Immunomodulation as rescue for chronic atonic skin wounds

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Abstract

In aging populations chronic skin wounds constitute increasing medical problems. Irrespectively whether these are caused by arterial or venous insufficiency or by physical pressure, granulation tissue of wet wounds yields better healing than atonic chronic wounds. Recently, prominent roles in the immunopathology of chronic wounds were attributed to specific cytokines, chemokines, matrix metalloproteinases and their substrates. Often it is uncertain whether their presence is cause or effect of wound pathology. At the cellular level, neutrophils and macrophages provide the necessary defense against infections, initiate angiogenesis and prepare tissue reconstitution orchestrated by resident and progenitor cells. We here review the current status and include the concept that, aside from surgery and stem cell therapy, healing may be enhanced by immunomodulating agents.

Chronic wounds: time does not heal

Traumatic, infected and even surgical wounds in humans heal spontaneously by innate mechanisms. Four hallmarks of normal wound healing are essential to consider, because these are helpful to understand the ongoing processes, to unravel healing in molecular terms, to define pathological healing and to intervene medically with optimal treatments. These hallmarks include sequentially: (i) blood coagulation (hemostasis), (ii) inflammation with removal of dead cells and debris by phagocytosis, (iii) formation of granulation tissue, rich in new blood vessels and, finally, (iv) fibroblast and epithelial cell proliferation for wound closure. In general, these mechanisms are quite similar for most tissues [1], but here we will restrict the issue to skin wounds. The sequence of normal wound healing is a dynamic process with gradual transitions from one stage to another. However, deficient control or exaggerated reactions may lead to problems with wound healing. An important example of deficiency that will be elaborated here is insufficient blood supply, this represents a typical endogenous host factor leading to various forms of cell death and defective wound healing. Common exogenous factors are infections, which provoke innate and adaptive immune reactions superimposed on the healing process and may cause collateral damage.

The cellular and molecular players of normal skin wound healing have been well studied [1,2] and, over a period of about two weeks, assist in repair of all skin layers in small-sized and disinfected wounds. These players include leukocytes, epithelial and endothelial cells and fibroblasts as cellular components, whereas cytokines, chemokines and proteases regulate extracellular matrix (ECM) remodeling and fibrosis at the molecular level. Within the ECM, a dynamic interplay between signaling molecules (cytokines and chemokines) and enzymes (proteases and other modifying enzymes) regulates the on/off switching of the four hallmarks of wound healing. For example, chemokines are induced by cytokines and other alarm signals in skin wounds, may be activated and controlled by various enzymatic posttranslational modifications and, at a later stage, destroyed by local proteases [3]. Therefore, discoveries in the cytokine [4], chemokine [5] and protease [6] fields not only are key elements to understand the immunobiology of acute wound healing, but these may be applied in a useful way for the immunomodulation and healing of chronic wounds.

Chronic atonic skin wounds do not heal spontaneously, are known for centuries and remain a major healthcare problem in elderly subjects. Chronic wounds lead to much patient suffering, come at high health care costs for all societies and are characterized by a notorious absence of solid basic research. Thankfully, this situation is changing by recent efforts by both fundamental and clinical researchers (Box). A key point is the diversification of chronic wounds into three subcategories based on etiology: arterial, venous and pressure ulcers. These three subcategories of chronic wounds are all driven by necrosis and insufficient removal of dead tissue.

In aging populations, the effects of slowly progressing multifactorial diseases, including atherosclerosis and diabetes become more evident and constitute a first etiological source of chronic wounds. These common metabolic diseases lead to progressive narrowing at the arterial side of the circulation (Figure 1a). With reduced downstream circulation in large atherosclerotic arteries, called *macro-angiopathy*, the skin tissue may become insufficiently irrigated with blood and may die [7]. With modern imaging techniques the absence of arterial blood circulation may be easily visualized in the skin [8]. In the case of diabetes, ulcers develop by additional thickening of the basement membrane of small capillaries (*micro-angiopathy*), which further hampers

exchanges of oxygen and nutrients into the skin layers. In addition, the peripheral nerves are damaged by this process, leading to local sensory loss, higher susceptibility to trauma, burns and wound infections. A typical example of such chronic arterial wound is called the diabetic foot [9] (Figure 1a).

A second common cause of chronic skin wounds are venous leg ulcers [10]. In this case, the pathogenesis is different (Figure 1b). As the name suggests, these lesions are caused by venous insufficiencies, most commonly by leakage of the venous and lymphatic valves. These valves ensure a centripetal blood flow into the large vein (*vena cava*) by passive pumping, driven by leg muscle action. During muscle relaxation, under normal conditions blood tries to fall down to the feet, but is retained by the function of venous vessel valves. However, when these valves fail in venous leg ulcer patients in upright position, the local venous hydrostatic blood pressure is increased, causing tissue edema (Figure 1b), vascular leakage and blood stasis. Eventually, this situation leads to superficial or deep vein thrombophlebitis. The classical picture is a swollen ankle and the involved skin may show signs of severe inflammation and may ultimately die. [10].

A third cause of chronic wounds is external pressure causing the skin circulation to be locally stopped, e.g. by pressure between bone and a solid surface [11,12]. These are commonly called pressure ulcers or bed sores. Such chronic wounds may be caused by paralysis, typically observed in young patients with spinal cord lesions. However, this disease occurs most commonly in elderly, who sit too long in the same position in a wheelchair or lay for too long time immobilized in bed and “forget” to move. This type of chronic skin wounds happens in both over-weighted and underweighted patients. Common factors that determine the initiation and progression of this type of skin wounds are extrinsic (e.g. skin pressure, moisture, shear stress, body posture) or intrinsic (e.g. immobility, sensory loss, high age, lowered consciousness, malnutrition). (Figure 1c). In all three pathogenic mechanisms (arterial, venous or pressure) and in sharp contrast with small acute skin lesions, including first and second degree burns, the paradigm is that these chronic skin wounds do not heal but instead worsen with time.

Preventive measures are always more cost-efficient than curative ones and differ depending on the three causes of chronic skin wounds. Prevention of atherosclerosis and diabetes

complications may be by healthy food, weight control, sufficient physical activity and medication [9]. Patients, susceptible to develop venous leg ulcers or after healing of such sores may wear graded compression stockings [10]. The prevention of pressure ulcers is by multiple daily controls of immobilized patients and relief of pressure.

Once a lesion occurs or develops, wound care is critical. We here review what is presently known about the immunobiology of chronic skin wounds (Figure 2) and illustrate three aspects of molecular interventions: controlled extracellular remodeling by immune cells and molecules, glycobiological aspects of the immune system and how to deal with immune molecules in a pharmacological approach. We will not address the important present-day approaches of (stem) cell-based therapies, which are included in or the subject of other reviews [13, 14, 15], but instead we will illustrate the importance of basic and applied molecular immunology research in this area towards better future treatments.

Chronic skin wounds

Although the medical problem of atonic skin wounds is well recognized and surgeons and wound care nurses reach medical wonders, occasional glimpses of success may not be generalized yet. One major reason is a lack of insight into the complex immunobiology and the variations of host genotypes and phenotypes. Furthermore, it is increasingly important to precisely define both the type of wound, and the stage at which the wound healing is arrested, to understand the pathology in a given subject.

In first instance, chronic skin wounds need to be related to the above mentioned three pathogenetic classes, albeit already for the reason of different ways of prevention and therapy. Beneficial and detrimental immune cells in each class of chronic wounds, as well as cytokines, chemokines and enzymes which improve or delay chronic wound healing constitute essential immunological parameters for wound staging and may become targets to inhibit or factors to be substituted for better, faster and permanent healing. It is important to understand that specific cell types or molecules are beneficial in one class or phase and detrimental in other ones.

Secondly, if a normal/acute skin healing process may be co-determined by host factors, it is logical to relate also differences in chronic skin wounds to the genetic background of the host.

This aspect has been investigated mainly in monogenetic diseases with skin involvement, in which structural or functional molecules define the pathology. Recent literature examples illustrate this aspect. Junctional epidermolysis bullosa in humans is caused by defects in a heterotrimeric ECM protein, named laminin-332. Patients with this skin fragility develop chronic wounds leading to skin cancer. These patients can now be cured by autologous transgenic keratinocytes encoding the wild type laminin [16]. Once wound healing is started, a host with a specific genetic constitution may be more susceptible to develop exaggerated reactions, for instance extreme skin fibrosis, called keloid [17]. Similarly, genetic differences in immune molecules, including cytokines, receptors and signal transducers may influence the inflammation or the granulation phases of wound healing. As a consequence and in analogy with other types of chronic inflammations, e.g. autoimmune diseases, sarcoidosis and amyloidosis, chronic wounds may thus be viewed as multifactorial diseases in which genetic, environmental (microbiota, *vide infra*) and host immune factors act together. The relative contribution of each of these determinants may be quite different. Illustrations of possible genetically determined factors, linking the pathology of chronic skin wounds with inflammation induction (*vide supra*) and operating in a multifactorial context are the transcription factor FOXC2 and the gap junctional protein (GJC2). Defects in such functional or structural molecules may lead to shortened vessel valve leafs [18] and carriers of these genetic defects may be more susceptible to develop venous leg ulcers. With rather simple present-day genetic tests (e. g. PCR analysis) it is possible to trace susceptible subjects. With the development of unbiased broad-spectrum genome analysis and a good understanding of genetic networking, it will become possible to determine dominant immune molecules in chronic wound healing. In approaches of preventive medicine it will be possible to determine better who is genetically more prone to develop chronic skin wounds. In addition, basic immunology research in this area will define immune molecules to target or to substitute for better wound healing.

Time phasing is the fourth dimension in chronic wound healing. Normal wound healing (Figure 2) develops over a time interval of about 2-3 weeks, although the following tissue-remodeling may proceed thereafter for years. Chronic wounds develop into pathologies with different colors (black, yellow, red)(Figure 3), moisture (wet, dry) and odors (often based on the infection). These

characteristics represent specific phases within the dynamic (non-)healing process. In simple phrasing, atonic chronic skin wounds may remain arrested within one phase with specific colonization of microbiota, limited blood supply and even external care (which can be wet or dry). The recognition of the phase will also determine the correct treatment. For instance, in the case of infection with a pathogenic bacterium, correct disinfection and antibiotic treatment are imperative. When dead tissue containing red blood cells dries out, a black crust is formed. Persistence of such material provokes a similar innate immune reaction as the triggering by a (sterile) foreign body. After a few weeks, macrophages, which can not cope with the removal of the material, will start to fuse into multinucleated giant cells and numerous lymphocytes will enter the site only slowly, because these cells are less equipped for active cell movement. This immune reaction is called a granulomatous reaction or cold abscess, to discriminate it from any acute inflammation with dilated blood vessels, warm and reddish skin and fast neutrophil infiltrations. The treatment of granulomatous chronic skin wounds starts with surgical removal of dead tissues, called “*débridement*”. By this procedure, an open wound is created and the cold abscess is turned into an acute inflammation in which nociceptive nerve endings are triggered, necessitating pain treatment and antimicrobial therapy. It is less obvious, whether and which specific inflammatory mediators, such as cytokines, chemokines or matrix metalloproteinases (MMPs) need to be blocked or induced. Indeed, the inhibition of a specific MMP may be beneficial in one phase, whereas such treatment may be detrimental in another phase of the healing process (*vide infra*). Having good immunological markers and knowing which cytokines, chemokines, immune proteases are to be substituted or which immune receptors and signaling cascades are to be inhibited for each phase in arterial, venous or pressure ulcers is a real challenge today. Here we provide the status of molecular immunology and define research opportunities for basic and clinical immunologists to obtain better insights before double-blind and controlled clinical trials are started.

Ingredients for healing chronic wounds

Enzymatic digestion, glycobiological aspects and pharmacological immunomodulating agents are all important ingredients within the setting of chronic wound healing.

1. *Controlled enzymatic extracellular remodeling*

The surgical approach of “*débridement*” is intended to remove detrimental dead tissue. Like a sterile foreign body causes direct tissue damage and interferes with normal functions, dead tissue in skin wounds induces similar granulomatous reactions. In such wounds, all normal skin functions, in particular the immunological barrier activity, are disturbed and infections become imminent and will persist. It needs to be well recognized that (i) chronic wounds are in fact tertiary lymphoid tissues resembling granulomatous reactions against late removal of skin suture material and that (ii) granulomatous diseases – the so-called cold abscesses - constitute the opposite of acute inflammations and do not heal, as long as the (sterile) debris is not removed. Finding pathogenic molecules of granulomas therefore seems essential in finding new solutions for chronic wound healing. One important cause and maintaining factor of granulomas is matrix metalloproteinase-9 (MMP-9) or gelatinase B [19] (*vide infra*). In chronic skin wounds MMP-9 seems a maintaining factor and therefore detrimental. However, this molecule has many other functions [20] and in acute inflammation of the skin specific functions of MMP-9, for instance its angiogenic potency, may even be beneficial (*vide infra*). This illustrates that for skin wound healing, like for other chronic diseases [21], MMPs are detrimental or beneficial depending on the phase of the disease or healing process.

Atonic skin wounds may be characterized at the molecular and cellular levels as the absence of the four mechanisms that govern acute inflammation: vasodilation with increased blood flow, recruitment of polymorphonuclear phagocytes, antithrombosis and increased immune surveillance (Figure 2). Acute inflammation is governed by the cytokine interleukin-1 (IL-1), which provides a primary signal for the acute phase reaction, for increased blood flow, systemic bone marrow activation, induction of interferons against viruses and of multiple other antimicrobial actions [22]. IL-1 also induces chemotactic cytokines, designated chemokines, for granulocytes [5] and monocytes [23], which, in turn, affect the production of MMPs. The enzymes that govern matrix remodeling in acute wound healing are, in the first place, matrix metalloproteinases and serine proteases including plasminogen activators and complement enzymes. These enzymes are

interconnected in a network and play different roles in skin healing processes [24, 25], including angiogenesis, re-epithelialization and even fibrosis. Aside IL-1, other inflammatory cytokines in chronic wounds include IL-6, colony stimulating factors (CSF), tumor necrosis factor (TNF) family members as well as IL-8 and related chemokines. These cytokines and chemokines are important biomarkers and immunomodulators in wound healing [25, 26] and regulate within the ECM the proteinase balances between enzymes and enzyme inhibitors [6, 21].

The cytokine-chemokine-proteinase axis is rather complex [3]. Consequently, before turning specific elements of this system into therapies, a good understanding of all molecules, their appearance and disappearance in time, their interconnections and their relative importance or “network hierarchy” need to be charted and understood. This becomes possible with the present unbiased approaches of research. For example, the mentioned regulated proteinases may potentiate [27], destroy or convert chemokines into chemokine receptor antagonists [28]. This implies that to develop drugs for chronic skin wound healing, the relative contributions and interconnections between these classes of mediators need to be well understood before individual molecules are clinically investigated or practically used. Therefore, we briefly explain these interactions.

MMPs possess many functions, from matrix wreckers, over immunomodulating proteases to signaling molecules [3, 21, 27-29]. The role of interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), collagenase 3 (MMP-13) and membrane-type 1 MMP (MMP-14), of the gelatinases A (MMP-2) and B (MMP-9) in skin re-epithelialization and resolution of scar tissue in acute wound healing have been reviewed previously [30]. MMP-1 at the wound edge results in lowering the affinity between $\alpha_1\beta_2$ integrin and collagen type 1 and thus enables keratinocytes to migrate from the wound edge to close the hole. It is established that for wound healing a whole set of interactions between stem cells, growth factors and scaffolds is needed [31], with MMPs also being involved. Once scar tissue is formed, collagenases including MMP-8 from neutrophils supposedly help in the removal of excess collagen. The role of MMP-2 and MMP-9 is even less clear, although

these gelatinolytic enzymes further degrade dermal collagen, after execution of a single cut in collagen by one of the collagenases (MMP-1, MMP-8, MMP-13 or MMP-14). So far, most wound studies are only correlative [30, 31] and a minority of these are based on functional research in MMP knockout mice. Moreover, rather than explaining healing of chronic skin wounds and indicating which MMPs are good or bad players, most of the gene knockout studies deal with acute inflammation phenotypes. For instance, impaired, respectively prolonged, skin-contact hypersensitivity was observed in MMP-3^{-/-} and MMP-9^{-/-} animals. Lower epithelial cell apoptosis and reduced transepithelial neutrophil migration was found in MMP-7^{-/-} mice, whereas decreased inflammatory cell invasion into skin and less efficient bacterial clearance was noticed in MMP-9^{-/-} animals and reduced matrix degradation after subcutaneous implantation of macrophages was seen in MMP-12^{-/-} mice [reviewed in ref 6]. When an acute skin disease does not heal, it will become chronic and eventually cancerous, as outlined above for junctional epidermolysis bullosa involving laminin-332 defects. Bullous pemphigoid is an example of a chronic non-healing autoimmune disease of the skin. In an experimental mouse model of bullous pemphigoid, neutrophil-derived gelatinase B/MMP-9 was pathogenic and MMP-9^{-/-} mice did not develop the blistering phenotype [32]. Delayed wound closure was observed in mice deficient in neutrophil collagenase/MMP-8. During the first days after wounding neutrophil infiltration was delayed and at later time points a persistent inflammation was observed [33]. This last example illustrates further the important role played by neutrophils in skin wound healing and that with good experimental animal models, including knockout mice, critical molecules may be identified as anchors or arrows for drug development programs (see below).

MMPs are produced as inactive zymogen forms and are tightly controlled by the tissue inhibitors of metalloproteinases (TIMPs) and α_2 -macroglobulin in the circulation. How these enzymes become activated in the skin and whether they are active is not known for most MMPs and remains a matter of speculation for wounds. A breakthrough in the field of chronic wound healing was reached once the active forms of the MMPs could be studied thanks to the development of an inhibitor-tethered resin that exclusively binds

active MMPs [34]. MMP-9 was found to be upregulated in and constituted an excellent marker of diabetic wounds. MMP-8 was detected in wounds of both diabetics and non-diabetics [35]. Furthermore, wound healing was accelerated in a mouse model of diabetes by inhibition of MMP-9 (and MMP-14) and by treatment with exogenous MMP-8. A combined treatment with MMP-8 and with an MMP-9 inhibitor yielded additive results. This study was not only an eye-opener, it also illustrated that novel therapies based on pharmacological MMP inhibition and exogenous enzyme substitution are possible (*vide infra*)[35-36]. Similar studies on chronic wounds by venous insufficiency and pressure sores are urgently needed. Broad profiling of MMPs and enzyme activity tests and solid preclinical work will establish a clear view about detrimental and beneficial MMPs. Then, the best selective MMP inhibitors [6, 36] and the ideal glycoforms of recombinant enzymes may be combined for the treatment of specific chronic wounds. Such rigorous scientific approach will avoid the failures observed with MMP inhibitors in cancer research [6, 37].

An exploratory study was recently published about venous leg ulcers [38]. In this study, the levels of 9 MMPs and the four human tissue inhibitors of metalloproteinases (TIMPs) in wound exudates from patients were analyzed with the use of multiplex ELISA and inflammatory and granulating wound fluids were compared. Furthermore, the fold-induction changes of these MMPs by inflammatory or granulating wound fluids were analyzed on a myelomonocytic cell line (THP-1). It was also verified whether Sulodexide, a glycosaminoglycan (GAG) mixture (*vide infra*), approved for the treatment of venous leg ulcers, had an influence on wound fluid-elicited MMP profile changes in THP-1 cell cultures *in vitro*. The levels of the collagenases MMP-1 and MMP-13 were found to be higher in granulating versus inflammatory wound fluids, whereas the amounts of MMP-2, MMP-9 and MMP-12 were significantly higher in inflamed versus granulating wound fluids of venous leg ulcer patients. TIMP-1 and TIMP-2 were high in inflammatory wound exudates, whereas TIMP-4 levels were higher in granulating wound fluids. When human myelomonocytic THP-1 cells were stimulated with inflammatory or granulating wound fluids, MMP-1, MMP-8 and MMP-9 levels were increased by inflammatory exudates,

whereas MMP-3, MMP-7, MMP-12 and MMP-13 expression levels were higher when the cells were stimulated with granulation exudates. Limited effects of Sulodexide *in vitro* were detected. This glycosaminoglycan mixture increased MMP-2 and decreased MMP-7 levels after stimulation with granulation wound fluids and it provoked a significant decrease of MMP-8 expression after stimulation with inflammatory wound fluids, respectively. This study [38] illustrates phase-specific differences in MMP levels and provides a screening method to obtain biochemical markers for different disease states and criteria to evaluate, in an objective way, clinical effects of treatments. Whether any of the analytes are cause of consequence of non-healing venous wounds is not yet known. However, this study also illustrates pharmacological effects of glycosaminoglycans on MMP modulation in a leukocytic cell line (*vide infra*). Unfortunately, these molecules and treatment options can not yet be investigated *in vivo*, because no animal model exists to test for the effect on chronic venous ulcers.

In another recent study [39], various molecular forms of MMP-9 were analyzed by ultrasensitive gelatin zymography [40] and the levels of all forms of this specific enzyme were upregulated in patients with pressure ulcers in comparison with control subjects. Genotyping of a specific single nucleotide polymorphism (SNP) in the MMP-9 gene promotor region (-1562 C->T), in which the T allele yields higher expression levels [41], illustrated a protective effect of the T allele. This study was also interesting because the changes in microbiota, which are gradually better understood to contribute to chronic inflammation [21], changed with time of skin lesion evolution. Gradually Gram-positive cocci in “young” pressure wounds were replaced by Gram-negative enterobacteriaceae [39].

2. Glycobiological aspects of wound healing

As named above, typical proteins of skin ECM include collagens, laminins and proteoglycans, all these being glycoprotein substrates of MMPs. Although their proteolytic remodeling by MMPs has been studied *in vitro*, glycobiological aspects of ECM remodeling are less appreciated. For instance, the core proteins of proteoglycans are

proteolyzed by stromelysins (MMP-3, MMP-10 and MMP-11) and other MMPs (*vide supra*). Importantly, the proteoglycan glycosaminoglycans (GAGs) (which are not cleaved by MMPs, but instead by glycan-modifying enzymes) possess many immunomodulating functions [42,43], as already mentioned for the GAG mixture Sulodexide [38]. Glycan-based scaffolding and anchoring of bound mediators has been described for cytokines [44-45] and chemokines [3, 43], but how these glycans are modified by host and microbiota enzymes in chronic wounds is not known. It is easy to envisage that immunology research with the present sequencing and synthesis tools of glycobiology [46] will boost new developments in the field of healing of (acute inflammation and) chronic wounds. In a recent study, the binding of “excessive” chemokines to a GAG-based hydrogel was found to improve wound healing [47]. These practical examples [38, 47] and the knowledge about immunomodulating roles of GAGs [42-45] reinforce the idea that (glyco)targeting of the extracellular matrix is an innovative avenue for research, under the condition that beneficial *versus* detrimental effects are clearly delineated. Immunomodulation by GAGs for chronic wound healing deserves more basic research and good clinical studies.

3. *Pharmacological immunomodulation as hope for new treatment*

A considerable advantage for the development of new therapies for wound healing is the fact that topical application is possible. To enhance re-epithelialization and restore barrier functions, autologous skin transplantation is widely used and may now be extended with transgenic skin transplantation [16]. Within the focus area of the present review, many recent studies deal with growth promoting cytokines [48], including gel-based platelet-derived growth factor (PDGF), the only FDA-approved growth factor for chronic wound healing (Regranex®). Here, we specifically discuss immunomodulation by cytokines, chemokines and proteinases and provide a model how - with insights from discoveries in acute inflammation - a chronic wound may be turned into a healing acute wound. For treatment of diabetic foot an excellent review is available [49] and new insights [35-36] may in the future complement existing therapies for venous and pressure wounds.

MMPs, including MMP-9, are important immunomodulators [6, 30, 50, 51]. Topical use of proteases (e.g. collagenases) and their inhibitors mimics the natural process of normal wound healing (Figure 2) and may constitute a new form of pharmacological immunomodulation for enzymatic *débridement* and therapy against exaggerated fibrosis [35]. Such local skin treatment may be devoid of systemic side-effects. A second way of immunomodulation is local application of MMP inducers to ignite, for instance, endogenous collagenase expression. In principle, this may be accomplished with pro-inflammatory IL-1, although systemic acute phase induction and fever might develop if levels of this cytokine are slightly increased in the circulation [22, 52]. A more clever approach might be with chemokines, such as IL-8, the major neutrophil chemoattractant in humans [5, 53]. Indeed, this chemokine binds locally to GAGs [3, 43] and recruits [5] neutrophils. By the activation of neutrophils, both neutrophil collagenase (MMP-8) [35] and all forms of pro-angiogenic inhibitor-free MMP-9 [54, 55] are provided. In the past, vascular endothelial growth factor (VEGF) has been tested to enhance angiogenesis in wound healing [15]. The beneficial effects of VEGF are probably also mediated via neutrophils [56].

The prescriptions for better chronic wound healing protocols may now be inspired by the general picture of the normal healing phases and of the molecular players involved (Figure 2). The sequence how nature copes with this process is understood and one can try to mimic some of these aspects to break out of the locked chronic conditions. It is obvious that novel immunomodulating pharmacological compounds will gain a place aside present-day cell-based therapies. For instance, once the phase of sufficient vascularization is reached, protease activities for angiogenesis need to be stopped [57]. At this stage, subsets of macrophages arrive and replace the neutrophils [58-59] for clearance of cell debris [60-62]. In small wounds this natural “*débridement*” will be sufficient. In large and chronic wounds, the helping hand of the surgeon will be needed to remove dead tissue, otherwise granulomatous reactions with fusions of macrophages into giant cells surrounded by abundant lymphocytes will develop tertiary lymphoid tissue (*vide supra*).

To establish better protocols for wound healing, drug-based modulation of the cellular players may constitute additional options. The phase-dependent occurrence of cells in a normal skin wound healing process (Figure 2) may be mimicked for chronic wound healing by specific and rightly timed pharmacological substances. Unfortunately, disease- and phase-specific leukocyte types and components are not yet well described for chronic wounds. Technically, it is possible with the use of depletion experiments to define whether the presence of a particular cell type is cause or consequence or is beneficial or detrimental. Although out of the scope of this review, it is clear that before cell-based therapy protocols are clinically studied, preclinical investigations with good animal models are needed to enhance progress.

A need for better animal models

“If there is a wound, we must try to heal it” constitutes a holistic approach for all medicine and is certainly applicable in the context of the present review. Current cutting-edge research on chronic wounds in humans is the unbiased “omics” analysis of the composition of wound edges [24,25]. The former manuscript by auf dem Keller and colleagues at the University of British Columbia in Vancouver, Canada, is exemplary for future work about chronic skin wounds. The authors quantified the proteome and proteolytic events (degradome) of normal mouse skin with that of animals in which acute inflammation was induced for 48 h by phorbol ester painting on shaved skin. With this approach, systems-level quanta of data were generated and these included relative abundancies of proteins and qualitative information about most posttranslational modifications. This study forms a solid basis for all future comparisons. For instance, about half of the proteins in normal skin are constantly turned-over by proteases and this proportion further increases after skin inflammation. Novel cleavage sites were discovered in chemokines and complement molecules. With the use of MMP-2^{-/-} mice, it was found that MMP-2 inactivates the complement C1 inhibitor, leading to increased complement activation and bradykinin generation and, hence, inflammation. The fact that elimination of a single protease enhances inflammation has implications for the use of selective and broad spectrum protease inhibitors in wound healing.

In a follow-up study at the ETH in Zurich, Sabino and co-workers have extended systemic protein profiling of deep “full thickness” wounds (with 3 cm diameter) in pig skin tissues. Wound fluids were collected into sterile foam wound dressings at 1 and 3 days and from 4 till 7 days. In this way, an unbiased and complete view about the proteome evolution during the first week of normal porcine skin healing has been obtained (see Figure 2). Consequently, this study yielded information about molecules in hemostasis, the early inflammation (day 1) and granulation phase (day 3) and the proliferation and early remodeling phases (days 4-7) (Figure 2). For instance, in the early phase inflammation (day 1) remnant proteins from red blood cells, complement C3, von Willebrand factor and molecules for detoxification of reactive oxygen species were detected. From 3 to 7 days, the detection of another cluster of proteins, including ECM collagen and keratins indicated dermal fibroblast activity and the onset of re-epithelialization. With dedicated proteomics technology, the presence and activity of secreted serine proteases, e.g. of the complement and coagulation systems, and of metalloproteinases MMP-1, MMP-2, MMP-8 and MMP-9 were detected and complemented with the presence of intracellular caspases between days 3 and 7 (Figure 2) [25].

It is thus technically feasible to define in wound exudates all proteins, including complement molecules, cytokines and chemokines and to define at once their proteolytic processing. These systems biology approaches with mouse [24] and porcine skin [25] are the references to benchmark similar data sets from human skin wound healing. It will also be possible with this technology to define the molecules that are upregulated or defective in specific phases of chronic skin wounds. Next, the molecules that are upregulated in chronic wounds need to be classified as the cause or result of non-healing. In the case of proteins, causative factors can be defined with the use of knockout mouse models [24, 33, 63], under the condition of extensive control of genetic and environmental factors [64]. Causal factors, different from (glyco)proteins, may be discovered and studied with the use of specific antagonists and defective factors may be substituted [33]. All these approaches represent steps forward in wound healing research. First-generation descriptive proteome studies are already available for venous [65], arterial [66] and pressure ulcers [67] in humans. In a comparison of normal healing with chronic venous wounds, Eming and colleagues detected biomarker signatures in human wound exudates. The nonhealing

exudates showed a picture of persistent inflammatory and tissue destructive molecules, including fibrinogen chains, α_2 -heparan sulphate-glycoprotein-2 and MMP-9, as well as neutrophil markers (lactotransferrin, myeloperoxidase and S100 calcium binding proteins as antimicrobials and annexin A1 from neutrophil apoptosis), but not annexin A5 [65]. Krisp and co-workers used filtered human arterial wound exudates. In their study, inflammatory response S100 proteins and MMPs were also found to be increased in diabetic foot exudates and the presence of antiangiogenic molecule suggested inhibition of the granulation phase and increased cell death, demonstrated by high levels of annexin A5, a marker for late apoptotic cells [66]. Third, in a joint effort, Italian and American investigators compared protein profiles in different focal tissue section areas (wound bed, adjacent dermis and hypertrophic epidermis) from 15 human pressure ulcers. Wound beds, adjacent dermis and hypertrophic epidermis contained as markers, respectively, antimicrobial defensin-6, thymosins/calcyclin, and calgranulins/calgizzarin [67]. Calcyclin, calgranulins and calgizzarin belong to the S100 protein family [67]. Of course, these data need to be corroborated in other studies of chronic stalled wound types from additional patients. They are also useful to be compared with animal studies and to define stage-specific, tissue-specific, pathogenetic and prognostic markers of healing.

The development of preclinical animal models remains a challenge, but is necessary to test new treatment options. Good animal models exist for normal wound healing in mice and pigs [24,25] of diabetic wound healing in mice [35] and for pressure ulcers in rats [68,69]. These models enable researchers to perform broad profiling for the definition of diagnostic, staging and prognostic markers, aside preclinical drug testing. Also weaknesses exist with the use of (large) animal models. First, wound contraction observed in many large mammals, does not exist in humans. Secondly, particular structural elements of the skin, such as body hair, sweat and sebaceous glands and pigmentation are different between humans and other mammals. These critical elements are known in the field and imply that drug testing is ideally performed in animals with similar skin histology and healing properties (over about 1 week) as observed in human skin. In this sense, pig skin [25] may have more similarities with human tissues than mouse or rat skin. We advocate that classical autologous transplantation of full-thickness skin tissue is a good surrogate for pressure ulcer healing research. Indeed, the skin graft is denervated and in need of

new blood circulation. The transplanted full thickness skin graft may even be physically pressed in a controlled way before transplantation. It remains an open question whether a small animal model for venous leg ulcers is possible.

Normal wound healing relies on a sequence of cellular and inherent molecular events, and one goal is to restart this stalled process as a way to initiate the healing of chronic skin wounds (Figure 3). Indeed, barrier formation with hemostasis, platelet aggregation and leukocyte influx and subsequent death of living cells first leads to the formation of a wound crust. In normal wound healing this dead tissue is undermined and rejected by normal skin underneath. In chronic wounds, these host processes fail and the dead debris needs to be removed by surgical debridement. This leads to acute suppurating inflammation and an influx of neutrophils (Figure 3), which provide antimicrobial defense and release angiogenic factors while dying. Different subsets of macrophages take over the organization of the wound during the granulation phase and initiate rebuilding the tissue through the release of signals that promote resolution [70, 71]. Analysis of the wound edges [72, 73] and exudates [25, 74] and comparisons with the use of all available “omics” technologies tell what happens in the debris, what is ongoing in the wound edges and what is needed for optimal healing. Such profiling studies will lead to novel diagnostics and will have therapeutic consequences.

Conclusions

Gradually it becomes understood that immunomodulators, such as cytokines, chemokines and MMPs, may become useful drugs or drug targets for the healing of common chronic wounds, such as venous leg ulcer and diabetic foot. Finding which protease is a target in which healing phase of chronic wounds is now technically doable with approaches which define tissue modifying enzymes and their substrates. For instance, MMP-9 is a beneficial neutrophil-derived molecule in the early inflammatory phase (first two days), but once granulation is ignited, it becomes a detrimental and maintenance factor for chronic wounds. Direct angiogenic approaches have failed so far, but in animal model studies treatment with topical proteases and protease inhibitors leads to faster healing of diabetic wounds. Substitution with pro-

inflammatory cytokines and chemokines may help to turn atonic granulomatous skin wounds into inflamed vessel-rich tissues, in a complementary way to surgical debridement. Topical treatment with GAGs also constitutes a promising approach. However, more research is necessary to define direct GAG effects on cells and indirect functional aspects of the binding of wound exudate components to topical GAGs. At the cellular level, neutrophil and macrophage subsets provide sequentially angiogenic and matrix remodeling molecules with important wound-healing properties. Similarly, chemokine modulation of skin wounds with GAG-containing wound-dressings illustrates that we are on the verge of finding solutions for chronic wound healing by immunomodulation. Present-day technologies of RNAseq and proteomics are used to profile normal and abnormal wound healing and with animal models it is possible to define useful immunomodulators and targets. The combination of efforts across disciplines and the replacement of case-reports by dedicated research in basic, preclinical and clinical molecular immunology will transform the field of chronic wound healing.

References

- [1] Aurora, A.B. and Olson, E.N. (2014) Immune modulation of stem cells and regeneration. *Cell Stem Cell* 15, 14-25.
- [2] Yildirimer, L. *et al.* (2012) Skin regeneration scaffolds: a multimodal bottom-up approach. *Trends Biotechnol* 30, 638-648.
- [3] Proost, P. *et al.* (2017) Chemokine isoforms and processing in inflammation and immunity. *J. Autoimmun.* 85, 45-57.
- [4] Vilcek, J. and Feldmann, M. (2004) Historical review: Cytokines as therapeutics and targets of therapeutics. *Trends Pharmacol. Sci.* 25, 201-209 (2004).
- [5] Van Damme, J. *et al.* (1988) A novel, NH₂-terminal sequence-characterized human monokine possessing neutrophil chemotactic, skin-reactive, and granulocytosis-promoting activity. *J. Exp. Med.* 167, 1364-1376.
- [6] Hu, J. *et al.* (2007) Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat. Rev. Drug Discov.* 6, 480-498.
- [7] Zhao, R. *et al.* (2016) Inflammation in chronic wounds. *Int. J. Mol. Sciences* 17, 2085.
- [8] Samies, J.H. *et al.* (2015) Use of a fluorescence angiography system in assessment of lower extremity ulcers in patients with peripheral arterial disease: a review and a look forward. *Sem. Vasc. Surg.* 28, 190-194.

- [9] Gibbons, G.W and Shaw, P.M. (2012) Diabetic vascular disease: characteristics of vascular disease unique to the diabetic patient. *Sem. Vasc. Surg.* 25, 89-92.
- [10] Alavi, A. *et al.* (2016) Wat's new: management of venous leg ulcers *J. Am. Acad. Dermatol.* 74: 643-664.
- [11] Kuffler, D.P. (2015) Improving the ability to eliminate wounds and pressure ulcers. *Wound Rep. Reg.* 23: 312-317.
- [12] Greenhalgh, D.G (2015) Management of the skin and soft tissue in the geriatric surgical patient. *Surg. Clin. N. Am.* 95: 103-114.
- [13] Bertozzi, N. *et al.* (2017) The biological and clinical basis for the use of adipose-derived stem cells in the field of wound healing. *Ann. Med. Surg. (Lond).* 20: 41-48.
- [14] Kirby, G.T. *et al.* (2015) Stem cells for cutaneous wound healing. *Biomed Res Int.* 2015: 285869, 11 pages.
- [15] Rennert, R.C. *et al.* (2013) Biological therapies for the treatment of cutaneous wounds: phase III and launched therapies. *Expert Opin. Biol. Ther.* 13: 1523-1541.
- [16] Hirsch, T. *et al.* (2017) Regeneration of the entire human epidermis using transgenic stem cells. *Nature* 551: 327-332.
- [17] Halim, A.S. *et al.* (2012) Keloid scarring: understanding the genetic basis, advances, and prospects. *Arch. Plast. Surg.* 39: 184-189.
- [18] Lyons, O. *et al.* (2017) Human venous valve disease caused by mutations in FOXC2 and GJC2. *J. Exp. Med.*, 214: 2437-2452.
- [19] Volkman, H.E., *et al.* (2010) Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. *Science* 327: 466-469.
- [20] Vandooren, J., *et al.* (2013) Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): the next decade. *Crit. Rev. Biochem. Mol. Biol.* 48, 222-272.
- [21] Opdenakker, G. *et al.* (2016) Microbiomic and posttranslational modifications as preludes to autoimmune diseases. *Trends Mol. Med.* 22, 746-757.
- [22] Van Damme, J. *et al.* (1985) Homogeneous interferon-inducing 22K factor is related to endogenous pyrogen and interleukin-1. *Nature* 314, 266-268.
- [23] Van Damme, J. *et al.* (1992) Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J. Exp. Med.* 176, 59-65.
- [24] auf den Keller U. *et al.* (2013) Systems-level analysis of proteolytic events in increased vascular permeability and complement activation in skin inflammation. *Sci. Sign.* 6(258):rs2.

- [25] Sabino, F., *et al.* (2015) In vivo assessment of protease dynamics in cutaneous wound healing by degradomics analysis of porcine wound exudates. *Mol. Cell. Proteom.* 14, 354-370.
- [26] Ligi, D., *et al.* (2016) Chronic venous disease - Part I: Inflammatory biomarkers in wound healing. *Biochim. Biophys. Acta.* 1862, 1964-1974.
- [27] Van den Steen, P.E. *et al.* (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO- α and leaves RANTES and MCP-2 intact. *Blood* 96, 2673-2681.
- [28] Proost, P. *et al.* (1998) Posttranslational modifications affect the activity of the human monocyte chemotactic proteins MCP-1 and MCP-2: identification of MCP-2(6-76) as a natural chemokine inhibitor. *J. Immunol.* 160, 4034-4041.
- [29] Garcia-Pardo, A. and Opdenakker, G (2015) Non-proteolytic functions of matrix metalloproteinases in pathology and insights for the development of novel therapeutic inhibitors. *Metalloproteinases in Med.* 2: 19-28.
- [30] Rohani, M.G. and Parks W.C. (2015) Matrix remodeling by MMPs during wound repair. *Matrix Biol.* 44-46, 113-121.
- [31] Futrega, K. *et al.* (2014) Threatening the whole not the hole: necessary coupling of technologies for diabetic foot ulcer treatment. *Trends Mol. Med.* 20, 137-142.
- [32] Liu, Z. *et al.* (1998) Gelatinase B-deficient mice are resistant to experimental bullous pemphigoid. *J. Exp. Med.* 188, 475-482.
- [33] Gutierrez-Fernandez, A. *et al.* (2007) Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *FASEB J.* 21, 2580-2591
- [34] Ikejiri *et al.* (2000) Potent mechanism-based inhibitors for matrix metalloproteinases. *J. Biol. Chem.* 280, 33992-334002.
- [35] Gao, M. *et al.* (2015) Acceleration of diabetic wound healing using a novel protease-anti-protease combination therapy. *Proc Natl Acad Sci U S A.* 112, 15226-15231.
- [36] Meisel, J.E. and Chang M. (2017) Selective small-molecule inhibitors as chemical tools to define the roles of matrix metalloproteinases in disease. *Biochim. Biophys. Acta.* 1864, 2001-2014.
- [37] Vandenbroucke, R.E and Libert, C. (2014) Is there new hope for therapeutic matrix metalloproteinase inhibition? *Nat. Rev. Drug Discov.* 13, 904-927.
- [38] Ligi, D. *et al.* (2016) Chronic venous disease - Part II: Proteolytic biomarkers in wound healing. *Biochim. Biophys. Acta* 1862, 1900-1908.

- [39] Latifa, K. *et al.* (2016) Evaluation of physiological risk factors, oxidant-antioxidant imbalance, proteolytic and genetic variations of matrix metalloproteinase-9 in patients with pressure ulcer. *Sci. Rep.* 6:29371.
- [40] Vandooren, J. *et al.* (2013) Zymography methods for visualizing hydrolytic enzymes. *Nat. Meth.* 3, 211-220.
- [41] Zhang, B. *et al.* (1999) Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. *Circulation* 99, 1788-1794.
- [42] Gulati, K. and Poluri, K.M. (2016) Mechanistic and therapeutic overview of glycosaminoglycans: the unsung heroes of biomolecular signaling. *Glycoconj. J.* 33:1-17.
- [43] Händel, T., *et al.* (2005) Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. *Annu. Rev. Biochem.* 74, 385-410.
- [44] Künze, G. *et al.* (2016) Identification of the glycosaminoglycan binding site of interleukin-10 by NMR spectroscopy. *J. Biol. Chem.* 291, 3100-3113.
- [45] Sebollela, A., *et al.* (2005) Heparin-binding sites in granulocyte-macrophage colony-stimulating factor. Localization and regulation by histidine ionization. *J. Biol. Chem.* 280, 31949-31956.
- [46] Zhang, P. *et al.* (2016) Challenges of glycosylation analysis and control: an integrated approach to producing optimal and consistent therapeutic drugs. *Drug Discov. Today* 21, 740-765.
- [47] Lohmann, N., *et al.* (2017) Glycosaminoglycan-based hydrogels capture inflammatory chemokines and rescue defective wound healing in mice. *Sci. Transl. Med.* 9(386) eaai9044.
- [48] Vranckx, J.J. *et al.* (2007) Cell suspension cultures of allogenic keratinocytes are efficient carriers for ex vivo gene transfer and accelerate the healing of full-thickness skin wounds by overexpression of human epidermal growth factor. *Wound Rep. Regen.* 15, 657-664
- [49] Baltzis, D., *et al.* (2014) Pathogenesis and treatment of impaired wound healing in diabetes mellitus: new insights. *Adv. Ther.* 31, 817-836.
- [50] Parks, W.C. *et al.* (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat. Rev. Immunol.* 4, 617-629.
- [51] Khokha, R. *et al.* (2013) Metalloproteinases and their natural inhibitors in inflammation and immunity. *Nat. Rev. Immunol.* 13, 649-665.
- [52] Dinarello, C.A. (1984) Interleukin-1. *Rev. Infect. Dis.* 6, 51-95.

- [53] Yoshimura, T. *et al.* (1987) Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. U S A.* 84, 9233-9237.
- [54] Ardi, V.C., *et al.* (2007) Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 104, 20262-20267.
- [55] Vandooren *et al.* (2015) Circular trimers of gelatinase B/matrix metalloproteinase-9 constitute a distinct population of functional enzyme molecules differentially regulated by tissue inhibitor of metalloproteinases-1. *Biochem. J.* 465, 259-270.
- [56] Christoffersson G. *et al.* (2012) VEGF-A recruits a proangiogenic MMP-9-delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue. *Blood.* 120, 4653-4662.
- [57] Serena, T.J. *et al.* (2016) Defining a new diagnostic assessment parameter for wound care: Elevated protease activity, an indicator of nonhealing, for targeted protease-modulating treatment. *Wound Repair. Regen.* 24, 589-595.
- [58] Phillipson, M. and Kubes, P. (2011) The neutrophil in vascular inflammation. *Nat. Med.* 17, 1381-1390.
- [59] Kolaczowska, E. and Kubes, P. (2013) Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13, 159-175 (2013).
- [60] Nicolas-Avila, J.A. *et al.* (2017) Neutrophils in homeostasis, immunity and cancer. *Immunity* 46: 15-28.
- [61] Wynn, T.A. and Vannella, K.M. (2014) Macrophages in tissue repair, regeneration, and fibrosis. *Immunity.* 44, 450-462.
- [62] McInnes R.L *et al.* (2013) Contrasting host immune-inflammatory responses to bacterial challenge within venous and diabetic ulcers. *Wound Rep. Regen.* 22, 58-69.
- [63] Bullard, K.M. *et al.* (1999) Impaired wound contraction in stromelysin-1-deficient mice. *Ann. Surg.* 2, 260-265.
- [64] de Bruyn, M. *et al.* (2017) Inhibition of gelatinase B/MMP-9 does not attenuate colitis in murine models of inflammatory bowel disease. *Nat. Commun.* 8: 15384.
- [65] Eming, S.A. *et al.* (2010) Differential proteomic analysis distinguishes tissue repair biomarker signatures in wound exudates obtained from normal healing and chronic wounds. *J. Proteome Res.* 9, 4748-4766.
- [66] Krisp, C. *et al.* (2013) Proteome analysis reveals antiangiogenic environments in chronic wounds of diabetes mellitus type 2 patients. *Proteomics.* 13, 2670-2681.

- [67] Taverna, D. *et al.* (2015) Imaging mass spectrometry for assessing cutaneous wound healing: analysis of pressure ulcers. *J. Proteome Res.* 14, 986-996.
- [68] Kurose, T. *et al.* (2015) Analysis of gene expression in experimental pressure ulcers in the rat with special reference to inflammatory cytokines. *PLoS One.* 10, e0132622.
- [69] Ahmed, A.K., *et al.* (2016) A non-invasive method to produce pressure ulcers of varying severity in a spinal cord-injured rat model. *Spinal Cord* 54, 1096-1104.
- [70] Anders, H.J. *et al.* (2013) Pathomechanisms: homeostatic chemokines in health, tissue regeneration, and progressive diseases. *Trends Mol. Med.* 20, 154-165.
- [71] Boniakowski, A.E. *et al.* (2017) Macrophage-mediated inflammation in normal and diabetic wound healing. *J. Immunol.* 199, 17-24.
- [72] Eriksson, E. and Vranckx, J. (2004) Wet wound healing: from laboratory to patients to gene therapy. *Am. J. Surg.* 188, 1A Suppl., 36-41.
- [73] Forbes, S.J. & Rosenthal, N. (2014) Preparing the ground for tissue regeneration: from mechanism to therapy. *Nat. Med.* 20, 857-869 (2014).
- [74] Thamm, O.C. *et al.* (2015) Acute and chronic wound fluids influence keratinocyte function differently. *Int. Wound J.* 12, 143-149.
- [75] Kroeze, K.L. *et al.* (2012) Simple wound exudate collection method identifies bioactive cytokines and chemokines in (arterio) venous ulcers. *Wound Rep. Regen.* 20, 294-303.
- [76] Ramsay S. *et al.* (2015) Wound samples: moving towards a standardized method of collection and analysis. *Int. Wound J.* 13, 880-891.
- [77] Serra, R. *et al.* (2016) Extracellular matrix assessment of infected chronic venous leg ulcers: role of metalloproteinases and inflammatory cytokines. *Int. Wound J.* 13, 53-58.
- [78] Filkor K. *et al.* (2015) The expression of inflammatory cytokines, TAM tyrosine kinase receptors and their ligands is upregulated in venous leg ulcer patients: a novel insight into chronic wound immunity. *Int. Wound J.* 13, 554-562.
- [79] Barrientos, S. *et al.* (2014) Clinical application of growth factors and cytokines in wound healing. *Wound Rep. Regen.* 22, 569-578.
- [80] Serra R. *et al.* (2015) Doxycycline speeds up healing of chronic venous ulcers. *Int. Wound J.* 12, 179-184.
- [81] Majtan, J. *et al.* (2013) Fir honeydew honey flavonoids inhibit TNF- α -induced MMP-9 expression in human keratinocytes: a new action of honey in wound healing. *Arch. Dermatol. Res.* 305, 619-627.

- [82] Fox, J.D. *et al.* (2016) Adalimumab treatments leads to reduction of tissue tumor necrosis factor-alpha correlated with venous leg ulcer improvement: a pilot study. *Int. Wound J.* 13, 963-966.
- [83] Barrois, B., *et al.* (2007) Efficacy and tolerability of hyaluronan (ialuset) in the treatment of pressure ulcers: a multicentre, non-randomised, pilot study. *Drugs R D* 8, 267-273.
- [84] Henshaw, F.R. (2014) Topical application of the bee hive protectant propolis is well tolerated and improves human diabetic foot ulcer healing in a prospective feasibility study. *J. Diab. Compl.* 28, 850-857.
- [85] Bucekova, M. *et al.* (2017) Bee-derived antibacterial peptide, defensin-1, promotes wound re-epithelialisation in vitro and in vivo. *Sci. Rep.* 7, 7340.
- [86]. Krishnaswamy V.R. *et al.* (2017) Matrix metalloproteinases: the sculptors of chronic cutaneous wounds. *Biochim. Biophys. Acta.* 1864, 2220-2227.

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Box 1: Immunobiology of chronic skin wounds: 10 commandments

- A clear distinction between 3 pathogenic entities of chronic wounds is critical: arterial wounds *versus* venous leg ulcers *versus* pressure sores.
- For these three distinctive entities, phase-specific and grade-specific immunological and biochemical markers are urgently needed for correct diagnosis and prognosis and for critical evaluation of existing and novel therapies [62,75-76].

- Present-day technologies for broad-spectrum molecular and cellular profilings [24, 25, 57, 75] and for non-invasive imaging [8] need to be applied to obtain an unbiased view of useful analytes, including MMPs, TIMPs, cytokines and chemokines [24-26, 38, 76-79].
- More and better preclinical (mouse) models are needed. Models of pressure ulcers [68,69] and of diabetic wounds [35] exist already and these need to be complemented with models of skin transplantation after denervation. The contribution of and modulation by microbiota to impairment of wound healing needs to be studied carefully, as micro-organisms generate toxins and may induce or deliver cytokine, chemokine or protease activities.
- Glycosaminoglycan (GAG) research is expanding towards the development of wound dressings with biological and biochemical properties, which enhance wound repair [38, 47].
- Cytokines, in particular growth factors (Platelet-derived growth factor/PDGF, epidermal growth factor/EGF, vascular endothelial growth factor/VEGF, placental growth factor/PIGF) have been studied for healing of chronic wounds [15], but with the exception of PDGF, more and better controlled studies are needed to enhance the phase-specific and beneficial use of such immunomodulators in the clinic [79].
- Novel matrix scaffolds and bio-engineered synthetic templates with growth factors and other cytokines/chemokines are being tested for chronic wound healing. In order to avoid unnecessary use and even side-effects, it is imperative to perform unbiased broad spectrum RNA-sequencing and proteomic analysis. By such approaches, the critical factors that are deficient will be determined and can be tested, first in preclinical models and subsequently in trials of substitution therapy.
- In cell-based therapies, the products are often ill-defined or need to be patient-tailored. This is much less a problem with recombinant enzymes, e.g. MMP-8, or pharmacological enzyme inhibitors and molecules blocking the signal transduction of cytokines and chemokines. It is expected that with such new products, clinical trials will be straightforward and the results more reproducible because the products are well defined.

- For each type of chronic wound ulcers, consensus protocols for sampling and analysis [76] need to be established for validations to provide formal evidence of activity in comparison with standard therapies.
- Innate immune cells, in particular neutrophil subsets carrying beneficial antifibrotic MMP-8 [35] and TIMP-1-free angiogenic MMP-9 [54,55], deserve attention in chronic skin wound research, aside adaptive immune cells about which no reports in relation to wound healing exist.

Box 2 Recommendations for interdisciplinary approaches

- *Basic scientists* need to develop standardized and robust analytical methods to plan, evaluate and compare clinical studies on wound research for the three major types of ulcers: chronic venous ulcers, diabetic foot and pressure ulcers [75-76].
- *Histopathologists* help with the the analysis full-thickness biopsies of the wound bed and with samples from wound edges (3-6 mm punch biopsies) to define the burden [67,76].
- *Immunologists* need to broaden studies about and beyond immunomodulation by innate immune cells (neutrophils, macrophages) [58-61, 71] and molecules (cytokines, chemokines, MMPs) towards adaptive immune cell subsets and lymphocyte mediators. Indeed, the roles of T cell subsets and glycoforms of immunoglobulins have not yet been studied in any of the three chronic wound healing mechanisms.
- In view that detrimental bacteria impair wound healing, the identification of skin microbiota is essential. The *microbiologists* gradually replace culture-based techniques by quantitative PCR-based methods and spectrum-broad rRNA sequencing techniques, which provide both quantitative and qualitative information. Classical *laboratory medicine* approaches with antibiogram determinations in combination with microbiota analysis will provide the ideal ways for disinfection and converting infected wounds to sterile granulomatous tissues.
- Acute wound fluid is different from chronic wound fluid [66, 74]. Wound exudates can be collected by *dermatologists* and *surgeons* with different methods. Each method has

strengths and weaknesses (reviewed in ref 76). For instance, to define substrate degradomes at a specific phase of wound healing, broad spectrum protease inhibitors need to be added to the samples, whereas this should not be done as a procedure, if one wants to measure the activity of specific proteases.

- Elevated protease activity [57], mainly originating from neutrophils and measured by substrate conversion tests are indicative for non-healing wounds. *Biochemists* may measure enzymes levels and activities. These enzymes need to include the serine protease human neutrophil elastase (HNE) and a broad spectrum of MMPs. It is clear that knowing the phase-specific contribution of MMPs to healing and non-healing of wounds will improve therapies [35].
- For cell treatment of chronic skin ulcers, no consensus exists among *biologists* [13, 14]. However, immunomodulating cytokines, including platelet-derived growth factor (PDGF), granulocyte/monocyte-colony stimulating factor (GM-CSF), G-CSF, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are used on and off label for wound healing [79]. More comparative studies by *stem cell researchers* with larger cohorts are needed.
- Last but not least, more rewards need to go to the field workers, the *wound care nurses*. Until now, much progress in the area of wound healing has come from their daily observations with various types of wound dressings and from their observations of specific clinical cases.

Box 3. Immunomodulation as therapeutic option for wound healing

- Substitution of cytokines yielding growth of keratinocytes, fibroblasts or endothelial cells [79].
- Inhibition of granuloma formation by MMPs [19, 72, 80, 86] and substitution of collagenolytic MMP-1 [30] and MMP-8 [35].
- Inhibition of detrimental microbiota [80,81].
- Inhibition of tumor necrosis factor/TNF-induced expression of chemokines and MMPs by flavonoids [81] or antibodies [82].

- Glycosaminoglycan/GAG-based immunomodulation of cytokines and chemokines [47, 83].
- Novel wound dressings, containing natural products with suggested immunomodulating activities [84, 85], need always to be biochemically defined.

Glossary (now in alphabetical order)

Diabetic foot presents as a chronic skin wound based on arterial clogging, called macro-angiopathy, on thickening of small vessels (micro-angiopathy) and on loss of sensory neural function (diabetic neuropathy).

Decubitus wounds or bed sores are necrotic skin wounds and originate from compression of all skin layers between bone and hard surfaces.

Full-thickness biopsy is a diagnostic biopsy in which all layers of skin are contained: the epidermis with keratinocytes, the dermis with small blood vessels and fibrous tissue and the subcutis making the connection with fat tissue or muscles.

Granulation tissue is the tissue regeneration before re-epithelialization is accomplished in early normal wound healing or after surgical removal of dead tissue in chronic wounds. It consists of vessel sprouts embedded within extracellular matrix and has a red color. In fact it is an open wound and suffering patients need careful painkilling/sedation at this stage.

Granulomas and granulomatous tissues consist of abnormal accumulations of lymphocytes and other mononuclear cells within tissues. Typically granulomas are caused by foreign bodies and are synonymous for “foreign body reactions”. Common causes of granulomas are asbestos inhalation, small loose cartilage fragments in an overstrained tennis elbow and slowly growing infections, such as tuberculosis and worm (egg) infestations. Other classical causes are forgotten sterile surgery materials, including late removal of suture material, and a number of diseases of unknown origin, such as amyloidosis and sarcoidosis. Granulomas are also called “cold abscesses”, as a contrast with neutrophil-rich acute inflammations. Granulomas typically consist

of antigen-presenting tissue macrophages, often fusing into multinucleated giant cells and lymphocytes.

Matrix metalloproteinases (MMPs) are a family of more than 20 proteases with a catalytic zinc ion. MMPs are produced as latent pro-enzymes and need to be activated by removal of the propeptide. Aside the active site, MMPs possess additional protein domains conferring additional functions, including membrane anchoring, receptor binding, signaling *etc.* Whereas the acronym MMP points to remodeling of extracellular matrix components, these enzymes have also potent immunomodulating functions, including the processing of cytokines and chemokines. In the context of wound healing, MMPs play multiple roles, both beneficial and detrimental ones [78]. For example, MMP-3 plays a role in wound contraction, MMP-8 seems to possess multiple beneficial effects and MMP-9 is angiogenic. Beneficial effects of an MMP in one phase of wound healing may be turned into negative effects in another phase.

Tertiary lymphoid tissues are accumulations of leukocytes within tissues where these normally do not belong. In contrast with the thymus and bone marrow as primary lymphoid organs and in contrast with the spleen, lymph nodes and mucosa-associated lymphoid tissues, which all consist of developmentally normal leukocyte accumulations, abnormal lymphoid accumulations in tissues, including granulomas and tumor-associated leukocytes, form tertiary lymphoid tissues.

Tissue inhibitors of metalloproteinases (TIMPs), aside having signaling functions, regulate the activity of MMPs and other metalloproteinases. In humans four TIMPs exist. Except neutrophils [49, 50], all other cell types produce TIMPs in a regulated way.

Venous leg ulcers are the result of defective venous valves, edema formation and skin inflammation.

Figure 1

Figure 1. Three major causes of chronic skin wounds

- a. Arterial narrowing by atherosclerosis (macro-angiopathy or arterial plaque) and diabetic micro-angiopathy are causes of diabetic foot. Macro-angiopathy is indicated by an arterosclerotic plaque in a large artery. In diabetes, additional thickening of the endothelial basement membrane occurs by the chemical addition of glucose to ECM molecules, named glycation, and deposition of glycated serum proteins; The latter process is called micro-angiopathy.
- b. Chronic venous ulcers develop by valve insufficiencies of superficial or deep leg veins. By leaking venous valves (inset), the hydrostatic pressure is increased and leads to extensive ankle edema. Insufficient draining of venous blood leads to coagulation and inflammation, called thrombophlebitis.

- c. Pressure ulcers develop after spinal cord lesions or by capillary occlusion of the skin by long-term pressure between bones and hard surfaces. Red areas indicate common locations of this type of chronic wounds.

Figure 2

Dynamics of cells and molecules in normal wound healing

Within exponential time lines (minutes, days, weeks and years) the appearance of leukocyte types and a limited number of validated molecular processes or molecules are

indicated for the different phases of skin wound healing. Bleeding of a skin wound is stopped by platelet aggregation and fibrin clotting during hemostasis. This coagulation phase by platelets and thrombosis leads to co-deposition of erythrocytes and generates a black wound crust. Already at this point complement molecules are observed in the exudates. Together with neutrophil invasion, TIMP-free MMPs appear within the first days. When mononuclear leukocytes arrive, the MMPs are first temporarily blocked, and then a second wave of fibrinolysis, complement and MMP activity turns-over and resolves excessive ECM, whereas the presence of caspases probably ensures that in about one week cellular elements are removed. Although the molecular information is still limited, recent proteomic approaches are yielding better and broader insights to describe normal skin healing [24,25]. With future functional data, including cellular and molecular depletion/inhibition and substitution experiments, it will be possible to define phase-specific determinants, which keep a wound stalled in a detrimental chronic state or which will enhance healing. As indicated, the inflammation phase of normal wound healing fades out around the fourth day and overlaps with the granulation phase of new blood vessel formation (angiogenesis). The proliferation phase starts by macrophage invasion and lasts from 3 days to 3 weeks and is characterized by fibroblast and endothelial cell proliferation. Tissue remodeling is observed between 2 weeks till 2 years. The involvement of lymphocytes is barely studied, but follows the logarithmic increase phase of macrophages and declines to almost zero levels in about 3 weeks. Time scales are indicated, respectively in minutes, days, weeks and years. Cell dynamics in normal wounds are well defined, but molecular aspects are still fragmentary. The color intensity represents the level of process or activity, white color being undetectable activity. The molecular processes beyond 7 days represent a grey zone.

Figure 3 Sequence of idealized chronic wound healing

(optional, if found appropriate)

- a. Chronic wounds contain necrotic tissue (black). Profiling analysis of molecules and cells and research into causal factors may lead to novel approaches for skin wound healing.
- b. One way of inducing healing is by removal of dead/necrotic tissue and by turning an atonic chronic wound into wet inflamed tissue, rich in neutrophils (yellow). This may be by enhancing neutrophil chemotaxis.
- c. Neutrophils provide TIMP-free neutrophil collagenase/MMP-8 and gelatinase B/MMP-9, the latter of which induces angiogenesis and granulation tissue (red).
- d. When a skin autograft is placed on top of such granulation tissue or when the defect is gradually re-epithelialized from the wound edges, the wound may heal completely.