Differences in Cellular Infiltrate and Extracellular Matrix of Chronic Diabetic and Venous Ulcers *Versus* Acute Wounds

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In diabetic patients, wound healing is impaired. We studied the pathogenesis behind this clinical observation by characterizing the pattern of deposition of extracellular matrix (ECM) molecules and the cellular infiltrate in chronic (>8 wk) diabetic wounds, compared with chronic venous ulcers and an acute wound healing model. Punch biopsies were obtained from the chronic ulcer margins and control samples were collected from upper leg skin 5, 19, 28 d and 12 and 18 mo postwounding (p.w.). T cells, B cells, plasma cells, granulocytes and macrophages, and the ECM molecules fibronectin (FN), chondroitin sulfate (CS), and tenascin (TN) were visualized using immunohistochemical techniques. Expression of FN, CS, and TN was detected in dermal tissue early in normal wound healing (5–19 d p.w.). Abundant staining was seen 3 mo p.w., returning to prewounding levels after 12–18 mo p.w. In the dermis of chronic diabetic and venous ulcers with a duration of 12 mo or more, a prolonged presence of these ECM molecules was noted. Compared with normal wound healing: (i) the CD4/CD8 ratio in chronic wounds was significantly lower ($p < 0.0027$) due to a relatively lower number of $CD4^+$ T cells; (ii) a significantly higher number of macrophages was present in the edge of both type of chronic ulcers ($p < 0.001$ *versus* day 29 p.w.); and (iii) more B cells and plasma cells were detected in both type of chronic wounds compared with any day in the acute wound healing model ($p < 0.04$ for $CD20^+$ and $p < 0.01$ for $CD79a^+$ cells). These data indicate that important differences exist in the cellular infiltrate and ECM expression patterns of acute, healing *versus* chronic wounds, which may be related to the nonhealing status of chronic wounds. ***Key words: diabetes mellitus/extracellular matrix molecules/immunophenotyping/wound healing. J Invest Dermatol 111:850–857, 1998***

Clinical and experimental evidence shows that diabetic patients as a group experience impaired wound healing (Goodson and Hunt, 1979; Spanheimer, 1991, 1992; Bennet and Schultz, 1993; Knighton and Fiegel, 1993). The mechanisms that contribute to poor wound healing are not fully understood. Wound healing normally proceeds through general stages such as hemostasis, inflammation, proliferation, epithelialization and tissue remodelling. Many chronic wounds fail to complete all these stages in healing. Diabetic ulcers are usually localized on pressure points, such as the metatarso-phalangeal joints, ankles, or heel region. The underlying pathology consists of neuropathy, ischemia (both macro- and microcirculatory) and infection (Levin, 1993). Infection plays an important role in the chronicity of these ulcers, partially due to the adverse effect of diabetes on leukocyte chemotaxis (Pereira *et al*, 1987; Thomas *et al*, 1991). Venous ulceration is characterized by a specific pathophysiology consisting of venous stasis and microcapillary pathology (Leu *et al*, 1980; Browse and Burnand, 1982; Fagrell, 1982; Coleridge Smith *et al*, 1988; Thomas *et al*, 1988; Herrick *et al*, 1992). Due to the prolonged hydrostatic pressure, the capillaries, which are originally not designed for high pressures, become dilated, twisted, and elongated and they start to leak plasma, proteins (including fibrin), and erythrocytes in the surrounding tissue. Some capillaries become occluded, while the blood flow in the remaining dysfunctional capillaries is hampered. Generally, after a period of strict bedrest, these capillary changes are reversed, and the ulcers show a healing tendency, similar to acute wounds.

Based on the clinical observation that epithelialization can proceed normally in these patients as soon as the quality of the wound bed has improved, we hypothesize that the disturbances are located in the two preceding phases, in the proliferation phase or the inflammation/debridement phase.

The predominant cell types in early wound healing, especially in the inflammation and debridement phase, are lymphocytes, granulocytes and macrophages. The major function of granulocytes in wounds is to eliminate contaminating bacteria. It has been known for long that lymphocytes are present in healing wounds (Ross and Bendit, 1969) but their function remains a topic of interest (Martin and Muir, 1990; Barbul, 1992). Macrophages play a crucial regulatory role in the transition between wound inflammation and the next phase of wound repair, granulation tissue formation (Clark, 1985; Reed and Clark, 1985). This phase is characterized by proliferation of endothelial cells and fibroblasts and the deposition of extracellular matrix (ECM) molecules. During the repair process the ECM is sequentially remodelled and rebuilt by the action of different cell types and their products.

Fibronectin (FN) promotes adhesion of cells to the fibrin matrix, acting as a scaffold for new matrix deposition, and also has chemotactic capacities that regulate cell movement (Clark *et al*, 1982; Knox *et al*, 1986; Clark, 1988; Kim *et al*, 1992). Its expression is highly upregulated in the dermis after wounding. Chondroitin sulfate (CS) is a disaccharide

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Abbreviations: CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycans; FN, fibronectin; p.w., postwounding; TN, tenascin.

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forming glycosaminoglycan (GAG) chains of different length. They are covalently attached to core proteins forming chondroitin sulfate proteoglycans (CSPG) (Ruoslahti, 1989; Hardingham and Fosang, 1992). In normal skin CSPG are mainly found in the basement membrane (McCarthy *et al*, 1989; Daugaard *et al*, 1991) but in healing wounds they are upregulated throughout the granulation tissue (Yeo *et al*, 1991) during the second week of wound repair, when they provide a temporary matrix with high hydrative capacity. Tenascin (TN) is an ECM glycoprotein which occurs in the basement membrane and in granulation tissue. It is known to have an influence on cell shape, and *in vitro* studies showed that TN is important for maintaining tissue homeostasis by interfering with cell migration and proliferation and by inhibiting cell adhesion to FN (Kanno and Fukuda, 1994).

Very little is known about the expression of these above mentioned ECM molecules and the composition of the cellular infiltrate in chronic diabetic wounds.

The objective of this study was to investigate the expression of different ECM molecules and to characterize the infiltrate composition in chronic diabetic wounds *versus* chronic venous ulcers and an acute wound healing model, in order to come to a better understanding of the process of delayed wound healing in diabetes mellitus.

MATERIALS AND METHODS

Clinical profile This study was approved by the medical ethical committee of the Academic Medical Center, Amsterdam, The Netherlands. Patients were fully informed about the purpose and consequences of this study, and subsequently gave their informed consent. Thirteen patients with diabetic ulcers (noninsulin-dependent diabetes mellitus) with a mean age of 69.2 y admitted to the clinic or visiting the outpatient department of our hospital were included in this study. As a reference we compared the results to those of 12 patients with a venous leg ulcer, with a mean age of 76 y, and an acute wound healing model. A chronic ulcer was defined as existing for 8 wk or longer. Punch biopsies of 4 mm were taken from the margin of chronic diabetic and venous ulcers with a median ulcer duration, respectively, 11 (range 2–60) and 6 mo (range 2.5–60). The biopsies were taken in such a manner that the epithelial margin was included in the central part of the biopsy. The epithelial margin can be considered as a reference point because normally the epithelium will grow from here into the wound bed, only if the tissue underneath it has reached a certain level of quality. When biopsies are taken at random from the wound bed large differences can be encountered in the composition of the tissue.

The acute wound healing model was studied in three groups of patients. In healthy agematched volunteers, acute wounds were made on the upper leg and wound healing was studied at day 5, 19, and 28 postwounding (p.w.) in four patients (mean age of patients 78.8, SD 13.3). During the study period the wound area was covered with Opsite (Smith and Nephew, York, U.K.) to establish a moist wound environment. The donor site (upper leg) for grafting of chronic ulcers was rebiopsied 12 and 18 mo p.w. in one patient (age 64). Wound healing 3 and 12 mo after breast reduction was studied in seven patients (mean age 24.4, SD 4.4). All punch biopsies were taken under local anesthesia. The biopsies were either fixed in 4% formalin phosphate-buffered saline (PBS) solution, processed by routine histologic procedures and embedded in paraffin, or snap-frozen and stored at –80°C until further processing.

Immunohistochemical staining

ECM molecules Polyclonal rabbit antibodies were used to detect FN (1:800, Dako, Copenhagen, Denmark) and TN (1:400, Life Technologies, Breda, The Netherlands) using a three-step labeling procedure with biotinylated polyclonal swine-antibodies anti-rabbit IgG (1:400, Dako) as second antibody followed by streptavidin biotinylated horseradish peroxidase (Hrp) complex (1:200, Dako). For the detection of CS a mouse monoclonal antibody (1:300, Sigma, St. Louis, MO) was used in a two step labeling procedure with as second step Hrp conjugated goat antibodies antimouse IgM (1:200, Dako). Diaminobenzidine (DAB) was obtained from Sigma.

Sections of 5–6 μm thickness were mounted on polylysine coated glass slides. The sections were deparaffinized in xylol and hydrated through graded series of ethanol. To remove endogenous peroxidase activity the slides were incubated for 30 min in a 0.3% H_2O_2/methanol solution, washed with water and PBS. Aspecific binding of antibodies was minimized by a 15 min preincubation with 10% normal goat serum (NGS) in PBS. The sections were incubated for 1 h at room temperature with the first antibodies and washed three times with PBS. Subsequently, the appropriate second antibody was applied, diluted in PBS/10% NGS. If the second antibody was biotinylated a third incubation step was performed with the streptavidin biotin complex conjugated with horse radish peroxidase (streptABComplex/Hrp). After extensive washing to remove nonbound antibodies, the color reaction was performed with DAB substrate. Finally, the sections were counterstained with hematoxylin, mounted in glycergel and examined. As recommended by the manufacturers, the fixed tissues used for the FN staining were predigested with a 0.25% pepsin 10 mM HCl solution pH 2.5 for 30 min at 37°C. A pepsin predigestion of sections for TN and CS staining diminished the specific staining signal. The sections were examined microscopically and photographs of representative staining were taken using an Olympus SC35 camera (Tokyo, Japan) with 64T (EPY-135) Ektachrome film (Kodak, The Netherlands). Sections of human normal skin served as positive controls. As negative controls, adjacent sections of the wound biopsies were stained with nonimmune IgG from the same species and in the same dilution as the primary antibody. No staining was noted in the negative controls.

ECM quantitation FN, CS, and TN expression in the dermis of all chronic and acute wounds was quantitated using a score system with a five point scale (0, no expression; 1, weakly positive; 2, moderate; 3, strong; 4, very strong expression). All specimens were evaluated blindly. In order to standardize the results, the intensity of the ECM staining was correlated to the expression found in the positive control sections. To check reproducibility, the slides were examined by two independent observers, which yielded a correlation coefficient of $\geqslant 0.83$.

Lymphocytes, granulocytes, and macrophages Immunohistochemical single staining was performed for the identification of T cells (CD3; pan T cells; 1:50; Becton Dickinson), B cells (CD20 1:1000; Dako), plasma cells (CD79a 1:50; Dako), granulocytes (CD15 1:50; Becton Dickinson) and monocytes, macrophages (CD68 1:2000; Dako) based on a three-step indirect peroxidase technique. Cryostat sections (6 μm) were allowed to dry overnight before fixing in acetone for 10 min at room temperature. Endogenous peroxidase activity was blocked with 0.1% sodium azide and 0.3% H_2O_2 in PBS for 10 min at room temperature. For plasma cells, paraffin-embedded tissue sections were deparaffinized in xylol and hydrated through a graded series of ethanol. To remove endogenous peroxidase activity the slides were incubated for 30 min in a 0.3% H_2O_2/methanol solution, washed with water and PBS. Antigen retrieval was achieved by boiling the sections in 10 mM Citrate buffer; PH 6.0 (Dako). Aspecific binding of antibodies was minimized by a 15-min preincubation with 10% NGS in PBS. Briefly, the sections were incubated sequentially with primary antibody for 60 min followed by an incubation with biotinylated secondary monoclonal rabbit antimouse antibody (30 min). This was followed by an incubation of 30 min with streptABComplex/Hrp (1:400, Dako). Before counterstaining with hematoxylin, the horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole (AEC; Sigma) as chromogen for cryostat sections and with DAB substrate for the paraffin sections.

For immunohistochemical double staining the following steps were performed: (i) incubation of 60 min with a cocktail of rabbit antihuman CD3 plus either mouse antihuman CD4 (1:20; Becton Dickinson) or mouse anti-human CD8 (1:20, Becton Dickinson); (ii) an incubation of 30 min with a cocktail of biotin-conjugated goat anti-mouse (1:200, Dako) plus alkaline phosphatase-conjugated goat anti-rabbit (1:10, Dako) and an incubation of 30 min with streptABComplex/Hrp (1:400, Dako). Alkaline phosphatase was detected as blue color, using naphthol-AS-MX-phosphate as a substrate and fast blue BB (Sigma) as azo dye. Peroxidase activity was detected as an orange red color, using the chromogen AEC. Double staining cells were detected by their purple color. The staining reaction was visually controlled and stopped by washing. Sections were finally fixed in formaldehyde (4%) and mounted with glycerin-gelatin without counterstaining. Sections of human tonsils served as positive controls. The negative controls were stained in the same manner as all specimen without incubation with the primary antibodies.

Cell enumeration The number of single or double stained cells of three different serial sections of each wound were counted (blinded) up to a depth of 1 mm. These values of each biopsy specimen were adjusted to 10 mm width. The mean value of each biopsy was used for further analysis.

Statistical evaluation The unpaired Student's t test was used for statistical evaluation of differences in the composition of the cellular infiltrate between each group. To detect the significance of differences in the CD4/CD8 ratio's and the number of B cells and plasma cells between acute and chronic wounds, the Mann–Whitney U test was used. p values < 0.05 (two-tailed) were considered significant.

RESULTS

ECM characterization

Fibronectin In normal skin FN could only be detected in bloodvessels (**Fig 1*A***). In sections of biopsies taken 19 d p.w. FN (**Fig 1*B***) was clearly present in the dermis. In time, FN staining increased markedly until 3 mo p.w. (**Fig 1*C***) to gradually decline towards 12–18 mo p.w.

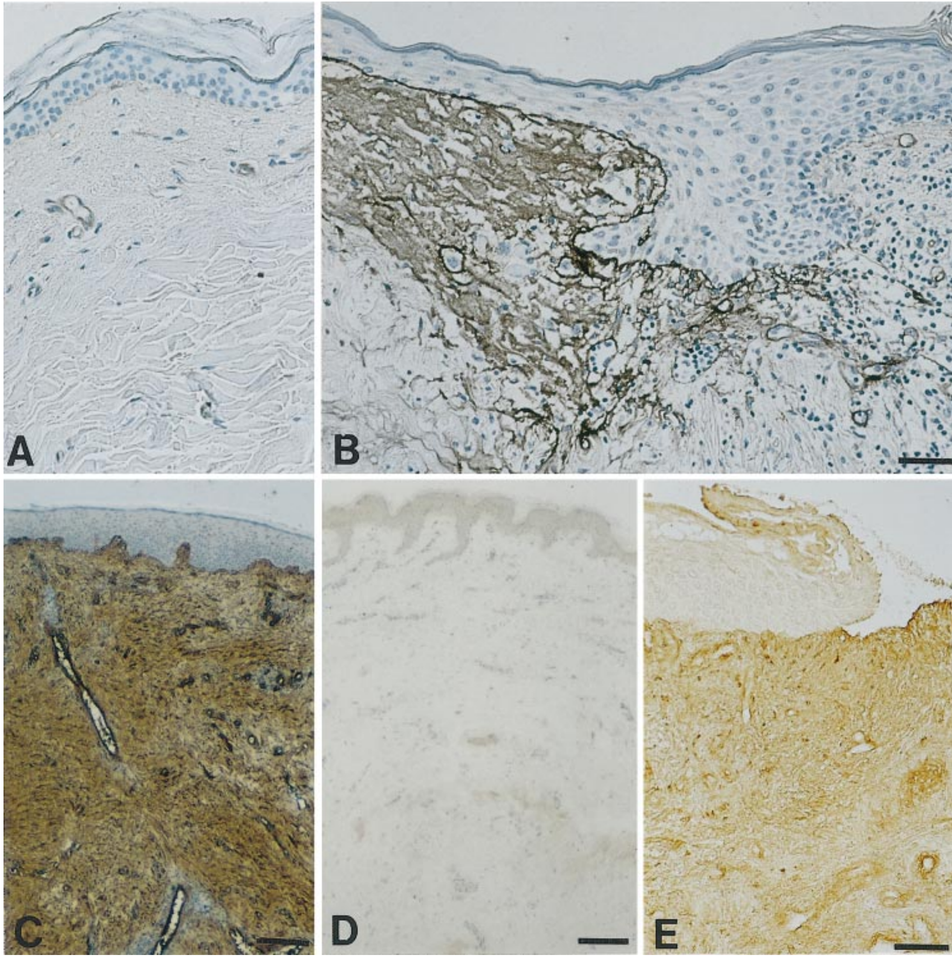

Figure 1. Prolonged expression of FN in chronic diabetic and venous ulcers compared to the acute wound healing model. Sections of normal skin and acute wounds (day 19, 3 and 12 mo p.w.) as well as from the edge of diabetic and venous ulcers were embedded in paraffin and cut in 5–6 μm sections. FN was detected as described in *Materials and Methods*. In normal skin FN could only be detected in blood vessels (*a*). Nineteen days p.w. expression of FN was seen in the re-epithelialized ulcer area and at the edge (*b*) to reach an intense expression at 3 mo p.w. (*c*) and return to prewounding levels 12 mo p.w. (*d*). In chronic diabetic ulcers an intense staining for FN was seen in the whole dermis of the ulcer area (*e*) as well as in venous ulcers with a duration of 12 mo or more (not shown). *Scale bars*: (*a*, *b*) 70 μm; (*c*) 200 μm; (*d*) 300 μm; (*e*) 100 μm.

(**Fig 1*D***). In chronic diabetic ulcers with a ulcer duration of more than 12–18 mo, and in two venous ulcers, expression of FN was still noted in the whole dermis of the ulcer area (**Fig 1*E***).

Chondroitin sulfate CS labeling in normal skin was found in the basement membrane (BM) and in the periphery of vascular structures (**Fig 2*A***). CS was detected after 19 d p.w. in the dermal tissue of acute wounds (**Fig 2*B***) and its expression became more intense up to 3 mo p.w. (**Fig 2*C***). At 12–18 mo p.w. CS staining in the dermis of all acute wounds was back to prewounding levels (**Fig 2*D***). All 25 chronic wounds showed high CS expression in the dermis and basement membrane (**Fig 2*E*, *F***).

Tenascin In normal skin, TN staining was seen as a patch wise distribution in the BM zone (**Fig 3*A***). At 19 d p.w. TN started to be expressed in the wound edge with the BM zone of the healthy skin being positive (**Fig 3*B***). At 3 mo p.w. strong expression of TN was seen in the dermis (**Fig 3*C***) which returned to prewounding levels at 12 mo (**Fig 3*D***). The chronic wounds showed a light, moderate or strong expression of TN in the dermis with great variability among the ulcers. For diabetic ulcers two showed no (**Fig 3*E***) and two showed weak expression, four moderate, three strong and one very strong TN expression (**Fig 3*F***) in the dermis. Among venous ulcers three showed no expression, two light, two moderate, and two strong expression.

Figure 4(***A***) shows the quantitated FN expression in all individual chronic ulcers as well as the acute wound healing model. In general there was still high FN expression in the chronic diabetic and venous wounds with a duration of more than 12 mo, when in normal wound healing the level should be reduced again. In **Fig 4**(***B*, *C***), all chronic ulcers and their dermal CS and TN expression are visualized *versus* the acute wound healing model. There was a trend of prolonged CS and TN expression in the dermis of venous and diabetic wounds with a duration of more than 12 mo. **Table I** shows the original data for ECM quantitation by two independent observers which yielded a correlation coefficient of 0.88 for FN, 0.84 for CS, and 0.92 for TN.

Lymphocytes, granulocytes, and macrophages A common observation in chronic wounds was a hyperkeratotic epidermis at the wound edge with necrotic wound tissue heavily infiltrated with inflammatory cells.

Figure 5 shows the kinetics of T and B cells, plasmacells, macrophages and granulocytes in acute and chronic wounds represented by CD3 (Pan T cells), CD4 (helper-inducer T cells) CD8 (suppressor-cytotoxic T cells), CD15 (granulocytes), CD20 (B cells), CD79a (plasma cells) and CD68 (monocytes, macrophages) positive cells. In the early stage of wound healing, there is an influx of T cells which were predominantly of the $CD3^+$ $CD4^+$ type (**Fig 5*A*, *B***). In chronic wounds T cells were present although in a lower number than in the acute wound healing model at any day ($p < 0.0048$ for venous ulcers and $p < 0.02$ diabetic ulcers). This was mainly caused by a significantly lower number of CD3 CD4 positive T cells in chronic wounds ($p < 0.0028$ venous, $p < 0.0029$ diabetic ulcers) whereas the number of $CD8^+$ T cells did not change dramatically (**Fig 5*C***). CD4/CD8 ratio's in acute wounds varied between 4.9 and 9.7 (**Table II**). In chronic wounds the CD4/CD8 ratio was significantly depressed, $p < 0.0027$, compared to any day in the acute wound healing model. Granulocytes increased rapidly in number during the early phase after wounding and decreased again after 5 d p.w.; they remained present in chronic wounds (**Fig 5*D***). B cells were detected at all time points in acute wounds with a peak at day 19. B cells were present in significantly higher numbers in both chronic wounds. In venous ulcers a mean B cell number of 750 was found whereas at day 5, 19 and 28 p.w. there was a maximum of 200 B cells ($p < 0.04$). This was also true for the diabetic ulcers ($p < 0.006$) (**Fig 5*E***). Plasma cells were

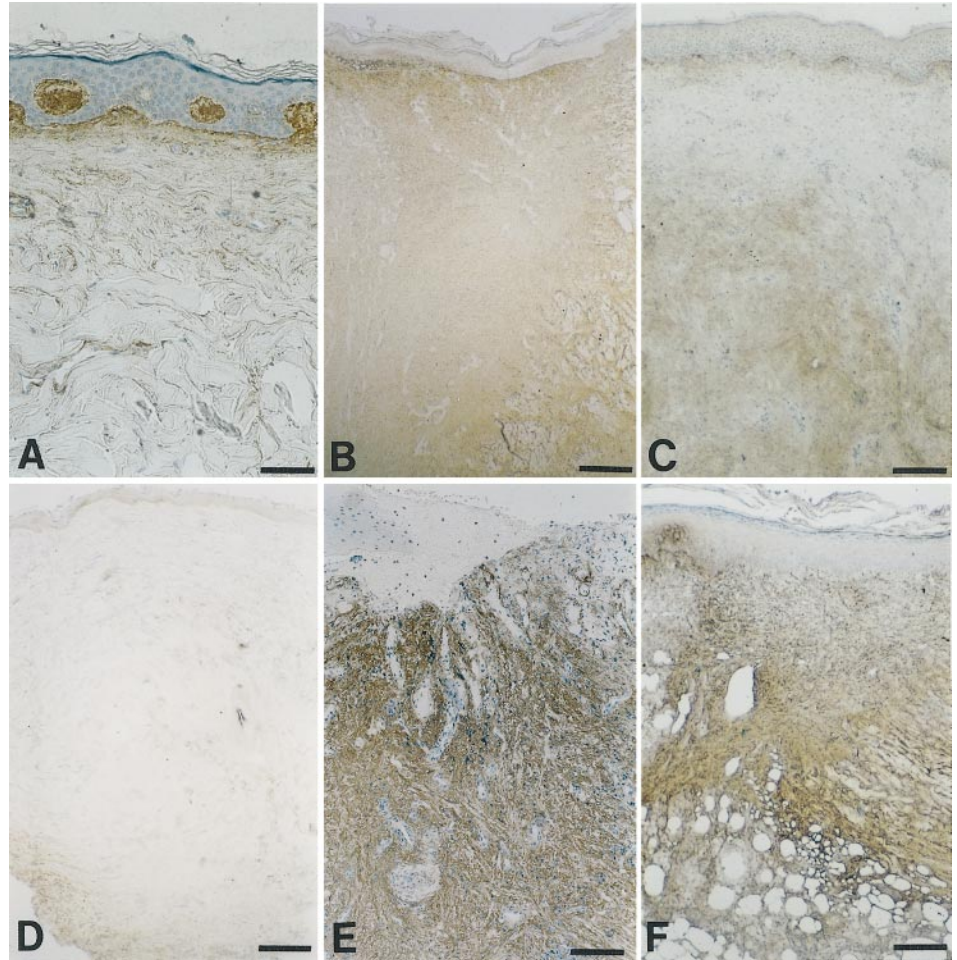

Figure 2. Prolonged expression of CS in chronic diabetic and venous ulcers compared to the acute wound healing model. Sections of normal skin and acute wounds (day 19 and 3 and 12 mo p.w.) as well as from the edge of diabetic and venous ulcers were fixed and stained for CS. CS labeling in normal skin was found in the basement membrane zone and in the periphery of vascular structures (*a*). Nineteen days p.w. CS was detected in the dermal tissue of acute wounds (*b*) as well as at 3 mo p.w. (*c*). At 12 mo p.w. CS staining in acute wounds was back to prewounding levels (*d*). Both diabetic ulcers (*e*) and venous ulcers (*f*) showed abundant expression of CS in the dermis of the ulcer bed and edge. *Scale bars*: (*a*) 70 μm; (*b*) 260 μm; (*c*) 200 μm; (*d*) 300 μm; (*e*) 160 μm; (*f*) 200 μm.

detected in significantly higher numbers ($p < 0.0121$) in both types of chronic wounds compared to any day in the acute wound healing (**Fig 5*F***). Macrophages appeared together with T lymphocytes in the initial phase of wounding in high numbers and in time their number decreased. The number of macrophages in both chronic wounds was significantly higher (diabetic ulcers, $p < 0.0011$ and venous ulcers, $p < 0.00001$) compared to the last time point (day 28 p.w.) of the acute wound healing model (**Fig 5*G***).

DISCUSSION

The purpose of this study in a broader perspective was to find an explanation on the cellular or molecular level for the clinical observation that wound healing is delayed in diabetes mellitus.

In normal wound healing, the consecutive phases such as hemostasis, inflammation and debridement, proliferation and remodelling, can be identified by characteristic patterns of cellular infiltrate and ECM deposition. Most chronic diabetic and venous ulcers fail to complete all these stages of normal wound healing. We hypothesized that the disturbances are located in the proliferation phase and/or the inflammation and debridement phase. Once these phases have been completed successfully, uncomplicated epithelialization will usually follow.

We observed a prolonged expression of FN, CS, and TN in patient material from 13 chronic diabetic ulcers and 12 venous ulcers, with a duration of more than 12 mo. Although a semiquantitative evaluation method was used to detect these ECM molecules, the statement that this is an abnormal finding is valid because normally these matrix molecules should only be present early in wound healing.

CS expression, investigated using a monoclonal antibody that can detect all CSPG because it recognizes the GAG chains attached to the core protein, e.g., decorin, CS-basement membrane proteoglycan, and versican, was present far beyond the normal time frame of expression seen in normal wound healing. CSPG expression has been shown to be indicative for different stages of ECM regeneration and remodelling in a porcine wound model (Lamme *et al*, 1996).

Abnormal patterns of FN and TN expression have also been observed by other groups. Latijnhouwers *et al* (1996) reported a variable, but mostly upregulated TN expression in the papillary dermis adjacent to the ulcer base of chronic venous ulcers. Ferguson *et al* (1996) demonstrated the presence of small abnormal blood vessels at the wound edge and base of diabetic ulcers, sometimes cuffed with collagen, laminin, FN and fibrin. Herrick *et al* (1992) showed a upregulated TN and FN expression in the ulcer margin and a weak positive ulcer base for TN and the absence of FN in the ulcer base of venous ulcers. The distribution of TN during wound healing has been studied in animal models and to a lesser extent in human skin. TN has been reported to be upregulated primarily in the first two weeks p.w., first in the dermal areas adjacent to the wound bed and later in the wound bed (Latijnhouwers *et al*, 1996), to gradually disappear after 1.5 mo (Betz *et al*, 1993). A strong but transient increase in TN expression (maximum at 4 d p.w., towards baseline at day 11) has been described in rats (Luomanen and Virtanen, 1993).

Studies on the composition of the cellular infiltrate in chronic wounds are scarce. An explanation might be that patient material is difficult to obtain for practical and ethical reasons.

Several hypotheses have been proposed to explain delayed wound healing in diabetes, such as glycosylation of important structural proteins and growth factors, a possible deficit in specific growth factor production or release into the wound (Knighton and Fiegel, 1993) and impairment of leukocyte (Thomas *et al*, 1991) or macrophage function (Moore *et al*, 1997). Some of these mechanisms may be related.

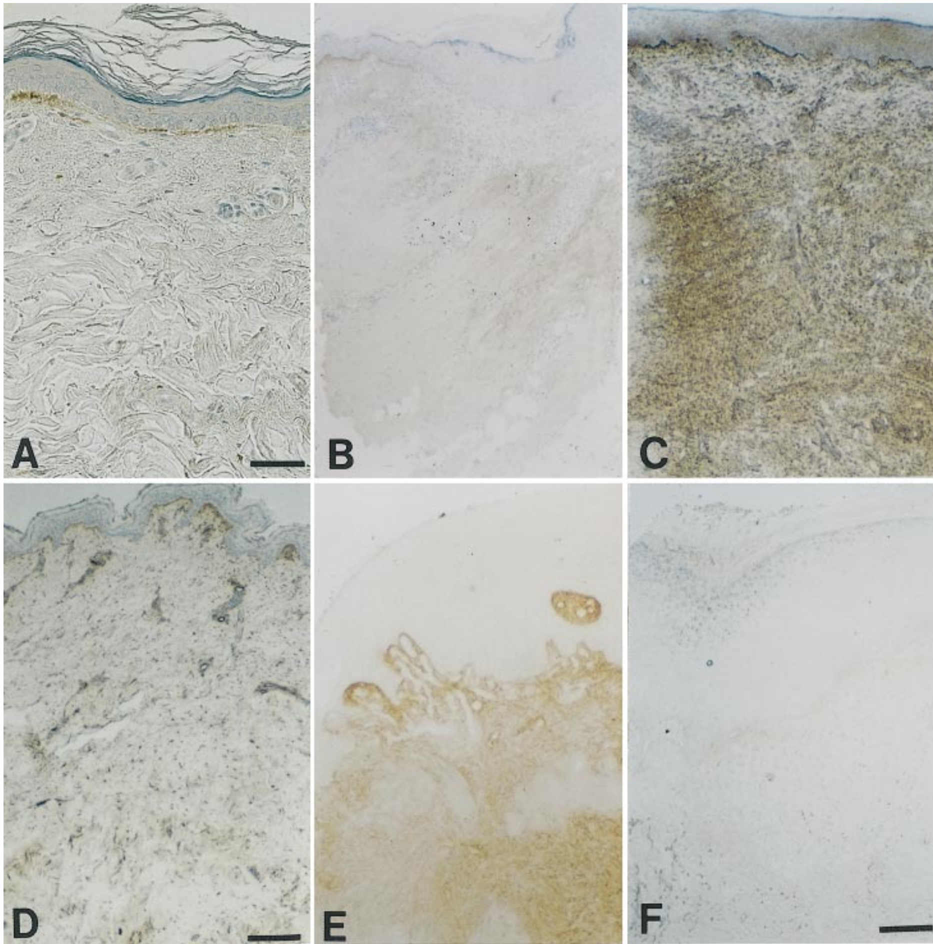

Figure 3. Prolonged expression of TN in chronic diabetic and venous ulcers compared to the acute wound healing model. Sections of normal skin and acute wounds (day 19, 3, and 12 mo p.w.) as well as from the edge of diabetic and venous ulcers were fixed and stained as described in *Materials and Methods***.** In normal skin TN expression was seen as a patch wise distribution in the BM zone (*a*). At 19 d p.w. TN started to be expressed in the ulcer edge of acute wounds with a positive BM zone of the healthy tissue (*b*). At 3 mo p.w. the most prominent expression of TN was seen in the dermis (*c*) and 12 mo p.w. TN expression was back to prewounding levels (*d*). Both chronic diabetic and venous ulcers showed variability in TN expression in the dermis varying from absence of expression (diabetic ulcer f) to, in most cases, prolonged expression (diabetic ulcer e). *Scale bars*: (*a*) 70 μm; (*b–d*) 200 μm; (*e*, *f*) 120 μm.

Glycosylation In diabetes it has been postulated that excessive formation of advanced glycosylated end products in the presence of continuously elevated blood glucose may overwhelm the body's ability to remove them, resulting in a net excess of advanced glycosylated end products on most of the structural proteins, leading to an altered recognition by their cellular receptors (Makita *et al*, 1991). The absence of a negative feedback mechanism may lead to overproduction of ECM. It has been shown *in vitro* that exposure of mesangial cells to advanced glycosylated end products was followed by an increase in ECM mRNA and FN production, which is the hallmark of glomerulosclerosis, and a decrease in cellular proliferation (Skolnik *et al*, 1991; Doi *et al*, 1992). The mechanisms that lead to nephropathy in diabetes may be similar to those leading to delayed wound healing.

Growth factors/cytokines The observed expression of FN, CS and TN in the edge of chronic wounds shows that there is a potential to heal since it is a natural pattern of acute wounds to express these molecules at the onset of the healing process. Normally, fibroblasts eventually cease to produce these provisional ECM molecules, possibly by inhibitory growth factors that are secreted in the next phase of wound healing, granulation tissue formation. An imbalance of cytokines in the chronic wound bed or edge and the presence of inflammatory cells could be responsible as well for the continued production of ECM molecules. ECM degradation is also taking place: FN degradation products have been observed in chronic skin ulcers (Wysocki and Grinell, 1990; Grinnell *et al*, 1992; Palohlahti *et al*, 1993) as well as elevated levels of several proteinases-gelatinases in wound fluid (Stacey *et al*, 1993; Wysocki *et al*, 1993). It seems that in these chronic nonhealing ulcers both excess deposition of ECM molecules and increased proteolytic activity against ECM molecules are present. TGF-β and activated $CD4^+$ T lymphocytes have been shown to trigger synovial fibroblasts and epitenon cells to increase their production of FN (Wojciak and Crossan, 1993, 1994).

Impairment of leukocyte or macrophage function The previously mentioned leukocyte impairment may be responsible for delays in the inflammation phase in diabetic patients. Compared to normal wound healing, in chronic venous and diabetic ulcers the CD4/CD8 ratio was significantly lower due to a relatively lower number of $CD4^+$ T cells compared to the acute wounds. Moore *et al* (1997) found a comparable CD4/CD8 ratio for chronic venous ulcers (1.5). Apparently, diabetic ulcers have a similar depressed CD4/CD8 ratio (1.25). It has been suggested in the literature that the continued presence of lymphocytes, and especially the predominance of $CD8^+$ T cells may impede certain stages of the healing process (Breslin *et al*, 1988; Barbul and Regan, 1990). This theory is supported by the accelerated healing process following the depletion of $CD8^+$ cells in acute wounds in mice (Barbul *et al*, 1989).

In the acute wound healing model B cells were detected with a peak at day 19. A significantly higher number of B cells was present in chronic wounds. According to the literature B cells are not present in normal skin. Rosner *et al* (1995) found a relatively low number of B cells in the edge of venous ulcers comprising less than 3% of the dermal infiltrate. We biopsied chronic ulcera in a nonhealing stage, and a continuous exposition to bacteria might be responsible for the increased number of B cells. The observation that significantly more plasma cells were present in the chronic ulcers also supports this hypothesis, because a plasmacellular infiltrate is often associated with the presence of microorganisms.

Macrophages appear together with T cells in the initial phase of wound healing. They play a key role in the transition from the inflammatory phase to the proliferating phase (Falanga *et al*, 1990; Hammer, 1993). Although macrophages dominate the ulcer edge of both diabetic and venous ulcers, they seem to be unable to direct the repair process towards the proliferative phase. Our finding is in concordance with the results of one other study on phenotyping of

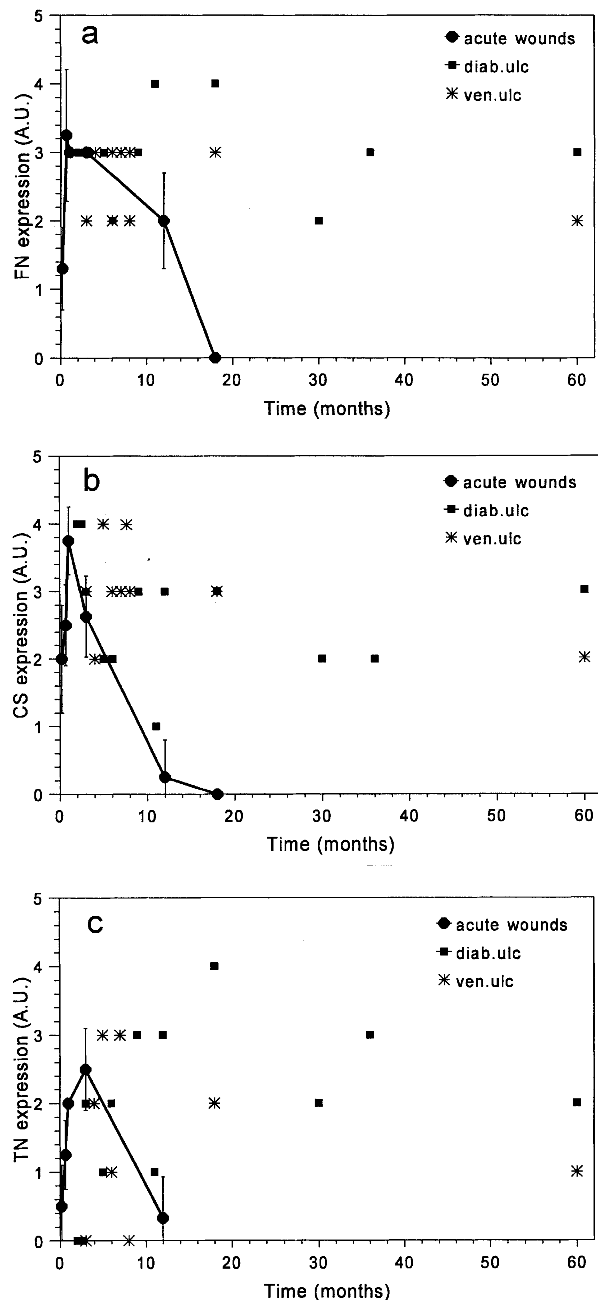

Figure 4. Prolonged expression of FN, CS, and TN in chronic ulcers *versus* acute wounds. FN (*a*), CS (*b*), and TN (*c*) expression in the dermis of sections of acute and chronic wounds was scored in one session on a five point scale by two independent observers (c.c 0.83). Their expression in acute wounds (5 and 19 d p.w., 4 wk, 3 mo, 12 mo, and 18 mo p.w.) was visualized as a reference line for uncompromised wound healing. The majority of the chronic diabetic and venous ulcers showed strong and or prolonged expression of these ECM molecules in the dermis as indicated outside the reference line. *Error bars*: SD (n = 4).

Table I. ECM quantitation by two independent observers[a]

	FN	CS	TN
Diabetic ulcers			
#1	3/3	2/2	1/1
#2	2/3	3/3	3/3
#3	4/4	3/4	4/4
#4	3/3	3/4	3/3
#5	2/3	2/3	2/2
#6	3/3	4/4	0/0
#7	3/3	4/4	0/0
#8	2/2	2/2	2/2
#9	4/4	1/1	1/1
#10	3/3	2/3	2/2
#11	3/3	2/2	3/3
#12	3/4	3/4	2/2
Venous ulcers			
#1	2/2	4/4	0/0
#2	3/3	3/4	0/0
#3	2/2	3/3	0/0
#4	2/3	3/3	1/1
#5	3/3	4/4	3/3
#6	3/3	3/2	2/2
#7	3/3	3/3	3/3
#8	3/2	2/2	2/2
#9	2/2	2/2	1/2
Acute wound model			
day 5			
#1	1/1	2/2	1/1
#2	2/2	2/2	0/1
#3	1/2	3/3	1/2
#4	–/–	1/1	0/0
day 19			
#1	4/4	2/3	1/2
#2	4/4	3/3	1/1
#3	3/3	3/3	1/1
#4	2/2	2/2	2/2
4 wk			
#1	3/3	4/3	2/2
#2	3/2	4/2	2/2
#3	3/3	3/3	2/3
#4	3/3	4/3	2/3
3 mo			
#1	4/4	4/3	3/3
#2	4/3	3/2	3/2
#3	4/3	3/2	4/4
12 mo			
#1	2/2	1/0	1/0
#2	2/2	1/0	1/0
#3	3/3	0/0	0/0
#4	1/1	0/0	0/1
18 mo	0/0	0/0	–/–

[a]Correlation coefficient: FN 0.88, CS 0.84, TN 0.92.

immunocompetent cells in venous leg ulcers (Rosner *et al*, 1995). These authors also showed that the venous ulcer edge was mainly populated by macrophages. Recently, Moore and coworkers reported that monocytes appeared to be active perivascular in venous ulcers but while penetrating the wound bed as macrophages they lost their activation markers (Moore *et al*, 1997).

In summary, there appears to be a disturbance in the phase of inflammation and debridement and in the proliferation phase. Distinct patterns of ECM deposition and a different composition of the cellular infiltrate were observed in chronic diabetic and venous ulcers. Although both chronic wound types have a different pathophysiology, the observed abnormalities in ECM quantitation and infiltrate immunophenotyping are very comparable. While in acute wounds the inflammatory phase is of short duration and directed at removal of bacteria and dead tissue, with polymorphonuclear leukocytes and macrophages as the predominant cell types, in chronic wounds there is a prolonged and increased presence of a T cell infiltrate, with an abnormal CD4-CD8 balance, and an increased number of macrophages, without any evidence of increased autodigestion and phagocytosis. These chronic venous and diabetic ulcers seem to be frozen in a chronic low-grade inflammatory state, in the sense that completion of the digestive tasks and transition into the next phase, proliferation of fibroblasts and endothelial cells, is not taking place.

Similar remarks can be made regarding the proliferation phase: large amounts of ECM molecules are being produced but the composition resembles the pattern seen in late phases (2–3 mo) of normal wound healing. The molecules are not removed or remodelled within a normal timeframe as in acute wounds. In fact, the composition of the ECM and cellular infiltrate share properties of both the inflammatory phase

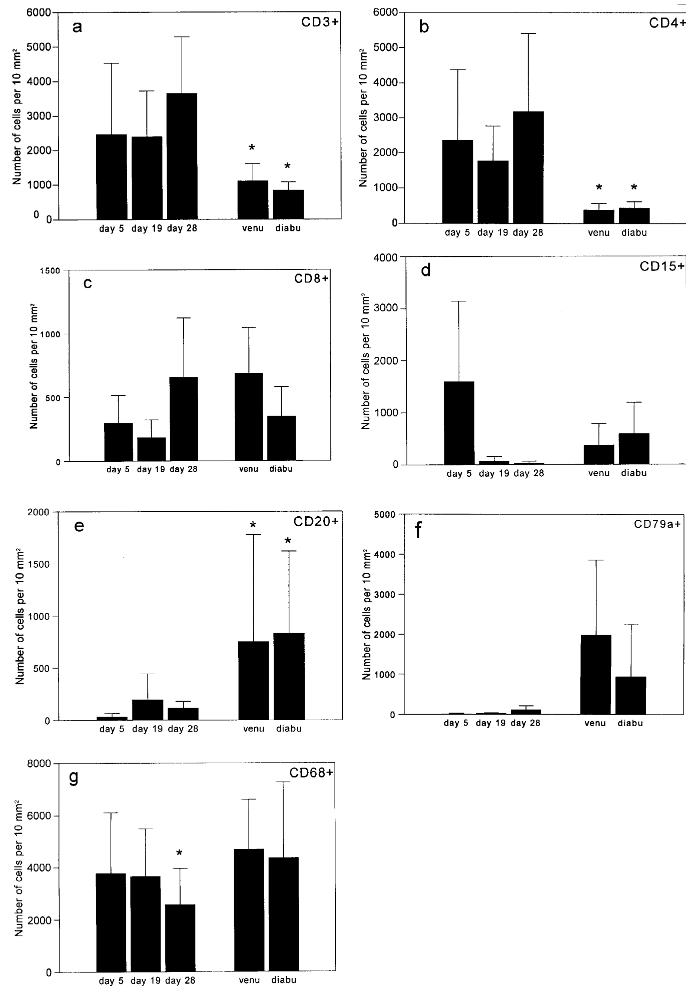

Figure 5. Reduced numbers of CD4$^+$ T cells and high numbers of B cells, plasma cells and macrophages in chronic diabetic and venous ulcers compared with the acute wound healing model. Kinetics of T cells represented by CD3$^+$, CD4$^+$ and CD8$^+$ cells (*a–c*), granulocytes (CD15$^+$, *d*), B cells (CD20$^+$, *e*), plasma cells (CD79a$^+$, *f*) and macrophages (CD68$^+$, *g*) in the acute wound healing model and in chronic diabetic and venous ulcers. Skin biopsies were taken from the upper leg at day 5, 19, and 28 p.w. as well as from the edge of diabetic and venous ulcers and snap-frozen. Immunohistochemical single and double staining techniques and cell counting were performed as described in *Material and Methods*. Venu, venous ulcers; Diabu, diabetic ulcers. *Error bars*: SD (n = 4–7), triple countings per patient. Day 5, 19, and 28 p.w. *Error bars*: SD (n = 1–4), triple countings for each time point per patient. (*a*, *b*, *e*, *f*) *$p < 0.05$ for number of positive cells per 10 mm^2 in diabetic and venous ulcers compared to any day in the acute wound healing model. (*g*) *$p < 0.05$ for number of positive cells per 10 mm^2 in diabetic and venous ulcers compared with day 28 acute wounds.

Table II. Depressed CD4/CD8 ratios in chronic *versus* normal wound healing

Wound type	CD4/CD8 ratio
Venous ulcers	0.59 ($p < 0.0015$)[a]
Diabetic ulcers	1.25 ($p < 0.0027$)
Day 5 acute wound	8.6
Day 19 acute wound	9.7
Day 28 acute wound	4.9

[a]p values are in parentheses and compare venous and diabetic ulcers to any day in the acute wound healing model.

and proliferation phase, and it is likely that the classification criteria that we use to define healing stages in acute wounds are not applicable at all in chronic leg ulcers.

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