Cutaneous Structural and Biochemical Correlates of Foot Complications in High-Risk Diabetes

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OBJECTIVE—Impairment of skin quality may contribute to diabetic foot ulceration (DFU). Our goal was to determine whether high-risk patients exhibited specific skin structural and metabolic deficits that could predispose to foot complications.

RESEARCH DESIGN AND METHODS—A total of 46 patients comprising 9 diabetic control subjects, 16 with diabetic peripheral neuropathy (DPN) alone, and 21 with recurrent DFUs (including 9 with Charcot neuroarthropathy [CNA]) were recruited and compared with 14 nondiabetic control (NDC) subjects. DPN was assessed using the Michigan Neuropathy Screening Instrument (MNSI). Skin punch biopsies (3 mm) were performed on upper and lower leg skin for measurements of intraepidermal nerve fiber density (IENFD), structural analysis, type 1 procollagen abundance, tissue degrading matrix metalloproteinases (MMPs), and poly(ADP-ribose) (PAR) immunoreactivity.

RESULTS—MNSI scores were comparable across DPN groups. IENFD was decreased by diabetes and DPN but did not differ between neuropathic groups. Skin structural deficit scores were elevated in all neuropathic subjects, particularly in the DFU group. Type 1 procollagen abundance was reduced in DFU subjects 387 ± 256 units (mean ± 1 SD) compared with NDC subjects (715 ± 100, $P < 0.001$). MMP-1 and MMP-2 were activated by diabetes. PAR immunoreactivity was increased in DFU (particularly in the CNA group; $P < 0.01$) compared with other DPN subjects.

CONCLUSIONS—Increased PAR, reduced type 1 procollagen abundance, and impaired skin structure are associated with foot complications in diabetes. The potential of therapies that improve skin quality to reduce DFU needs to be investigated.

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The foot complications of diabetes remain a cause of considerable morbidity (1,2). Although early identification of foot insensitivity, vascular insufficiency, and deformities has helped reduce the incidence of foot complications, chronic ulceration remains one of the most common and most serious consequences of diabetes.

Foot ulceration is frequently recurrent, reflecting in part the increased susceptibility of lower limb skin to trauma. Skin structural and biochemical deficits, such as dermal atrophy, reduced fibroblast numbers and proliferative capacity, reduced procollagen synthesis, and increased levels of connective tissue–degrading matrix metalloproteinases (MMPs), have been implicated in the pathogenesis of chronic wounds (3,4). Increased oxidative/nitrosative stress may also impair skin structure and disrupt skin microvascular function by overactivation of poly(ADP-ribose) (PAR) polymerase (PARP) (5–7). Therapeutic approaches aimed at reversing these biochemical and structural deficits, thereby improving the overall quality of the skin, in diabetes may reduce foot complications.

Subjects with diabetes exhibit a range of vascular and inflammatory complications that may affect skin quality. For example, compared with subjects with diabetic peripheral neuropathy (DPN) alone, subjects with Charcot neuroarthropathy (CNA) demonstrate distinctive small nerve fiber neurologic deficits and skin vascular responsiveness that may predispose to ulceration (8–10). However, it is unknown whether small nerve fiber loss or changes in skin quality differentiates these subjects from other neuropathic patients.

Understanding the effect of diabetes on skin structure and function is important for the detection of those subjects at highest risk of developing foot complications and the development new preventative therapies. We hypothesized that some subjects with diabetes and DPN may be predisposed to develop diabetic foot ulceration (DFU) or CNA as a result of specific deficits in skin innervation and skin quality that would therefore represent an additional risk factor in these subjects. Thus, we sought to compare skin biochemical and structural deficits in subjects with and without specific diabetic foot complications.

RESEARCH DESIGN AND METHODS

A cross-sectional study was performed of randomly selected adults with diabetes recruited from diabetes and foot clinics of a hospital-based diabetes center in the U.K. The project was approved by the Black Country Research Ethics Committee (REC 08/H1202/137). All subjects provided written informed consent.

DPN was assessed using the Michigan Neuropathy Screening Instrument (MNSI) (11). DPN was diagnosed if the MNSI examination (MNSIe) score was >2 and/or the MNSI questionnaire (MNSIq) score was ≥7 (11). CNA was confirmed by radiology. Significant peripheral vascular disease (PVD) was excluded by review of the medical records, the absence of symptoms of intermittent claudication, the detection of all peripheral pulses, and the presence of normal Doppler wave forms in the foot. Media and tissues were analyzed blinded to subject group.

Human skin organ cultures

Organ cultures of human skin were prepared as described previously (8). Punch

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biopsies (3-mm full thickness) of upper and lower leg skin were obtained. Tissue was immersed in MCDB-153 (Sigma-Aldrich, Gillingham, Dorset, U.K.) culture medium containing 1.4 mmol/L $CaCl_2$ and 6 mmol/L glucose. Cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 8 days.

MMP and tissue inhibitor of metalloproteinase-1 assays

Cultured media were assayed for MMP-1, MMP-2, and MMP-9 by casein and gelatin zymography, respectively, as described previously (3,4). Zymographic images were digitized and quantified by scanning densitometry. Culture fluids were assayed for tissue inhibitor of metalloproteinase-1 (TIMP-1) (12) by ELISA (R&D Systems, Abingdon, Oxfordshire, U.K.).

Skin structural deficit score

Tissue was fixed in 10% formal saline and processed for paraffin histology. Sections (4 μm) were stained with hematoxylin-eosin and blinded. To assess collagen structure, four parameters were evaluated—fibril thickness, space between fibers, degree of organization, and depth of any disorganization—using a scale of 1–9 for each parameter, where 1 is normal and 9 is maximal fiber damage.

Type 1 procollagen immunohistochemistry

Paraffin sections (4 μm) were dewaxed and endogenous peroxidases were removed. After antigen retrieval, nonspecific staining was blocked followed by incubation with antibodies to type 1 procollagen (Millipore, Hertfordshire, U.K.; 1:400). Immunoreactivity was revealed using a VECTASTAIN Universal Elite ABC Kit (Vector Laboratories Ltd., Cambridgeshire, U.K.) and diaminobenzidine (DAKO, Cambridgeshire, U.K.). Sections were counterstained with Mayers hematoxylin, dehydrated, and mounted. Images were captured using a Ziess Axiostar Plus microscope and an Axiovision 4.4 program. The area and density of staining within an area of 1,000 × 1,000 pixels was determined by image analysis after subtraction of background staining, and the product was calculated and presented as the overall abundance.

Intraepidermal nerve fiber density

Four randomly selected 50-μm sections were immunohistochemically stained using a free-floating protocol with rabbit anti-human polyclonal PGP9.5 antibody (Biogenesis; 1:1,200) (13). Individual epidermal nerve fibers were counted as they passed through the basement membrane. Negative controls comprised replacing the PGP9.5 antibody with rabbit immunoglobulin. Epidermal nerve counts were expressed as the number of fibers per millimeter of epidermis.

PAR

Paraffin sections (4 μm) were dewaxed and endogenous peroxidases were removed and blocked. After antigen retrieval, mouse monoclonal anti-PAR antibody (1:400; Enzo Life Sciences, Exeter, U.K.) was applied and then washed. Sections were incubated with ImPRESS universal reagent (Vector Laboratories). Immunoreactivity was revealed using diaminobenzidine (Vector Laboratories), and sections were counterstained with hematoxylin. The number of stained and unstained endothelial nuclei were counted in 10 fields from each section using ×400 magnification and resulting in 300–400 nuclei being assessed. The results are expressed as percent PAR–stained nuclei (6).

Statistical analysis

Data analysis was performed using SPSS 15.0 for Windows (LEAD Technologies Inc.). Continuous data are presented as mean ± SD. The two-tailed independent *t* test was used to assess the impact of diabetes (vs. no diabetes) on the study outcomes. To assess outcomes across multiple independent groups, one-way ANOVA was used. Equality of group variances was tested using the homogeneity of variance test. If the assumption of equal variance was violated, the Welch statistic was used to test for the equality of group means. Post hoc analysis was performed using the Tukey method if equal variances assumed and the Games-Howell method if equal variances not assumed. ANCOVA was used to examine whether certain scale variables have an effect on the differences observed between multiple independent groups. $P \leq 0.05$ was considered significant. An analysis was conducted in which all patients with DFUs were grouped together whether or not they had CNA. A subgroup analysis comparing patients with DFU and CNA and those with DFU without CNA was also performed.

RESULTS

Patient population

In total, 49 patients with diabetes (mean age 58 ± 8 years, range 42–73 years, 63.5% male) were recruited (Table 1). These comprised 9 diabetic control (DC) subjects, 16 with DPN alone, and 21 with recurrent DFUs (including 9 with CNA). A group of 14 nondiabetic control (NDC) subjects was recruited for comparison. Patients' characteristics are summarized in Table 1. Subject groups with diabetes were of similar age and diabetes duration. HbA_{1c} values tended to be higher in subjects with DPN and foot complications. The MNSIq and MNSIe scores did not differ between subjects with DPN alone or those with foot complications. None of the patients had evidence of PVD. Skin biopsies did not result in any major adverse events; one patient developed bruising around the biopsy site, which completely recovered without the need for treatment.

Effect of diabetes and its complications on intraepidermal nerve fiber density

Intraepidermal nerve fiber density (IENFD; lower leg scores shown in parentheses) was significantly reduced in DC compared with NDC subjects, 12 ± 1 (10 ± 2) vs. 17 ± 3 (14 ± 1) fibers/mm ($P = 0.001$ and $P < 0.001$ for upper and lower leg, respectively), a difference that remained significant ($P < 0.001$) after adjustment for age. The presence of DPN alone 10 ± 2 (9 ± 1) fibers/mm or DFU 10 ± 1 (8 ± 1)

Table 1—*Summary of baseline characteristics*

	NDC (*n* = 14)	DC (*n* = 9)	DPN (*n* = 16)	DFU (*n* = 21)
Age (years)	45 ± 15	57 ± 10	60 ± 7*	57 ± 8*
Male (%)	57	56	44	81
Diabetes duration (years)	N/A	15 ± 9	19 ± 13	18 ± 9
HbA_{1c} (%)	N/A	7.4 ± 1.1	8.9 ± 1.2**	8.9 ± 0.9**
MNSIq score	N/A	2.2 ± 1.7**	5.9 ± 3.0	6.4 ± 2.2
MNSIe score	N/A	1.7 ± 0.8**	5.5 ± 1.8	6.6 ± 2.0
Retinopathy (proliferative) (*n*)	N/A	0	8	16
Nephropathy (*n*)	N/A	0	2	6

Data are mean ± SD. N/A, not applicable. *$P < 0.05$ vs. NDC. **$P < 0.05$ vs. other groups.

fibers/mm was associated with a further reduction of IENFD ($P = 0.003$ and $P = 0.095$ for DPN vs. DC subjects and $P < 0.001$ and $P = 0.007$ for DFU vs. DC subjects in upper and lower leg, respectively). These differences were significant after correction for age ($P \leq 0.01$) except for the difference in IENFD between DC and DPN groups in the lower leg ($P = 0.18$). No significant differences, however, were detected between DPN and DFU groups.

Effect of diabetes and its complications on skin structural deficit scores

Diabetes disrupted skin structure, with the mean skin structural deficit scores (SSDSs) higher in diabetic compared with NDC subjects (3.6 ± 1.0 vs. 2.8 ± 0.7, $P = 0.005$), which remained significant ($P = 0.02$) after age adjustment. All parameters of collagen structure in upper and lower leg biopsies differed between subjects with diabetes and NDC subjects (Table 2). Analysis of individual groups of subjects with diabetes demonstrated that mean SSDS was significantly ($P < 0.001$) worse in DFU subjects compared with NDC subjects. The difference between DC and DFU subjects was also significant ($P = 0.024$). Scores in DC subjects, however, were not significantly different from NDC subjects. Analysis of individual parameters of collagen structure demonstrated significant deficits in fibril thickness in DFU subjects compared with DC subjects.

Effect of diabetes and its complications on type 1 procollagen abundance

Overall in the upper leg skin, the abundance of type 1 procollagen was reduced by 34% by diabetes (715 ± 100 vs. 476 ± 252 units, $P < 0.001$, respectively). This reduction principally reflected reduced levels in DFU subjects (387 ± 256 units), which were decreased by 46% ($P < 0.001$). The reduction of type 1 procollagen in DC (569 ± 219 units) and DPN (542 ± 237 units) subjects was not statistically significant. In lower leg skin, levels of type 1 procollagen were on average 27% ($P < 0.001$) lower than in the upper leg. Type 1 procollagen levels were also reduced in the lower leg in subjects with diabetes compared with NDC subjects (614 ± 104 vs. 321 ± 135 units, $P < 0.001$). All categories of patients with diabetes had lower type 1 procollagen levels compared with NDC subjects, with the greatest deficits evident in DFU subjects in which levels were significantly below DC ($P < 0.001$) and DPN ($P < 0.01$) subjects (Fig. 1).

Effect of diabetes on MMP-1, MMP-2, MMP-9, and TIMP-1

Levels of total (active and latent forms) MMP-1, MMP-2, and MMP-9 were not different in NDC and diabetic subjects (data not shown). However, in the upper leg, compared with NDC subjects, subjects with diabetes exhibited marked activation of MMPs. For example, in NDC subjects, 33 ± 12, 28 ± 15, and 30 ± 28% of skin MMP-1, MMP-2, and MMP-9, respectively, was in the active form. In patients with diabetes (all diabetic groups combined), these percentages had increased to 50 ± 17 ($P < 0.01$), 39 ± 14 ($P < 0.05$), and 50 ± 31% ($P < 0.05$). Active MMP-1 levels were significantly higher in DFU (52 ± 17, $P < 0.05$) than NDC subjects (which was not significant after age adjustment). No differences of active MMP abundance, however, were detected between individual patient groups with diabetes (data not shown). Levels of TIMP-1 were reduced by 29% ($P = 0.02$) in subjects with diabetes (154 ± 45 vs. 106 ± 69 ng/mL in NDC and diabetic subjects, respectively) a difference that was not significant ($P = 0.09$) after adjustment for age. Levels of TIMP-1 were most reduced in subjects with DPN alone (75 ± 43 ng/mL) compared with NDC ($P = 0.006$) and DC (160 ± 68, $P = 0.028$) subjects, which remained significant after age adjustment ($P = 0.037$ and 0.048 for NDC and DC subjects, respectively). In the lower leg, levels of MMP activity were insignificantly reduced compared with upper leg values, but the differences between patient groups remained similar to findings in the upper leg (data not shown).

PARP

In the upper leg skin, the percentage of skin PAR–stained nuclei was increased by 32% in diabetic compared with NDC subjects (70 ± 11 vs. 53 ± 9%, $P < 0.001$), an effect that persisted after adjustment for age ($P < 0.001$). Analysis of individual groups of subjects with diabetes demonstrated that this increase of PARP activity was observed in the DC (67 ± 16), DPN (67 ± 10), and DFU (72 ± 9%) subject groups. After adjustment for age, the increase in PARP remained highly significant in the subjects with DFU ($P = 0.001$). Analysis of lower leg skin differentiated between neuropathic groups, with levels in DFU subjects being significantly greater (Fig. 2).

Comparison of DFU patients with and without CNA

The DFU group comprised 9 subjects with and 12 without CNA. We therefore explored whether any of the differences reported above were related to CNA rather than DFU. Age (56 ± 7 vs. 58 ± 8 years, $P = 0.677$) and diabetes duration (19 ± 10 vs. 18 ± 10, $P = 0.750$) were similar between patients with CNA and without CNA, but HbA_{1c} (9.3 ± 0.8 vs. 8.0 ± 0.8, $P = 0.028$) was higher in the CNA group. We compared measurements of IENFD, SSDS, MMPs, TIMP, and PAR in the upper and lower leg between patients with DFU and CNA and those with DFU without CNA. No differences in any of these parameters were detected between these two groups except for PAR staining in the lower leg, which was significantly higher in patients with CNA (86 ± 4 vs. 76 ± 1%, $P = 0.001$)

Table 2—*Assessment of skin structure in hematoxylin-eosin–stained sections*

	Fibril thickness	Space between fibrils	Degree of organization	Depth of disorganization	Mean
NDC	2.2 ± 0.6 (2.3 ± 0.4)	2.6 ± 0.8 (2.5 ± 0.4)	3.2 ± 1.0 (3.1 ± 0.4)	3.2 ± 0.9 (3.1 ± 0.4)	2.8 ± 0.7 (2.7 ± 0.3)
DC	2.3 ± 0.6 (2.4 ± 0.4)	2.7 ± 1.0 (3.0 ± 0.7)	3.4 ± 0.7 (3.8 ± 0.8)	3.6 ± 0.3 (3.6 ± 0.7)	3.1 ± 0.8 (3.2 ± 0.5)
DPN	2.5 ± 0.5 (3.0 ± 0.7)	2.8 ± 0.6 (3.6 ± 1.2)*	4.0 ± 1.0 (4.5 ± 1.1)*	4.0 ± 1.2 (4.2 ± 1.0)*	3.3 ± 0.6 (3.8 ± 1.0)*
DFU	3.1 ± 0.8**†^ (3.5 ± 0.8)*†	3.9 ± 1.7* (3.9 ± 0.7)**	3.7 ± 1.5**§ (5.2 ± 0.8)**†	4.9 ± 1.5* (5.1 ± 0.9)**§^	4.1 ± 1.1**^ (4.4 ± 0.6)**§

Each of the four parameters was given a score of 1–9 (1 = normal, 9 = maximum damage). Values for the lower leg are shown in parentheses. Data are mean ± SD. *$P < 0.05$ vs. NDC. **$P < 0.01$ vs. NDC. †$P < 0.05$ vs. DC. §$P < 0.01$ vs. DC. ^$P < 0.05$ vs. DPN.

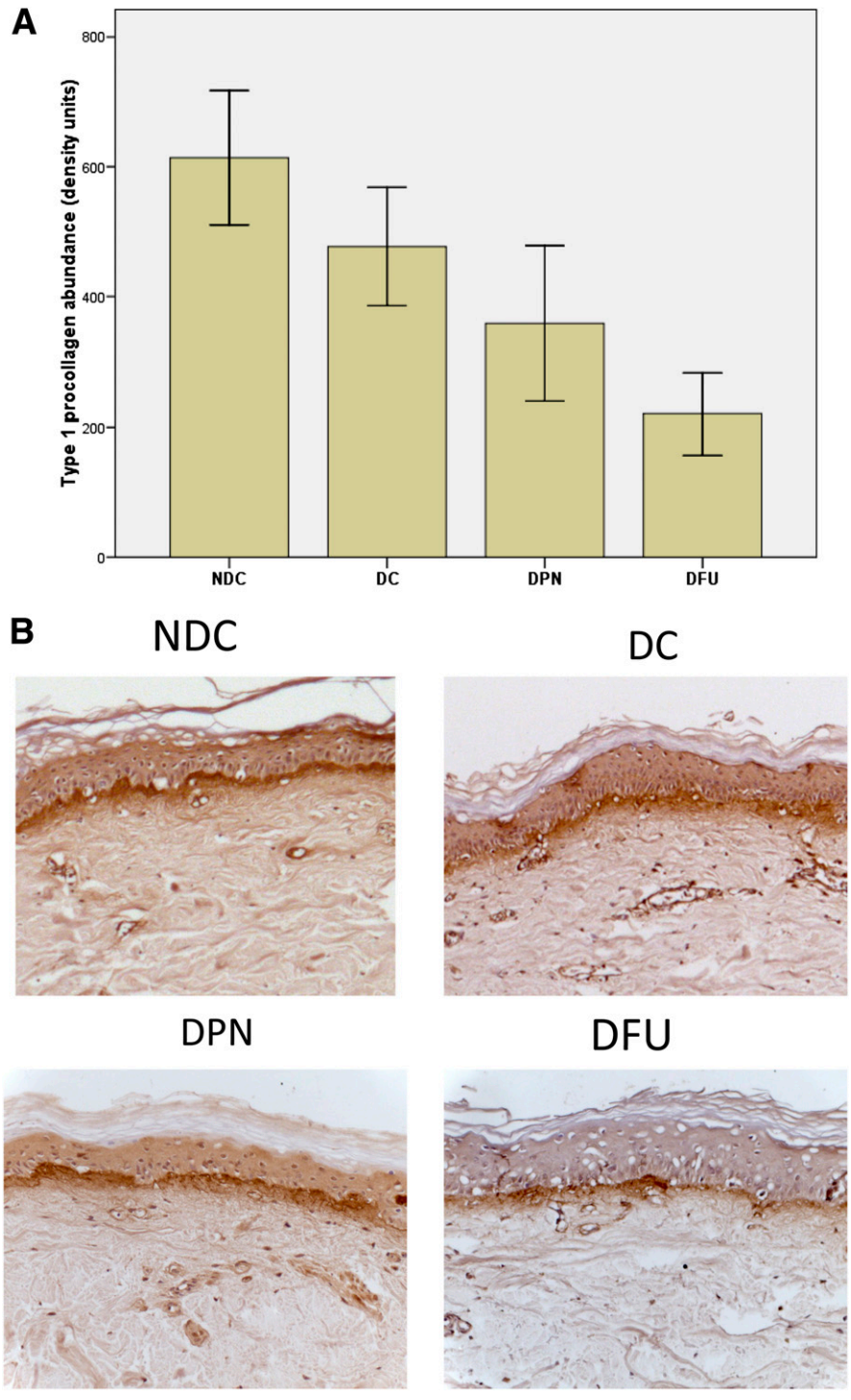

Figure 1—A: *Comparison of type 1 procollagen abundance in the lower leg in different subject groups. Skin tissue from nondiabetic and diabetic subjects was maintained in organ culture for 8 days. At the end of the incubation period, sections were dewaxed, blocked with 10% horse serum, and incubated with antibodies to type 1 procollagen, and the immunoreactivity was revealed using a VECTASTAIN Universal Elite ABC Kit and diaminobenzidine. The area and density of staining was determined by image analysis using Scion Image. Data are mean ± SD.* P *< 0.001 for DFU vs. NDC;* P *= 0.002 for DFU vs. DC;* P *= 0.025 for DFU vs. DPN;* P *< 0.001 for DPN vs. NDC.* B: *Representative immunohistochemical staining for type 1 procollagen in the skin of healthy subjects and subjects with and without DPN and DFU. Original magnification ×200. (A high-quality color representation of this figure is available in the online issue.)*

and which remained significant after adjusting for HbA_{1c} ($P = 0.002$).

CONCLUSIONS—Diabetes can be complicated by a range of neurologic, biochemical, inflammatory, and vascular skin deficits that may increase the risk of foot complications (6,9,10,14). Our goal was to determine whether high-risk patients exhibited specific skin structural and functional deficits that could serve as potential biomarkers and may ultimately predispose to foot complications. We found that increased PARP, reduced type 1 procollagen, and impaired skin structure are associated with the development of foot complications. There were no differences in diabetes control or DPN severity among the DPN groups to account for our findings. In addition, none of the subjects had clinical evidence of PVD.

Measurement of IENFD is widely accepted as a highly reproducible (13) tool for the quantitative assessment of DPN (15–17). Our findings that IENFD is reduced in all subjects with diabetes and that the presence of neuropathy further exacerbates this deficit is consistent with other reports (17). This is the first report to assess IENFD in CNA, and the lack of difference of IENFD in subjects with CNA and in subjects with DFU indicates that the skin biochemical and structural differences detected between patient groups does not primarily reflect differences in the severity of small fiber neuropathy. Moreover, these findings suggest that the selective neuropathy and altered skin blood flow regulation observed in some subjects with CNA (9) may not result from changes in IENFD.

In the upper leg, SSDSs were not significantly impaired by diabetes or DPN alone but were significantly increased in DFU subjects. In the lower leg, multiple aspects of skin structure were disrupted in subjects with DPN alone, although the greatest deficits were observed in subjects with DFU. Collagen structure was assessed using four parameters that have proven useful in the past to document connective tissue fiber damage in aged skin (4). This is the first report of the application of this score to patients with diabetes, DPN, or foot complications. In contrast to skin structure, active MMP levels were higher in diabetes irrespective of the presence of complications. Increased elaboration of activated MMPs has been proposed to precede changes in skin structure (4). Our findings therefore challenge this view and indicate that differences in MMP elaboration

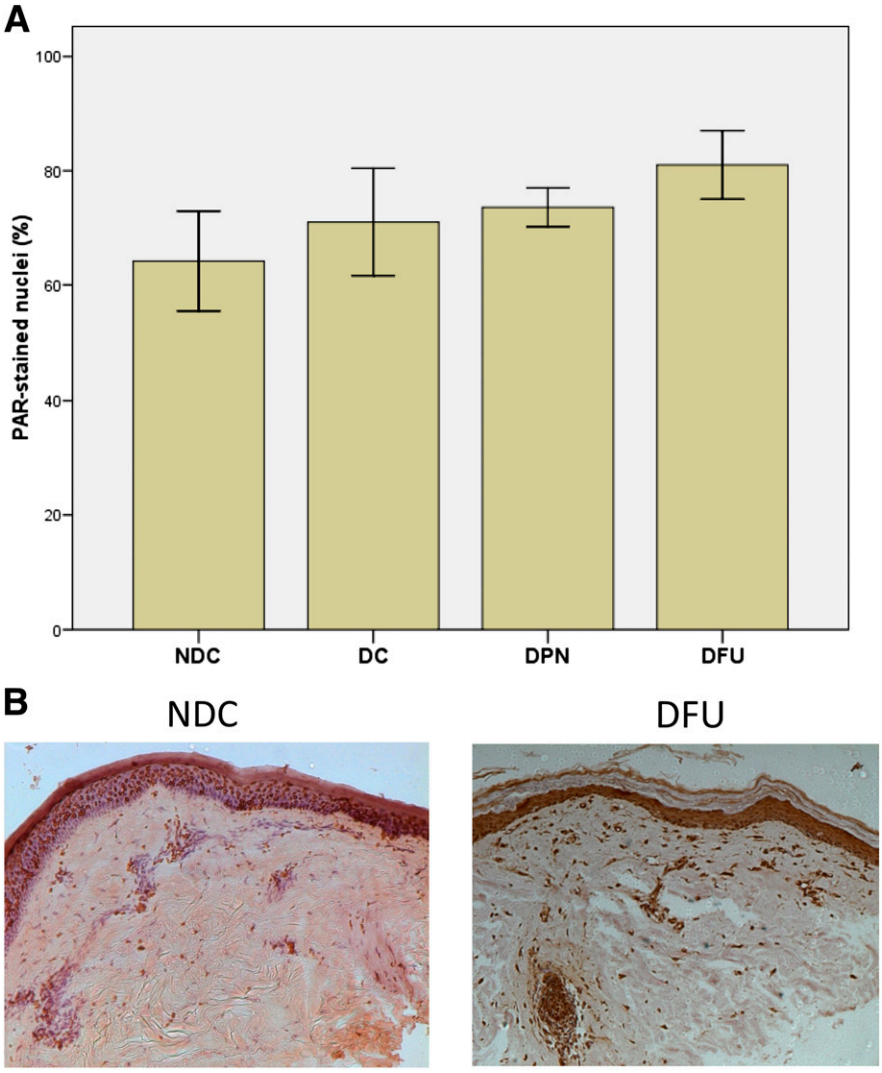

Figure 2—A: *Comparison of percentage of PAR + nuclei in different subject groups. Lower leg skin tissue from nondiabetic and diabetic subjects was maintained in organ culture for 8 days. Paraffin sections (4 μm) were prepared. Mouse monoclonal anti-PAR antibody (1:400) was applied to sections. Immunoreactivity was revealed using diaminobenzidine, and sections were counterstained with hematoxylin. Data are mean ± SD.* $P < 0.001$ *for DFU vs. NDC;* $P = 0.006$ *for DFU vs. DPN;* $P = 0.018$ *for DPN vs. NDC.* B: *Representative immunohistochemical staining for PAR in the skin in an NDC subject and a patient with DFU (and CNA). (A high-quality color representation of this figure is available in the online issue.)*

alone do not appear to be the principal mechanism leading to the differences in skin structure observed across the different subject groups.

Type 1 collagen protein is the most abundant skin structural protein (12). Thus, we sought to determine whether type 1 procollagen was particularly depleted in subjects with DFU and could serve as a potential biomarker of subjects most at risk. The reduction of type 1 procollagen was statistically significant in the DC subjects and was significantly greater in DFU than DC subjects. This finding raises the possibility that subjects with DPN may be predisposed to the development of DFU in part because of specific impairment of skin quality. Analysis of skin structure in high-risk subjects with DPN therefore may offer the opportunity to identify subjects at the highest risk. Nevertheless, since our study was of cross-sectional design, prospective studies will be required to assess whether the presence of skin structure deficits can predict the development of foot complications.

The mechanism whereby type 1 procollagen becomes depleted in subjects with DFU is unknown. Collagen synthesis is regulated at a transcriptional and posttranslational level (18). Increased reactive oxygen species has been reported to increase gene expression and the activity of MMP-1, reduce the expression of pro-α1 (I) collagen and pro-α1(III) collagen (18), and decrease collagen production (19) in human dermal fibroblasts. Therefore, increased oxidative stress, which is known to complicate both DPN (20) and DFU (21), could play an important role in this finding.

We sought to obtain evidence for a downstream consequence of increased oxidative/nitrosative stress on skin structure and function by measuring the activation of PARP (6). An increase of skin PARP activity has previously been demonstrated in patients with uncomplicated diabetes and associated with impaired skin microvascular reactivity (6). In the upper leg, PARP activity was elevated in subjects with DFU. However, in the lower leg, the increase in PARP activity in the DFU group was greater in the group that had CNA and DFU. The increase of PARP in CNA is consistent with a recent report that the receptor for advanced glycosylation end products defense mechanisms are impaired in patients with CNA (22), a finding that may be etiologically important in the pathogenesis of skin microvascular blood flow deficits (14) as well as bony fractures (22,23) and implies that multiple downstream targets of oxidative stress are activated in these subjects.

A limitation to our study was the younger age of our NDC group as well as the relatively small sample sizes. We have adjusted our data to account for this age difference; however, such adjustment assumes a linear relationship between age and the parameters tested, which may not always be the case. Nonetheless, such a linear relationship does exist in our cohort, and previous reports indicate a linear reduction in skin thickness and elasticity with age (24,25). Moreover, the subjects with DPN alone and those with foot complications were of similar age and, thus, this factor could not account for the differences observed. For safety reasons, skin biopsies were taken from the leg rather than the plantar surface of the foot, which is a frequent site of neuropathic ulceration and, therefore, it is unclear whether our results extend to this location. Nevertheless, our data comparing the upper and lower leg demonstrate that although the quality of skin is decreased in the distal limb in all subjects, the differences between subject groups are maintained and for some end points (e.g., type 1 procollagen) augmented.

Indeed, preliminary studies of plantar skin taken from amputation specimens from DFU patients indicate that skin structure is similarly impaired and procollagen abundance equally reduced at this location.

In conclusion, increased PARP, reduced type 1 procollagen, and impaired skin structure are associated with the development of foot complications in diabetes and may constitute novel biomarkers to identify patients at maximal risk. Therapies aimed at improving skin quality also warrant consideration as an approach to reduce DFU.

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A.A.T. recruited patients; performed skin biopsies, laboratory studies, and statistical analysis; and wrote and reviewed the manuscript. W.Z. and S.H. performed laboratory studies and reviewed the manuscript. J.S. performed skin biopsies and reviewed the manuscript. M.K.P. recruited patients, performed skin biopsies, and reviewed the manuscript. K.D. recruited patients, performed skin biopsies and laboratory studies, and reviewed the manuscript. M.J.S. conceptualized and designed the study, obtained funding, performed skin biopsies, recruited patients, and wrote and reviewed the manuscript. M.J.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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