Invited Review

The molecular biology in wound healing & non-healing wound

Chun Qing

Institute of Burn, Ruijin Hospital, Medical School, Shanghai Jiaotong University, Shanghai 20025, China

A B S T R A C T

The development of molecular biology and other new biotechnologies helps us to recognize the wound healing and non-healing wound of skin in the past 30 years. This review mainly focuses on the molecular biology of many cytokines (including growth factors) and other molecular factors such as extracellular matrix (ECM) on wound healing. The molecular biology in cell movement such as epidermal cells in wound healing was also discussed. Moreover many common chronic wounds such as pressure ulcers, leg ulcers, diabetic foot wounds, venous stasis ulcers, etc. usually deteriorate into non-healing wounds. Therefore the molecular biology such as advanced glycation end products (AGEs) and other molecular factors in diabetes non-healing wounds were also reviewed.

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In the past 30 years, the development of molecular biology and other new biotechnologies helps us to recognize the wound healing on normal skin that damaged by external factors such as physical, chemical, thermal, biological and so on. At first, we can only know the progress is composed of hemostasis, inflammation, proliferation and maturation, during which the platelets, neutrophils, monocytes/macrophages, lymphocytes, granulation tissue, fibroblasts, collagen, epidermal cells, etc. are observed involving in under microscope, followed by soluble mediators such as chemokines, cytokines (including growth factors) and other molecular factors. Many new observed molecular factors help us to further know the mechanism in the process of wound healing at damaged skin.

Mechanism of wound healing

Molecular biology in platelets on wound healing

The platelets were activated in the initial stage of injury and play a crucial role in clot formation during hemostasis after aggregation and attachment to the exposed collagen surfaces. The cytokines (including growth factors) such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and other factors released by the platelets mediate the process. Cytokines (including growth factors) can reach their target cells by endocrine, paracrine, autocrine, or intracrine routes and stimulate target cell response after binding to cell surface receptors and activating the special signal pathway system and silent genes of non-stimulating state. Some growth factors function diversely, such as PDGF, is not only stimulation to neutrophils and macrophages but also a mitogen and chemotactic agent for fibroblasts and smooth muscle cells that stimulate angiogenesis, collagen synthesis, collagenase, and so on. Then one cytokine or growth factor can play multiple roles because of different target cells and receptors.

Molecular biology in the inflammatory cells on wound healing

Chemokines (or chemotactic cytokines) are small heparin-binding proteins that induce the movement of circulating inflammatory cells to the injury sites via interaction with specific membrane-bound receptors. Neutrophils are the predominant cell type in the first inflammation phase (48 h after injury) and begin to wane after 24—36 h by apoptosis in the time of circulating monocytes enter the wound and mature into tissue macrophages that play the very important role in the wound healing. This process is mediated by the chemokines IL-8 released by neutrophils, which attracts the macrophages and other cells to the wound site. Chemokines are classified into CC, CXC, CX3C, and XC families depending on the spacing or presence of four conserved cysteine residues. CXC chemokines primarily attract neutrophils and lymphocytes and are believed to orchestrate the early phases of wound healing.[1]

Following the neutrophils, the monocytes migrate to the wound site and become the macrophages, which play a central role in both the inflammatory phase and all stages of repair. Macrophages phagocytose debris and bacteria, meanwhile produce and orchestrate inflammatory cytokines (including growth factors) such as TNF, IL-6, IL-1, bFGF, etc. IL-1 stimulates the proliferation of inflammatory cells and promotes angiogenesis through endothelial cell replication. TNF-α is a mitogen for fibroblasts. bFGF is a chemotactic and mitogenic factor for fibroblasts, endothelial cells and other mesenchymal cells, which are the stimulus for angiogenesis. Besides, bFGF stimulates wound contraction, epithelialization and production of collagen, fibronectin, and proteoglycans. Macrophages also secrete collagenases and elastases, which break down injured tissue and debride the wound.

In recent years activation of macrophages has been classified into classical M1 and M2 states depending on the stimulus. The M1 activation results in a highly pro-inflammatory macrophage phenotype, which is mediated by Toll-like receptor (TLR)-4 ligands and IFN-γ and becomes the sauce of pro-inflammatory cytokines. The M2 activation is mediated by IL-4 and/or IL-13 which release some growth factors such as macrophage-derived growth factors, PDGF, αFGF, bFGF, TGFα, TGFβ, etc., suggesting an important role for alternatively (M2) activated macrophages in the phase of wound healing.

Besides, M1 and M2 promote Th1 and Th2, which plays cellular immunity and humoral immunity, respectively. Products of Th1 such as lL-2, IFN and Th2 such as IL-10, IL- 4 also down regulate M1 and M2 activity, respectively. The balance of the products of Th1 and Th2 is the balance of cellular immunity and humoral immunity. Thus, the balance of M1/M2 also demonstrates the importance of innate immunity.[2–5]

Recent studies have showed another factor, autophage, may play role of molecular biology in the repair process, because autophage has a lot of functions that influence infection, inflammation and immunity. Autophage is induced by pattern recognition receptors and, through autophage adaptors, provides a mechanism for the elimination of intracellular microorganisms. Autophage controls inflammation through regulatory interactions with innate immune signaling pathways, by removing endogenous inflammasome agonists and through effects on the secretion of immune mediators. Moreover, autophage contributes to antigen presentation and to T cell homeostasis; it also affects T cell repertoires and polarization.[6]

Lymphocytes migrate into the wound during the inflammatory phase, approximately 72 h following injury and secrete lymphokines, EGF, bFGF and so on. T lymphocytes are attracted to the wound by IL-1, which also contributes to the regulation of collagenase. Therefore, lymphocytes play a role in cellular immunity and antibody production.

Toward the end of the inflammatory cycle, the macrophage-derived growth factors and macrophage-derived angiogenic factors reach the optimal levels, which strongly influence the influx of fibroblasts, endothelial cells and then keratinocytes into the wound. Subsequently, macrophages can stimulate proliferation of connective, endothelial and epithelial tissue directly and indirectly by changing the composition of the extracellular matrix (ECM) both during angiogenesis and in the remodeling phase by release of degrading enzymes and by synthesizing ECM molecules.[2,3] Therefore, in the adult body, no macrophages, no wound healing. As mononuclear cells continue to replace macrophages and other inflammatory cells, the proliferation phase begins.

Molecular biology in repair cells on wound healing

In response to macrophage-synthesized growth factors such as PDGF, FGF, VEGF, TGF-α, TGF-β, KGF, etc., the fibroblasts, a critical component of granulation tissue, begin to migrate, proliferate and produce the components of ECM, such as glycosaminoglycans and proteoglycans, as well as collagen, a critical event in the proliferation phase and wound healing in general. Collagen is secreted to the extracellular space in the form of procollagen and then cleaved of its terminal segments, which is called tropocollagen. Tropocollagen can aggregate with other tropocollagen molecules to form collagen filaments that are rich in hydroxylysine and hydroxyproline moieties and enable it to form strong cross-links. The stability of the collagen fiber depends on the intermolecular cross-links that make collagen fiber resistant to destruction. The more cross-links in intramolecular and intermolecular of collagen, the more increased bursting strength in wound healing. Therefore, collagen forms tight cross-links to other collagen and with protein molecules, increasing the tensile strength of the healing wound. We further know the hydroxylation of proline and lysine residues depends on the presence of oxygen, vitamin C, ferrous iron, and α-ketoglutarate. Collagen fibers are deposited in a framework of fibronectin that also serves as an anchor for the myofibroblast, which migrates in some wound healing.

Endothelial progenitor cells (EPCs) express markers of both hematopoietic stem cells (CD34 and CD133) and endothelial cells (CD146, vWF, and VEGFR2),[7] which helps us to know the revascularization relies on mobilization of EPCs from the bone marrow to areas of regenerating or healing tissue. EPCs are critical for maintenance and repair of endothelial cells. They play an important role in angiogenesis as they proliferate, migrate and differentiate, and are a source for proangiogenic cytokines. During normal wound healing, EPCs are effectively recruited to the remodeling microcirculation, thus leading to wound revascularization and timely healing. Therefore, in wounding healing, many progenitor cells also play very important roles.

Cell differentiation is a process, characterized by the loss of intrinsic and special phenotype of a cell and the transformation of a new phenotype into another, which is characterized by a change in phenotype, morphology and function.[8] The growth factors are important messengers for cell transition such as mesenchymal-to-mesenchymal transition and endothelial-mesenchymal transition that could occurred by TGF-β signal pathway or by Notch signal pathway which inhibits the expression of endothelial cell adhesion molecule VE-cadherin.[9,10] The epithelial-mesenchymal transition (EMT) is the communication especially for establishing the emerging basement membrane and subsequent reepithelialization. It plays an important role in wound healing. Moderate EMT can accelerate wound healing, and the abnormal regulation of EMT is closely related with hypertrophic scar and tissue fibrosis.[11] The results showed that the epithelial cells gained mesenchymal phenotypic feature and stronger ability in movement and migration. β2-AR is a critical molecule which mediated EMT process.[12]

The proliferation and migration function of epidermal cells are the most important repair processes in wound healing. Epidermal cells at the wound edges undergo structural changes, allowing them to detach from their connections to other epidermal cells and to their basement membrane. The migration of epidermal cell is commonly perceived as the movement of individual cells that undergo cycles of polarized extension-contraction of their actin cytoskeleton coupled with adhesion and subsequent de-adhesion from the surrounding substrate. Cellular movement relies on the establishment of physical forces by means of protrusive forces that lead to membrane extensions and traction forces allowing the cell to contract and slide forward,[13] i.e, single cell migration requires an initial step of cell polarization, during which intracellular actin polymerizes to form ruffles or leading pseudopodia. The cell generates these deformations of its body via the actin cytoskeleton. The

Rho family small guanosine triphosphate (GTP)-binding proteins (GTPases) are pivotal regulators of actin organization and control of lamellipodia and filopodia formation. Protrusions rely on polymerization and depolymerization of actin filaments while the traction is generated by myosin-based motors which pull actin filaments past one another. In order for a cell to move, its actin-based protrusions must be polarized. Indeed, a cell that simultaneously extends protrusions in opposite directions, or even more dramatically all around its body, will be immobilized. Cell polarization is a requirement for cellular movement.

ECM is the molecular micro-environment of the repair cells. At the sites where contact with the ECM occurs, big protein complexes are assembled through the recruitment and clustering of receptors of the integrin families. These big protein structures are known as focal adhesions or focal contacts. In order to provide space for the forward expanding cell body, pericellular matrix molecules are locally broken down by surface proteases, such as MT1-MMP. Shortly after integrin binding with ECM, cytoplasmic actin filaments engage with contractile proteins, such as myosin II, which stabilize and shorten the membrane-tethered actin filaments. This results in local cell contraction, generally at the opposite pole respect to the leading edge. The Integrin/MMP-dependent mode of cell migration is known as mesenchymal. However, cells may be able to migrate across connective tissue by simply squeezing themselves within pre-existing ECM pores. This mode of migration is Integrin/MMP-independent and known as ameboid.

Intracellular actin microfilaments are formed, allowing the epidermal cells to creep across the wound surface. Epidermal cells secrete collagenases that break down collagen and plasminogen activator, which stimulates the production of plasmin. Plasmin promotes clot dissolution along the path of epithelial cell migration. Migrating epithelial cells interact with a provisional matrix of fibrin cross-linked to fibronectin and collagen. In particular, fibronectin seems to promote keratinocyte adhesion to guide these cells across the wound base. This epithelial layer provides a seal between the underlying wound and the environment. The interfollicular stem cells should only be required to contribute to epidermal tissue. The stem cells are found in the deep rete ridges, suggesting that this site may provide protection for the long-lived stem cell population from harmful environmental mutagens. The sebaceous glands and hair follicles contribute to reepithelialization.

EGF can promote the proliferation of epithelial cells, fibroblasts, enhance the vitality and delay the aging of skin cells, so that the optimized composition of the skin maintains the best physiological state. After trauma, a large number of EGF expression is helpful to the early epithelium of the wound. EGF was the first growth factor described and is a potent mitogen for epithelial cells, endothelial cells, and fibroblasts. EGF stimulates fibronectin synthesis, angiogenesis, fibroplasia, and collagenase activity.

When epithelialization is complete, the epidermal cell assumes its original form, and new desmosomal linkages to other epidermal cells and hemidesmosomal linkages to the basement membrane are restored. Superficial to this activity, epithelial cells continue to migrate inward from the wound edge until the defect is covered.

The above all shows mechanism of molecular biology and the other new biotechnologies in wound healing on normal skin after injury. But many common chronic wounds, which are often considered as a specific type of non-healing wounds such as pressure ulcer, leg ulcers, diabetic foot wounds, venous stasis ulcer, surgical and malignant wounds as well as lymphoederma and dermatological conditions, are associated with skin breakdown.[14] Here we mainly discuss the mechanisms of the molecular biology and the other new biotechnologies in diabetes non-healing wounds.

Advance in refractory wound

Diabetes has multiple effects on molecular biology in non-healing wounds

Great influence of molecular biology in non-healing wounds has been known in the past several years. The effects include hyperglycemia, decreased or impaired production of cytokines (including growth factors) and their receptors that interfere the function of cells such as macrophage, angiogenic response, collagen accumulation, quantity of granulation tissue, keratinocyte and fibroblast migration and proliferation, number of epidermal nerves and balance between the accumulation of ECM components and their remodeling by MMPs,[15] which damages the epidermal barrier function in the end. Diabetic wound is a special wound, for diabetes itself can cause oxidative stress through a variety of ways. Research on the molecular biology mechanism of diabetic wound healing focuses on harmful substances deposited, such as high glucose, AGEs, etc. AGEs mainly accumulate some large molecular weight of proteins with a long half-life, such as collagen protein. The easy accumulation of AGEs in skin collagen results in the formation of glycosylated collagen that functions differently compared with normal collagens in the skin. Another mechanism of AGEs interfering wound healing is characterized by the evidence of increased oxidative stress. Extended exposure to reactive oxygen species (ROS) is believed to lead to cellular dysfunction and organism death via the destructive oxidation of intracellular proteins, lipids, and nucleic acids. Extracellular superoxide dismutase (ecSOD/SOD3) is a prime antioxidant enzyme in the extracellular space that eliminates ROS. Fujiwara et al[16] confirmed that reduced SOD3 levels contribute to healing impairments in aged mice. Long term hyperglycemia may lead to the production of a large number of AGEs in the body. Here we mainly discuss the issue that AGEs influence the non-wound healing in skin.

The molecular biology of neutrophils in non-healing wound

Study of Coltison et al[17] found that AGEs could be high affinity with the human neutrophil AGER (AGE Receptor) and lead to increased intracellular calcium and actin polymerization, which will depress the transendothelial cell migration and sterilization ability of neutrophil. Tian et al's[18,19] research found that neutrophils could not reach the basal part of the wound in time and form a dense inflammatory zone. A large number of neutrophils are scattered around the wound. Immunohistochemistry showed that AGE is distributed in the skin tissue of diabetic rats. Neutrophil migration test is shown in vitro that AGEs can inhibit the migration of the neutrophil by binding its receptor on the surface of the neutrophil. At the same time, neutrophils bind with AGEs outside the vascular tissues, then a large number of inflammatory cytokines are released and induce oxidative stress of neutrophils. This release and oxidative stress burst are delayed and last longer than the normal wound. But early inflammatory cells such as neutrophils and macrophages infiltration decreased and maintain a longer time in the wound site and formed the chronic or refractory wounds.[20]

Neutrophil plays an important role in the normal healing process, but abnormal neutrophil may contribute to the pathogenesis of non-healing wounds present in diabetic patients. Tennenberg et al[21] found that neutrophils from patients with diabetes are prone to apoptosis, which may be related with hyperglycemia. This would cause decreased functional longevity of neutrophils and increased neutrophil clearance from infectious sites, possibly contributing to the increased susceptibility and severity of infections in diabetic patients.

Gustke et al[22] found that the average neutrophil chemotaxis index was significantly lower than that of the control group ($p < 0.02$) in type 1 diabetic patients. The changed cell function of neutrophils was dependent on HLA-DR3, DR4, and DR5 genes. Impaired wounds such as diabetic wounds and chronic venous ulcer were found abnormal inflammatory retention and reduced granulation tissue state.[23] A better understanding of the molecular mechanisms and cellular interactions of neutrophils in diabetic patients is critical for the development of novel therapeutic strategies to promote diabetic wound healing.

The molecular biology of macrophage in non-healing wound

During the process of refractory wound healing, the function of macrophages is abnormal such as M1/M2 macrophage imbalance. The diabetic wound healing model using db/db mice showed the absence of expression of iNOS, the marker of caM(M1) and the increased Arg-1(arginase-1), the marker of aaM(M2). Miao et al[24] found a reduction in iNOS level on days 1 and 3 after wounding in STZ-induced diabetic rat lesion, especially on day 3, compared with the normal rats. Compared with normal rats, the expression of iNOS in the early stage of diabetic rats was decreased, Arg-1 and anti-inflammatory factors such as IL-4, IL-10 increased, indicating that Th1/Th2-M1/M2-iNOS/Arg-1 adjustment mechanism of normal healing was inclined to the side of Th2-M2-Arg-1 in diabetic wound,[25] that is, the pro-inflammatory and anti-inflammation disordered and the balance of the inflammation process disordered.

The accumulation of AGEs induces excessive TNF-α production from macrophages at the late stages of posting injury 7, 9, 11 days, which impairs epithelialization and causes non-healing wound. Dong et al[26] also found that activating α7 nicotinic acetylcholine receptor (α7nAChR) can promote diabetic wound healing by suppressing AGE-induced TNF-α production, which may be closely associated with the blockage of NF-κB activation in macrophages.

Macrophages undergo a transition from pro-inflammatory to healing-associated phenotypes that is critical for efficient wound healing. The switch in macrophage phenotypes during skin wound healing was associated with up-regulation of the peroxisome proliferator-activated receptor (PPAR)γ and its downstream targets, along with increased mitochondrial content. Miraz et al[27] reported that in the setting of diabetes, up-regulation of PPARγ activity was impaired by sustained expression of IL-1β in both mouse and human wounds. In addition, experiments with myeloid-specific PPARγ knockout mice indicated that loss of PPARγ in macrophages was sufficient to prolong wound inflammation and delay healing. Furthermore, PPARγ agonists promoted a healing-associated macrophage phenotype both in vitro and in vivo, even in the diabetic wound environment.

The molecular biology of endothelial cells (ECs) in non-healing wound

It has been reported that ECs suffered increased apoptosis, up-regulation secreting of adhesion molecule such as ICAM-1 and VCAM-1, increased ROS, MDA, decreased SOD level and activated cell signal pathway of MAPK, NF-kb under the high glucose or AGEs in vitro,[28,29] which could help leukocytes to gather on the wall of vascular and cells more easily migrate from vessel to the injured area. It may be one of the reasons for the presence of lymphocytes in diabetic skin being susceptible to infection and showing the sub-inflammatory response after injury, which may be one of the mechanisms of non-healing in diabetic foot.

High-glucose environment can activate oxidative stress in ECs by AGEs, the classical polyol pathway, PKC, the sorbit pathway, the acetylglucosamine pathway, etc. The activated oxidative stress can increase the active oxygen species in blood, reduce vascular diastole factor such as endothelial cell nitric oxide (NO) and prostacyclin, and raise vascular contraction factors such as ET-1, thromboxin A2. ET-1 can activate inflammatory reaction to promote leukocyte adhesion and TNF-α secretion. This may also be one risk factor of non-healing wound. Besides, it is known that under diabetic conditions there are increased oxidative stress levels such as the increased ROS that prompts EPCs to produce pathologic cytokines like MCP-1 (monocyte chemoattractant protein-1), TNF-α, NF-κB, IL-8, elevated levels of iNOS and decreased eNOS. The reduced functional activity of EPCs during hyperglycemia involves the Akt/eNOS pathway, where signaling is down-regulated under diabetic conditions.[30,31]

The molecular biology of the fibroblasts in non-healing wound

Wang et al[32] confirmed that in vitro AGEs can inhibit the proliferation of fibroblasts and induce cell apoptosis in a dose-dependent way. Many scholars reported that when cultured in high glucose or AGEs medium, primary dermal fibroblasts presented as inhibited proliferation, decreased collagen synthesis, reduced synthesis of hyaluronic acid, abnormal expression or activity of proinflammatory cytokines or growth factors (such as IL-1, IL-6, TNF-a, PDGF and CTGF) and matrix metalloproteinase (MMP-2, 3, 9, 13). It has confirmed that the molecules that can bind to AGEs are P60, P90, galectin-3, macrophage scavenger receptors and AGEs specific receptors (such as RAGE, AGER1, AGER2 and AGER3). The high expressed AGEs receptor on the membrane of fibroblasts was found in diabetic patients.

The fibroblasts of diabetic mice show a severe impairment in VEGF production under normoxic and hypoxic conditions in addition to an increased pro-degradative activity due to the high expression of matrix metalloprotease type 9 (MMP-9).[33] Studies with human fibroblasts have confirmed the pro-degradative phenotype by the increased MMP-2 and MMP-3 production and reduced collagens gene expression.[34,35] Human diabetic fibroblasts also exhibit a failure in NO production, which is concomitant to elevations in MMP-8 and -9.[36] The fact that these fibroblasts fail in secreting NO is particularly negative given its role for wound healing. Conversely, NO donors' administration has shown to stimulate cell proliferation and restore the balance of MMPs.[37]

The molecular biology of keratinocytes in non-healing wound

There are many factors such as NF-κB signal transduction pathway that regulate the proliferation and apoptosis of keratinocytes. Takao et al's study[38] has showed that NF-κB activity in the keratinocytes of diabetic rats was significantly higher than that in normal rats. In vitro studies showed that AGEs could activate the activity of NF-κB in normal keratinocytes and the activity was correlated with the concentration of AGEs. The more AGEs concentrated, the less keratinocytes will be activated. AGEs hamper two key stages of cell cycle and inhibit the keratinocyte proliferation function of keratinocytes. When the NF-κB activity is inhibited, the proportion of cell cycle in G2/M phase can be increased obviously, but the changes in the percentage of cell cycle in S phase is not obvious. This suggests that the AGEs can inhibit the cell transition from S to G2/M phase by activating NF-κB pathway. However, inhibiting the activity of NF-κB can partly enhance the activity of keratinocytes. At the same time, the proportion of cells in G2/M phase increases and cell proliferation ability enhance when the activity of NF-κB pathway is inhibited. But the inhibitory NF-κB pathway does not promote G0 cells into S phase. It is showed that AGEs can inhibit the S phase transition from the G1 phase to the S

phase through other pathways. Similar results have been obtained in the study of the apoptosis of keratinocytes. AGEs can promote the apoptosis proportion of keratinocytes by regulating the NF-κB signal.

A study found that in impaired diabetic wound the migration ability of epidermal keratinocytes is enhanced at the same time.[39] In vitro research revealed AGEs intervention can significantly promote the migration; and the ability of cell migration returned to normal when inhibiting the activity of NF-κB.

Enhanced AGEs deposition in the nerve tissue can lead to increased cell skeleton protein, which can damage the axial plasma transport and affect the intracellular signal transduction and protein phosphorylation or phosphorylation, so that axonal degeneration. At the same time, AGEs deposition in nutrient vessels of the nerve intima not only makes the lumen stenosis, occlusion, but also combines with RAGE on the endothelial cells to reduce the formation of NO, affect the nerve blood supply and lead to neuropathy. Recently Duran-Jimenez et al[40] found glycosylated ECM can also affect the nutrient supply of nerve fibers and bud proliferation and impact regeneration ability after nerve injury. As the support cells of peripheral nerve cells, Schwann cells play a crucial role in the process of injury and repair and regeneration of nerve.

The causes of non-healing wound such as in diabetic patients are very complex, which involves many molecular biology and much cross-linkage in mechanisms. The diabetic skin is greatly different from the normal skin such as AGEs accumulation, which makes it more susceptible to infection and injury. Once the wound is formed in diabetic skin, the wound may be non-healing.

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