Expression of matrix metalloproteinases, cytokines and connexins in diabetic and non-diabetic human keratinocytes before and after transplantation into an ex-vivo wound healing model

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Running title: Molecular factors of keratinocytes in diabetic wounds

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Abstract

Object: Wound healing is known to require a well-organized balance of numerous factors, e.g. cytokines, matrix metalloproteinases (MMPs) and their inhibitors, as well as direct cell-cell communication (connexins, Cx). Disruption of this balance may lead to the formation of chronic wounds such as diabetic foot ulcers. The transplantation of autologous keratinocytes is a promising therapy for diabetic foot ulcers, however little is known about their characteristics on molecular level. Therefore, we intended to characterize transplanted keratinocytes from diabetic and non-diabetic origin before and after transplantation.

Research Design and Methods: We isolated human keratinocytes from diabetic and non-diabetic origin and transplanted them into an ex-vivo wound healing model. To characterize the keratinocytes we investigated mRNA expression of MMP1, 2, and 9 TIMP1 and 2, IL-1β, TNFα, Cx26 and Cx43 and, for Cx, immunolocalization.

Results: We found no significantly increased expression of the molecules investigated in cultured keratinocytes from diabetic compared to non-diabetic origin, even though there were significant differences for MMP-2, IL1-β and TNFα in skin biopsies. Expression of IL-1β was significantly lower in keratinocytes from diabetic origin. In the course of wound healing, differences in the dynamics of expression of MMP-1, IL-1β and Cx43 were observed.

Conclusion: Our results suggest that keratinocytes from diabetic origin are as capable for transplantation into chronic wounds as keratinocytes from healthy origin at the starting point of therapy. However, differences in expression dynamics later on might reflect the systemic influence of diabetes resulting in a memory of the transplanted keratinocytes.
Diabetic foot syndrome represents a major complication of diabetes causing considerable mortality and substantial lower limb amputation rates of about 44,000 per year in Germany (1). To comprehend the microenvironment of acute and chronic wounds, the pathophysiology of impaired wound healing has been increasingly investigated. Elevated levels of matrix metalloproteinases (MMPs) and reduced levels of their endogenous tissue inhibitors (tissue inhibitors of MMPs, TIMPs) have been shown in chronic wounds, including diabetic foot lesions, and can result in excessive proteolysis of tissue, as well as of growth factors and their receptors, (2, 3, 4). MMPs are responsible for controlled degradation of the extracellular matrix as well as migration in normal wound healing. They also affect angiogenesis and remodelling of the dermis. MMPs are produced by several types of cells including fibroblasts, keratinocytes, macrophages, and eosinophils.

Inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα), (5) have also been shown to be increased in non-healing wounds. They stimulate synthesis and secretion of MMPs but inhibit the production of TIMPs (6; 7). In addition, TNFα is autostimulative and can induce the secretion of IL-1β, resulting in a persisting cycle of inflammation (2). Thus, uncontrolled interaction of these wound factors may contribute to wound chronification.

Direct cell-cell communication via gap junctions (GJ) also seems to play an important role for regular wound healing. GJ consist of connexins (Cx) Connexin 43 (Cx43) which is the predominant connexin in human epidermis is down-regulated at the wound margins shortly after wounding, followed by a re-induction later on (8-10). Transgenic mice deficient for Cx43 in the epidermis as well as mice treated with Cx43 antisense gel exhibit accelerated wound healing (11; 12). In chronic wounds, Cx43 is present at the wound margins (10). Recent results suggest an involvement of Cx43 down-regulation in migration and proliferation of keratinocytes and fibroblasts as well as in inflammatory response (13). Cx26 protein expression is induced in the course of wound healing (10).

A promising therapy for chronic wounds, including diabetic foot ulcers, is the transplantation of autologous keratinocytes. The cells are thought to produce growth factors and cytokines important for the re-induction of wound healing (14-17). However, it is important that the transplanted keratinocytes are able to produce growth factors in a normal range and are not predisposed to produce altered concentrations due to e.g. their diabetic origin. Not much is known about the characteristics of keratinocytes from diabetic origin. Acikgoz and colleagues found no significant difference between diabetic and non-diabetic patients in the mitotic index of the oral-gingival keratinocytes (18). Blakytny and colleagues demonstrated a lack of IGF-1 in keratinocytes in the basal cell layer of diabetic skin (19). In addition, a reduction of NGF and an elevation of tyrosine kinase receptors (trk) was found in the diabetic epidermis (20).

The aim of this study was to compare the characteristics of cultured keratinocytes from diabetic and non-diabetic origin in order to draw conclusions for the capability of diabetic keratinocytes to positively affect the wound healing process. Therefore, we investigated the expression of MMPs, TIMPs, cytokines and connexins in cultured keratinocytes of diabetic and non-diabetic origin before
and after transplantation into wounds. To determine the time course of expression, we applied cultured keratinocytes to our ex-vivo wound healing model (WHM) (21) and analyzed their mRNA expression at 0-7-18/24 hours via RT-PCR as well as Cx staining after 24 h.

**Research Design and Methods**

**Patients.** Our study was approved by the Human Studies Committee of the University of Magdeburg, Germany. Informed consent was obtained from 14 diabetic patients (12 males, 2 females; age 61±10 yrs; diabetes duration 11±7; HbA1c 7.32±1) and 11 non-diabetic healthy volunteers (7 males, 4 females; age 52±10 yrs; diabetes duration 0; HbA1c 5.61±0.3).

**Tissue biopsies.** 8 mm punch biopsies were taken from the lateral intact upper thigh of each patient under sterile conditions and transferred into Dulbecco’s modified Eagle’s medium (DMEM). The biopsies were directly transported to Hamburg where keratinocytes were cultured.

**Primers and antibodies.** Primers amplifying MMP1 (fw-5’-TGTGGACCATGCCATTGAGA-3’, rev-5’-TCTGCTTGACCCTCAGAGACC-3’, probe-FAM-5’-CCAACTCTGGAGTAATGTCACACCTCGACATTCACC-3’) and MMP9 (fw-5’-GGCCACTACTGTGCCTTTGAG-3’, rev-5’-GATGGCGTCGAAGATGTTCAC-3’, probe-FAM-5’-TTGCAGGCATCGTCCACCGG-3’) were designed in the laboratory of Dr. Pap, (Münster, Germany), and synthesized by MWG Biotech (Ebersberg, Germany). Primers amplifying MMP2 (Hs00234422_m1), TIMP-1 (Hs00171558_m1), TIMP-2 (Hs00234278_m1), IL1β (Hs00174097_m1), TNFα (Hs00174128_m1), Cx26 (Hs00269615_s1), Cx43 (Hs007748445_s1), and GAPDH (4310884E) were purchased from Applied Biosystems (Weiterstadt, Germany). Rabbit polyclonal antibodies directed to Cx43 (71-0700) were purchased from Zymed Laboratories (San Francisco, CA), polyclonal antibodies directed against Cx26 were produced as described (10)).

**Cell culture.** Keratinocytes were cultured as described (10). Briefly, skin samples were trypsinized overnight at 4°C and then placed in keratinocyte basic medium supplemented with epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone, and glutamine. They were transplanted in the 2nd passage.

**Ex-vivo wound healing model (WHM).** After excising a 6 mm diameter punch biopsy of porcine skin, another 3 mm diameter punch biopsy including epidermis and the upper dermis was removed from its center. Each piece was placed on a culture disk filled with DMEM, which was supplemented with hydrocortisone, 5 % fetal calf serum, penicillin, and streptomycin. The model was incubated in air-liquid interphase (21). Cultured keratinocytes (750 000 cells/wound) were transplanted into the wound followed by incubation at 37 °C with 10 % CO₂. 11 samples from diabetic patients and 9 samples from healthy volunteers yielded a sufficient number of keratinocytes for transplantation into duplicates of WHM from three different pigs. In 6 samples from diabetic patients and 4 samples from healthy volunteers, it was possible to perform additional WHM at 24 h for immunohistochemical evaluation. For mRNA evaluation, models were stopped by scratching the cells out of the wound, transferring them into RNA Later Solution (Qiagen, Hilden, Germany) and storing them at -80°C. For immunohistochemistry, models were snap-frozen and stored at –80°C.
RNA-extraction, reverse transcription and real-time PCR. RNA-extraction was accomplished by using RNeasy-Mini-Kit (Qiagen, Hilden, Germany). Reverse transcription of 0.25-0.5 µg isolated RNA to cDNA was performed using a Reverse Transcriptase Hex Primer Kit (Applied Biosystems, Weiterstadt, Germany). Real time PCR was performed according to the manufacturer’s protocols (TaqMan Assay, Applied Biosystems, Weiterstadt, Germany) by using ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, USA). Triplet reactions were performed. After analysis of the real-time PCR data by the SDS Software, the resulting threshold cycle values (CT) were used to establish quantitative relationships between the initial template concentration of the unknown samples and those of the endogeneous standard GAPDH (∆CT).

Immunofluorescence microscopy. Cryostat sections (6 µm) from the central parts of the WHM were fixed in –20°C acetone for 10 min. Staining was performed as previously described (10).

Statistical methods. Statistical analysis was performed by using SPSS 13.0 for Windows. Means ± SD were determined for descriptive calculations. A general linear model, as well as independent and paired T-Tests, was used for statistical evaluation. Dynamic changes were determined with the general linear model for repeated measures. A value of p < 0.05 was considered to indicate statistical significance.

Results
In order to determine the time course of the expression of MMPs, cytokines and connexins in the keratinocytes transplanted into the ex-vivo WHM, we analyzed the mRNA expression at 0 h (before transplantation), and after 7h and 18/24 h of wound healing by using quantitative real-time PCR: the first half of experiments were stopped after 0 h, 7 h and 18 h. Because of organizational reasons 18 h could not be realized anymore and we had to switch to 24 h as the maximal incubation time. In order to analyze whether there were any statistically significant differences between samples from 18 and 24 h incubations, parameters were analyzed and no significant differences were observed

Thus it was feasible to pool data from 18 and 24 h incubation (defined as ‘endpoint’) for further statistical analysis.

MMPs and TIMPs
In non-lesion skin biopsies from diabetic origin, there was no significant difference in expression of MMP1, MMP9, TIMP-1 and TIMP-2 compared to skin from healthy origin (Table 1). In contrast, expression of MMP2 was statistically significantly higher in skin biopsies from diabetic patients compared to controls (Table 1). In cultured keratinocytes, expression of MMP1 was increased; expression of MMP2 and MMP9 was decreased compared to skin biopsies (see Table 1). For MMP2, the significant differences between diabetic and non-diabetic origin were no longer seen in cultured keratinocytes. There were no significant differences in TIMP-1 and TIMP-2 expression between diabetic and non-diabetic origin, neither in skin biopsies, nor in cultured keratinocytes (see Table 1). In the course of wound healing, expression of MMP1 increased in transplanted keratinocytes of both, diabetic and healthy origin, but while increase was more pronounced in the first 7 h in non-diabetic keratinocytes, the highest increase in diabetic keratinocytes was observed in the second phase of our experiment (Figure 1). This difference in the dynamic progress of MMP1 expression during wound healing was
statistically significant (p=0.007). However, there was no significant difference between the various time points. MMP-2 expression slightly decreased during wound healing in healthy and diabetic transplanted keratinocytes (Figure 1). MMP9 expression increased in the course of wound healing in diabetic and non-diabetic keratinocytes with no significant differences at the various time points (data not shown). There was no significant difference between diabetic and non-diabetic keratinocytes in the ratio of investigated MMPs/TIMPs, except for MMP2/TIMP-2 which exhibited a significant increase at 7 h after transplantation (Table 1).

**Cytokines**
In skin biopsies, there was a significantly higher expression of IL-1β and TNFα in diabetic patients compared to healthy volunteers (Table 1). In contrast, IL-1β expression was significantly lower in cultured keratinocytes of diabetic compared to healthy origin. There was no difference in expression of TNFα. Compared to skin biopsies, IL-1β expression was increased and TNFα strongly decreased in cultured keratinocytes (Table 1). In the course of wound healing, IL-1β expression constantly decreased in keratinocytes of healthy origin (Figure 1). In contrast, expression significantly increased in keratinocytes of diabetic origin in the first 7 hours of the experiment, while it significantly decreased in the second part (Figure 1). The difference in dynamics of IL-1β expression during wound healing between keratinocytes of diabetic and non-diabetic origin was significant (p=0.007). TNFα expression significantly increased in the first 7 h in cultured keratinocytes of diabetic and healthy origin. Subsequently, a slight decrease could be observed which was significant for diabetic keratinocytes, but not for healthy keratinocytes. There was no significant difference in the dynamic of TNFα expression in the course of wound healing between the two groups.

**Connexins**
The amount of Cx26 was not significantly different in skin biopsies of diabetic origin and non-diabetic origin. The same was true for cultured keratinocytes and keratinocytes after transplantation (Table 1, Fig. 1). There was a slight increase of Cx26 in cultured keratinocytes compared to the skin biopsies. In transplanted keratinocytes, Cx26 was slightly down-regulated at 7 h after transplantation and clearly induced 18 h/24 h after transplantation. Evaluation of Cx26 immunohistochemistry showed an intensive staining of transplanted keratinocytes 24 h after transplantation in most diabetic and non-diabetic samples (Fig 2).
Also for Cx43, there was no statistical significant difference in the amount of mRNA in skin biopsies of diabetic and non-diabetic origin. The same was true for cultured keratinocytes even though much less Cx43 was present in cultured keratinocytes compared to skin biopsies (Table 1). In transplanted keratinocytes from non-diabetic origin, Cx43 was significantly down-regulated 7 h after transplantation. Later on, there was a slight reinduction (Figure 1). In contrast, in keratinocytes from diabetic origin, a delayed down-regulation of Cx43 was observed. 7 h after transplantation, Cx43 was only moderately decreased, but down-regulation continued even 18/24h after transplantation, resulting in a statistically significant reduction at 18/24 h compared to 0 h (p = 0.001). Consequently, at the endpoint of observation a 3.5-fold decrease in expression of Cx43 (p=0.029) was found.
in diabetic keratinocytes compared to non-diabetic controls (Figure 1). Diabetic and non-diabetic keratinocytes showed significant differences in dynamic changes (p=0.024).

Evaluation of Cx43 immunohistochemistry showed a low staining intensity in transplanted keratinocytes from healthy and diabetic origin in wound models 24 h after transplantation (Fig 2).

Discussion

Diabetic foot syndrome represents a major complication of diabetes (1). Chronic wounds, including the chronic foot ulceration are characterized by a disturbed relation of pro-inflammatory cytokines (TNFα, IL1β), an elevated concentration of MMPs and altered expression of connexins (2; 10; 22, 23)). Levels of MMP-1, MMP-2, and MMP-9 are elevated in chronic leg ulcers compared to acute wounds of patients with non-diabetic origin; concentrations of TIMPs are reduced (3). Our data show that there is no significant difference between diabetic and non-diabetic origin for MMP-1 and MMP-9 expression in skin and cultured keratinocytes before and after transplantation. This agrees with results found by Saarialho-Kere who demonstrated that there is no difference in MMP-1 expression between epidermis of chronic and acute wounds while the number of MMP-1 expressing stromal cells was greater in chronic wounds (24). We observed a difference in the dynamic of MMP-1 expression in the course of wound healing. However, even though the increase of MMP-1 in healthy keratinocytes after 7 h is more pronounced than in diabetic keratinocytes, the level of MMP-1 reached at 7 h is quite similar. Therefore, one might suggest that the different dynamic should not have a major contribution to the efficiency of the cultured keratinocytes during wound healing.

We found that, in contrast to MMP-1, the MMP-2 concentration is significantly higher in diabetic skin compared to non-diabetic skin. This significant difference was abolished in cultured keratinocytes where we observed a decreased concentration of MMP-2 in both, keratinocytes from diabetic and non-diabetic origin, compared to skin samples. Wall et al. found an elevated production of MMP-2 in fibroblasts from diabetic origin (25). This suggests that the differences observed in skin samples for MMP-2 are due to differences in fibroblasts, not in keratinocytes. We also found a significant elevated ratio of MMP-2 to TIMP-2 in skin of diabetic origin compared to healthy skin, but no difference in cultured keratinocytes. However, 7 h after transplantation, a significantly elevated ratio of MMP-2 to TIMP-2 was observed. It has been shown, that in the fluids of chronic wounds, TNFα and IL-1β concentration are elevated (2; 26). We were able to demonstrate that TNFα and IL-1β mRNA levels are significantly increased in skin from diabetic origin compared to non-diabetic origin. This might reflect a generally increased inflammatory activity in patients with diabetes. Increased concentrations of TNFα and IL-1β might be responsible for the increased expression for MMP-2. However, as wound healing is a very complex process and as various pathways are able to cross-talk with each other, it is likely that also other factors upstream of MMP expression are involved (23). In cultured keratinocytes, the difference in IL-1β concentration between diabetic and non-diabetic cells was not only abolished but even reversed. IL-1β concentration was significantly lower in cells from diabetic origin compared to non-diabetic origin. After transplantation, IL-1β expression initially increased then...
decreased in transplanted keratinocytes of diabetic origin in the course of wound healing. For non-diabetic keratinocytes, a continuous decrease was observed. However, the intial increase of IL-1β in diabetic keratinocytes only resulted in an IL-1β level comparable to that of keratinocytes of healthy origin.

Also the significant difference in TNFα concentration between diabetic and non-diabetic skin was abolished in cultured keratinocytes. In general, TNFα expression in cultured keratinocytes was much lower than in skin samples. This might reflect that TNFα is not mainly produced by keratinocytes, but by macrophages and neutrophils. Also after transplantation, diabetic and non-diabetic cells behaved similar with respect to TNFα production.

An important event during normal wound healing seems to be the down-regulation of Cx43 at the wound margins shortly after wounding (8, 9; 10). Ex-vivo investigation showed that in keratinocytes of healthy origin, Cx43, which is found in moderate amounts at the cell-cell borders of primary keratinocytes in cell culture, is lost after transplantation of the cells into wounds and regained later on in the course of wound healing (10). Comparing non-diabetic and diabetic skin samples, we found no difference in Cx43 expression. The same was true for cultured keratinocytes, but the concentration of Cx43 markedly decreased compared to skin samples. This might reflect the increased proliferation of cells in culture and therefore confirm the correlation between Cx43 expression and proliferation described before (13). After transplantation in a statistically significant difference of Cx43 concentration between diabetic and non-diabetic keratinocytes could be observed at the late time point. However, this difference was not reflected at that time point at the level of Cx43 staining at cell-cell borders of transplanted keratinocytes.

Cx26, a connexin often found in hyperproliferative epidermis, is up-regulated in normal wound healing at the wound margins and is also induced in transplanted healthy keratinocytes after an initial lag phase (8, 9, 11). We found the same result for transplanted keratinocytes from diabetic origin.

The normal expression of MMP-1, MMP-2, MMP-9, TNFα, Cx26 and Cx43 and the – compared to keratinocytes from healthy origin - even reduced expression of IL-1β in cultured keratinocytes of diabetic origin shows that these cells do not aggravate the distressed environment found in the chronic wound. This might account for their capability to induce wound healing in diabetic foot ulcers. Furthermore, the transplantation of keratinocytes increases the number of (target) cells in the wound for several factors (cytokines, MMPs) which are found in higher levels in chronic wounds than in normal wounds, supposedly resulting in an improved ratio of factors to target-cells and a less destructive action of several factors. Nevertheless, the elevated MMP-2/TIMP1 ratio at 7 h after transplantation and the altered dynamic of IL-1β expression in the course of wound healing, as well as the delayed reinduction of Cx43 might indicate a slight disadvantage of diabetic keratinocytes compared to normal keratinocytes. This altered behavior of keratinocytes from diabetic origin might indicate a memory of keratinocytes of their origin which might only be evident in a three-dimensional environment. However, even though we used a three-dimensional model which is advantageous to many other (2-D) wound healing models, we have to point out that the resemblance of our ex-vivo WHM to chronic wounds – as is also true for other wound models - is only limited. Especially alterations in local blood microcirculation
Molecular factors of keratinocytes in diabetic wounds might have some influence. Consequently, our results concerning the dynamics of expression of the several factors in the course of wound healing should be interpreted with this in mind. In summary, our results suggest that, at the starting point of therapy, diabetic keratinocytes are as capable for transplantation into chronic wounds as keratinocytes from healthy origin. Later on they show differences in expression dynamics which might reflect a memory of the transplanted keratinocytes for their diabetic origin.

Basic mechanisms of wound healing in chronic wounds require further analysis to clarify the pathogenesis and to develop innovative treatment strategies.

**Acknowledgements**
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References
Tables legends:
Table 1: Expression of MMPs, TIMPs, cytokines and connexins in skin biopsies and cultured keratinocytes. Units: \( \Delta C_t \)-values between the various MMPs, TIMPs, cytokines and connexins respectively and GAPDH.
Table 1: Expression of MMPs, TIMPs, Cytokines and Cx in skin biopsies and cultured keratinocytes

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<th>Diabetic</th>
<th>Control</th>
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<tr>
<td>MMP-1, skin biopsy</td>
<td>13.4 +/- 1.6</td>
<td>11.8 +/- 2.6</td>
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<td>MMP-1, cultured keratinocytes</td>
<td>5.6 +/- 1.0</td>
<td>7.7 +/- 3.7</td>
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<td>MMP-2, skin biopsy</td>
<td>0.8 +/- 0.6</td>
<td>3.4 +/- 3.3</td>
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<td>5.8 +/- 0.4</td>
<td>6.6 +/- 3.6</td>
<td>n.s.</td>
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<td>7.7 +/- 0.9</td>
<td>7.0 +/- 0.6</td>
<td>n.s.</td>
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<td>11.7 +/- 1.7</td>
<td>8.8 +/- 0.4</td>
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<td>2.7 +/- 0.7</td>
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<td>3.2 +/- 0.9</td>
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n.s.: not significant
**Figure Legends**

**Figure 1:** Expression (ΔCT) of MMP-1, MMP-2, IL-1β, TNFα, Cx26 and Cx43 before (0h), 7h, and 18/24 h after transplantation into an ex-vivo WHM. Black rhomb: Keratinocytes of diabetic origin, grey square: Keratinocytes of non-diabetic origin * significant differences between the various time points of the same origin. ** significant differences between keratinocytes of diabetic and non-diabetic origin at the same point in time. Cave: High ΔCT indicates low expression!

**Figure 2:** Immunofluorescence localization of Cx26 (red, A, B) and Cx43 (red, C, D) in transplanted keratinocytes of non-diabetic (A, C; nd) and diabetic (B, D; d) origin 24 h after transplantation. Blue: DAPI-stained nuclei. The “magnifying glass” in the schematic illustration of the wound model (yellow: epidermis, beige: dermis) at the bottom of the figure indicates the location of the regions shown above. Bar: 50 µm
Figure 2

Molecular factors of keratinocytes in diabetic wounds