Neutral Endopeptidase Activity is Increased in the Skin of Subjects with Diabetic Ulcers

Marcos A. Antezana, Stephen R. Sullivan, Marcia L. Usui, Nicole S. Gibran,* Michelle L. Spenny,* Jerrie A. Larsen,† John C. Ansel,‡ Nigel W. Bunnett,§ and John E. Olerud

Departments of Medicine (Dermatology) and *Surgery, University of Washington, Seattle, WA, U.S.A.; †Department of Orthopaedics, Veterans Affairs Puget Sound Health Care System, Seattle, WA, U.S.A.; ‡Department of Dermatology, Emory University, Atlanta, GA, U.S.A.; §Departments of Physiology and Surgery, UCSF, San Francisco, CA, U.S.A.

Cutaneous sensory nerves mediate inflammation and wound healing by releasing neuropeptides, such as substance *P*, which stimulates pro-inflammatory responses by keratinocytes, fibroblasts, and endothelial cells. The cell surface enzyme, neutral endopeptidase, degrades substance *P*, thereby regulating its biologic actions. We hypothesized that neutral endopeptidase enzymatic activity is increased in chronic wounds and skin from subjects with diabetes. We compared cutaneous neutral endopeptidase expression and enzymatic activity between normal controls and diabetic subjects with neuropathy and chronic wounds. Skin samples from subjects with diabetes were taken at the time of amputation for nonhealing ulcers. Skin taken from the ulcer margin, 1 cm from the ulcer (adjacent), and from the most proximal region of the amputated leg were studied. Skin biopsies from the leg of healthy control subjects were also studied. Neutral endopeptidase was localized by immunohistochemistry in all tissue sections. Neutral endopeptidase activity was measured using a fluorimetric assay. The median neutral endopeptidase activity of the ulcer margin was 1.21 × higher ($p > 0.2$) than adjacent skin, 5.26 × higher ($p < 0.001$) than proximal skin, and 15.22 × higher ($p < 0.001$) than control skin. Adjacent skin had a median neutral endopeptidase activity 4.34 × higher ($p < 0.001$) than proximal skin and 12.58 × higher ($p < 0.001$) than control skin. The median neutral endopeptidase activity of proximal skin was 2.90 × higher ($p < 0.001$) than control skin. This elevated neutral endopeptidase activity in the skin and chronic ulcers of subjects with diabetes combined with peripheral neuropathy may contribute to deficient neuroinflammatory signaling and may impair wound healing in subjects with diabetes. ***Key words: inflammation/nerves/neuropeptides/substanceP/wound healing. J Invest Dermatol 119:1400–1404, 2002***

The cutaneous sensory nervous system carries afferent signals originating in the periphery towards the central nervous system for interpretation of pain, pressure, and thermal stimuli. Sensory nerves extend into the epidermis as finely branching filaments that appear to originate from either small dermal nerve bundles (Hilliges *et al*, 1995) or a single dermal trunk (Reilly *et al*, 1997). These nerve fibers are noted by light (Hilliges *et al*, 1995), electron (Hilliges *et al*, 1995), and confocal microscopy (Reilly *et al*, 1997) to be distributed throughout the basal, spinous, and granular layers of the epidermis and make close contact with keratinocytes via membrane–membrane apposition. Nerve fibers also appear to contact Langerhans cells (Hosoi *et al*, 1993) and human dermal microvascular endothelial cells (Ansel *et al*, 1997). Many epidermal and dermal sensory nerves stain positively for substance P (SP) (Levy *et al*, 1989; Lindberger *et al*, 1989; Schulze *et al*, 1997). The presence of SP in free nerve fiber endings and the proximity of these endings to a variety of cells in the skin seems to implicate the cutaneous sensory nervous system not merely for its role in sensation but in other biologic actions as well (Ansel *et al*, 1996).

Neuropeptide involvement in cutaneous inflammation has been extensively demonstrated (Payan, 1989). By means of its actions on the vasculature, cutaneous epithelium, and connective tissue, SP may mediate events involved in cutaneous inflammation and wound healing (Ansel *et al*, 1996). Through both direct action on endothelial cells and stimulation of increased nitric oxide release, SP stimulates vasodilatation and microvascular permeability (Khalil and Helme, 1989; Baraniuk *et al*, 1990). Expression of adhesion molecules on endothelial cells (Quinlan *et al*, 1998, 1999; Lindsey *et al,* 2000), monocyte chemotaxis (Helme *et al*, 1987), and inflammatory cell activity (Lambert and Granstein, 1998) are all upregulated by SP. SP stimulates neutrophil and mast cell activity directly and induces human-dermal microvascular endothelial cells to produce interleukin-8 (Kramp *et al*, 1995). SP also stimulates DNA synthesis (Tanaka *et al*, 1988) and proliferation (Tanaka *et al*, 1988; Paus *et al*, 1995) by keratinocytes, and DNA synthesis by fibroblasts (Nilsson *et al*, 1985) as well as stimulating proliferation by endothelial cells and inducing neovascularization (Ziche *et al*, 1990).

The effects of SP are mediated primarily through a neurokinin receptor, NK-1R, a G-protein coupled receptor expressed in nervous and peripheral tissues. NK-1R mRNA can be found in keratinocytes, fibroblasts, dermal endothelial cells, and circulating immune cells (Scholzen *et al*, 1998). The major regulatory

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Reprint requests to: John E. Olerud, MD, Department of Medicine (Dermatology), University of Washington, 1959 NE Pacific St, Box 356524, Seattle, WA 98195-6524, U.S.A. Email: olerudje@u.washington.edu

Abbreviations: SP, substance P; NEP, neutral endopeptidase; MNA, 4-Methoxy-2-napthylamine.

mechanism of SP is enzymatic degradation. A zinc metalloprotease known as neutral endopeptidase (NEP) is anchored, by a small hydrophobic amino acid chain with its active site facing the extracellular space (Roques *et al*, 1993), and is present in the cell membrane of the same cells that express NK-1R. NEP competes with NK-1R and hydrolyzes peptide bonds on the amino terminal end of hydrophobic substrates such as SP (Roques *et al*, 1993). Enzymatic degradation of SP requires high concentration and coexpression of NEP with NK-1R (Okamoto *et al*, 1994).

NEP expression is immunolocalized to keratinocytes, dermal appendages, and the microvasculature of the skin (Olerud *et al*, 1999), and is found on cultured skin fibroblasts (Bou-Gharios *et al*, 1995). Fluorimetric assays confirm NEP enzymatic activity in the skin of mice (Paus *et al*, 1994) and guinea pigs (Iwamoto *et al*, 1989). NEP inhibitors increase SP-induced plasma extravasation (Iwamoto *et al*, 1989), and NEP knockout mice exhibit spontaneous plasma extravasation and cutaneous edema (Ansel *et al*, 1996; Scholzen *et al*, 2001). Synthesis and secretion of SP, interaction of SP with the SP receptor on the cell membrane and degradation of SP by NEP are key elements of neuroinflammatory signaling in wound repair (Ansel *et al*, 1996).

In normal unwounded skin, NEP is immunolocalized to the cell membranes of basal keratinocytes (Olerud *et al*, 1999); however, the NEP staining pattern in standardized human incisional wounds (Olerud *et al*, 1995) extends to cover the wound matrix, full thickness of the epidermis, and to a "transitional" zone 30–50 cells lateral to the wound margin by 7 d postwounding (Olerud *et al*, 1999). Increased NEP mRNA was also demonstrated in normal 3 d and 7 d wounds by northern blot analysis. Immunolocalization of NEP returns to normal basal cell staining by 28 d postwounding (Olerud *et al*, 1999).

Chronic, nonhealing foot ulcers are a significant problem for subjects with diabetes, accounting for one-quarter of the days spent in the hospital by this population (Bild *et al*, 1989). Evidence shows that there is a disruption in neural signaling pathways, which is important for wound healing in subjects with diabetes. Subjects with diabetes with both neuropathy and significant peripheral vascular disease show no evidence of SP immunoreactive nerves in the skin (Levy *et al*, 1989). The purpose of this study was to compare cutaneous NEP expression and enzymatic activity in the lower extremities of diabetic subjects with normal controls. Skin samples from various locations on the lower extremity of subjects with diabetes were also studied, including the margin of chronic ulcers, unwounded adjacent skin as well as proximal leg skin.

MATERIALS AND METHODS

Tissue collection Eight subjects with diabetes undergoing lower extremity amputation at the Veterans Affairs Puget Sound Health Care System were recruited. Ten age-matched healthy control subjects were recruited by an advertisement in a daily newspaper. Informed consent was obtained from all subjects in accordance with procedures approved by the University of Washington Institutional Review Board for Research on Humans. The normal volunteers were screened and excluded if they had a history of cardiovascular disease, hypertension, diabetes mellitus, smoking in the past 10 y, or alcoholism. To confirm that the subjects had no evidence of diabetes mellitus, peripheral vascular disease, or neuropathy, a physical examination, including testing for light touch (Semmes–Weinstein filament) and vibration sensation, and a laboratory examination, including fasting blood glucose and hemoglobin A_{1c} levels, were carried out.

Skin samples from diabetic subjects were harvested in the operating room immediately following amputation. Samples were collected from: (i) the ulcer margin; (ii) unwounded skin free of inflammation 1 cm away from the ulcer margin (adjacent skin); and (iii) unwounded skin from the most proximal part of the amputated lower extremity (proximal skin). Unwounded skin samples from control subjects (control skin) were removed 10 cm distal to the patella from a region on the lateral leg comparable with where the proximal sample taken from the diabetic subjects. Samples were taken from control subjects using a 6 mm skin biopsy punch after local anesthesia with 1–2 ml of 1% lidocaine with epinephrine. Samples were trimmed of subcutaneous fat, and frozen as quickly as possible (usually within 1–2 min of excision) in OCT (Sakura Finetek, Torrance, CA) for immunohistochemistry or on a block of dry ice for NEP enzymatic assays. All skin was stored at −70°C.

Immunohistochemistry Six micron frozen tissue sections were immunolabeled using standard ABC methods as previously published (Olerud *et al*, 1999). Briefly, slides were postfixed in 4% paraformaldehyde and incubated with NEP anti-serum at 1:200 dilutions. Biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlington, CA) at 1:200 dilution was used as a secondary antibody followed by streptavidin–biotin complex (SABC Universal Kit, Zymed Laboratories, San Francisco, CA) at 1:200 dilution. 3,3′ diaminobenzidine (Sigma, St Louis, MO) was used as chromogen, control slides were incubated without.

Tissue preparation for NEP enzymatic assay Frozen skin samples were weighed and kept on ice throughout preparation for the enzymatic assay. Skin was placed in a small amount of 50 mmol per l. Tris–HCl, pH 7.5, and cut into smaller pieces using a sterile razor blade. To minimize protein degradation during tissue preparation, 1 mmol per l phenylmethylsulfonyl fluoride (Sigma) and 1 μmol per l Pepstatin A (Peninsula Laboratories, Inc., Belmont, CA) were used. Skin samples were homogenized for 1 min using a Tissue Mizer (Sakura Finetek, Torrance, CA) followed by manual homogenization using a ground-glass homogenizer. Samples were then aliquoted and stored at −70°C. This method was adapted from that previously described by Terashima *et al* (1992).

Determination of protein concentration Protein concentration was measured using Bradford dye reagent (Bio-Rad Laboratories, Richmond, CA) (Bradford, 1976). Twenty microliter aliquots of sample homogenate were thawed, diluted with 20 μl of 1 M NaOH, dissolved at room temperature for 30 min and centrifuged at $14{,}000 \times g$ for 2 min. The supernatant was diluted 1:10 in 50 mmol per l Tris–HCl, pH 7.5. Twenty microliters of the diluted supernatant was added to 780 μl of distilled water. Two hundred microliters of dye reagent was added to the 800 μl of diluted sample, mixed gently and allowed to sit for 5 min. Absorption was read at 595 nm (Beckman DU-50, Beckman Coulter, Inc., Fullerton, CA). Skin samples were run in triplicate and spectrophotometric readings were averaged. The protein concentration (μg per μl) of each homogenate was then determined by comparison with a BSA (bovine serum albumin) standard curve.

Fluorimetric assay NEP activity (pmol 4-Methoxy-2-napthylamine MNA per h per μg) of tissue homogenates was assayed fluorimetrically using a previously described method (Erdos *et al*, 1989; Terashima *et al*, 1992; Bunnett *et al*, 1993; Hwang *et al*, 1993). Aliquots of sample homogenates were thawed and 50 μg of protein, as determined by spectrophotometry, was used per reaction. The homogenates were incubated at 37°C for 10 min in 50 mmol per l Tris–HCl, pH 7.5 containing 4 μg aminopeptidase M per ml (Calbiochem, San Diego, CA). These homogenates were then incubated with or without 25μmol per l DL-thiorphan (Sigma), an inhibitor specific to NEP activity. To each sample, 40 μmol per l glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamine was added (Enzyme Systems Products, Livermore, CA) and incubated at 37°C for 30 min. Samples were centrifuged at $14{,}000 \times g$ for 2 min. Two hundred microliters of supernatant was added to 1.8 ml of 1.1 M dithiothreitol (Sigma) to stop the reaction. Fluorescence of the samples was measured using a fluorometer (Shimadzu, Scientific Instruments, Inc., Columbia, MD) with excitation wavelength of 340 nm and emission wavelength of 425 nm. Skin samples were run in triplicate and fluorescent readings were averaged. The amount of fluorescent product generated by NEP activity was determined by subtracting the arbitrary fluorescence units produced by samples incubated with DL-thiorphan from the arbitrary fluorescence units produced by samples incubated without DL-thiorphan. Only activity that was inhibited by DL-thiorphan was attributed to NEP. Arbitrary fluorescence units of each sample were compared with an MNA (Sigma) standard curve. NEP enzymatic activity was then expressed as pmol of MNA per h per μg of total protein.

Statistical analysis To examine potential differences in protein concentration and cutaneous NEP activity among the skin samples, a regression analysis with indicator variables for the site of biopsy was performed using Stata 6.0 software (StataCorp, College Station, TX 1999). A logarithmic transformation of NEP activity was used to adjust for heteroscedasticity in the distribution of the data. Results, expressed as mean ± SD and 95% confidence intervals (CI), were corrected for multiple comparisons using Bonferroni's method (Kleinbaum *et al*, 1998).

RESULTS

All eight diabetic subjects were males undergoing lower extremity amputation. The fact that all subjects were males reflected the Veterans Administration Hospital population with diabetic ulcers requiring amputation during the study period. Age of patients ranged from 66 to 79 y (mean = 68.7). All patients were insulin dependent at the time of their operation and hemoglobin A_{1c} levels varied from 6.1 to 12.1% (mean = 8.25%). The normal range for hemoglobin A_{1c} is 4.4–6.4%. Neurologic testing revealed an inability to detect a 5.07 Semmes–Weinstein filament consistent with severe diabetic neuropathy in the feet of all but one subject tested.

Control subjects included seven males and three females with ages that ranged from 52 to 79 y (mean = 65.7). History and physical examination established that all normal volunteers were free of clinically apparent cardiovascular disease, peripheral neuropathy, or diabetes mellitus. All control subjects had a normal fasting blood glucose that varied from 83 to 108 (mean = 93) at the time of biopsy and had been previously shown to have normal hemoglobin A_{1c} levels that varied from 4.9 to 6.8% (mean = 5.7%). Neurologic testing with the Semmes–Weinstein filament and tuning fork revealed normal sensation in the feet of all control subjects. None of the control subjects had wounds on the lower extremities.

A total of eight ulcer margins, eight adjacent skin, and six proximal skin samples were taken from diabetic subjects. Two subjects with diabetes had ankle disarticulations rather than below knee amputations, hence no proximal skin specimen was available. Ten control skin specimens were harvested.

Immunohistochemistry showed increased NEP in ulcer margin and adjacent skin of diabetic subjects. NEP was immunolocalized to basal keratinocytes in control skin (**Fig. 1*A***), whereas NEP was dramatically increased throughout epidermal and dermal layers of ulcer margin (**Fig. 1*B***) and adjacent skin (**Fig. 1*C***), and variable in the proximal skin (**Fig. 1*D,E***) of subjects with diabetes. NEP immunostaining of proximal skin was, however, more extensive than control skin. For the most part, immunostaining of NEP was both cytoplasmic and membranous. NEP also appeared to stain extracellular matrix in ulcer margin and adjacent skin samples (**Fig 1*B***, asterisk). Immunohistochemistry without primary antibodies showed no staining (data not shown).

Protein concentration of the skin samples was determined. Protein content of the ulcer margin was 3.85 ± 0.88 μg per μl, adjacent skin was 1.98 ± 0.69 μg per μl, proximal skin was 1.08 ± 0.78 μg per μl, and normal control skin was 0.79 ± 0.20 μg per μl. The statistically significant differences in protein concentration in these skin samples were as follows: the ulcer margin had significantly greater protein concentration than all other skin samples ($p < 0.05$), adjacent skin was significantly higher in protein concentration than control skin ($p < 0.05$), and no other differences in protein concentrations of the skin samples were statistically significant.

The NEP enzymatic assay showed the highest cutaneous NEP activity to be in ulcer margin samples (**Fig. 2**). The cutaneous NEP activity of the ulcer margin was 47.54 ± 17.18 pmol MNA per h per μg. NEP activity in adjacent skin was 41.44 ± 20.48 pmol MNA per h per μg. Proximal skin showed considerably less cutaneous NEP activity than both ulcer margin and adjacent skin with 9.06 ± 3.60 pmol MNA per h per μg. In control skin, cutaneous NEP activity was 3.42 ± 1.84 pmol MNA per h per

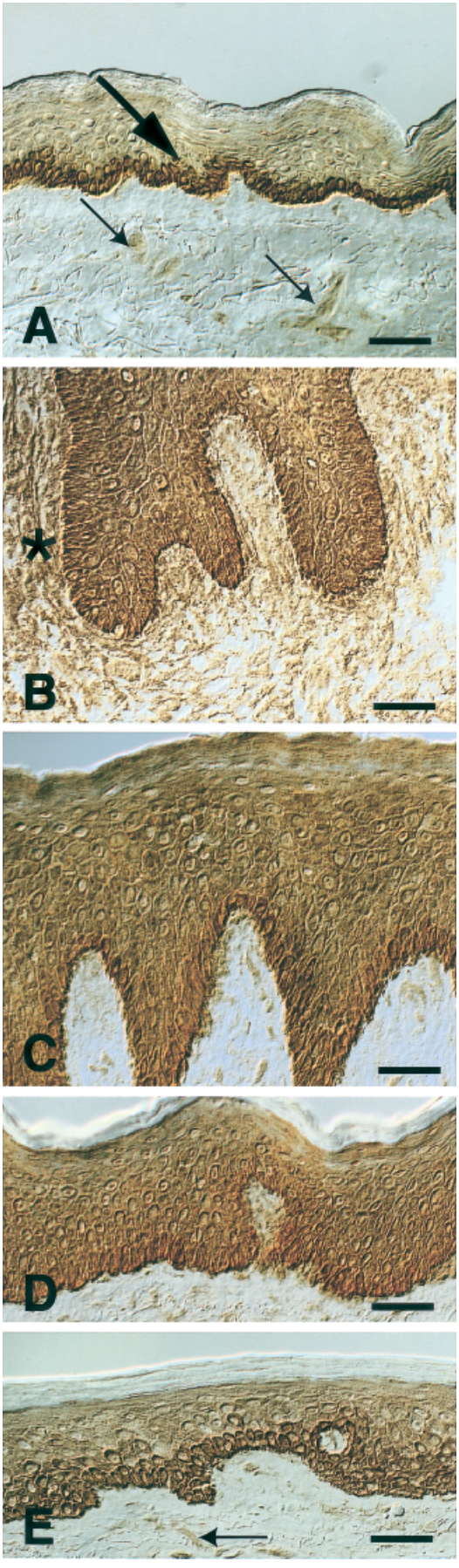

Figure 1. NEP immunohistochemistry of normal and diabetic skin. (*A*) Normal, unwounded skin with *large arrow* indicating NEP staining of basal keratinocytes and *small arrows* showing NEP staining of blood vessels. (*B*) Ulcer margin from a patient with diabetes with asterisk (*) indicating ulcer bed. Note strong NEP staining throughout the epidermis and strong dermal matrix staining. (*C*) Unwounded skin adjacent to the ulcer from a subject with diabetes showing strong NEP staining throughout the epidermis. (*D,E*) Unwounded skin from the proximal regions from two different patients with diabetes showing variability of NEP epidermal staining where *D* shows strong epidermal staining and *E* shows a staining pattern approaching that of normal skin. *Scale bar* = 50 μm

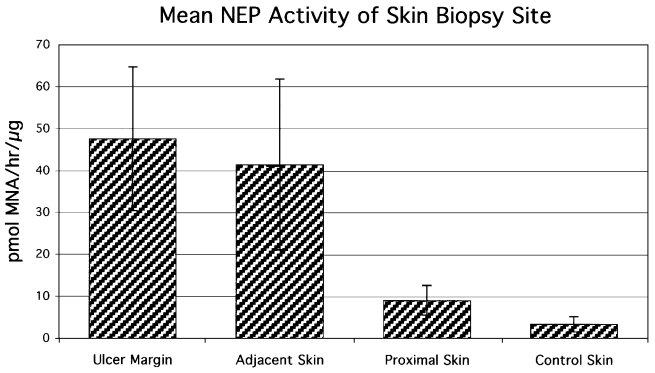

Figure 2. Cutaneous NEP activity (pmol MNA per h per µg) of skin homogenates determined with fluorimetric assay. For each skin sample, three fluorimetric readings were averaged and compared with a standard curve to determine NEP activity. Results are mean ± SD.

Table I. Regression analysis comparing NEP activity with skin sample location

Skin sample comparisons	Median difference in NEP Activity	p-value[a]	95% CI
Ulcer margin (U)/Adjacent skin (A)	1.21 × (U/A)	> 0.2	(1.70–2.49)
Ulcer margin (U)/Proximal skin (P)	5.26 × (U/P)	< 0.001	(2.42–11.45)
Ulcer margin (U)/Control skin (C)	15.22 × (U/C)	< 0.001	(7.68–30.15)
Adjacent skin (A)/Proximal skin (P)	4.34 × (A/P)	< 0.001	(1.99–9.46)
Adjacent skin (A)/Control skin (C)	12.58 × (A/C)	< 0.001	(6.35–24.92)
Proximal skin (P)/Control skin (C)	2.90 × (P/C)	< 0.001	(1.38–6.10)

[a]Adjusted using Bonferroni's correction for multiple comparisons.

µg. Analysis of NEP activity in controls showed no significant differences between male and female subjects. When female subjects were removed from the analysis, cutaneous NEP activity for control men was 3.61 ± 1.90 pmol MNA per h per µg.

When the median cutaneous NEP activity level of the ulcer margin was compared with the median cutaneous NEP activity level for adjacent skin samples 1 cm from the ulcer, the ulcer margin was found to be 1.21 times higher (95% CI: 1.70–2.49 times higher). This difference, however, was not greater than that which could be explained by random sampling error in the absence of a true difference with respect to the effect of site of skin biopsy ($p > 0.2$). The median cutaneous NEP activity of the ulcer margin was 5.26 times higher than the activity level of proximal skin from those same patients (95% CI: 2.42–11.45 times higher, $p < 0.001$) and 15.22 times higher than control skin (95% CI: 7.68–30.15 times higher, $p < 0.001$) (**Table I**).

When the median cutaneous NEP activity level of adjacent skin in subjects with diabetes was compared with the proximal skin samples from those same patients, the adjacent skin was found to be 4.34 times higher than the proximal skin (95% CI: 1.99–9.46 times higher, $p < 0.001$). The median cutaneous NEP activity level for adjacent skin in subjects with diabetes was estimated to be 12.58 times higher (95% CI: 6.35–24.92 times higher, $p < 0.001$) than control skin. The median cutaneous NEP activity level of the proximal skin was estimated to be 2.90 times higher (95% CI: 1.38–6.10 times higher, $p < 0.001$) than control skin (**Table I**).

DISCUSSION

This study documents high levels of NEP enzymatic activity in the skin and high immunoreactivity in the epidermis and dermis of the ulcer margin and adjacent skin of subjects with diabetes compared with normal control subjects. Likewise, median cutaneous NEP activity levels are significantly increased in unwounded skin of subjects with diabetes compared with skin samples taken from normal control subjects at a comparable location on the lateral leg. Progressively and significantly higher levels of cutaneous NEP activity were observed in the skin of subjects with diabetes with values for NEP increasing from proximal to distal, with the ulcer margins having the highest values. The cutaneous NEP activity of the adjacent skin samples tended to be lower than the samples from the ulcer margins but this difference was not statistically significant. These adjacent samples were removed from areas of intact skin without clinically significant inflammation. Hence, we may not conclude from these experiments that chronic ulcers in subjects with diabetes have greater NEP activity than unwounded adjacent skin even though both areas were more than 10-fold higher than skin from normal control subjects. We did not biopsy the legs and feet of diabetic subjects without leg ulcers to evaluate the effect of diabetes alone on NEP expression in the skin. We were concerned that such biopsies by themselves could result in nonhealing wounds. We have, however, observed in diabetic db/db mice that the cutaneous NEP enzymatic activity in unwounded skin is 2–3 × ($p = 0.02$) that of control nondiabetic littermates (Spenny, 2002).

Unwounded skin from the proximal leg of diabetic subjects, however, has much lower NEP activity levels than the more distal sites. One could speculate that these differences in NEP activity between the proximal and distal leg may account for the impaired neuroinflammatory signaling and poor healing of these chronic ulcers. The differences in healing may also be due to regional variation in a variety of other factors, including decreased innervation, decreased skin perfusion, and increased likelihood of trauma. If these differences are due to regional variation, it is interesting to note that higher NEP activity occurs in a region most prone to developing neuropathy and chronic ulcers. Clearly, neuropathy leads to decreased release of neuropeptides. Whereas factors regulating the production of NEP in the skin have not been defined, it is tempting to speculate that somehow the decrease in neuropeptides may be linked to an increase in the production of NEP. It is not known whether the high levels of NEP in the adjacent skin and ulcers of subjects with diabetes proceeds or follows the development of chronic wounds. Previous studies in normal subjects demonstrated an increase in wound NEP during the first week following an acute wound. Thereafter, the NEP immunostaining returned to a pattern and intensity of staining resembling the prewounded state (Olerud *et al*, 1999). The increase and subsequent decrease in NEP after wounding is presumably a mechanism for downregulation of the neuroinflammatory signaling pathways activated during the normal healing response. As NEP degrades SP, a mediator of neuroinflammatory signaling for the repair response in keratinocytes, fibroblasts, endothelial cells, and inflammatory cells (reviewed in Olerud *et al*, 1999), it follows that the high level of NEP observed in the skin of patients with diabetic ulcers could lead to diminished or absent neuroinflammatory signaling and impaired wound healing.

Homogenates of ulcer margin samples have a significantly higher protein concentration than adjacent and proximal homogenates. Given the presence of a significant amount of exudate in the ulcers, the percentage of protein in the ulcer margin homogenates attributable to NEP may actually be decreased relative to that found in adjacent and proximal homogenates. As NEP activity is expressed in terms of total protein used in the reaction, the NEP activity in the viable tissue of the ulcer margin may therefore be even higher than what was reported in this study.

NEP activity was measured in homogenates of full thickness excisional skin biopsies from subjects with diabetes. Whereas the results from this study do not determine which cells in the skin were the source of high levels of NEP, the immunohistochemistry studies showed staining throughout all layers of the epidermis and dermis, suggesting that keratinocyte, fibroblast, and endothelial cell NEP expression is increased and may be the source of high NEP activity. Fibroblasts, endothelial cells, and other cells have been previously shown to express NEP (Ansel *et al*, 1996; Olerud *et al*, 1999).

Whereas this study must be viewed as preliminary because of the limited number of patients and control subjects, the differences observed in NEP enzymatic activity and immunostaining were very large and uniformly observed in all patients. Additionally, diabetic db/db mice show similarly large increases in cutaneous NEP enzymatic activity compared with nondiabetic littermates (Spenny, 2002). We suggest that in diabetic patients with peripheral neuropathy, the combination of decreased neuropeptides resulting from the loss of cutaneous innervation and the increase in cutaneous NEP observed in this study leads to a loss of the neuroinflammatory signaling seen in normal wound healing. We hypothesize that this loss of neuroinflammatory signaling contributes to the poor wound healing observed in diabetes and that treatment may potentially be directed at correcting this deficit.

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