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ORIGINAL ARTICLE

Acute and chronic wound fluids influence keratinocyte function differently

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Key words
Acute wound; Chronic wound; Gene expression; Keratinocytes; Migration; Proliferation; Wound healing; Wound fluid

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Abstract

Wound healing requires a proper functioning of keratinocytes that migrate, proliferate and lead to a competent wound closure. Impaired wound healing might be due to a disturbed keratinocyte function caused by the wound environment. Basically, chronic wound fluid (CWF) differs from acute wound fluid (AWF). The aim of this study was to analyse the effects of AWF and CWF on keratinocyte function. We therefore investigated keratinocyte migration and proliferation under the influence of AWF and CWF using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test and scratch assay. We further measured the gene expression by qRT-PCR regarding growth factors and matrixmetalloproteinases (MMPs) involved in regeneration processes. AWF had a positive impact on keratinocyte proliferation over time, whereas CWF had an anti-proliferative effect. Keratinocyte migration was significantly impaired by CWF in contrast to an undisturbed wound closure under the influence of AWF. MMP-9 expression was strongly upregulated by CWF compared with AWF. Keratinocyte function was significantly impaired by CWF. An excessive induction of MMP-9 by CWF might lead to a permanent degradation of extracellular matrix and thereby prevent wounds from healing.

Introduction

Wound healing is a multicellular process occurring in the following three stages: inflammation, new tissue formation and remodelling (1). Epithelialisation is the resurfacing of a wound to restore anatomy and function of the skin (2). Migration and proliferation of keratinocytes (KC) at the periphery of the wound play a mandatory role in this process (2–4). Proliferation of KC principally occurs in the basal lamina of the epithelium and these basal KC undergo terminal differentiation as they migrate to the surface (5). Growth factors that are known to stimulate wound healing include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), keratinocyte growth factor and others (4). Disorders in this well-orchestrated repair process lead to an impaired wound healing, which often affects patients with diseases, such as diabetes mellitus and atherosclerosis (6). Of over 150 million diabetic patients worldwide, more than 15%

Key Messages

- the aim of this study was to analyse the effects of acute and chronic wound fluids on keratinocyte function in a comparative way
- harvested fluids from acute and chronic wounds were added to the medium of keratinocyte cell cultures.
- proliferation, migration and gene expression patterns were examined
- keratinocyte function is negatively impaired by chronic wound fluid
- excessive induction of MMP-9 by chronic wound fluid might lead to a permanent degradation of extracellular matrix and thereby prevent wounds from healing

[†]Both authors contributed equally to this work.

suffer from chronic wounds (7). Thus, non-healing wounds represent a growing medical and socioeconomic problem. However, treatment strategies remain limited and largely ineffective (8). Furthermore, these wounds are often accompanied by odour, exudation and chronic pain associated with a reduction in quality of life (9). Previous work has demonstrated significant differences between acute and chronic wound environments (4,10). Compared with acute wound fluid (AWF), chronic wound fluid (CWF) is characterised by elevated local levels of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1). Proteases such as matrixmetalloproteinases (MMP) and neutrophil elastase are also elevated, whereas the levels of tissue inhibitors of metalloproteinases are reduced (11). These mediators of the chronic wound environment might disturb KC function, leading to impaired wound healing (4). Protein composition of AWF may, however, stimulate KC and thereby positively influence epithelialisation. The aim of this study was to analyse the differences in KC function in vitro under the influence of AWF and CWF. We examined the effect of AWF and CWF on proliferation, migration and gene expression of KC.

Methods

Cell culture

Human adult low calcium high temperature (HaCaT) cells, an immortalised human keratinocyte cell line developed by Deutsches Krebsforschungszentrum Heidelberg (DKFZ), were purchased from Cell Lines Service (CLS, Eppelheim, Germany). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Biochrom, Berlin, Germany) containing 10% foetal calf serum (FCS) or 2% FCS in experimental settings, respectively. HaCaT cells were used between passages 45 and 54.

Preparation of wound fluids

Ethical approval was obtained from the ethics committee of the Witten/Herdecke University (No 39/2007). Informed consent was obtained from all patients for wound fluid collection. Patient-specific data are shown in Table 1.

Acute wound fluid

AWF was collected from five patients after elective abdominoplasty. Wound fluid subcutaneously drained during the first 8 hours after operation was discarded to exclude blood contamination. Wound fluid drained within the following 8 hours was collected. After centrifugation, the supernatant was diluted with DMEM and filter sterilised. Protein concentrations of all samples were analysed using Bradford test according to the manufacturer's instructions and pooled afterwards.

Chronic wound fluid

CWF was harvested from chronic sacral decubitus. All decubitus existed for at least 6 weeks. Patients with prior vacuum therapy were excluded. Wound fluid was collected by applying an occlusive dressing for 24 hours. After wound fluid centrifugation, the supernatant was diluted with DMEM and passed through a sterile filter to prevent cell cultures from bacterial contamination. Wound fluids from five patients were subjected to Bradford assay for protein quantification and pooled for further experiments.

To evaluate the most suitable wound fluid concentration for this study, we investigated different wound fluid concentrations with respect to their impact on HaCaT proliferation using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Based on these results, we chose a concentration of 2% AWF and 2% CWF.

MTT assay

MTT test was performed to assess cell proliferation. Therefore, HaCaT cells were seeded on 96-well microplates. They were allowed to attach overnight and then incubated with 2% AWF or 2% CWF. The MTT assay was performed every 24 hours from day 1 until day 4 using MTT reagent (5 µg/ml; Sigma-Aldrich, Hamburg, Germany) according to the manufacturer's instructions. Extinction was measured at 570 nm using the ELISA reader µQuant (Biotek, Bad Friedrichshall, Germany).

Scratch assay

HaCaT cells were plated onto six-well microplates at a density of 700,000 cells per well. After 24 hours, cells were incubated with 10 µg/ml mitomycin C (Serva, Heidelberg, Germany) for 2 hours to inhibit cell proliferation. The HaCaT monolayer was then scratched with a plastic pipette tip in a standardised manner. Culture medium was changed by 2% AWF or 2% CWF. In vitro epithelialisation was documented by photography using Leica CTR400 microscope and Leica Application Suite V3.6 software. Wound closure was evaluated by measuring the remaining cell-free area using Adobe Photoshop 12.0 and expressed as percentage of the initial cell-free zone.

Quantitative real-time RT–PCR

A total of 400,000 HaCaT were placed on the six-well microplates and cultured according to the standard protocols. After 24 hours, they were exposed to 2% AWF or 2% CWF for 2, 12 and 24 hours or left untreated. Cells were dissolved in RLT buffer and RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis of 1 µg of total RNA was performed with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). qRT-PCR was performed using the Brilliant II SYBR Green QRT-PCR Master Mix (Agilent Technologies, Böblingen, Germany). Data were acquired with Stratagene Mx3005P QPCR System (Agilent Technologies). Primers were obtained from Biomers (Ulm, Germany) (Table S1). Expression was normalised to the housekeeping gene RPL. The comparative threshold cycle

Table 1 Patient data

Sample ID	Age (years)	Sex	BMI (kg/m^2)	Diabetes	Arterial hypertension	Vessel disease	Smoking	Microbiology of wound swab
AWF I	42	f	32·5	–	–	–	–	Negative
AWF II	34	m	19·2	–	–	–	–	Negative
AWF III	46	f	39·6	–	–	–	–	Negative
AWF IV	34	f	37·1	–	+	–	–	Negative
AWF V	65	f	26	+	+	–	–	Negative
Mean	44·2		30·88					
CWF I	61	f	35·7	+	+	PVD	+	Enterococci, pseudomonads
CWF II	87	m	16·3	–	–	–	+	Enterobacteria, enterococci, staphylococci
CWF III	63	f	35·6	+	–	–	–	Pseudomonads, staphylococci, streptococci
CWF IV	60	f	22·6	–	+	–	–	Enterobacteria, enterococci, pseudomonads, staphylococci
CWF V	61	f	26·9	–	+	–	–	Enterobacteria, enterococci
Mean	66·4		27·42					

f, female; m, male; AWF, acute wound fluid; CWF, chronic wound fluid; PVD, peripheral vascular disease.

(C_t) method was applied to determine relative expression differences (12).

Statistical analysis

For statistical reasons, all experiments were performed in triplets. Data are shown as mean ± standard deviation unless otherwise indicated. Continuous variables were compared using Student's *t*-test. A two-tailed *P*-value <0·05 was considered significant.

Results

Protein concentration of wound fluids

Protein concentrations of AWF obtained from five different patients ranged from 35·82 to 41·72 g/l (38·77 ± 4·17 g/l), whereas the protein concentrations of CWF samples were slightly lower, varying from 28·64 to 38·25 g/l (33·45 ± 6·80 g/l) as summarised in Table 2.

Table 2 Protein concentration of different wound fluid samples

Sample	Protein concentration (g/l)	Sample	Protein concentration (g/l)
AWF I	38·91	CWF I	31·34
AWF II	41·72	CWF II	35·81
AWF III	37·16	CWF III	34·30
AWF IV	36·17	CWF IV	32·26
AWF V	35·82	CWF V	38·25
Mean ± SD	38·77 ± 4·17	Mean ± SD	33·45 ± 6·80

AWF, acute wound fluid; CWF, chronic wound fluid.

HaCaT proliferation

After 24 hours of incubation with 2% AWF, KC proliferation decreased to 68·1% compared with the untreated control and then continuously increased to 121·1% after 4 days. Under the influence of 2% CWF, proliferation was reduced to 58·4% after 1 day and 38·3% after 2 days, followed by a slight increase to 54·9% on day 3 and 74·0% on day 4 (Figure 1).

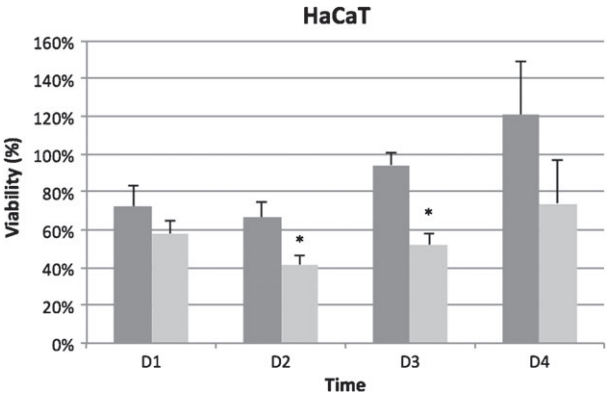

Figure 1 Proliferation of keratinocytes (KC) during incubation with acute wound fluid (AWF) and chronic wound fluid (CWF). Incubation with AWF is indicated as dark grey bars, with CWF as light grey bars. The proliferation is presented as percentage related to the untreated control. The results of three independent experiments plotted as mean ± SD are shown. Asterisks indicate significant differences between KC proliferation under the influence of AWF and CWF ($P \leq 0{\cdot}05$).

HaCaT migration

To assess KC migration without bias through proliferation, the scratch assay was performed after preincubation with mitomycin C. After 12 hours, there were only marginal differences regarding the size of the cell-free area between all three groups (control: 70%, AWF: 68·6%, CWF: 74·9%). After 24 hours of incubation, the remaining cell-free area decreased to 21·1% in the control group. Influenced by AWF and CWF, the cell-free area measured 30·6% and 49·1%, respectively. Although in vitro wound closure was nearly completed after incubation with AWF within 36 hours (control: 2·5%, AWF: 4·1%), the

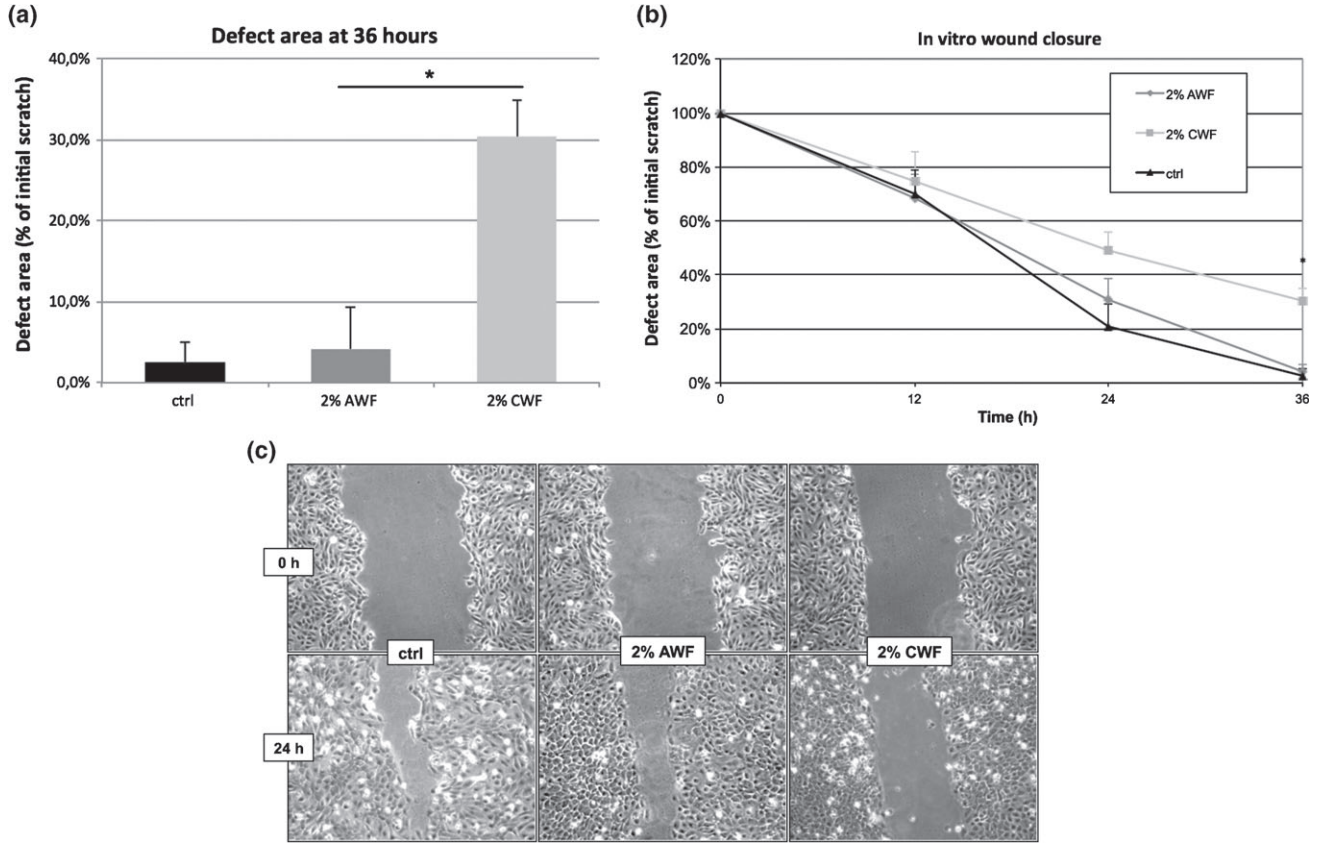

Figure 2 Scratch assay. Keratinocytes were plated onto microplates. After 24 hours, they were incubated with 10 µg/ml mitomycin C for 2 hours to inhibit cell proliferation. The monolayer was scratched with a plastic pipette tip. Culture medium was changed (black) or replaced by 2% acute wound fluid (dark grey) or 2% chronic wound fluid (light grey) and in vitro epithelialisation was documented. Wound closure was evaluated by measuring the remaining cell-free area and expressed as percentage of the initial cell-free zone. The results of three independent experiments as mean ± SD are shown. $*P \leq 0{\cdot}05$. (A) Remaining cell-free area after 36 hours. (B) Time course of epithelialisation. (C) Representative photo documentation at 24 hours.

cell-free area was significantly bigger (30·4%, $P < 0{\cdot}05$) with CWF. The results are shown in Figure 2.

Gene expression of HaCaT

For qRT-PCR, we focussed on two important growth factors, VEGF and b-FGF, during the physiological wound healing. We further measured MMP-2 and MMP-9, which are proteases involved in extracellular matrix (ECM) degradation and allow KC to migrate to the wound bed and resurface the denuded skin. After 2 hours of treatment, VEGF expression by AWF and CWF started and increased to a fold change of nearly 3 (AWF) and 5 (CWF) after 12 hours. Gene expression was almost stagnant within the next 12 hours. Expression of b-FGF increased after 12 hours and reached a fold change of 5 (AWF) and 6·5 (CWF) after 24 hours. MMP-2 was only minimally regulated by both wound fluids, whereas MMP-9 showed a strong induction. Under the influence of CWF, MMP-9 expression increased to a fold change of 24 after 2 hours and then slowly decreased to 13 after 24 hours. Upon incubation with AWF, MMP-9 was induced ninefold after 12 hours and then directly dropped to fourfold. Results are shown in Figure 3.

Discussion

Wound healing is an evolutionarily conserved process that aims the restoration of anatomic continuity and function of the skin and involves the interaction of various cell types, including KC (13,14) Environmental conditions such as the absence or surplus of certain mediators might have an impact on KC function and thereby impair wound healing. The wound itself represents a reactive microenvironment (15), which we were able to transfer into an in vitro wound model. The wound fluids have been widely studied but rarely used in cell cultures. Previous studies have shown considerable differences in protease levels with much higher levels in CWF (16). Analysis of wound fluids regarding their growth factor content is discussed controversially in the literature. In most studies, the average level of growth factors in CWF was shown to be significantly lower than that in AWF (16,17). However, the negative impact of the chronic wound environment can certainly not be reduced to a single factor but must be explained by a disadvantageous combination of all effectors. The competence of KC to close cutaneous defects of skin in vivo rests upon their ability to migrate and proliferate (2) To gain insight into disturbed wound

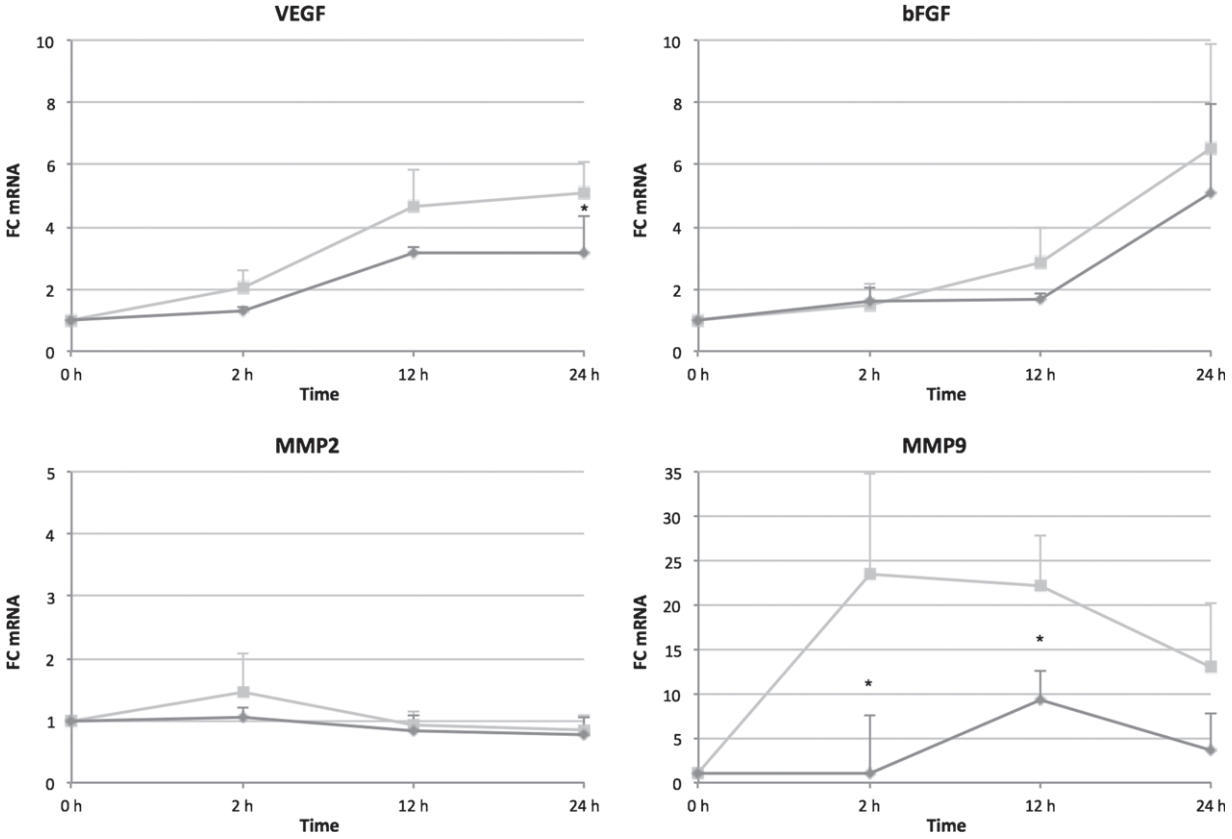

Figure 3 Gene expression changes by acute wound fluid (AWF) and chronic wound fluid (CWF). Keratinocytes were treated with 2% AWF (dark grey lines) or 2% CWF (light black lines) for 2, 12 and 24 hours or left untreated. Fold-changes of gene expression were analysed by qRT–PCR and plotted as mean ± SD. Asterisks indicate significant differences in gene expression between AWF and CWF with $P \leq 0{\cdot}05$ ($n = 3$).

healing processes, we therefore investigated the proliferation and migration capacity of KC under the influence of AWF and CWF. The proliferation assay demonstrated that initially, both AWF and CWF reduced the proliferation capacity of KC. However, AWF stimulated proliferation over time, whereas CWF impaired KC proliferation continuously. These findings fit into our understanding of a compromised KC function in the healing process of chronic wounds. The initial inhibition of proliferation mediated by AWF and CWF might be because of adaption processes to new culture conditions. CWF has previously been shown to impair proliferation of different cell types, including KC (18,19) and the proliferation of fibroblasts and endothelial cells has been reported to be stimulated by AWF (20). A lack of distinct growth factors or the surplus of cytotoxic agents in CWF might explain these differences. Undisturbed migration of KC is an important requirement in resurfacing the wound and plays a decisive role in the very early stage of wound healing. This directed migration is critical to epithelialisation and depends on a complex balance of mediators (14). The composition of mediators in CWF might diminish KC migration and therefore impair or even prevent undisturbed wound closure. As demonstrated by the scratch assay results, wound closure was significantly delayed by CWF. As cell proliferation was inhibited by mitomycin C, these results cannot be explained by the proliferation-inhibiting effect of CWF. They clearly demonstrate a negative impact of CWF on KC migration, which may be a relevant factor explaining the low tendency of chronic wounds to heal. Wound healing mechanisms are enhanced by a subset of growth factors secreted by various cell types (4). Resident and infiltrating cells interact with these growths factors and thereby orchestrate the wound healing process. A poor growth factor production or a lack of their availability has been shown to be a major cause of a compromised regeneration process in chronic wounds (21). Wound infection might play another important role in impaired wound healing. All chronic wounds are secondarily colonised by bacteria from the surrounding skin or the local environment (22). Lipopolysaccharides (LPS) are found in the outer membrane of Gram-negative bacteria and act as endotoxins. Loryman and Mansbridge showed that LPS decreased keratinocyte migration in vitro (23). We analysed the gene expression profile of KC with regard to VEGF and b-FGF under the influence of AWF and CWF. VEGF is known to have a stimulating effect on wound healing mainly through angiogenesis, but likely promotes epithelialisation and ECM deposition as well (24). As angiogenesis is a key component of the physiological repair process, diminished VEGF production and therefore angiogenesis are likely to contribute to impaired tissue regeneration (24,25). In this study, VEGF was strongly induced by CWF than AWF, which might be because of tissue hypoxia in chronic wounds,

activating the hypoxia-inducible factor (HIF)-1α pathway and thereby inducing VEGF expression (26). So a lacking expression of angiogenic factors by KC seems not to be responsible for an insufficient angiogenesis in chronic wounds. Deficiencies might be located in maturation and remodelling processes, which are also required to achieve a functional and stable vascular network (27–30). b-FGF is another angiogenic factor that has also been shown to stimulate KC proliferation (31). Under the influence of AWF and CWF, KC showed a significantly elevated expression of b-FGF, but CWF had a slightly stronger effect. However, as b-FGF activity is primarily regulated by its receptor level, the sole consideration of b-FGF expression does not necessarily reflect its potential effects (29). MMPs are mandatory during the physiological wound healing process as they are involved in ECM remodelling and epithelialisation (32,33). During epithelialisation, KC have to loosen their cell-cell and cell-ECM contacts at the wound margin (33). MMPs have been shown to lessen these contacts allowing KC to migrate across the wound (34,35). We therefore investigated the expression of MMP-2 and MMP-9, which are overrepresented in CWF probably causing a constant ECM degradation in chronic wounds (17,36,37). In our study, MMP-2 expression of KC was only marginally affected by AWF and CWF, respectively. The role of MMP-2 during wound healing is discussed controversially in the literature. Although some groups relate an important role of MMP-2 during physiological wound healing (38), others claim that MMP-2 activity is not essential for this process (39). MMP-9, however, was upregulated by both wound fluids with CWF having an earlier and much stronger effect. This might be explained by the higher levels of proinflammatory cytokines in CWF such as IL-1 and TNF-α that are well known to stimulate MMP-9 expression (36). Although MMP-9 is mandatory during the wound healing process (40), excessive MMP-9 production is deleterious (41). So an excessively increased MMP-9 expression by KC in the chronic wound environment might cause continuous self-digestion of ECM and thereby impair wound healing. Furthermore, MMP-9 leads to degradation of growth factors and their receptors (42), and is likely to have a negative impact on cell proliferation (33).

These results give an insight into impaired KC function in chronic wounds and identify possible targets for intervention. As proliferation and migration are the two key skills that give KC the ability to resurface a wound, impaired wound healing might partially be explained by deficiencies in these functions. Further studies are needed to identify major effectors within CWF that cause the detected effects. This understanding might then help to develop new treatment strategies that aim reactivation of impaired KC function by establishing a more physiological wound environment through inhibiting these adverse mediators.

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Supporting Information

The following supporting information is available for this article:

Table S1. Primer sequences used for qRT-PCR

Additional Supporting Information may be found in the online version of this article.

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