

Chapter 4 Comparison of cytokine,
chemokine and growth factor
profiles in burn wounds, chronic
wounds and surgical excision
wounds

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ABSTRACT

Full thickness burn wounds often heal with hypertrophic scar formation and chronic wounds are difficult to close. Knowledge on the inflammatory status of the different wound beds will support the development of advanced medical therapies. Wound exudates were isolated from full thickness burns, venous ulcers and surgical excision wounds. Inflammatory status was studied by determining the expression levels of pro-inflammatory cytokines (IL-1 α , IL-6, IL-8, IL-10, TNF- α , IFN- γ), inflammatory chemokines (CCL2, CCL5, CCL17, CCL18, CCL20, CCL27, CXCL1, CXCL12) and growth factors (bFGF, HGF, TGF- β 1) by ELISA. Burn wounds secrete low amounts of cytokines and high amounts of chemokines and growth factors compared to surgical wounds. Chronic wounds secrete high amounts of cytokines similar to surgical wounds, and higher amounts of chemokines and growth factors than surgical wounds. When comparing with surgical wounds TNF- α was low in burn and chronic wounds, CCL5/RANTES and CCL27/ CTACK was high only in burns, and CCL20/ MIP-3 α and CXCL1/ Gro- α were high only in chronic wounds. This study has identified wound healing mediators potentially suitable for targeting and provides information on the wound bed status which may be exploited when implementing wound healing therapies where further over-activation or under-activation of the wound bed should be avoided.

INTRODUCTION

Full thickness skin wounds can be categorized into 3 types: surgical wounds (e.g.: tumor excision surgery and reconstruction surgery), trauma induced wounds (e.g.: deep thermal burns) and chronic wounds (e.g.: lower leg ulcers, diabetic foot, decubitus, abdominal wall defects). Surgical wounds are generally clean, non-infected wounds, which are devoid of necrotic tissue. Since they heal rapidly and usually with an acceptable scar, they are considered as good healing wounds. In contrast, full thickness burn wounds and chronic wounds are regarded as difficult to heal wounds. Deep burn wounds heal with more than 90% chance of forming a hypertrophic scar. This is thought to be initiated by the extensive necrosis, edema and hypoxia in the wound bed (1). Chronic wounds have an inert wound bed making them therapy resistant. Such wounds may remain open for many years (2). Current therapies for closing all 3 types of deep skin wounds include application of autografts (full thickness, split thickness or punch biopsies). More advanced therapies include application of novel tissue engineered skin constructs (3). Since autografts and skin substitutes (SS) are placed directly onto the wound bed, it would be beneficial to have prior knowledge of the inflammatory status of the wound bed. This would permit treatments to be modified in order to ensure maximal take and optimal wound healing. Knowledge of the wound bed status can be achieved by analyzing wound healing (WH) mediators present in wound exudates (4). The aim of this study was to analyze the WH mediators, such as cytokines, chemokines and growth factors, present in the wound bed of the 3 types of wounds at the time when an autograft or SS would be applied.

A number of studies have shown that WH mediators derived from wound exudates can indeed influence cell proliferation; e.g. exudates from acute wounds stimulate fibroblast and endothelial cell growth (5); burn blister fluids increase keratinocyte proliferation (6) and chronic wound exudates inhibit keratinocyte and fibroblast proliferation (7). Therefore, wound exudates reflect the extracellular environment of the wound and may be used to gain information on the status of different wound beds. Comparison of wound exudates obtained from surgical wounds, burn wounds and chronic wounds may provide information as to why for example a burn wound heals readily but with hypertrophic scarring whereas some leg ulcers are inert non-healing wounds, which if they do heal, tend to heal with a relatively good scar quality. Wound healing mediators which are over or under expressed in one wound type compared to other wound types may be targeted in novel advanced wound healing therapies.

A variety of cytokines, chemokines and growth factors are differentially expressed during cutaneous wound healing (8). They are produced by several cell types, such as skin residential cells, immune cells and the bacterial flora present in the wound bed. These wound healing mediators play an essential role in the 3 phases of wound healing; inflammation, wound closure and tissue remodeling (8). Cytokines regulate the intensity and the duration

of the inflammatory response. Chemokines are important for attracting 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 maintenance of repair of tissues and tissue remodeling.

In this study we determined the profile of a panel of cytokines, chemokines and growth factors present in 3 different types of cutaneous wounds at the point in time when an autograft or SS would be applied to the wound. Surgical wound exudates were obtained from tumor excision wounds and were collected from occlusive dressings which were applied for 7 days to the full thickness skin wound. This 7-day interval is the period during which the excision wound is left open awaiting pathological confirmation that the tumor is excised completely. Burn exudates were extracted from the living tissue interface of freshly excised eschar tissue, which was removed surgically from deep burn wounds at 11-21 days post burn. Exudates from chronic, therapy resistant leg ulcers were extracted from debridement tissue, which was removed surgically during the weekly visit to the outpatient clinic.

The amounts of cytokines (IL-1 α , IL-6, IL-8, IL-10, IFN- γ and TNF- α), chemokines (CCL2, CCL5, CCL17, CCL18, CCL20, CCL27, CXCL1, CXCL12) and growth factors (bFGF, HGF, TGF- β 1) are described in the wound bed of surgical wounds, full thickness burn wounds and chronic leg ulcers. Such profiles may identify potential WH mediators suitable for targeting and will provide information on the wound bed status, which may be exploited when implementing advanced wound healing therapies where further over-activation (e.g.:burn) or under-activation (e.g.: chronic wound) of the wound bed should be avoided.

METHODS AND MATERIAL

Samples

The wound bed of patients with acute surgical wounds (n=6), chronic leg ulcers (n=12) and deep burn wounds (n=10) were analyzed at the time when an autograft or SS would be applied as therapy for healing the wound. All procedures were performed with consent of the medical ethics committee of our hospital and in agreement with the ethical guidelines of the 1975 Declaration of Helsinki.

Surgical wounds A tumor excision was performed in 6 patients diagnosed with various skin malignancies. The tumor was totally removed with an adequate margin of surrounding healthy appearing skin. The surgical wounds were then covered by gauze under a plaster for 7 days until further treatment could be applied once the wound margins had been confirmed tumor free by histopathologic examination. Upon confirmation the gauze was removed and WH mediators were extracted from the gauzes and autograft was applied to the wound bed. The gauze was soaked in 1ml PBS with protease inhibitor cocktail (1:100) and gently shaken

at 4°C for 1 hour. After incubation samples were centrifuged and supernatant was stored at -80°C until further analysis.

Leg ulcers Therapy resistant chronic venous ulcers (n=12), which had been present for more than 1-year and showed no tendency to heal for more than 12 weeks of adequate dermatological treatment were included in this study. Surgical debridement was conducted during the weekly visit to our out patient clinic. Debridement tissue was taken up in 1ml phosphate buffered saline (PBS) (B.Braun, Melsungen, Germany) with protease inhibitor cocktail (PIC) (Sigma- Aldrich, Steinheim, Germany) (1:100). Hereafter it was processed as described above.

Burn wounds In 10 patients with full thickness burn wounds removal of eschar tissue was conducted within 11-21 days post burn. Eschar was removed to the depths where viable tissue was reached. Wound exudates were directly scraped off from the viable, superficial lower layer of eschar tissue and added to 1ml PBS with protease inhibitor cocktail (1:100). Hereafter it was processed as described above.

Since the final concentration of WH mediators present in the exudates was influenced by the dilution factor introduced by the buffer, supernatant of all samples were normalized for total protein content using Bio-Rad Protein Assay (BioRad Laboratories, Hercules, California) essentially as described by supplier.

Enzyme-linked immunosorbent assay for chemokine production

For cytokine, chemokine and growth factor quantification in wound bed samples, enzyme-linked immunosorbent assays (ELISA) reagents were used in accordance to the manufacturer's specifications. IL-1RA, bFGF, IL-1 α , CCL2, CCL5, CCL17, CCL18, CCL20, CCL27, HGF, CXCL1, CXCL12, 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. Protein levels of all wound healing factors were corrected for total protein present in the wound bed sample, and results are expressed as amount of wound healing factor/ total protein.

Data analysis

All data are presented as mean \pm standard error mean. Differences were evaluated by one-way ANOVA post hoc dunnet's, using computer program GraphPad Prism (San Diego, CA, USA).

RESULTS

Characterization of wounds

The 3 different types of wounds are shown in figure 1. In full thickness surgical wounds the underlying adipose tissue is exposed. From these wounds, exudates are collected from under an occlusive dressing. For deep full thickness burns, the eschar tissue is surgically removed and exudates are scraped off from the interface of dead-living tissue. For leg ulcers, exudates are extracted from the debridement tissue routinely scraped from the inert wound bed. Collection of wound exudates is described in detail in Material and Methods.

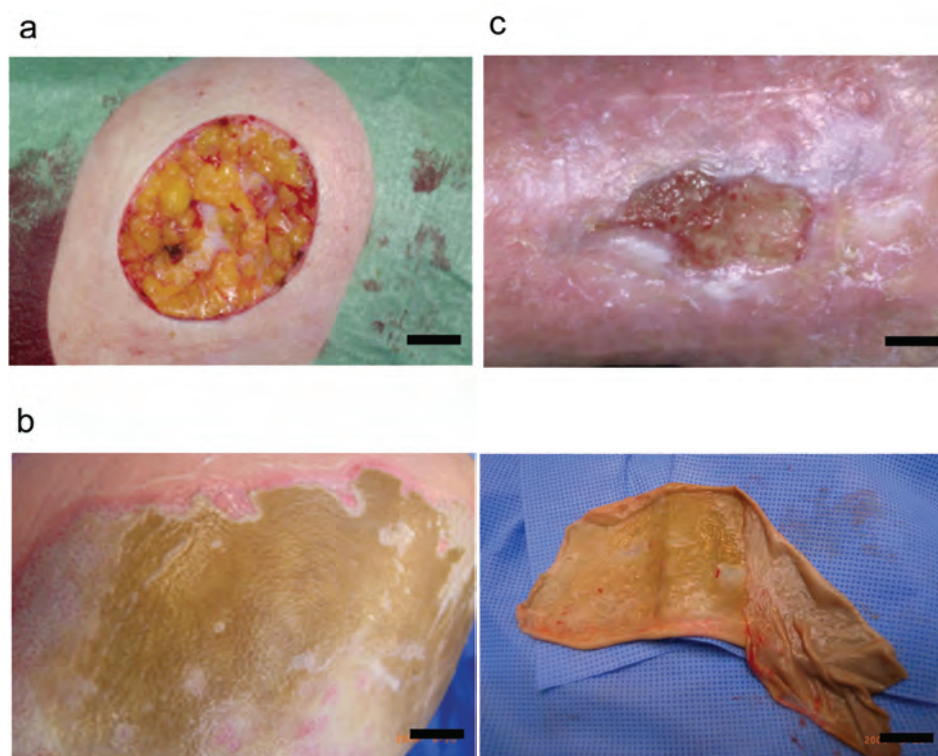


Figure 1: Difficult to heal wounds.

Photograph of a representative a) surgical wound (bar = 2cm), b) burn wound (upper) (bar = 1cm) and excised eschar tissue (lower) and c) chronic leg ulcer (bar = 2 cm) are shown.

Cytokines

First, the levels of cytokines (IL-1 α , IL-6, IL-8, IL-10, INF- γ and TNF- α) present in burn wounds and chronic ulcers was compared with surgical wounds (Figure 2). All cytokine levels were

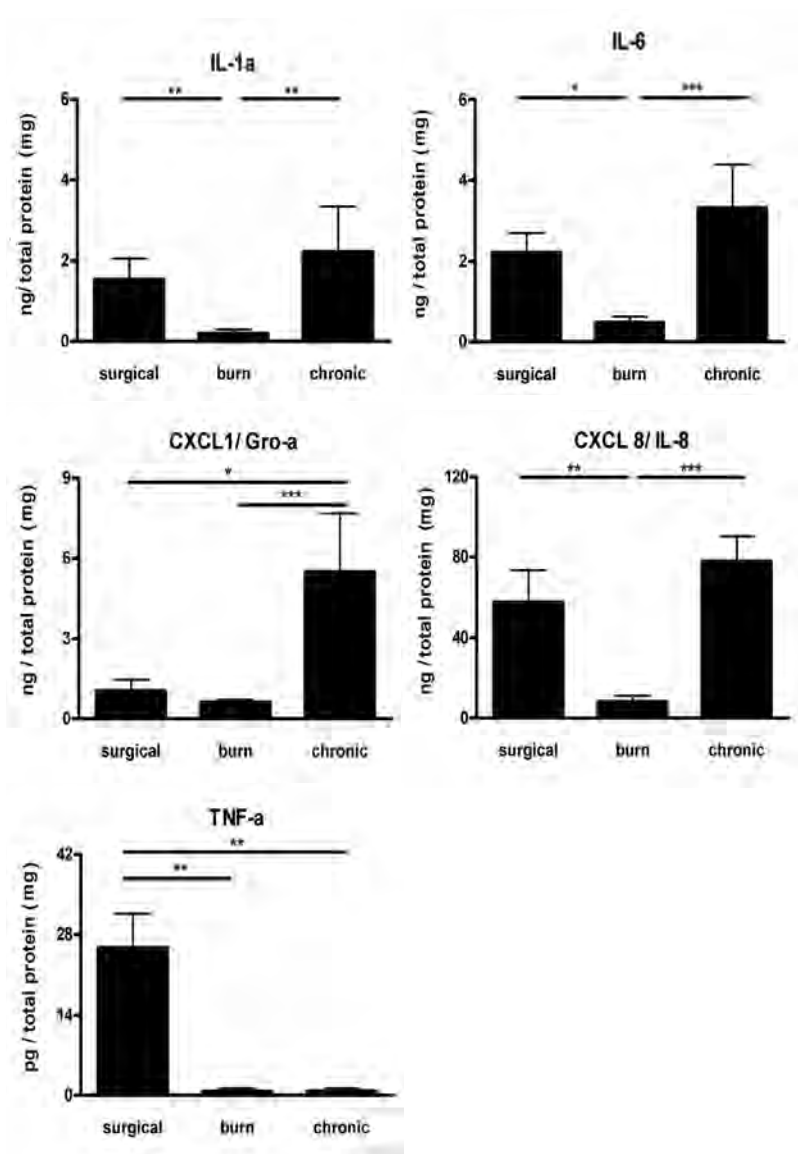


Figure 2: Cytokines.

Comparison of pro-inflammatory cytokines in wound exudates isolated from the wound bed of surgical wounds (n=6), deep burn wounds (n=10) and chronic leg ulcer (n=12). Cytokine amounts were quantified by enzyme-linked immunosorbent assay and expressed as ng or pg/mg total protein. Mann-Whitney -test. * P<0.05, ** P<0.01, *** P<0.001. Mean +/- SEM is shown.

extremely low in burns when compared to surgical wounds. These results are not an artifact due to sampling since chemokines and growth factor levels were high in burns compared to surgical wounds (See figure 3, 4 and below). In chronic wounds the cytokine levels were comparable to surgical wounds with the exception of TNF- α , which, as with burns wounds, was very low.

In conclusion, cytokine levels were extremely low in burns when compared to surgical wounds or chronic wounds. TNF- α was the exception since it was low in both burns and chronic wounds

Chemokines

Next, the level of chemokines (CCL2, CCL5, CCL18, CCL20, CCL27, CXCL1 and CXCL12) present in burn wounds and chronic ulcers was compared with surgical wounds (Figure 3). In contrast to cytokine levels, in general chemokine levels were remarkably high in burn and chronic wounds compared to surgical wounds. In burn wounds higher levels of CCL2 (trend), CCL5,

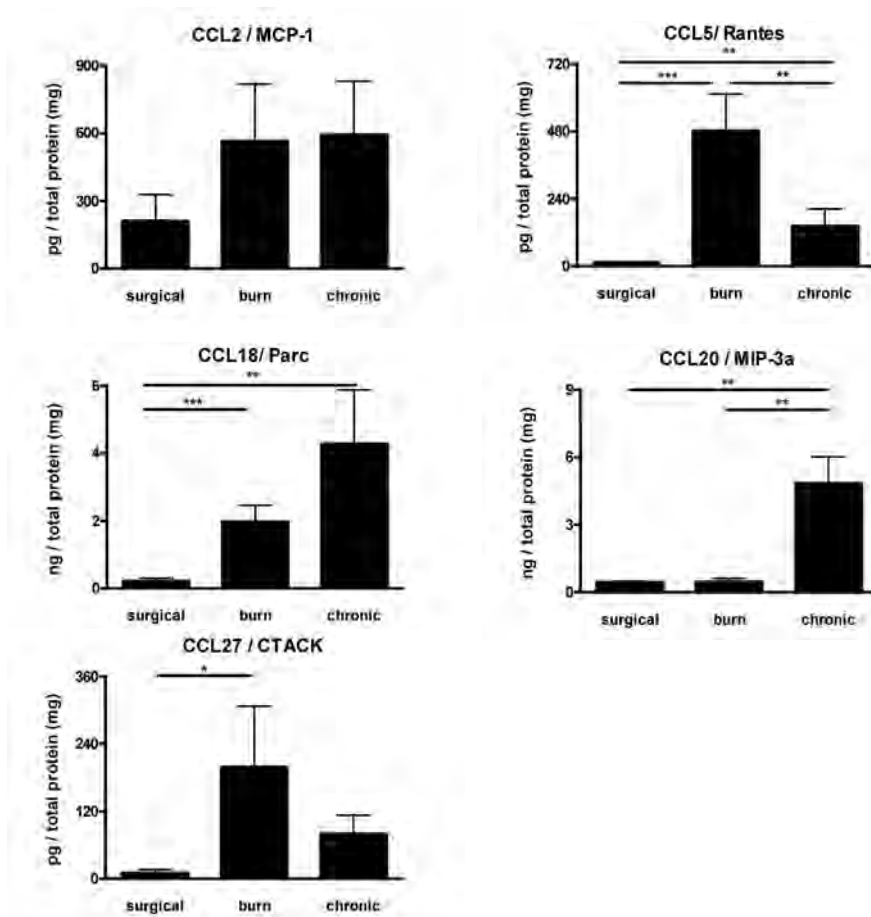


Figure 3: Chemokines.

Comparison of inflammatory chemokines in wound exudates isolated from the wound bed of surgical wounds (n=6), deep burn wounds (n=10) and chronic leg ulcer (n=12). Chemokine amounts were quantified by enzyme-linked immunosorbent assay and expressed as ng or pg/ mg total protein. Mann-Whitney -test. * P<0.05, ** P<0.01, *** P<0.001. Mean +/- SEM is shown.

CCL18, CCL27 and CXCL12 (trend) were observed when compared to surgical wounds. CCL20 and CXCL1 were detected at similar low levels in burn and surgical wounds. For chronic wounds, higher levels of CCL2 (trend), CCL5, CCL18, CCL20, CCL27 (trend) and CXCL12 (trend) were observed when compared to surgical wounds. CCL17 was not detectable in any of the 3 wounds described in this study.

In conclusion, inflammatory chemokine levels were higher in both burn and chronic wounds compared to surgical wounds. Notably, when comparing the 3 wound types, CCL5 levels were high in burn wounds, whereas CCL20 and CXCL1 levels were high in chronic wounds.

Growth factors

In addition to cytokines and chemokines, the level of major growth factors were studied. As with chemokines and in contrast to cytokines, growth factors (bFGF, HGF and TGF- β 1) showed remarkable increases in burn wounds when compared to surgical wounds (Figure 4). In contrast, only the level of HGF was increased in chronic wounds compared to surgical wounds. Notably, bFGF and TGF- β 1 levels were higher in burn wounds compared to either surgical or chronic wounds.

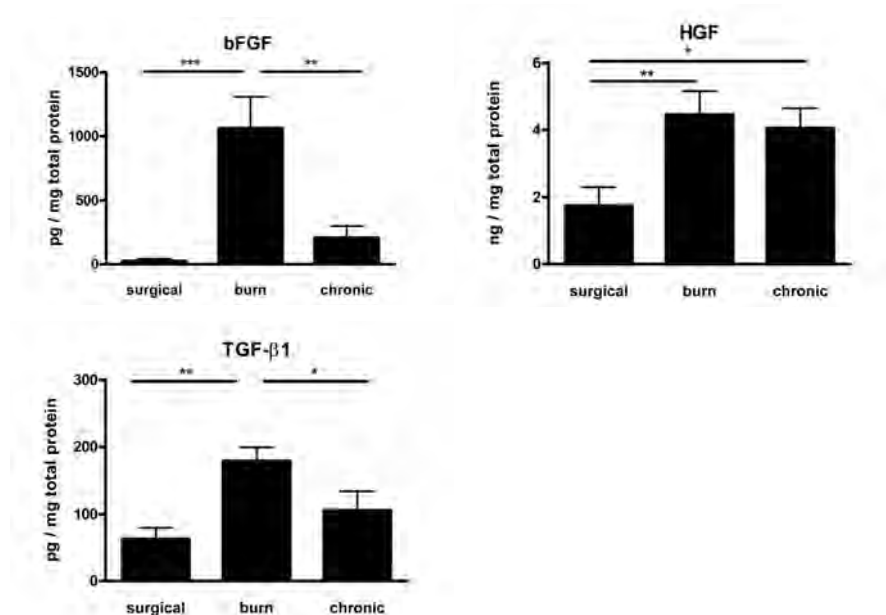


Figure 4: Growth factors.

Comparison of growth factors in wound exudates isolated from the wound bed of surgical wounds (n=6), deep burn wounds (n=10) and chronic leg ulcer (n=12). Growth factor amounts were quantified by enzyme-linked immunosorbent assay and expressed as ng or pg/ mg total protein. Mann-Whitney -test. * P<0.05, ** P<0.01, *** P<0.001. Mean +/- SEM is shown.

DISCUSSION

This study describes the inflammatory factor profiles of 3 different types of deep cutaneous wounds. Two are difficult to heal wound types (full thickness burn wounds and chronic leg ulcers) and the third is the relatively good healing surgical excision wound. The focus of the study was on the WH mediator profile present in the wound bed at the time a SS or autograft was applied in order to close the wound. The differential expression of cytokines, chemokines and growth factors in difficult to heal wounds is summarized in Table 1 and discussed in detail below.

Table 1: Differential WH mediator expression in surgical, burn and chronic wounds

Group	WH mediator	Surgical	Burn	chronic
cytokine	IL-6	↑↑	↑	↑↑↑
	IL-8 / CXCL8	↑↑↑↑	↑↑↑	↑↑↑↑
	IL-10	↑	↑	↑
chemokines	CCL2 / MCP-1	↑	↑↑	↑↑
	CCL5 / RANTES	↑	↑↑	↑
	CCL18 / Parc	↑	↑↑	↑↑↑
	CCL20 / MIP-3α	↑	↑	↑↑↑
	CCL27 / CTACK	↑	↑	↑
	CXCL1 / Gro-α	↑↑	↑	↑↑↑
	CXCL12 / SDF-1	-	↑	↑
Growth factors	bFGF	↑	↑↑	↑
	HGF	↑↑	↑↑↑	↑↑↑
	TGF-β1	↑	↑	↑

Overview of WH mediator expression in wound exudates isolated from surgical wounds (n=6), deep burn wounds (n=10), and chronic venous ulcers (n=12). ↑↑↑↑: >10 ng / total protein (mg); ↑↑↑: > 3<10 ng / total protein (mg); ↑↑: > 0.5 <3 ng / total protein (mg); ↑: < 0.5 ng / total protein (mg); -: not detected.

Upon infliction of a wound, pro-inflammatory cytokines are released from the damaged tissue. These are taken up by infiltrating cells (macrophages/ neutrophils) and surrounding residential cells, which in turn produce inflammatory chemokines. Chemokines are chemotactic for inflammatory cells and in addition regulate migration and proliferation of skin residential cells thus inducing wound closure (8). The extent of the (pro) inflammatory response is proportional to the extent of the initial tissue damage.

In the surgical wound profile we observed a relatively and generally higher level of cytokines compared to chemokines and growth factors. This profile is indicative of an acute skin wound where no excessive inflammation or granulation tissue synthesis is occurring. Therefore the profile is in agreement with the clinical description of such an excision wound.

In burn wounds, relatively low levels of pro-inflammatory cytokines and higher levels of inflammatory chemokines were detected. In burn wounds, in which extensive tissue damage occurs (e.g.: more than in surgical excision wounds) the initial cytokine response would be expected to be very high. Cytokines result in chemokines being secreted from residential cells which in turn result in a massive influx of infiltrating cells into the wound bed compared to surgical excision wounds. The massive influx of infiltrating cells remove cytokines from the wound bed along with increasing their chemokine production and in turn attracting even more inflammatory cells into the wound bed. In line with this, in burn wounds the (pro-) inflammatory response is greater than in surgical wounds in which necrotic tissue is negligible.

In chronic wounds, comparable levels of pro-inflammatory cytokines and high levels of inflammatory chemokines were detected. This is in addition with the persistent inflammation which is a feature of chronic wounds (9). Other reports have reported substantial amounts of TNF- α in wound fluid from chronic wounds, while in this study it is only present in acute wounds (already discussed in chapter 2 page 40). Whether TNF- α plays a role in optimal healing in surgical wounds and whether TNF- α neutralization or administration to burn-and chronic wounds respectively would improve healing needs further investigation.

In this study, the expression of some chemokines appeared to be wound specific. A high level of CCL5/ RANTES and CCL27/ CTACK was noted in burn exudates compared to both surgical and chronic wound exudates. Recently we have shown that CCL5/ RANTES is a potent chemo-attractant molecule for mesenchymal stem cells derived from adipose tissue (12). Also, it has been shown that adipose derived mesenchymal stem cells (known as adipose derived fibroblasts) express high levels of alpha-smooth muscle actin (α -SMA) which is related to adverse scar contraction and formation (13). It is therefore possible that CCL5/ RANTES in the wound bed of burns may play a role in hypertrophic scar formation. This is supported by the knowledge that hypertrophic scars are rarely observed in surgical or chronic wounds. CCL5/ RANTES is therefore a potentially interesting chemokine to investigate when developing advanced wound healing therapies aiming for improved scar formation. As mentioned, in addition to CCL5/ RANTES, CCL27/ CTACK was also increased in burns compared to surgical wounds. CCL27/ CTACK is constitutively expressed by keratinocytes, has been reported to be up regulated upon skin inflammation and induces migration of keratinocyte precursors from bone marrow cells (14,15). Taken together, our findings and those published by others indicate that the high presence of CCL27 in burn wounds may influence the quality of wound healing and scar.

With regards to chronic wounds, a noticeably high level of chemokines CCL20 and CXCL1/ Gro- α was observed compared to both surgical and burn wound exudates. CCL20 is a strong chemo-attractant for inflammatory and regulatory T-cells and is secreted within minutes after tissue damage (16). CXCL1 is a strong chemo-attractant for neutrophils and is secreted already within one day after wounding. In addition, CXCL1 is reported to stimulate keratinocyte migration and neovascularization (17). Until now, neither of these chemokines has been

implicated with therapy resistant non-healing wounds. CCL20 and CXCL1 are therefore potentially interesting chemokines to target when considering the development of advanced wound healing therapies aimed at closing therapy resistant ulcers.

In addition to cytokines and chemokines, the levels of major growth factors were studied. The higher expression of growth factors observed in the burn exudates compared to the surgical or chronic wound exudates may be related to the extensive degree of acute tissue damage observed only in burn wounds. Growth factors are required for tissue repair and remodeling in order to restore skin integrity. Our results on TGF- β 1 are in agreement with those reported by Ferguson et al., who confirmed the role of TGF- β 1 in scar formation by showing that neutralizing TGF- β 1 in adult rodents results in scar free healing (18). Our results on bFGF are in contrast to those reported by others, who demonstrated low levels of bFGF in partial thickness burn wounds (6, 19). Application of bFGF to these partial thickness wounds accelerated WH and resulted in better scar quality (20). This indicates that the amount of growth factor detected in a wound may be dependent on the severity of the wound and / or the time at which the exudates are analysed, and this should be taken into account when a therapy is administered.

In conclusion, our results indicate that the wound bed of surgical excision wounds is healing in a normal manner as described in a number of articles (4,10,20). Therefore one would expect an autograft or SS with characteristics of healthy skin to be most suitable for closing surgical wounds. For burn wounds however, our results indicate a highly inflamed, granulating wound bed. Chemokines and in particular growth factors which are potent stimulators of granulation tissue are highly expressed. During treatment, care should be taken not to over activate the wound bed further since this may result in excessive granulation tissue formation and adverse scar formation. Indeed a custom designed SS that reduces the inflammation status of the wound bed would be preferred and may prove to be even more suitable than classic autograft which currently heals with poor scar quality. In contrast, chronic wounds lack transition to different phases leading to complete WH. The stagnation observed in the healing process of chronic wounds is possibly partly due to an imbalance in pro- and anti-inflammatory mediators (9). A SS suitable for chronic wounds should be very potent in secreting granulation-stimulating factors in order to stimulate the inert wound bed into healing. Indeed, we have previously shown that our in house autologous full thickness SS (reconstructed epidermis on fibroblast populated dermis) secretes significantly greater amounts of (pro)-inflammatory mediators compared to an autograft and is able to heal therapy resistant ulcers which had previously been unresponsive to autograft (2,11). Our data suggest that chronic wound beds require activation, while care should be taken not to further activate the surgical and in particular the burn wound bed. Furthermore, our study has identified key WH mediators (TNF- α , CCL5, CCL27, CCL20, CXCL1, bFGF, TGF β 1) which warrant further investigation when developing advanced wound healing therapies.

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