TISSUE ENGINEERING: Part A
Volume 20, Numbers 1 and 2, 2014

DOI: 10.1089/ten.tea.2013.0123

Differential Response of Human Adipose Tissue-Derived Mesenchymal Stem Cells, Dermal Fibroblasts, and Keratinocytes to Burn Wound Exudates: Potential Role of Skin-Specific Chemokine CCL27

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Many cell-based regenerative medicine strategies toward tissue-engineered constructs are currently being explored. Cell–cell interactions and interactions with different biomaterials are extensively investigated, whereas very few studies address how cultured cells will interact with soluble wound-healing mediators that are present within the wound bed after transplantation. The aim of this study was to determine how adipose tissue-derived mesenchymal stem cells (ASC), dermal fibroblasts, and keratinocytes will react when they come in contact with the deep cutaneous burn wound bed. Burn wound exudates isolated from deep burn wounds were found to contain many cytokines, including chemokines and growth factors related to inflammation and wound healing. Seventeen mediators were identified by ELISA (concentration range 0.0006–9 ng/mg total protein), including the skin-specific chemokine CCL27. Burn wound exudates activated both ASC and dermal fibroblasts, but not keratinocytes, to increase secretion of CXCL1, CXCL8, CCL2, and CCL20. Notably, ASC but not fibroblasts or keratinocytes showed significant increased secretion of vascular endothelial growth factor (5-fold) and interleukin-6 (253-fold), although when the cells were incorporated in bi-layered skin substitute (SS) these differences were less pronounced. A similar discrepancy between ASC and dermal fibroblast mono-cultures was observed when recombinant human-CCL27 was used instead of burn wound exudates. Although CCL27 did not stimulate the secretion of any of the wound-healing mediators by keratinocytes, these cells, in contrast to ASC or dermal fibroblasts, showed increased proliferation and migration. Taken together, these results indicate that on transplantation, keratinocytes are primarily activated to promote wound closure. In contrast, dermal fibroblasts and, in particular, ASC respond vigorously to factors present in the wound bed, leading to increased secretion of angiogenesis/granulation tissue formation factors. Our findings have implications for the choice of cell type (ASC or dermal fibroblast) to be used in regenerative medicine strategies and indicate the importance of taking into account interactions with the wound bed when developing advanced therapies for difficult-to-close cutaneous wounds.

Introduction

Many different cell types (e.g., dermal fibroblasts, keratinocytes, endothelial cells, macrophages, monocytes, and granulocytes) are involved in wound healing. Extensive crosstalk via secretion of soluble mediators takes place between these cells in order to heal deep cutaneous wounds.[1] Adipose tissue-derived mesenchymal stem cells (ASC) are also very likely to be involved in wound healing and skin regeneration due to their subcutaneous location as well as their ability to self-renew, their multi-lineage differentiation potential, and their migration capacities.[2–4] However, although much is known about ASC contribution to chondrogenic, osteogenic, and adipogenic regeneration,[5,6]

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surprisingly little is known about the ability of ASC to contribute to wound healing and skin regeneration. In addition, a wide variety of literature is available describing how ASC interact with different biomaterial scaffolds used in tissue engineering, whereas[7,8] surprisingly very little information is available describing how ASC interact with soluble mediators secreted into the wound bed. This information is essential if custom-designed, tissue-engineered constructs and regenerative strategies are to be developed for different types of difficult-to-heal cutaneous wounds. For example, a deep burn wound needs to heal without stimulating excessive inflammation and granulation tissue formation, as this will enhance hypertrophic scar formation; whereas a chronic wound can only be stimulated to heal if, in contrast, granulation tissue formation is promoted.[9]

Earlier, we have shown that ASC and dermal fibroblasts both display a mesenchymal stem cell phenotype ($CD31^-$, $CD34^+$, $CD45^-$, $CD54^+$, $CD90^+$, $CD105^+$, and $CD166^+$) and show similar multi-lineage differentiation potential.[3] In addition, we have shown that both ASC and dermal fibroblasts migrate predominantly toward chemokine CCL5,[3] which is present in the wound fluid of chronic cutaneous wounds.[10] However, it is still unclear how the migrated ASC and dermal fibroblasts respond to wound-healing mediators that are present in the wound bed. Generally, skin substitutes (SS) contain dermal fibroblasts and keratinocytes.[11] Trottier *et al.* showed that SS containing ASC look similar to SS containing dermal fibroblasts.[12] However, it can be expected that cultured ASC will react differently to dermal fibroblasts when applied to a wound bed, as, for example, ASC express high levels of alpha smooth muscle actin, which is characteristic of scar-forming myofibroblasts whereas dermal fibroblasts express only very low amounts of this biomarker.[13]

Bone marrow-mesenchymal stem cells (BM-MSC) have been described as a possible potential therapeutic tool in wound healing and skin regeneration due to their ability to secrete paracrine factors and to differentiate into skin cells.[14] ASC have also been described to stimulate healing of chronic wounds.[15] In contrast to BM-MSC, ASC can be harvested in large numbers by relatively non invasive techniques.[16]

The aim of this study was to determine how different cell types (ASC, dermal fibroblasts, and keratinocytes) that are currently being investigated for their future use in tissue-engineered products and regenerative medicine strategies will react when they come in contact with the deep cutaneous wound bed. Here, we use the deep third degree cutaneous burn wound as an example of a difficult-to-heal cutaneous wound. Healing of such burn wounds generally occurs with formation of an adverse hypertrophic scar.[17] Therefore, new treatment strategies should be aimed at fast wound closure without stimulation of excessive granulation tissue, which will lead to hypertrophic scar formation. Predicting how transplanted cells will react when placed on the wound bed is, therefore, of vital importance before entering clinical trials.

In this study, we first identified the profile of wound-healing mediators that are present in burn wound exudates. The panel of 17 wound-healing mediators was selected for this study based on the fact that taken together, they have been reported in literature to be involved in most aspects of wound healing: for example, inflammation, granulation tissue formation, angiogenesis, mitogen, epithelialization (closure), stem cell attraction, and tissue remodeling.[1,18–20] Next, the ability of burn wound exudates to activate ASC, dermal fibroblasts, and keratinocytes to further stimulate the release of mediators involved in granulation tissue formation was assessed. Keratinocytes as well as mesenchymal cells were studied, as they form the epidermis of SS and, ultimately, are responsible for wound closure.

Within the burn wound exudates, the skin-specific chemokine CCL27 was detected.[21] Chemokine CCL27 has been reported to be secreted only by skin-derived keratinocytes,[21] and it is up-regulated in the wounded skin of mice.[20] CCL27 was first described to play a role in skin homing of CLA^+ $CD4^+$ memory T cells expressing CCR10 during immune surveillance.[22,23] The only known receptor for CCL27 is CCR10, a G-protein-coupled seven-transmembrane domain receptor.[21] This receptor is expressed in many cells, including keratinocytes, dermal fibroblasts, and ASC.[3,24] Since we previously detected this chemokine also in chronic wound exudates,[10] it is most probable that it is related to skin trauma in general. However, the function of this skin-specific chemokine during wound healing is still unknown. Therefore, we next studied in detail the effect of CCL27 on ASC, dermal fibroblasts, and keratinocytes with regard to activating these cells to release mediators involved in granulation tissue formation and also proliferation and migration.

Materials and Methods

VU University Medical Center approved all the experiments described in this article. The study was conducted according to the Declaration of Helsinki 1975.

Wound exudates

Burn wounds. In 10 patients with third-degree burn wounds, eschar tissue was removed 7–21 days postburn. Eschar was removed to the depth where viable tissue was reached, and wound exudates were collected from the viable tissue interface. Exudates were further handled as described next.

Leg ulcers. Therapy-resistant chronic venous ulcers ($n=12$) that 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 material was collected from which wound exudates were extracted during the weekly visit to our outpatient clinic.

Surgical excision wounds. A tumor excision was performed in six patients. 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. At day 7, wound exudates were collected from the gauze covering the open skin wound.

One milliliter phosphate-buffered saline (PBS) containing protease inhibitor cocktail (1:100) was directly added to the exudates followed by gentle shaking at 4°C for 1 h. After incubation, samples were centrifuged and supernatant was stored at −80°C until further analysis. For the wound

exudates, the supernatant was normalized for total protein content using Bio-Rad Protein Assay (BioRad Laboratories, Hercules, CA) essentially as described by the supplier. Total protein content was used in the normalization, as 1 mL of buffer was added independent of the amount of exudate collected. This meant that the concentration of the diluted wound exudate was not standardized. The protein concentration was 4.18 ± 1.14 mg/mL (mean ± SEM) for all burn wound, ulcer, and excision wound exudates described earlier taken together. In contrast, total protein content in the samples was representative of the total amount of wound exudate in the original sample.

Ex vivo *wound model*

Human adult skin was obtained from healthy individuals undergoing abdominal dermolipectomy and was used directly after surgery. Skin (0.8 mm thick removed with a dermatome, Acculan II; Braun, Tuttligen, Germany) was cut into pieces of 4 cm^2. For full-thickness burn injury, a metal device attached to a Weller® soldering station (WSD 81Cooper Tools, Besigheim, Germany), heated continuously at 128°C, was applied for 10 s to the epidermis as previously described.[25] Unwounded skin was used as a control. The (damaged) skin samples were placed dermis side down on a transwell (0.4 μm pore size; Costar Corning Incorporated, Corning, NY) and cultured at the air–liquid interface as described earlier. Culture medium was renewed twice a week. Cultures were harvested, and culture supernatants were collected at different time intervals (day 0, 1, 2, and 7) for histological analysis, CCL27 and CCR10 immunostaining, and CCL27 ELISA.

Cell isolation and culture

Dermal-derived fibroblasts and ASC were isolated from human adult skin exactly as previously described.[3] Cells were cultured in DMEM (Lonza, Verviers, Belgium) containing 1% UltroSerG (UG) (BioSepra SA, Cergy-Saint-Christophe, France) and 1% penicillin/streptomycin (P/S) (Invitrogen, Gibco, Paisley, United Kingdom). Cells derived from the dermis and adipose tissue were cultured under identical conditions and used for experiments at passage 3.

Epidermal keratinocytes were isolated from human adult skin, essentially as described earlier.[26] Keratinocytes were cultured in keratinocyte medium consisting of DMEM:-Ham's F-12 (Invitrogen, Gibco) (3:1), 1% UG, 1% P/S, 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, and 1 ng/mL keratinocyte growth factor (KGF). Unless otherwise stated, all culture additives were obtained from Sigma-Aldrich (St. Louis, MO). Keratinocytes were used for further experiments at passage 1.

Granulocytes were obtained from fresh human blood using Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation and further isolated as described by Ellerbroek et al.[27] Freshly isolated granulocytes were used for further experiments as described next. The isolated granulocytes were $CD15^+$ and $CD45^{low+}$ as determined by flow cytometric analysis.

Monocytes were isolated from fresh human blood using Lymphoprep (Nycomed Pharma AS) density gradient centrifugation and further isolated as previously described.[28] Monocytes were used immediately for experiments as described next and were $CD14^+$, $CD86^+$, $HLA\text{-}DR^+$, and $CD1a^-$ as determined by flow cytometric analysis.

Human microvascular endothelial cells (HMVEC) were isolated from neonatal foreskin as previously described.[29] HMVEC were cultured in plates precoated with 1% gelatin (Sigma-Aldrich) in M199 (Lonza), 10% newborn calf serum (Invitrogen, Paisley, United Kingdom), 10% human serum (Sanquin, Amsterdam, The Netherlands), 1% P/S, 2 mM L-glutamine (Invitrogen), 5 U/mL heparin (Leo Pharmaceutics Products, Weesp, The Netherlands), and 0.0375 mg/mL endothelial cell growth factor (ECGF) (prepared from bovine brain). For all experiments, cells were used between passage 6 and 10.

SS (donor matched) consisting of reconstructed epidermis on ASC or dermal fibroblast populated dermis were constructed as previously described.[30] Keratinocytes (5×10^5 cells/SS) were seeded on top of acellular human dermis (prepared from glycerol-preserved donor skin [Euro Skin Bank, Beverwijk, The Netherlands]) and cultured, submerged, in keratinocyte medium. ASC and dermal fibroblasts were cultured in 0.4 μm pore size transwells (Cat. No. 3450; Costar Corning Incorporated, Acton, MA) until they were 70% confluent in DMEM containing 1% UG and 1% P/S. After ~7 days of culturing the keratinocyte cultures, the dermis containing keratinocytes was placed onto the fibroblasts or ASC in order to allow fibroblast and ASC migration into the reticular side of the dermis. The SS was further cultured, exposed to the air, in DMEM:Ham's F12 (3: 1) containing 0.2% UG, 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 10 μM carnitine, 10 mM L-serine, 1 μM DL-α-tocopherol acetate, and 1% P/S 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. KGF (4 ng/mL) and EGF (1 ng/mL) were added to the culture medium for the next 7 days. Culture medium was renewed twice weekly. Unless otherwise stated, all culture additives were obtained from Sigma-Aldrich.

Exposure of cultures to burn wound exudate and relevant stimulatory factors

Cells were exposed to stimulatory factors in their standard culture medium described earlier (with the omission for KGF and EGF with keratinocyte cultures and SS and the omission for ECGF with hMVECs). Granulocytes were exposed in Roswell Park Memorial Institute-1640 Medium (RPMI-1640) (Lonza) containing 1% heat-inactivated fetal calf serum (FCS) (Thermo Fischer Scientific, Waltham, MA) and 1% P/S. Monocytes were cultured in Isocove's-modified Dulbecco's medium (Lonza) containing 1% heat-inactivated FCS, 1% P/S, and 50 μM dithiothreitol (Merck, Darmstadt, Germany). When indicated, ASC and fibroblasts, both 150,000 cells/1.5 mL/well in six-well plates (Cat. No. 3506; Costar Corning Incorporated, Corning, NY) and keratinocytes 100,000 cells/1 mL/well in 12-well plates (Cat. No. 3512; Costar Corning Incorporated, Corning, NY) precoated with 0.5 μg/cm^2 human placental collagen IV (Sigma-Aldrich) were exposed to different concentrations of burn wound exudates (0%, 1% and 2% v/v) or recombinant human (rh)-CCL27 (R&D Systems, Minneapolis, MN) (0, 62.5, 125, 250, and 500 ng/mL). When indicated, cells were stimulated with potent and relevant factors as described in the literature for each different

cell type: ASC,[31] fibroblasts,[32] and keratinocytes[21] 10 ng/mL TNF-α (R&D Systems); granulocytes 10 nM *N*-Formylmethionyl-leucyl-phenylalanine (fMLP) (GenScript, Piscataway, NJ)[33]; monocytes 100 ng/mL Lipopolysaccharides (LPS) (Sigma-Aldrich)[34]; or HMVEC 10 ng/mL vascular endothelial growth factor (VEGF) (PrepoTech, Rocky Hill, NJ).[18] Culture supernatants were harvested (granulocytes after 4 h, monocytes after 16 h, and ASC, fibroblasts, keratinocytes, and HMVEC after 24 h) and stored at −20°C for further analysis by ELISA. Viability of the exposed cells was determined as described next.

Exposure of SS. After 3 weeks of standard culture required to generate the SS, the culture medium was supplemented with burn wound exudates for 24 h (200 μg total protein, corresponding to 2–5% burn wound exudates, in 1.5 mL standard air-exposed SS culture medium, but without KGF and EGF). Cultures were harvested, and culture supernatants were collected for histological analysis, vimentin staining, ELISA, and MTT assay (as previously described[32]).

In all exposure experiments described earlier, addition of burn wound exudate, rh-CCL27, or other stimulatory factors did not influence cell viability or proliferation when compared with unexposed control cultures within the duration of the experiment (data not shown).

Cell proliferation and viability

For ASC and fibroblast cultures, proliferation and viability were determined with an MTT assay that measures mitochondrial activity which correlates to cell number.[35] The assay was performed as described by the supplier (Sigma-Aldrich). Keratinocyte and hMVEC proliferation and viability were determined by quantifying intracellular lactate dehydrogenase (LDH) activity released into the culture medium from the cytosol on total cell lysis with 0.1% triton in PBS.[36] LDH activity was measured with an LDH detection kit (Roche Applied Science, Mannhein, Germany) in accordance with the manufacturer's specifications. Viability of monocytes and granulocytes was determined using propidium iodide staining (Invitrogen).

Proliferation experiments with ASC, fibroblasts, and keratinocytes were performed as follows. Cells were cultured in their standard medium in the absence of growth factors for 24 h. Then, cells were exposed to different concentrations of rh-CCL27 (see earlier) or relevant stimulation media. Cultures were harvested after 3 days, and the number of cells present was determined relative to parallel control unexposed cultures using the MTT (ASC and fibroblasts) or LDH (keratinocytes) assay.

CCL27-EN4 endothelial cell immunostaining and CCR10 analysis

Frozen cryostat tissue sections (5 μm) were stained for ligand CCL27 as described earlier.[22] For CCL27–EN4 double staining, cryostat frozen sections were fixed in ice-cold acetone for 8 min and incubated with goat serum for 10 min before incubation for 1 h with primary monoclonal antibodies directed against CCL27 (IgG2a, Clone 124302, 1:200; R&D Systems) and EN4 (IgG1, 1:20; Monosan, Uden, The Netherlands). After washing in PBS for 10 min, sections were incubated for 30 min with goat anti-mouse IgG2a-Alexa555 (1:400; Invitrogen) and goat anti-mouse IgG1-Alexa488 (1:400, Invitrogen). The sections were washed thrice in PBS and then examined using a fluorescence microscope (Nikon Eclipse 80i, G-2a Ex510–560, DM575, BA590). Paraffin tissue sections (5 μm) were stained for receptor CCR10 as described by Kroeze *et al*.[36] CCR10 expression was determined on the cell surface by flow cytometry as described earlier.[3] Cell staining was performed using rat anti-human CCR10-PE (314305; R&D Systems) and corresponding isotype to assess nonspecific binding rat IgG2a-PE (R&D Systems).

Chemotaxis assay

Chemotactic migration of cells toward CCL27 was assessed with the aid of a modified Boyden well chamber technique using a 24-transwell system with 8 μm poresize transwells (Costar Corning Incorporated, Corning, NY) essentially as described by Kroeze *et al*.[3] For all cell types, a relevant positive chemoattractant was included in the assay: ASC = 250 ng/mL CCL5 (R&D Systems); fibroblasts = 250 ng/mL CCL5; and keratinocytes = 5 ng/mL EGF. Chambers were incubated for 24 h at 37°C in a humidified atmosphere.

The number of ASC, fibroblasts, and keratinocytes were quantified as previously described.[3] For ASC and fibroblasts, the number of nuclei present in 40-fold magnification on the underside of the transwell were counted. For keratinocytes, total fluorescence intensity of the cells within 40-fold magnification was measured as described earlier,[36] as it was not possible to count individual nuclei in the migrated keratinocyte clones.

Enzyme-linked immunosorbent assay for cytokine and growth factor production

All reagents were used in accordance to the manufacturer's specifications. For CCL2, CCL5, CCL17, CCL18, CCL20, CCL27, CXCL1, CXCL12, bFGF, VEGF, HGF, INF-γ, TGF-β, interleukin (IL)-1α, IL-6 and IL-10 commercially available paired ELISA antibodies and recombinant proteins obtained from R&D System were used. For CXCL8 and TNF-α quantification, a Pelipair reagent set (CLB, Amsterdam, The Netherlands) was used.

Statistical analysis

Unless otherwise stated, at least three independent experiments were performed, with each experiment having an intra-experimental duplicate. Mann–Whitney U test, Wilcoxon-matched pairs test, or repeated-measures ANOVA test followed by a Dunnett's multiple-comparison test were used to determine statistical significance as indicated in the figure legends. Differences were considered significant when $p < 0.05$.

Results

Cytokines, chemokines, and growth factors are present in burn wound exudates

Since living tissue-engineered products are transplanted onto the open burn wound bed directly after excision of burn eschar, we first identified which cytokines, chemokines, and growth factors were present in exudates isolated from these

Table 1. Cytokines, Chemokines, and Growth Factors Detected in Burn Wound Exudates

Mediator	*Function*	*Amount in burn wound exudates (pg/mg total protein)*[a]
Abundant		
CXCL8/IL-8	Inflam; Ang; Epith; Fib	8300 ± 2600
HGF	TR; Epith; Gran; Mitogen	4500 ± 700
CCL18/PARC	Inflam; Collagen; Fib	2000 ± 500
bFGF	Ang; Gran	1062 ± 246
Moderate amount		
CXCL1/GRO-α	Inflam; Ang; Epith; TR; Fib	630 ± 90
CCL2/MCP-1	Inflam; Ang; Epith; TR	569 ± 253
CCL5/RANTES	Inflam; Mitogen; Chemotactic for stem cells	482 ± 130
IL-6	(Anti-)inflam; Gran; Ang; Mitogen	476 ± 152
CCL20/MIP-3α	Inflam; Antibacterial; Fib	474 ± 150
CCL27/CTACK	Inflam, Chemotactic for BMDSC; Fib	199 ± 108
IL-1α	Pro-inflam; Mitogen; Hematopoiesis	191 ± 108
TGF-β	Anti-inflam; ECM; TR; Gran	179 ± 20
Low amount		
CXCL12/SDF-1	Inflam; Ang; LC; Fib	9.6 ± 5.8
Minimal amount		
IL-10	Anti-inflam	0.6 ± 0.3
TNF-α	Pro-inflam	0.7 ± 0.5
Not detectable		
IFN-γ	T cell mediated immune responses, keratinocyte apoptosis, TNF- α prod.	Not detected
CCL17/TARC	T-cell homing	Not detected

[a]Amount in burn wound exudates (pg/mg total protein). Mean ± SEM of 10 third degree burn wound exudates is shown. Functions are given as stimulating angiogenesis (Ang), collagen production (Collagen), extracellular matrix production (ECM), epithelialisation (Epith), fibroblast migration (Fib), granulation tissue (Gran), inflammatory mediator production (Inflam), Langerhans cell migration (LC), tissue remodelling (TR), chemotaxis of bone marrow derived stem cells (BMDSC).

IL, interleukin; GRO-α, growth related oncogene; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; MIP, macrophage inflammatory protein; PARC, pulmonary and activation regulated chemokine; HGF, hepatocyte growth factor; MCP-1, monocyte chemoattractant protein-1; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; CTACK, cutaneous T cell attracting chemokine; RANTES, regulation upon activation, normal T-cell expressed and secreted; SDF-1, stromal cell-derived factor 1; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; TARC, thymus and activation regulated chemokine.[1,29,34]

wound beds. Seventeen mediators known to influence wound healing were identified by ELISA (Table 1). There were five categories related to protein abundance: (1) abundant: CXCL8, CCL18, HGF, and bFGF were detected at 1.1–8.3 ng/mg total protein; (2) moderate amount: CXCL1, CCL2, CCL5, CCL20, CCL27, IL-6, IL-1α, and TGF-β were detected at 170–630 pg/mg total protein; (3) low amount: CXCL12 was detected at 9.6 pg/mg total protein; (4) minimal amount: IL-10 and TNF-α were detected at 0.6–0.7 pg/mg total protein; and (5) CCL17 and INF-γ were not detectable. Obviously, a wide variety of soluble wound-healing mediators can be found in exudates collected from deep third-degree burn wounds.

ASC respond more vigorously to soluble mediators present in the burn wound bed than either dermal fibroblasts or keratinocytes

Since the burn wound exudates contained an extensive cocktail of factors related to inflammation and wound healing,[18,19,37] we next determined whether this wound exudate could directly influence cell types often used in tissue engineering and regenerative medicine strategies. Therefore ASC, dermal fibroblasts, and keratinocytes were exposed to burn wound exudate (0%, 1% and 2%), and it was determined whether the wound exudate was able to influence the activity of the cells with regard to enhanced secretion of mediators related to angiogenesis, granulation tissue formation, and inflammation (VEGF, IL-6, CXCL1, CXCL8, CCL2, CCL5, and CCL20) (Fig. 1). Burn wound exudates stimulated ASC and dermal fibroblasts to secrete an enormous amount of CXCL1, CXCL8, CCL2, and CCL20 (e.g., just 2% burn wound exudate resulted in secretion of ~110 ng/mL CXCL1, which is more than a 220-fold increase). Interestingly, ASC but not dermal fibroblasts significantly increased secretion of VEGF (4.75-fold, $p < 0.05$) and IL-6 (253-fold, $p < 0.05$) on exposure to burn wound exudate. In contrast to ASC and dermal fibroblasts, no increased cytokine secretion was observed by keratinocytes after exposure of burn wound exudates.

Tissue-engineered skin constructs aimed at healing cutaneous wounds often do not consist of just one cell type but are bi-layered constructs consisting of reconstructed epidermis on a fibroblast populated dermis.[12,29] Therefore, we next constructed SS with a fully differentiated epidermis containing either ASC or dermal fibroblasts in the dermal compartment and exposed these SS to 0 and 2% burn wound exudates for 24 h (Fig. 2a). SS containing ASC significantly increased relative secretion of IL-6, CXCL8, and CCL2 and showed a trend to increase CXCL1, CCL5, CCL20, and VEGF. In contrast, SS-containing dermal fibroblast showed only a trend to increase secretion of 6 out of the 7 cytokines studied (Fig. 2b). Notably, the clear differences observed

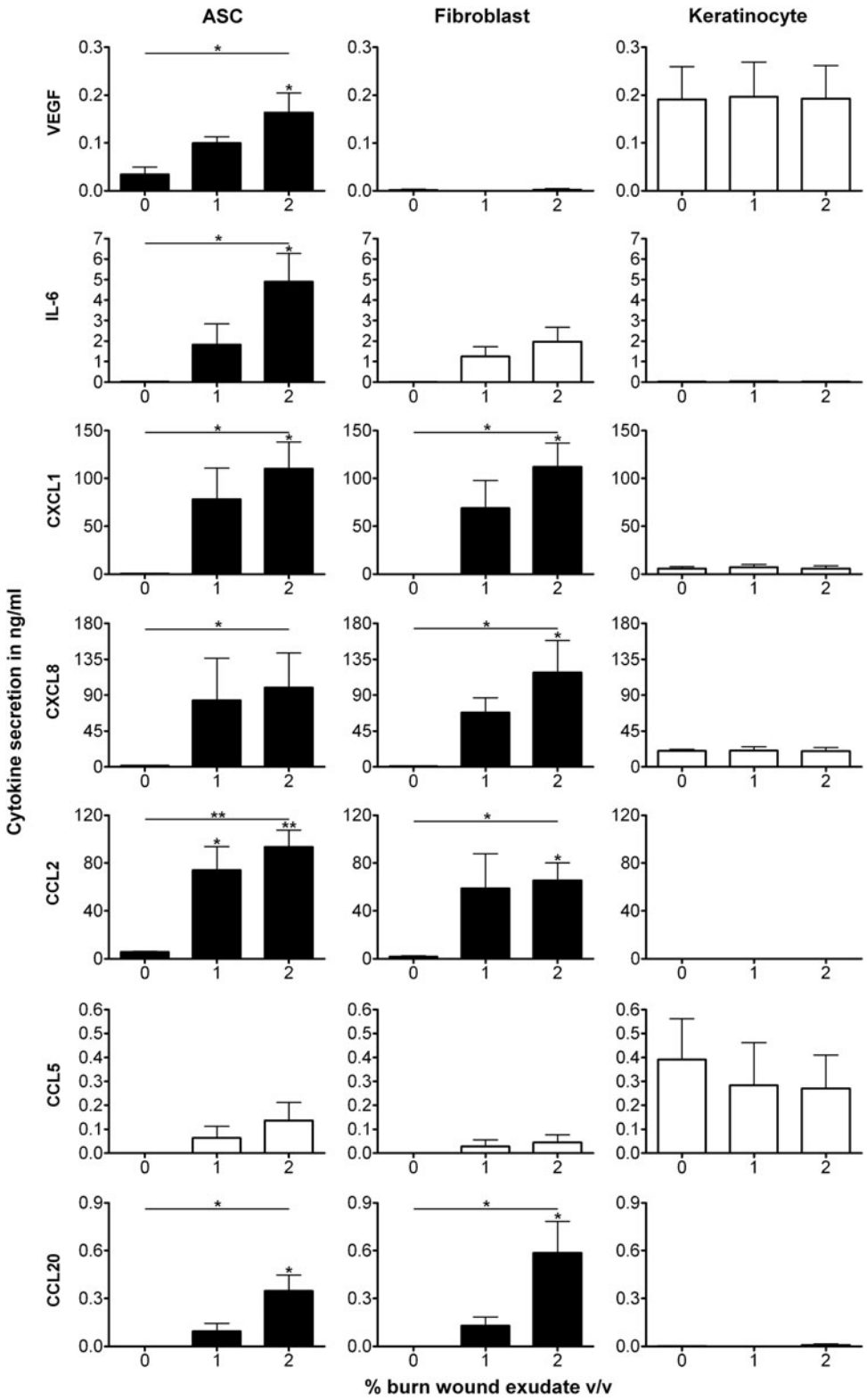


FIG. 1. BWE stimulate ASC, but not dermal fibroblasts and keratinocytes to secrete VEGF and IL-6. ASC, dermal fibroblast, and keratinocyte cultures were supplemented with BWE (0%, 1% or 2%) for 24 h. Amounts of wound-healing mediator secreted into culture supernatants were analyzed by ELISA. Data represent the mean ± SEM in ng/mL per 24 h and per 100,000 seeded cells of four independent experiments, each performed with an intra-experiment duplicate (*$p < 0.05$ and **$p < 0.01$ by repeated-measures ANOVA test followed by a Dunnett's multiple-comparison test). Black bars represent significant increase; White bars represent no significant increase. Basal amounts of mediators in medium containing 2% BWE: VEGF: not detectable; IL-6: 0.93 pg/mL; CXCL1: 14.8 pg/mL; CXCL8: 1776 pg/mL; CCL2: 2.4 pg/mL; CCL5: not detectable; CCL20: not detectable. ASC, adipose tissue-derived mesenchymal stem cells; VEGF, vascular endothelial growth factor; IL, interleukin; BWE, burn wound exudate.

between ASC and dermal fibroblasts with regard to VEGF and IL-6 secretion were no longer emphasized in the SS.

Taken together, these results indicate that ASC respond more vigorously to factors present in the burn wound bed than either dermal fibroblasts or keratinocytes; however, when incorporated into a bi-layered SS, differences were less pronounced.

Skin-specific chemokine CCL27 is present in wound exudates and is secreted by stimulated keratinocytes, monocytes, and HMVEC but not by ASC, fibroblasts, or granulocytes

To further investigate the differential behavior of ASC and dermal fibroblasts when these cells come into contact

with the cutaneous wound bed, we focused on the effect of the unique skin-specific chemokine CCL27, as this chemokine was detected in third-degree burn eschar exudates (199 ± 108 pg/total protein in mg; mean ± SEM), in chronic wound exudates (80 ± 33 pg/total protein in mg; mean ± SEM), and in surgical wound exudates (11 ± 5.8 pg/total protein in mg; mean ± SEM). In line with CCL27 secretion into wound exudates, CCL27 secretion was also increased in culture supernatants collected during the first 24 h after inflicting a full-thickness burn wound into *ex vivo* healthy adult skin compared with control unwounded skin and remained slightly elevated for approximately 7 days (Fig. 3a, b).

Since CCL27 is up-regulated on infliction of major skin trauma, we first determined which cells present in the wound bed (infiltrating or residential) were able to produce CCL27. Immunostaining of CCL27 in the *ex vivo* re-epithelializing burn wound showed increased CCL27 expression throughout the migrating epidermal front compared with basal layer expression in adjacent unwounded areas of the skin (Fig. 3c) and in control not-burned skin (data not shown). CCL27 also stained endothelial cell clusters in the dermis in agreement with Homey *et al.*, (arrows, double staining CCL27/EN4; Fig. 3c, d).[24]

Next, it was determined whether other cells present in the wound bed, in addition to keratinocytes, were able to produce CCL27. In contrast to keratinocytes (basal secretion of 23 pg/mL CCL27), ASC, dermal fibroblasts, HMVEC, granulocytes, and monocytes did not secrete detectable amounts of CCL27 (Fig. 4). In order to determine whether under extreme conditions representative of skin trauma the cells would be able to secrete CCL27, the different cell types were next stimulated with relevant and potent stimuli as described in the literature (see Materials and Methods section). Notably, monocytes and HMVECs were able to secrete CCL27 on stimulation with LPS and VEGF, respectively (Fig. 4). In contrast, ASC, dermal fibroblasts, and granulocytes did not secrete CCL27 on appropriate triggering. Taken together, these results indicate that CCL27 can be secreted by keratinocytes, monocytes, and HMVEC after skin injury in line with the presence of CCL27 in wound exudates derived from deep cutaneous wounds.

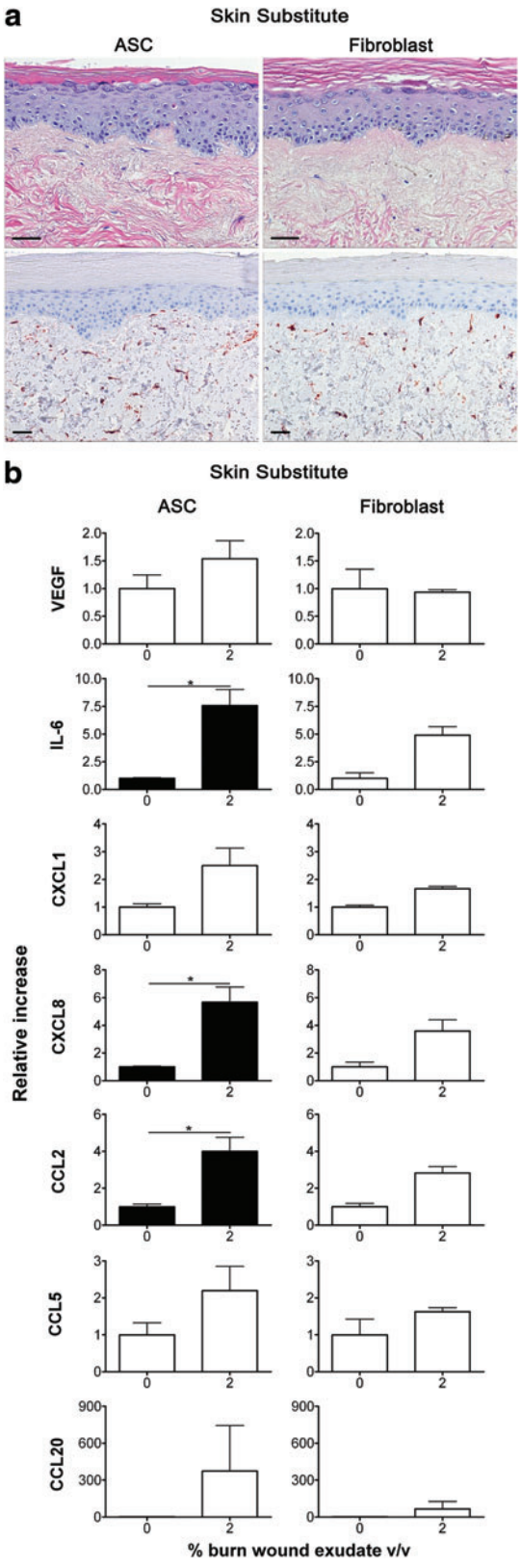
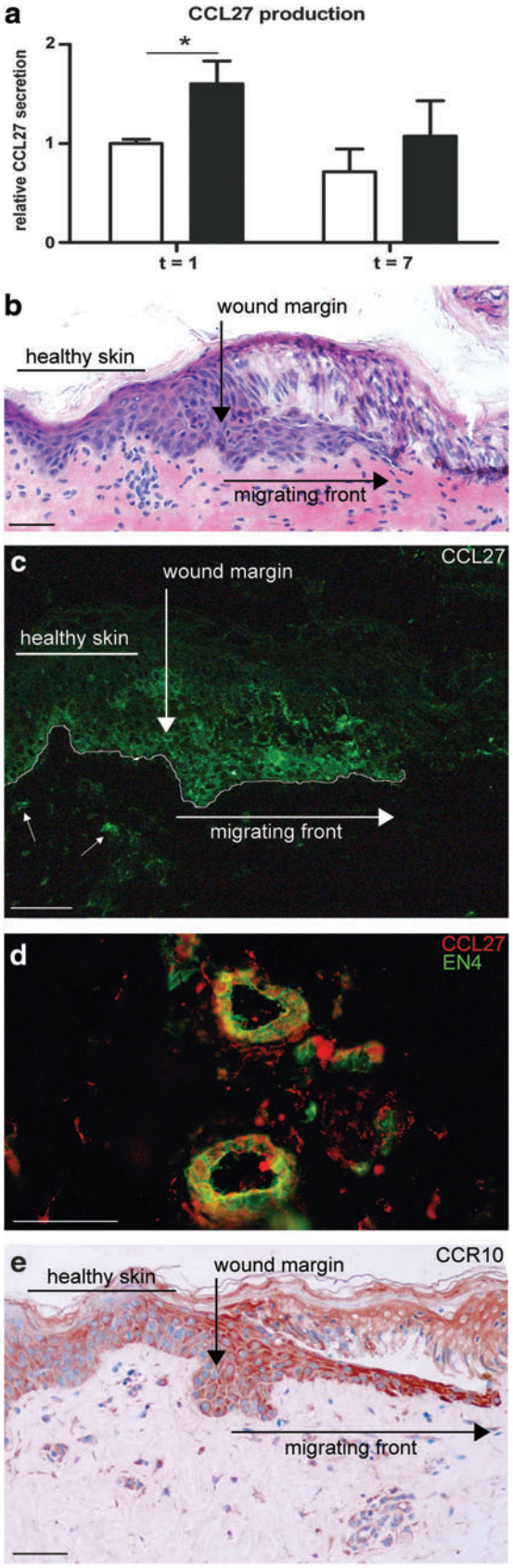


FIG. 2. BWE stimulates SS containing ASC, more than SS containing dermal fibroblasts to secrete IL-6, CXCL8, and CCL2. **(a)** SS containing either ASC or dermal fibroblasts in the dermis were stained with hematoxylin/eosin to show tissue histology (upper panel) or vimentin to visualize ASC and fibroblasts (lower panel); bar = 50 μm **(b)** SS culture media were supplemented with 2% wound exudates for 24 h. Amounts of wound-healing mediators secreted into culture supernatants were analyzed by ELISA and represented as the mean ± SEM relative increase compared with not supplemented SS of three independent experiments, each performed with an intra-experiment duplicate. (*$p < 0.05$ by a Wilcoxon-matched pairs test). Black bars represent significant increases; white bars represent no significant increase. Basal secretion of SS containing ASC: VEGF: 12.5 ± 3.06 ng/mL; IL-6: 454 ± 30 pg/mL; CXCL1: 24.4 ± 2.82 ng/mL; CXCL8: 7.76 ± 0.38 ng/mL; CCL2: 10.3 ± 1.42 ng/mL; CCL5: 220 ± 71 pg/mL; CCL20: 1.6 ± 1.6 pg/mL or dermal fibroblast: VEGF: 11.0 ± 3.67 ng/mL; IL-6: 612 ± 26 pg/mL; CXCL1: 31.9 ± 2.33 ng/mL; CXCL8: 10.6 ± 3.05 ng/mL; CCL2: 14.3 ± 2.15 ng/mL; CCL5: 273 ± 116 pg/mL; CCL20: 2.42 ± 2.42 pg/mL (mean ± SEM). SS, skin substitutes. Color images available online at www.liebertpub.com/tea



CCL27 increases secretion of inflammatory and angiogenic factors from ASC but not from dermal fibroblasts or keratinocytes

Next, we determined whether ASC, dermal fibroblasts, and keratinocytes were triggered by CCL27, in a similar manner to that observed by burn wound exudates, to secrete mediators that could influence angiogenesis, granulation tissue formation, and inflammation (VEGF, IL-6, CXCL1, CXCL8, CCL2, CCL5, and CCL20). Since for a cell to be able to respond to CCL27 it is essential that the receptor CCR10 is present on the cell surface, we first determined whether CCR10 was present on ASC, dermal fibroblasts, and keratinocytes. CCR10 was expressed in all epidermal cell layers in human skin and was increased in the migrating front of the epidermis after burn injury (Fig. 3e). Staining was also observed in single cells within the dermis and on blood vessels. Flow cytometry confirmed receptor expression on the cell surface of ASC and dermal fibroblasts, although keratinocytes had, by far, the highest CCR10 expression (Table 2).

Having confirmed that the receptor CCR10 was indeed present in all 3 cell types, we next determined whether the cells could respond to rh-CCL27 (Fig. 5). Notably, rh-CCL27 stimulated ASC to increase secretion of VEGF, CXCL1, CXCL8, and IL-6, but not CCL2, CCL5, or CCL20. In contrast to ASC, no increased cytokine, chemokine, and growth factors secretion was observed by either dermal fibroblasts or keratinocytes even though they expressed the receptor.

CCL27 stimulates cell migration but not proliferation

Since mobilization of cells into the wound bed is required for wound healing, we next determined whether ASC, dermal fibroblasts, and keratinocytes could migrate in a chemotactic transwell assay toward rh-CCL27. Keratinocyte migration was increased 4-fold toward rh-CCL27, and a dose-dependent trend was observed for both ASC and dermal fibroblast migration toward CCL27 (Fig. 6). The response was chemotactic rather than chemokinetic, as no significant increase in migration of these three cell types was observed when CCL27 was placed in both the upper and lower well.

FIG. 3. Increased CCL27 in epidermis of freshly excised skin after full-thickness burn injury. Full-thickness burn wounds were introduced into excised skin. Unwounded skin (control) and burnt skin were then cultured air-exposed. New culture medium was added 24 h before collecting culture supernatants. Cultures were harvested, and supernatants were collected at day 0, 1, 2, and 7 for ELISA and/or (immuno-) histological analysis of tissue sections. **(a)** Relative CCL27 secretion by burnt skin (black bar) compared with unwounded excised skin (white bar). CCL27 secretion per 4 cm^2 excised skin per 24 h per mL culture supernatant as determined by ELISA is shown for day 0–1 and 6–7 after burning (mean ± SEM; $n = 4$ in duplicate; *$p < 0.05$ by Wilcoxon-matched pairs test); **(b)** hematoxylin/eosin staining of the migrating epidermal front 2 days after burning; **(c)** CCL27 staining of re-epithelializing wound 2 days after burning, small arrows indicate clusters of endothelial cells **(d)** CCL27–EN4 endothelial cell double staining of dermal part human skin **(e)** CCR10 staining of re-epithelializing wound 3 days after burning **(b–d)** scale bar = 50 μm. Color images available online at www.liebertpub.com/tea

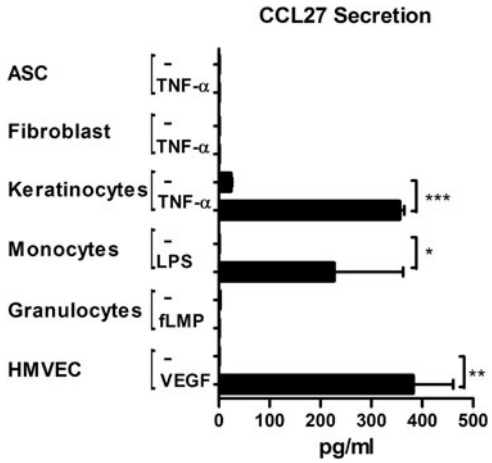


FIG. 4. Secretion of CCL27 by cells involved in wound healing. ASC, fibroblasts, keratinocytes, monocytes, granulocytes, and HMVEC were cultured in the presence or absence of relevant stimuli TNF-α (10 ng/mL) LPS (100 ng/mL), fLMP, or VEGF (5 ng/mL) as indicated, respectively, for 24 h. Culture supernatants were isolated and analyzed for CCL27 by ELISA. Data are represented as the mean ± SEM secretion of CCL27 in pg/mL corrected per hour and per 100,000 cells of at least three independent experiments, each performed with an intra-experiment duplicate. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by a Wilcoxon-matched pairs test). HMVEC, human micro vascular endothelial cells.

In order to determine the influence of CCL27 on cell proliferation, ASC, dermal fibroblasts, and keratinocytes were incubated in the absence or presence of different concentrations rh-CCL27 (0, 62.5, 125, 250, and 500 ng/mL) for 24 and 72 h. No difference in cell number for ASC, dermal fibroblasts, or keratinocytes was observed after the 24 h incubation period, confirming that the increase in mediator secretion described earlier for ASC and keratinocyte migration was a direct effect of CCL27 exposure and not an indirect effect due to increased cell numbers. After 3 days of exposure to rh-CCL27, only keratinocytes showed a slight but significant increase in proliferation compared with unexposed keratinocytes (1.3-fold by rh-CCL27 ($n = 4$, $p < 0.05$) compared with 2-fold by EGF. ASC and dermal fibroblasts did not increase proliferation in response to rh-CCL27 (data not shown).

Table 2. CCR10 Expression on Cells

Cell type	*% Cells expressing CCR10*[1]	*Significance*	
ASC	5.15 ± 0.25	ASC vs. fibroblast	ns
Fibroblast	4.48 ± 0.05	ASC vs. keratinocyte	[a]
Keratinocyte	38.00 ± 7.00	fibroblast vs. keratinocyte	[a]

The mean number of cells expressing CCR10 is shown as a percentage of total cell number ± standard deviation ($n \geq 3$). Wilcoxon signed rank was used to determine statistical significance between ASC versus dermal fibroblast (donor matched). Mann–Whitney test was used to determine statistical significance between ASC versus keratinocyte and dermal fibroblast versus keratinocyte (not donor matched) ([a]$p < 0.05$).

ASC, adipose tissue-derived mesenchymal stem cells; ns, not significant.

Discussion

In this study, we show that wound exudates isolated from deep burn wounds greatly influence the behavior of three important cell types (ASC, dermal fibroblasts, and keratinocytes) that are being investigated for their use in tissue-engineered constructs and regenerative medicine strategies. It is also shown that these three commonly used cell types react differently to burn wound exudates, with ASC reacting more vigorously than dermal fibroblasts or keratinocytes with regard to secretion of potent angiogenic factors, granulation tissue formation factors, and inflammatory cytokines/chemokines. These results demonstrate the importance of taking into account the interactions between the cells to be transplanted and the wound bed environment, as these interactions can be expected to influence wound healing and the final quality of the resulting scar tissue.

When monolayers of ASC and dermal fibroblasts were exposed to burn wound exudate, a huge increase in CXCL1, CXCL8, CCL2, and CCL20 secretion was observed in both cell types but only ASC showed substantial increases in secretion of the angiogenic factor VEGF and the wound-healing cytokine IL-6. This finding indicates that both ASC and dermal fibroblasts will stimulate granulation tissue formation when applied to a burn wound bed but that ASC are much more potent in stimulating angiogenic and inflammatory stages of wound healing than dermal fibroblasts. Since excess granulation tissue formation is thought to result in hypertrophic scar formation,[38] our results may have serious implementations when ASC are considered in therapeutic strategies for burns patients. These results are also in line with Hong *et al.*, who recently showed in a rabbit ear model that ASC increased granulation tissue formation in wounds whereas dermal fibroblasts and BM-MSC did not.[39] Of note, keratinocytes did not respond to the burn wound exudate. This latter finding is in agreement with clinical data in which cultured keratinocytes are used to close burn wounds and where wound closure (re-epithelialization) has been reported to suppress excessive granulation tissue forming in the burn wound bed.[40] When ASC or dermal fibroblasts were incorporated into SS and exposed to burn wound exudates, the increase in secretion of the cytokines was less pronounced than with monolayer experiments, and also the differences between ASC and dermal fibroblasts were less pronounced. This is most probably due to the huge increase in cytokine release caused by the synergistic cross-talk between keratinocytes and ASC or fibroblasts in the SS. Indeed, SS basal secretion for cytokines was generally in the order of ng/mL; whereas for monolayer cultures, it was only in the order of < 50 pg/mL. This finding is in line with our previous results[32] and indicates that SS are very potent secretors of wound-healing mediators and, therefore, are suitable for healing chronic wounds.[10,41] Incorporation of ASC rather than fibroblasts may increase the potency of SS even further.

In the present study, we investigated the skin-specific chemokine CCL27 in greater detail, as its current function in wound healing is unknown even though it was detected in the wound exudates of 3 different types of difficult-to-heal full-thickness cutaneous wounds. It was found that in addition to the previously reported keratinocytes,[21,24] other cell types present in the wound bed (monocytes and HMVEC)

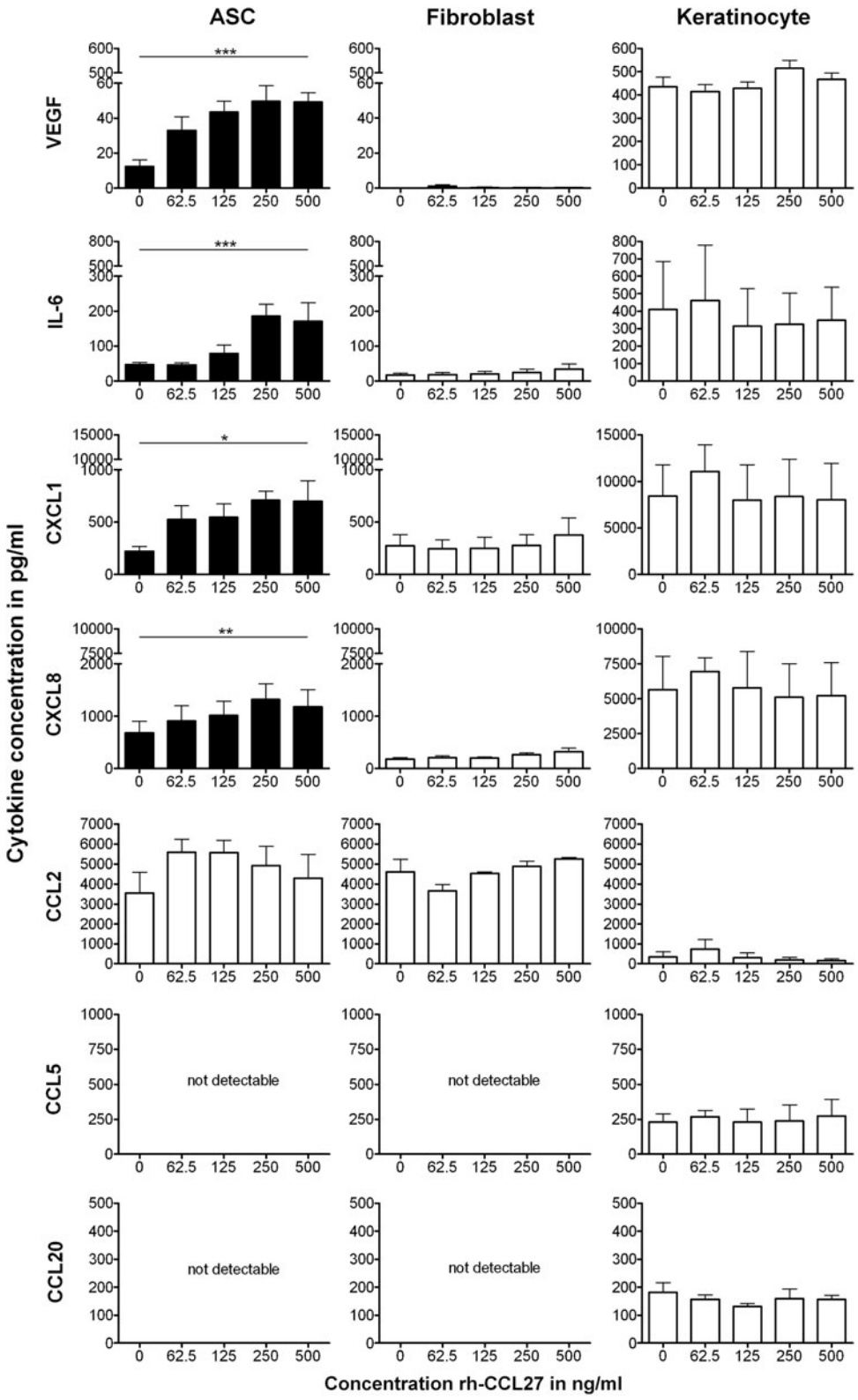


FIG. 5. rh-CCL27 stimulates ASC, but not fibroblasts to secrete wound-healing mediators. ASC and fibroblasts were cultured in the presence or absence of rhCCL27 for 24 h. Mediators involved in angiogenesis, granulation tissue formation, and inflammation secreted into culture supernatants were analyzed by ELISA. Data are represented as the mean ± SEM production of mediators in pg/mL per 24 h and per 100,000 seeded cells of 4 independent experiments, each performed with an intra-experiment duplicate. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by repeated-measures ANOVA test followed by a Dunnett's multiple-comparison test). Graphs with black bars show a significant dose-dependent increase compared with graphs with white bars that show no significant increase. Similar to fibroblasts, keratinocytes did not increase cytokine secretion in response to rhCCL27. rh, recombinant human.

were also able to secrete CCL27 when stimulated with factors related to extreme skin trauma. Monocytes were able to increase secretion of CCL27 on stimulation with LPS, a molecule present in the outer membrane of gram-negative bacteria that frequently infects open wounds.[42] HMVEC staining positive for CCL27 were also able to increase secretion of CCL27 on stimulation with VEGF, an important factor mediating angiogenesis.[37] Our results are in line with Homey *et al.*, who showed CCL27 immunostaining of endothelial cells in the papillary dermis, possibly derived from

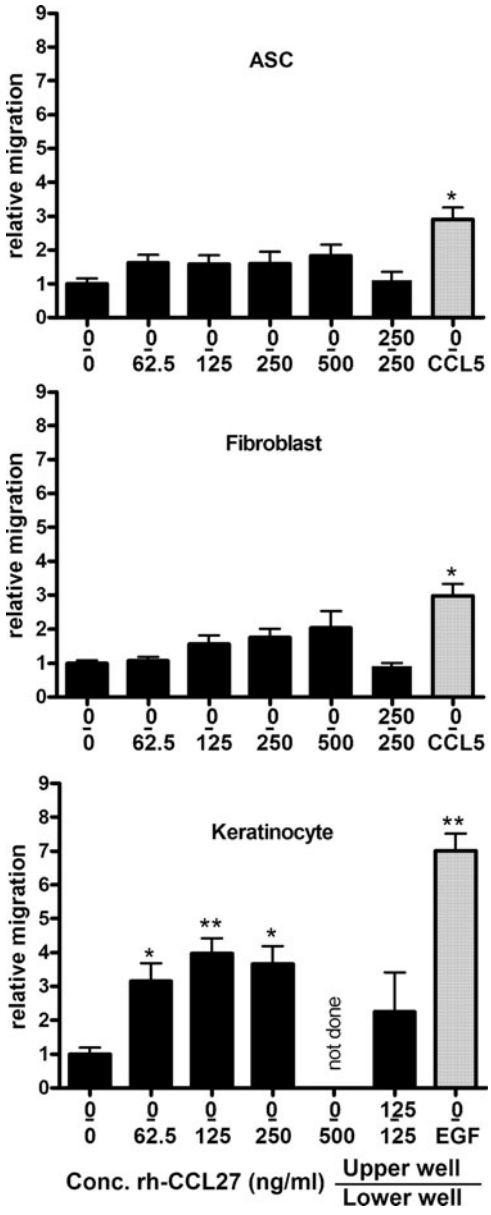


FIG. 6. Chemotactic migration of keratinocytes but not of ASC or fibroblasts toward rhCCL27. Cells were incorporated into a chemotaxis transwell assay in the presence or absence of rh-CCL27 in the lower well. Chemotaxis rather than chemokinesis is confirmed by placing an equal concentration of rh-CCL27 in upper and lower wells. Positive controls were CCL5 for ASC and fibroblasts and EGF for keratinocytes. Data are represented as the mean ± SEM relative cell migration in 24 h to the underside of the transwell filter of 3 independent experiments, each performed with an intra-experiment duplicate (*$p < 0.05$ and **$p < 0.01$ by Wilcoxon-matched pairs test).

keratinocytes or from secretion by endothelial cells themselves.[24]

Importantly, CCL27 increased the secretion of granulation tissue-stimulating mediators (VEGF, IL-6, CXCL1, and CXCL8) from ASC but not from dermal fibroblasts or keratinocytes, in line with our results for total burn wound exudate. All of these mediators have been reported to play a role in granulation tissue formation and angiogenesis.[1,18,19] The cause of this differential response is unknown, as both ASC and fibroblasts express CCR10 to similar extents on their cell surface. We also found that CCL27 was not able to stimulate ASC to secrete potent inflammatory mediators CCL2, CCL5, and CCL20.[1,18,19,43] Further reports in line with our findings describe that ASC exhibit strong angiogenic potential (e.g., greater release of angiogenic factors VEGFa, HGF, and Ang-1) compared with dermal fibroblasts.[44,45]

Notably, CCL27 was not able to stimulate the secretion of any of the wound healing mediators by keratinocytes, even though keratinocytes highly expressed the CCR10 receptor. Instead, keratinocyte migration was enhanced by CCL27. This effect was significantly observed for keratinocytes, and only a slight trend was observed for ASC and dermal fibroblasts. Similarly, only keratinocytes showed a slight increase in proliferation after stimulation by CCL27. This finding further supports our observation that burn wound exudate does not influence keratinocyte cytokine, chemokine, and growth factor secretion and explains clinical data in which keratinocytes are applied to close burn wounds without stimulating granulation tissue formation.

In our studies, it was noticed that the amount of endogenous secreted CCL27 was in the order of 200–400 pg/mL in the vitro studies. This is much lower than the concentration of rh-CCL27 required to cause a response in our *in vitro* studies, which was in the order of 100 ng/mL. This discrepancy is most possibly explained by the endogenous protein having a higher activity than the recombinant protein or due to a higher concentration existing directly in the cell environment compared with that present in the culture supernatants. Since the wound exudate could not be assessed in pg/mL but in pg/total protein in mg due to the extraction method required,[10] it is not possible to determine from our results whether this discrepancy was also observed between endogenous CCL27 in wound exudate and rh-CCL27 in *in vitro* studies.

The present findings may have implications in the use of stem cells and skin constructs for clinical use. ASC may be more suited for use in skin constructs designed for chronic wounds in which activation of the inert wound bed is essential for healing.[30] This is in line with others who showed that ulcers treated with ASC in rats showed increased capillary density and granulation tissue thickness.[46] In contrast, dermal fibroblasts may be more suited for use in skin constructs designed for the treatment of deep burns where overactivation of the wound bed and stimulation of excessive granulation tissue needs to be avoided in order to reduce the chance of hypertrophic scar formation.

In addition to implications with regard to applying tissue-engineered products, our findings imply that full-thickness wounds, which penetrate the adipose tissue containing ASC, may respond more vigorously to wound-bed-derived CCL27 than superficial dermal wounds containing dermal fibroblasts. This is in line with the clinical observation that excess granulation tissue formation resulting in adverse scar formation (e.g., hypertrophic scar) mainly occurs during healing of deep wounds which penetrate below the viable dermis,[38] such as third-degree burns, and suggests a putative role for CCL27 in scar formation. From an evolutionary point of view, the difference between ASC and dermal fibroblasts may be considered to be related to the function of the ASC,

which, in part, is to close life-threatening deep wounds rapidly by strongly promoting the formation of granulation tissue and angiogenesis even at the expense of adverse scar formation.

In this study, we show that factors present in the wound bed influence cells commonly used in tissue-engineered constructs. Our results suggest that burn wound exudates may trigger ASC more than fibroblasts to produce granulation tissue-forming factors on severe full-thickness skin trauma where adipose tissue is exposed. When ASC or dermal fibroblasts are incorporated into SS, this difference is less pronounced, indicating that cross-talk with keratinocytes may decrease ASC potency. However, application of ASC mono-cultures to burn wounds may be expected to increase scar formation by promoting angiogenesis and granulation tissue formation.

Acknowledgments

The authors would like to thank the Department of Physiology of the VU Medical Center for technical assistance with HMVEC cell culture; and J.A. Rezende (ACTA, Amsterdam, The Netherlands) for supplying freshly isolated immune cells. This study was financed by the Dutch Burns Foundation grant number 08.103.

Disclosure Statement

No competing financial interests exist.

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Received: February 17, 2013
Accepted: July 17, 2013
Online Publication Date: October 3, 2013