Review Article

Analytical Approaches to Wound Healing Process

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Abstract

Wound healing is a complex domain that needs to solve abundant parameters. Changed protein concentration, pH effect on wound environment, mediators of immune system, and aging process are fight subjects of wound healing. Therefore, in modern approaches to improve of challenges are tending to analytical techniques for lighting dark side. Research of genetic markers, micro RNAs expression or post-translational modifications of proteins was recently evaluated in wound healing. In review article, we focused on some parameters of the healing system which are affected processes of wound healing and some analytical approaches which are using to find connect of the network.

Introduction

A wound is described as the disruption of a tissue's normal anatomical structure and function. It also signifies the destruction of the body's natural defenses, which facilitates the invasion of microorganisms [1]. The intricate combination of matrix destruction and reconfiguration that occurs during wound regeneration necessitates [2] well regulated mechanisms that result in the healing of damaged tissues [3]. These procedures are fusions of intricate molecular and biological processes that result in proliferation, cell migration, and remodeling and extracellular matrix deposition of scar tissues [4].

According to the type of skin damage, potential underlying disorders (such as diabetes and peripheral artery occlusive disease), local wound factors and systemic mediators, healing processes are induced. Depending on how delicately the various elements are balanced, either a physiological mode of healing (healing of acute wounds) or a pathologically delayed mode of healing (healing of chronic wounds) takes place [5].

Acute (syn. physiological) wound healing is a Four-phased, coordinated process that includes the (i) Hemostasis phase, (ii) inflammatory phase, (iii) proliferative phase (neoangiogenesis, granulation, and re-epithelialization), and (iiii) remodeling phase [Extracellular Matrix (ECM) remodeling] (Figure 1).

During hemostasis, platelets clump together and a fibrin clot forms at the area of endothelial damage. By sticking to the damaged endothelium and releasing chemokines, platelets draw in the inflammatory phase's cellular elements [6].

Following cutaneous injury, cytokines, chemokines, growth factors, and their effects on cellular receptors are the main mediators of the inflammatory phase. Various cell types, including granulocytes and macrophages, are attracted to the wound site by chemoattractant factors, which start the healing process. The wound milieu's pH value gradients, which include proteinases, cytokines, chemokines, etc., may have a significant effect on cellular processes [7].

Following this, the proliferative phase that begins with an overlap in time. This phase includes re-epithelialization, granulation tissue production, and neoangiogenesis, as well as the formation of ECM.

One of the most crucial steps in wound healing is neoangiogenesis because the increased metabolic activity during the proliferative phase limits the availability of nutrients and oxygen. After a suitable wound bed has been formed, an epithelial layer is applied to the wound surface, which encourages fibroblast proliferation and dramatically increased collagen synthesis and deposition. Epidermal keratinocytes, which are triggered and moved into the wound site starting from the wound margins, differentiate, proliferate, and migrate in order to re-epithelialize [8].

Beginning a few days after the injury, the tissue remodeling phase can continue for up to two years. Different proteinases participate in this phase of coordinated wound healing. When compared to physiological conditions, the wound milieu itself, such as pH value variations during the various stages of wound healing, affects how these proteinases behave and how active they are [9].

Chronic wounds show inadequate repair processes that prevent the creation of a sustained anatomical and functional outcome in a reasonable amount of time [5]. Chronic wounds are described as wounds that do not heal physiologically, instead becoming stuck in an uncontrolled and self-sustaining phase of inflammation, and do not follow the well-known step-by-step process of physiological healing. This damages anatomical and functional integrity over a physiologically acceptable period of time [10].

Effect of pH on Wound Healing

Changes in pH can influence or be affected by the complicated process of wound healing at each stage [11-13]. According to early research, a chronic wound has a pH between 7.11 and 8.9 [14,15] and Compared to wounds with a pH closer to neutral, wounds with a high alkaline pH has a slower rate of healing [16].

During wound healing, the environment around acute and chronic wounds changes from being alkaline to neutral to acidic [17]. Since pH can be used as an indicator of how well a wound is healing, it can also be utilized as a diagnostic tool, according to various researchers [11-13,16].

Effect of pH on Skin Cells in Wound Healing

Changing of pH is affected skin cells behavior in during wound healing. Using *ex vivo* and *in vitro* skin cell models, researchers
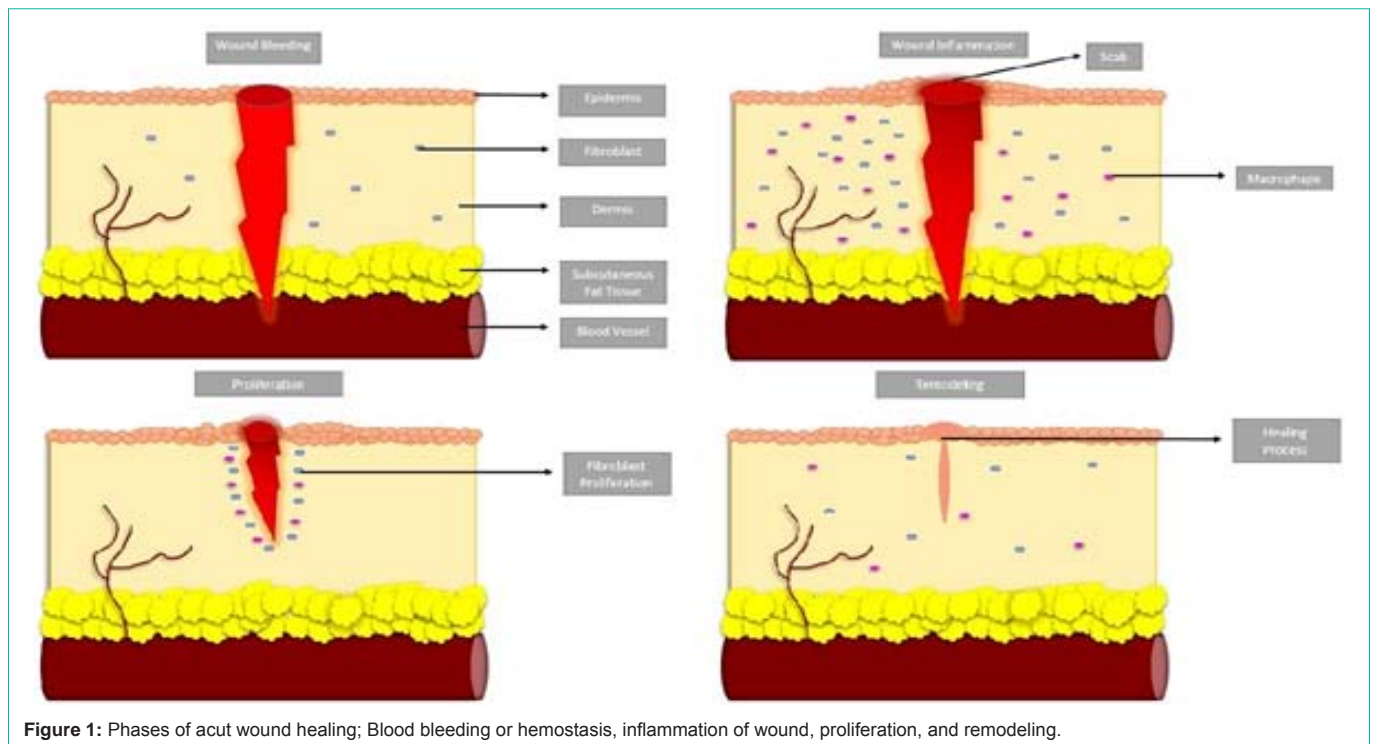
Figure 1: Phases of acut wound healing; Blood bleeding or hemostasis, inflammation of wound, proliferation, and remodeling.

looked at how pH affected keratinocyte and fibroblast attachment, proliferation, and migration.Additionally, the effect of pH on keratinocyte differentiation was measured by the expression of cytokeratins, the proteins of keratin intermediate filaments found between epithelial cells, in particular cytokeratins 1 and 5. It was determined that low pH values promote a differentiated keratinocyte phenotype. Furthermore, the best pH range for fibroblast and keratinocyte is between 7.2 and 8.3, on the other hand the optimal pH value for ex vivo skin explants development was 8.4. Indicates that skin cells and explants reproduce and move at pH level above the physiological range [18].

Described how pH influences cell migration and how cell density creates changes in pH suited to healing: Studies have demonstrated that cellular proliferation is less susceptible to pH lowering in sparse chick embryo cell cultures than dense cell culture. Experiments on wounds showed similar outcomes, with low pH preventing cell migration. But those cells that migrated into the wound area proliferated just as quickly at low pH as they did at high pH, illustrating the combination impacts of pH and cell density [19,20].

Effects of Aging on Wound Healing

The alterations in aging skin are a result of both intrinsic and extrinsic aging. The alterations in skin that take place in areas shielded from the sun without reference to external aggressors are referred to as intrinsic aging. Extrinsic aging is the result of long-term environmental exposure, especially to sunlight's UV radiation, which causes cumulative alterations in the body [21]. Increased sensitivity to the environment, a decline in homeostatic capacity, and a cumulative loss of function are the overall effects of intrinsic and extrinsic aging [22].

Comparing older humans to younger ones, there is a noticeable decrease in cutaneous blood flow [23]. Age-related changes in blood flow are accompanied by a decrease in cutaneous lymphatic drainage, which impairs the ability to rid the wound of pathogens and also prevents wound contraction [24].

Age-Related Changes in Phases of Healing

Alterations in Hemostasis and Inflammation

With endothelial damage exposes collagen, which helps platelets attach to the damaged endothelium. Aged subjects have increased platelet adhesion to the endothelium [25,26].

Age also causes platelets to release more alpha-granules, which are composed of TGF-β, TGF-α, and Platelet-Derived Growth Factor (PDGF) [27].

Elderly endothelial cells release less nitric oxide, a vasoactive mediator [28]. As a result, the diapedesis of neutrophils is reduced, and capillary permeability is reduced at the site of damage. On the other hand, leukocytes exhibit an age-related increase in the secretion of and responsiveness to several inflammatory mediators [29,30]. Macrophage and B-lymphocyte infiltration into wounds is postponed in middle-aged and elderly mice using wound healing models. Aged animals similarly have a delay in T-lymphocyte entry at the wound bed, but the overall level is higher than in young animals. Lymphocytes from elderly animals exhibit a reduced proliferative response, a reduced number of naive cells, and an increased number of memory cells [31,32].

Alterations in Proliferation

In older animals, the proliferative response of fibroblasts, keratinocytes, and vascular endothelial cells is diminished [33,34].

Age-related delays can be seen in angiogenesis, collagen production, and re-epithelialization [31]. Dermal fibroblast size and number generally decline with age [35]. Additionally, maturated fibroblasts are representedless sensitivity to growth factors and have less replication potential than young fibroblasts [35,36]. Both in animal models and in human wounds, these changes cause an age-related delay in the healing of the wound [37,38]. In aged animals [39, 40] as well as humans [41,42], whole wound studies have revealed lower rates of epithelialization and contraction.

The process of angiogenesis is thought to be crucial for the best possible healing of wounds because the epidermis needs nutrients to move and multiply [43].

Alterations in Resolution

Collagenase activity is higher in young animals than in older animals, allowing for more collagen remodeling and turnover [44]. In older wounds, keloids, hypertrophic scars and other hyperproliferative wound-healing disorders are uncommon. This may be caused by lower amounts of circulating TGF-β, according to some studies [45]. In adults, severe scarring can be avoided by blocking TGF-, a protein that promotes the synthesis of collagen [46].

Have demonstrated that aged animals have diminished strength and gain wound strength more slowly than younger animals. It has been demonstrated that these findings also apply to human beings; incisional wounds in individuals older than 70 years old showed lower tensile strength than those in patients younger than 70 years old [47].Infection rates and medical consequences rise as a result of the delayed wound closure [48-51].

Analytical Approaches to Wound Healing

When trying to understand wound healing, it is important to monitor changes due to comorbidities (e.g., diabetes, vascular diseases). More biological materials are needed for these studies [52]. Analyzing wound fluid helps us understand the microenvironment of the wound.

In recent years, inflammatory mediators, growth factors, cytokines [53,54], proteases [55,56] and oxidative stress proteins [57] have been analyzed in wound fluid, and in this way the effects of proteins in different phases of wound healing have been tried to be characterized. However, since the mechanism underlying wound healing has not yet been fully elucidated, a diagnostic marker cannot be recommended in routine clinic.

In wound healing studies, immunoblotting, microbeads arrays, and immunoassays methods are used. These methods allow the analysis of a relatively small fraction of selected proteins.

With mass spectrometry based proteomics, a diagnostic point of view can be developed by detecting potential biomarkers that will allow us to monitor wound healing [58]. Proteins from different tissues can be used in these studies [59-60].

However, despite the large number of proteins, which are the handicaps of proteomic studies, issues such as insufficient concentrations and the predominance of abundant protein groups limit these studies [61,62].

A gold standard method cannot currently be recommended for reproducible analyzes of proteins that are modified or expressed/depressed in wound healing and derived from wound fluid.

In addition, chronic ulcers, diabetic wounds and pathological features due to venous stasis ulceration cause delay in the healing process. At the pathological level, abnormal formations such as fibrotic responses and adhesions also occur. When we add the contribution of cytokines and enzymes, controlling and optimizing the physiological responses to wound healing becomes complicated. Therefore, analytical research methodologies developed are of great importance

Microdialysis for Following of Wound Healing

Microdialysis is a method used to analyze low molecular weight proteins from the extracellular interstitium. A semipermeable membrane is designed like a blood capillary (mimic) and inserted into the wound. Dialysate obtained as wound fluid is used for protein analysis in situ studies. [59,61,63,64]. Samples collected from a specific site can help explain a dynamic physiological and pathophysiological process. For this reason, samples taken from tissue, skin, brain, eye and liver by microdialysis method are increasingly used to understand the molecular mechanism [59, 61].

The samples consist of molecules that cross the semi-permeable membrane with a concentration gradient. In fact, although the biological activities of cytokines are high, their concentrations in body fluids are low [64]. Therefore, analysis of cytokines and chemokines is useful as a direct method. It can be said that the most important benefit of microdialysis is that it allows the removal of wound fluid from the same place with a non-invasive method and the continuation of sample collection during the healing process [64].

The resulting micro dialysate is subjected to further investigation with traditional analysis methods such as radioimmunoassay or Enzyme Linked Immunosorbent Assays (ELISAs) [65].

These analytical methods have some limitations. However, characterization of many molecules can be done with "omics" techniques. It makes it possible to analyze molecules <2 kDa with metabolomics, which is among the omics techniques [66].

Microdialysates collected from samples not from the skin, but especially from various tissues, can be applied to metabolomics techniques. Among these analytical techniques, separation methods such as mass spectrometry liquid and gas chromatography or Nuclear Magnetic Resonance (NMR) spectroscopy are used [67].

Omics for Following of Wound Healing

Because the complex molecular mechanisms of wound healing have not been fully elucidated, we need tools to optimize the analysis of the healing process. To understand the molecular pathways involved in wound healing and to see the cascading links of different stages of the healing process, we need to know the regulatory mechanisms. Regulatory mechanisms of wound healing include genes, proteins, microRNAs, metabolites and drug molecules. All these molecules interact with each other through chemical reactions. In addition, the concentrations and interactions of molecules increase the complexity of the healing process.

MicroRNAs (miRNAs): miRNAs are important post transcriptional regulators and are being investigated as potential

Table 1: miRNA regulation of major proteins involved with wound inflammation [72].

Cytokine	miRNA	References
TNFα	miR-125b	28
TRAF6	miR146a	36
IRAK	miR146a	36
TGF-βR1	miR128a	47
IL-10	miR-466l	50
MCP-1	miR124a	32

markers in the diagnosis and treatment of diseases. Our knowledge of miR-mediated gene regulation in wound healing is limited and "omics" research is gaining in importance.

miRs bind to the 3' untranslated region of target mRNAs, causing destabilization and translational repression in mRNA [68].

Since miRNAs are regulators of the complex gene network, it has been suggested that they have essential biological functions in wound healing [69,70].

In addition, miR-based therapeutics constitutes a promising approach in the wound healing process [71].

Imbalance in the response to inflammation during wound healing may cause chronic inflammation and delay healing. It is suggested that miRNAs regulate inflammation in wound healing by target specific genes (Table 1). Table 1 lists 6 key mediators involved in the inflammation stage during wound healing and miRNAs regulating these proteins; TNF, transforming growth factor receptor 1, IL10, TNF receptor-associated factor 6, interleukin receptor-associated kinase, monocyte chemotactic protein 1, miR-125b, miR-128a, miR-466l, miR-146a, and miR-124a [72].

TNF-α may have beneficial or detrimental effects on tissue repair depending on the concentration. Suppression of miR-125b expression controls TNF-α production [73]. miR-146 negatively affects Toll-Like Receptors (TLRs) by targeting TRAF and IRAK [74]. The gene expression of Macrophage Chemoattractant Protein (MCP-1) increases approximately 70-times when the wound is formed [75]. IL-10, on the other hand, inhibits the pro-inflammatory response by inhibiting the STAT3-dependent pathway. On the other hand, when chronic and non-chronic wounds are evaluated to decide a biomarker, there was indicated that a lot of some İnterleukines (IL-) entity such as IL-11, IL-1A, IL-1B, IL-12B, IL-8, IL-15, IL-23. However, target gene analyzes of these interleukins have not been performed [76].

The route used for proteomic analysis of proteins in wounds is generally described in (Figure 2).

Potential biomarkers are determined by proteome analyzes applied for proteins that are involved in wound healing and that are expressed or depressed. Proteomics enables the analysis of specific proteins using Mass Spectrometry (MS), a fast and reliable method. It is possible to quantify the analytes in the analyzes made with MS, at the same time the analysis of protein mixtures is carried out sensitively [77]. Protein analyzes in biological tissue samples were presented in studies using the Imaging MS (MALDI IMS) technique [78].

While analysis is performed on minimal samples in biological
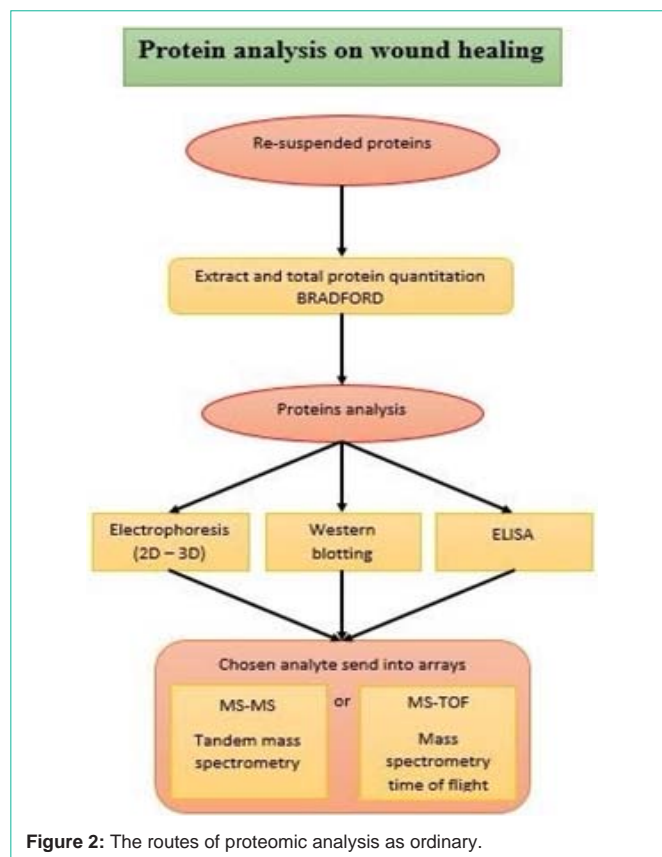
Figure 2: The routes of proteomic analysis as ordinary.

processes, protein analysis is also possible in frozen or paraffin-fixed tissue samples, thus detecting the healing process of the damaged tissue. MALDI IMS studies on chronic wounds caused by pathological features are promising for therapeutic approaches [79].

Nanofibrous biomaterial for wound healing: Nano-technological approaches emerge as one of the current methodologies in the healing of acute and chronic wounds [80].

Studies on the variety of synthetic or natural nanomaterials such as nanofiber mats and scaffolds in wound healing have come to the fore in recent years. The aim here is to develop a treatment approach that will assume the role of a blood clot in the wound area. In natural functioning, during the healing of the wound under in vivo conditions, fibrinogen, a plasma protein, is formed by blood coagulation, and then fibrin degradation process (fibrinolysis) occurs with enzymatic degradation [81,82].

Meanwhile, the fibrous blood clot behaves like an Extracellular Matrix (ECM) and fibroblasts and endothelial cells migrate to the wound site [83,84].

Because of this relationship, the use of nanoscaffolds as an accelerating factor in blood coagulation and wound healing, that is, the use of fibrinogen nanofibers, has gained importance. Although it is possible to use nanofibrous fibrinogen in different sizes with electrospining, this method has not yet become optimal because it can cause changes in the natural protein structure [85,86].

One of the important handicaps is that the biological function of nanofibrous scaffolds can change due to electro spinning and

protein degradation occurs. For the use of nanofibrous scaffold in wound healing, it would be appropriate to eliminate problems such as organic solvents and acidic conditions [87]. Changed total protein concentrations is other problem because of collection process of fluid of wound affects total protein concentration. We know that total protein concentration is not change just depend on sample collection method, meanwhile there is occurred protein oxidation. Oxidation on proteins of skin or fluid of wound is formed loss of total protein concentration, actually carbonylated proteins are formed after oxidation.

Conclusion

Wound healing process has complex molecular pathways, intermediators, and it is open to different pathological challenges at the same time. We need to understand all parameters such as pathological conditions, loss of total protein concentration, pH effect, changed aging condition to improve healing process of wound. Modern approaches are focused on omics technology. These approaches will allow the identification of biomarkers of molecular pathways.

Author Disclosure

The authors declare have no commercial associations and no competing financial interest.

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