The Dipeptidyl Peptidase-4 Inhibitor Linagliptin Attenuates Inflammation and Accelerates Epithelialization in Wounds of Diabetic ob/ob Mice

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Linagliptin Accelerates Epithelialization in Mice

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Abbreviations

Cox-2 Cyclooxygenase-2
DPP-4 Dipeptidyl peptidase-4
ECL Enhanced chemiluminescence
eIF2 Eucaryotic initiation factor-2
GAPDH Glyceraldehyde phosphate dehydrogenase
GIP glucose-dependent insulinotropic polypeptide
GLP-1 Glucagon-like peptide-1
Mφ Macrophages
MIP-2 Macrophage inflammatory protein-2
nt Nucleotides
PMN Polymorphonuclear neutrophils
SDF Stromal-derived factor
VEGF Vascular endothelial growth factor

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ABSTRACT

In recent years, new and effective therapeutic agents for blood glucose control have been added to standard diabetes therapies—dipeptidyl peptidase (DPP)-4 inhibitors which prolong the bioavailability of the endogenously secreted incretin hormone glucagon-like peptide-1 (GLP-1). Full-thickness excisional wounding was performed in wildtype (C57BL/6J) and diabetic (C57BL/6J-\textit{ob/ob}) mice. DPP-4 activity was inhibited by oral administration of linagliptin during healing. Wound tissue was analyzed using histological, molecular, and biochemical techniques. In healthy mice, DPP-4 was constitutively expressed in keratinocytes of non-wounded skin. Following skin injury, DPP-4 expression declined and was lowest during the most active phase of tissue re-assembly. In contrast, in \textit{ob/ob} mice, we observed increasing levels of DPP-4 at late time points, when delayed tissue repair still occurs. Oral administration of the DPP-4 inhibitor linagliptin strongly reduced DPP-4 activity and stabilized active GLP-1 in chronic wounds, and improved healing in \textit{ob/ob} mice. At day 10 post-wounding, linagliptin-treated \textit{ob/ob} mice showed largely epithelialized wounds characterized by the absence of neutrophils. In addition, DPP-4 inhibition reduced the expression of the proinflammatory markers cyclooxygenase-2 and macrophage inflammatory protein-2, but enhanced the formation of myofibroblasts in healing wounds from \textit{ob/ob} mice. Our data suggest a potentially beneficial role of DPP-4 inhibition in diabetes-affected wound healing.
Introduction

In 1887, the British surgeon T Davis Pryce recognized for the first time that “diabetes itself may play an active part in the causation of perforating ulcers” (Pryce, 1887). Yet despite more than a century of research, few pharmacologic approaches enhance the healing of diabetes-related wounds. Diabetes-related wounds continue to impose considerable clinical, societal, and economic burdens. The annual incidence of diabetic foot ulcers ranges from 1% to 4% suggesting that up to 25% of people with diabetes will develop foot ulcers sometime during their lives (Setacci et al., 2009). Moreover, 3- to 5-year mortality rates in diabetic patients may reach 74% among those that undergo lower-extremity amputation. To put this into context, the prognosis for people with diabetic ulcers is worse than that associated with several malignancies including prostate, breast and colon cancers, and Hodgkin’s disease (Robbins et al., 2008).

Recent research has identified numerous changes in diabetic wounds that contribute to a delay in healing: markedly abnormal patterns of growth factor, cytokine, chemokine, enzyme or protease expression and activity are associated with impaired tissue repair (Blakytny and Jude, 2006; Eming et al., 2007). Moreover, an observed dysfunctional insulin signaling, the primary abnormality in diabetes, may further compromise the disordered and disturbed wound healing as shown in obese/obese (ob/ob) mice. Activated insulin receptors were virtually absent in impaired wounds from these severely diabetic animals (Goren et al., 2006). On the other hand, monoclonal antibodies against tumor necrosis factor (TNF)-α enhance diabetic wound healing, in part by influencing insulin signaling (Goren et al., 2006). TNF-α promotes insulin resistance (Nieto-Vazquez et al., 2008) representing another link between the clinical hallmarks of diabetes and impaired wound healing. In addition, insulin enhances the release of vascular endothelial growth factor (VEGF) from keratinocytes in skin wounds (Goren et al., 2009b). Targeting insulin release and resistance (two common therapeutic strategies employed by antihyperglycemic drugs) may therefore modulate impaired wound healing conditions.
Antihyperglycemic drugs may also alter patterns of wound cytokine and chemokine expression (Schiefelbein et al., 2008; Fadini et al., 2010), which could partly determine wound healing rates.

Classical pharmacologic therapies to improve diabetes-associated insulin resistance involve the administration of sulfonlyureas, thiazolidinediones, glinides, metformin, or insulin. Within the past few years, the incretin hormone glucagon-like peptide (GLP)-1 has been the molecular target for developing novel antidiabetic drugs: the GLP-1 receptor agonists and the dipeptidyl peptidase (DPP)-4 inhibitors. GLP-1 is rapidly cleaved by DPP-4, leading to inactivation and abrogation of its insulinotropic activity (Deacon et al., 1995; Pauly et al., 1996). As a consequence, inhibitors of DPP-4 activity maintain the biological activity of GLP-1 and exert glucose-lowering effects in type 2 diabetes mellitus (Pratley and Salsali, 2007). The effect of new treatments for diabetes on tissue regeneration may be of interest, although this aspect of antihyperglycemic pharmacology is relatively neglected. However, pharmacologic activation of GLP-1 receptors has been shown to exert an anti-inflammatory effect on skin disease (Drucker and Rosen, 2011; Hogan et al., 2011).

According to the above-mentioned classical antidiabetic pharmacologic strategies, very limited data characterize the effect of DPP-4 inhibitors on wound healing. We therefore assessed the effect of linagliptin, a DPP-4 inhibitor with a unique xanthine-based structure (Thomas et al., 2008), on the regulation of DPP-4 and wound healing in the ob/ob mouse, a well-established animal model for studying type 2 diabetes.
Materials and Methods

**Animals.** Female C57BL/6J and C57BL/6J-*ob/ob* mice were obtained from Charles River Wiga (Sulzfeld, Germany) and maintained under a 12 h light/12 h dark cycle at 22°C. At the age of 12 weeks, animals were caged individually, monitored for body weight, and wounded as described below.

**Wounding of Mice.** Wounding of mice was performed as described previously (Frank et al., 1999). Briefly, mice were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg body weight)/xylazine (10 mg/kg body weight). Six full-thickness wounds (5 mm in diameter, 3 to 4 mm apart) were made on the back of each mouse. Skin biopsy specimens were obtained from the animals at day 10 after injury. As a control, a similar amount of skin was taken from the backs of non-wounded mice. All animal experiments were performed according to the guidelines and approval of the local Ethics Animal Review Board.

**Treatment of Mice.** Each experimental group (vehicle or linagliptin treatment) consisted of 10 individual *ob/ob* mice (*n* = 10). Animals were treated orally once a day (8:00 AM) by gastrogavage using vehicle (1% methylcellulose) or linagliptin (3 mg/kg body weight in 1% methylcellulose) beginning 2 days (day –2) before wounding. After wounding, animals were subsequently treated once a day throughout the 10-day healing period.

**Oral Glucose Tolerance Test (OGTT).** *ob/ob* mice were analyzed after 12 days of vehicle or linagliptin treatment. They were starved for 16 h and subsequently administered glucose orally (1.5 g/kg body weight) by gastrogavage. Blood glucose levels were determined before and 20, 60, 90, 120, and 180 min following glucose application.

**DPP-4 Activity and GLP-1 Assay.** EDTA plasma (20 µl) was diluted with 30 µl of DPP-4 assay buffer (100 mmol/l Tris, 100 mmol/l NaCl, adjusted to pH 7.8 with HCl) and mixed with 50 µl H-Ala-Pro-7-amido-4-trifluoromethylcoumarin (Bachem, Bubendorf, Switzerland). The 200 mmol/l stock solution in dimethylformamide was diluted 1:1000 with water to yield a final concentration of 100 µmol/l. The plate was incubated at room temperature for 10 min and
fluorescence in the wells determined using a Victor™ 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA) at an excitation wavelength of 405 nm and an emission wavelength of 535 nm. For detection of DPP-4 activity in wound lysates, 100 µg of protein from the respective wound lysates were used instead of 20 µl of plasma. Active GLP-1 was also detected from 100 µg of respective wound tissue samples and analyzed using the Mouse/Rat Total Active GLP-1 Assay Kit (Meso Scale Discovery, Gaithersburg, MD, USA).

**RNA Isolation and RNase Protection Analysis.** RNA isolation from wounds and RNase protection assays were performed as described previously (Chomczynski and Sacchi, 1987). All samples were quantified using phosphorimager photostimulated luminescence counts per 15 µg of total wound RNA and normalized for glyceraldehyde phosphate dehydrogenase (GAPDH)-specific mRNA hybridization. The murine cDNA probes were cloned using reverse transcriptase-polymerase chain reaction and corresponded to nucleotides (nt) 796 to 1063 (for cyclooxygenase-2 [Cox-2], GeneBank™ accession number M64291), nt 181 to 451 (for macrophage inflammatory protein-2 [MIP-2], NM009140) and nt 163 to 317 (for GAPDH, NM002046) of the published sequences.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).** Predesigned primers to assess gene expression in wounds were purchased from Applied Biosystems (Darmstadt, Germany): Mm00478374_m1 (for Cox-2), Mm00436450_m1 (for MIP-2), Mm00475988_m1 (for arginase-1) and Mm00657889_mH (for Ym1). qRT-PCR was performed as described previously (Schürmann et al., 2009).

**Immunohistochemistry.** Biopsies of non-wounded tail skin and 10-day back skin wounds were isolated, fixed in formalin, and subsequently embedded in paraffin. Six-micrometer sections were counterstained either with hematoxylin or subsequently incubated overnight at 4°C with antisera raised against DPP-4 (Abcam, Cambridge, MA, USA), Ly-6G (Gr-1), F4/80 (AbD Serotec, Düsseldorf, Germany), processed active caspase-3 (DCS Inc., Hamburg, Germany), or α-smooth muscle actin (α-SMA; Sigma, Taufkirchen, Germany). Specificity of
staining was controlled for all primary antibodies by single incubation with the detection antibody alone. The slides were subsequently stained with the avidin-biotin-peroxidase complex system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using 3,3-diaminobenzidine-tetrahydrochloride or with the Fast Red Substrate-Chromogen System (Dako, Hamburg, Germany) as chromogenic substrates.

**Preparation of Protein Lysates and Western Blot Analysis.** Skin tissue biopies and cultured cells were homogenized in lysis buffer (1% Triton X-100, 20 mM Tris/HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 10 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonylfluoride, 5 ng/ml aprotinin, 5 ng/ml leupeptin). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Inc., Rockford, IL, USA). Fifty micrograms of protein lysates were separated using SDS gel electrophoresis. Specific proteins were detected using antisera raised against Ly-6G (Gr-1), F4/80 (AbD Serotec), Cox-2 (Cayman, Ann Arbor, MI, USA), eukaryotic initiation factor (eIF)-2 and phospho-eIF-2 (p-eIF-2) or eIF-4E binding protein (4E-BP1) and phospho-4E-BP1 (Cell Signaling, Frankfurt, Germany). A secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system were used to visualize the proteins. Quantification of developed immunoblots was carried out using the ‘Quantity One’ Software (Biorad, München, Germany). Phenylmethylsulfonylfluoride, dithiothreitol, aprotinin, NaF and Na<sub>3</sub>VO<sub>4</sub> were from Sigma (Taufkirchen, Germany). Leupeptin and ocdaadic acid were from BioTrend (Köln, Germany). The ECL detection system was obtained from Amersham (Freiburg, Germany).

**Enzyme-Linked Immunosorbent Assay (ELISA).** Quantification of murine MIP-2, DPP-4 or glucose-dependent insulinotropic polypeptide (GIP) protein was performed using the respective murine ELISA kits (for MIP-2 and DPP4: R&D Systems, Wiesbaden, Germany and for GIP: Millipore, Billerica, Massachusetts, USA) according to the manufacturer’s instructions.
Keratinocyte Cell Culture. HaCaT keratinocytes were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FCS), 2 mmol/l L-glutamine, and 1% penicillin/streptomycin (GIBCO/Life Technologies Inc., Karlsruhe, Germany). Confluent cells were starved for 24 h using DMEM and subsequently treated using epithelial growth factor (EGF; 10 ng/ml) in the presence or absence of linagliptin (10 nM), GLP-1 (10 nM), or exendin-4 (10 nM) for 8 h.

Keratinocyte Proliferation Assay. 4.0 x 10^7 keratinocytes per well were seeded into 24-well plates. After reaching 50% confluency, cells were starved for 24 h with DMEM. Proliferation of cells was assessed using 1 µCi/ml of [3H]-methyl-thymidine in DMEM in the presence of 10% FCS and increasing concentrations of linagliptin (3, 30, 300, or 600 nM) for 24 h. Cells were then washed twice with PBS, incubated in 5% trichloroacetic acid at 4°C for 30 min, and the DNA was solubilized in 0.5 M NaOH for 30 min at 37°C. Finally, [3H]-thymidine incorporation was determined.

Statistical Analysis. Data are shown as means ± SEM as indicated. Data analysis was carried out using the unpaired student’s t-test on raw data.
Results

Expression of DPP-4 in Non-Wounded Skin and Wound Tissue. First, we investigated the expression of DPP-4 in murine skin tissue. Non-wounded skin of healthy (C57BL/6J) as well as diabetic (C57BL/6J-ob/ob) animals expressed DPP-4 protein (Fig. 1b [Control skin]). We performed an immunohistochemical (IHC) staining against DPP-4 protein in skin tissue sections using a DPP-4-specific antibody. We did so to assess the cellular sources of the observed prominent DPP-4 expression in non-wounded skin (Fig. 1b [Control skin]). Here, the bay-colored signals in sections determined epidermal keratinocytes and dermal fibroblasts as the DPP-4 expressing cell types in non-wounded skin (Fig. 1a). Upon wounding, DPP-4 protein expression in healthy mice was high on day 1 but decreased to a minimum level during the acute repair phase (days 3 and 5 post-wounding; Fig. 1b, left panel). Importantly, the high DPP-4 levels in 1-day wounds of healthy mice (Fig. 1b, left panel) suggested that low DPP-4 levels in acute wound tissue were not simply due to tissue loss. DPP-4 protein reappeared when wound tissue repair progressed beyond the acute and inflammatory phase (Figure 1b, left panels; Fig. 1c). By contrast, DPP-4 protein expression in diabetic ob/ob mice was not detectable in early wounds (days 1 to 5) but appeared from day 7 post-wounding (Fig. 1b, right panel; Fig. 1c). This pattern of DPP-4 protein expression suggests that healing conditions in 7-day wound tissue in healthy and diabetic ob/ob mice were different, although the observed pattern of DPP-4 expression seen at day 7 (Fig. 1b) appeared to be similar. The observed appearance of DPP-4 protein expression at day 7 in ob/ob mice (Fig. 1b, right panel) clearly paralleled a persistent and ongoing wound inflammatory process that has been described in earlier studies for the animals at that time point (Goren et al., 2003; Seitz et al., 2010). Therefore, the observed comparable patterns of wound DPP-4 expression (Fig. 1b) were associated with different wound conditions in normal and diabetic ob/ob mice. Re-increasing DPP-4 levels in healthy C57BL/6J mice (Fig. 1b, left panel; Fig. 1c) encountered conditions of a resolved wound inflammation (Wetzler et al., 2000; Goren et al., 2003; Seitz et al., 2010),
whereas DPP-4 levels re-increased in ob/ob mice (Fig. 1b, right panel; Fig. 1c) in the presence of augmented wound inflammatory conditions (Goren et al., 2003; Seitz et al., 2010).

Inhibition of DPP-4 Activity During Skin Repair. DPP-4 inhibitors are currently in use as oral antidiabetic agents for the treatment of type 2 diabetes. We treated diabetic ob/ob mice with the DPP-4 inhibitor linagliptin (Thomas et al., 2008). As shown in Fig. 2a, linagliptin significantly improved the clearance of elevated blood glucose levels during an OGTT, reducing glucose AUC by 25% (Fig. 2b). Accordingly, DPP-4 activity in serum was reduced by approximately 80% following linagliptin treatment of ob/ob mice, although body weight remained unchanged (Figs. 2c and d). A notable finding was that linagliptin strongly reduced the observed high DPP-4 activity in chronic wound tissue in ob/ob mice (Fig. 2e), resulting in markedly increased levels of non-cleaved (and thus active) GLP-1 in wound lysates (Fig. 2f). We also assessed wound levels of GIP, as this peptide represents an additional target for DPP-4 (Kieffer et al., 1995). As assays detecting active GIP were not commercially available, we determined total GIP levels in 10-day wounds by ELISA. Notably, total wound GIP levels were up to 10-fold lower as compared to levels of active GLP-1 (Fig. 2g). Thus, it is tempting to argue that GIP might not be the prime target of DPP-4 activity in wound repair. Inhibition of DPP-4 activity by linagliptin improved the re-epithelialization process of impaired wounds in ob/ob mice as shown by the reduced distances of wound margin epithelia compared with vehicle-treated mice when assessed using histology (Fig. 2h). This finding is also highlighted in median wound sections from vehicle- and linagliptin-treated animals which showed that linagliptin-treated ob/ob mice had a reduction in non-epithelialized wound areas or a complete epithelial coverage of wound areas (Fig. 2h; Fig. 3; see also Figs. 4a and d). We also assessed α-SMA expression as a marker of myofibroblast differentiation, a process that essentially contributes to normal wound contraction (Hinz, 2007). Linagliptin-treated mice showed an enhanced presence of myofibroblasts throughout the regenerating tissue at day 10 of repair (Fig. 3b). As 10-day wound tissue in ob/ob mice revealed the presence of DPP-4 protein (Fig. 1c) and activity (Figs.
2e, f) and the most pronounced disparities between vehicle- and linagliptin-treated ob/ob mice, we focused on that experimental time point for a more detailed analysis of the effect of linagliptin treatment on wound healing.

**Analysis of Wound Neutrophil and Macrophage Populations Upon Linagliptin Treatment.** To determine immune cell numbers in wound tissue, we used the expression of distinct cellular markers restricted to polymorphonuclear neutrophils (PMN) or macrophages (Mφ). The surface markers Ly6-G/Gr-1 (for PMN) (Daley et al., 2008) and F4/80 (for Mφ) (Austyn and Gordon, 1981) represent constitutively expressed surface antigens which are specific for the above mentioned types of immune cells. These markers allow the biochemical quantification of respective cell numbers at wound sites (Wetzler et al., 2000; Goren et al., 2003; Goren et al., 2009a; Seitz et al., 2010). Wounds from vehicle-treated ob/ob mice showed high numbers of PMN; however, re-epithelialized wounds in linagliptin-treated ob/ob mice had much lower PMN infiltration (Figs. 4a and b). Immunoblots of wound lysates against PMN-specific Gr-1 protein confirmed the observed reduction of wound PMN upon linagliptin treatment (Fig. 4c). In contrast to the PMN data, histologic (Figs. 4d and e) as well as immunoblot (Fig. 4f) analyses showed equal numbers of Mφ in wounds of vehicle- and linagliptin-treated mice. In addition, linagliptin treatment did not alter the caspase-3–assessed viability (Fig. 4g) or the M1/M2 ratio of the wound Mφ infiltrate compared with vehicle, as assessed by the expression of the M2 markers arginase-1 (vehicle [mean ± SEM]: 3.31 ± 0.40 vs linagliptin 3.47 ± 0.71 (x-fold increase compared with non-wounded skin; \( p = \text{not significant} \)) and Ym1 (vehicle: 44.75 ± 12.00 vs linagliptin 23.22 ± 8.10 (x-fold increase compared with non-wounded skin; \( p = \text{not significant} \)) (Raes et al., 2005).

**Effects of Linagliptin on Cox-2 and MIP-2 Expression.** Cox-2 and MIP-2 are rapidly induced upon injury and serve as molecular markers of acute wound inflammation. These markers are not direct targets of linagliptin action but indicate chronically impaired wound
conditions. Persistently elevated expression of these proinflammatory molecules (Goren et al., 2003; Seitz et al., 2010) were observed in the diabetes-impaired skin wounds of ob/ob mice (Fig. 5a). Notably, linagliptin induced a down-regulation of Cox-2 (Fig. 5b, right panel, Fig. 5d, upper panel) and MIP-2 (Fig. 5c, right panel) protein expression in the presence of unaltered levels of the respective mRNA species (Fig. 5b and c, left panels as indicated by Y axes), suggesting a post-transcriptional effect on Cox-2 and MIP-2 gene expression. Therefore, we investigated the levels of phosphorylated eIF-2 and 4E-BP1, which are central factors in the control of eukaryotic translation. Phosphorylation inhibits eIF-2 but stimulates 4E-BP1 activation (Jackson et al., 2010; Armengol et al., 2007). As shown in Fig. 5d, linagliptin reduced p-eIF-2 levels but increased p-4E-BP1 levels, indicating an overall translational activation in impaired wounds. This finding also suggested that linagliptin-increased wound GLP-1 (Fig. 2f) is a potential mediator of mRNA translation at wound sites. To support this notion, the GLP-1 receptor agonists GLP-1 and exendin-4 enhanced de-phosphorylation and thus activation of eIF-2 in EGF-stimulated human keratinocytes (Fig. 5e). Thus, the linagliptin-induced decrease in Cox-2 and MIP-2 expression (Fig. 5b and c, right panels as indicated) may therefore be responsible for the loss of PMN infiltrate (Fig. 4a-c) observed upon linagliptin treatment.

DPP-4 Activity is Not Functionally Connected to Keratinocyte Proliferation. The constitutive DPP-4 expression observed in the keratinocytes of non-wounded skin (Fig. 1a and b) was lost in developing wound margin epithelia of acute 5-day wound tissue (Fig. 6a). This finding suggested a potential inhibitory function of DPP-4 in the control of keratinocyte proliferation. However, increasing concentrations of linagliptin had no direct effect on the proliferation rate of exponentially growing human HaCaT keratinocytes (Fig. 6b), arguing against a major role for DPP-4 in the regulation of keratinocyte proliferation. ELISA analysis revealed that HaCaT cells constitutively expressed DPP-4 protein (535 ± 116 pg/100 µg total cellular protein). This finding was most likely due to the absence of GLP-1 in this cell culture system. Therefore, we stimulated keratinocytes with EGF in the presence of the GLP-1 receptor
agonists GLP-1 and exendin-4. Linagliptin was also used to inhibit a potential GLP-1 degradation by the cells. Interestingly, GLP-1 and exendin-4 markedly reduced the EGF-induced phosphorylation of the p42/44 MAPK in human keratinocytes (Fig. 5e). These results again do not support an effect of GLP-1 receptor activation in the control of keratinocyte proliferation.
Discussion

A global increase in diabetes mellitus was predicted more than 10 years ago (Zimmet et al., 2001). Obesity clearly represents the major causative factor that enables a global progression of this disease (Finucane et al., 2011), leading to an expected rise in diabetic ulcerations in the near future (Boulton et al., 2005). Unfortunately, the diabetic ulceration still represents an unresolved clinical condition associated with a high mortality (Robbins et al., 2008). Pharmacologically improved insulin resistance and glycemic control in humans by classical antidiabetic strategies such as insulin, thiazolidinediones, glinides, and metformin do not resolve the clinical problem of diabetic ulcerations. This practical knowledge strongly suggests that glycemic control alone does not necessarily account for improved wound healing. Accordingly, wound inflammation and collagen accumulation were not normalized by dietary restriction in \textit{ob/ob} mice (Goren et al., 2003).

It is now even tempting to argue that only those approved antidiabetic drugs may exert beneficial pleiotropic ‘side effects’ under disturbed wound conditions that potentially act on defined molecular targets at the wound site. This notion is supported by the finding that a TNF-\(\alpha\)-neutralizing antibody, which depleted activated M\(\phi\) directly from diabetes-impaired wound tissue, profoundly advanced disturbed wound repair in \textit{ob/ob} mice. The robust healing was paralleled by persisting hyperglycemia, again suggesting independence of repair processes from glycemic control (Goren et al., 2006). A second example is represented by the pleiotropic action of the antidiabetic thiazolidinediones which directly stimulated VEGF expression in wound keratinocytes in \textit{ob/ob} mice (Schiefelbein et al., 2008).

Because linagliptin bioavailability has been proven in skin tissue in rats (Fuchs et al., 2009), we decided to investigate the activity of this glucose-lowering drug to interfere with diabetes-induced cutaneous wound healing disorders. Linagliptin is a very potent inhibitor of DPP-4 with an IC\(_{50}\) of 1 nM (Thomas et al., 2008). DPP-4 normally cleaves the insulinotropic hormone GLP-1, which is released postprandially from the intestine. However, DPP-4
expression is not restricted to the intestine and a major membrane-bound form (Rasmussen et al., 2003) can be found throughout almost all tissues, surface epithelia (Hartel et al., 1988), and particularly in skin (Fuchs et al., 2009). Thus, the ubiquitous expression of DPP-4 suggests additional functions of this enzyme besides its role in the control of endogenous glucose levels.

In light of taking DPP-4 inhibitors as antidiabetic drugs, long-term DPP-4 inhibition may exert yet unknown pleiotropic actions in those tissues which also express DPP-4, including the skin.

Our study confirmed a constitutive expression of DPP-4 in skin of healthy and diabetic mice. Wounding of animals caused a down-regulation of DPP-4 expression. The persisting DPP-4 signal in early 1-day wounds of healthy mice suggested that the injury-induced reduction of DPP-4 protein expression was not a simple consequence of tissue loss upon wounding. The loss of DPP-4 expression in very early wounds in diabetic mice clearly argues for a distinct pathophysiological condition. We suggest that the early loss of DPP-4 from wound tissue may contribute to a deregulation of inflammatory mediators at the wound site, because DPP-4 is known to cleave additional peptide substrates such as cytokines and chemokines (Brandt et al., 2006). However, DPP-4 protein prominently reappeared at day 7 post-wounding in both healthy and diabetic mice. Here it is important to note that the overall similarity of the DPP-4 expression kinetic between normal and diabetes-impaired wound healing does not reflect similar healing conditions in the animals. In healthy mice, DPP-4 expression was lowest at day 3 and 5 post-wounding, during that phase of repair when acute but tightly controlled inflammation, the developing granulation tissue, and the formation of large proliferating wound margin epithelia constitute a robust tissue repair process (Stallmeyer et al., 1999; Kämpfer et al., 2003; Goren et al., 2009a). By contrast, healing of wound tissue in ob/ob mice is known to be markedly delayed and the appreciable formation of new tissue does not become visible before day 7 post-wounding (Frank et al., 2000; Goren et al., 2003; Kämpfer et al., 2003; Seitz et al., 2010). Thus, during the regeneration process, the reappearance of DPP-4 expression in ob/ob mice occurred at a time when DPP-4 was reduced in healthy animals. It was tempting to hypothesize that
linagliptin, besides its antihyperglycemic effects, may exert additional direct effects on impaired wound tissue.

Indeed, linagliptin improved wound healing in ob/ob mice. The drug accelerated wound re-epithelialization, reduced the numbers of infiltrating PMN, attenuated Cox-2 and MIP-2 protein expression, and advanced myofibroblast differentiation. But what may be the direct actions of this DPP-4 inhibitor at the wound site? The first and foremost observed action of linagliptin was the reduction of DPP-4 activity. As the subsequent increase in wound GLP-1 levels appeared to be of functional importance, we would expect comparable effects using other DPP-4 inhibitors and GLP-1 receptor agonists (e.g. exendin-4) provided that those drugs were efficiently bioavailable in skin. Initially, it was tempting to argue that linagliptin-protected active GLP-1 at the wound site may have contributed to the observed advanced epithelialization process, as activation of the GLP-1 receptor results in ERK1/2 activation in murine skin (List et al., 2006). However, active GLP-1 as well as exendin-4 appeared to interfere with mitogen (EGF)-induced activation of p42/44 MAPK in keratinocytes in vitro. Nevertheless, a possible but as yet unknown mechanism of linagliptin action may be mediated through active GLP-1 in wounds that potentially acts as a regulatory mediator of translation in wound tissue in vivo and also keratinocytes in vitro.

Second, antihyperglycemic drugs may uncover yet unknown pleiotropic effects, for example, by directly altering cytokine or growth factor expression (Schiefelbein et al., 2008; Fadini et al., 2010), which could contribute to wound healing rates. Inhibition of DPP-4 in type 2 diabetic patients increased plasma levels of stromal-derived factor (SDF)-1α, representing a direct substrate of DPP-4, and numbers of circulating endothelial progenitor cells (Fadini et al., 2010). SDF-1α may promote leukocyte migration and potentially contribute to wound healing (Badillo et al., 2008).

Third, the observed down-regulation of Cox-2 protein expression occurred in the presence of unaltered mRNA levels. Wound Mφ were the major source of Cox-2 expression in
wounds of ob/ob mice (Kämpfer et al., 2005). Cox-2 represents a marker for classically activated proinflammatory M1-type Mφ, but not for their alternatively-activated M2-type Mφ counterparts (Mosser and Edwards, 2008). The observed reduction in Cox-2 protein expression upon linagliptin treatment may therefore indicate an increase in alternatively activated M2-type Mφ numbers, as overall wound Mφ numbers remained unchanged. These changes may have a direct impact on repair, as M2-type Mφ, in particular, are currently valued as beneficial wound healing Mφ (Mosser and Edwards, 2008). The reduced Cox-2 protein expression in the presence of unaltered Cox-2 mRNA levels may reflect a similar condition that has been described for interleukin (IL)-1β, which is also expressed in classically activated Mφ. Full and efficient translation of IL-1β protein does not necessarily occur from its robustly induced mRNA but is dependent on additional mediators that control or interfere with Mφ activation such as cell adherence or the complement factor C5a (Dinarello, 1992). Our data could not exclude a contribution of glucose-lowering effects of linagliptin in improved repair, but the observed differential expression of DPP-4 strongly argues for direct effects of the drug in wound tissue. However, understanding this question needs additional animal experiments using a non-systemic, topical application of linagliptin directly on wound tissue.

In summary, our findings demonstrate that a DPP-4 inhibitor can improve healing, closure, and morphology of wounds in a model of diabetes-disturbed wound healing. These findings suggest that inhibition of DPP-4 enzymatic activity may support diabetes-impaired tissue regeneration processes.
Acknowledgments

Data from this study was presented, in part, at the American Diabetes Association’s Scientific Sessions in 2009 (Linke A, et al. 596-P) and 2010 (Schürmann C, et al. 576-P).

Authorship Contributions

Participated in research design and/or acquisition analysis and interpretation of the data:
Schürmann, Linke, Engelmann-Pilger, Steinmetz, Mark, Pfeilschifter, Klein, and Frank

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Detection of DPP-4 plasma and wounds as well as detection of active GLP-1 from wound lysates: Mark and Klein

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References


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Footnotes

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Conflict of Interests
Christoph Schürmann: no conflict of interest
Andreas Linke: no conflict of interest
Kerstin Engelmann-Pilger: no conflict of interest
Cornelia Steinmetz: no conflict of interest
Michael Mark: employee of Boehringer Ingelheim
Thomas Klein: employee of Boehringer Ingelheim
Josef Pfeilschifter: no conflict of interest
Stefan Frank: no conflict of interest
Figure Legends

Fig. 1. DPP-4 in skin and wound tissue. (a) Immunohistochemical staining of DPP-4 protein in non-wounded murine tail skin. Immunopositive signals (brown) are indicated by arrows. A negative control is shown in the upper panel. (b) Immunoblots for DPP-4 protein expression in non-wounded skin (Control skin) and wound tissue at indicated time points after wounding are shown for healthy C57BL/6J control animals (left panel) or diabetic ob/ob mice (right panel). Every single data point depicts six wounds (n = 6) isolated from three individual animals. Equal loading was confirmed by Ponceau S (PonS) staining. (c) Immunoblots (left panel) for DPP-4 protein expression in 10-day wounds of non-treated C57BL/6J control mice or vehicle- and linagliptin-treated ob/ob mice as indicated. Numbers represent individual mice. Quantification (right panel) of wound DPP-4 protein expression (grey bar: C57Bl/6J; white bar: vehicle- and black bar: linagliptin-treated ob/ob mice) Data represent means ± SEM from animals (n = 3). DPP-4, dipeptidyl peptidase-4.

Fig. 2. Treatment of wounded ob/ob mice with linagliptin. Mice were treated with vehicle or linagliptin (3 mg/kg) 2 days before surgery and 10 days following wounding (white lines and bars represent vehicle and black lines and bars represent linagliptin). (a) Blood glucose levels (mg/dl) in ob/ob mice during an oral glucose tolerance test (OGTT). (b) Area under the curve (AUC) value for blood glucose levels of the OGTT. (c) Body weight before (day -2) and after 12-day vehicle or linagliptin treatment. DPP-4 ex vivo activity in serum (d) or 10-day wound lysates (e) of vehicle- or linagliptin-treated ob/ob mice. (f) Active GLP-1 levels in 10-day wound lysates of vehicle- or linagliptin-treated ob/ob mice. (g) Total GIP levels in 10-day wound lysates of vehicle- or linagliptin-treated ob/ob mice. (h) Distances of wound margin epithelia in chronic wound tissue of vehicle- and linagliptin-treated mice after 10 days. Data (a–h) represent means ± SEM obtained from 10 mice (n = 10). *p < 0.05; **p < 0.01 compared with vehicle-treated
mice. DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide.

**Fig. 3.** Wound morphology upon linagliptin treatment. (a) Hematoxylin-stained 10-day wound sections of *vehicle*- and *linagliptin*-treated *ob/ob* mice. (b) 10-day wound sections of *vehicle*- and *linagliptin*-treated *ob/ob* mice were stained for the myofibroblast differentiation marker α-SMA. Immunopositive signals (*yellow*) are indicated by arrows. The *yellow lines* define the epithelial margin. gt, granulation tissue; ne, neo-epithelium; sc, scab; sf, subcutaneous fat; we, wound margin epithelia.

**Fig. 4.** Wound neutrophil and macrophage infiltration. 10-day wound sections from *vehicle-* and *linagliptin*-treated *ob/ob* mice were stained for neutrophils (a) or macrophages (d). The *yellow lines* define the epithelial margin. Wound neutrophil (PMN) (b) and macrophage (e) cell numbers from respective sections (white bars represent *vehicle* and black bars represent *linagliptin*). *p < 0.05 compared with *vehicle*-treated mice. Data represent means ± SEM obtained from animals (*n* = 3). Gr-1 (c) or F4/80 (f) protein in 10-day wounds. **p < 0.01 compared with *vehicle*-treated mice. Data represent means ± SEM from animals (*n* = 3). Equal loading was confirmed by Ponceau S (PonS) staining. Individual numbers (vehicle/linagliptin) in (a, d) and (c, f right panels) indicate directly adjacent wounds isolated from the identical individual animal for histology or immunoblot analyses. (g) Staining of wound macrophages (*red*) for active caspase-3 (*brown*) indicated by *yellow arrows*. gt, granulation tissue; ne, neo-epithelium; sc, scab; sf, subcutaneous fat; we, wound margin epithelia.

**Fig. 5.** Expression of inflammatory and translation markers at the wound site. (a) Cox-2 (*left panel*) and MIP-2 (*right panel*) mRNA expression in healthy and diabetic *ob/ob* mice (circles represent healthy C57BL/6J mice and squares represent diabetic C57BL/6J-*ob/ob* mice). *p <
0.05, **p < 0.01 compared with healthy C57BL/6J mice. (b-d) Analysis of 10-day wound tissue from vehicle- and linagliptin-treated ob/ob mice (white bars represent vehicle and black bars represent linagliptin). Quantification of wound Cox-2 mRNA expression (b, left panel). Data represent means ± SEM from animals (n = 10). Immunoblot analysis for wound Cox-2 protein expression (right panel). **p < 0.01 compared with vehicle-treated mice. Data represent means ± SEM from animals (n = 3). (c) Quantification of wound MIP-2 mRNA expression (left panel). Data represent means ± SEM from animals (n = 10). ELISA analysis for MIP-2 protein in wounds (right panel). *p < 0.05 compared with vehicle-treated mice. Data represent means ± SEM from animals (n = 3). (d) Immunoblot for Cox-2, p-eIF2, or p-4E-BP1 in 10-day wounds. Numbers in blot represent individual mice. eIF2, 4E-BP1 and Ponceau S (PonS) staining were to confirm equal loading. (e) Activation of p42/44 MAPK and eIF2 in EGF (10 ng/ml) -stimulated HaCaT keratinocytes in the absence or presence of linagliptin (10 nM), GLP-1 (10 nM) and exendin-4 (10 nM) as indicated. p42/44 MAPK and eIF2 to confirm equal loading. Cox-2, cyclooxygenase-2; MIP-2, macrophage inflammatory protein-2; MAPK, mitogen-activated protein kinase; eIF2, eukaryotic initiation factor 2; EGF, epidermal growth factor; GLP-1, glucagon-like peptide-1; Lin, linagliptin; Ex, exendin-4.

Fig. 6. Linagliptin does not increase keratinocyte proliferation rates. (a) Histologic staining for DPP-4 protein in 5-day wound tissue of C57BL/6J mice. Immunopositive signals (brown) are indicated by arrows. The red line defines the epithelial margin. (b) Serum-stimulated proliferation of HaCaT keratinocytes (³H-thymidine incorporation, counts per min) in the absence (Control) or presence of increasing concentrations of linagliptin (3 nM to 600 nM) after 24 h as indicated (white bar represents control and black bars represent linagliptin). Data represent means ± SEM obtained from three independent cell culture experiments (n = 3). we, wound margin epithelia; n.s., not significant.
Figure 1
Figure 3
Figure 4
Figure 5