap1	Synapse-associated protein 1	sp Q9D5V6 SYAP1_MOUSE	3	0.59 $\pm$ 0.19	1.36 $\pm$ 0.46	1.22	0.036

Jun and Lau (2017) demonstrated that the fibrosis associated gene, *Ccn2* induce fibroblast senescence through integrin $\alpha\beta 1$ -mediated accumulation of ROS that in turn activates p53 signalling. The senescent-associated secretory phenotype (SASP) in fibroblasts, in turn, upregulates the expression of MMPs and downregulate collagen production, which restricts fibrosis in normal cutaneous wound healing (Jun & Lau 2010), but excessive signalling could hamper regeneration as was evident in the DM wounds. During angiogenesis, integrin $\alpha 6$ heterodimerise with the $\beta 1/4$ subunit to regulate endothelial cell–matrix interactions. In functional angiogenesis, integrin $\alpha 6$ must be downregulated during the early stages but is essential for vessel maturation during the later stages and is therefore dynamically regulated (Desai *et al.* 2013). In the current study, integrin $\alpha 6$ was overexpressed during the initial stages postwounding and could potentially be implicated in the lack of revascularisation in DM wounds. Although cause–effect could not be established in the current study, the role of integrin $\alpha 6$ dysregulation in impaired healing is supported by the observations from Desai *et al.* (2013).

The two other structural proteins identified in this study include Protein 4.1 (Epb41) and alpha-actinin 3 (Actn3), neither of which have been studied in the context of either DM nor wound healing. Epb41 plays a role in intracellular signal transduction with very little additional data available. Actn3 mediates the interaction between cytoskeletal proteins and the sarcomeres (Z-line) within skeletal muscle (Hsu *et al.* 2018). It is known to affect skeletal muscle function and glycogen metabolism with deficiency linked to a loss of force generation and increased susceptibility to damage (Seto *et al.* 2011). This protein has been mainly studied in the context of sports performance and muscular pathologies, with only one study showing a link between Actn3 and insulin resistance (Mason *et al.* 2011). In chronic DM wounds (involving the underlying muscle layer), Actn3 is, therefore, most likely involved in cytoskeletal disorganisation and altered glycogen metabolism.

Initial stages neurofilament proteins (Sncg, Nefm)

Two proteins involved in the neurofilament network integrity and synaptic transmission, namely gamma synuclein (Sncg) and neurofilament medium polypeptide (Nefm) were differentially expressed in the acute and DM wounds in this study. Even though Sncg is considered a neuropeptide (peripheral neurons) it is also highly expressed in white adipose tissue (WAT), (Oort *et al.* 2008) and share functional links with leptin and

fatty acid-binding protein 4 (Oort *et al.* 2008, Bag *et al.* 2015, Dunn *et al.* 2015). Its prominent role in lipid metabolism was confirmed in a study indicating that Sncg knockout mice are protected against high-fat diet-induced metabolic complications (Millership *et al.* 2012). The increased levels of Sncg detected in the DM wounds in the current study is thus likely related to the abundance of WAT within the wound edges. The overexpression of Nefm in the DM wounds is, however, consistent with observations from a streptozotocin-induced DM rat model, in which the accumulation of the Nefh and Nefm subunits in proximal axons was suggested to be contributing factors in nerve degeneration leading to DM neuropathy (Sayers *et al.* 2003).

Initial stages proteins involved in ribosomal function, transcriptional translation or protein modification (Rsp18, Rsp19, Rpl22, Macroh2a1, Rpn1, Ppib, Snrnp70, Ddx5, Eif3g)

In the DM wounds, a large cluster of ribosomal-interacting proteins were differentially expressed, of which nine proteins (Rsp18, Rsp19, Rpl22, Macroh2a1, Rpn1, Ppib, Snrnp70, Ddx5, Eif3g) were upregulated more than two-fold on day 2 postwounding. Ribosomal proteins are RNA-binding proteins that function in ribosome biogenesis and protein translation by acting as RNA chaperones, stabilising rRNA and promoting the correct folding of transcribed proteins. These proteins also have extra-ribosomal functions associated with apoptosis, cell cycle arrest, proliferation and migration mediated by MDM2/p53 dependent and p53-independent mechanisms (Xu *et al.* 2016). Ribosomal proteins furthermore play an important role in the maintenance of genomic integrity by modulating the DNA damage repair response (Xu *et al.* 2016). Their function has been mainly studied in the context of tumorigenesis where it has been shown that either impairment or hyperactivation of ribosomal biogenesis is associated with deregulated cell growth (Barna *et al.* 2008, Xu *et al.* 2016). Patterns of dysregulated ribosomal proteins are also thought to be predictive of disease progression in cancer and has highly specific effects on mRNA translation (Guimaraes & Zavolan 2016). To our knowledge, this is the first study to identify dysregulated ribosomal proteins in chronic DM wounds and warrants further investigation, especially since the extra-ribosomal functions (i.e. DNA damage repair, proliferation, migration) are all processes known to be impaired in DM wounds.

Initial stages proteins involved in cellular growth (Tpt1)

Consistent with the overexpression of ribosomal proteins, the growth-associated protein Tpt1 was also overexpressed more than two-fold in the DM wounds. Tpt1 is a cytoprotective protein known to promote cellular division/growth and plays a role in the oxidative stress response to either promote DNA damage repair or induce autophagy (Bommer 2017). In the context of DM, Tpt1 have been implicated in DM-associated dementia (Matsuura *et al.* 2018), nephropathy (Tessari *et al.* 2007) and the development of auto-immune DM (Yan *et al.* 2008), confirming its role in the pathogenesis of DM. Even though it has not been studied in the context of DM wounds, other proteomic studies have identified Tpt1 as protein of interest in burn wounds (Pollins *et al.* 2007) and nerve regeneration (Jiménez *et al.* 2005), indicating that it might have a potential role in dysregulated healing responses.

In addition to the proteins discussed previously, this study also identified differentially expressed proteins involved in metabolic regulation/lipid rafts (FABP5, Cavin1), co-factors/catalases/protease inhibitors (Stfa1, Stfa3, Cycs, Tkt) and vestibular bodies (Chmp2a) during the initial stages postwounding. The role of these proteins in wound repair is unknown. It is, however, clear from the current data, that the protein dysregulation in DM wounds evident during the early stages (day 2) postwounding is complex and involve multiple regulatory pathways and biological processes.

Late stage proteins involved in immune modulation (Cfd, Ptms, Hp), transcriptional regulation (Hmga1, Cbx3) and neural synapses (Syap1)

In the later stages postwounding (day 7), fewer proteins were differentially expressed between acute and DM wounds compared to the initial stage. On day 7 postwounding, two immune modulatory proteins namely, complement factor D (Cfd) (also known as adipsin) and parathymosin (Ptms) were downregulated in DM wounds. Cfd is thought to be one of the links between energy metabolism and the innate immune response (White *et al.* 1992). It has a prominent role in lipid accumulation by mediating adipogenesis within WAT and simultaneously plays a role in the cleavage of C3 in the alternative immune complement pathway to induce inflammation (White *et al.* 1992, Song *et al.* 2016,

Vasilenko *et al.* 2017). Elevated serum Cfd levels have been detected in DM patients with retinopathy (Hase *et al.* 2017) and it has been implicated in age-associated deterioration of the dermal skin layer (Ezure *et al.* 2019). In the current study, Cfd was, however, downregulated in the DM wounds on day 7 postwounding. Similarly, Ptms known to inhibit the transcriptional activity of pro-inflammatory NFκB (Okamoto *et al.* 2016) was also downregulated in the DM wounds, whilst haptoglobin (Hp) which has anti-inflammatory properties (Krzyszczuk *et al.* 2020) was upregulated. Krzyszczuk *et al.* (2020) recently demonstrated that haptoglobin elicit strong anti-inflammatory effects (involving the downregulation of Hmga1 signalling) in LPS-stimulated macrophages *in vitro* and that Hp treatment of DM wounds (db/db mice) improved healing outcomes. Despite a similar profile (increased Hp and decreased Hmga1) being observed in the current study, there were no signs of healing in the DM wounds suggesting that cellular targets within the wounds might be unresponsive to signalling in this more severe wound model. The diverse regulation of different inflammatory mediators observed during the late stages of healing in this study further highlights the complexity of interactions between the underlying molecular processes in chronic wounds. In addition to these immune modulatory proteins of interest, Cbx3 and Syap1 was also diversely expressed in acute vs DM wounds on day 7, the role of these proteins in either DM or wound healing is, however, unknown.

Conclusion

This study highlighted the complex nature of obese DM wounds and suggest that early intervention immediately postinjury in high risk type 2 DM patients should be considered. It furthermore identified numerous proteins that play a potential role in the development of chronic wounds. Future studies with more in-depth investigation are, however, required to determine cause-and-effect. Validation of the proteomic findings using antibodies to target proteins of interest overtime during wound healing is required in future studies using either Western blotting or immunohistochemistry. A further limitation of the current study was not being able to distinguish between the contribution of obesity and hyperglycaemia and the results are therefore only applicable to obesity-associated type 2 diabetic patients. Given the complexity of dysregulation, therapeutic agents targeting a single pathway is unlikely to be successful (as have been evident to date with the lack of effective treatments). Biological

agents such as engineered mesenchymal stem cells or inflammatory cells (macrophages) that can 'sense' and restore the microenvironment through paracrine signalling and facilitate the transition into the proliferative phase of healing should be the focus of regenerative strategies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability

<http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD020134>

Author contribution statement

K B performed all the experimental procedures on the animals, sample analysis and wrote the first draft of the manuscript. M V assisted with sample preparation and performed the LC-MS/MS experiments. D L T did the quality control of mass spectrometry data and performed all the bioinformatic analysis. K H M contributed to the original study design and editing of the manuscript. M v d V contributed to the study design, data interpretation, statistical analysis and writing of the manuscript. All authors contributed to the editing of the final manuscript.

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