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Macrophage Differentiation in Normal and Accelerated Wound Healing

Girish J. Kotwal and

Noveratech LLC, Louisville, KY, USA

Sufan Chien

Department of Surgery, University of Louisville, School of Medicine, Louisville, KY, USA;

Noveratech LLC, Louisville, KY, USA

Abstract

Chronic wounds pose considerable public health challenges and burden. Wound healing is known to require the participation of macrophages, but mechanisms remain unclear. The M1 phenotype macrophages have a known scavenger function, but they also play multiple roles in tissue repair and regeneration when they transition to an M2 phenotype. Macrophage precursors (mononuclear cells/monocytes) follow the influx of PMN neutrophils into a wound during the natural wound-healing process, to become the major cells in the wound. Natural wound-healing process is a four-phase progression consisting of hemostasis, inflammation, proliferation, and remodeling. A lag phase of 3–6 days precedes the remodeling phase, which is characterized by fibroblast activation and finally collagen production. This normal wound-healing process can be accelerated by the intracellular delivery of ATP to wound tissue. This novel ATP-mediated acceleration arises due to an alternative activation of the M1 to M2 transition (macrophage polarization), a central and critical feature of the wound-healing process. This response is also characterized by an early increased release of pro-inflammatory cytokines (TNF, IL-1 beta, IL-6), a chemokine (MCP-1), an activation of purinergic receptors (a family of plasma membrane receptors found in almost all mammalian cells), and an increased production of platelets and platelet microparticles. These factors trigger a massive influx of macrophages, as well as in situ proliferation of the resident macrophages and increased synthesis of VEGFs. These responses are followed, in turn, by rapid neovascularization and collagen production by the macrophages, resulting in wound covering with granulation tissue within 24 h.

14.1 Introduction

Wounds in aging or diabetic patients can be difficult to heal or can become chronic, resulting in considerable public health challenges. The development of a wound in response to an injury is followed by a healing process carried out predominantly by white blood cells (leukocytes) known as macrophages, which mediate phagocytosis to remove pathogens, as well as dead and dying cells, from the wound site. Besides this scavenger function, however, macrophages play multiple other roles in wound healing. Macrophages or their monocyte

precursors infiltrate and reside in the wound site, where they are activated by pro-inflammatory cytokines, interferons, pathogen-associated modifying proteins (PAMPs), or damage-associated modifying proteins (DAMPs).

The two most predominant forms of macrophages are the M1 form, the classical activated form that acts in pathogen phagocytosis and destroys/removes damaged cells, including neutrophils, and the M2 form, which has repair and regeneration functions. The M1 to M2 polarization, or switch, reflects the macrophage differentiation process that shifts the cells from inflammation to proliferation functions. This polarization is a vital step in wound healing and is brought about by IL-4, IL-10, glucocorticoids, prostaglandins, and modulators of glucose and lipid metabolism (Landen et al. 2016). The M2 form resides in tissues and secretes anti-inflammatory mediators and growth factors, along with suppressors of cytokine-signaling (SOCS) proteins (Gordon et al. 2016), and it switches off the damage-causing pro-inflammatory activities of the M1 macrophages. The transition to the M2 form can be amplified by IL-4, and the increased numbers of M2 cells then result in elevated levels of IL-10, TGF-beta, and IL-12 (in minute quantities).

Both the M1 and M2 macrophages are critical for wound healing. During the natural wound-healing process (without intervention), blood-derived polymorpho-nuclear neutrophils enter the tissue, followed after 48 h by CXCR4-bearing mono-nuclear cells/monocyte precursors that transition to macrophages to become the major cells at the wound tissue site. The four-phase natural wound-healing process is characterized by a lag of approximately 3–5 days and requires an intermediate step of activation of resident fibroblasts and myofibroblasts during the remodeling phase for collagen production. Prolonged inflammation can result in fibrotic wound healing (Zhu et al. 2016).

More than 100 factors are known to lead to the formation of nonhealing wounds, such as diabetic foot ulcers (DFUs) (Valls et al. 2009; Davidson 1998; Ovington and Schultz 2004; Pugh and Ratcliffe 2003; Harding et al. 2002), but one known critical pathophysiology is the ischemia associated with a deficient blood supply (Silver 1980; Niinikoski et al. 1991; Ehrlich et al. 1972; Theoret 2005; Im and Hoopes 1970). Ischemia may not be the initiating factor for wounds like DFUs, because most DFUs start from a combination of neuropathy, pressure loading, infection, and/or trauma (Medina et al. 2005; Fang and Galiano 2008). However, tissue ischemia is the main hindrance to healing—wounds do not heal in tissue that does not bleed, whereas they always heal in tissue that bleeds extensively (Gottrup 2002; Schaffer et al. 2002; Niinikoski et al. 1991).

The patterns of DFUs seen in Western countries have changed in the last two decades, with the neuroischemic ulcer now replacing the previously predominant neuropathic type as the most frequently seen DFU in many clinics (Boulton 2013). Strategies for increasing the wound oxygen supply with hyperbaric oxygen therapy have not shown consistent results (Berendt 2006; Cohen et al. 1999; Hunt and Pai 1972; Niinikoski 2003), because oxygen alone cannot adequately reverse ischemic damage. The most critical consequence of ischemia is a decrease in cellular energy supply (Im and Hoopes 1970; Smith et al. 1999), as energy is required for every aspect of the wound-healing process. For example, the linking of individual amino acids through peptide bonds to form a protein requires a significant

input of chemical energy (Hunt and Pai 1972). Therefore, a protein such as collagen cannot be synthesized by fibroblasts (Fine and Mustoe 2001), nor can these cells proliferate, under ischemic or hypoxic conditions (Hunt and Pai 1972; Stadelmann et al. 1998). Other cellular processes, such as cell migration and proliferation, membrane transport, and growth factor production, also consume cellular energy and are also essential for wound healing (Wang et al. 1990). Thus, although small-scale clinical trials have shown the effectiveness of addition of growth factors such as bFGF to nonischemic wounds, these beneficial effects disappear in hypoxic dermal ulcer models (Harding et al. 2002; Lindblad 2007).

No one has yet studied the energy status of diabetic wounds. A Medline search retrieved only one article that reported ATP and PCr (phosphocreatine) contents in dorsal foot skin in 10 normal and 16 diabetic adults and noted a fourfold difference in the ATP/PCr ratio between diabetic and normal foot skin (Smith et al. 1999). We have measured the high-energy phosphate content in 34 diabetic and 21 postoperative wounds. Although both wounds had a similar appearance, we observed a two- to threefold decrease in the high-energy phosphate content of the diabetic wounds.

Ischemic wounds cannot be effectively treated by a direct infusion of free ATP because the bilayer cell membrane is impermeable to most water-soluble molecules (Cooper 1997). Specific transport proteins (carrier or channel proteins) permit the selective passage of small molecules across the membrane, but larger molecules, especially charged forms like ATP, cannot cross the cell membrane under normal conditions. An added complexity is that even when infused into the bloodstream, the half-life of ATP in the blood is less than 40 s, which poses technical difficulties for maintaining a stable ATP supply by intravenous administration (Puisieux et al. 1994).

These problems could be potentially overcome by encapsulating the ATP in specially formulated and highly fusogenic nanoscale unilamellar lipid vesicles (ATP vesicles or ATP nanoliposomes). If the chemical composition of the nanoliposome shell is similar to that of cell membranes, these vesicles will fuse with the cell membrane upon contact and deliver their ATP contents into the cytosol (Chien 2010). The current composition of the ATP nanoliposomes used in our research is 100 mg/ml L- α -phosphatidylcholine (Soy PC)/1, 2-Dioleoyl-3-trimethylammonium-propane (DOTAP) (50:1), Trehalose/Soy PC (2:1), 10 mM KH₂PO₄, and 10 mM Mg-ATP. The diameters of the lipid vesicles range from 85 nm to 150 nm. The preparation can be freeze-dried and then reconstituted with deionized water and mixed with a neutral cream (Velvachol, Coria Laboratories, Fort Worth, TX) for use as a local wound dressing.

The use of nanoliposome-encapsulated Mg-ATP alters and accelerates the entire wound-healing process by triggering a novel pathway in which the activation and polarization of macrophages to the M2 form is absolutely critical and central (Howard et al. 2014; Kotwal et al. 2015). This novel alternative pathway is characterized by an increase in pro-inflammatory cytokines (TNF, IL-1 beta, IL-6), chemokine (MCP-1) release, activation of purinergic receptors (a family of plasma membrane receptors found in almost all mammalian cells), and increases in the numbers of platelets and platelet microparticles. This results in a massive influx of macrophages, as well as in situ proliferation of the incoming macrophages

as early as 12 h after ATP-nanoliposome application, followed by increases in vascular endothelial growth factors that promote neovascularization and collagen production.

14.2 Infection Control by Macrophages in Natural and Assisted Wound-Healing Processes

Although the issue is still somewhat debated, macrophages are thought to play essential direct and indirect roles in the complicated wound-healing process (Martin and Leibovich 2005). This process requires that any microbial contamination of the wound site be eliminated by antimicrobial action and by phagocytosis and opsonization by macrophages. Phagocytosis takes place when macrophage receptors recognize PAMPs on the surfaces of bacteria or fungi, prompting the macrophages to attach to and engulf the invading microbes. A phagolysosome is then formed inside the macrophage by the fusion of the engulfed bacterium with the lysosome. This is followed by a proteolytic breakdown of the bacterium and the release of the breakdown material by exocytosis. The process of opsonization, by contrast, is triggered by an activation of the alternate complement pathway, due to recognition of the lectins and sugar chains on bacterial surfaces. This then results in deposition of the breakdown products of the third complement component onto the microbe surfaces. Macrophages have receptors for these breakdown products (C3b and iC3b), so they attach specifically to bacteria bearing the complement C3b and iC3b. This initiates opsonization, which is phagocytosis due to the specific targeting of macrophages to bacteria. Besides infection control, the role of macrophage phagocytosis is to clear cell components that are formed after cell death; these components have their own molecular patterns, called DAMPs.

The classical wound-healing process, which includes hemostasis, inflammation, proliferation, and remodeling, is characterized by a 3–5 day lag (Delavary et al. 2011). During the inflammatory phase, pro-inflammatory cytokines (IL-1 β , IL-6, and TNF α) are secreted to recruit peripheral circulating white blood cells and monocytes, which differentiate into macrophages. Stimulation with interferon gamma and lipopolysaccharide causes classically activated macrophages to polarize into cytotoxic phenotypes. During the reepithelialization stage, while phagocytosing surrounding dead cells and debris, macrophages secrete vascular endothelial growth factors and promote the proliferation of endothelial cells, skeletal myoblasts, and fibroblasts, while also secreting IL-10 to suppress further influxes of macrophages. This phase is followed by angiogenesis, myotube formation, and collagen production. The final remodeling phase involves collagen remodeling (Novak and Koh 2013).

14.3 The Role of Alternately Activated Macrophages in Accelerated Wound Healing

Extensive studies on mice now indicate a temporal variability during the wound-healing process, which suggests that selected phenotypic traits are promoted during the classically activated early phase of wound healing, followed by later alternatively activated phases. This aspect of wound healing has been reviewed by Brancato and Albina (2011), who suggest

that the lack of a pure macrophage form in vivo indicates that activated macrophages could appropriately be described as a continuous spectrum of distinct and continuous phenotypic features. The wound macrophages express the alternatively activated M2 phenotype, so they have been proposed to represent *wound-healing macrophages*.

14.4 Macrophage Phenotype Switching

The macrophage phenotype changes as the wound heals, progressing from the M1 (pro-inflammatory) to the M2 (anti-inflammatory) forms, indicating that the wound-healing phenotype is influenced by the local microenvironment (Ferrante and Leibovich 2012). The M2 phenotype is characterized by distinct surface receptors or cell differentiation (CD) markers (CD68, CD163, CD206) and shows several subphenotypes (M2a, M2b, M2c, and M2d). The switch from the M1 to the M2 phenotype occurs in stages in response to downregulation of IL-10 and upregulation of IL-4 and IL-13 and in response to specific mediators. The M1 to M2a transition is induced by activation by IL-4R-alpha.

Macrophage polarization, which is the process that leads to the acquisition of distinct phenotypes, must occur in an orderly manner for effective wound healing. Macrophage polarization is regulated by a complex process in which adenosine-mediated “switching” regulates angiogenesis during the final stages of the repair process that results in wound healing (Ferrante and Leibovich 2012). Another layer of complexity is added by the presence of specific macrophage phenotypes associated with each tissue type (Davies et al. 2013a, b).

14.5 Accelerated Wound Healing Following Intracellular ATP Delivery

In the past half century, numerous interventions have been proposed for either facilitating or accelerating the wound-healing process (Garash et al. 2016; Ogle et al. 2016). In sharp contrast to the classical wound-healing process that occurs with all presently used interventions, the fast-track process occurs in response to the direct introduction of nanoliposome-encapsulated Mg-ATP to the wound. This intracellular ATP delivery via fusogenic unilamellar nanoliposomes induces a significant wound-healing enhancement in the skin of rodent models (Chiang et al. 2007). This enhanced healing was reaffirmed in a rabbit model, where full-thickness skin wounds treated with ATP nanoliposomes showed accelerated wound sealing as well as extremely rapid tissue regeneration. The traditional 3–6 day lag was eliminated, and granulation tissue started to appear within 24 h, a phenomenon never observed previously or reported with any other therapeutic treatment modality.

The early growth is composed mainly of macrophages, suggesting active proliferation. Collagen is produced, neovascularization is enhanced at the wound site, and re-epithelializing tissue tunnels through the granulation tissue (Wang et al. 2009). The top of the granulation tissue ultimately peels off, revealing a healed wound. A similar growth pattern is not observed following treatment with either unencapsulated Mg-ATP, empty nanoliposomes, or with the only FDA-approved prescription growth factor for wound dressing—Regranex. Most notably, the growth has a self-limiting characteristic, so that it

leaves no hypertrophic scars or any other unusual growth, even 2 years after the treatment (Howard et al. 2014).

This type of healing contrasts sharply with the conventional process typically observed by those engaged in wound care, where fibrin, platelets, and erythrocytes are the major components of the provisional matrix seen early after injury. This matrix is then gradually replaced by granulation tissue during a proliferation phase that begins after a 3–5-day lag (Levenson and Demetriou 1992; Schaffer et al. 2002).

The individual steps that lead to this novel wound-healing process have been assessed by testing several individual activities triggered by the delivery of ATP (Howard et al. 2014). In vitro studies showed an increase in the chemokine MCP-1, accompanied by a reduction in the anti-inflammatory cytokine IL-10 levels, in human macrophage cells treated with ATP nanoliposomes (Wang et al. 2009). These chemokine and cytokine changes could explain the large influx of macrophages observed following ATP-nanoliposome administration (Kieran et al. 2013). The predominant macrophage phenotype was M2, which was consistent with the observed increase in IL-13R and the detection of the CD163 cell differentiation surface marker (Howard et al. 2014). The level of vascular-specific growth factors also increased, consistent with the observed neovascularization (Chiang et al. 2007). Early increases were also observed in platelet numbers at the wound site (Wang et al. 2010). The inference made at the time was that the influx, accumulation, and phenotypic differentiation of the incoming stem cells and macrophages were a result exclusively of the increased ATP energy supply (Chien 2006, 2010).

Recent studies have shed more light on the mechanism underlying this rapid wound healing in response to ATP. The rapid granulation and the accompanying tissue generation and macrophage proliferation (as determined by increased PCNA synthesis and BrdU staining) appear to drive collagen synthesis without an intermediate fibroblast generation at the wound site (Howard et al. 2014). These multiple activities seen in response to treatment with nanoliposome-encapsulated ATP are summarized below. Previous studies by Wang et al. (2009) indicated that the phenomenon of macrophage accumulation in response to the ATP-nanoliposome treatment could be attributed to the ATP-driven rearrangement of the chromatin structure within the 12-component cell proliferation SWI/SNF complex, which includes the BRG1 or Brm ATPase subunits. They also suggested that the increased levels of BRG1 and BRM in the wounds treated with ATP nanoliposomes could be associated with ATPase activity that contributed to the in situ proliferation of the macrophages, thereby suggesting a possible mechanism for the fast-track wound-healing pathway (Yang et al. 2015). The current understanding of the mechanism of wound healing following intracellular ATP delivery illustrated in Fig. 14.1 can be summarized as follows:

1. Stem/progenitor cell recruitment, in response mostly to purinergic receptor activation.
2. Leukocyte chemotaxis toward the wound.
3. Enhanced platelet accumulation at the wound site.

4. Monocyte accumulation and activation from processes 1 to 3; the platelet-derived growth factors further enhance monocyte activation.
5. Monocyte transformation to macrophages via platelets and platelet microparticles and the MCP-1 pathways.
6. Massive cell accumulation caused by in situ macrophage proliferation.
7. Changes in RNA expression patterns to generate building blocks to support proliferation.
8. Activated macrophages perform their phagocytic functions, but also secrete MCP-1, resulting in further cell accumulation.
9. Macrophages activated by the alternate pathway also produce collagen directly in response to nanoliposome-encapsulated ATP. This process results in much faster collagen production than normally occurs indirectly by fibroblasts.
10. Upregulated apoptosis keeps the growth in check and maintains the balance between proliferation and regression.

The elucidation of the steps involved in the proliferation phase that follows the inflammation phase after an injury will provide a better understanding of the rapid wound-healing mechanism associated delivery of nanoliposome-encapsulated ATP. Other possibilities could include the increased expression of small RNAs (Kotwal and Chien, unpublished) and other building blocks that would facilitate M2 macrophage proliferation.

14.6 Regulation of Macrophage Polarization

Autoimmune damage can theoretically occur from the immune system itself due to unregulated complement activation or phagocytic killer cell activation, such as is induced by M1 macrophages. Therefore, timely regulation of these activations is critical. The complement system has multiple regulatory proteins that can bind key complement proteins and limit the release of chemotactic factors that normally draw an influx of macrophages and pro-inflammatory cells. The transition from inflammation to proliferation also requires some form of control or regulatory system that regulates macrophage activation.

The macrophage polarization and soluble mediator gene expression occurring during inflammation are under epigenetic control (Kapellos and Iqbal 2016). Specifically, M1 or M2 activation occurs by the posttranslational modifications of DNA-binding histones found adjacent to genes encoding proteins that function as inflammatory response mediators. The posttranslational modifications include methylations and acetylations catalyzed by methyltransferases, dimethyltransferases, acetyltransferases, and deacetyltransferases. These posttranslational modifications contribute to the epigenetic control of macrophage polarization to either the M1 or M2 phenotypes, which, in turn, modulates the type of cytokines and chemokines that are generated and the types of immune cells that are present at the wound site.

14.7 Conclusion

Wound healing is a complex process driven by the actions of macrophages (Snyder et al. 2016). The inflammatory phase occurs following the influx of neutrophils, and then monocytes, and their activation to macrophages of the inflammation, or M1, phenotype. These M1 macrophages then transition to the M2, or the proliferative phenotype, and the M2 macrophages then drive the natural wound-healing process. Extensive studies in a rabbit wound model developed by the Chien group have indicated that the pathway of wound healing that occurs in response to treatment with nanoliposome-encapsulated ATP is unique, as gross healing commences within 24 h via macrophages that proliferate and then transform directly into the extracellular matrix to rapidly fill in the wound cavity. This contrasts strongly with the natural form of wound healing, where gross healing starts only after about a 3–6-day lag, and the wound space is filled with a provisional matrix consisting of red cells trapped in a fibrin mesh. The elimination of the lag time and the very early ability to support cell survival and proliferation in a wound cavity without any blood supply are two features never achieved with any other wound treatment.

The rapid tissue generation following intracellular ATP delivery is a major clinical advance that awaits full elucidation of its detailed mechanism. At present, the possible explanations for the hastened wound healing include the provision of energy to the wound microenvironment, the overexpression of noncoding RNAs such as 5S rRNA and U4 spliceosome RNA (Kotwal and Chien, unpublished) that contribute to a more efficient buildup of the biochemical building blocks needed for proliferation, the adenosine-mediated macrophage phenotype switching, or all of the above.

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Fig. 14.1. Flow diagram of Steps following wound treatment with or without Intracellular ATP delivery