Expression of Matrix Metalloproteinases, Cytokines, and Connexins in Diabetic and Nondiabetic Human Keratinocytes Before and After Transplantation Into an Ex Vivo Wound-Healing Model

Johanna M. Brandner, phd[1]
Steffi Zacheja, md[2]
Pia Houdek[1]
Ingrid Moll, md[1]
Ralf Lobmann, md[2]

OBJECTIVE — 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). 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 a molecular level. Therefore, we intended to characterize transplanted keratinocytes from diabetic and nondiabetic origin before and after transplantation.

RESEARCH DESIGN AND METHODS — We isolated human keratinocytes from diabetic and nondiabetic origins and transplanted them into an ex vivo wound healing model. To characterize the keratinocytes, we investigated mRNA expression of MMP-1, MMP-2, and MMP-9; tissue inhibitor of MMP (TIMP)-1 and TIMP-2; interleukin (IL)-1β, tumor necrosis factor (TNF)-α; Cx26 (connexin 26) and Cx43; and, for connexins, immunolocalization.

RESULTS — We found no significantly increased expression of the molecules investigated in cultured keratinocytes from diabetic compared with nondiabetic origin, even though there were significant differences for MMP-2, IL-1β, and TNF-α 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.

CONCLUSIONS — 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.

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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 MMP [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 (2–4). MMPs are responsible for controlled degradation of the extracellular matrix as well as migration in normal wound healing. They also affect angiogenesis and remodeling of the dermis. MMPs are produced by several types of cells including fibroblasts, keratinocytes, macrophages, and eosinophils.

Inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α (5), have also been shown to be increased in nonhealing wounds. They stimulate synthesis and secretion of MMPs but inhibit the production of TIMPs (6,7). In addition, TNF-α is autostimmulative 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 also seems to play an important role for regular wound healing. Gap junctions consist of connexins; Cx43 (connexin 43), which is the predominant connexin in human epidermis, is downregulated at the wound margins shortly after wounding, followed by a reinduction 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 downregulation 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

From the [1]Department of Dermatology and Venerology, University Hospital Hamburg-Eppendorf, Hamburg, Germany; and the [2]Department of Endocrinology and Metabolism, Otto-von-Guericke University Hospital, Magdeburg, Germany.

Address correspondence and reprint requests to Associate Professor Ralf Lobmann, MD, Department of Endocrinology and Metabolism, Magdeburg University Medical School, Leipziger Strasse 44, 39120 Magdeburg, Germany. E-mail: ralf.lobmann@med.ovgu.de.

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J.M.B. and S.Z. contributed equally to this article and are mentioned alphabetically.

Abbreviations: IL, interleukin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP; TNF, tumor necrosis factor; WHM, wound healing model.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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 et al. (18) found no significant difference between diabetic and nondiabetic patients in the mitotic index of the oral-gingival keratinocytes. Blakytny et al. (19) demonstrated a lack of IGF-1 in keratinocytes in the basal cell layer of diabetic skin. In addition, a reduction of nerve growth factor and an elevation of tyrosine kinase receptor (trk) was found in the diabetic epidermis (20).

The aim of this study was to compare the characteristics of cultured keratinocytes from diabetic and nondiabetic 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 nondiabetic 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, and 24 h via RT-PCR as well as their connexin staining after 24 h.

RESEARCH DESIGN AND METHODS

— Our study was approved by the Human Studies Committee of the University of Magdeburg, Germany. Informed consent was obtained from 14 diabetic patients (12 men and 2 women aged 61 ± 10 years, diabetes duration 11 ± 7 years, A1C 7.32 ± 1%) and 11 nondiabetic healthy volunteers (7 men and 4 women aged 52 ± 10 years, A1C 5.61 ± 0.3%).

Tissue biopsies

Eight-millimeter 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. The biopsies were directly transported to Hamburg, where keratinocytes were cultured.

Primers and antibodies

Primers amplifying MMP-1 (forward: 5′-TGTGGACCATGCCATTGAGA-3′, reverse: 5′-TCTGCTTGACCCTCAGAGACC-3′, probe-FAM: 5′-CCAACTCTGGAGTAATGTCACACCTCTGACATTCACC-3′) and MMP9 (forward: 5′-GGCCACTACTGTGCCTTTGAG-3′, reverse: 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 MMP-2 (Hs00234422_m1), TIMP-1 (Hs00171558_m1), TIMP-2 (Hs00234278_m1), IL-1β (Hs00174097_m1), TNF-α (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), and 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 second passage.

Ex vivo WHM

After excising a 6-mm punch biopsy of porcine skin, another 3-mm punch biopsy including epidermis and the upper dermis was removed from its center. Each piece was placed on a culture disk filled with Dulbecco's modified Eagle's medium, which was supplemented with hydrocortisone, 5% FCS, 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% CO_2 (patent no. DE 10317400). Eleven samples from diabetic patients and nine samples from healthy volunteers yielded a sufficient number of keratinocytes for transplantation into duplicates of WHM from three different pigs. In six samples from diabetic patients and four 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 an 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 an ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA). 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 endogeous standard GAPDH (ΔCT).

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

— 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 7, 18, and 24 h of wound healing by using quantitative real-time PCR; the first half of experiments was stopped after 0 , 7, and 18 h. Due to organizational issues, we were no longer able to use 18 h as the maximal incubation time and had to switch to 24 h. Parameters were analyzed to ascertain whether there were any statistically significant differences between samples from 18- and 24-h incubations, and no signif-

Table 1—*Expression of MMPs, TIMPs, cytokines, and connexins in skin biopsies and cultured keratinocytes*

	Diabetic	Control	*P*
MMP-1			
Skin biopsy	13.4 ± 1.6	11.8 ± 2.6	NS
Cultured keratinocytes	5.6 ± 1.0	7.7 ± 3.7	NS
MMP-2			
Skin biopsy	0.8 ± 0.6	3.4 ± 3.3	<0.05
Cultured keratinocytes	5.8 ± 0.4	6.6 ± 3.6	NS
MMP-9			
Skin biopsy	7.7 ± 0.9	7.0 ± 0.6	NS
Cultured keratinocytes	11.7 ± 1.7	8.8 ± 0.4	NS
TIMP-1			
Skin biopsy	2.7 ± 0.7	3.5 ± 2.8	NS
Cultured keratinocytes	3.4 ± 0.7	3.2 ± 0.9	NS
TIMP-2			
Skin biopsy	0.5 ± 0.4	1.6 ± 2.3	NS
Cultured keratinocytes	5.5 ± 1.6	5.6 ± 2.7	n.s
MMP2/TIMP2 7 h after transplantation	1.2 ± 0.3	1.8 ± 0.4	<0.01
IL-β			
Skin biopsy	8.6 ± 1.3	10.4 ± 1.2	<0.05
Cultured keratinocytes	4.6 ± 1.0	3.0 ± 1.4	<0.05
TNF-α			
Skin biopsy	5.3 ± 0.7	7.3 ± 2.0	<0.05
Cultured keratinocytes	11.0 ± 3.7	11.0 ± 3.5	NS
Cx26			
Skin biopsy	3.3 ± 0.7	3.5 ± 1.6	NS
Cultured keratinocytes	2.0 ± 0.3	2.0 ± 0.9	NS
Cx43			
Skin biopsy	0.5 ± 0.4	0.3 ± 0.2	NS
Cultured keratinocytes	4.1 ± 1.0	3.1 ± 1.2	NS

Data are means ± SD. Units are ΔC_t values between the various MMPs, TIMPs, cytokines, and connexins and GAPDH. NS, not significant.

icant differences were observed. Thus, it was feasible to pool data from 18- and 24-h incubation (defined as "end point") for further statistical analysis.

MMPs and TIMPs

In nonlesion skin biopsies from diabetic origin, there was no significant difference in expression of MMP-1, MMP-9, TIMP-1, and TIMP-2 compared with skin from healthy origin (Table 1). In contrast, expression of MMP-2 was statistically significantly higher in skin biopsies from diabetic patients than in those from control subjects (Table 1). In cultured keratinocytes, expression of MMP-1 was increased, and expression of MMP-2 and MMP-9 was decreased compared with skin biopsies (Table 1). For MMP-2, the significant differences between diabetic and nondiabetic origin were no longer seen in cultured keratinocytes. There were no significant differences in TIMP-1 and TIMP-2 expression between diabetic and nondiabetic origin, neither in skin biopsies nor in cultured keratinocytes (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 nondiabetic keratinocytes, the highest increase in diabetic keratinocytes was observed in the second phase of our experiment (Fig. 1). This difference in the dynamic progress of MMP-1 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 (Fig. 1). MMP-9 expression increased in the course of wound healing in diabetic and nondiabetic keratinocytes with no significant differences at the various time points (data not shown). There was no significant difference between diabetic and nondiabetic keratinocytes in the ratio of investigated MMPs to TIMPs, except for MMP2-to-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 TNF-α in diabetic patients than in healthy volunteers (Table 1). In contrast, IL-1β expression was significantly lower in cultured keratinocytes of diabetic compared with healthy origin. There was no difference in expression of TNF-α. Compared with skin biopsies, IL-1β expression was increased and TNF-α strongly decreased in cultured keratinocytes (Table 1). In the course of wound healing, IL-1β expression constantly decreased in keratinocytes of healthy origin (Fig. 1). In contrast, expression significantly increased in keratinocytes of diabetic origin in the first 7 h of the experiment, while it significantly decreased in the second part (Fig. 1). The difference in dynamics of IL-1β expression during wound healing between keratinocytes of diabetic and nondiabetic origin was significant ($P = 0.007$). TNF-α 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 but not for healthy keratinocytes. There was no significant difference in the dynamic of TNF-α 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 and nondiabetic origin. The same was true for cultured keratinocytes and keratinocytes after transplantation (Table 1 and Fig. 1). There was a slight increase of Cx26 in cultured keratinocytes compared with the skin biopsies. In transplanted keratinocytes, Cx26 was slightly downregulated at 7 h after transplantation and clearly induced at 18 and 24 h after transplantation. Evaluation of Cx26 immunohistochemistry showed an intensive staining of transplanted keratinocytes 24 h after transplantation in most diabetic and nondiabetic samples (Fig. 2).

Also for Cx43, there was no statistical significant difference in the amount of mRNA in skin biopsies of diabetic and nondiabetic origin. The same was true for cultured keratinocytes, even though much less Cx43 was present in cultured keratinocytes than in skin biopsies (Table

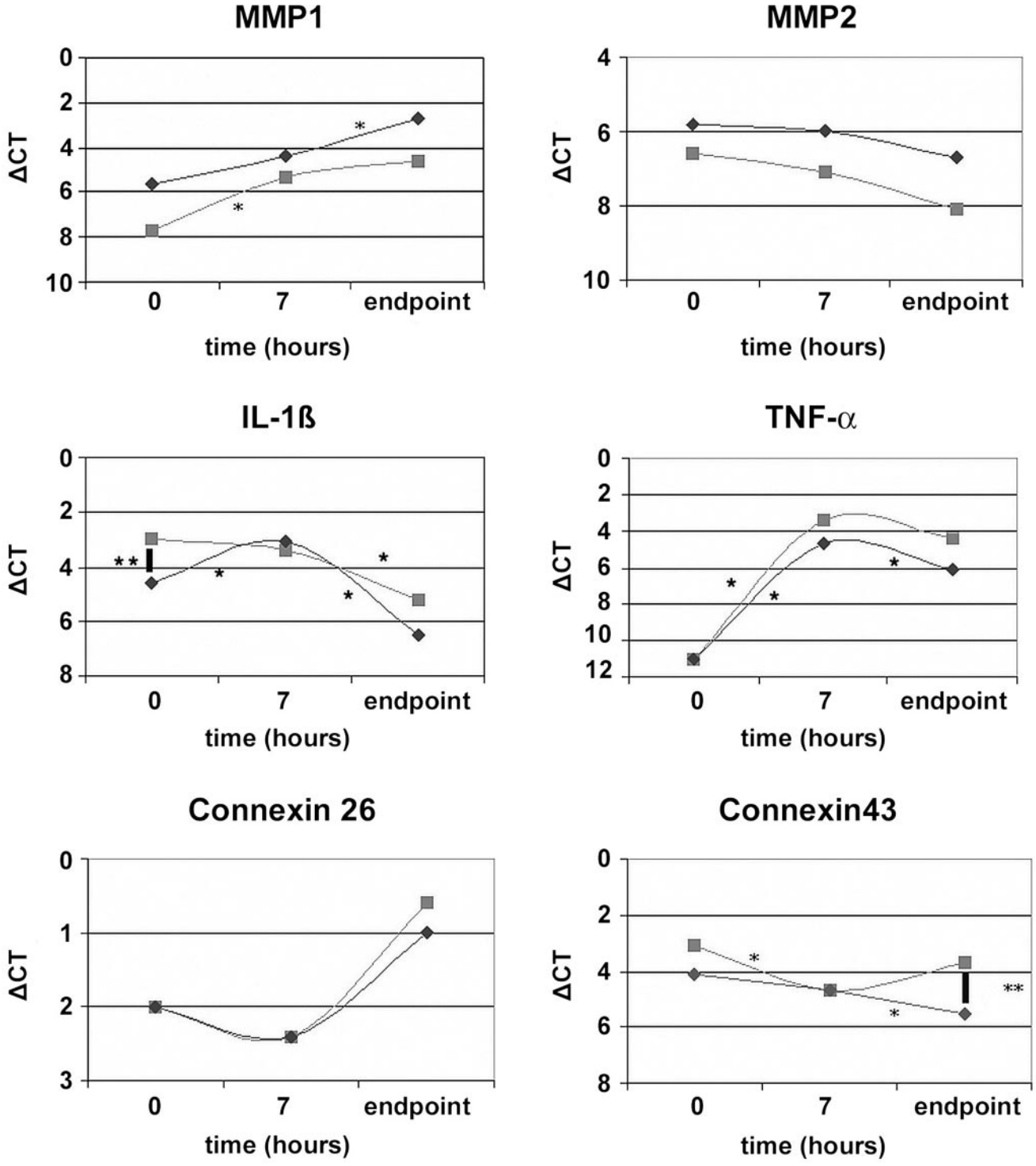

Figure 1—*Expression (ΔCT) of MMP-1, MMP-2, IL-1β, TNF-α, Cx26, and Cx43 before (0 h) and 7 h, as well as 18 and 24 h (end point), after transplantation into an ex vivo WHM. Diamonds, keratinocytes of diabetic origin; squares, keratinocytes of nondiabetic origin. *Significant differences between the various time points of the same origin. **Significant differences between keratinocytes of diabetic and nondiabetic origin at the same point in time (rectangles). High ΔCT indicates low expression.*

1). In transplanted keratinocytes from nondiabetic origin, Cx43 was significantly downregulated 7 h after transplantation. Later on, there was a slight reinduction (Fig. 1). In contrast, in keratinocytes from diabetic origin, a delayed downregulation of Cx43 was observed. Seven hours after transplantation, Cx43 was only moderately decreased, but downregulation continued even 18–24 h after transplantation, resulting in a statistically significant reduction at 18–24 h compared with 0 h ($P = 0.001$). Consequentially, at the end point of observation, a 3.5-fold decrease in expression of

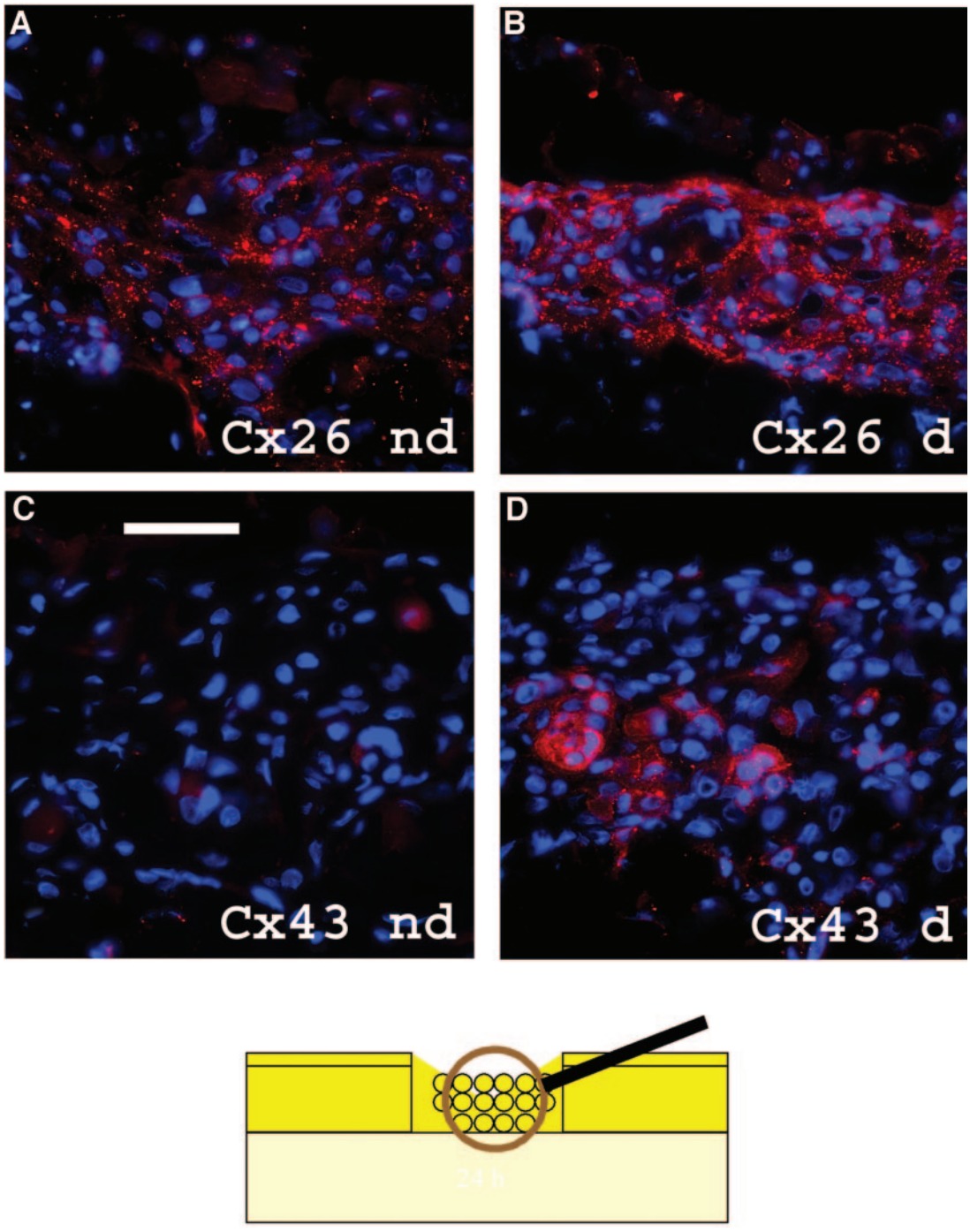

Figure 2— *Immunofluorescence localization of Cx26 (red,* A *and* B*) and Cx43 (red,* C *and* D*) in transplanted keratinocytes of nondiabetic (nd) (*A *and* C*) and diabetic (d) (*B *and* D*) origin 24 h after transplantation. Blue: DAPI-stained nuclei. The "magnifying glass" in the schematic illustration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

Cx43 ($P = 0.029$) was found in diabetic keratinocytes compared with nondiabetic controls (Fig. 1). Diabetic and nondiabetic 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).

CONCLUSIONS — Diabetic foot syndrome represents a major complication of diabetes (1). Chronic wounds, including chronic foot ulceration, are characterized by a disturbed relation of proinflammatory cytokines (TNF-α and IL-1β), 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 with acute wounds of patients with nondiabetic origin; concentrations of TIMPs are reduced (3). Our data show that there is no significant difference between diabetic and

nondiabetic 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 (24), who demonstrated that there was 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. 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 than in nondiabetic skin. This significant difference was abolished in cultured keratinocytes, where we observed a decreased concentration of MMP-2 in both keratinocytes from diabetic and nondiabetic origin compared with skin samples. Wall et al. (25) found an elevated production of MMP-2 in fibroblasts from diabetic origin. 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 with 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, TNF-α and IL-1β concentrations are elevated (2,26). We were able to demonstrate that TNF-α and IL-1β mRNA levels are significantly increased in skin from diabetic compared with nondiabetic origin. This might reflect generally increased inflammatory activity in patients with diabetes. Increased concentrations of TNF-α and IL-1β might be responsible for the increased expression of 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 other factors upstream of MMP expression are involved (23). In cultured keratinocytes, the difference in IL-1β concentration between diabetic and nondiabetic cells was not only abolished but even reversed. IL-1β concentration was significantly lower in cells from diabetic compared with nondiabetic origin. After transplantation, IL-1β expression initially increased then decreased in transplanted keratinocytes of diabetic origin in the course of wound healing. For nondiabetic keratinocytes, a continuous decrease was observed. However, the initial increase of IL-1β in diabetic keratinocytes only resulted in an IL-1β level comparable with that of keratinocytes of healthy origin.

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

An important event during normal wound healing seems to be the downregulation of Cx43 at the wound margins shortly after wounding (8–10). 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 nondiabetic 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 with that in 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). A statistically significant difference of Cx43 mRNA concentration between diabetic and nondiabetic keratinocytes could be observed 18–24 h after transplantation. However, this difference was not reflected in the level of Cx43 staining at cell-cell borders of transplanted keratinocytes.

Cx26, a connexin often found in hyperproliferative epidermis, is upregulated 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, TNF-α, Cx26, and Cx43 and the reduced expression (compared with keratinocytes from healthy origin) 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 and MMPs) that are found in higher levels in chronic 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–to–TIMP-1 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 re-induction of Cx43, might indicate a slight disadvantage of diabetic keratinocytes compared with normal keratinocytes. This altered behavior of keratinocytes from diabetic origin might indicate a keratinocyte memory 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 (two-dimensional) WHMs, we must point out that the resemblance of our ex vivo WHM to chronic wounds is limited, as is true for other wound models. Alterations in local blood microcirculation, in particular, 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 that 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.

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