The epithelialisation phase in wound healing: options to enhance wound closure

ABSTRACT: This review highlights epithelialisation and therapeutic options to optimise and speed the epithelialisation process. To influence this process therapeutically, it is important for clinicians to understand the underlying principles of epithelialisation. The role of growth factors and the hostile local wound environment can explain why epithelial wound closure is so difficult to speed up in some chronic wounds. Clinicians should be aware of the different surgical techniques of skin grafting and more advanced technologies, such as skin substitutes, as options for wounds which fail to respond to standard protocols. Finally, novel dressing-based concepts are discussed, including macromolecular crowding, a concept which aims at boosting growth factor activities produced in the wound space once wound healing is normalised and underway.
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epithelialisation ● growth factors ● proteases ● skin grafting ● skin substitutes

Epithelial wound closure is central in the wound healing process because it re-establishes the integrity of the epidermal barrier, which blocks uncontrolled fluid loss and prevents the invasion of microbes from the surrounding environment.

Epithelial wound closure is more complex than it appears at first glance.[1,2] It involves growth factor signalling, keratinocyte migration and proliferation, cell-matrix degradation during migration, as well as *de novo* synthesis when the basement membrane zone matures. Finally, it comprises the barrier restoration, which is the ultimate goal of the wound healing process. In normal wound healing, these processes depend on a healthy, productive granulation tissue. Complex cell-cell interactions among keratinocytes, fibroblasts, endothelial cells and inflammatory cells coordinate epithelialisation.[3]

In chronic wounds, wound bed preparation (WBP) has been comprehensively studied resulting in many options for clinicians.[4] However, preparation of the wound edge is more elusive. Epithelialisation proceeds when the conditions of the matrix and local microenvironment are optimal. The harsh environment of chronic wounds prevents epithelial wound closure.[1] Theclinical approach is focused on stimulating granulation tissue formation, whereas epithelialisation is left to progress on its own.

Growth factor regulation in wound healing

Growth factor regulation and cellular interactions with the extracellular matrix (ECM) are key to understanding the complex biology underlying normal and impaired epidermal wound closure. There have been excellent reviews on the molecular and cellular events in wound healing,[5] and in this paper we attempt to intergrate the underlying scientific body of evidence with the clinical persepctive.

Wound healing, as depicted in Fig 1, proceeds through several overlapping phases involving multiple cell types and functions. During normal wound healing, these cellular functions are regulated and coordinated by growth factors, cytokines and soluble mediators.[6] In the inflammatory phase, pro-inflammatory cytokines are predominant. Later, during granulation tissue formation and epithelialisation, the wound environment switches from inflammation to angiogenesis, connective tissue production and epithelial cell proliferation and migration.[7] Consequently, cells in the granulation phase produce a different set of growth factors compared with the early inflammatory phase.

Platelet activation and degranulation during the coagulation phase release high concentrations of preformed growth factors at the site of injury.[8] When activated, platelets release large amounts of chemokines, CCL5/RANTES, CXCL1/2/3 (aka GROα/β/γ)[9] attracting neutrophils, keratinocytes, endothelial cells and fibroblasts (Table 1). At the early stages of the inflammatory phase, neutrophils and macrophages produce pro-inflammatory cytokines, such as interleukin-1 and tumour necrosis factor (TNF)-α.[10] This 'wave' of strong pro-inflammatory signals triggers a negative feed-back loop of anti-inflammatory signals,

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Fig 1. Cellular interactions during inflammation and granulation tissue formation in wound healing. The early inflammatory phase (**a**) is characterised by an influx of inflammatory cells, such as neutrophils and macrophages. A diffuse interstitial oedema reflects vascular leakiness. Apart from the inflammatory cell infiltration, there are relatively few other morphological changes. On the molecular level, the cytokine microenvironment is dominated by interleukin 1 (IL-1α, -1β), tumour necrosis factor-α (TNF-α) and reactive oxygen species (ROS). Slightly later, the production of anti-inflammatory factors, such as interleukin-1 receptor antagonist (IL-1RA) and interleukin-10, begins. When the granulation tissue has formed (**b**) the microenvironment is dominated by growth factors from the transforming growth factor (TGF)-β family, fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and granulocyte-macrophage colony stimulating factor (GM-CSF). At this stage, production of extracellular matrix, angiogenesis and keratinocyte migration reach their maximum. The number of neutrophil granulocytes declines and macrophages assume an M2-like phenotype. Communication between cells in the wound space occurs via soluble, diffusible mediators

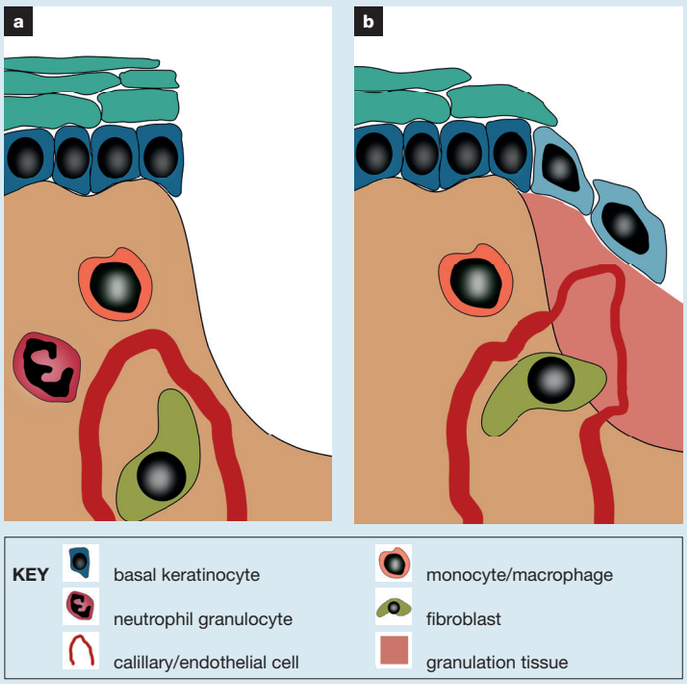


Table 1. Factors released from activated platelets, adapted from Martínez et al.[8] and Fekete et al., 2012[9]

Interleukin (IL)-1α
Interleukin (IL)-1β (traces)
Tumor necrosis factor (TNF)-α
Interleukin (IL)-6 (traces)
Interleukin (IL)-7
Interleukin (IL)-8
Interleukin (IL)-10 (traces)
Interferon (IFN)-γ
Transforming growth factor (TGF)-β1
Fibroblast growth factor 2 (bFGF)
Transforming growth factor (TGF)-α
Platelet-derived growth factor (PDGF)-AA
Platelet-derived growth factor (PDGF-AB/BB)
Vascular endothelial growth factor (VEGF)
Insulin-like growth factor (IGF)1
Insulin-like growth factor (IGF)2
Insulin-like growth factor binding protein (IGF-BP)3
Granulocyte–colony-stimulating factor (G-CSF)
CXCL1/2/3 (GRO)
CCL3/MIP-1α
CCL4/MIP-1β
CCL5/RANTES
Soluble CD40 ligand (sCD40L)
Soluble vascular cell adhesion protein 1 (sVCAM-1)
Soluble intercellular adhesion molecule 1 (sICAM-1)
Platelet factor 4 (PF4)
Beta-thromboglobulin (β-TG)

such as interleukin-1 receptor antagonist, interleukin-10, and cortisol.[6–11] There is evidence that infiltrating macrophages change their polarisation from the M1 to the M2 phenotype.[12] Conceptually, M1 macrophages are predominantly inflammatory with antimicrobial defense mechanisms, tissue destruction, and debridement, while the M2 macrophage polarisation is associated with granulation tissue build-up including collagen synthesis, angiogenesis and healing.[13] Anti-inflammatory cytokines are required so that inflammation is contained and granulation can become productive. This phase is dominated by growth factors which stimulate connective tissue production, transforming growth factor (TGF)-β family members, and connective tissue growth factor, angiogenesis vascular endothelial growth factor (VEGF), and growth factors which stimulate keratinocyte proliferation and migration, fibroblast growth factor (FGF)-2, 7, 10, TGF-α, hepatocyte growth factor (HGF) (further reviewed in reference 7). Eventually, epithelial wound closure is achieved, the epidermal barrier re-established and wound healing is completed. The young scar is remodelled for the next few weeks/months until a mature scar, with altered tissue texture and reduced capillary density, ensues.[14]

Specificity of growth factor signals in the wound space

How can growth factors elicit specific cellular responses

at a certain time and place in the wound space? How can cells integrate the wide range of different external stimuli? There are several regulatory mechanisms.

Growth factors and cytokines are powerful regulators of tissue homeostasis, growth and maintenance. Following the appropriate stimulus, cells synthesise growth factors and process them to convert inactive pro-forms into active growth factors.[15] Growth factors can be produced in advance, stored in intracellular granules and, upon activation, released within minutes. Platelets are a good example; when activated, a complex mixture of stored growth factors are released into the extracellular environment (Table 1).[9] Growth factors can also be synthesised as inactive precursors. TGF-α is produced and then stored on the surface of keratinocytes as a large precursor molecule.[16–17] These membrane-bound precursors can stimulate the EGF receptors of nearby cells but cannot diffuse. Limited proteolysis is required to generate the diffusible, 50 amino acid active form. The required protease was subsequently identified as TACE/ADAM (tumor necrosis factor-alpha converting enzyme/a disintegrin and metalloproteinase).[17,18] ADAMs proteases have a wide spectrum of substrates such as cell membrane-bound growth factors, cytokines, cell adhesion molecules and receptors (reviewed in reference 19). In addition, growth factors can be exported in extracellular vesicles (exosomes) that can further regulate cellular behaviour in both the local tissue environment and systemically as circulating factors.[20]

Diffusion gradients, with diminishing growth factor concentrations the further away from the producing cell, are a powerful means to regulate growth factor- or cytokine-derived effects. Many growth factor and cytokines bind to high-affinity receptors on the cell surface.[6] Receptor activation occurs after binding through changes in the three-dimensional structure and/or clustering of several receptors. At the receptor level, there is some promiscuity. Some cytokines and growth factors can activate several different receptors. Other growth factor cytokine-receptor pairs are highly specific and exclusive. Through up- or down-regulation of receptor expression cells can become more or less sensitive to low growth factor levels, a cell population can have cells highly expressing a given receptor making these cells extremely responsive to that growth factor. Other cells do not express the receptor or at such low levels, rendering these cells little-to-non-responsive. Some cells use another mechanism to regulate growth factor/cytokine sensitivity through receptor shedding. The protease-dependent controlled shedding of receptors from the cell surface makes these cells insensitive to the respective growth factor or cytokine. For example, TNF receptor is cleaved and released, and the released fragments can still bind and neutralise TNF-α.[21]

Inside the cell, the signalling machinery integrates all incoming signals by regulating gene expression that leads to the specific cellular response. Signals can be antagonistic or synergistic, depending on the context of the cellular milieu and environment. Of clinical interest is the example of pro-inflammatory signals which can override concurrent TGF-β signals required for granulation tissue formation.[22–23] This correlates well with findings in chronic wounds where TGF-β signals and granulation tissue formation are attenuated.[24] On the other hand, ECM binding through integrin receptors can co-stimulate growth factor receptors even in the absence of growth factors.[25–27]

A fourth level in the regulation of growth factor/cytokine effects involves binding of the factors to the ECM either directly or via extracellular vesicles. In this case growth factors are produced, secreted and bind with high affinity to ECM molecules of the connective tissue. This is a very elegant way to localise growth factors and their activities to defined areas in a tissue compartment. These growth factors can be dormant and only become active once released from their matrix reservoir by ECM remodelling or breakdown.[28] It is noteworthy that some growth factor activity can be potentiated by interaction with ECM molecules or their breakdown products (e.g. mediating tighter binding to their respective receptors, co-stimulation)[29–30] or they become protected from degradation (e.g. by extracellular proteases).[30]

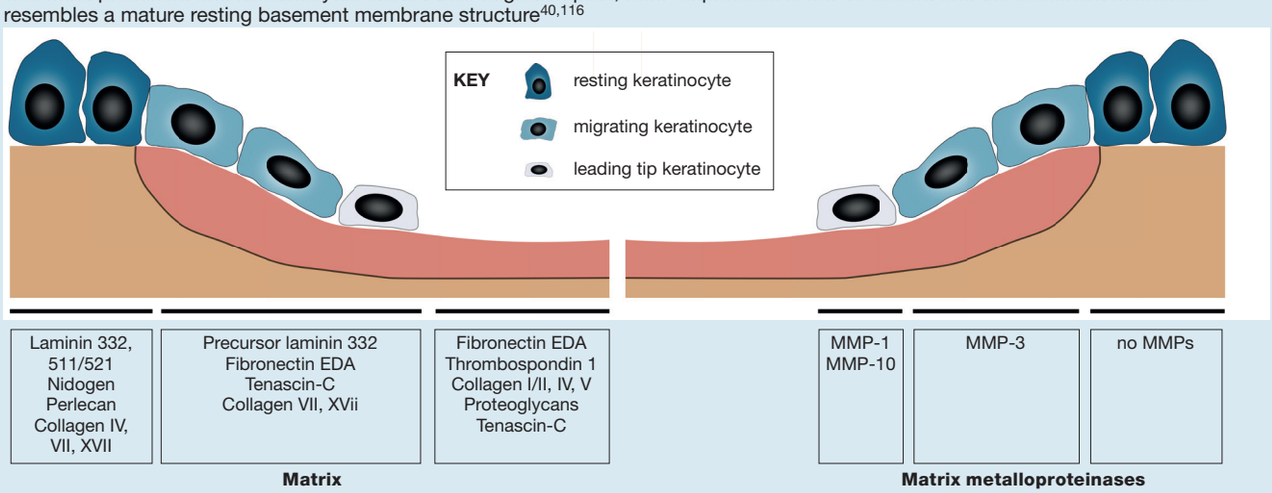
Epithelialisation: basic mechanisms

Epithelialisation requires keratinocyte migration and proliferation that is orchestrated by activated keratinocytes.[31] Both processes are driven by growth factors, ECM components, intracellular modulation of the keratin scaffold to allow migration and balanced protease expression.

Keratinocyte proliferation depends on interactions with an activated granulation tissue. Activated in this context reflects that a fully functional granulation tissue as opposed to a friable granulation tissue often seen in chronic wounds, brightly red, bleeding on light touch and rich in exudate production. Keratinocytes and underlying mesenchymal cells interact via paracrine mediator loops.[32] Keratinocytes secrete factors such as IL-1α which stimulate growth factor expression in fibroblasts (e.g. FGF7, FGF10, HGF) which in turn have their effect on keratinocyte proliferation and migration.[33–34] These amplification loops are highly effective. In genetic animal models blocking HGF function results in severe wound healing defects.[35] In other instances, deficiency of one growth factor or cytokine can be compensated by related or overlapping growth factor effects.[36–38] This redundancy illustrates multiple safety and backup mechanisms. Yet, they cannot make up for the loss of normal, physiological tissue repair mechanisms in chronic wounds.[6–39]

Keratinocyte migration also depends on growth factor signals. These mediators that stimulate keratinocyte proliferation are largely the same. Some are produced by neighbouring keratinocytes (e.g. TGFα, FGF22), some are synthesised by mesenchymal cells

Fig 2. Extracellular matrix remodelling in the migrating epithelium. When keratinocytes migrate towards the wound centre, a complex cascade of extracellular remodelling events occur underneath the cells. On the right-hand side, the expression of matrix metalloproteinases (MMP) is shown. On the left-hand side, the distribution of extracellular matrix (ECM) components in the regenerating basement membrane zone is shown. MMP-1 and -10 are produced by the most outward keratinocytes. EMC is remodelled and replaced by laminin 332 precursors, the fibronectin EDA isoform, tenascin-C and several collagen types. Keratinocytes further away from the leading edge express MMP-3 and continue to remodel the basement membrane zone. At the former wound border, where proliferation was most prominent and keratinocytes started their migration path, MMP expression is low-to-absent and the basement membrane resembles a mature resting basement membrane structure[40,116]



(e.g. FGF7, FGF10, HGF) or by both cellular compartments (e.g. heparin binding-epidermal growth factor (HB-EGF)) and the above mentioned paracrine loops. Table 2 lists the most prominent examples.

Keratinocyte migration also includes a mechanical component, both inside and outside of the cell. Reorganisation of the cytoskeletal components inside a cell, that is regulated by growth factor signals, results in the formation of filopodia and lamellipodia, as well as diminishing stress fibres that allow for directional cell migration. Cells at the wound edge are tightly anchored to the underlying basement membrane through hemidesmosomes. This adhesion structure provides a mechanical link from the basement membrane through the cell membrane to the intracellular keratin scaffold. To become motile, keratinocytes need to reduce the stiffness of the intracellular scaffold.

To reduce adhesion strength, hemidesmosome numbers need to decrease. This is achieved by proteases, most notably matrix metalloproteinases (MMP). There is a distinct pattern of MMP expression in the migrating epithelial tongue (Fig 2).[40–41] When the keratinocytes migrate towards the wound centre they receive further signals from ECM components they normally do not encounter. Migrating keratinocytes produce fibronectin variants (EDA-fibronectin) underneath the leading epithelial tip.[42] The leading tip keratinocytes also produce a laminin isoform (laminin-5/laminin 332) which forms, together with EDA-containing fibronectin, the provisional ECM. Laminin 332 is produced as a precursor, not proteolytically processed at their c-terminal end (reviewed in 43). This conveys preferential binding by the α3β1 integrins found on migrating keratinocytes.[44] The contact with interstitial type I collagen triggers MMP-1 expression in the keratinocytes of the migrating epithelial front.[41] Provisional matrix and MMP-1 expression at the leading front are essential for epithelial wound healing. As cells move in from behind, the provisional basement membrane matrix matures and eventually assembles into the pre-wounding basement membrane. This includes proteolytical processing of laminin 332 and binding to α6β4 integrins found in hemidesmosomes and stationary keratinocytes.[45–46] Adhesion strength of keratinocytes increases with the maturation of the basement membrane. Long before these molecular details were discovered, clinicians already implemented the findings into correct care—gently, avoiding shear stress, which disrupts cell adhesion and loss of the newly formed epithelium.

Epithelialisation in different wound types

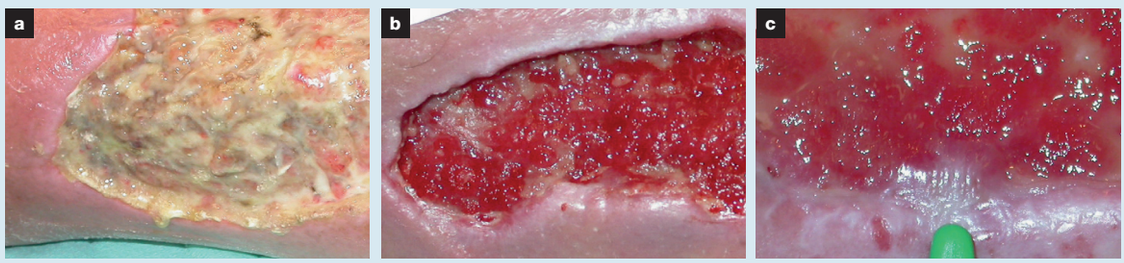
Normal epithelialisation can be expected in split-thickness donor sites. These wounds are artificially created when autologous skin transplantation is performed. Shallow wounds of <1.5mm are created with a dermatome. The excised split-thickness skin graft (STSG) is transplanted directly or after meshing to close large wounds. The resulting donor site will heal within 2–3 weeks. Key to those short healing times is that epidermal wound closure occurs from the wound borders and most importantly from the skin appendage remnants within the wounds. Depending on the density of skin appendages at the donor site keratinocytes have closed the defect a little faster or closer to the three weeks. There are multiple epithelial islands from which

Table 2. Factors influencing keratinocyte proliferation and migration (adapted from Seeger et al.[117])

			Keratinocyte	
Growth factor	**Receptor**	**Cellular source**	**Proliferation**	**Migration**
Epidermal growth factor family				
Epidermal growth factor (EGF)	EGF-receptor (EGFR)	Platelets, macrophages, fibroblasts	Yes[118]	Yes[119]
Transforming growth factor (TGF) α	EGFR	Keratinocytes, fibroblasts, macrophages, platelets, leukocytes	Yes[120]	Yes[121,122]
Heparin binding-epidermal growth factor (HB-EGF)	EGFR, HER2, HER3	Keratinocytes, fibroblasts	Yes[123]	Yes[124,125]
Betacellulin	ERFR, ERBB4	Keratinocytes	Yes[126]	
Epiregulin	EGFR, HER2, HER3	Keratinocytes, fibroblasts	Yes[127]	Yes[128,129]
Neuregulin	HER2, HER3	Keratinocytes, fibroblasts		Yes[130,131]
Insulin family				
Insulin	Insulin receptor, IGF-1 receptor	β-cells pancreas	Yes[132]	Yes[133]
Fibroblast growth factor				
Fibroblast growth factor-1 (aFGF)	FGF-receptor 1c, 2c, 2b, 3c	Fibroblasts	Yes[134]	Yes[135,136]
Fibroblast growth factor-2 (bFGF)	FGF- receptor 1c, 2c	Fibroblasts	Yes[134]	Yes[137,138]
Fibroblast growth factor-7 (KGF-1)	FGF-receptor 2b	Fibroblasts, mesenchymal cells	Yes[139,140]	Yes[141,142]
Fibroblast growth factor-10 (KGF2)	FGF-receptor 2b	Fibroblasts, mesenchymal cells	Yes[143]	Yes[143,144]
Fibroblast growth factor-22 (FGF22)	FGF- receptor 2b	Keratinocytes	Yes[145,146]	No[145,146]
Vascular endothelial growth factor (VEGF) A	Vascular endothelial receptor 1, 2	Keratinocytes, macrophages	Yes[147]	Yes[147,148]
Scatter factor family				
Hepatocyte growth factor (HGF)	c-Met	Fibroblasts, mesenchymal cells, keratinocytes	Yes[149]	Yes [35,150]
Macrophage stimulating protein (MSP)	Ron	Hepatocytes	Yes[151]	Yes[151,152]
Granulocyte macrophage-stimulating factor (GM-CSF)	GM-CSF-receptor (GM-CSFR)	Fibroblasts, mesenchymal cells, keratinocytes, macrophages, leukocytes	Yes[153]	Yes[154]
High mobility group protein β1 (HMGB1)	multiple	Leukocytes, macrophages	Yes[155]	Yes [156]
Heat shock protein 90 (HSP90)	LDL receptor-related protein 1 (LRP-1)	Keratinocytes	Yes[157]	Yes[158,159]

epithelialisation starts. This is also the reason why split-thickness donor site healing is surprisingly independent of the wound size. Distances between skin appendages, the internal starting points for epithelialisation, are fairly constant.[47–48] Epithelialisation in this system reflects the basic mechanisms summarised before. Missing epithelialisation in chronic wounds is the other extreme. Here, normal mechanisms are perturbed and epithelialisation is impaired, primarily due to lack of keratinocyte migration.[3–49] Moreover, if these wounds are deep and skin appendages are missing inside the wound, keratinocytes need to migrate large distances; from the wound border to the wound centre until epithelial wound closure can occur. There are many differences to normal epithelialisation. Inflammation and chronicity will result in a local wound environment characterised by excessive protease production,[50–52] matrix destruction,[53–54] and perturbed growth factor regulation. Chronic wound fluid had been shown to inactivate growth factors through proteolytic degradation.[55–56] Taken together, keratinocytes are missing growth factor cues to stimulate migration and the underlying provisional matrix which is required for migration is degraded. At the border, however, keratinocytes assume a hyperproliferative state characterised by impaired keratinisation,[57] parakeratosis

Fig 3. Typical wound conditions and their relation to wound epithelialisation. The wound in (**a**) shows a wound bed in which wound bed preparation has not occurred yet. Massive fibrin deposits, most likely hosting large numbers of bacteria/biofilm stimulate inflammation. Often exudate levels are high indicating leakiness of vessels as a consequence of deep-seated inflammation. The microenvironment can be expected to be dominated by excessive levels of proteases. In (**b**) the granulation tissue developed, yet epithelialisation is poorly progressing. The upper wound border is bulging but keratinocytes are missing the correct cues and conditions to migrate. Obviously, the granulation tissue formed but it seems that there are qualitative differences in the functionality of different granulation tissue states not easily assessed by their visual appearance. In comparison, the wound in (**c**) shows the beginning of keratinocyte migration in the lowermost part. The granulation tissue aspect is very similar to (**b**), yet, the wound bed supports epithelial migration. From a clinical point, it is difficult to convert the wound in (**b**) to the state in (**c**) in an active manner. Keratinocyte grafting through various methods can be a therapeutic approach



and macroscopically by a bulging phenotype (reviewed in reference 1). Fig 3 illustrates typical wound conditions encountered in daily clinical practice.

Histologically and ultrastructurally, aberrations in the migrating epithelium of chronic wounds have been described. There are numerous experimental systems available to study impaired epithelialisation in animal models. Here, pathology can be experimentally manipulated and new therapies formulated. Yet, from a clinical perspective, these models represent certain aspects but not the full spectrum of human disease including ageing, long-term complications of venous insufficiency or diabetes. An illustration in diabetes or hyperglycaemic and epithelialisation centres around the basement membrane zone. In hyperglycaemic rats, epithelialisation can be normalised by the administration of acylated homoserine lactone.[58] The authors observed in laminin 5 stainings abnormalities including fragmentation and immaturity of the basement membrane in hyperglycaemic rats. In human diabetic foot ulcers (DFU), the precursor form of laminin 3A32, associated with cell migration, was found to be variable and weaker expressed than in normal healing wounds.[59] Yet, the mature, processed laminin 332 form and integrin ligands for both the precursor form of laminin 3A32, α3β1 integrin, and the mature laminin 332 form, α6β4, did not show major differences.[59] This might explain some of the decreased epithelialisation. For venous leg ulcers (VLU), systematic data are missing. More emphasis was laid on the fibrin cuff concept around the capillaries in the connective tissue.[60–61] In pressure ulcers (PU), morphology is more associated with capillary vessel occlusion and necrosis.[62–63]

Surgical options to speed epithelialisation

Speeding up wound healing, particularly the epithelialisation phase has been attempted through numerous approaches. Surgical techniques are most commonly used. Transplanting autologous skin is a technique employed for more than 100 years. Reverdin pinch grafts include the epidermis as wells as upper parts of the dermis. Several small grafts are placed on the wound. They can take and stimulate epithelialisation from the grafts but also activate keratinocytes in the original wound borders to migrate. Öien et al. recently reported results on 126 chronic leg ulcers, including arterial and multifactorial ulcers, treated with Reverdin pinch grafting.[64] They anaesthetised the donor site, lifted the skin with a small calibre canula and cut the base of the lifted skin with a scalpel. The grafts were immediately transplanted on the ulcers, each a few millimetres apart from the other. Ulcers were 13.5cm^2 on average and ulcer duration was 15.9 months. The overall healing rate was 33% after three months and 60% after 12 months. A larger series was reported by Christiansen et al.[65] In their retrospective study of 412 leg ulcers, healing rates were best in vasculitic ulcers (56%), VLUs (38%), arterial ulcers (33%), mixed ulcers (33%) and other ulcers (20%). The overall healing rate was 38%.

Ollier-Thiersch grafting employed STSG with 0.2–0.25mm thickness. This includes the epidermis, papillary dermis and superficial parts of the reticular dermis. The grafting procedure was analysed in 1940 by Rank and Melb.[66] They reported take rates for the 144 secondary healing wounds of 14.6% for complete take, 25.7% for take rates >75%, 38% for take rates of <75% and take rate failure in 18%. Meshing STSGs helps to expand the graft and use smaller donor sites. When expanded keratinocytes migrate from the graft to the wound bed and promote epithelial wound closure, exudate can drain and accumulation of serum or blood underneath the graft is avoided. Negative pressure wound therapy (NPWT) can be combined to fix the graft to the wound bed and provide effective drainage of exudate or serum. This results in better take rates.[67–69]

The advantage of STSGs is that they are tolerant to a wider range of wound bed conditions and applications are broader than full-thickness skin grafts. The major drawback is the cosmetic appearance and contraction of scars. Failure of skin graft take is associated with infection, underlying haematoma, seroma, poor fixation and shear forces, as well as inadequate wound bed preparation which does not permit graft take.[70] Fig 4 illustrates the different steps in STSG preparation.

Specialised STSG techniques for burn wounds as well as full-thickness transplants are less used in the surgical closure of chronic wounds while flap procedures are commonly considered in decubitus surgery (most commonly fasciocutaneous and myocutaneous flaps) and in lower extremity wounds where blood supply is a concern.

Some procedures deserve mentioning. For leg ulcers with dermatoliposclerosis, Schmeller introduced the 'shave therapy procedure'[71,72] building on the early work of Hynes[73] and Quaba et al.[74] These ulcers are notoriously recalcitrant. A productive granulation tissue rarely forms and most wounds do not heal. This operation technique tangentially shaves off dermatoliposclerotic tissue down to the fascia or to a level where the wound bed appears rich in capillaries and bleeding is diffuse. STSGs are transplanted in the same operation without further conditioning of the wound bed. Hermanns reported healing rates of 79% in 249 wounds operated with this technique.[75]

Recently an old technique, epidermal grafting derived from suction blisters,[76] has been revived.[77] The basic technique requires the generation of suction blisters, removal of the blister roof and grafting of the blister roof on to the wound bed.[78] Epidermal grafts contain proliferation-competent basal keratinocytes[79–80] and the advantage of this technique is that the tissue defect of donor sites is smaller and the procedure can be repeated quicker. Comparative studies of the novel device-generated epidermal grafting procedure against conventional STSG are outstanding.[81]

Topical growth factor application to speed wound closure

The concept of topical application of growth factors became widespread with the availability of recombinant growth factors.[39] One of the first studies in humans was the use of recombinant EGF in STSG donor site healing in 12 patients.[82] Wound healing of 25% and 50% were reached one day earlier and 75% and 100% wound closure 1.5 days earlier; the differences were statistically significant. These results were in line with earlier animal experiments and the optimal dose of 10µg/ml was determined.[83] As encouraging as these results were the effect in VLUs was less clear. A positive trend was reported by Brown,[84] while Falanga reported safety of EGF in chronic wounds but no statistically significant difference in healing versus the vehicle.[85] Later work established that topical application of recombinant growth factors in chronic wounds is at risk of proteolytic degradation[55–56,86] which could explain the disappointing results of several trials.[87–90] One way to circumvent proteolytic degradation is periwound injection of the growth factor.[91–92] Still, of the numerous growth factors tested, only becaplermin (PDGF-BB) is commercially available for non-healing neuropathic DFUs and only in the US. Although only minimal doses are systemically absorbed, a follow-up of study participants from two placebo-controlled randomised clinical trials of becaplermin indicated a higher occurrence of newly diagnosed cancers in becaplermin-treated subjects (2.7%) versus participants in the placebo group (1.0%). This led to a warning statement in the drug information[93–94] while a matched cohort study analysing cancer risk in becaplermin users did not find significant differences.[93] Despite much enthusiasm and great expectations, recombinant growth factor application did not change clinical practice as much as anticipated.

Skin substitutes

Autologous culture-expanded keratinocyte grafting saved lives in burn patients with high total body surface

Fig 4. Illustration of split-thickness skin graft (STSG) preparation (meshed). STSG preparation starts with anaesthetised skin. A dermatome is used to remove superficial skin parts consisting of epidermis and superficial parts of the dermis (**a**). Skin appendages remain in the wound bed underneath; they are the starting points for re-epithelialisation of the donor site. The STSG can be meshed next (**b**). Specially designed apparatuses create alternating rows of incisions which, when pulled at, create the typical reticular pattern of meshed skin grafts (**c**, **d**). Depending on the expansion level, keratinocytes have to migrate shorter of larger distances from the graft to fully close the defect. It is important that the graft is optimally attached to the wound bed during the first few days and that the wound bed fully supports keratinocyte migration. Shear forces should be avoided at any cost. The graft take rate is a measure of how much of the grafted surface has taken the graft and supports the survival of the outgrowing cells

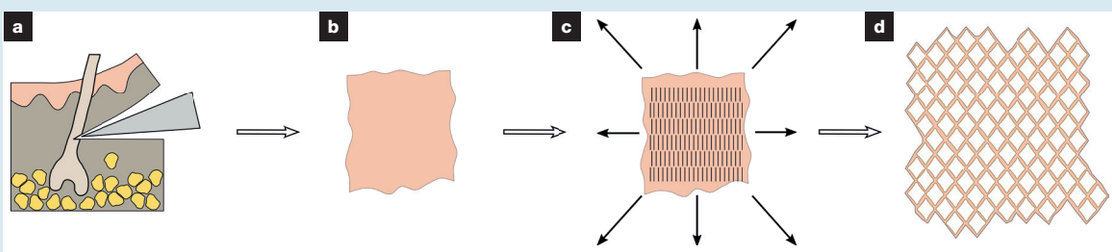
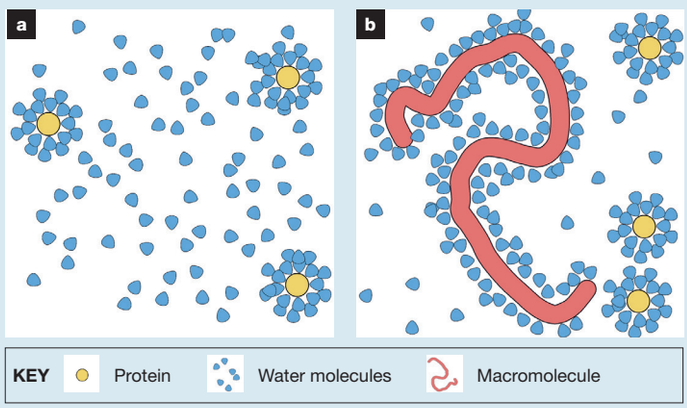


Fig 5. A simplified illustration of molecular crowding effects in wound fluid. Proteins or growth factors are solubilised in wound fluid. They interact with the surrounding water molecules and, in the near vicinity, water becomes attached to the protein like a coat. Electrostatic forces are the basis for the stability of the water 'coat'. This is shown in (**a**). Also, proteins and water 'coat' are randomly distributed and over time wander around in the solution (diffusion) maintaining a random distribution pattern. If large macromolecules are added to this solution (**b**), they also hydrate and create their water 'coat'. Due to their enormous size, they organise a large proportion of the volume with ordered structure. The 'free' water volume is decreased. As a consequence, growth factors or proteins become compartmentalised in smaller areas, they come closer together. Binding to the respective growth factor receptor becomes more likely or, in the case of chemical reactions, the efficiency of the reaction increases. It is important to realise that the number of growth factors or proteins has not increased in total, yet the biological effects are increased



area burns.[95–96] Starting with small biopsies of unaffected skin, keratinocytes can be expanded in tissue culture so that large areas of the body can be covered. The culturing step requires 2–3 weeks. The autologous keratinocytes are not rejected so that permanent wound closure is achieved. Use of cultured epidermal autografts was approved by the US Food and Drug Administration (FDA) in 2007 for use in deep dermal or full-thickness burns with more than 30% total body surface area burns.[97] Advantages over STSG are avoidance of painful donor sites, repeated transplantation starting from frozen keratinocyte stocks with high cost, variable graft take, and cosmetic outcomes were mentioned by some authors (reviewed in 98). This limited the use of cultured epidermal autografts to large total body surface area burn patients. Newer cell-based products try to circumvent the tissue culture expansion step and improve the delivery of cells to the wound. In one system, skin biopsies are incubated in an enzyme solution. Keratinocytes are released by gentle mechanical manipulation and delivered to the wound in a spray. The yield and quality of the isolated cells were analysed in a recent study. Per cm^2, an average of 1.7×10^6 of cells with a viability of 75.5% was obtained. The suspension contained keratinocytes, fibroblasts, and melanocytes. Colony forming efficiency was 0.3% of viable cells, corresponding to approximately 3825 cells with colony forming potential per $1cm^2$ of donor skin.[99]

For chronic wounds, a slightly different approach was explored. The use of ready to use allogeneic cell transplants had been investigated. There are two major product classes in clinical use: bilayered living skin constructs consisting of a differentiated epidermis, and collagen lattice-embedded fibroblasts in the dermal compartment.[100–101] Recently, a genomic approach was used in a first clinical trial to study its mechanisms of action that revealed multipronged effects of therapeutic reprogramming that shifts a chronic wound into an acute wound by modulating inflammatory response, signalling of growth factors, keratinocyte activation and attenuation of Wnt/β-catenin signalling.[102] The second consists of fibroblasts embedded in their own matrix and supported by a bioresorbable carrier.[103] The original concept was published in 1993.[104] These cell-based therapies are FDA-approved and marketed in the US. They can improve healing of selected wounds, with VLUs and DFUs being the most common indications.

Another allogeneic cellular graft technology had been pursued with HP802-247. Neonatal human keratinocytes and fibroblasts were expanded in tissue culture, mixed in a defined ratio, growth arrested by low-dose gamma irradiation (80Gy) and cryopreserved.[105] Delivery was through spray application of the thawed cells in a fibrin matrix. This approach reflects earlier observations that keratinocyte grafts enhance endogenous wound keratinocyte migration from the wound border or skin appendages, independent of take rates. An initial multicentre, randomised controlled, dose-finding study demonstrated promising outcomes.[106] Yet, the following phase III study showed no significance to the control arm[107] and differences in the production process.[107] Patient baseline characteristics may underlie the incongruent study outcomes.[108] The relative simplicity of this approach would have provided an attractive therapeutic option for hard-to-heal wounds not responding to any other form of treatment.

Detailed analysis revealed that skin substitutes containing both keratinocytes and fibroblasts secrete TNFα, IL-1α, IL-6, CCL2, CXCL1, CXCL8, sST2, CCL5, HGF, VEGF and TIMP-2.[109] This involved paracrine loops between epithelial and mesenchymal cells originally identified in co-culture models.[110,111] On the other hand, fibroblast cultures or keratinocyte culture transplants can secrete some of this spectrum.

Novel dressing-based concepts

From a clinical perspective, how many more patients with hard-to-heal wounds could benefit from more advanced technologies in daily practice? Compared with the large number of patients with complex wounds it is obvious that only a minor fraction has access to the therapeutic options mentioned above, particularly in the outpatient and nursing home setting.

When looking at standard dressing materials many only control the moisture of the wound. There are

several concepts on how to deliver growth factors through advanced dressings, yet none of these prototypes have been made available for clinical use.

A novel, technically more modest approach emerged in the last few years. It aims at boosting endogenously-expressed growth factors once wound bed preparation has been achieved and the granulation tissue has formed. If the bioactivity of growth factors at this stage could be enhanced, epithelialisation would be stimulated.

One option to do this works through a process called macromolecular crowding. In essence, macromolecular crowding is about a reduction of free water molecules in a solution. Protein molecules in solution are coated with layers of water molecules around them. The inner layers are tighter, the outer layers less tightly associated. The whole complex can diffuse in the solution, protein plus water mantle. If large macromolecules are added to this solution they will bind very large amounts of free water. As a consequence, the amount of free water (water not associated with either protein or macromolecule) decreases. While the actual concentration of proteins (molecules per volume) does not change, diffusion and chemical reactions become faster and more efficient,[112] and biological processes take place more efficiently, for example, connective tissue loaded with hydrated macromolecules such as collagen and hyaluronan. Enzymatic processing and the deposition of new collagen molecules are highly efficient in connective tissue while very inefficient in tissue culture where most of the water is free, not bound to macromolecules. Adding certain macromolecules to tissue cultures of fibroblasts mimics the macromolecular crowding effect of the connective tissue and collagen deposition increases dramatically.[113]

This mechanism can also be used in dressings. Recently, we could show that hydrated polyurethanes absorb fluid in a selective manner. Water is preferentially absorbed while larger molecules, such as proteins, are excluded and remain in solution. Their concentration in the remaining solution increases and biological effects of, for example, growth factors increase. Testing these effects on HGF and epithelialisation, the concentration was increased almost three-fold and re-epithelialisation *in vitro* increased by 2.6-fold.[114,115] Epithelialisation in porcine split-thickness donor site wounds increased by more than 20% versus an inert silicone interface dressing (data presented at the EWMA 2016, Bremen, Germany).

Conclusion

Epithelialisation in the wound healing process depends on many cues from the surrounding microenvironment. This involves interactions between many cell populations, growth factors and ECM-derived signals. In most wounds, epithelialisation proceeds well but is vulnerable to multiple interferences. The clinical approach tries to prevent any negative influence on epithelialisation, such as exsiccation or infection. If epithelialisation is impaired, surgical procedures such as STSG are the most often used interventions. Cell therapies provide a source of sustained release of wound healing stimulating signals that promote epithelialisation. Recombinant growth factor application to speed wound closure is only available in the US for DFUs in the form of a PDGF BB-containing gel. In terms of wound dressings, there is a lack of specialised technologies to speed epidermal wound closure. Novel concepts, such as increasing the biological effectiveness of endogenous growth factors, may help facilitate epithelialisation and improve clinical outcomes. **JWC**

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