The Differences of Cell Biology in the Repair Process of Wound and Refractory Wound Surface

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Abstract In the following paragraph we will discuss the differences of the cell biology in the repair process of wound and refractory wound surface. In the repair process of wound surface the cell biology in hemostasis phase, in inflammation phase, in proliferation, angiogenesis, fibroplasia and epithelialization phase and in contraction, maturation and remodeling phase in the normal organ or tissue such as skin after injury will be shown. The cell biology in the repair process of refractory wound surface, we mainly discuss the cell biology in refractory wound surface of the diabetes such as the effect of diabetes on the biological function of fibroblasts, M1/M2 macrophage imbalance in the repair process of refractory wound surface of diabetic, the effect of glycosylated extracellular matrix on fibroblasts and so on.

Keywords The cell biology · Repair process · Wound surface · Refractory wound surface · Diabetes

Skin repair after injury such as scald includes a complex programmed sequence of cellular and molecular progresses that involves hemostasis, inflammation, proliferation, and maturation, which include multiple cell populations, the extracellular matrix (ECM) and the action of soluble mediators such as cytokines (including growth factors). In this paragraph we mainly talk about the differences of cell biology in the repair process of wound and refractory wound surface.

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1 The Cell Biology in Hemostasis Phase

The platelets that can release cytokines (including growth factors), chemokines, and hormones play a crucial role in clot formation during hemostasis after aggregation and attachment to exposed collagen surfaces and activated in the initial stage of injury. Cytokines (including growth factors) have emerged as important mediators in repair process. As we know, cytokine is released from various cells, which can bind to target cell surface receptors to stimulate a cell response by endocrine, paracrine, autocrine, or intracrine routes. Platelets elaborate a number of proinflammatory substances and growth factors such as platelet-derived growth factors (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and so on. PDGF is released from the alpha granules of platelets and is responsible for the stimulation of neutrophils and macrophages, and also a mitogen and chemotactic agent for fibroblasts and smooth muscle cells, which can stimulate angiogenesis, collagen synthesis, and collagenase. VEGF contributes to angiogenesis by stimulating the mitosis of endothelial cells. TGF-β promotes proliferation of fibroblasts, regulates its own production in an autocrine manner and produces proteoglycans, collagen, and fibrin. It also promotes accumulation of ECM and fibrosis. All these cytokines (including growth factors) act on surrounding cells and stimulate chemotaxis of neutrophils, monocytes, and fibroblasts to the area of injury. So chemokines released by platelet activation attract inflammatory cells to the area, leading to the next phase i.e. inflammatory phase in the repairing process in the adult body.

1.1 The Cell Biology in Inflammation Phase

Neutrophils, monocytes/macrophages and lymphocytes are the main cells in the inflammatory phase in the wound surfaces area.

Neutrophils cleanse the wound site of bacteria and necrotic matter and release the chemotaxis such as interleukin 8 (IL-8) that chemotactic the macrophages and other cells involved into the wound site. Chemokines (or chemotactic cytokines) are small heparin-binding proteins that direct the movement of circulating leukocytes to sites of inflammation or injury via interaction with specific membrane-bound receptors and, as such, contribute to the pathogenesis of a variety of diseases [1]. Depending on the spacing or presence of four conserved cysteine residues, chemokines are classified into CC, CXC, CX3C, and XC families. CXC chemokines primarily attract neutrophils and lymphocytes and are believed to orchestrate the early phases of wound healing [2].

On the other hand, neutrophils produce reactive oxygen species (ROS) and proteases and also function to debride devitalized tissue. These functions are required in a timely manner. Neutrophils are produced in the bone marrow from stem cells that proliferate and differentiate to mature neutrophils fully equipped with

an armory of granules. Neutrophils are dormant in the blood circulation. Once trauma and infection occurred the neutrophils are activated and the first to arrive the wound site. Neutrophils can release the particles of granules not only to fight microorganisms but also to cause great tissue damage. In the burn wounds, neutrophil infiltrates in the skin tissue in 4 h after the injury and reached the peak level 24 h later. The number of neutrophils decreased after 48 h of injury. At sites of infection and trauma, endothelial cells capture bypassing neutrophils and guide them through the endothelial cell lining whereby the neutrophils are activated and tuned for the subsequent interaction with microbes.

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 site. In the adult body, no macrophages, no wound repair.

Following the neutrophils the monocytes involve in the wound site and become the macrophages. Macrophages play a central role not only in the inflammatory phase but also in all stages of repairing. Their functional phenotype is dependent on the wound microenvironment. During the early and short inflammatory phase macrophages phagocytose debris and bacteria and produce and orchestrate inflammatory cytokines (including growth factors) such as Tumor Necrosis Factor (TNF), Interleukin-6 (IL-6), Interleukin-1 (IL-1) and basic fibroblast growth factor (bFGF) and so on. IL-1 stimulates inflammatory cell proliferation and promotes angiogenesis. TNF-α is secreted from macrophages and as a mitogen for fibroblasts. bFGF is a chemotactic and mitogenic factor for fibroblasts and endothelial cells and other mesenchymal cells and also is an important stimulus for angiogenesis, that facilitate the repairing process.

Then let's talk about macrophages. Macrophages are known to produce collagenases and elastases, which remove the damage tissue by phagocytosis and make the wound clean.

Depending on the stimulus in vitro, activation of macrophages has been classified into two populations. The classical (M1) activation results in a highly pro-inflammatory macrophage phenotype, with microbicidal activity and pro-inflammatory cytokine production, and is mediated by like Toll-like receptor (TLR)-4 ligands and interferon-γ (IFN-γ). The alternative (M2) activation can reduce inflammatory reaction, promote tissue repair and humoral immunity, and is mediated by IL-4 and/or IL-13.

The phenotype of wound macrophages in this phase is probably the classically activated or the so-called M1 phenotype. During the proliferative phase, macrophages stimulate proliferation of connective, endothelial and epithelial tissue directly and indirectly. M2-type macrophages release some growth factors such as PDGF, acid fibroblast growth factor (αFGF) and bFGF, transforming growth factor α (TGFα), macrophage-derived growth factors. Especially fibroblasts, keratinocytes and endothelial cells are stimulated by macrophages during this phase to induce and complete ECM formation, reepithelialization and neovascularization. Subsequently, macrophages can change the composition of the ECM both during

angiogenesis and in the remodeling phase by release of degrading enzymes and by synthesizing ECM molecules [3, 4].

Besides, M1 and M2 promote T help 1 cells (Th1) that play the main role in cellular immunity and T help 2 cells (Th2) that play the main role in humoral immunity responses, respectively. Products of Th1 such as interleukin-2 (lL-2), interferon (IFN) and Th2 such as interleukin-10 (IL-10), interleukin-4 (IL-4) responses 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, M1/M2 also demonstrated the importance of Innate Immunity [5, 6]. This suggests an important role for alternatively (M2) activated macrophages in this phase of wound healing.

Recent studies have been showed another factor, autophage, may play role of the cell 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 regulates inflammation through controlling interactions with innate immune signaling pathways, by removing endogenous inflammasome agonists and through effects on the secretion of immune mediators. At the same time, autophage participate in antigen presentation and to T cell homeostasis, and it can affect T cell polarization and repertoires [7].

During the inflammatory phase, lymphocytes migrate into the wound area approximately 72 h post injury. Lymphocytes produce lymphokines such as bFGF, heparin-binding epidermal growth factor (EGF) and so on. T lymphocytes arrive to wound through IL-1 induced, which also contributes to the regulation of collagenase. Therefore, lymphocytes also play an important role in antibody production and cellular immunity. As mononuclear cells, T lymphocytes continue to replace macrophages and other inflammatory cells, their proliferation phase begins. They take wound repair into the end of inflammatory phase, the evolving milieu of eicosanoids in the wound interact with the cell types present, resulting in fibroblast synthesis of collagen and other substance. Additionally, the macrophage-derived growth factors are now at optimal levels, strongly influencing the influx of fibroblasts and then endothelial cells and keratinocytes into the wound.

2 The Cell Biology in Proliferation, Angiogenesis, Fibroplasia and Epithelialization Phase

Angiogenesis, fibroplasia and epithelialization occur during the proliferation phase. Formation of granulation tissue is a central event during the proliferation phase. Its formation occurs 3–5 days following injury and overlaps with the preceding inflammatory phase. A rich blood supply is vital to sustain newly formed granulation tissue. The macrophage is essential to the stimulation of angiogenesis and

produces macrophage-derived angiogenic factor in response to low tissue oxygenation. This factor functions as a chemoattractant for endothelial cells. Besides, the macrophages secrete bFGF and vascular endothelial growth factor (VEGF), which are also important to angiogenesis. Endothelial expansion contributes to angiogenesis, as intact vessels generate buds in granulation tissue. Neovascularization facilitates growth of the advancing line of fibroblasts into the wound, providing them with necessary nutrients and cytokines. The fibroblasts is a critical component of granulation tissue, which grow in the wound as the number of inflammation cells decrease. Two to three days after injury, the fibroblasts migrate inward from wound margins over the fibrinous matrix, which has been established during the inflammatory phase. During the first week, fibroblasts begin to migrate, proliferate and produce glycosaminoglycans and proteoglycans, the ground substance for granulation tissue, as well as collagen, in response to macrophage-synthesized growth factors such as PDGF, FGF, VEGF, TGF-α, TGF-β and etc. Type III collagen is the primary component of early granulation tissue. Fibroblasts soon become the dominant cell type, peaking at 1–2 weeks. The synthesis and deposition of collagen is a critical event in the proliferation phase and to wound healing in general. They generate not only collagen molecules but also growth factors such as PDGF, TGF-ß, bFGF, insulinlike growth factor-1(IGF-1), keratinocyte growth factor (KGF) and so on. Angiogenesis results in greater blood flow to the wound and, consequently, increased perfusion of repairing factors. Degradation of the fibrin clot and provisional matrix is accompanied by the deposition of granulation tissue (ground substance, collagen, capillaries), which continues until the wound is covered. Angiogenesis ceases as the demand for new blood vessels ceases. New blood vessels that become unnecessary disappear by apoptosis.

Fibroplasia starts on 3–5 days following injury and may sustain as long as 14 days. Fibroblasts produce the collagen, fibronectin, glycosaminoglycans, and other components of ECM. Fibroblasts are able to assemble cross-linked and fascicular fibers using collagen molecules. This synthesis work would last about 2–4 weeks. In normal skin there is approximately 80 % of the collagen identified type I collagen; the remaining is mostly type III. Collagen is the major component of acute wound connective tissue, it will continue to produce in the next 6 weeks. The accumulation of wound collagen is related to the increase of tensile strength. Collagen is rich in hydroxyproline and hydroxylysine moieties, which promote to form a strong cross-link structure. The hydroxylation of lysine and proline residues depends on the presence of oxygen, vitamin C, ferrous iron, and α-ketoglutarate. Particularly, deficiencies of vitamin C and oxygen result in under-hydroxylated collagen that is less capable of forming strong cross-links and is easier to breakdown. The formation of collagen is carried out on extracellular. First cells secret procollagen. Then procollagen is cleaved of its terminal segments and called tropocollagen. Collagen filaments can be formed through aggregate of tropocollagen molecules. Moreover, the cross-linked structure of intermolecular makes collagen fiber stabilize and resistant to destruction. Collagen fibers are deposited in a framework of fibronectin, which is closely connect with fibronectin In addition,

fibronectin can paly a role of an anchor to make the myofibroblast migrate into the wound. At the moment, granulation tissue is gradually formed, and the wound begins to contract.

Epithelialization is the formation of epithelium that involves cell migration and covering the wound area. Firstly, epidermal cells at the wound edges, under their structural changes, detach from their basement membrane. 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 [8]. The polar change of actin cytoskeleton intracellular causes the cell generate these deformations. 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 a word, cell movement is based on the direction of polarity of the cells.

The initial step of cell polarization is that intracellular actin polymerizes to form ruffles or leading pseudopodia. The Rho family small guanosine triphosphate (GTP)-binding proteins (GTPases) are pivotal regulators of actin organization and control the formation of lamellipodia and filopodia. At the sites where contact with the extra cellular matrix (ECM) occurs, big protein complexes are assembled through the recruitment and the clustering of receptors of the integrin families. These large protein molecule structures are known as focal adhesions or focal contacts. There are known two types of migration mode: “Integrin/MMP dependent mode” and “Integrin/MMP-independent mode”.

“The dependent mode of cell migration” is called “mesenchymal”. Surface proteases, such as MT1-MMP, break down pericellular matrix molecules locally to provide sufficient space of cell expanding. Shortly after integrin binding with ECM, contractile proteins connect with cytoplasmic actin filaments, such as myosin II, which can stabilize and shorten the membrane-tethered actin filaments. This results in local cell contraction, generally at the opposite pole respect to the leading edge. Another mode is called “ameboid”. Cells also can migrate across connective tissue within pre-existing ECM pores by simply squeezing.

The formation of Intracellular actin microfilaments makes the epidermal cells crawl across the wound surface. Epidermal cells can secrete collagenases and plasminogen activator, collagenases break down collagen, plasminogen activator stimulates the production of plasmin, which promotes clot dissolution along the pathway 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. Besides, as the cells migrate, they dissect the wound and separate the overlying eschar from the underlying viable tissue. The stem cells are found in the deep rete ridges, leading them to propose 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.

When epithelialization is complete, the epidermal cell restores its original morphology, and forms new desmosomal linkaging to other epidermal cells, and

hemidesmosomal linkages to the basement membrane are restored. At the same time, epithelial cells continue to migrate inward from the wound edge until the defect is covered. The transformation of fibroblasts into myofibroblasts which contain contractile actin fibers, is contact inhibition induced. Then the new tissue replaces injured tissue volume.

2.1 The Cell Biology in Contraction, Maturation and Remodeling Phase

Contraction, defined as the centripetal movement of wound edges that facilitates closure of a wound defect, is maximal 5–15 days after injury. The result of contraction is decreased wound size which depends on the degree of tissue laxity and shape of the wound. The process of wound contraction is usually accompanied by collagen synthesis. During this phase, collagen remodeling depends on continued collagen synthesis in the presence of collagen destruction. For the first 6 weeks, new collagen production dominates the wound healing process, deposited randomly in acute wound granulation tissue. As the wound matures, collagen is remodeled into a more organized structure with increased tensile strength. With collagen synthesis, matrix metalloproteinase collagenolysis achieves a steady state.

Collagen forms tight cross-links to other collagen and with protein molecules, increasing the tensile strength of the healing wound. Stress, age, pressure and tension affect the rate of collagen synthesis. Loose tissues contract more than tissues with poor laxity, and square wounds tend to contract more than circular wounds. Wound contraction does not seem to depend on collagen synthesis but depends on the myofibroblast located at the edge of the wound, its connection to myofibroblast proliferation and components of the ECM.

In remodeling phase, collagen becomes organized increasingly. During this phase, a balance exists between formation of new collagen and removal of old collagen depending on collagenases and matrix metalloproteinases in the wound to assist removal of excess collagen while synthesis of new collagen persists. Fibronectin gradually disappears, and proteoglycans instead of hyaluronic acid and glycosaminoglycans. Gradually, type I collagen replaces type III until the normal skin ratio of 4:1 is achieved. The cross-links of Intramolecular and intermolecular collagen result in increased wound bursting strength. Remodeling begins approximately 21 days after injury, when the net collagen content of the wound is stable. Remodeling may continue indefinitely. Bursting strength varies with skin thickness. The tensile strength of the wound reached its peak at about 60 days after injury.

2.2 The Biology of Stem Cell in the Repair Process of Wound

Stem cell research has become one of the hot points in the repair process of wound because the stem cells have the characteristic of self-renewing, differentiated into multiple types of total specialized cells of the body [9]. But it still exist many problems because we still don't know how many stem cells which include many types still existing in our organism when we leave the uterus. Such as what kind of damage and microenvironment can "home" the stem cells and induce them to differentiate into the appropriate cells to remodel damaged tissue. Nevertheless, the stem cell research has helped our mankind to understand how single cell can grow and develop into tissue and organ, and how the damaged cells can be replaced by healthy cells in adult body, which guides us to explore the cytological pathway to treat disease.

Kucia et al. have found and identified a population of stem cells in the BM, they are small (about 2–4 μm), but have large nuclei surrounded by a narrow rim of cytoplasm, and contain open-type chromatin (euchromatin), express several markers such as SSEA-1, Oct-4, Nanog and Rex-1, they are typical embryonic stem cells (ES). These cells can differentiate into all three germ-layer lineages in vitro. So they are also called very small embryonic-like (VSEL) stem cells. These cells have the characteristics of age-dependence. With the increase of age, the number of them is gradually reduced. They are barely detectable in 1 year old mice which correspond to a 50 year old human. This feature may be one of the reasons why the regeneration of young individuals is more effective than aged. It has been provided that as the organ is damaged, non-hematopoietic stem cells (including VSELs) are enter the peripheral blood circulation from the BM to "home" to the damaged tissues and participate in tissue repair. These cells may efficiently differentiate and regenerate into special tissue cells to replace the damaged cells in injuries. During this time damaged tissues up-regulate the expression of several chemotactic factors, which may participate in the homing and inducing differentiation of VSELs. But, if these cells migrate to the wrong place or/and migrate at the wrong time, they may lead to the formation of pathological diseases, such as tumor formation.

Adult stem cells are present in tissues and organs of the body, which have the potential to self-renew and differentiate into various types of cells. The process of differentiation is regulated by multiple genes. So that they can develop into specific structures and perform special biological functions. Some local adult stem cells are differentiated to supply new cells that effectively replace senescent ones or those undergoing apoptosis such as epidermal stem cells differentiated and developed into several layers of epidermis in maintaining normal metabolism condition of our skin [10]. In some injuries such as second-degree burns, some adult stem cells that are located in the wound or wound edge can be rapidly differentiated, proliferated and migrated with still healthy terminated cells to replace the damaged tissue cells, even in the microenvironment of chemoattractants that may express and secrete by damaged tissue cells or/and healthy cells, and finally restore the damaged tissue to

the normal condition. Because there are no healthy cells, structures and even adult stem cells in site used to wound repair, such as the local area of severe burns or three-degree burns, bone marrow stem cells as precursor cells of other stem cells, play an important role in the reconstruction of various kinds of trauma. For example: many fibroblasts are derived from the blood delivered cells harvested from BM in the early stage of granulation formation of the repairing process in the local severe wound surfaces area.

Now the hypothesis of "Stem cell Niche" has become the hot point to stem cell research. Some scholars believe that "Niche" may be a habitat, such as the limbal SC niche, in which SCs could remain stable in this environment or microenvironment that not to differentiation. Adult SCs are regulated by microenvironment of their "niche", i.e., the adult-specific SCs in "niche" are maintained in undifferentiated state, and their biological functions, consisting of other cellular and extracellular components have been adjusted accordingly in the vicinity of the area. So, the differentiation of stem cells may be synergistically regulated by various factors of micro-environmental, such as gene expression, cell-cell contacts, cell-matrix interactions and etc. The existence of niche and surrounding cells may guard stem cells to have a stable reserve force, by avoiding stimulation of differentiation and apoptosis. Stem cells, in the niche, not only have the ability of self-renewal, but also when the niche or the surrounding environment is stimulated from outside, they are able to differentiate. At that time the niche would modify to ensure that SC activity parallels the organism's needs for particular differentiated cell types. However, if the adult stem cells are differentiated or/and differentiated cells migrate and proliferate into the inappropriate region, such as defection or without of "dermal template" or niche, it may result in "abnormal repair", e.g. scar formation. But there are still a lot of issues that need to be explored, such as the multiple signal-pathways relative to stem cell differentiation, and the corresponding microenvironment of niche and surrounding that is suit for stem cell biological changes.

In view of the characteristics of bone marrow stromal cells (BMSCs), which are easily harvested from bone marrow, easy to culture in vitro and could be re-introduced into patients as autografts without serious ethical and technical problems, many researchers have used them as the ideal seed cells to transplant onto various medias such as denuded human amniotic membrane (AM). It has been used in many surgical procedures, such as skin equivalent and vaginal reconstructive surgery, and also for human embryonic SC differentiation into neural cells as well as for supporting chondrocyte proliferation and phenotype maintenance in vitro and the regeneration of osteochondral defect in rabbits [9]. Some researchers have confirmed that the amnion-derived cellular cytokine solution can promote the migration of macrophages during wound repair [11]. So the bio-scaffold with appropriate three-dimensional structure e.g. niche or "dermal template" and their components such as collagen of ECM may have a special influence on SC differentiation or "homing" SC and then assisting them to differentiate to form a functional tissue or organ. And the problem we are facing now is that due to the structure and content of bio-scaffold is always suffered a certain degree of damage

in the process of production, we can't create an ideal bio-artificial scaffold that is completely consisted with natural structure and environment.

Above all shows the cell biology in normal organ or tissue such as skin after injury. But many common chronic wounds such as diabetic foot ulcer, pressure ulcer, venous stasis ulcer and etc. Considers specific type of nonhealing wounds such as pressure ulcer, leg ulcers, diabetic foot wounds, surgical and malignant wounds as well as lymphoedema and dermatological conditions associated with skin breakdown [12]. Here we mainly discuses the diabetes and cell biology in refractory wound surface.

3 Diabetes Has Multiple Effects on Cell Biology in Refractory Wound Surface

Chronic wounds include vasculitis, non healing ulcer, pyoderma gangrenosum, and disease that cause ischemia. There are so many physiologic factors which contribute to wound healing deficiencies in individuals, such as decreased, hyperglycemia or impaired growth factor production, cytokine receptor, angiogenic response, macrophage function, collagen accumulation, epidermal barrier function, 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 [13–18].

Diabetic patients with spontaneous rupture of skin, such as diabetic lower extremity ulcers, wound or trauma is difficult to heal, all is a hotspot and difficulty in clinical research.

In recent years, the research on the mechanism of diabetic wound healing focus on harmful substances deposited, such as advanced glycation end products (AGE), high glucose deposition, cell signal transduction, cell apoptosis and cell function, blood vessels and extracellular matrix etc. Histological observation also showed that the thickness of the epidermis and the dermis of skin tissue of diabetic rats was significantly thinner, the epidermal cell layer is not clear, partial epidermis lack of multiple layer arrangement, significant reduction in the number of heckle cells, dermal collagen arrangement disordered, partial collagen degeneration, fracture, focal infiltration of chronic inflammatory cells was seen in the area of collagen degeneration. This shows that the skin tissue of diabetic patients in the absence of injury has been the existence of the changes in histology and cell biology behavior; this is a kind of "**Underling Disorder**", that does not result in the integrity and continuity damage of the skin. This damage is endogenous, although it does not cause skin defects or damage to the visibility of the damage, but because of the change of histology and cell function, can make the skin tissue to increase the vulnerability of exogenous damage [19].

3.1 Neutrophils

The normal healing process can be defined by a number of overlapping events: clot formation, inflammation, reepithelialization, angiogenesis, granulation tissue formation, wound contracture, scar formation, and tissue remodeling. Diabetic wounds are characterized by functional defects in the majority of these events, leading to impaired wound healing, in addition to local ischemia caused by well-recognized macro- and microvascular occlusive disease. Usually, impaired wound healing in diabetic patients is accompanied by decreased early inflammatory cell infiltration but persistence of neutrophils and macrophages in the chronic, impaired wounds [20].

3.1.1 The Biological Characteristics of Neutrophils in the Diabetic Impaired Wound Healing

A series of function changes of neutrophil will occur in diabetic state. Insulin levels in patients with diabetes have a certain effect on the function of neutrophils. In the research [21] of 8 healthy volunteers showed that after treated with insulin, neutrophil chemotaxis, phagocytic ability and sterilizing ability were improved, and the control group showed significant differences. At the same time, Okonchi's [22] research found, high concentrations of insulin can promote neutrophil transmembrane swimming and the expression of the platelet endothelial cell adhesion molecule-1 increased. When used with Gliclazide drug, can inhibit the abnormal function of neutrophils. From the above research, it can be speculated that the level of insulin in the body can directly affect the function of neutrophils. Then, the secretion of insulin in patients with type 1 diabetes is insufficient. This is one of the reasons that diabetes patients are susceptible to infection and impaired wound healing. Although there is no reduction in insulin secretion in patients with type 2 diabetes, neutrophil receptor glycosylation also affects the binding of neutrophils to insulin, which affect neutrophil function.

Tennenberg et al. [23] found that neutrophils from patients with diabetes are prone to apoptosis, the authors believe that this 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.

Long term hyperglycemia may lead to the production of a large number of advanced glycation end products (AGEs) in the body. Study of Collison et al. [24] found that AGEs could be high affinity with the human neutrophil AGER (AGE Receptor) and lead to increase in intracellular calcium and actin polymerization, which will depress the transendothelial cell migration and sterilization ability of neutrophil. Tian et al.'s research found [25, 26] that neutrophils couldn't reach the basal part of the wound in time and form a dense inflammatory zone. A large number of neutrophils were scattered around the wound. Immunohistochemistry

showed that AGE was distributed in the skin tissue of diabetic rats. Neutrophil migration test is shown in vitro that AGE can inhibit the migration of the neutrophil by binding its receptor on the surface of the neutrophil. At the same time, neutrophil and AGE combined outside the vascular tissue leads to a large number of inflammatory cytokines are released and oxidative stress burst by neutrophils. This release and burst are delayed and lasts longer than the normal wound.

Some studies have also similar views, Osar et al. [27] confirmed that the neutrophil oxidative burst index decreased significantly compared with the control group ($p < 0.05$), coenzyme I (NADPH) activity decreased by studying 30 type 2 diabetes patients. Gustke et al. [28] found that the average neutrophil chemotaxis index was significantly lower than that of the control group ($p < 0.02$) in type 1 diabetic patients. This changed cell function of neutrophil were dependent on HLA-DR3, DR4, and DR5 genes.

Wound healing involves many complex, interrelated processes that involve multiple cell types. Neutrophil plays an important role in the normal healing process, but abnormal neutrophil function may contribute to the pathogenesis of nonhealing wounds present in diabetic patients. A better understanding of the molecular mechanisms and cellular interactions of neutrophil in diabetic patients, is critical for the development of novel therapeutic strategies to promote diabetic wound healing.

3.2 Macrophage

Abnormal macrophage function in process of wound healing may not be conducive to the normal development of wound and lead to adverse results, such as the formation of ulcers, chronic wounds, hypertrophic scars and keloids. During the process of impaired wound healing, macrophage activation phase and degree were abnormal, wound repair process cannot be in accordance with the conversion from severe inflammation to mild inflammation state. Compared with the normal repair of acute wound, impaired wound is usually stuck in inflammatory phase and it was found that there was an in situ retention of macrophages.

3.2.1 M1/M2 Macrophage Imbalance in the Repair Process of Refractory Wound Surface of Diabetic

Impaired wounds such as diabetic wounds and chronic venous ulcer were found abnormal inflammatory retention and reduced granulation tissue state [29]. The first evidence is the number of aaM in wound area more than caM in diabetic wound healing model using db/db mice. Miao et al. [30] found a decrease in iNOS level, which is marker of caM(M1), on days 1 and 3 after wounding in STZ-induced diabetic rat lesion, especially on day 3, compared with the normal rats. The expression of Arg-1, which is marker of aaM(M2), in the diabetic group was lower

than in normal group on day 7, but increased sharply and significantly higher on day 13. The study showed that the M1 in the non diabetic SD rats mainly appeared in the inflammatory phase and gradually replaced by the M2 in the repair of the proliferative phase. The infiltration of macrophages ($CD68^+$) in the scald wound of STZ-induced diabetic rats was "slow in and slow out" which insufficient at early stage, and detained at late stage. Compared with normal rats, the expression of iNOS in the early stage of diabetic rats was decreased, Arg-1 was increased in expression, IL-4, IL-10 and other anti-inflammatory factors were relatively higher, 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, that is, changes in performance for insufficiently pro-inflammatory at early stage, at late stage the pro-inflammatory and anti-inflammation disordered, and with anti-inflammatory as the main.

So the balance of caM/aaM is very important in the process of wound healing. It is found Th2-aaM-Arg-1 increased in streptozotocin-induced diabetic wounds. But, abnormal caM may also lead to bad results. Unrestrained proinflammatory caM induced by iron and too many TNF-a positive macrophages, which are considered as caM cells, impairs wound healing in humans and mice [31]. An imbalance of caM/aaM in wound healing may delay and even hinder skin defect restoration. It appears that successful healing requires the activation of macrophages at an appropriate phase and a suitable extent.

There is large number of accumulated AGEs founded in diabetes mellitus, and AGEs might induce macrophages to product TNF-α to influence wound healing. Goren et al. [32] found that in the dorsum of ob/ob mice full thickness concise was observed in the number of abnormal TNF-a positive caM, and at the late stages of inflammation (post injury 7, 9, 11 days) in removal wound caM secreting TNF-a, thus launched a fast impaired wound epithelialization process. Dong et al. [33] 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. Suggesting that, there are inflammatory disorders during the process of some impaired wound healing such as diabetes, in this environment due to anomaly time phase of activated macrophages, macrophages cannot successfully complete from the early inflammation M1 based active state to late inflammatory M2 based activation state transition. Trem2 is a cell surface receptor that is specifically induced in macrophages by IL-4/IL-13 and is important in injury responses. Wound healing in Trem2−/− mice showed an increased expression of caM markers, decline aaM markers. This wound also demonstrated diminished burst of epithelial proliferation and wound closure rate [34].

One of the key factors of effective wound healing is proper phenotypes transformation of macrophages from the phase of pro-inflammatory to 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. [35] 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 is 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.

In short, many studies confirm the importance of macrophage activation in wound healing. The imbalance of CaM and aaM may cause impaired wound. Moreover, regulation of the balance between caM and aaM may be regarded as a therapeutic strategy to promote wound healing.

3.3 Endothelial Cells

Wound healing process can be split to 3 distinct phases [36], endothelial cells participate in all the phases, and play the deferent roles in each one. That can be summarized as the excessive activation of ECs in inflammation phase (lost or declined vascular barrier function), but the weakened or obstacled ability of angiogenesis in late phase.

It has been reported that ECs were suffered from increased apoptosis, up-regulation of secreting of adhesion molecule (such as ICAM-1, 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 [37, 38]. All that could help leukocytes to gather on vascular wall, and cells more easily migrate from vessel to the injured area. It may be the one of the reasons for the presence of sub-inflammatory phenomena, being susceptible to infection and the sustained inflammatory response after trauma in diabetic skin.

As we all known, diabetic skin inflammatory cells infiltration excess normal skin. The disordered ability of re-establish blood supply in injury area can ultimately lead to the occurring of chronic wounds. Many researchers have confirmed that some of the biological functions of ECs, such as, migration, angiogenesis, and secretion of angiogenic-relative cytokine, have been down-regulated in high glucose or AGEs environment. Dr. Qiao. investigated the degree of neo-vascularization in STZ-induced diabetic rats suffered partial thickness scalding of 20 % total body surface area (20 % TBSA) on back, and the quantity of vascular ECs by double-labeling immunofluorescence. He found that vascular ECs can proliferate actively in the diabetic wound with burns, but it is still poor in blood supply due to lack of functional capillaries. The mechanism may be related to sustained abnormal high expression of Ang-2 and down-regulated VEGF [39].

New evidence-based medical research shows that: if the patient in hyperglycemic state for a certain time, even after the blood glucose levels returned to normal, still prone to vascular complications, this phenomenon called “hyperglycemia metabolic memory” (metabolic memory) [40]. Dr. Li et al. established endothelial cells memory model of high glucose to evaluate cell proliferation,

apoptosis, ROS, SOD, MDA, eNOS mRNA, and NO levels in supernatant of endothelial cell culture media. These results suggest that in hyperglycemia memory cell model, transient hyperglycemia may lead to persistent imbalance in oxidative stress and reduce endothelium—derived relaxing factor NO level, indicating that hyperglycemia memory may play an important role in persistent vascular endothelial cell injury.

3.3.1 Endothelial-Mesenchymal Transition, EnMT

Recently, the concept of EnMT has attracted more and more attention of researchers. Cell differentiation is a process which is characterized by the loss of the 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 [41]. Once ECs are subjected to mechanical tension, oxidative stress, abnormal lipid metabolism, inflammation, hypoxia and other external stimuli, will change their composition, structure to adapt to these changes. EnMT could be occurred by TGF-ß signal pathway, and also by Notch signal pathway which inhibits the expression of endothelial cell adhesion molecule VE-cadherin [42–44]. Endothelial cells which were occurred EnMT, the adhesion junctions were disrupted and the destruction of this continuity makes the vascular wall surface to become rough, exposed sub-endothelial collagen, platelet adhesion enhancement, coupled with the increased expression of endothelial cell adhesion molecule, promoting the adhesion of leukocyte-endothelial and the adhered leukocyte can also injury endothelial cell through the release of elastase, forming a vicious circle. High-glucose environment can activate oxidative stress in ECs by AGEs, the classical polyol pathway, PKC, the sorbitol pathway, the acetylglucosamine pathway, and et al. The activated oxidative stress can increase the active oxygen species in blood, reduce vascular diastole factor such as endothelial cell NO and prostacyclin, raise vascular contraction factor such as ET-1, thromboxin A2. ET-1 can active inflammatory reaction to promote the formation of leukocyte adhesion, TNF-α and so on. This may also be one of risk factors of wound healing.

3.3.2 Endothelial Progenitor Cells

Another important factor for revascularization in wound area is successful mobilization of endothelial progenitor cells (EPCs) from the bone marrow to injury area and participate in vasculogenesis. EPCs proliferate, migrate, differentiate, and produce proangiogenic cytokines during the process of angiogenesis. They are also charge of maintenance and repair of endothelial cells. EPCs express markers of both hematopoietic stem cells (CD34 and CD133) and endothelial cells (CD146, vWF, and VEGFR2) [45–47]. Dysfunction of EPC may promote to vascular pathological disease. Also, the decrease in number of EPCs from peripheral blood and dysfunction EPCs were found in patients with diabetes and cardiovascular disease.

In the normal process of wound healing, EPCs are effectively recruited to participate the remodeling microcirculation, and leading to wound repair in time. But in diabetic ulceration, this situation could be partial inhibited. Such as, it is recently reported that EPCs from normal but not diabetic patients contribute to postischemic revascularization. The number and function of diabetic EPCs were 5 times lower than that of normal EPCs, and show a tendency to pro-inflammatory phenotype. The bone marrow derived EPCs in diabetes mellitus are dysfunctional, due to oxidative stress, they produce fewer endothelial cells with reduced proliferative, and migratory potential. The number and function of EPCs act as a surrogate marker of vascular health and indicate cardiovascular risk in healthy persons [48–50]. Feng et al. [51] showed that oxidized low-density lipoprotein (OxLDL) can inhibit survival of EPCs that derived from umbilical cord, and impair their function, thus inhibiting the production of eNOS.

As we know there are increased oxidative stress levels in the body of diabetes, ROS can promote EPCs to secret many pathologic cytokines to increase iNOS level and decrease eNOS level. The reduced functional activity of EPCs during hyperglycemia involves the Akt/eNOS pathway, where signaling is downregulated under diabetic conditions [52, 53]. Ii et al. [54] have attributed the phenotypic differences of EPCs during diabetes to decreased thrombospondin-1 expression. There is an indication that upregulation of cyclin-dependent Kinase (CDK) inhibitors p16 and p21 leads to a reduction in proliferating EPCs under hyperglycemic conditions [55]. Although we have got a lot of evidence as above, but we still don't known clearly what molecular mechanisms affect the number and function of EPC in diabetes mellitus.

3.4 Fibroblast

The role of fibroblast (FB) in wound healing process is extremely important. They are involved in establishing the emerging basement membrane and subsequent reepithelialization. So any dysfunction of FBs will affect wound healing and ultimately lead to chronic, nonhealing wounds. FBs in diabetic skin and mellitus have been threaten by high glucose environment, and their biological function also occurred corresponding change, therefore we frequently find the cases of impaired wound healing with diabetes in clinical.

3.5 *The Effect of Diabetes on the Biological Function of Fibroblasts*

3.5.1 Fibroblast Proliferation

FB is a higher proportion in the number of cells in the dermis and a kind of dominant repair cells, the proliferation of normal metabolism and repair of dermal tissue after trauma depends mainly on the FB, cell cycle analysis is to understand the direct index of cell proliferation. Cell proliferation is a multi stage, multi factor involved in the orderly regulation process, which is achieved through the cell cycle. In this process, the cells were followed by phase G1, S, G2 and M to complete the proliferation. The most critical stage is the S phase because DNA multiplication and chromosome replication in this stage. The study of Wang et al. [56] found that FB were increased significantly in S phase but decreased obviously in phase G0/G1 and in G2/M phase in diabetic skin of rats compared with normal skin of rats (showing in Table 1). The results indicated that FB have the ability to self renew but can't effectively complete the proliferation in the diabetic state. This phenomenon of "DNA replication but can't effectively enter the G2/M phase and finish chromosome replication and cell division and proliferation" may be one of the important reasons of having complication such as diabetic foot. This phenomenon of "DNA replication but not division or proliferation" may be one of the important reasons of delayed or impaired wound healing in diabetes. But further biological effects in this phenomenon should be explored. Other results showed [57] that the number of FB in phase S were all significantly lower than those in normal rats in diabetic skin with deep II degree burns, on the 3, 7, 14 and 21 days of post injury. This indicated FB proliferation was inhibition completely during the repair process of diabetic wound. In the diabetic skin, FB due to the long-term in a composite environment of high glucose, high AGEs or other substances, its proliferation state has been affected (percent of S phase was increased) and entered self-renew stage. When the diabetic skin tissue was injured, the original should enter the repair process (the percent of S phase of FB in normal group was observably increased in 3 days post injury, and gradually decline in the late) has not been started, FB has unexpectedly been in state of inhibited proliferation, which affected the process of wound healing. It suggests that the changes of diabetic wound microenvironment may be one of the causes of impaired diabetic wound healing.

3.5.2 Skeleton Structure of Fibroblast

Many functions of the cell such as chemotaxis, movement and proliferation are mediated through the cytoskeleton, cell migration, differentiation and proliferation during the process of wound healing are accompanied by significant changes in the cytoskeleton system. Chen et al. [58] observed FB with transmission electron microscope and found that there is the expansive endoplasmic reticulum, the less

Table 1 Percentage of cell cycle of dermal FB in wounds after burns

Group	Before injury	Time after injury (day)			
		3	7	14	21
Normal rats (n = 6)					
G0/G1 phase (%)	82 ± 10	42 ± 13	56 ± 13	70 ± 7	77 ± 4
S phase (%)	17 ± 10	55 ± 16	44 ± 16	29 ± 6	23 ± 4
Diabetic rats (n = 6)					
G0/G1 phase (%)	66 ± 5**	70 ± 4**	88 ± 5**	82 ± 6*	84 ± 5*
S phase (%)	33 ± 5**	30 ± 4*	11 ± 5**	17 ± 6*	12 ± 4*

There is significant difference compared with normal group, $^{*}p < 0.05$, $^{**}p < 0.01$

developed Golgi complex, the lack of actin filaments and microtubules, and obviously increased lysosome in the cytoplasm in the dermal fibroblast of diabetic group But FB on normal control group within the dermis can be seen in different forms and maturity of FB, mostly fusiform, cytoplasm with abundant rough endoplasmic reticulum and well-developed Golgi complex, the nucleus was oval, nuclear chromatin pale staining, uniform distribution, and can be seen with FB and smooth muscle cells with characteristics of myofibrlblast (MFB), the cytoplasm appeared cells arranged parallel to the long axis of the microfilament bundle visible electron dense plaques (Fig. 1).

α-SMA can be connected to FB through “fibronexus”, a kind of transmembrane complex, to regulate cell contraction. It has been confirmed that there is a positive correlation between tissue contraction and the expression of a-SMA. MFB not only has strong synthesis function as FB, but also because of its expression of a-SMA

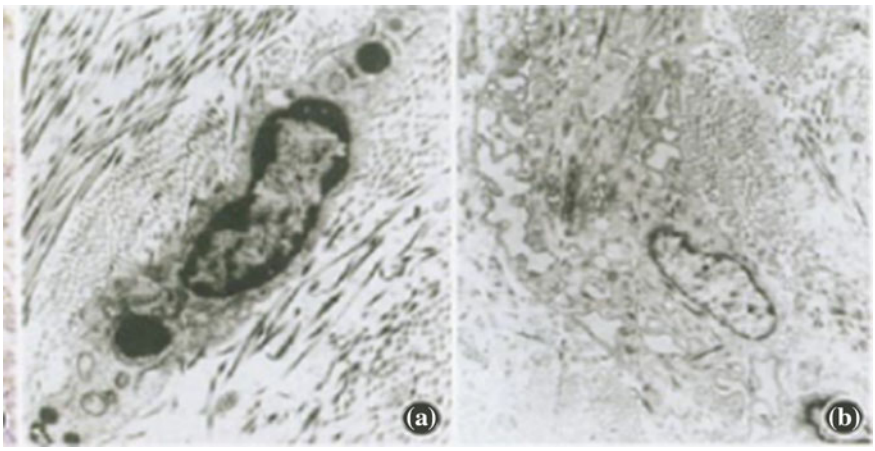

Fig. 1 Ultrastructural changes of FB in the 14 days after the burn injury of the rat model of diabetes mellitus and normal rats. **a** In diabetic rats, the cytoplasm of dermal fibroblasts lysosomes was significantly increased. TEM × 15000. **b** The normal rat dermis were observed in mature FB with spindle shaped. TEM × 800

and has stronger contraction ability than FB, is the main power of wound contraction. The lack of MFB and/or a-SMA inadequate expression in diabetic wound, which suggested that delayed or impaired diabetic wound healing may be related to the deficiency of wound matrix synthesis and contraction disorder. More important is that in the FB cytoplasm of diabetic wounds, in addition to the endoplasmic reticulum expansion and the lack of actin filaments, the lysosomal increased obviously, this might suggest the FB synthetic function of diabetic wounds was impaired, but the degradation function was enhanced.

3.5.3 The Effect of Glycosylated Extracellular Matrix on FB

AGE mainly accumulated in a long half-life, large molecular weight of protein, skin tissue is rich in collagen protein, AGE is easy to accumulate those parts, and more in the form of glycosylated collagen. Niu et al. mode the glycosylated ECM model in vitro, they confirmed that glycosylated matrix induced cell-cycle arrest and apoptosis of dermal fibroblast, while application of RAGE blocking antibody redressed these changes [59]. 3-deoxyglucosone (3DG) is one of the AGEs precursors. The migration ability of FBs can be efficiently reduced when cultured on 3DG-treated collagen [60]. Two years later this group provided that the inhibition in fibroblast migration, proliferation, and collagen expression by exposure to 3DG-collagen was mediated via extracellular regulated kinase 1/2 (ERK1/2) and Akt downregulation through activation of p38 MAPK (Mitogen-Activated Protein Kinase). They also indicated that 3DG-modified collagen can lead to oxidative stress, endoplasmic reticulum stress, and induce apoptosis via caspase-3 activation [61]. Therefore, Glycosylated ECM has an critical influence on the function of FBs in diabetic skin.

3.5.4 Secretion and Synthesis Disorder of FB

The synthesis of collagen in the matrix is the basis of tissue repair after skin burns. Lin et al. indicated the declined ability of collage synthesis and secretion of FB in diabetic wound tissue by measuring the contents of hydroxyproline and the ratio of collagen types I and III [58]. As the main cells of collagen synthesis, the proliferation disorder of FB obviously had a negative effect on collagen synthesis and wound repair.

It's reported that diabetic mice fibroblasts were detected with highly expression of matrix metalloprotease type 9 (MMP-9), and a severe impairment in VEGF production under normoxic and hypoxic conditions, showed an increased prodegradative activity [62]. The ability to synthesize NO was failure with the increase of MMP-8 and MMP-9 in human diabetic fibroblasts [63]. Also in human fibroblasts, they were confirmed the prodegradative phenotype by the increased MMP-2 and MMP-3 production and reduced collagens gene expression [64, 65]. In the process of wound healing dysfunction of producing NO is detrimental to wound

repair. NO donors' administration is considered to be a controller to restore the balance of MMPs and has the ability of stimulating cell proliferation [66].

3.5.5 The Effect of AGEs on Fibroblast

Any endogenous or exogenous factors that may lead to changes in the biological function of dermal fibroblasts may affect the biological process of wound healing. Wang et al. [56] confirmed that in vitro AGEs can inhibit the proliferation of FB, and induce cell apoptosis with dose dependent. And compared with diabetic rats, the sensitivity of FB to AGEs was higher in normal rats. Many scholars reported that primary dermal FB cultured in high glucose or AGEs medium, that was found inhibited the proliferation, decreased collagen synthesis, decreased 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). AGEs receptor (RAGE) was found on the membrane of fibroblasts,and persistent high expression of RAGE on FB in diabetic patients.

3.5.6 Effect of Oxidation Stress on FB in Diabetic Condition

AGEs was not conducive to wound repair, and it is found that there was an increase in oxidative stress in the site of AGEs deposition. Extended exposure to reactive oxygen species (ROS) is thought to lead to cellular dysfunction and organismal death via the destructive oxidation of intracellular proteins, lipids, and nucleic acids.

Diabetic wound is a special wound, diabetes itself can cause oxidative stress through a variety of ways. Ge et al. [67] detected out the significantly increased plasma H_2O_2 in STZ-induced diabetic rat model on the 14th day after deep II degree scald, and decreased GSH (with antioxidant capacity), that may also one of the reason to cause impaired wound healing. In vitro, H2O2 could decrease FB vitality and induce cell apoptosis, these damage could be partly reversed by treatment with antioxidant aminoguanidine [68]. Extracellular superoxide dismutase (ecSOD/SOD3) is a prime antioxidant enzyme in the extracellular space that eliminates ROS. Fujiwara et al. [69] confirmed that reduced SOD3 levels contribute to healing impairments in aged mice. SOD3 deficiency and aged fibroblasts both display reduced production of TGF-β1, leading to decreased differentiation of fibroblasts into myofibroblasts. These impairments include delayed wound closure, reduced neovascularization, impaired fibroblast proliferation, and increased neutrophil recruitment.

3.6 Keratinocytes

Diabetes is an important health issue because of its increasing prevalence and association with the development of serious complications include impaired wound healing [70].

In the process of wound healing, epidermal keratinocytes is a new final epidermal layer to cover the wound formed by migration, proliferation and differentiation, as an important symbol of wound healing. The biological behavior of normal play a very important role on wound healing process and quality plays. However, in diabetic skin tissue, due to the influence of the microenvironment of the skin tissue, the biological behavior of epidermal keratinocytes will have a series of changes and will affect wound re epithelization.

3.6.1 The Changes of Epidermal Keratinocytes in Cell Proliferation Capacity

The proliferation of epidermal keratinocytes is one of the most important repair processes in the wound healing process. There are many factors regulating epidermal keratinocyte cell proliferation and apoptosis. The NF-κB can regulate a variety of epidermal keratinocytes and genes related to cell proliferation and signal transduction pathways, such as apoptosis pathway and TNF receptor promoter Fas.

The normal operation of the cell cycle is largely dependent on the precise regulation of the signal transduction pathway. Takao et al. [71] study confirmed that the PMA (BU quinoline alcohol myristate acetate) can significantly activate the NF-κB activity, with the increase of the dose of PMA, will have more epidermal keratinocyte cells to enter the G0 phase. Takao speculated that PMA can inhibit the growth of epidermal cells by launching the cell cycle of the epidermal keratinocyte. Studies [71] also showed that NF-κB activity in the epidermal 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 epidermal keratinocytes, while the activity was decreased, and the activity was correlated with the concentration of AGEs. S phase and G2/M phase epidermal keratinocytes percentage were significantly lower than normal group rats epidermal cell in AGEs intervention in vitro 48 h. However, inhibiting the activity of NF-κB can partly enhance the activity of epidermal keratinocytes. The classical theory believes that there are two decisive phases in the cell cycle, the transition from G1 phase to S phase when DNA replication; another decisive stage is G2 phase transition to M phase, at chromosome condensation and mitotic stage.

This experiment shows that 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 cells cycle in G2/M phase can be increased obviously, but the changes in the percentage of cells cycle in S phase is not

obvious. This suggests that the AGEs can inhibit the cells transition from S to G2/M phase by activating NF-κB pathway.

At the same time, the proportion of cells in G2/M phase increases and cell proliferation ability enhance when inhibited the activity of NF-κB pathway. however, the inhibitory NF-κB pathway does not promote G0 cells into S phase. It 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 epidermal keratinocytes. AGEs can promote the apoptosis proportion of epidermal keratinocytes by regulating the NF-κB signal.

3.6.2 The Changes of Epidermal Keratinocytes in Cell Migration Ability

The normal play of epidermal keratinocyte migration function is an important repair behavior in the process of re-epithelial and wound healing. It plays an important role in the process and quality of wound healing. The formation of skin wound can stimulate the migration of epidermal keratinocytes to the wound surface, and participate in wound healing.

A study found [72], in impaired diabetic wound the migration ability of epidermal keratinocytes is enhanced, at the same time. In vitro research, AGEs intervention can significantly promote the migration, the ability of cell migration returned to normal when inhibiting the activity of NF-κB.

3.6.3 The Changes of Epidermal Keratinocytes in Other Biological Functions

The adherent ability of epidermal keratinocytes in culture medium reflects the activity of cells. Cell proliferation and division can only be carried out after the cell is adherence to the culture flask bottom. Tian et al. found that [73], the epidermal keratinocytes come from diabetic skin, were cultured in normal medium in 48 h, its adherence rate was significantly lower than the epidermal keratinocytes come from normal rat. AGEs in vitro can significantly reduce the adherent rate of epidermal keratinocytes, but the inhibition of NF-κB activity could not increase the rate of adherence of epidermal keratinocytes.

EGF (epidermal growth factor) can promote the proliferation of epithelial cells, fibroblasts, enhance the vitality and delay the aging of the skin cells, so that the composition of the skin to maintain the best physiological state. After the trauma, a large number of EGF expression is helpful to the early epithelium of the wound. Studies have found that [72] AGE can hinder the use of epidermal keratinocytes in EGF in the culture medium. Epidermal keratinocytes derived from diabetic rats reduced the use of EGF in culture medium. In vitro experiment showed that AGE

intervention of epidermal keratinocytes will reduce its use of EGF, inhibit the activity of NF-κB, the utilization ability of EGF to improve.

3.7 Neuropathy and Diabetic Wound Healing

Some scholars believe that the function changes of neuroendocrine system may be related with impaired wound healing. The relationship between the nerves and immune or cutaneous cells is closely, nerves can effect wound healing through inflammatory pathway, even in early and complication of diabetes [74]

Diabetic neuropathy (DNP) is one of the most common chronic complications of diabetes, and is the leading cause of diabetic foot ulcer impaired healing. As a part of peripheral nerve system, cutaneous nerve plays an important role in the protection, defense, metabolism, temperature regulation and sensation of the skin. But because of the anatomical and structural characteristics, make it become the most easily involved tissue in the course of diabetes. Long-term complication of diabetes is characterized by the progressive loss of somatic and autonomic nerve fibers, so DNP is often easy to be unnoticed [75]. In recent years, the study found that DNP could damage intercellular information exchange transfer between skin tissue and central nervous system, and cause abnormal structure and function of skin, also may be an important cause of wound healing [76].

3.7.1 The Pathological Changes of Skin Nerve in Diabetic State

Skin is rich in peripheral nerve fibers, according to the function, that can be divided into two categories: sensory nerve fibers and autonomic nerve fibers. In diabetic state, the pathological change of skin nerve is mainly including two aspects: structural change and function change. Structural change is mainly manifested as a reduction in the number of nerve fibers, myelinated nerve myelin edema, degeneration, dissolution, axon squeezed, degeneration of Schwann cell, exposed fibers; unmedullary nerve edema, vacuoles, actin filaments and microtubules are not arranged neatly. Function changes mainly include somatosensory and autonomic dysfunction, mainly manifested as pain, feeling of allergy and loss of one or several senses, as well as blood vessels, vertical hair muscle contraction dysfunction and abnormal sweat gland secretion. The risk of foot amputation was greatly increased in patients with peripheral sensory neuropathy [77].

3.7.2 The Pathogenesis of DPN

The pathogenesis of diabetic neuropathy is complex, and many factors may be involved in its occurrence and development. The metabolic factors, oxidative stress,

vascular factors, neurotrophic factors and immune factors were discussed in this paper.

3.7.3 Metabolic Factors

Diabetes mellitus is a metabolic disease characterized by persistent hyperglycaemia. There are many abnormal pathways and the accumulation of metabolites in the course of disease, diabetes cause a series of metabolic disorders, which interfere with the nervous material and energy metabolism, resulting in damage to its structure and function.

3.8 Excessive Activation of Polyol Metabolic Pathway

Aldose reductase and sorbitol dehydrogenase is a key enzyme in the polyol metabolic pathway. The polyol metabolic pathway is excessive activation in diabetic state, fructose and sorbitol accumulation in nerve cells, leading to the cell degeneration, edema, and segmental demyelination and axonal necrosis. The polyol pathway can also induce the depletion of glutathione by overexpression of aldose reductase, and then activate NF-κB, leading to Schwann cells secrete neurotrophic factors decreased, resulting in repair disorders after nerve injury [78]. Treated with aldose reductase inhibitors (ARIs) to animal models can effectively reduce the levels of sorbitol in nerve cells, Na^+-K^+-ATP enzyme activity recovery and improve of nerve conduction velocity and abnormality morphology [79].

3.9 Inhibition of Inositol Metabolism Pathway

In diabetic state, the over-activation of the polyol pathway makes the synthesis of inositol decrease, at the same time glucose competitive inhibition of inositol transport, which further increases the intracellular inositol decreased state. Inositol is necessary for the Na^+-K^+-ATP enzyme, the decrease of the inositol can lead to the decline of Na^+-K^+-ATP enzyme activity, which in turn can affect the carrier transport of inositol, and form a vicious circle. Inositol is also a component of the myelin sheath, its metabolic disorder, which will certainly affect the energy metabolism and structural integrity of the nervous tissue.

3.10 Accumulation of Advanced Glycation End Products

Enhanced AGEs deposition in the nerve tissue can lead to the increase of the 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, is not only make the lumen stenosis, occlusion, but also combine with RAGE on the endothelial cells, reduce the formation of NO, affect the nerve blood supply and lead to neuropathy. Recently Duran-Jimenez and et al. found glycosylated extracellular matrix can also be affecting the nutrient supply of nerve fibers and bud proliferation and impact regeneration ability after nerve injury [80, 81]. 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. Chen etc. observed the effects of high glucose and AGE-HSA on Schwann cells in vitro, results showed that, high glucose, AGE-HSA could obviously inhibit the proliferation of Schwann cells, promote the apoptosis, and the effect of AGE-HSA is closely related to its concentration. This view is further validated theory of "diabetes of Schwann cell disease". It also showed that repair and regeneration barriers after nerve injury in diabetic patients and diabetic neuropathy had a common cause in the pathogenesis [82].

3.11 Others

Currently, it is found that the essential fatty acid metabolic disorders, vitamin deficiency, accumulation of homocysteine, abnormal cytokine and growth factors secretion and et al. have a certain relationship with occurrence and development of DPN [83].

4 Oxidative Stress Enhancement

High glucose environment in diabetic patients can damage the mitochondrial electron transport chain, so that the oxygen free radical (ROS) such as hydrogen peroxide, ultra hydrogen peroxide and so on production increased. ROS can cause lipid, nucleic acid, protein oxidation, reduce the nerve blood vessels and increase the apoptosis of nerve cells. At the same time, oxidative stress was also enable to reduce neurotrophic factor level by 64 %, and produces 8 hydroxy-2′-deoxy guanine, damage the DNA and Na^+-K^+-ATP enzyme activity decreased, resulting in nerve function and abnormal structure. Thermal pain sensitivity of motor nerve was significantly improved in the animal model of treatment with anti oxidation.

5 Abnormal Neurotrophic Factor

Neurotrophic factor is a kind of protein molecules secreted by the body, maintaining the survival, growth and differentiation of nerve cells. It plays an important role in the maintenance of nerve morphology, regeneration and the release of the transmitter. It was found that the nerve growth factor (NGF), brain derived neurotrophic factor, IGF-I, IGF-II and other neurotrophic factors decreased in diabetic state. Chu et al. [84] found in animal models, To improve systemic IGF-I levels can recovery hypoalgesia. But there are also contrary reports, Kim HC and other found that skin tissue of diabetic patients NGF mRNA expression increased, but the level of serum NGF was elevated [85].

6 Vascular Factors

Diabetic nerve biopsy showed nerve ischemia, infarction, endothelial cell proliferation, capillary basement membrane thickening. Microvascular structure and function abnormalities, abnormal blood rheology, anticoagulation and fibrinolysis imbalance, and induced nerve ischemia, hypoxia, is an important cause of neuropathy. Moreover microvascular disease can cause nerve aregeneratory after damage. Recently, Doupis et al. [86] also found in diabetic patients with peripheral neuropathy that the function of endothelium-related vasodilatation and C fiber-mediated vasoconstriction were impaired.

7 Immune Factors

The research confirmed that the existing autoantibodies to nerve tissue in patients with type I and type II diabetes, such as ß-microglobulin antibodies, anti microglobulin antibody, can cause nerve damage. Peripheral nerve myelin protein combined IgG and IgM in diabetic patients was respectively 4 times and 14 times of in non diabetic patients [87].

7.1 The Relationship Between DPN and Impaired Wound Healing

Wound healing is a complex network process with inflammatory cells, repair cells, extracellular matrix and multiple factors, which is highly coordinated and controlled. Post trauma, skin nerve endings can be involved in wound repair process through the release of a variety of neuropeptide. Diabetic skin sensory neuropathy

patients are easy to suffer from injury, delayed wound healing and even nonunion. Diabetic skin neuropathy through the secretion of neuropeptides may play an important role in wound healing in diabetes mellitus.

7.2 The Effect of DNP on Inflammatory Cells

A variety of noxious stimulus can induce skin nerve endings release neuropeptides and cause neurogenic inflammation in the local. CGRP and Substance P are the most common neuropeptide released from the cutaneous nerve endings, they are often released at the same time, all belongs to a strong vasodilator. Different neuropeptides play different roles in the inflammatory process. Now that, the substance P, bradykinin and vasoactive intestinal peptide are as proinflammatory mediators, CGRP is considered as a potent anti inflammatory mediator. CGRP in skin tissue in early stage of STZ induced diabetic rats, vasoactive intestinal peptide positive fibers was increased. In diabetic mice model, the early inflammatory response of the wound was delayed, and the total inflammatory reaction time was prolonged, which may be related to the abnormal release of the neuropeptide in the skin nerve endings [88].

7.3 The Effect of DNP on Cytokines

Cytokines play an important role in regulating the proliferation, migration and differentiation of various repair cells. Richards AM found that substance P can promote the synthesis of keratinocyte interleukin, TGF-α, and increased INF-γ mediated expression of keratinocyte adhesion molecules, their expression and synthesis can promote wound healing. In addition to the direct effect, but also the neuropeptide synthesis and expression of indirect effects of various cytokines related to wound repair [89]. Some neuropeptides are downregulated (SP, NPY, CGRP) in diabetes and others upregulated (CRF, α-MSH and NT) with the net effect being that downstream cytokines in the skin are dysregulated. This disruption in the balance of cytokines may cause impaired wound healing.

7.4 The Effect of DNP on Repair Cells

Although there is no direct evidence that neuropathy can lead to impaired wound healing, but the neuropeptides do play an important role in this connection. Neuropeptides exert their biological effects by binding to specific receptors on the cutaneous cells, that such as immune cells, Langerhans cells, ECs, mast cells, fibroblasts and keratinocytes, or through direct activation of intracellular G-protein

signaling cascades [90]. Known neuropeptides that related to wound repair are SP, NPY, CGRP, corticotropin releasing factor (CRF), α-melanocorticotropin releasing hormone (α-MSH) and neurotensin (NT). The roles for NT and α-MSH in diabetic wound need in further verification [91, 92].

8 Summary and Outlook

There is no doubt that DNP is one of the important causes of diabetes impaired wound healing. The neuropeptide is an important material in the process of wound healing. Peripheral vascular disease with neuropathy may result in impaired wound healing. The homeostasis maintained by the nerve-immune system is disrupted in diabetic skin. Because of the limited cognition and condition, we have a lot of unknown function in the process of wound healing of diabetic skin, so much knowledge is worthy of depth study. Such as, we know that the skin nerve endings can secrete a variety of neuropeptide and expression of related receptors, but we don't know what changes in their expression, binding, and subsequent reactions in diabetes. We know that many kinds of neuropeptides may be involved in wound healing, but it is still not clear what kinds of neuropeptides and through which signal transduction pathways are involved in wound healing, as well as in diabetic state, what changes have occurred. Believe that with the solution of these problems will help to deepen our understanding for the role of cutaneous neuropathy in diabetic wound healing, provide new ideas and methods for the treatment of diabetic wound healing.

9 Summary of All

The causes of refractory diabetic wound is so complex, it involves many links and cross-linking. Before the trauma diabetic skin has been different from the normal skin of some changes, including the decline in the sense of nerve,sub inflammatory state, some diseases of micro blood vessels and so on, making it more susceptible to infection and injury. And once the wound is formed, it is difficult to heal. Treatment of diabetic refractory wound is a difficult clinical problem. The summary of the relevant mechanism in this paper is just "the tip of the iceberg", the more clear mechanism is to be further explored by researchers.

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