Chapter 2 Simple wound exudate collection method identifies bioactive cytokines and chemokines in (arterio) venous ulcers

Kim Kroeze
Liselotte Vink
Edith de Boer
Rik Scheper
Bibi van Montfrans
Susan Gibbs

Wound Repair and Regeneration; In Press

ABSTRACT

A major challenge for clinicians treating (arterio-)venous leg ulcers is to decide between standard therapy or advanced interventions. Here, we developed a simple method to collect human material representative of the ulcer wound-bed which can be used to identify biomarkers for prognostic test development. Superficial surgical debridement was performed using a small vidal curette during the weekly visit to the outpatient clinic. Moist, easily removable debridement material essentially blood free (including necrotic and non-viable slough) was collected from the surface of the ulcer. The amount ranged from 5.5 - 78 mg material per ulcer. Seventeen cytokines, chemokines and growth factors were extracted and analysed by ELISA (concentration range: 0.0005 - 78 ng/mg total protein). Notably, CXCL8 was by far the most abundant protein present. Inflammatory mediators were more abundant than anti-inflammatory mediators (e.g.: IL-10, TGF-β1). Bio-activity assays showed chronic wound extracts to be capable of stimulating fibroblast migration in a chemokine dependent manner, and also capable of stimulating healthy cells within skin substitutes to secrete wound healing mediators (CCL2, CXCL1, CXCL8, IL-6) in an IL-1α dependent manner. Collection of debridement tissue enables investigation of the ulcer environment in an easy non-invasive manner which may be suitable for prognostic test development.

INTRODUCTION

Chronic cutaneous wounds, usually occurring on the lower legs, are a large problem in our society. It is estimated that approximately 1% (up to 3% for the elderly) of people in the western population will develop a venous leg ulcer (1). Optimal treatment strategies, including the timing for a particular intervention, remain a challenge for clinicians. Despite many different treatments being available, approximately 8% of leg ulcers are still open after 5 years and for many ulcers, if the ulcer does heal, multiple recurrences occur frequently (2,3).

Ideally, an easy to implement, robust prognostic test should be developed that can predict at an early stage, before visual signs of healing are apparent, whether an ulcer has a good chance of healing by continuation of standard therapy alone or whether a more advanced therapy is required. In the past, some success has been achieved by developing diagnostic tests based on measuring clinical signs of healing (wound duration, wound surface area and decrease in surface area over a 4 week period) (4). These diagnostic tests predict closure in approximately 75 % of ulcers already showing signs of healing. In order to develop sensitive prognostic tests, next to diagnostic tests, it is important to gain more information on the etiology of the ulcer. Much can be learnt from studying the wound environment. Normal cutaneous wound healing is a complex sequence of partially overlapping phases comprised of inflammation, proliferation, (neoangiogenesis, granulation, re-epithelialization) and tissue remodeling (extra-cellular matrix remodeling). The transition into these phases is regulated by many cytokines, chemokines and growth factors (5). These wound healing mediators are produced by several cell types, such as skin residential cells and immune cells present in the wound bed. In general, cytokines regulate the intensity and the duration of the inflammatory response. Chemokines are important for the trafficking of inflammatory cells (e.g. neutrophils, monocytes, macrophages) into the wound bed and for stimulating wound closure (residential cell migration, proliferation, angiogenesis). Growth factors play a role in the repair of tissues and tissue remodelling (5,6). Such wound healing mediators are potential biomarkers for a prognostic test that will determine whether or not an ulcer has the tendency to heal.

The expression profile of cytokines, chemokines and growth factors is normally tightly regulated and time dependent. In chronic wounds it is believed that the inflammatory phase is prolonged due to an increased ratio of inflammatory versus anti-inflammatory factors (7). Previous studies found that pro-inflammatory cytokines such as IL-1α and TNF-α were present at significantly higher concentrations in chronic wound fluid compared to acute wound fluid (8), and it has already been suggested that increased levels of these cytokines cause prolonged inflammation at the wound site (9-12). Fivenson et al. suggest that increased levels of inflammatory chemokines in the wound bed of chronic venous ulcers play a critical role in the pathogenesis by preventing the progression to the proliferative phase of wound healing at the site of injury (13). In animal studies, it has been shown that lack of growth factors e.g.: FGF, HGF and TGF can cause impaired wound healing (6).

Attempts to determine which molecules are involved in chronic wound development, failure to heal and eventual closure have been limited since animal- and *in vitro* models do not represent effectively the human chronic wound situation. Thus far biopsies and wound exudate obtained from patients with chronic wounds have offered the only means for analyzing molecules involved in the pathogenesis of chronic human wounds. The major disadvantage of taking biopsies from the wound bed or wound margin is the invasive nature of this sampling method which involves the creation of an additional wound in patients at a site where already impaired wound healing is apparent. The use of wound exudate is non-invasive and has been accepted as a means of examining the chronic wound environment (14). Wound exudate collection methods include prolonged vacuum assisted closure therapy (VAC) to draw fluid from the wound bed; direct aspiration from under occlusive dressings (15,16) which in one study was preceded by fasting followed by rehydration (11,17); extraction directly from dressing materials (13) and collection into porous filters placed within a porous mesh (18). Clearly these methods are time consuming, logistically complicated, are a burden to the patient and often involve admittance into hospital together with the accompanying costs. Also, it is possible that expression levels of potential molecules of interest may be influenced by these collection methods.

The aim of this study was to develop a simple method to collect human material representative of the chronic wound environment which can be used for prognostic test development. The methodology should be minimally invasive and easily applicable to standard out patient time schedules. To meet this aim, debridement material routinely removed directly from the surface of chronic wounds undergoing standard wound care therapy was collected. This material was analysed and found to contain a wide panel of cytokines, chemokines and growth factors. Furthermore, bio-activity assays showed that the debridement material contained active chemokines which were able to stimulate healthy fibroblast migration, and also active IL-1α (but not TNF-α) which was able to stimulate secretion of wound healing cytokines and chemokines from healthy skin substitutes. Notably, CXCL8 was by far the most abundant protein present. We show that analysis of debridement tissue provides a simple means to investigate the human chronic wound bed in a robust non-invasive manner.

METHODS AND MATERIALS

Patients

Fourteen patients with therapy resistant leg ulcers of duration 0.3 – 40 years and size 0.25 - 96 cm^2 were enlisted in this study (Table 1). For the venous and (arterio)-venous ulcer groups (12/14 patients) the ulcers showed no tendency to heal for at least 12 weeks despite receiving standard ulcer treatment according to the FDA guidelines were (19). Patients with infected

Table 1: Patient information

Patient and ulcer type	[1]Gender	Age (yrs)	Ulcer location	duplex /doppler evaluation	[2]Ulcer Size (cm^2)	Ulcer Duration (yrs)	Ankle/brachial index	Debridement tissue (mg)
Venous								
I	M	57	shin	insuffient deep system insufficient perforantes	12.9	35 (recurring)	> 1	20
II	M	42	medial lower leg	insufficient superficial systeem insufficient perforantes	30	1	-	42.2
III	F	76	dorsal foot	insufficient superficial system	6	0.5	-	78
IV	M	61	ankle	deep and superficial system sufficient	49	7	> 0.8	26
V	F	91	heel	insufficient superficial system	3	0.5 (recurring)	-	7.5
[3]XI	F	65	dorsal foot and toes	-	> 50	8 (recurring)	-	6.8
(arterio) venous								
VII	F	88	lateral lower leg (3 ulcers)	-	1.5, 8.8, 5	> 0.3 (recurring)	0.6	55 (pooled)
VIII	F	72	ankle	insufficient superficial system insufficient perforantes	3.2	40 (recurring)	0.6	7.9
IX	F	87	ankle	insufficiënt superficial system	4.2	10 (recurring)	> 0.8	7.6
X	F	87	lateral lower leg	insufficient superficial system	2.6	0.7	> 1	5.2
XI	F	62	lower leg and heel (3 ulcers)	-	0.25, 0.05, 1.5	1	-	5 (pooled)

Table 1: Patient information (continued)

Patient and ulcer type	[1]Gender	Age (yrs)	Ulcer location	duplex /doppler evaluation	[2]Ulcer Size (cm^2)	Ulcer Duration (yrs)	Ankle/brachial index	Debridement tissue (mg)
XII	F	83	lower leg	insufficient deep system	4	> 0.3	-	40
Others								
[4]XIII	F	89	lateral ankle, outside	-	3	-	-	5.5
[5]XIV	F	66	lower shin	no insufficiency	96	4	-	36

[1] Gender: Male = M; Female = F; [2] Ulcer size was estimated from maximum length x width measurement; [3] Patient XI: ulcer type was venous and lymphedematous; [4] Patient XIII: ulcer type was hydroxycarbamide induced ulcer; [5] Patient XIV: ulcer type was unknown; patients were not known to suffer from known diabetis or pedal neuropathy; - = information not known.

ulcers and/ or underlying malignancies were excluded. The etiology of the ulcers was assessed by history, ulcer examination, and / or duplex investigation of the venous system and ankle brachial pressure index to rule out arterial insufficiency (Table 1). Informed consent was obtained from each patient, and ethics approval was given by the medical ethics committee of our hospital in agreement with the ethical guidelines of the 1975 Declaration of Helsinki.

Collection of debridement tissue from chronic wounds

Standard superficial surgical debridement of ulcers was conducted by trained staff using a small vidal curette during the weekly visit to the outpatient clinic. Moist, easily removable debridement material essentially blood free (including necrotic and non-viable slough) was collected from the surface of the ulcer. Dry crust was excluded. This amount ranged from 5.5 - 78 mg material per ulcer (Table 1). Phosphate buffered saline (PBS) (1 ml) (B.Braun, Melsungen, Germany) containing protease inhibitor cocktail (PIC) (Sigma- Aldrich, Steinheim, Germany) (1:100) was added immediately to debridement tissue. Next the mixture was gently shaken at 4ºC for 1 hour to elute proteins into the PBS / PIC solution. After incubation samples were centrifuged and their supernatants were stored at -80ºC until further analysis. Samples used in tissue culture experiments were filter sterilized using 22µm filters before adding to culture medium. These samples are further referred to as chronic wound extract.

Enzyme-linked immunosorbent assay for chemokine production

For cytokine, chemokine and growth factor quantification enzyme-linked immunosorbent assay (ELISA) reagents were used in accordance to the manufacturer's specifications. IL-1α, TNF-α, CXCL1, CXCL8, IL-6, IL-10, CCL2, CCL5, CCL17, CCL18, CCL20, CCL27, CXCL12, and IFN-γ, bFGF, HGF and TGF-β were measured by commercially available paired ELISA antibodies and recombinant proteins obtained from R&D System Inc. (Minneapolis, Minnesota). For CXCL8/ IL-8 and TNF-α quantification, a Pelipair reagent set (CLB, Amsterdam, The Netherlands) was used. Supernatant of all samples was normalized for total protein content using the Bio-Rad Protein Assay (BioRad Laboratories, Hercules, California) essentially as described by the supplier. ELISA results are expressed as amount of wound healing factor (ng)/ total protein (mg).

Wound-healing scratch assay to assess bio-activity in debridement tissue

In order to determine the bio-activity in the debridement tissue of chronic wounds a wound healing scratch assay was performed as previously described by Kroeze et al. (20). Confluent healthy human fibroblast monolayers in a 48-well plate were incubated with serum free medium (DMEM, 1% P/S, 0.1% bovine serum albumine (BSA) (Sigma-Aldrich, Steinheim, Germany) for 4 days. After 4 days a scratch was drawn with a plastic disposable pipette tip.

Cultures were washed with PBS to remove detached cells and then exposed to different concentrations of chronic wound extract (1%, 10% and 50% v/v DMEM, 1% P/S, 0.1% BSA) (R&D, Oxon, UK) in the presence or absence of a chemokine receptor inhibitor (100 ng/ml pertussis toxin, From Bordetella pertussis)(Invitrogen corporation, Carlsbad, CA). The wound area was photographed (when the monolayer was damaged (t=0) and at 2 days (t=2) after scratching using phase contrast microscopy coupled to a digital camera (Coolpix 5400, Nikon Corporation, Japan). Cell migration in chronic wound extract-supplemented medium (with and without pertussis toxin) was compared to control medium and given as a percentage relative to control. All experiments were performed in duplicate using at least 3 different fibroblast donors.

Exposure of skin substitute to debridement tissue of chronic wounds

Autologous skin substitutes consisting of reconstructed epidermis on fibroblast populated dermal matrix were constructed as previously described (21). For these experiments skin substitutes were constructed using human adult skin obtained from healthy donors undergoing abdominal dermolipectomy.

Culture of skin substitutes in the presence of chronic wound extract: After 3 weeks of standard culture required to generate the skin substitutes, the culture medium was supplemented with chronic wound extract for 48 hrs (200 µg total protein in 1.5 ml standard SS medium).

Addition of neutralizing antibodies: Neutralizing antibodies for IL-1α (10 ug/ml, R&D Systems Inc., Minneapolis, MN) and TNF-α (100 ng/ml, R&D Systems Inc., Minneapolis, MN) were added to chronic wound extract supplemented medium for 30 minutes prior to exposure to skin substitutes for 48 hrs. Relevant control cultures with or without chronic wound extract or neutralizing antibodies were carried out in parallel. After 48 hours, culture supernatants (1.5ml/ culture) were stored at -20°C until further analysis by enzyme-linked immunosorbent assay (ELISA).

RESULTS

Cytokines, chemokines and growth factors are present in chronic wound debridement tissue

In order to determine whether it was possible to identify a broad panel of cytokines, chemokines and growth factors in debridement tissue isolated from leg ulcers, we first determined the presence and levels of 17 factors known to influence wound healing by ELISA. Notably, CXCL8 was by far the most abundant protein present. Six categories related to protein abundance were identified: i) CXCL8 was detected at >75 ng/ mg total protein; ii) IL-6, CCL18,

CCL20, CXCL1, HGF and IL-1α were detected at 2 – 6 ng/mg total protein; iii) CCL2, bFGF and TGF-β1 were detected at 0.1 - 0.6 ng/ mg total protein; iv) CCL5, CCL27, and CXCL12 were detected 10 - 90 pg/ mg total protein; v) IL-10 and TNF-α was detected at 0.5 - 5 pg/ mg total protein; and vi) IFN-γ and CCL17 were not detectable (Table 2). Clearly the methodology used to collect chronic wound exudate enabled many chemokines, cytokines and growth factors to be identified in concentrations ranging from picogram to nanogram amounts. Also, as seen from the SEM values, very little variation was observed between donors even though the therapy resistant ulcers were of different origins, size and duration (Table 1; Table 2). However, from these results it could not be concluded whether the factors were present in an active or inactive form.

Table 2: Cytokines, chemokines and growth factors detected in chronic wound debridement tissue

Mediator	Function	Amount in chronic wound debridement (ng/mg total protein)[a]
Highly abundant		
CXCL8 / IL-8	Inflam; Ang; Epith; Fib	77.8 +/- 12.7
Abundant		
CXCL1 / GRO-α	Inflam; Ang; Epith; TR; Fib	5.5 +/- 2.2
CCL20 / MIP-3α	Inflam; Antibacterial; Fib	4.8 +/- 1.2
CCL18 / PARC	Inflam; Collagen; Fib	4.3 +/- 1.6
HGF	TR; Epith; Gran; Mitogen	4.0 +/- 0.6
IL-6	(Anti-)inflam; Gran; Ang; Mitogen	3.3 +/- 1.1
IL-1α	Pro-inflam; Mitogen; Hematopoeises	2.2 +/- 1.1
Moderate amount		
CCL2 / MCP-1	Inflam; Ang; Epith; TR	0.6 +/- 0.2
bFGF	Ang; Gran	0.227 +/- 0.098
TGF-β	Anti-inflam; ECM; TR; Gran	0.112 +/- 0.042
Low amount		
CCL27 / CTACK	Inflam, Chemotactic for BMDSC; Fib	0.082 +/- 0.032
CCL5 / RANTES	Inflam; Mitogen; Chemotactic for stem cells	0.079 +/- 0.012
CXCL12 / SDF-1	Inflam; Ang; LC; Fib	0.013 +/- 0.007
Minimal amount		
IL-10	Anti-inflam	0.007 +/- 0.003
TNF-α	Pro-inflam	0.0005 +/- 0.0005
Not detectable		
IFN-γ	T cell mediated immune responses, keratinocyte apoptosis, TNF- α prod.	Not detected
CCL17 / TARC	T-cell homing	Not detected

[a] Amount in chronic wound debridement tissue (ng/mg total protein). Mean +/- SEM of 14 therapy resistant (arterio)venous ulcers is shown (for patient information see Table 1). Ang = stimulates angiogenesis; Collagen = stimulates collagen production; ECM = extracellular matrix production; Epith = epithelialisation; Fib = stimulates fibroblast migration; Gran = granulation tissue stimulating; Inflam = inflammatory mediator, LC = Langerhans Cell migration; TR = tissue remodelling, BMDSC = bone marrow derived stem cells.

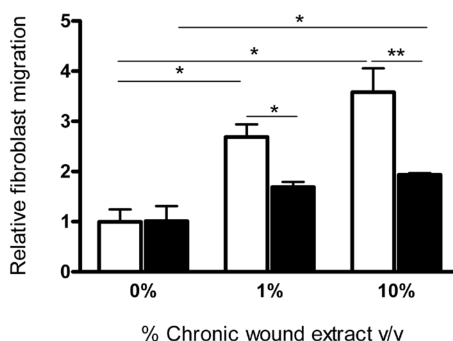

Figure 1: Chronic wound extract stimulates fibroblast migration in a chemokine dependent manner.
Healthy human dermal fibroblasts (p2) were incorporated into a wound healing scratch assay in the presence or absence of chronic wound extract (white bars) or in combination with 100 ng/ ml pertussis toxin (chemokine receptor blocker) (black bars) for 2 days. Data are presented as the mean number +/- SEM of migrated cells. Three experiments each using different fibroblast donors and performed in duplicate are shown. Three batches of chronic wound extract were tested, each batch consisting of 3 pooled donors. Differences were evaluated using one-way ANOVA with post-hoc Dunnett's test $**P<0.01$, $*P<0.05$

Chemokines in chronic wound debridement tissue increase fibroblast migration

In order to determine whether factors are present in an active form rather than being inactivated by proteases present in the chronic wound bed, we next determined whether supplementation of healthy fibroblast cultures with chronic wound debridement tissue extracts could influence migration. For this study, the previously described *in vitro* wound healing scratch assay was used (20). Indeed fibroblast migration increased by approximately 2.5 fold after supplementation of culture media with only 1% chronic wound extract and by approximately 3.5 fold after supplementation with 10% chronic wound extract (Figure 1; white bar). Next, we determined whether chemokines present in the chronic wound extract were responsible for the increased migration. Fibroblasts in the scratch assay were cultured with chronic wound extract in the presence of pertussis toxin which is a specific chemokine receptor blocker. Pertussis toxin strongly inhibited the increase in migration by 65% in cultures supplemented with 10% v/v chronic wound extract (Figure 1; black bars). Since migration was not completely reduced to basal levels, it is possible that additional factors, e.g.: growth factors and cytokines, may also contribute to fibroblast migration or that a higher concentration of chronic wound extract is required for full inhibition. However, we can conclude that chemokines are present in an active form in debridement tissue.

Functional activity of IL-1α but not TNF-α in chronic wound debridement tissue

Having determined that chemokines are active in the chronic wound extract we next determined whether the major pro-inflammatory cytokines IL-1α and TNF-α were also functional. In order to do this we made use of our previously described autologous full thickness skin substitute which consists of a reconstructed epidermis on a fibroblast populated dermis (Figure 2a) (21,22).

First, we determined whether incubation of the skin substitute with chronic wound extract influenced secretion of typical factors reported to stimulate wound healing (CCL2, CXCL1, CXCL8, IL-6) (Table 2). A huge dose dependent increase in secretion of CCL2, CXCL1, CXCL8 and IL-6 from the skin substitute, in response to chronic wound extract, was observed (Figure 2b). Already an approximately 10 fold higher secretion of wound healing factors was observed when skin substitutes were supplemented with only 2% chronic wound extract (which corresponds to 200µg chronic wound eschar total protein/ 1.5 ml culture supernatant). These results show that components within chronic wound extract are not only capable

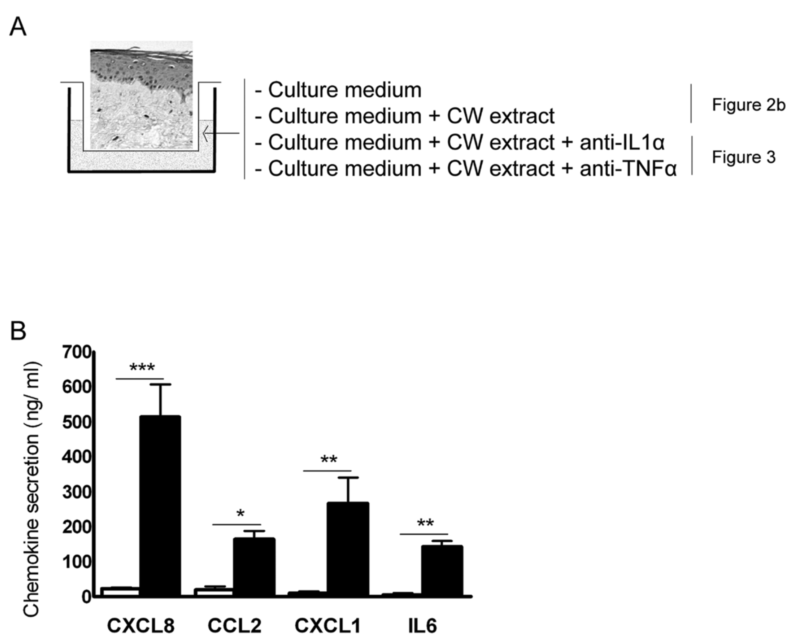

Figure 2: Chronic wound extract stimulates secretion of wound healing mediators from skin substitute.
(A) Experimental design for supplementation of culture medium of autologous full thickness skin substitute with chronic wound extract and / or neutralizing antibodies.
(B) Skin substitutes were exposed to chronic wound extract (200 µg total protein/ 1.5 ml culture medium) for 48 hrs. Control = white bars; chronic wound extract = black bars. Cytokines and chemokines secreted into culture supernatants were analysed by ELISA. Mean ± SEM of three experiments each using different donors and performed in duplicate is shown. Three batches of chronic wound extract were tested, each batch consisting of 3 pooled donors. Differences were evaluated using one-way ANOVA with post-hoc Dunnett's test $^{**}P<0.01$, $^{*}P<0.05$

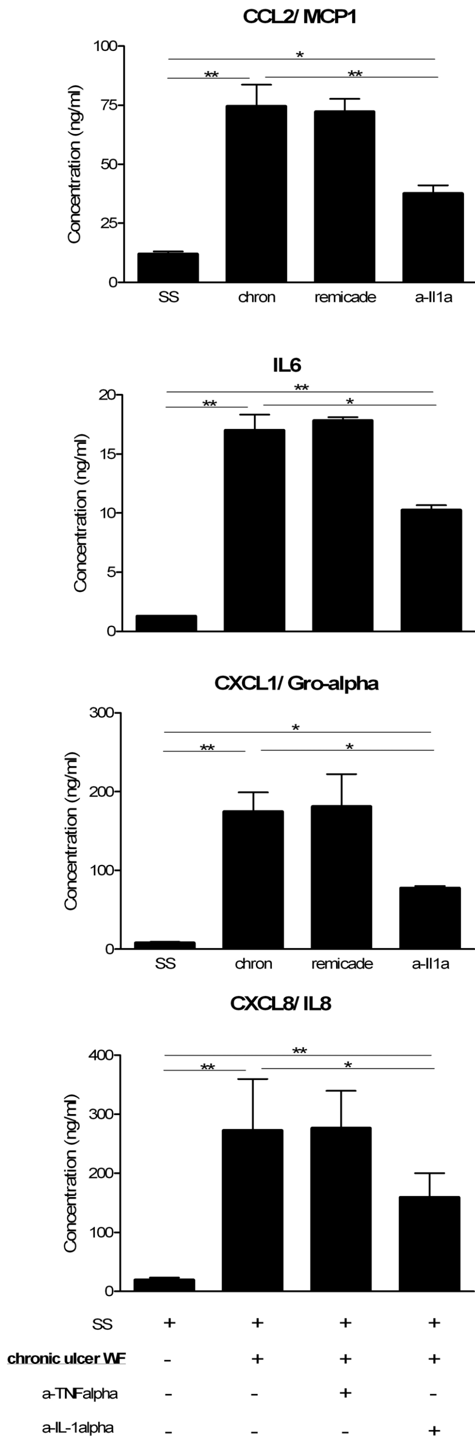

Figure 3: Increased secretion of wound healing mediators is regulated via IL-α.

Skin substitutes were exposed to chronic wound extract (200 µg total protein/ 1.5 ml culture medium) in the presence or absence of neutralizing antibodies to anti-TNF-α (100 ng/ml) or anti-IL-1α (10 µg/ml) for 48 hrs. Data is expressed relative to control unsupplemented skin substitutes. Cytokines and chemokines secreted into culture supernatants were analysed by ELISA. Mean ± SEM of three experiments each using different donors and performed in duplicate is shown. Three batches of chronic wound extract were tested, each batch consisting of 3 pooled donors. Differences were evaluated using one-way ANOVA with post-hoc Dunnett's test **P<0.01, *P<0.05

of stimulating fibroblast migration, but are also capable of stimulating healthy cells within skin substitutes to secrete wound healing mediators.

Previously we have shown that TNF-α and IL-1α are capable of increasing secretion of CCL2, IL-6, CXCL1 and CXCL8 from the skin substitute (22). We next determined whether these cytokines, which were also present in chronic wound extract, were responsible for the observed increase in secretion of the wound healing mediators (Figure 3). Skin substitutes were cultured for 48 hrs with chronic wound extract in the presence or absence of neutralizing antibodies for IL-1α or TNF-α. Notably, anti-IL-1α, but not anti-TNF-α, clearly reduced the increased secretion of wound healing mediators from the skin substitute cultured with chronic wound extract.

Taken together our results indicate that IL-1α is active in debridement material isolated from chronic wounds. In contrast, our results suggest that TNF-α is not an active component of debridement tissue isolated from chronic wounds.

DISCUSSION

This study describes a simple, non-invasive method to collect samples from the human chronic wound bed. Our results show that cytokines, chemokines and growth factors pool to form a potent cocktail of active mediators in chronic wound debridement tissue. Since this debridement tissue is in direct contact with the wound bed it closely reflects the wound environment. For the group of therapy resistant ulcers selected for this study, very little donor variation was observed despite differences in ulcer origin, size and duration. This suggests that it may be possible to select common markers which are representative of inert leg ulcers in general. Advantages of this sampling technique are that it is simple, inexpensive, non-invasive and compatible with standard wound care. It is widely accepted that wound fluid is a reflection of the environment of the wound bed as it contains locally produced molecules (5,13,14,23).

Results obtained from our sampling method are consistant with those obtained by others using different sampling techniques. We detect the presence of (pro-) inflammatory cytokines such as TNF-α, IL-1α, IL-6 and IL-8 which also have been detected in chronic wound fluid collected using other methods as described in the introduction (5,14,17,24). The expression of inflammatory cytokines in chronic wounds is in agreement with the clinically observed elongated inflammation phase of these wounds. We also show the presence of HGF (25), bFGF and TGFβ in agreement with reports describing chronic wound fluid collected using other methods (11,26). Importantly, our results describe for the first time the presence of CCL2, CCL5, CCL18, CCL20, CCL27, CXCL1, CXCL12 and IL10 and the absence of CCL17 and IFN-γ in the wound environment of a chronic wound. We chose ELISA as our method of analysis in this pilot study since the amount of protein obtained per ulcer was not limiting.

However, alternative methods such as luminex or multiplex analysis will enable screening of larger biomarker panels in the future.

Notably we show that cytokines and chemokines present in chronic wound debridement tissue are functionally active. This suggests that these cytokines and chemokines are not inactivated by the multiple proteases that have been reported to be present in chronic wounds. The chronic wound extract was able to clearly stimulate migration of healthy dermal fibroblasts in a chemokine dependent manner. Also, chronic wound extract was able to stimulate healthy skin substitutes to produce large amounts of cytokines and chemokines reported to be involved in normal wound healing, in particular granulation tissue formation, in an IL-1α dependent manner (27). This suggests that the reason for ulcers remaining open for long periods of time may not reside in the inactivity of crucial factors required to trigger the wound healing response, but may reside in the cells within the chronic wound bed not being able to respond to these factors. In line with our findings, others have described that fibroblasts isolated from chronic wounds are indeed senescent (28). Also in line with our findings, Raffetto et al. showed that when comparing healthy fibroblasts isolated from the thigh with those from the ulcer edge of the same patient, chronic wound exudate reduced mobility of the ulcer fibroblasts compared to healthy fibroblasts. (29). It has been described that neonatal foreskin derived fibroblasts become senescent (but not apoptotic) and showed reduced migration when exposed to chronic wound exudate extracted from foam wafer occlusive dressings (Allevyn) (29,30,31). The decrease in proliferation is not contradictory to our results since the enhanced migration of fibroblasts which we observed would be expected to coincide with decreased proliferative potential due to the necessary temporary arrest in the cell cycle needed to permit migration. However the decrease in migration observed by these authors is contradictory to our findings. It may be possible that the type of healthy fibroblast used in the study (neonatal foreskin versus adult abdomen) and/ or the method of extracting, isolating and storing (including addition of protease inhibitors) the chronic wound exudate may be responsible for this discrepancy.

In a previous study we demonstrated that the same skin substitute used in this study was able to initiate healing of therapy resistant chronic wounds whereas other therapies had failed (21). Here we show that when the skin substitute containing healthy fibroblasts and keratinocytes is brought into contact with chronic wound extract it starts to secrete large amounts of cytokines and chemokines reported to be involved in normal wound healing (see table 2; and the review by Barrientos et al., Baum et al. and Gillitzer et al.) (5,32). This finding, together with the mobilization of healthy fibroblasts, would explain the means by which skin substitutes are able to revitalize previously inert non-healing ulcers (21,33,34).

The active component in chronic debridement tissue was found to be IL-1α and not TNF-α. Our result was in contrast to that of Cowin et al. who showed that TNF-α in chronic wound fluid showed bioactivity in a cell line L929 fibroblast bioactivity assay and that this could be inhibited with Etanercept (anti-TNF-α) (8). The difference in the results could be due to the

degree of chronicity of the ulcers. In our study we have only investigated hard to heal ulcers. Cowin et al. made no comparison between IL-1α and TNF-α and did not determine IL-1α activity. We found that IL-1α, was detectable in higher amounts than TNF-α in the chronic wound extract. This finding was in agreement with Beidler et al. (35) who also detected higher amounts of Il-1α than TNF-α (normalized for total protein) in biopsies obtained from chronic wounds and with Trengove et al. who showed slightly higher amounts of IL-1α than TNF-α (pg/ml) in chronic wound exudates collected actively by fasting followed by rehydration (11) .

Regular wound debridement has been described to improve wound healing by not only removing dead, damaged and infected tissue from the wound bed but also by removing the excess of proteases, inflammatory mediators and bacterial biofilms (36,37). Proteases in chronic wounds have been described to degrade matrix proteins (38) and growth factors (e.g. PDGF, EGF, VEGF) (39). In line with this we detected only moderate amounts of bFGF and TGF-β1. However, our results show clearly that many cytokines and chemokines are not degraded by proteases. Indeed, CXCL8 has been reported to be processed by multiple MMPs leading to a considerable increase in CXCL8 activity (40). Notably, CXCL8 was by far the most abundant chemokine detected in our study. Some CXC chemokines e.g.: CXCL1 are reported to be completely resistant to cleavage. Interestingly, CXCL1 is the second most abundant chemokine detected in our study. In contrast to CXC chemokines, some CC chemokines have been reported to be processed by MMPs to form receptor antagonists. CCL2 is such a chemokine. Therefore the moderate amount of this chemokine detected in our study may reflect an antagonist rather than an agonist chemokine.

It has been reported by others that an imbalance between inflammatory and anti-inflammatory mediators in the wound bed may prolong the inflammatory phase of wound healing resulting in the failure of chronic wounds to close (13,41,42). We detected both inflammatory (TNFα, IL-1α, IL-6, CXCL8) and anti-inflammatory (IL-6, IL-10) mediators in chronic wound extract. Cytokine IL-6 has inflammatory properties by inducing an immune response after tissue damage as well as anti-inflammatory properties by inhibiting TNF-α and IL-1α (43). The inflammatory mediator CXCL8 was particularly abundant. Bio-active IL-1α, in addition to being able to stimulate production of inflammatory mediators such as CXCL8, CXCL1 and CCL20, also has been reported to stimulate production of antibacterial peptides e.g.: Lcn-2 (12,22). Our results suggest that the failure of ulcers to close is not due to the inactivity or absence of cytokines, chemokines or growth factors. Further implementation of this methodology will be required in order to determine whether a shift in the balance between inflammatory and anti-inflammatory mediators is related to ulcer healing. Such comparisons between healing and non-healing ulcers are underway in order to identify prognostic markers which in turn will aid the understanding of the pathogenesis of chronic wounds and treatment of ulcers.

In conclusion, we have developed a novel method to investigate the chronic wound environment in an easy non-invasive manner. The method that we used enabled a large panel of cytokines, chemokines and growth factors to be detected in concentrations ranging from 0.5

pg / mg total protein to 78 ng / mg total protein. Donor variation between samples was low. This indicates that the sampling technique is robust and very suitable for quantitative detection of wound healing factors (table 2). Also, importantly, it indicates that therapy resistant (arterio)venous leg ulcers have a characteristic profile of cytokines, chemokines and growth factors irrespective of patient age, ulcer size and duration (compare Table 1 and Table 2). This later is important when defining the applicability range for a prognostic test aimed at predicting whether or not an ulcer may heal by standard therapies alone.

REFERENCES

1. Margolis DJ. Bilker W. Santanna J. Baumgarten M. Venous leg ulcer: incidence and prevalence in the elderly. *J Am Acad Dermatol* 2002;46(3):381-386.
2. Nederlandse vereniging voor dermatolologie en venereologie. Diagnostiek en behandeling van het ulcus cruris venosum (in Dutch). 2010.
3. Gohel MS. Taylor M. Earnshaw JJ. Heather BP. Poskitt KR. Whyman MR. Risk factors for delayed healing and recurrence of chronic venous leg ulcers--an analysis of 1324 legs. *Eur J Vasc Endovasc Surg* 2005;29(1):74-77.
4. Gelfand JM. Hoffstad O. Margolis DJ. Surrogate endpoints for the treatment of venous leg ulcers. *J Invest Dermatol* 2002;119(6):1420-1425.
5. Gillitzer R. Goebeler M. Chemokines in cutaneous wound healing. *J Leukoc Biol* 2001;69(4):513-21.
6. Werner S. Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 2003;83(3):835-870.
7. Harding KG. Morris HL. Patel GK. Science, medicine and the future: healing chronic wounds. *BMJ* 2002;324(7330):160-163.
8. Cowin AJ. Hatzirodos N. Rigden J. Fitridge R. Belford DA. Etanercept decreases tumor necrosis factor-alpha activity in chronic wound fluid. *Wound Repair Regen* 2006;14(4):421-426.
9. Gohel MS. Windhaber RA. Tarlton JF. Whyman MR. Poskitt KR. The relationship between cytokine concentrations and wound healing in chronic venous ulceration. *J Vasc Surg* 2008;48(5):1272-1277.
10. Wallace HJ. Stacey MC. Levels of tumor necrosis factor-alpha (TNF-alpha) and soluble TNF receptors in chronic venous leg ulcers--correlations to healing status. *J Invest Dermatol* 1998;110(3):292-296.
11. Trengove NJ. Bielefeldt-Ohmann H. Stacey MC. Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcers. *Wound Repair Regen* 2000;8(1):13-25.
12. Pukstad BS. Ryan L. Flo TH. Stenvik J. Moseley R. Harding K. Thomas DW. Espevik T. Non-healing is associated with persistent stimulation of the innate immune response in chronic venous leg ulcers. *J Dermatol Sci* 2010;59(2):115-122.
13. Fivenson DP. Faria DT. Nickoloff BJ. Poverini PJ. Kunkel S. Burdick M. Strieter RM. Chemokine and inflammatory cytokine changes during chronic wound healing. *Wound Repair Regen* 1997;5(4):310-322.
14. Yager DR. Kulina RA. Gilman LA. Wound fluids: a window into the wound environment? *Int J Low Extrem Wounds* 2007;6(4):262-272.
15. Stechmiller JK. Kilpadi DV. Childress B. Schultz GS. Effect of Vacuum-Assisted Closure Therapy on the expression of cytokines and proteases in wound fluid of adults with pressure ulcers. *Wound Repair Regen* 2006;14(3):371-374.
16. Yager DR. Zhang LY. Liang HX. Diegelmann RF. Cohen IK. Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids. *J Invest Dermatol* 1996;107(5):743-748.
17. Trengove NJ. Langton SR. Stacey MC. Biochemical analysis of wound fluid from nonhealing and healing chronic leg ulcers. *Wound Repair Regen* 1996;4(2):234-239.
18. Schmidtchen A. Chronic ulcers: a method for sampling and analysis of wound fluid. *Acta Derm Venereol* 1999;79(4):291-295.
19. U.S.Department of Health and Human Services Food and Drug Administration, Center for Biologics Evaluation and Research (CBER), Center for Drug Evaluation and Research (CDER), and Center

for Devices and Radiological Health (CDRH). Guidance for industry; Chronic cutaneous ulcers and burn wounds – developing products for treatment. 1-6-2006. Online source.

20. Kroeze KL. Jurgens WJ. Doulabi BZ. van Milligen FJ. Scheper RJ. Gibbs S. Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/RANTES. *J Invest Dermatol* 2009;129(6):1569-1581.
21. Gibbs S. van den Hoogenband HM. Kirtschig G. Richters CD. Spiekstra SW. Breetveld M. Scheper RJ. de Boer EM. Autologous full-thickness skin substitute for healing chronic wounds. *Br J Dermatol* 2006;155(2):267-2274.
22. Spiekstra SW. Breetveld M. Rustemeyer T. Scheper RJ. Gibbs S. Wound-healing factors secreted by epidermal keratinocytes and dermal fibroblasts in skin substitutes. *Wound Repair Regen* 2007;15(5):708-717.
23. Trengove NJ. Stacey MC. MacAuley S. Bennett N. Gibson J. Burslem F. Murphy G. Schultz G. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen* 1999;7(6):442-452.
24. Fivenson DP. Faria DT. Nickoloff BJ. Poverini PJ. Kunkel S. Burdick M. Strieter RM. Chemokine and inflammatory cytokine changes during chronic wound healing. *Wound Repair Regen* 1997;5(4):310-322.
25. Buchstein N. Hoffmann D. Smola H. Lang S. Paulsson M. Niemann C. Krieg T. Eming SA. Alternative proteolytic processing of hepatocyte growth factor during wound repair. *Am J Pathol* 2009;174(6):2116-2128.
26. Mast BA. Schultz GS. Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Repair Regen* 1996;4(4):411-420.
27. Maish GO III. Shumate ML. Ehrlich HP. Vary TC. Cooney RN. Interleukin-1 receptor antagonist attenuates tumor necrosis factor-induced alterations in wound breaking strength. *J Trauma* 1999;47(3):533-537.
28. Wall IB. Moseley R. Baird DM. Kipling D. Giles P. Laffafian I. Price PE. Thomas DW. Stephens P. Fibroblast dysfunction is a key factor in the non-healing of chronic venous leg ulcers. *J Invest Dermatol* 2008;128(10):2526-40.
29. Raffetto JDR. Mendez MV. Marien BJ. Byers HR. Phillips TJ. Park HY. Menzoian JO. Changes in cellular motility and cytoskeletal actin in fibroblasts from patients with chronic venous insufficiency and in neonatal fibroblasts in the presence of chronic wound fluid. *J Vasc Surg* 2001;33:1233-41.
30. Mendez MV. Raffetto JD. Phillips T. Nenzoian JO. Park HY. The proliferative capacity of neonatal skin fibroblasts is reduced after exposure to venous ulcer wound fluid: A potential mechanism for senescence in venous ulcers. *J Vasc Surg* 1999;30:734-743.
31. Seah CC. Phillips TJ. Howard CE. Ponova IP. Hayes CM. Asandra AS. Park HY. *J Invest Dermato*l 2005;124:466-474.
30. Barrientos S. Stojadinovic O. Golinko MS. Brem H. Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008;16(5):585-601.
31. Marston WA. Hanft J. Norwood P. Pollak R. The efficacy and safety of Dermagraft in improving the healing of chronic diabetic foot ulcers: results of a prospective randomized trial. *Diabetes Care* 2003;26(6):1701-1705.
32. Falanga V. Sabolinski M. A bilayered living skin construct (APLIGRAF) accelerates complete closure of hard-to-heal venous ulcers. *Wound Repair Regen* 1999;7(4):201-207.
33. Beidler SK. Douillet CD. Berndt DF. Keagy BA. Rich PB. Marston WA. Inflammatory cytokine levels in chronic venous insufficiency ulcer tissue before and after compression therapy. *J Vasc Surg* 2009;49(4):1013-1020.

34. Cardinal M. Eisenbud DE. Armstrong DG. Zelen C. Driver V. Attinger C. Phillips T. Harding K. Serial surgical debridement: a retrospective study on clinical outcomes in chronic lower extremity wounds. *Wound Repair Regen* 2009;17(3):306-311.
35. Cornell RS. Meyr AJ. Steinberg JS. Attinger CE. Debridement of the noninfected wound. *J Am Podiatr Med Assoc* 2010;100(5):353-359.
36. Grinnell F. Ho CH. Wysocki A. Degradation of fibronectin and vitronectin in chronic wound fluid: analysis by cell blotting, immunoblotting, and cell adhesion assays. *J Invest Dermatol* 1992;98(4):410-416.
37. Yager DR. Chen SM. Ward SI. Olutoye OO. Diegelmann RF. Kelman CI. Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of proteinase inhibitors. *Wound Repair Regen* 1997;5(1):23-32.
38. Gill SE. Parks WC. Metalloproteinases and their inhibitors: regulators of wound healing. *Int J Biochem Cell Biol* 2008;40(6-7):1334-1347.
39. Schreml S. Szeimies RM. Prantl L. Landthaler M. Babilas P. Wound healing in the 21st century. *J Am Acad Dermatol* 2010;63(5):866-881.
40. Eming SA. Krieg T. Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol* 2007;127(3):514-525.
41. Tilg H. Trehu E. Atkins MB. Dinarello CA. Mier JW. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* 1994;83(1):113-118.