Published in final edited form as:

J Surg Res. 2011 May 15; 167(2): 336–342. doi:10.1016/j.jss.2009.09.012.

Gene Expression of Pro-Inflammatory Cytokines and Neuropeptides in Diabetic Wound Healing

Leena Pradhan, PhD¹, Xuemei Cai, BS¹, Szuhuei Wu, BS², Nicholas D Andersen, MD¹, Michelle Martin, MD¹, Junaid Malek, MD¹, Patrick Guthrie, BS¹, Aristidis Veves, MD, DSc², and Frank W LoGerfo, MD¹

¹Department of Surgery, Division of Vascular and Endovascular Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

²Department of Surgery, Microcirculation Laboratory, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

Abstract

The interaction between neuropeptides and cytokines and its role in cutaneous wound healing is becoming evident. The goal of the present study is to investigate the impact of diabetes on peripheral cytokine and neuropeptide expression and their role in diabetic wound healing. To achieve this goal, the effect of diabetes on wound healing along with the role of inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8) secreted in the wound microenvironment and neuropeptides, such as substance P (SP) and neuropeptide Y (NPY), secreted from peripheral nerves is monitored in non-diabetic and diabetic rabbits.

Rabbits in the diabetic group received alloxan monohydrate (100mg/kg i.v.). Ten days after diabetic induction, four full thickness circular wounds were created in both ears using a 6mm punch biopsy. Wound healing was monitored over ten days and gene expression of cytokines and neuropeptides was assessed in the wounds.

Compared to the non-diabetic rabbits, wounds of diabetic rabbits heal significantly slower. Diabetic rabbits show significantly increased baseline gene expression of IL-6 and IL-8, their receptors, CXCR1, CXCR2, GP-130 and a decrease of pre-pro Tachykinin-A (PP-TA), the precursor of SP whereas the expression of prepro-NPY (PP-NPY), the precursor of NPY is not different. Similarly, baseline protein expression of CXCR1 is higher in diabetic rabbit skin. Post-injury, the increase over baseline gene expression of IL-6, IL-8, CXCR1, CXCR2 and GP-130 is significantly less in diabetic wounds compared to non-diabetic wounds. Whereas, there is no difference in PP-TA gene expression between non-diabetic and diabetic rabbits post-injury, the gene expression of PP-NPY is reduced in diabetic rabbits.

In conclusion, diabetes causes dysregulation in the neuropeptide expression in the skin along with a suppressed focused inflammatory response to injury. This suggests that the chronic inflammation in the skin of diabetic rabbits inhibits the acute inflammation much needed for wound healing.

Diabetic foot ulceration (DFU) is a major problem that significantly impairs the quality of life of the patient, leads to prolonged hospitalization, and may require a major amputation. Pain insensitivity is the main feature of peripheral neuropathy that is associated with foot ulceration. Thus, diabetic patients are unaware of noxious stimuli or injury under their feet so they keep walking on the injured limb until and after an ulcer has appeared following which there is impaired wound healing which prevents ulcer resolution (1).

Normal wound healing can be divided into four overlapping phases: hemostasis, inflammation, proliferation and remodeling. Whereas acute wounds progress through the phases of wound healing linearly, chronic wounds become stalled in different phases at the same time and progression does not occur in synchrony (2). The first observations regarding the impact of diabetes on wound healing are focused on an impaired leukocyte function related to hyperglycemia that is associated with the aberrant expression and activity of inflammatory chemokines, cytokines and growth factors required for wound healing (3).

Tissue injury triggers an acute inflammatory response, involving neutrophils, monocytes/macrophages, and mast cells to the site of injury producing inflammatory cytokines and growth factors that coordinate wound repair (4, 5). Proper wound healing requires a sequential self-limited cytokine/immune cell interaction in order to achieve an adequate immune response, necessary for bacterial clearance and organized tissue breakdown and subsequent regeneration.

In the last decade it has been realized that numerous neuropeptides that are secreted by the small nerve fibers, both sensory and autonomic, play an important role during the inflammatory and proliferative phases of the wound healing. The receptors of these neuropeptides are present on various cells in the skin, including endothelial cells, mast cells, fibroblasts and keratinocytes (6). In addition, the skin is the target organ for numerous neuroendocrine, neurotrophic, neurotransmitter, and neuropeptide signals that are related to stress and underlie the development or flare of disorders like pruritis, atopic dermatitis, psoriasis and urticaria (7). The neuropeptides commonly involved in wound healing are Substance P (SP), Neurotensin (NT), and neuropeptide Y (NPY) whereas the roles for Neurotensin (NT) and the corticotropin releasing hormone (CRH) family of peptides are less well defined (8, 9). All these neuropeptides are known to regulate cytokine expression and/or function (10).

It is known that there is a chronic upregulation of pro-inflammatory cytokines in both Type I and Type II diabetes (11, 12). But it is often argued that the chronic systemic inflammation of diabetes may not translate into chronic peripheral inflammation. The goal of this study is to investigate the effect of diabetes on cytokine and neuropeptide expression and their role in diabetic wound healing. To achieve this goal, a rabbit model of diabetic wound healing is implemented. Peripheral expression of cytokines important in the wound healing pathways such as IL-8 and IL-6 is monitored along with the expression of their receptors such as CXCR1, CXCR2 and GP130. Also monitored are the gene expression levels of prepro-NPY (PP-NPY), the precursor of NPY and prepro-tachykinin A (PP-TA), the precursor of SP, the most commonly implicated neuropeptides in wound healing.

MATERIALS AND METHODS

22 New Zealand White rabbits weighing 3.0 – 3.2kg were obtained from Millbrook Farms (Amherst, MA) and were allowed to acclimate before dividing them into 2 treatment groups, non-diabetic and diabetic. Rabbits in the diabetic group received 2 doses of 50 mg/kg of alloxan monohydrate injection via the marginal ear vein 48 hours apart. Rabbits in the non-diabetic group received normal saline. Normal baseline blood glucose levels for rabbits varied between 100 mg/dl to 200 mg/dl. Rabbits with blood glucose over 250 mg/dl were considered diabetic. 10 days after diabetic induction, rabbits were anesthetized using Ketamine (25mg/kg I.M.) and Xylazine (3mg/kg I.M.). Four full thickness circular wounds were created in both ears using a 6-mm punch biopsy. The cartilage was kept intact to stent the wounds open, and minimize tissue contracture to less than 3%, allowing the wound to heal by new tissue formation. 10 days after wound creation, rabbits were euthanized and wound tissue was collected for further analysis. The Harvard Medical Area Standing Committee on Animals approved all procedures employed in this investigation.

Wound area measurement

Wound healing was monitored over a 10 day period. Wounds were photographed using Medical Hyperspectral Imaging camera (Hypermed Inc. Burlington MA). To ensure consistent high quality of the images, rabbits were sedated with Acepromazine (0.5mg/kg I.M.) prior to acquiring the images. Using the magic wand available in the Adobe Photoshop™ software, wound circumference was outlined and the wound area was measured in pixels. Data is presented as both, wound area in pixels and change in wound area over the 10 day period.

Tissue Analysis

After euthanizing the rabbits, 1 cm × 1 cm sections that included the wound were cut from the rabbit ear. Each wound was further divided into different sections for morphologic and gene expression analysis. For morphologic analysis, tissue was fixed in formalin and for gene expression analysis tissue was snap frozen in liquid nitrogen.

Gene Expression

Quantitative real-time RT-PCR (qRT-PCR) was used to quantitate and compare levels of specific RNA transcripts between RNA samples from non-diabetic and diabetic rabbit skin biopsies and wounds. Total RNAs were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was prepared from 1µg total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) prior to thermal cycling using a Stratagene MX3000P qRT-PCR machine (Stratagene, La Jolla, CA). For quantitative analysis, target gene levels were normalized to beta-actin levels and target and beta-actin gene amplification reactions were performed using Brilliant SYBR Green QPCR Master Mix Reagent (Stratagene, La Jolla, CA) in triplicate for each cDNA sample using 1µl cDNA per reaction. Primer sequences (Table 1) were obtained from Integrated DNA Technologies (Coralville, IA). Thermal cycling was performed under the following conditions: Stage 1: 10 minutes at 95°C; Stage 2 (40 cycles): 30 seconds at 95°C, 1 minute at 60°C, 30 seconds at 72°C. Gene expression was

measured in both non-diabetic and diabetic animals at baseline in the skin biopsies and in the wound sample as a percent change over the baseline gene expression.

Morphologic Analysis and Immunohistochemistry

Tissue samples were fixed in formalin and embedded in paraffin. Six micrometer sections were cut and stained with hematoxylin and eosin. Inflammatory cells were counted in each sample by a blinded observer. For immunohistochemistry (IHC), 6 μ m sections were deparaffinized in xylene, and rehydrated. Sections were treated with 3% hydrogen peroxide. Non-serum protein blocking was followed by incubation over-night with CXCR1 primary antibody (20 μ g/ml) obtained from R & D Systems (Cat#MAB330). Sections were incubated with biotinylated secondary antibody, streptavidin-peroxidase, and DAB (3,3'-diaminobenzidine)-chromagen, (Vectastain Mouse Kit, Vectorlabs, CA) then counterstained with hematoxylin. Negative controls were used. An arbitrary score on a scale of 1–4 was assigned to each section by a blinded observer.

Statistics

All data are expressed as the mean $\pm$ SD and were analyzed using ANOVA and Bonferroni's PostHoc test on Statview (Abacus Concepts, Inc., Berkeley, Calif.), with $p < 0.05$ considered statistically significant.

RESULTS

Wound Healing

Diabetic rabbits have a significantly reduced rate of healing compared to non-diabetic rabbits ($P < 0.01$) both at day 5 and day 10 (Fig. IA and IB).

Morphologic Analysis

Baseline skin inflammatory cell infiltration is similar in non-diabetic and diabetic rabbits. Post-injury, the infiltration is significantly higher in non-diabetic wounds compared to diabetic wounds (Fig. IIA and IIB).

Gene Expression

Baseline gene expression of IL-8, its receptors CXCR1 and CXCR2, IL-6, its receptor GP130 is significantly higher ($P < 0.01$) in the skin of diabetic rabbits compared to the non-diabetic rabbits. Baseline gene expression of PP-TA the precursor of SP is significantly reduced ($P < 0.05$) whereas that of PP-NPY, the precursor of NPY is not different between non-diabetic and diabetic rabbit ear skin (Fig. III).

Following injury the change over baseline gene expression of IL-8, CXCR1, CXCR2, IL-6 and GP130 is higher in the non-diabetic wounds compared to the diabetic wounds ($P < 0.05$). Although at baseline PP-TA is reduced in diabetic rabbits, post-injury, there is no difference in the change over baseline of gene expression of PP-TA in the non-diabetic and diabetic rabbits. Whereas, the PP-NPY gene expression is similar at baseline between non-diabetic and diabetic rabbits, there is significantly reduced gene expression over baseline of PP-NPY in the diabetic wounds compared to the baseline wounds (Fig. IV).

Protein Expression

Baseline protein expression of IL-8 receptor, CXCR1 is significantly higher in diabetic compared to non-diabetic skin whereas post-injury there is no difference in expression between non-diabetic and diabetic wound tissue (Fig. V). In both, non-diabetic and diabetic wound tissue the change over baseline protein expression did not achieve significance.

DISCUSSION

This study is first to demonstrate that in diabetic wound healing, the focused inflammatory cytokine response to injury is significantly reduced. Whereas, the diabetic rabbit ear skin, prior to injury has significantly higher expression of cytokines and their receptors, post-injury, the much needed acute inflammatory response is significantly blunted compared to the non-diabetic rabbits. This suggests that the chronic inflammatory condition of diabetes hinders the focused acute response needed for wound healing. In the present study, wound healing is significantly impaired in the diabetic rabbits and this is associated with a dysregulation in cytokine and neuropeptide gene expression. Leukocyte infiltration is similar at baseline whereas post-injury, there is more infiltration in non-diabetic wounds compared to diabetic wounds. Cytokines, IL-8 and IL-6 along with their receptors CXCR1, CXCR2 and GP130 are significantly higher at baseline in diabetic rabbit skin but post-injury their expression does not change over the baseline whereas in non-diabetic rabbits, there is a significant upregulation of these cytokines. Similar to the gene expression, the protein expression of atleast one of the cytokine receptors, CXCR1, is significantly elevated at baseline in diabetic skin whereas post-injury there is no difference between the non-diabetic and diabetic wound samples. Although the change over baseline protein expression of CXCR1 in non-diabetic wounds did not achieve significance, the trend is similar to gene expression. Future immunolocalization studies will be needed to determine the cellular sources of both, the cytokines and their receptors especially since the number of inflammatory cells is not significantly different at baseline between the skin of the non-diabetic and diabetic rabbits.

SP levels are significantly lower in diabetic rabbit ear skin at baseline and post-injury the gene expression in both the non-diabetic and diabetic wounds is similar suggesting injury itself reduces SP expression. On the other hand, NPY expression is not different at baseline between non-diabetic and diabetic rabbit ear skin but post-injury, there is a reduction in NPY expression in the diabetic wounds compared to the non-diabetic wounds.

In Type I and Type II diabetes, hyperglycemia and insulin resistance have been shown to be associated with a state of chronic low-grade inflammation (11, 12). The source of these cytokines is not limited to immune cells as these cytokines are produced by other cell phenotypes in the skin including keratinocytes and microvascular ECs (13, 14). Infact, in diabetes, the immune cell infiltration has shown to be reduced and immune cells are shown to be dysfunctional (15, 16). Thus, it is highly likely, that in our study, the source of higher expression of baseline cytokine gene expression might be from non-immune cells. In addition, the post-injury blunted cytokine response could be due to either insufficient immune cell infiltration in the wound area or infiltration with dysfunctional immune cells (10).

Neuropeptides, including SP and NPY are cutaneous neuroimmunomodulators that can exert their effect in the skin by modulating cytokine response and thereby affecting the outcome of wound healing (10). In the periphery, SP is found in C-fiber sensory neurons whereas NPY is found in the sympathetic nerves, where it is stored either alone in small vesicles or in large vesicles in combination with catecholamines. In both, animal and clinical studies, diabetes is shown to reduce SP nerves and exogenous treatment of wounds with SP shortened wound closure time (17, 18). Similar to SP, NPY levels are shown to be reduced in patients with Type I diabetes and in animal models of diabetes (19, 20). The present study clearly indicates that the expression of both SP and NPY is significantly affected by diabetes, but the underlying mechanisms are unclear. Advanced Glycation End products along with hyperglycemia caused by diabetes have been suggested as possible mechanisms that can cause nerve damage (21, 22).

This preliminary report is the first to detect that the focused inflammatory response to skin injury in diabetes is impaired in spite of a high baseline level of chronic inflammation. Since neuropeptides are known to affect inflammatory pathways, future mechanistic studies are warranted to investigate the relationship between cytokines and neuropeptides in diabetic wound healing.

Acknowledgments

This work part is supported by the Juvenile Diabetes Research Foundation (JDRF#5-2005-1006) to AV, the William J. von Liebig Foundation and the NIH T32-HL007734 to FWL.

References

1. Dinh TL, Veves A. A review of the mechanisms implicated in the pathogenesis of the diabetic foot. *Int J Low Extrem Wounds*. 2005; 4:154–159. [PubMed: 1610096]
2. Falanga V. Wound healing and its impairment in the diabetic foot. *Lancet*. 2005; 366:1736–1743. [PubMed: 16291068]
3. Bagdade JD, Root RK, Bulger RJ. Impaired leukocyte function in patients with poorly controlled diabetes. *Diabetes*. 1974; 23:9–15. [PubMed: 4809622]
4. Engelhardt E, Toksoy A, Goebeler M, Debus S, Bocker EB, Gillitzer R. Chemokines IL-8, GROalpha, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am J Pathol*. 1998; 153:1849–1860. [PubMed: 9846975]
5. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C. Macrophages and angiogenesis. *J Leukoc Biol*. 1994; 55:410–422. [PubMed: 7509844]
6. Roosterman D, Goerge T, Schneider SW, Bunnett NW, Steinhoff M. Neuronal control of skin function: the skin as a neuroimmunoendocrine organ. *Physiol Rev*. 2006; 86:1309–1379. [PubMed: 17015491]
7. Arck PC, Slominski A, Theoharides TC, Peters EM, Paus R. Neuroimmunology of stress: skin takes center stage. *J Invest Dermatol*. 2006; 126:1697–1704. [PubMed: 16845409]
8. Nakamura M, Kawahara M, Morishige N, Chikama T, Nakata K, Nishida T. Promotion of corneal epithelial wound healing in diabetic rats by the combination of a substance P-derived peptide (FGLM-NH2) and insulin-like growth factor-1. *Diabetologia*. 2003; 46:839–842. [PubMed: 12764579]
9. Movafagh S, Hobson JP, Spiegel S, Kleinman HK, Zukowska Z. Neuropeptide Y induces migration, proliferation, and tube formation of endothelial cells bimodally via Y1, Y2, and Y5 receptors. *Faseb J*. 2006; 20:1924–1926. [PubMed: 16891622]

10. Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A. Inflammation and neuropeptides: the connection in diabetic wound healing. *Expert Rev Mol Med*. 2009; 11:e2. [PubMed: 19138453]
11. Tilg H, Moschen AR. Inflammatory mechanisms in the regulation of insulin resistance. *Mol Med*. 2008; 14:222–231. [PubMed: 18235842]
12. Rosa JS, Flores RL, Oliver SR, Pontello AM, Zaldivar FP, Galassetti PR. Sustained IL-1alpha, IL-4, and IL-6 elevations following correction of hyperglycemia in children with type 1 diabetes mellitus. *Pediatr Diabetes*. 2008; 9:9–16. [PubMed: 18211631]
13. Laine PS, Schwartz EA, Wang Y, Zhang WY, Karnik SK, Musi N, Reaven PD. Palmitic acid induces IP-10 expression in human macrophages via NF-kappaB activation. *Biochem Biophys Res Commun*. 2007; 358:150–155. [PubMed: 17467667]
14. Ochoa O, Torres FM, Shireman PK. Chemokines and diabetic wound healing. *Vascular*. 2007; 15:350–355. [PubMed: 18053419]
15. Hatanaka E, Monteagudo PT, Marrocos MS, Campa A. Neutrophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. *Clin Exp Immunol*. 2006; 146:443–447. [PubMed: 17100763]
16. Stegenga ME, van der Crabben SN, Dessing MC, Pater JM, van den Pangaart PS, de Vos AF, Tanck MW, Roos D, Sauerwein HP, van der Poll T. Effect of acute hyperglycaemia and/or hyperinsulinaemia on proinflammatory gene expression, cytokine production and neutrophil function in humans. *Diabet Med*. 2008; 25:157–164. [PubMed: 18290856]
17. Gibran NS, Jang YC, Isik FF, Greenhalgh DG, Muffley LA, Underwood RA, Usui ML, Larsen J, Smith DG, Bunnett N, Ansel JC, Olerud JE. Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. *J Surg Res*. 2002; 108:122–128. [PubMed: 12443724]
18. Galkowska H, Olszewski WL, Wojewodzka U, Rosinski G, Karnafel W. Neurogenic factors in the impaired healing of diabetic foot ulcers. *J Surg Res*. 2006; 134:252–258. [PubMed: 16580687]
19. Ahlborg G, Lundberg JM. Exercise-induced changes in neuropeptide Y, noradrenaline and endothelin-1 levels in young people with type I diabetes. *Clin Physiol*. 1996; 16:645–655. [PubMed: 8937803]
20. Kuncova J, Svirglerova J, Tonar Z, Slavikova J. Heterogenous changes in neuropeptide Y, norepinephrine and epinephrine concentrations in the hearts of diabetic rats. *Auton Neurosci*. 2005; 121:7–15. [PubMed: 15955747]
21. Cellek S, Qu W, Schmidt AM, Moncada S. Synergistic action of advanced glycation end products and endogenous nitric oxide leads to neuronal apoptosis in vitro: a new insight into selective nitroergic neuropathy in diabetes. *Diabetologia*. 2004; 47:331–339. [PubMed: 14676945]
22. Cellek S, Foxwell NA, Moncada S. Two phases of nitroergic neuropathy in streptozotocin-induced diabetic rats. *Diabetes*. 2003; 52:2353–2362. [PubMed: 12941776]

A:

B:

Fig. I. Wound healing

A: Representative picture of non-diabetic and diabetic wounds.

Day 0 is before injury and days 2, 5 & 10, are post-injury. Arrows indicate healed wounds in non-diabetic rabbits.

B: Quantification of wound healing.

Data is expressed as (a): Wound area in pixels and (b): percent change in wound area pixels of non-diabetic and diabetic rabbits from days 2 to 10 (n=10, P<0.05). (Cross marks represent non-diabetic rabbits and black circles represent diabetic rabbits). Data are expressed as mean $\pm$ SD

A:

B:

Fig. II. Morphologic analysis of skin and wound tissue

A: Representative H&E staining picture of (a): Non-diabetic skin, (b): Diabetic skin, (c): Non-diabetic wound and (d): Diabetic wound tissue (Mag 10×).

B: Quantification of inflammatory cell infiltration of the skin and wound tissue of non-diabetic and diabetic rabbits. (Cross bars represent non-diabetic rabbits and black bars represent diabetic rabbits). Data are expressed as mean $\pm$ SD.

Fig. III. Baseline gene expression

Fold change in baseline gene expression of IL-6, IL-8, CXCR1, CXCR2, GP130, SP and NPY in diabetic skin compared to non-diabetic skin. (n=8, P<0.05). (Black bars represent diabetic rabbits). Data are expressed as mean±SD

Fig. IV. Post-injury gene expression

Post-injury change of IL-6, IL-8, CXCR1, CXCR2, GP130, SP and NPY gene expression over the baseline in non-diabetic and diabetic rabbit wounds (n=8, P<0.05). (Cross bars represent non-diabetic rabbits and black bars represent diabetic rabbits). Data are expressed as mean±SD.

A:

B:

Fig. V. CXCR1 protein expression

A: Representative CXCR1 immunohistochemistry images of (a): Non-diabetic skin (b): Diabetic skin (c): Non-diabetic wound (d): Diabetic wound tissue (Mag. 10×).

B: Quantification of CXCR1 protein expression using a arbitrary score (1 to 4). (Cross bars represent non-diabetic and black bars represent diabetic tissue samples). Data are expressed as mean $\pm$ SD.

Table 1

Q-RT-PCR primer sequences

Gene	Forward	Reverse
IL-6	5'-GTC AGC CTG ATG GAG AAC CT-3'	5'-GGA TGA AGT GGA TCG TGG TC-3'
IL-8	5'-CTC TGC TGG CTG CCC TAC-3'	5'-CTG ACA CGT CTC CTG GAT CA-3'
CXCR1	5'-GGC GCT GTC TCT GAT TTT GT-3'	5'-GGC TGG AAT TGT TTG GAG AA-3'
CXCR2	5'-AGC ATG TGG GGA GTG TCT TT-3'	5'-GGA AGA TGG CAT TAC GGA AC-3'
GP130	5'-TGG CCT AAT GTT CCA GAT CC-3'	5'-AAT TGT GCC TTG GAG GAG TG-3'
PP-TA (SP)	5'-TGT GTC TCA GGG CTG AAA TG-3'	5'-TAT GGA ACC ACA AAC CGT GA-3'
PP-NPY	5'-CCA GCC CAG AGA CAC TGA TT-3'	5'-ACA TTG CAG GGT CTT CAA GC-3'