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Cytokine stability in chronic wound fluid and its association to fibroblast proliferation

Ewa Anna Burian MD, PhD[1] | Christian Enevold PhD[2] | Tonny Karlsmark MD, DMSci[1,3] | Magnus S. Ågren DMSci[1,3,4]

[1]Department of Dermato-Venereology and Copenhagen Wound Healing Center, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark

[2]Institute for Inflammation Research, Center for Rheumatology and Spine Diseases, Rigshospitalet, University Hospital, Copenhagen, Denmark

[3]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

[4]Digestive Disease Center, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark

Correspondence
Magnus S. Ågren, Copenhagen Wound Healing Center, Bispebjerg Hospital, Nielsine Nielsens Vej 17, DK-2400 Copenhagen, Denmark.
Email: sven.per.magnus.aagren@regionh.dk

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Abstract

Cytokines are commonly deregulated in venous leg ulcers. We have investigated cytokine stability by incubating sterile-filtered wound fluids from chronic venous leg ulcers in vitro. Incubation of wound fluids for 24 h at 37°C decreased IL-1β levels by 88% and TNF-α levels by 64%. IL-1β was degraded by serine proteinases and metalloproteinases while the mechanism for reduced TNF-α remains elusive. The levels of the other peptides did not change significantly ($p > 0.05$). Normal human dermal fibroblasts exposed to five of the six wound fluids showed increased proliferation with the length of prior incubation using an assay optimised for evaluation of wound fluid bioactivity. Exogenous IL-1β and TNF-α unexpectedly increased ($p < 0.001$) cell proliferation at concentrations that were measured in the wound fluids. In conclusion, the stability of the eight investigated cytokines in wound fluids differed and presumably the loss of detrimental factors, unlikely IL-1β or TNF-α, resulted in increased fibroblast proliferation.

KEYWORDS
cytokines, fibroblasts, inflammation, leg ulcer, wound fluid

1 | INTRODUCTION

Venous leg ulcers (VLUs) are a common type of chronic wound and represent a huge unmet medical need. There is a paucity of documented treatments targeting any of the pathophysiological mechanisms that have been proposed; one of these is dysregulation of cytokines.[1] Our knowledge of the kinetics of cytokines in VLUs is limited.

The duration of the sampling of wound fluid (WF) greatly impacts the levels of certain cytokines/chemokines.[2] These time-related cytokine fluctuations in WF are presumably the result of concurrent molecular and biochemical processes occurring in the wound. To isolate the effects of proteolysis from the other processes, one procedure would be to incubate WF ex vivo. Such an experiment requires maintenance of sterility because the normal defence mechanisms in vivo are out of play.

Our aim was to study cytokine levels as a function of time of incubation of WFs from chronic VLUs in vitro. The bioactivity of the incubated WFs was also measured by the metric fibroblast proliferation. The results will shed light on the kinetics of a selected panel of cytokines in the microenvironment of chronic wounds.

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; IQR, interquartile range; NHDF, normal human dermal fibroblast; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; SEM, standard error of the mean; SRB, sulforhodamine B; TGF, transforming growth factor; TNF, tumour necrosis factor; TNF R1, tumour necrosis factor receptor 1; VEGF, vascular endothelial growth factor; VLU, venous leg ulcer; WF, wound fluid.

2 | MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Capital Region (H-23004870) and conducted at the Copenhagen Wound Healing Center, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark. Patients with VLU were consecutively recruited according to the inclusion and exclusion criteria described elsewhere.[3]

WFs, collected by a hydrophobic polyurethane foam for 24 h[2], were sterilised using Spin-X® filters with 0.22 μm pore cellulose acetate membrane (Costar®) centrifuged at 16,000*g* for 10–30 min. This method retained ($p = 0.028$) 7.1 (5.9–9.3)% of the WF (Table S1) and reduced the levels of the eight cytokines by 35.0 (28.4–43.7)% (Table S2).

WFs were dispensed into three 1.5 mL autoclaved polypropylene tubes (Cat. No.: 616261, Greiner Bio-One, Frickenhausen, Germany); the 1st tube was used for baseline measurements (0 h), the 2nd tube was incubated for 1 h at 37°C and the 3rd tube was incubated for 24 h at 37°C (Figure 1A).

After incubation, the tubes were immediately stored at −80°C until analysed on GM-CSF, IL-1α, IL-1β, IL-6, IL-8, PDGF-AA, TNF-α and VEGF-A concentrations using a bead-based multiplexed immunoassay system.[3] Values below the lowest standard concentration for respective cytokine (ranges are given in Table S1 in Burian et al.[3]) were excluded from the presentation and statistical analysis. Bioactivity was evaluated in normal human dermal fibroblasts (NHDFs).[4] In one study, the response to WFs from VLUs was similar for NHDFs, keratinocytes and endothelial cells.[5]

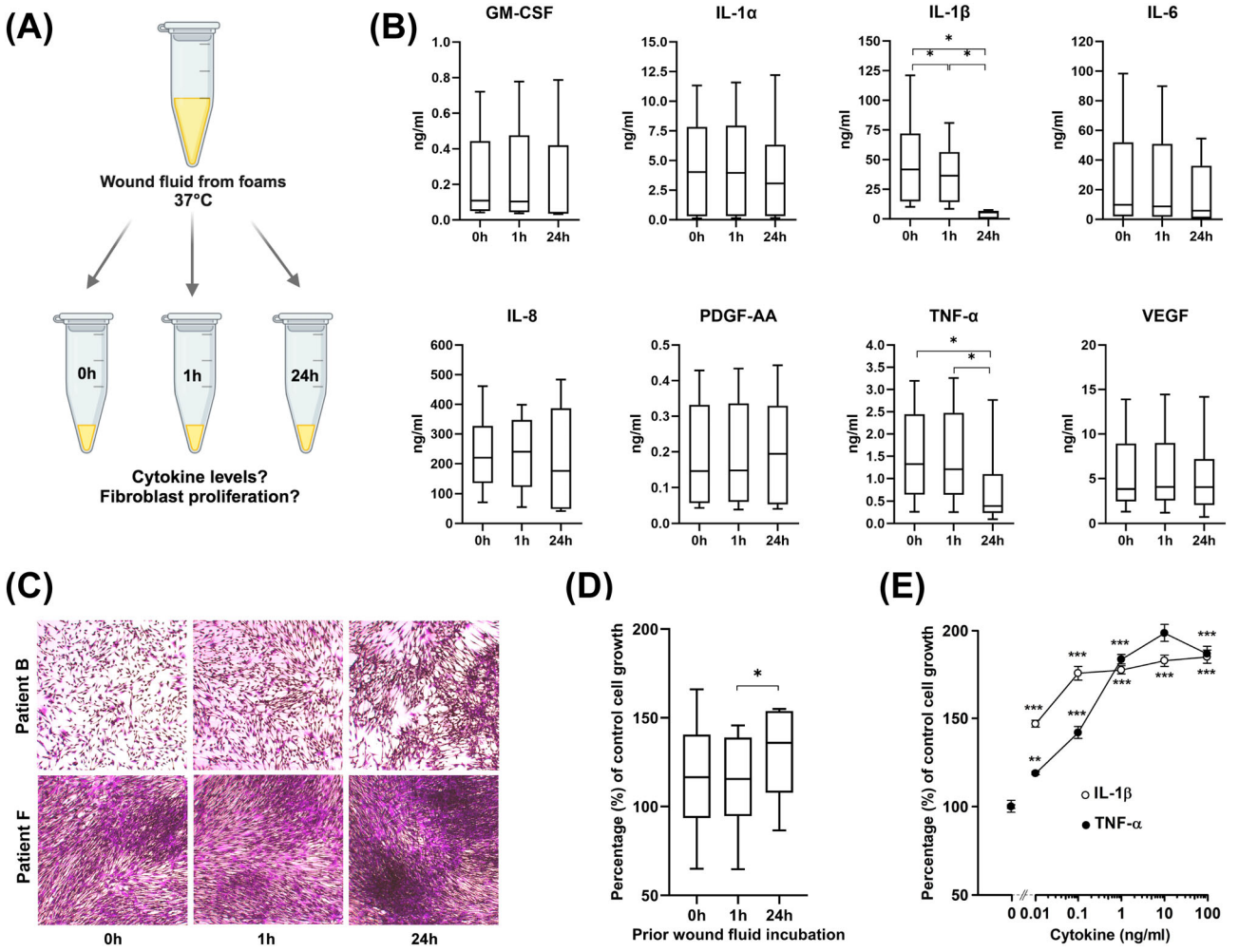

FIGURE 1 (A) WFs from each patient were analysed for cytokine levels and fibroblast proliferation after incubation of WFs for 0, 1 and 24 h at 37°C. (B) Cytokine levels in WFs incubated for 0, 1 or 24 h. Three (0, 1 and 24 h) GM-CSF measurements and one (24 h) PDGF-AA measurement in patient B were below the lowest standard concentration and were excluded. (C–E) Fibroblast (NHDF) proliferation measured by the SRB assay after treatment for 72 h with incubated WFs (C and D) using three wells per sample or with IL-1β and TNF-α (E) using six wells per group. Representative SRB-stained NHDFs (before elution) after 72 h of treatment with WFs that had been incubated for 0, 1 and 24 h at 37°C before being added to the NHDFs for two patients (C). (B and D) Box and whisker plots (boxes represent 25–75th percentile, whiskers are maximum and minimum, and horizontal bars within boxes are median values). (E) The dose-dependent effect of rhIL-1β and rhTNF-α on NHDF proliferation. Mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control (=100%). Control was DMEM plus 10% FBS (culture medium).

The effect of proteinase inhibition was studied in separate experiments using WFs from a subset of patients (C, D and F). Sterile-filtered WFs (46 μL per tube) were aliquoted into four tubes; two tubes contained proteinase inhibitors EDTA (20 mM, final concentration), EDTA (20 mM)/PMSF (10 mM) or E-64 (100 μM), and the other two tubes contained equal volumes of solvent (sterile water, ethanol) in a total volume of 50 μL per tube. The four tubes were incubated for 1 h at 4°C. Two tubes (with or without proteinase inhibitors) were then frozen directly at −80°C and the other two tubes were incubated for 24 h at 37°C, and then frozen at −80°C. IL-1β (DLB50) and TNF-α (DTA00D) concentrations were analysed by Quantikine® ELISA from R&D Systems (Minneapolis, MN). Samples for IL-1β determinations were diluted 1000× and for TNF-α 10× in calibrator diluent.

NHDFs were cultured in DMEM supplemented with 10% FBS (culture medium) in a humidified atmosphere of 5% CO_2/air at 37°C and subcultured as detailed by Ågren et al.[6] NHDFs were seeded into 96-well tissue culture plates (1×10^4 NHDFs in 110 μL culture medium per well), incubated for 24 h, and then exposed to 8% (vol/vol) of 0, 1 and 24 h WFs, or to equal volume of the control (culture medium) for 72 h.[5,7] Using the same set-up, we studied the effect of rhIL-1β (201-LB, R&D Systems) and rhTNF-α (210-TA, R&D Systems) at 0.01, 0.1, 1, 10 and 100 ng/mL. After incubations, NHDFs were fixed by 10% TCA, washed, air-dried and stained with 0.4% SRB (S1402, Sigma-Aldrich) in 1% acetic acid.[4] The wells were then washed with 1% acetic acid, air-dried and the dye solubilised by 200 μL of 10 mM Tris base solution (pH 10.5) per well.[4] The ODs were read at 490 nm and at 540 nm using a microplate reader.[4] Percentage (%) of cell growth of the control (DMEM with 10% FBS)-treated NHDFs was calculated.[5,7]

GraphPad Software (Version 9.4.1, San Diego, CA) or IBM SPSS® Statistics (Version 29.0.2.0, Armonk, NY) were used for statistical analyses. Comparisons were made using the Wilcoxon signed ranks test. The effect of rhIL-1β and rhTNF-α on NHDF proliferation was analysed by one-way ANOVA, followed by the Šidák post-hoc test. Data are presented as median (IQR) unless stated otherwise. Statistical significance was set to $p < 0.05$.

3 | RESULTS

Six patients with clinically determined chronic VLUs, three hospitalised and three outpatients, were included between May 2020 and June 2021 (Table 1). No VLU showed signs of clinical infection.[3]

Incubation of WFs for 24 h decreased IL-1β by 88 (81–97)% and TNF-α by 64 (43–75)% (Figure 1B). The levels of the other peptides did not change significantly ($p > 0.05$). To understand the mechanism for the reduced cytokine levels, WFs were incubated with or without proteinase inhibitors. The EDTA/PMSF mixture reduced the decline in IL-1β levels completely (100%) in the three WFs; EDTA alone 10 (8–13)%. EDTA/PMSF had no effect on the TNF-α levels and E-64 alone reduced the decline in TNF-α levels by 2.3 (2.0–3.6)% (Table S3).

NHDFs exposed to WFs with low bioactivity at baseline (A, B, C, D and F) showed increased proliferation with the duration of prior WF incubation (Figure 1C). In contrast, the WF with highest bioactivity at baseline (E), lost bioactivity with incubation time (Figure S1). NHDF proliferation in the presence of the 24 h WF was significantly higher ($p = 0.046$) than with 1 h WF added to the fibroblasts (Figure 1D).

Exogenous IL-1β and TNF-α increased ($p < 0.001$) NHDF proliferation compared to control in the SRB assay. IL-1β elicited a maximal response at 0.1 ng/mL and TNF-α at 10 ng/mL (Figure 1E). Lower initial seeding density (0.2×10^4 NHDFs per well) yielded similar results with IL-1β and TNF-α treatment compared to the default seeding density (Tables S4 and S5).

4 | DISCUSSION

The balance between beneficial and unfavourable cytokines is often disturbed in VLUs by unknown mechanisms.[1] In the present study, the influence of proteolysis on a selection of wound-healing related cytokines was investigated. This was accomplished by incubating WFs from VLUs at body temperature and thus eliminating the effect of production of cytokines normally occurring in a wound. The main findings of this report were that the degradation differs among these cytokines and that the bioactivity (NHDF proliferation) of WF was improved with the duration of prior incubation of WFs perhaps by reducing the levels of detrimental factors. Imperatively, inactivating the detrimental factors by controlled proteolysis could lead to improved wound healing; a therapeutic approach that has been assessed in vitro using chronic WFs.[8]

TABLE 1 Patient, VLU and WF characteristics.

Patient	Age (years)	VLU age (months)	VLU size (cm^2)	WF volume (mL)	WF protein (mg/mL)[a]	VLU status[b]
A	57	18	5.74	1.1	38.9	Status quo
B	80	18	5.95	1.7	48.5	Status quo
C	77	6	9.22	2.9	34.4	Status quo
D	72	5	14.5	7.3	63.9	Improving
E	58	48	18.7	1.8	46.9	Improving
F	75	11	76.0	12	33.6	Deteriorating

[a]Measurements from Burian et al.[3]
[b]Clinical judgement based on changes of wound sizes, and the quality and quantity of the granulation tissue.

IL-1β and TNF-α levels decreased significantly in our study, possibly due to proteolysis by a combination of intrinsic and exogenous proteinases.[9–12] Ito et al.[13] found that matrix metalloproteinases digest IL-1β but not IL-1α. Here, endogenous IL-1β was predominantly degraded by serine proteinases and to a lesser extent by metalloproteinases. In contrast, minimal degradation of TNF-α could be ascribed to cysteine proteinases only. Kirketerp-Møller et al.[5] found no correlation between human neutrophil elastase activity and TNF-α levels in WFs from VLUs. We cannot exclude the possibility that TNF-α in WF undergoes structural changes with the incubation at 37°C.[14] Because of the complex composition of WFs, numerous molecules can interfere with the ELISA assay; soluble form of TNF RI is one example.[15] The use of Western blot would possibly illuminate the mechanism for the reduced TNF-α levels.[16] Regardless, these findings confirm previous observations of divergent proteolytic susceptibilities of IL-1β and TNF-α.[8,17]

We expected that the improved NHDF proliferation was due to the decreased levels of IL-1β and TNF-α. In contrast to our prediction, both cytokines stimulated proliferation of NHDFs.[18,19] This is counterintuitive as one would expect that the decreased levels of proliferative cytokines were accompanied by reduced and not improved proliferation. Doerfler et al.[7] found that the addition of the IL-1α/IL-1β antagonist IL-1ra to chronic WFs did not impact NHDF proliferation using the same assay as in the present study. This indicates that at least IL-1β in WF does not directly contribute significantly to proliferation and that other factors were responsible for the negative response of NHDFs that was improved with prior incubation. Cytokine candidates are IFN-α and IFN-γ that both inhibit NHDF proliferation.[20] These were not measured here. Another possibility is that induction of detrimental factors in the WF-treated NHDFs was decreased.[1]

The WF with highest bioactivity showed reduced ability to stimulate NHDF proliferation with the length of incubation, which indicates that positive factor(s) were degraded; one could be TGF-β1.[9]

We found no significant decline in GM-CSF, IL-1α, IL-6, IL-8, PDGF-AA or VEGF-A levels of 24-h incubated WFs. It is possible that the cytokine levels would have decreased with prolonged incubation as Boink et al.[21] observed degradation of rhIL-6 and rhIL-8 after 96 h, but not after 24 h exposure to VLU debridement tissue extracts.

In conclusion, most of the cytokines in VLU WFs were remarkably resilient over time except for IL-1β and TNF-α that were significantly reduced in levels. The developed ex vivo model is the first step in identifying unfavourable cytokines for VLU healing. In future studies this model would preferably be combined with unbiased proteomic technologies.

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CONFLICT OF INTEREST STATEMENT

No company or organisation played any role in the initiation or design of the study, collection of WFs, analyses, or interpretation of the data, writing of the manuscript or decision to publish the results.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the article and in the Supporting Information of the article.

ORCID

Ewa Anna Burian https://orcid.org/0000-0003-0630-2694
Magnus S. Ågren https://orcid.org/0000-0002-2263-0826

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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