Semiquantitative Analysis of the Histopathological Features of the Neuropathic Foot Ulcer

Effects of pressure relief

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OBJECTIVE — This study was designed to evaluate the histopathology of neuropathic ulcers and whether pressure relief could change such histological patterns.

RESEARCH DESIGN AND METHODS — We compared neuropathic plantar ulcers tissue excised from 10 diabetic patients (group A) with those taken from 10 patients with comparable lesions and glycemic control after 20 days in a total contact cast (group B). Tissue specimens were blindly examined by two independent pathologists for hyperkeratosis, fibrosis, comparable lesions and glycemic control after 20 days in a total contact cast (group B). Tissue excised from 10 diabetic patients (group A) with those taken from 10 patients with comparable lesions and glycemic control after 20 days in a total contact cast (group B). Tissue excised from 10 diabetic patients (group A) with those taken from 10 patients with comparable lesions and glycemic control after 20 days in a total contact cast (group B). Tissue excised from 10 diabetic patients (group A) with those taken from 10 patients with comparable lesions and glycemic control after 20 days in a total contact cast (group B).

RESULTS — Patients in group B showed a marked reduction in ulcer size after 20 days of casting (P < 0.01). The histopathological features of the two groups markedly differed. Group A patients showed a predominance of inflammatory elements as well as matrix alterations, vessel disruptions, inflammation, and debris. Group B ulcers showed a shift toward a reparative pattern with prevalence of neoformed capillaries and fibroblasts. Semiquantitative analysis confirmed the prevalence of hyperkeratosis, fibrosis, inflammation, and cellular debris in group A patients (P < 0.05), whereas cutaneous annexes, capillaries, and granulating tissue were more prevalent in group B lesions (P < 0.01).

CONCLUSIONS — These results indicate that pressure relief with a total contact cast is associated with changes in the histology of neuropathic foot ulcers, indicating reduction of inflammatory and reactive components and acceleration of reparative processes.

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Diabetic neuropathic foot ulcers are a frequent late complication in diabetic patients and the most prevalent lesion of the diabetic foot. Neuropathy is the main determinant of plantar ulcers in diabetic patients (1), and the sequence of events leading to ulceration has been carefully described (2). A critical pathogenetic role is played by the loss of protective sensation due to sensitive neuropathy, which exposes the foot to abnormally high pressure. Concomitantly, modifications of the foot architecture due to motor neuropathy reduce the surfaces upon which increased forces persist. Hence, local hyperkeratosis develops that may be followed by an open lesion, the extension and depth of which are generally proportional to the postural stress (3,4).

Much less is known on the histopathology of the open lesion. The few available studies have come to nonunivocal conclusions, although a common agreement exists supporting substantial histological modification associated with an interruption of tissue repair processes (5,6). The main drawbacks of these studies rely on the lack of uniformity of the methodology, the inclusion of ulcers with different etiology, and the lack of appropriate control lesions (7,8). Even more important, in all of these studies, biopsies of the lesions were obtained under non-controlled conditions, in particular, without eliminating the postural trauma (9). This is important because recent studies have indicated that pressure relief from neuropathic ulcers can accelerate the healing process (10,11).

In the present study, a morphological analysis of foot ulcer has been carried out in carefully selected lesions. Moreover, the effect of pressure relief on the histological features has been determined.
Histopathology of neuropathic foot ulcers

purulent discharge, and odor) or systemic (fever, malaise, and leukocytosis) clinical signs, confirmed by culture specimen examination, peripheral macroangiopathy (ankle/brachial pressure index [ABPI] <0.9), serum creatinine >2 mg/dl, recent episodes of ketoacidosis, malignancies, and any chronic pathology or systemic therapy that could potentially interfere with the healing process were considered exclusion criteria. The study was approved by the local ethical committee, and all patients gave their written voluntary informed consent before participation.

A total of 26 patients met the inclusion/exclusion criteria, but only 20 were actually enrolled in the study: 3 patients did not give their consent, 2 developed a lesion on the contra-lateral foot before inclusion in the study, whereas 1 developed severe osteomyelitis requiring surgery, intravenous antibiotics, and hospital admission.

Study procedures
Upon collection of accurate medical history, patients underwent a focused clinical examination, mainly oriented to determine the size and features of the foot lesion. The lesion size was determined by tracing the surface on a polyurethane film (Opsite flexigrid; Smith and Nephew, Hull, U.K.) and measuring the corresponding area, whereas depth was assessed with a sterile probe. Lesions were staged by the University of Texas grading scale (12), and the feet were examined for the presence of localized or generalized hyperkeratosis, dishydrosis, or fissuration. A two projection X-ray film of the foot was obtained to exclude the presence of osteomyelitis or Charcot’s disease. All X-rays were interpreted by the same radiologist (V.Z.) according to the procedure described by Cavanagh et al. (13). Any modification of the profile of bones and joints, as well as cortical erosion or interruption, bone fragmentation, or fractures were all considered indicative of osteoarticular pathology and led to the exclusion of patients from the study. Patients were then admitted to the Day-Surgery of the Diabetes Section for complete surgical removal of localized hyperkeratosis. After completion of this procedure, patients were randomly assigned to two different treatment groups using a computer-generated randomization list.

Group A. Patients in this group underwent surgical ulcerectomy. Briefly, the entire lesion was excised by performing an elliptical incision including all the ulcerated tissue and sinuses, followed by the suture of the margins (14).

Group B. For patients allocated to this group, the ulcer was dressed with non-adherent parafilm gauze. Then, a total contact cast was manufactured to ensure complete pressure relief from the lesion. The cast covered the leg and the foot, up to the toes. All casts were provided with a plantar heel, so that patients could walk with it. After 20 days, the cast was removed and, upon measurement, ulcers were excised with the same technique adopted in group A patients.

After all surgical procedures, a protective bandaging was applied, and patients were given special postoperative shoes and crutches and were followed up weekly until stitch removal. Metabolic control was assessed at entry and during the study period by measurement of HbA1c, fasting plasma glucose, and home glucose monitoring at each control visit. Patients were advised to seek help for any problem they may have faced during the study by contacting a 24-h active phone number.

Morphological and histological measurements
All ulcers were excised completely in all their extension, and a reference stitch was placed at the distal edge of each ulcer. The tissue was immediately fixed in 10% formal solution, stained, and embedded in paraffin. A complete morphological analysis was performed at the local pathology department upon appropriate staining. Careful description of tissue structural features and cellular component was performed on each lesion analyzed. A semi-quantitative analysis of these parameters was then calculated to allow comparison between the two groups. The following parameters were used for morphological and histological description: 1) presence and severity of hyperkeratosis; 2) presence and severity of fibrosis; 3) number and integrity of cutaneous annexes; 4) number and integrity of capillaries; 5) presence and distribution of inflammatory reaction; 6) presence and distribution of cellular and bacterial debris; and 7) presence and distribution of granulating tissue. The morphohistological assessment was performed in three different thin sections taken respectively from the outer margin of the ulcer, center of the lesion, and midway point between the other two sections as illustrated in Fig. 1. Finally, the presence and distribution of leukocytes, single-layered endothelial cells, and fibroblasts were taken as an index for the degree of inflammation, neoangiogenesis, and tissue repair, respectively. All of these items were evaluated blindly by the same investigators (P.V. and G.N.), and the results were expressed as absent (no presence of any of the considered parameter), scarcely present (parameter detected on <33% of the lesions’ preparations), present (parameter found in 33–66% of preparations), and intensively present (parameter found in >66% of preparations).

Data analysis
A score was arbitrarily assigned (0, absent; 1, scarcely present; 2, present; 3, intensively present) to each of the above mentioned categories to allow numerical comparison. Results are expressed as mean ± SD, and statistical analysis was performed by means of ANOVA, χ², and Mann-Whitney U tests, using Staview 512, running on an iMac computer.

RESULTS — There were no differences for both demographic (age 64.4 ± 10.4 vs. 58.6 ± 12.2 years, duration of diabetes 24.8 ± 5.2 vs. 21 ± 7.1 years) and clinical (APBI 1.1 ± 0.2 vs. 1.1 ± 0.1, vibration perception threshold 37.3 ± 7.4 vs. 40.1 ± 6.2 V) parameters, as well as for the glycemic control at entry (HbA1c 10.7 ± 2.8 vs. 10.1 ± 1.6%) and at the end of the study period (10.5 ± 2.0 vs. 10.1 ± 1.4) between the two groups. Similarly, no differences were apparent both in terms of lesions’ dimensions (area 4.6 ± 1.2 vs. 4.4 ± 1.1 cm², depth 9.2 ± 3.7 vs. 10 ± 3.1 mm) and duration (181.7 ± 93.8 vs. 172.9 ± 97.9 days). All of the ulcers were localized under the metatarsal heads, and grades IA and IIA were equally represented (five IA/five IIA) among patients of both groups.

Before admission in the study, patients did not receive any antibiotic or anti-inflammatory therapy. All patients walked into the clinic while wearing their regular shoes and did not off-load the ulcers in any way before entering the study. Although no quantification for the level of activity was attempted, it should be noted that patients were active up to the very beginning of the study and that they did not report any variation in their activity pattern.
In group B, the casting was well tolerated and caused no complications. A significant reduction in the ulcer size was apparent at the removal of the cast (2.4 ± 0.7 vs. 4.7 ± 1.1 cm²; \( P < 0.01 \)).

**Morphological histology**

Lesions from group A were characterized by a diffuse and intense inflammatory reaction with the presence of all the cellular elements of the inflammatory reaction. Leukocytes, lymphocytes, and macrophages were dispersed all over the lesion, forming nodular conglomerates, mainly localized around small arteries, often infiltrating the vessel walls and the perivascular spaces in a panarteritis-like fashion. Many structures within the lesion, such as vessels, tendons, and sweat glands, were diffusely altered, interrupted, and sometimes grossly fragmented. Furthermore, cellular debris, as well as other fragments of degraded extracellular matrix, and necrotic tissues were present and they showed a progressive dehydration toward the surface of the lesion assuming the aspect of amorphous degenerated material (Fig. 2). Neoangiogenesis and granulating tissue were scarcely appreciated. On the contrary, hyperkeratosis was conspicuous, particularly at the edges of the lesion, and it exceeded the size of the underlying epidermal tissue, although acanthosis involving basal epidermal layer throughout the ulcers was well present. The dermis was hypertrophic with a greater degree of fibrosis, frequently disrupting the normal structure of the extracellular matrix.

The inflammatory cellular component was much less evident in the lesions from group B. Similarly, the amount of debris was lower as compared with group A. In these biopsies there was evidence for granulating tissue spurs rich in fibroblasts, neoangiogenetic vessels, mainly arising from the edges, the bottom, and close to residual cutaneous annexes (Fig. 3). Newly formed small vessels were clearly identifiable by a single-layered endothelial wall. They were mainly oriented from the edges of the lesions toward the inner spaces, following the fibroblasts spurs. Cells were often found to be in a mitotic stage. At the level of the epidermal layer, from the margins of ulcers, keratinocytes increased their replicative activity as well as their number. Sign of migration toward the center of the ulcer was observed in more than one-half of all sections at this level as compared with no more than 10% of sections in group A. The main cellular elements found in group B lesions were fibroblasts.

**Semiquantitative analysis**

When a semiquantitative analysis was used, it became readily apparent that a difference in the histological features was present between the two groups. Each of the determined parameters was significantly different (Table 1). In group A, hyperkeratosis, cellular debris, and inflammatory reaction scores were all significantly higher than in group B. Conversely, all the regenerative markers (cutaneous annexes, granulation tissue, capillaries) were more often found in the lesions from patients in group B. In particular, a highly significant difference was apparent for granulation tissue (2.8 ± 0.4 vs. 0.2 ± 0.4; \( P = 0.0001 \)).

**CONCLUSIONS** — This study describes the morphohistological features of neuropathic foot lesions in diabetic individuals and the changes associated with pressure relief obtained by fiberglass casting. The main finding of our study is that pressure not only seems to play a continuous mechanical stress to the injured foot, but it also supports an inflammatory condition that contributes toward slow-
ing the healing process and wound repair. On the contrary, pressure relief is associated with active repairing processes, as clearly indicated by more evident granulation tissue, more active neovascularization, cutaneous annex regeneration, and overall cell replication. These positive aspects are the likely consequence of pressure relief, because no difference occurred in terms of glycemic control at any phase of the study. A final proof of the impact of pressure relief, however, would require a specific control that was not available in this study. We have compared morphohistological features in separate groups of lesions, whereas a more direct comparison would require comparing the same lesion before and after pressure relief. The inherent difficulty of such an approach is readily apparent when a whole lesion analysis is planned. Nonetheless, we feel confident that the initial lesions were quite comparable between the two groups. Careful selection of the patients, precise definition of the lesions’ characteristics, identification of a common pathogenic mechanism (neuropathy), exclusion of infected ulcers, and comparable overall glycemic control all support quite a homogeneity of the initial lesions in the two groups. Therefore, there are good reasons to assume that the initial histological picture was similar in all patients with no difference between the two randomly generated groups. This assumption is further supported by the similarity of our findings and those reported in previous work on the same issue. In the past, Ferguson et al. (7) obtained biopsies from a heterogeneous sample of ulcers in diabetic patients. They described vessel wall thickening, elastic lamina disruption, and deposition of material forming “cuffs” around the capillaries. Those results were very similar to the ones the same authors found in venous leg ulcers’ biopsies. However, no definite conclusion could be drawn mainly because of the heterogeneity of their material. Loots et al. (8) performed a study on chronic ulcers, including diabetic and venous lesions, and compared them with acute wounds. They found that the former, although lasting for >8 months, contained more inflammatory cells and provisional extracellular matrix than the lesion 19 days after wounding. The authors interpreted their findings as the expression of the inability of chronic ulcers to progress from the acute inflammatory phase to a reparative stage. They described chronic lesions that seemed “frozen” in a chronic low-grade inflammatory state. The same authors, in a more recent work, found that fibroblasts taken from diabetic foot ulcers had a much lower proliferation rate than those from normal control subjects but also compared with those taken from a different point on the contralateral leg of the same patients, independently of the glucose concentration used in the culture medium. Moreover, the fibroblasts taken from the ulcers’ bed showed several structural abnormalities. The authors concluded that some signal had to be generated within the ulcers’ environment capable of affecting the behavior of fibroblasts of the diabetic patient (9).

A major difference, and a main advantage of our work, is that at variance with previous studies, where local biopsies were taken, the entire lesion was excised for thorough morphohistological assessment. Therefore, our description is more likely to reflect the histological nature of the wound rather than the focal lesions. In particular, our evaluation represents an integrated morphohistological description of the lesion for the very simple reason that it represents the weighed ob-

![Image](image-url)

**Figure 2**—Phlogistic infiltrate in a section of a lesion in group A, involving both arterioles and perivascular spaces, and dispersed in all of the subcutaneous layer (A). This is the edge of a lesion from group A. The interruption of the skin, the eschar covering the bottom of the ulcer, and the cellular debris more relevant near the edge of the ulcer (B) are evident.
evident aspects of reparative and reproductive processes are all signs consistent with the hypothesis of the continuous effect of a traumatizing factor. Our picture does not support a histological specificity for neuropathic lesions in diabetes as compared with other conditions (i.e., venous stasis). Nonetheless, pressure emerges as a main trigger for the inflammatory state, as indicated by the positive changes observed after 20 days of complete pressure relief with total contact cast. The reduction of inflammatory infiltrate was accompanied by signs of active tissue regeneration, such as granulating tissue, cell migration from the borders to the center of the lesion, and more importantly, a reduction of >50% in the size of the ulcers. A more direct and quantitative comparison was made possible by an arbitrary score system. By using this semi-quantitative analysis, the differences in the two groups were highlighted.

In group A, the histological features were all consistent with an active inflammatory phase, whereas in group B, an actively proliferating attitude with formation of granulating tissue from the margins and bottom of the ulcer was readily apparent. These changes characterize the evolutive pattern of acute lesions (15–17).

Because no previous studies have investigated the histology of ulcers after off-loading, this has to be considered novel information from the present study. Although no definitive conclusion can be drawn from our still limited number of patients, it is very likely that the relief of pressure from the lesion, obtained with a fiberglass cast, is the determinant of the switch from a phlogistic to a reparative pattern. Two recent studies have confirmed the efficacy of casting in promoting the healing of neuropathic ulcers (10,11). Now, we provide histological evidence for what may be responsible for

### Table 1—Semi-quantitative analysis of histological features of neuropathic ulcers

<table>
<thead>
<tr>
<th>Features</th>
<th>Group A</th>
<th>Group B</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperkeratosis</td>
<td>2.8 ± 0.4</td>
<td>1.8 ± 0.6</td>
<td>0.0018</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>2.8 ± 0.4</td>
<td>1.8 ± 0.9</td>
<td>0.0063</td>
</tr>
<tr>
<td>Cutaneous annexes</td>
<td>0.8 ± 0.6</td>
<td>1.9 ± 0.6</td>
<td>0.0018</td>
</tr>
<tr>
<td>Capillaries</td>
<td>0.5 ± 0.4</td>
<td>2.5 ± 0.8</td>
<td>0.0003</td>
</tr>
<tr>
<td>Inflammation</td>
<td>3 ± 0</td>
<td>1.1 ± 0.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>Cellular debris</td>
<td>2.8 ± 0.6</td>
<td>1.7 ± 1.3</td>
<td>0.0233</td>
</tr>
<tr>
<td>Granulating tissue</td>
<td>0.2 ± 0.4</td>
<td>2.8 ± 0.4</td>
<td>0.0001</td>
</tr>
</tbody>
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Date are means ± SD. *Mann-Whitney U test; z corrected for ties.
the switching from an inflammatory to a regenerative condition.

In conclusion, we postulate that the histology of neuropathic ulcers is conditioned by the application of pressure, which induces reactive modifications in all the tissues of the ulcer with hypertrophy of both the skin and the subcutaneous tissue. The continuous or subcontinuous application of pressure prevents the positive evolution of the lesion, blocking it in the inflammatory phase. The mere relief of pressure is sufficient for promoting the shift of the ulcer to the reparative phase. Our data also allow the conclusion that there are not peculiar histological features, which could distinguish the neuropathic ulcers from other models of chronic lesions.

References