Chapter 5  
Skin substitute optimized for healing chronic wounds causes hypergranulation in excision wounds – differential roles for wound bed derived IL-1α and TNF-α 

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ABSTRACT

In this study it was determined i) whether an autologous skin substitute (SS) which heals chronic wounds could heal excision wounds, and ii) whether soluble proteins present in chronic and excision wound exudates might influence the potency of the SS and in turn the final outcome of wound healing. For clinical applications, SS were applied to tumor excision wounds. For mechanistic studies, SS culture medium was supplemented with exudates from excision or chronic wounds, with or without anti-IL-1α or anti-TNF-α. To determine SS potency, granulation-stimulation-mediators (CCL2/MCP-1, IL-6, CXCL1/Gro-α, CXCL8/IL-8) in culture supernatants were analysed by ELISA. The SS caused hypergranulation within 1 week after application to excision wounds resulting in delayed wound healing. Wound exudates isolated from chronic wounds or excision wounds resulted in a huge increase in secretion of granulation-stimulatory-mediators from SS. Antibodies against IL-1α decreased this secretion in the case of chronic wound exudates whereas antibodies against TNF-α decreased this secretion in the case of excision wounds. In its current form the SS is not suitable for healing excision wounds. However, a potential combined therapy of anti-TNF-α and SS may provide a novel means to heal acute wounds since it might be expected to inhibit granulation tissue formation.
**INTRODUCTION**

Effective treatment of difficult to heal wounds, e.g. ulcers, large surgical excision wounds, trauma wounds and full thickness burn wounds, is still a major clinical challenge. Ulcer patients are conventionally treated with wound debridement followed by wound dressings and / or compression therapy and in some cases autografts are applied (punch biopsies or split-skin) (1). Wound closure is a lengthy process and a therapy resistant group of ulcers remains open (2). In the case of small, acute wounds (e.g. tumor- and scar excision wounds) and large burn/ trauma wounds an autograft (split or full thickness) is conventionally applied to directly close the wound (3). Use of autograft requires relatively large amounts of donor skin which is often a limiting factor and the resulting scar formation, particularly when meshed or split skin is used, is unsatisfactory.

New wound healing therapies are exploring the field of skin tissue engineering. Application of keratinocyte sheets according to the method of Green was already described in 1975 (4). However, application of only keratinocytes, in the absence of a dermal compartment resulted in poor take, blistering and fragile healing (5). Application of only a dermal matrix is usually successful in small wounds since epithelialisation has to occur from the wound margins. Two-step methods have been described in which donor dermis is first applied and allowed to attach followed by application of cultured keratinocytes (6). This method although looking very promising is hindered by logistics around timing of dermal take and culture of keratinocytes. Ideally, a full thickness skin substitute (SS) is required to close deep wounds in a one-step protocol. Such full thickness SS are being used more and more often to heal therapy resistant chronic wounds such as lower leg ulcers, decubitus and diabetic foot ulcers (1,7,8). They are thought to function by not only closing the wound but also by continuously secreting chemokines, angiogenic factors and growth factors which stimulate wound healing (9,10). In contrast to chronic wounds, reports describing SS application for healing full thickness acute wounds such as surgical wounds, trauma wounds or deep burns are scarce. Only 2 reports described application of an allogeneic SS (Apligraf®) to surgical excision wounds and only 3 case study reports described application of the same autologous SS to full thickness burn wounds (11-15). In all of these studies no added benefit of using SS above standard therapy (autograft or dressing) was observed. Lack of published data may indicate that indeed very few studies have been performed, or alternatively negative results predominated.

Recently we developed an autologous, full, thickness SS, which is currently being studied for treating therapy resistant chronic wounds (7). This SS consists of autologous reconstructed epidermis on acellular donor dermis repopulated with autologous fibroblasts. The wound healing capacity of this SS resides in the secretion of wound healing mediators (9). In the SS, epidermis derived TNF-α and IL-1α induce secretion of factors such as CCL2/ MCP1, IL6, CXCL1/ Gro-α and CXCL8/ IL8 by dermal fibroblasts (9). These factors are well known
granulation tissue stimulating factors since they are i) major chemoattractants for inflammatory cells (CCL2/MCP1: monocytes/macrophages; CXCL1/Gro-α and CXCL8/IL8: neutrophils) which are involved in the inflammation phase of normal wound healing (16, 17) and ii) major chemotactic and/or mitogenic factors for endothelial cells thereby stimulating angiogenesis (CCL2 (18); IL6 (19); CXCL1 (20); CXCL8 (21)). CCL2 and IL6 are also known to regulate collagen deposition by fibroblasts, thereby playing a role in wound closure and tissue remodelling (19, 22). In vitro experiments showed that CXCL1 and CXCL8 have a strong mitogenic effect on KC suggesting a role for these chemokines in re-epithelialization (23).

In a previous study, our autologous full thickness SS was applied to 19 therapy resistant lower leg ulcers (7). These wounds healed by direct take of the SS (n=13) and/or stimulation of epithelialisation (n=6) (7). SS were very well tolerated and pain relief was immediate after application. No adverse events were observed. In this study, we determined whether or not this autologous, full thickness SS might be suitable for healing acute wounds created by excision of tumors. We found that application of the SS to excision wounds resulted in hypergranulation. In order to study the different healing outcome observed for chronic wounds and excision wounds in more detail, we determined whether or not the wound bed of these wounds stimulated the secretion of granulation tissue inducing factors from SS. Previously we have demonstrated that TNF-α and IL-1α are present in chronic wounds and excision wounds (see figure 5) (manuscript submitted). Also TNF-α and IL-1α are known to induce secretion of granulation tissue factors from SS (9). In this study we describe how TNF-α and IL-1α present in the wound bed of excision wounds and chronic wounds influence the potency of SS and how this may influence the final outcome of wound healing.

METHODS AND MATERIALS

Construction of an autologous skin substitute

Epidermal sheets and dermal fibroblasts were used to construct autologous SS as previously described (7). For clinical application of SS to surgical excision wounds, 3 mm biopsies obtained from the patient’s upper leg were required to construct 6 cm² of autologous SS. These donor biopsy sites healed without complications, sutures were not required. Acellular human allogermis was prepared from glycerol preserved donor skin (Euro Skin Bank, Beverwijk, The Netherlands). Three patients were treated with SS from which informed consent was obtained. 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.

In short: Epidermis and dermis were separated from skin biopsies by incubation in dispase II overnight at 4°C. Epidermal sheets were placed on dead de-epidermized dermis and cultured air exposed. After one week of culturing the primary fibroblast and epidermal sheet apart,
they were combined and further cultured for 2 weeks in standard SS medium (Dulbecco’s modified Eagle medium [DME; ICN biomedicals, Irvine, CA] : Hams F12 [ICN biomedicals] 3:1 containing 0.2% UltroserG [BioSepa S.A, CergySaintChristophe, France], 1μM hydrocortisone, 1μM isoproterenol, 0.1μM insulin, 1x10 μM L-carnitine, 1x10 μM L-serine, 50IU/ml penicillin-G, and 50 g/ml streptomycin and enriched with a lipid supplement containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid, and 24 μM bovine serum albumin. SS were ready for application 3 weeks after removal of the skin biopsies from the

**Figure 1: Comparison of full thickness skin substitute and autograft.**

Upper panel: Macroscopic view of autologous full thickness SS and autograft (bar = 1cm). Lower panel: Tissue architecture of SS and autograft (bar = 50μm). Haematoxylin and eosin staining of 5-μm paraffin-embedded sections is shown.
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A quality control involved histological analysis of a 3 mm punch biopsy obtained from the cultured SS before application (hematoxylin and eosin staining of 5 μm paraffin embedded tissue section) and analyses of cell viability by MTT assay (see below). Figure 1 compares the SS with an autograft (native, human skin), which is the classic treatment for closing cutaneous wounds. Both SS and autograft consist of a fully differentiated epidermis in which a basal layer, spinous layer, granular layer and stratum corneum are present. The dermis of the SS contains only fibroblasts, whereas the autograft contains predominantly fibroblasts and endothelial cells (small capillaries).

Clinical application on surgical excision wounds

A tumor excision was performed in 3 patients diagnosed with various skin neoplasm’s. The tumor was fully removed with an adequate margin of healthy appearing skin. The surgical wounds were then covered by gauze under a plaster for 7 days until the SS could be applied when the wound margins had been confirmed tumor free by histopathologic examination. Upon confirmation the gauze was removed and a SS was applied to the wound bed. A single SS completely covered the wound and was held in place by sutures. Three patients undergoing a surgical excision of a skin malignancy were treated with the SS.

Patient 1, a 52-year old man, with a tumor (3.5cm diameter) on his right shin. The clinical aspect was that of a keratoacanthoma, supported by histology. The tumor was radically excised (margin 0,5 cm) and the SS was applied to tumor free margins of the excision wound.

Patient 2, a 48-year old woman, with a basal cell carcinoma (BCC) (2.5cm diameter) on her right shoulder. The BCC was radically excised (margin 0,5cm) and the SS was applied to tumor free margins of the excision wound.

Patient 3, a 79-year old man, with an atypical fibroxanthoma (1,5cm diameter) on his forehead. The neoplasm was radically excised (margin 0,3cm) and the SS was applied to tumor free margins of the excision wound.

Collection of wound exudate

Surgical excision wounds Wound exudates (WE) was collected from tumor excision wounds, that were performed in 6 patients diagnosed with various skin neoplasm’s. The tumor was totally removed with an adequate margin (0,3 – 0,5cm) of surrounding healthy appearing skin. The surgical wounds were then covered by gauze under a plaster for 7 days until further treatment once tumor free margins had been confirmed by histopathologic examination. After 7 days the gauze was removed and wound healing mediators were extracted from the gauze and an autograft or SS was applied to the wound bed. The gauze was soaked in 1 ml PBS with protease inhibitor cocktail (Sigma- Aldrich, Steinheim, Germany) (1:100) and gently shaken at 4ºC for 1 hour. After incubation samples were centrifuged and supernatant was
stored at -80°C until further analysis. Wound exudate isolated from the wound bed of tumor excision wounds is referred to as *excision* WE.

*Ulcers* Wound exudate was collected from therapy resistant chronic venous ulcers (n=12), which had been present for more than 1 year duration and which showed no tendency to heal for more than 12 weeks. Surgical debridement was conducted during the weekly visit to the out patient clinic. Debridement tissue was taken up in 1ml phosphate buffered saline (PBS) (B. Braun, Melsungen, Germany) containing protease inhibitor cocktail and shaken gently at 4ºC for 1 hour. Hereafter the WE was processed as described above. Wound exudate isolated from the wound bed of chronic venous ulcers is referred to as *chronic* WE. For more detailed description see chapter 2 of this thesis.

Supernatant of all samples were normalized for total protein content using Bio-Rad Protein Assay (BioRad Laboratories, Hercules, California) essentially as described by the supplier.

**Culture of SS in the presence of wound exudates and/or neutralizing antibodies**

For these experiments SS were constructed using human adult skin obtained from healthy donors undergoing abdominal dermolipectomy.

*Culture of SS in presence of wound exudate: Excision* WE and *chronic* WE was added to standard SS medium (200μg total protein/ml). WE-supplemented medium was then filtered through a 0.22μm pore sized filter (MILLEX-GV; Millipore Co., Bedford, Mam USA). After 3 weeks of standard culture required to generate the SS, SS were cultured in WE-supplemented medium for 48 hrs.

*Addition of blocking reagents:* Blocking antibodies for IL-1α (10ug/ml, R&D Systems Inc., Minneapolis, MN) and TNF-α were added to WE-supplemented medium for 30 minutes prior to exposure to SS for 48 hrs. After 48 hrs culturing the SS in the presence or absence of WE or neutralizing antibodies, the culture supernatant (1.5ml/culture) was stored at -20°C until further analysis by enzyme-linked immunosorbent assay (ELISA).

**Culture of autograft and SS in presence of rhTNF-α or rhIL-1α**

Full thickness adult human abdominal skin was used to represent autograft. After removal of subcutaneous adipose tissue, skin was cut into 6 cm² pieces and placed in a transwell system identical to that of cultured 6 cm² SS. rhTNF-α or rhIL-1α was added to standard SS medium (0, 50, 100, 200 U/ml). Culture supernatants (1.5ml/culture) were harvested 48 hrs later and stored at -20°C until further analysis by ELISA.
Enzyme-linked immunosorbent assay for quantification of chemokine production

For CCL2/ MCP-1, IL-6, CXCL1/ Gro-α and CXCL8/ IL-8 quantification in culture medium, enzyme-linked immunosorbent assays (ELISA) reagents were used in accordance to the manufacturer’s specifications. CCL2/ MCP-1, IL-6 and CXCL-1/ Gro-α were measured by commercially available paired ELISA antibodies and recombinant proteins obtained from R&D System Inc. (Minneapolis, Minnesota). For CXCL8/ IL-8 quantification, a Pelipair reagent set (CLB, Amsterdam, The Netherlands) was used.

IL-1α quantification in chronic and excision WE was measured by commercially available paired ELISA antibody and recombinant protein obtained from R&D System Inc. (Minneapolis, Minnesota). For TNF-α quantification, a Pelipair reagent set (CLB, Amsterdam, The Netherlands) was used. Protein levels are expressed as amount of protein/ ml.

MTT assay

The viability of the SS was routinely determined as part of the quality control procedure with the aid of a MTT assay. The MTT assay was performed essentially as described by Mosmann (24). Briefly, 3mm punch biopsies were taken from the SS and transferred to multiwell plates, each well containing 200µl of 2mg/ml freshly prepared MTT solution (Roche, Mannheim, Germany). After 2 hrs at 37°C, the biopsies were gently blotted on tissue paper and transferred to a multiwell plate containing 200µl isopropanol (acidified with 0.04M HCl, 3:1) per well, and further incubated overnight at room temperature. Optical density was measured at 550 nm, using acidified isopropanol as a negative control.

Data analysis

All data are presented as mean ± standard error mean. Differences between 2 groups were evaluated by one-way ANOVA with post-hoc Dunnett’s test, using computer program GraphPad Prism (San Diego, CA, USA).

RESULTS

Application of the autologous skin substitute to excision wounds

In a previous study, we demonstrated that SS could be used to heal therapy resistant chronic ulcers (7). An example, which illustrates healing of a therapy resistant ulcer after application
of SS is shown in figure 2. Complete wound healing occurred 22 weeks after application to the venous ulcer which had been open for 1.5 years.

In order to determine whether full thickness excision wounds could also be closed with autologous SS we elected patients undergoing removal of tumors in this pilot study. Initially 6 patients were elected, but due to adverse events (described below) occurring in the first 3 patients, it was considered unethical to continue the study. In contrast to observations made

**Figure 2: Application of the SS.**
Representative example of application of SS to a chronic venous ulcer. See results of Gibbs et al. for detailed study (7). Complete healing occurred after 22 weeks. (bar = 1 cm) (left). Application of SS to a tumor excision wound results in hypergranulation after 1 week (bar = 2cm). The healed wound and resulting scar is shown one year post surgery (right). SS, skin substitute.
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After application of the SS to ulcers (attachment, outgrowth of the epidermis, vascularization, enhanced healing, good scar quality), one week after applying the SS to tumor excision wounds, all three wounds began to hypergranulate indicating over-activation of the wound bed (Figure 2). In all 3 cases the SS remained in place and was incorporated into the wound bed. Patients showed a delayed wound healing with greatly increased secretion of wound exudate for up to 8 weeks. Autograft treatment used in regular practice does not result in hypergranulation or secretion of excessive amounts of wound exudates during wound healing (data not shown). Despite the observed hypergranulation when SS was applied, the final quality of the scar one year later was similar to that observed by primary or secondary wound closure. Therefore, long-term effects of SS application were not detrimental to the patient involved.

Figure 3: Wound exudate derived from excision wounds increases secretion of granulation tissue stimulating factors from the skin substitute.

After a three-week culture period, SS were exposed for 48 hrs to medium supplemented with increasing amounts of excision WE. SS-derived granulation tissue factors secreted into the culture medium were quantified by immunsorbent enzyme-linked assay. The amount of granulation tissue factors present in the excision WE was negligible compared to that secreted by SS. Data is expressed as relative to control unexposed cultures (SS). Mean ± SEM of three experiments each using different donors and performed in duplicate is shown. Differences were evaluated using one-way ANOVA post hoc dunnets’s ***P<0.001, **P<0.01, *P<0.05 vs. unexposed (control) SS. EWE, excision wound exudates; SS, skin substitute; WE, wound exudate.
Wound exudate derived from chronic- and excision wounds increases the secretion of granulation tissue stimulating factors from SS

Our previous clinical study showed that application of the SS to the wound bed of therapy resistant ulcers results in healing, and here we demonstrate that application to the wound bed of excision wounds resulted in hypergranulation (7). This may indicate that factors in the wound bed of excision wounds may stimulate granulation tissue-inducing factors from SS (CCL2, CXCL1, CXCL8 and IL-6). We therefore studied the interaction between the wound bed of excision wounds and the SS by culturing the SS for 48 hrs in presence of excision WE. A huge dose dependent increase in secretion of CCL2, CXCL1, CXCL8 and IL-6 from the SS, in response to excision WE, was observed (Figure 3). Already an approximately 10 fold higher secretion of granulation tissue factors was observed when SS were supplemented with only 2% excision WE (which corresponds to 200μg excision WE total protein/ml culture supernatant). Supplementation with 10% excision WE resulted in more than 300 fold increase in secretion of granulation tissue factors.

![Graphs showing increase in secretion of CCL2, CXCL1, CXCL8, and IL-6](image)

Figure 4: Wound exudate derived from chronic wounds also increases secretion of granulation tissue stimulating factors from the skin substitute.

After a three-week culture period, SS were exposed for 48 hrs to medium supplemented with chronic WE or excision WE (200 μg total protein/ml culture medium). SS-derived granulation tissue factors secreted into the culture medium were quantified by immunosorbent enzyme-linked assay. The amount of granulation tissue factors present in the chronic –and excision WE was negligible compared to that secreted by SS. Data is expressed as relative to control unsupplemented cultures (SS). Mean ± SEM of three experiments each using different donors and performed in duplicate is shown. Differences were evaluated using one-way ANOVA with post-hoc Dunnett’s test ***P<0.001, **P<0.01, *P<0.05 vs. unexposed (control) SS. SS, skin substitute; CWE, chronic wound exudates; EWE, excision wound exudates; WE, wound exudate.
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Next, the effect of excision WE on the SS was compared with the effect of chronic WE (cultures were supplemented with 200μg WE total protein/ ml culture supernatant). Figure 4 shows that SS cultured for 48 hours in presence of chronic WE also resulted in an increased secretion of granulation tissue factors from SS (Figure 4). Excision WE and chronic WE increased the secretion of CCL2 (excision WE and chronic WE: 6 fold), IL-6 (excision WE: 30 fold, chronic WE: 12 fold), CXCL1 (excision WE: 10 fold, chronic WE: 20 fold) and CXCL8 (excision WE: 20, chronic WE: 40 fold) from the SS. Culture of the SS in presence of WE for 48 hrs did not influence the viability of the SS (MTT assay: data not shown).

In conclusion, both excision – and chronic wounds stimulate the secretion of granulation tissue factors from SS.

**Differential effect of wound bed derived IL-1α and TNF-α on SS potency**

Already we have demonstrated a comparable IL-1α level between venous ulcers and surgical wounds and a low amount of TNF-α in venous ulcers compared to high levels in surgical wounds (Figure 5) (chapter 4 of this thesis). We have also demonstrated that epidermal TNF-α and IL-1α amplify the secretion of CCL2/ MCP-1, IL-6, CXCL1/ Gro-α and CXCL8/IL-8 from SS (9). Therefore here we next studied whether TNF-α and IL-1α in the wound bed could be responsible for this huge increase in secretion of granulation tissue factors from the SS (Figure 6).

In order to determine whether wound bed derived IL-1α and/ or TNF-α were responsible for the increased secretion of granulation tissue factors from the SS, SS were cultured for 48 hrs with excision- and chronic WE in the presence or absence of a neutralizing antibody for IL-1α or TNF-α. Anti-IL-1α, but not anti-TNF-α, reduced the increase in secretion of granulation tissue factors from SS cultured with chronic WE. This indicated that IL-1α in the wound bed of

**Figure 5: TNF-α and IL-1α are present in the wound bed of chronic and excision wounds.**

TNF-α and IL-1α were quantified in chronic WE (n=12) and excision WE (n=6) by enzyme-linked immunosorbent assay. Data are presented as mean ± SEM. Differences were evaluated using one-way ANOVA with post-hoc Dunnett’s test **P<0.01, comparing chronic WE with excision WE. WE, wound exudate.**
Figure 6: Differential role for wound bed derived TNF-α and IL-1α in chronic wounds and excision wounds.

After the three-week culture period, SS were exposed for 48 hrs to medium supplemented with chronic WE (left) or excision WE (right) with or without addition of anti-TNF-α or anti-IL-1α. For both types of wound WE proteins were added (200μg total protein/ml culture supernatant). SS-derived granulation tissue factors secreted into the culture medium were quantified by immunosorbent enzyme-linked assay. Data is expressed as relative to control unsupplemented cultures (SS). Mean ± SEM of three experiments each using different donors and performed in duplicate is shown. Differences were evaluated using one-way ANOVA with post-hoc Dunnett's test **P<0.01, *P<0.05 vs. unsupplemented skin substitutes. SS, skin substitute; WE, wound exudates.
chronic wounds, rather than TNF-α, may result in an increased secretion of granulation tissue factors from SS once placed onto a chronic venous ulcer.

In contrast, anti-TNF-α, but not anti-IL-1α, reduced the increase in secretion of granulation tissue factors from SS cultured with excision WE. This indicates that the high levels of TNF-α in the wound bed of excision wounds, rather than IL-1α, may result in an increased secretion of granulation tissue factors from SS when placed onto a excision wound.

**Autograft vs. skin substitute**

So far our results describe an autologous SS which heals chronic ulcers and causes hypergranulation in excision wounds. In contrast, an autograft results in inefficient healing of ulcers (1), whereas in regular practice it heals excision wounds. IL-1α and TNF-α in the wound bed increase the secretion of granulation tissue factors from the SS and therefore most likely also from an autograft. Since SS and autograft preferentially heal different wounds (SS-chronic ulcers, autograft-acute wounds), we next studied whether recombinant human IL-1α and TNF-α have a different effect on SS and autograft regarding the secretion of granulation tissue factors (Figure 7).

**Figure 7: Influence of rhTNF-α and rhIL-1α on secretion of granulation tissue factors by autograft and SS.**

Autograft and SS were exposed for 48 hrs to medium supplemented with 100U/ml rhIL-1α or rhTNF-α. SS-derived secretion of CCL2/ MCP1, IL6, CXCL1/ Gro-α and CXCL8/IL8 into culture supernatant was quantified by immunosorbent enzyme-linked assay. Data are presented as mean ± SEM of three experiments each using different donors and performed in duplicate. Differences were evaluated using one-way ANOVA with post-hoc Dunnett’s test ***P<0.001, **P<0.01, *P<0.05 vs. unsupplemented skin substitutes. Black bar represents autograft and white bar represents skin substitute.
In agreement with our previous study it can be seen that all wound healing mediators secreted by autograft are also secreted by SS, although basal levels differ; SS>autograft: CCL2/ MCP-1 (13 fold) and CXCL1/ Gro-α (10 fold), autograft>SS: IL-6 (40 fold) and CXCL8/ IL-8 (5 fold) (9). In figure 7 we show that independent of basal secretion levels (control) the SS secretes greater amounts of CCL2/ MCP-1, CXCL1/ Gro-α and CXCL8/IL-8 compared to autograft when culture medium is supplemented with 100U/ml rhTNF-α or rhIL-1α. The high basal secretion of IL-6 from autograft is not further increased by 100U/ml rhTNF-α or rhIL-1α, whereas the low basal IL-6 secretion from SS increases approximately 5 fold. These results show that TNF-α and IL-1α increase the secretion of granulation tissue factors from both SS and autograft, although the effect on the SS is significantly larger. This indicates that the SS is a more active construct compared to an autograft when applied to a chronic – and excision wound bed. Addition of rhIL-1α and rhTNF-α to culture medium of the SS for 48 hrs did not influence the viability of the SS (MTT assay: data not shown).

DISCUSSION

We have developed a full thickness autologous SS, consisting of an autologous reconstructed epidermis on autologous fibroblast re-populated donor acellular dermis. Our previous study already reported that this autologous SS is suitable for healing therapy resistant leg ulcers (7). This study describes its application to excision wounds. Application to tumor excision wounds resulted in excessive secretion of wound exudate in the first two months of healing, which in turn delayed wound healing compared to autograft. Therefore the only benefit of using a SS with this study protocol was that less donor skin is required compared to autograft (± 20 fold less). It should be noted that follow up studies at 4 months and 12 months showed that the resulting scar was good and of the same quality as secondary intention wound closure.

Very few studies have been described in which SS are applied to excision wounds. Apligraf®, an allogeneic full thickness SS, has been FDA-approved to promote the healing of ulcers that have failed standard wound care (10). The safety and efficacy of Apligraf® has also been studied off-label in full thickness surgical wounds (11,12,25). Results shown by Gohari et al. 21 days after applying Apligraf® to a full thickness excision wound resembled our results shown at 4 weeks after applying SS (12). Compared to autograft, Apligraf® was described to show no difference in a limited follow-up study in which both constructs resulted in complete healing (11,12). Eaglstein et al. however reported that wound healing outcome scored 50% worse for Apligraf® treated excision wounds compared to autograft (n=107) (11). Boyce et al. have developed an autologous full thickness SS using bovine collagen glycosaminoglycan as dermal matrix. In 2 independent studies at 28 days after application to deep burn wounds their SS demonstrated an increased secretion of exudates, decreased engraftment and lower rate of wound healing compared to autograft (13,14). In line with our study, and the Apligraf® stud-
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In this study, in addition to describing clinical outcome after SS application to excision wounds, we described the means by which the wound bed and SS interact to influence SS potency. Exposure to wound exudate isolated from chronic leg ulcers or tumor excision wounds resulted in an extremely large increase in secretion of wound healing mediators (CCL2/ MCP-1, IL-6, CXCL1/Gro-α, CXCL8/ IL-8) from the SS. These mediators are chemokines involved in the formation of granulation tissue (16-23). For chronic leg ulcers the interaction between the wound bed and SS is most likely strongly contributing to wound healing in that therapy resistant ulcers are revitalized when the SS comes into contact with the inert wound bed. However, since the wound bed of surgical excision wounds already have a good tendency to heal, application of the SS, resulting in the massive release of granulation tissue factors most probably results in over-activation of the wound bed, increased secretion of wound exudate and hypergranulation.

Interestingly, in chronic wounds IL-1α amplifies the secretion of granulation tissue factors from the SS, whereas in excision wounds TNF-α in the wound bed is responsible for this induction. Our results suggest that inhibition of TNF-α in excision wounds might prevent excessive secretion of granulation tissue factors from SS and might therefore reduce hypergranulation. Therefore a combined therapy of SS and topical anti-TNFα is a potential novel means for healing acute wounds, large trauma wounds, full thickness burns and scar excision wounds and warrants further investigation.

Notably, autograft and SS differ in wound healing potency. Indeed our pilot study showed that ulcers, which remained inert after prior treatment with autograft, healed by application of SS (7). In contrast to chronic wounds autograft heals full thickness excision wounds in regular practice within days, while our SS caused hypergranulation and a delayed wound healing. Also to be noted is that TNF-α and IL-1α have a larger increasing effect on the secretion of granulation tissue factors from SS than from autograft. This indicates that the SS is a more active construct compared to an autograft when applied to a chronic - and excision...
wound bed. This makes SS a suitable therapy for resistant chronic ulcers, but care should be taken not to over activate the wound bed when applying to excision wounds. The differential effect of TNF-α and IL-1α on both constructs suggests that cells within SS are more responsive to soluble factors in the wound bed than cells within an autograft. During culture, cells within SS are stimulated to migrate and proliferate and are activated in a similar manner to normal wound healing, which may make them more sensitive to soluble factors, in contrast to autograft in which the tissue is in homeostasis when excised (9, 26).

This manuscript describes healing of chronic wounds and surgical excision wounds with our autologous SS. It cannot be automatically concluded that our findings apply to all cultured SS. Our results underline that soluble factors present in the wound bed of tumor excision and chronic wounds can strongly influence activity of cells within SS and in doing so the rate and the quality of wound healing. Notably, TNF-α and IL-1α play a key role. Whereas IL-1α may mediate activation of chronic wounds and is beneficial to healing, TNF-α may induce hypergranulation and in doing so delay wound healing. A potential combined therapy of topical anti-TNF-α and SS may provide a novel means to heal acute wounds since it would be expected to inhibit excess granulation tissue formation therefore warrants further investigation.
REFERENCES