WOUND HEALING

2012

CONCEPTS

Wound healing is a complex process that normally occurs in the postnatal setting through scar tissue formation, with regenerative healing limited to the liver and bone.

In contrast, the fetus in the mild-gestational period heals cutaneous wounds without scarring by regeneration of the normal dermal architecture, including restoration of dermal appendages and neurovasculature, in all mammalian species. This period of regenerative healing is followed by a transitional period in which wounds heal with a normal extracellular matrix but fail to regenerate its dermal appendages. Lastly, near the end of gestation, progression to the postnatal phenotype exists in which wounds heal with an excess of collagen, a loss of dermal appendages and a flattened epidermis.

Many studies have shown that stimulating inflammation enhances the extent of scarring in fetal wounds. Moreover, a more substantial inflammatory response to injury is seen in late-gestational fetal skin that heals with a scar. These findings suggests an intrinsic property of fetal skin that is permissive of scarless wound healing.

Limb regeneration is one of the best examples of organ/appendage regeneration in vertebrates and has been called *"epimorphosis"* or *"epimorphic regeneration"*since it requires blastema formation and proliferation. Urodele amphilibians, such as newts and salamanders, can regenerate amputate appendages (limbs and tail). Typically, wound healing proceeds incredibly quickly in these animals. Within 4 to 12 hours, the stump is covered with a layer called the *"wound epidermis"*.

Unlike urodele amphibians, the regenerative ability of anuran amphibians (frogs and toads) depends on their developmental stage. For instance, before

metamorphosis can completely regenerate its developing hindlimb buds, this regenerative capacity declines as metamorphosis proceed.

The skin of mammalian adults can neither heal scarlessly nor regenerate skin derivates, such as sweat glands or hair follicles. Wound healing in postnatal mammal's skin (organ) could be considered an unusual regeneration process since regeneration of the epithelium (parenchyme) is incomplete while an excessive production of connective tissue (stroma) is associated. Therefore, skin wound repair is defective, since a tissue forms lacking the structural and functional characteristics of normal skin. Scar formation, unfortunately presents the main form of repair in adult skin wound healing.

Every tissue disruption of normal anatomic structure with consecutive loss of function can be described as a wound. Tegmental injuries are defined as open or outer wounds, whereas inner or closed wounds are injuries or ruptures of inner organs and tissues with the skin still intact.

It is well known that the difference between superficial and deep wounds is of great clinical importance and largely determines how these injures heal and the degree of scarring to be expected. Superficial wounds usually heal with a minimum of scarring. Superficial injury less than 0.56 mm in depth or 33% of normal hip skin thickness, results in regeneration rather than scar, whereas deeper injury results in increasing scar formation. This result suggests that injury beyond a critical depth leads to scar formation rather than regeneration.

The stages of wound repair

The multiple pathophysiological mechanisms that overlap during the progression of the skin wound-healing reaction may explain the lack of consensus on the number of phases involved in this reaction. Some researches argue that wound healing involves three phases: Inflammation, proliferation and tissue remodeling whereas other researchers believe there are four stages in wound healing: Hemostasis, inflammation, proliferation and tissue remodeling with scar formation; and others even defend that there are five phases: Hemostasis, inflammation, cellular migration and proliferation, protein synthesis

and wound contraction and remodeling. However, everyone agrees that these phases are interrelated, suggesting that the wound-healing process is a continuum.

In the 3-stage model of wound healing, inflammation includes coagulation and inflammatory cell recruitment. The different clotting cascades are then initiated by clotting factors from the injured skin (extrinsic system). Then, thrombocytes get activated for aggregation by exposed collagen (intrinsic system). At the same time, the injured vessels follow a 5 to 10 minute vasoconstriction, triggered by the platelets, to reduce blood loss and fill the tissue gap with a blood clot comprised of cytokines and growth factors. Furthermore, the blood clot contains fibrin molecules, fibronectin, vitronectin and thrombospondins, forming the provisional matrix as a scaffold structure for the migration of leukocytes, keratinocytes, fibroblasts and endothelial cells and also acts as a reservoir of growth factors. The life-saving vasoconstriction with clot formation accounts for a local perfusion failure with a consecutive lack of oxygen, increased glycolysis and pH-changes. The vasoconstriction is then followed by a vasodilation in which the traumatized tissue suffers a reperfusion phenomenon. Both platelets and leukocytes release cytokines, chemokines and growth factors to activate the inflammatory process, stimulate the collagen synthesis, activate the transformation of fibroblasts to myofibroblasts, start angiogenesis and support the reepithelialization process. The vasodilatation can also be recognized by a local redness (hyperemia) and by wound edema (tumor).

Neutrophil recruitment is crucial within the first days after injury because their ability in protease secretion and phagocytosis kills local bacteria and helps to degrade necrotic tissue. They start their debridement by releasing highly active antimicrobial substances i.e. cationic peptides and eicosanoids, and proteinases, i.e. elastase, cathepsin G, proteinase 3 and an urolinase-type plasminogen activator. Approximately 3 days after injury, macrophages enter the zone of injury and support the ongoing process by performing phagocytosis of pathogens and cell debris and by secreting growth factors, chemokines and cytokines. Macrophages have many functions including host defense, the promotion and resolution of inflammation, the removal of apoptotic cells and the support of cell proliferation and tissue restoration following injury. Beside their immunological functions as antigen-presenting cells and phagocytes during wound repair, macrophages supposedly play an integral role in a successful healing response through the synthesis of numerous potent growth factors, such as transforming growth factor (TGF- β , TGF- α , basic FGF, platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which promote cell proliferation.

During the proliferative phase, granulation tissue deposition provides a wound bed for re-epithelialization. In the phase of proliferation, that is 3 to 10 days after wounding, the main focus of the healing process lies in covering the wound surface, the formation of granulation tissue and restoring the vascular network. The proliferative phase in wound healing is characterized by angiogenesis, granulation tissue formation, epithelialization and wound contraction. Granulated tissue basically consists in fibroblast and new blood vessels. The first step in new vessel formation is the binding of growth factors to their receptors on the endothelial cells of existing vessels, thereby activating intracellular signaling cascades. The endothelial cells proliferate and migrate. This is a process also known as *"sprouting"*. The newly built sprouts form small tubular canals that interconnect with others forming a vessel loop. Thereafter, the new vessels differentiate and mature by stabilizing their vessel wall via the recruitment of pericytes and smooth muscle cells.

Towards the end of this stage, fibroblasts, attracted from the edge of the wound or from the bone marrow, are stimulated by macrophages, and some differentiate into myofibroblasts. Fibroblasts begin synthesizing collagen and proliferate to form granulation tissue. TGF- β induces fibroblasts to synthesize type I collagen and reduce matrix metaloproteinases (MMPs) production. Fibroblasts produce collagen but also extracellular matrix substances, like fibronectin. glycosaminoglycans, proteoglycans and hyaluronic acid. Myofibroblasts are contractile cells that, over time, bring the edges of a wound together. Wound contraction is a biological means whereas the edges of an open wound are pulled together by forces resulting from the wound-healing process. The environmental cytokines have a significant effect on the contractile process. TGF- β increases the rate and degree of contraction without upregulating proliferation and it has been postulated that this may be through the induction of PDGF. At the end of this phase, the number of fibroblasts is reduced by myofibroblast differentiation and terminated by consecutive apoptosis.

Remodeling is the last phase of wound healing and occurs from day 21 to up 1 year after injury. During the remodeling phase, formation of granulation tissue ceases through apoptosis of the responsible cells. This process is important because its aberration leads to hypertrophic scarring and keloids. A mature wound is therefore characterized as avascular and acellular. With wound maturation, the composition of the extracellular matrix undergoes change. The type III collagen deposited during the proliferative phase is slowly degraded and replaced with stronger type I collagen. This type of collagen is oriented in small parallel bundles and is, therefore, different from the basket-weave collagen in healthy dermis.

Later on, myofibroblasts cause wound contractions by their multiple attachments to collagen while they help decrease the surface of the developing scar. The angiogenic processes also diminish; the wound blood flow declines and the acute wound metabolic activity slows down and finally stops. Scar contraction is the shrinkage that occurs in an already healed scar. Well-known clinical risk factors include hypertrophic scarring and the most predominant theories involve myofibroblasts. The results using fibroblast and myofibroblast isolated from hypertrophic scars, suggests that fibroblasts are the primary cells involved in wound contracture whereas myofibroblasts are the primary cells involved in scar contracture.

Connective tissue growth factor (CTGF) is a downstream regulator of fibrosis that is induced by TGF γ . It seems, that although TGF β is important in initiating pathologic scarring, it is CTGF that sustains the fibrotic process. Insulin like growth factor (IGF-1) modulates growth hormone effects on fibroblasts. IGF-1 is expressed locally in injured tissue formation up to 5 weeks post-injury. In addition IGF-1 has been shown to act as a TGF- β stimulating factor and both play an important role in the pathogenesis of abnormal scarring.

Inflammatory phenotypes related to wound repair

In a broader perspective, the inflammatory response would include all phases that constitute wound healing. We have therefore proposed that inflammation could be the basic mechanism that drives the nature of the different stages of wound repair. In essence, the post-traumatic local acute inflammatory response is described as a succession of three functional phases of possible trophic significance: nervous or immediate (ischemia-reperfusion phenotype), immune or intermediate (leukocytic phenotype) and endocrine or late (angiogenic phenotype) (Figure 1). Thus, one could also hypothesize that the sequence of transformations undergone by traumatized tissue in the three above-mentioned phases represents a metamorphic phenomenon. The inflammatory response induced in the wounded skin could be a result of three overlapping phases during which metabolic phenotypes featuring progressive complexity of oxygen use are expressed.



Figure 1: The Inflammatory Response.

1. The ischemia-reperfusion phenotype

In the first or immediate phase is referred to as the nervous phase because the sensory (pain and analgesia) and motor alterations (contraction and relaxation) respond to the injury. Wounds produce a pathological neuromuscular response that induces systemic and local ischemia-reperfusion through sensory changes (stress, inflammatory pain, analgesia) and motor alterations with skeletal muscle reactions (fight-to-flight and withdrawal reflexes); myocardium changes (tachycardia) and vascular smooth muscle impairment (vasoconstriction and vasodilation). Sudden hydroelectrolytic change is a common and basic pathogenic mechanism of this response. Through this mechanism, the intense local and systemic inflammatory responses are associated with abnormal ion transport.

In the early neurogenic stress response, the activation of the hypothalamic-pituitary-adrenocortical, sympathetic-adrenal medullary and renin-angiotensin-aldosterone axes occur, with the release of catecholamines, glucocorticoids and mineralocorticoids. Consequently, selective accumulation of these substances in the interstitial space of tissues suffering from ischemia-reperfusion is produced because endothelial permeability is increased, especially in postcapillary venules (Figure 2).

Damage inducible alarm signals from injured tissue, such as those exposed to mechanical damage, may determine the subsequent activation of this pathological neuro-motor response. Tissue injury is a critical initiator of the acute inflammatory response. Cell components released by necrotic cells elicit local, regional and systemic inflammatory responses. Accordingly, the inflammatory activity of dying cells decays over time. As soon as these alarm or "danger" signals cannot be replenished, they are degraded. Endogenous inducers of inflammation include ATP, K⁺ ions, the nuclear protein HMGB1 (high-mobility group box 1 protein), heat shock proteins, the end product of purine catabolism, uric acid, galectins and several members of the S100 calcium-binding protein.



Figure 2:

An early pathological motor response, where the smooth muscular fibers is prominent, particularly in the vascular system, is triggered. The vasomotor response with vasoconstriction, which collaborates in the production of ischemia and vasodilation, causes the redistribution of the local vascular and systemic blood flow. The intensity and duration of this ischemia-reperfusion phenomenon determines the evolution of the subsequent inflammatory response phases.

Vasoconstriction occurs in this vasomotor response and produces ischemia and cellular edema. This is then followed by vasodilation with reperfusion injury, which in turn causes exudation, followed by an increase in endothelial permeability that induces interstitial edema. Both cellular, by ischemia, as well as interstitial, by reperfusion, edema could represent an ancestral mechanism to feed cells by diffusion. Nevertheless, disturbance of ion transport has been associated with cellular dysfunction. There is increasing evidence that conditions characterized by an intense local inflammatory response are associated with abnormal ion transport. Inflammatory mediators which influence ion transport are bradykinin, leukotrienes, cytokines and TGF. They trigger the release of specific messengers like prostaglandins, nitric oxide and histamine, which alter ion transport function through specific receptors, intracellular second messengers and protein kinases. There is an additional influence of thrombin, and the complement system.

Interstitial edema causes a steady separation of the cells from the capillaries, which would favor the persistence of an ischemic phenotype (anoxia) and the defective use of oxygen (hypoxia) represented by excess production of reactive oxygen and nitrogen species or oxidative and nitrosative stress during reperfusion. Oxidative and nitrosative tissue damage could also increase lipid peroxidation with increased membrane permeability, increased degradation of extracellular matrix and edema. The accumulation of glycosaminoglycans fragments has been proposed as an important mechanism for edema formation because of its hydrophilic properties. Glycosaminoglycans are long unbranched polyssacharide that tend to adopt highly extended random coil conformation and occupy a huge volume for their mass. They attract and entrap water and ions, thereby forming hydrated gels, while permitting the flow of cellular nutrients. Under inflammatory conditions, hyaluronan, a nonsulphated glycosaminoglycan, is more polydisperse with a preponderance of lowermolecular forms: it favors edematous infiltration of the tissues as well as the interstitial fluid flow and the tissue lymph pressure gradient. Likewise, while the progression of interstitial edema reduces the blood capillary function, it simultaneously enhances lymphatic circulation (circulatory switch). Also, interstitial flow is important for lymphangiogenesis. The interstitial fluid flow associated with edema, even though it can be extremely slow, can have important effects on tissue morphogenesis and function, cell migration and differentiation and matrix remodeling, among other processes. Abnormally increased interstitial flow rates can occur during inflammation and can also trigger fibroblasts to differentiate or remodel the extracellular matrix, contributing

9

to the development of tissue fibrosis. Also, upon activation, mast cells, major effector cells in host defense responses and immunity, not only release vasoactive substances, i.e. histamine and serotonin, but also proteolytic enzymes favoring interstitial edema. Curiously, the functional impotence of the somatic motor system, which controls voluntary movements, favors vascular blood stasis and interstitial edema. Limiting swelling is extremely important, because the injured area cannot return to normal until swelling is gone. In musculoskeletal injuries, this is best accomplished with the "RICE" technique, which involves "Rest, Ice, Compression and Elevation".

The traumatized tissue seems to adopt an ischemic phenotype (anoxichypoxic). Chronic extreme hypoxia leads to tissue loss. In contrast, generally acute mild to moderate hypoxia supports adaptation and survival. The traumatized tissue probably suffers a metabolic hypoxia, a state where, although oxygen is available, the cell is unable to utilize it for respiration. In this critical situation, HIF- (hypoxia inducible factor) independent mechanisms of energy conservation, could promote survival under very low oxygen conditions but they are not compatible with the formation of new tissue, as required during wound healing. On the contrary, during the subsequent phases of the posttraumatic evolution, HIF-dependent pathways for survival and vascularization can function under conditions where hypoxia is moderate and not extreme. HIF- 1α enhances the expression of hypoxia responsive genes and therefore, allows improved cell survival in conditions of limited oxygen availability. HIF-1 α activates the transcription of genes involved in diverse aspects of cellular and integrative physiology, including energy metabolism, cell growth, survival, invasion, migration and angiogenesis.

Hence, in this initial phase of the inflammatory response, it could be considered that hypometabolism, anerobic glycolysis with lactate production, low temperature and decreased energy expenditure are associated with primitive cellular trophic mechanisms, which may be favored by neuroendocrine-stress response substances to arrive to the interstitial space of the traumatized patient. This environment could favor the cell dedifferentiation process through which cells adapt embryonic characteristics and stem cell recruitment with origin in the bone marrow.

2. The leukocytic phenotype

Nowadays, the inflammatory bone marrow-related response induced by wounds is considered both a key and complementary arm of the stress response. The inflammatory activation of the bone marrow stem cell niche indicates the stimulation of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), both multipotent stem cells. HSCs are the progenitors signaling molecules, including interferons, that appear to stimulate HSC proliferation in response to acute and chronic inflammation.

The leukocytic phenotype of the acute post-traumatic inflammatory response is characterized by the infiltration of the traumatized tissue, which has previously suffered ischemia-reperfusion, by inflammatory cells and bacteria. Acquiring an active immune phenotype through the traumatized tissue involves both parenchymal (epithelial cells) and non-parenchymal cells (endothelial cells, fibroblasts and tissue-resident macrophages, mast cells and lymphocytes) as well as blood cells that migrate to the tissue interstitium. This interstitial infiltration occurs in an oxygen-poor environment and one of its purposes could be trophism of the traumatized tissue (Figure 3).

Cellular interstitial infiltration is favored by the action of intrinsic and extrinsic components of the coagulation cascades. This results in the production of thrombin and conversion of fibrinogen of intravascular origin to fibrin. In most pathophysiological situations, it seems that the activation of both the coagulation and complement cascades occur simultaneously. The complement and coagulation systems are organized into proteolytic cascades composed of serine proteases of the chymotrypsin family. An interesting hypothetical explanation for the structural and functional similarities between the complement and clotting systems is that they originate from a common ancestral developmental-immune cascade. Thus, the functional linkages between development, immunity and hemostasis in vertebrates would be explained. Complement activation could express successive and predominant functions related to the inflammatory post-traumatic phenotypes. Therefore, the different functions of the multiple components of the complement activation could be integrated into the acute inflammatory response phases which would facilitate the comprehension of the implied biochemical mechanisms, like the complement-coagulation interactions. Hence, an early provisional would matrix of fibrin and extravased plasma fibronectin is formed, which also includes other components, such as the extracellular matrix proteins, vitronectin and thrombospondin. Blood clots facilitate extravascular migration, first of platelets and later of leukocytes.





Within minutes of wounding, inflammatory cells are attracted by complement activation, degranulation of platelets and products of bacterial degradation. Neutrophils arrive first, followed by mast cells and monocytes that subsequently differentiate into tissue macrophages. In the tissue suffering oxidative stress, symbiosis of the inflammatory cells and bacteria for extracellular digestion by enzyme release (fermentation) and by intracellular digestion (phagocytosis) could be associated with enzymatic stress. Furthermore, lymphatic circulation plays a major role in which macrophages, dendritic cells and mast cells migrate to the lymph nodes and activate lymphocytes.

Accumulating evidence demonstrates that platelets contribute to the initiation and propagation of the inflammatory process. These cells are replete with secretory granules, α -granules, dense granules and lysosomes. The α granule is the most abundant and its content includes both membrane bound proteins that become expressed on the platelet surface and soluble proteins that are released into the extracellular space. Whereas platelet dense granules contain high concentrations of low molecular weight compounds that potentiate platelet activation i.e. ADP, serotonin and calcium, α -granules concentrate large polypeptides i.e. fibrinogen and von Willebrand factor, which contributes to hemostasis. Platelet α -granules also influence inflammation both by expressing receptors that facilitate adhesion of platelets with other vascular cells, i.e. Pselectin, and by releasing a wide range of chemokines, among which CXCL4 and CXCL7 are the most abundant. Perhaps the strongest evidence that platelet release promotes wound healing is the use of the "platelet-derived wound healing factor" (PDWHF) in the treatment of chronic wounds. Lastly, platelet α -granules contain a variety of both pro- and anti-angiogenic proteins. Growth factors stored in α -granules include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGE) fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin-like growth factor (IGF).

In the post-traumatic local inflammatory response, the activation of the innate immune system is not only based on the recognition of danger signals or danger-associated molecular patterns (DAMPs), but also relies on the presence of pathogen-associated molecular patterns (PAMPs). DAMPs and PAMPs are recognized by pattern-recognition receptors (PRRs), that are cytoplasmic, membrane-bound or secreted. The most intensely studied PRRs are the Toll-like receptors (TLRs), the nucleotide-binding and oligomerization domain

(NOD)-like receptors (NLRs) and the retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs). In particular, NLRs from central molecular platforms that organize signaling complexes, such as inflammosome, were coined to describe the high molecular weight complex that activates inflammatory cascades and cytokine IL-1B. All these receptors activate signaling cascades that lead to the activation of MAP kinases and NF-kB. The host can discriminate between danger and pathogen-associated molecular patterns.

Once activated, TLRs induce different signaling cascades depending on the adaptor protein, ultimately leading to the activation of the transcription factors NF-kB, AP-1 and interferon-regulatory factor (IRF). TLRs recognize PAMPs and DAMPs. Thus TLRs appear to regulate inflammatory responses during wound healing under both sterile and non-sterile conditions. Ischemiareperfusion injury with oxidative stress, represents the scenario in which a profound injury-promoting role of TLR-2 and TLR-4 has been most thoroughly established. Available data suggests that the presence of TLR ligands during wound healing responses promote healing by scarring rather than healing by regeneration. The regulatory event of NF-kB activation is the phosphorylation of IkB proteins by the IkB kinase (Ikk) complex, which leads to IkB protein ubiquitylation and subsequent degradation. This results in the release of cytoplasmic NF-kB complexes, which then translocate to the nucleus and drive the expression of target genes. Thus, the expression of cytokines, cytokine receptors, chemokine, chemokine receptors, adhesion molecules and autacoids in the traumatized tissue is induced. Although NF-kB fulfills its expected role in inducing pro-inflammatory gene-expression programs, surprisingly it also plays an important role in the resolution of inflammation and tissue repair.

Leukocytes transverse the subendothelial basement membrane during their immunological surveillance patrol through tissues. This process, called "diapedesis" is strongly enhanced under the influence of inflammation. The preferred sites of leukocytes are the venules, which are characterized by a special composition of the subendothelial basement membrane. Leukocyte migration into the interstitium occurs in three stages: rolling along the endothelial surface, firm adhesion and transmigration. In the interstitium, the recruited and activated neutrophils begin debridement of devitalized tissue and attack infectious agents. To perform this task, they release a large variety of active antimicrobial substances i.e. ROS, cationic peptides, eicosanoids; and proteases, i.e. elastase, cathepsin G, proteinase 3 (PR-3), urokinase-type plasminogen activation (uPA). The most probable mechanism by which neutrophils delay the healing process is through the production of excessive toxic molecules, like ROS and proteases, which damage the cells of the wound site and thereby delay the healing process.

Tissue injury is a critical initiator of the acute phase response. Inflammatory cytokines trigger peutraxin generation. Pentraxins are superfamily of acute phase reactants. The C-reactive protein (CRP) and serum amyloid Pcomponent are well-characterized short pentraxins. Both resident and innate immunity cells produce pentraxin 3 in peripheral tissues in response to inflammatory signals and TLR activation. Neutrophils store pentraxin 3 in specific granules and release it in response to inflammatory signals. Besides a protective effect, pentraxins also influence the resolution phases of the inflammatory response later on.

As monocytes extravasate from the blood vessel they become activated and differentiate into mature tissue macrophages. The differential activation of macrophages is involved in many facets of tissue injury and inflammation. "Classically activated macrophages", or M1 macrophages, express proinflammatory cytokines (IL-1, IL-6, IL-23, IFN-8) and reactive oxygen/nitrogen species which are involved in the phagocytosis and killing of microbes. They also promote type I immune responses. "Alternatively activated macrophages", or M2 macrophages, fail to express pro-inflammatory mediators and are involved in angiogenesis, tissue remodeling and inflammation resolution, and therefore are supposed to promote repair functions.

T-helper cells play critical roles in modulating the differential activation of macrophages. Type 1 T-helper (Th1) cells produce pro-inflammatory cytokines (IFN- γ , TNF- α) which skew macrophages into the M1 phenotype. Type 2 T-helper (Th2) cells belong to a larger spectrum of distinct Th responses that have evolved to protect the host against a spectrum of pathogens. Traditionally, Th2 cells have been defined as the T cells that produce IL-4, IL-5, IL-13, IL-9 and IL-

10 and express the transcription factors GATA-binding protein 3 (GATA-3), signal transducer and activator of transcription-5 (STAT-5) and STAT-6. However, single-cell analysis has revealed a marked degree of heterogeneity in the cytokine profiles of Th2 cells. Many endogenous molecules released by tissue damage are potent inducers of type 2 responses. HMGB-1, a non-histone chromatin binding protein that is released upon cell death and matrix metalloproteinase (MMP)-2 activate dendritic cells via a TLR-4 dependent pathway to induce Th2 cell response. It has been proposed that type 2 responses represent a rapid repair response to tissue damage i.e. synthesis of collagen I and III, suppression of IL-17 production, enhanced expression of IL-10 and generation of anti-inflammatory macrophages, all of which lead to rapid tissue repair.

Although neutrophils, macrophages and T lymphocytes are considered central in the pathogenesis of post-traumatic inflammation, recent studies also imply the involvement of dendritic cells, mast cells, basophils and B lymphocytes to modulate the inflammatory response and wound healing. In addition, when leukocytes infiltrate inflamed tissues, they express metabolic anatomy as well as neuroendocrine ability. Particularly the potential role of leukocyte-derived neuropeptides and hormones in inflammation as a localized hypothalamic-pituitary-like axis has been proposed. However, the incorrect use of oxygen persists in this phase. Activated phagocytes would require anaerobic glycolisis as the main source of ATP to function, suggesting that activated leukocytes can metabolically adapt to the hypoxic environment in this evolutive phase of inflammation.

3. The angiogenic phenotype

Angiogenesis permits numerous substances, including hormones, to be transported by the blood circulation. For this reason it is considered that the predominance of angiogenesis during the last phase of the inflammatory response would allow for naming this period, the "endocrine" phase. Angiogenesis is defined as the growth of new vessels from preexisting ones and occurs by sprouting, intussusceptions (non-sprouting) and looping from preexisting vessels. The angiogenic switch which stimulates new vessel formation is induced under conditions of hypoxia, low pO₂ and mechanical stress i.e. shear stress. Hypoxia induces angiogenesis through HIF-1 pathway with growth factor expression elevated in hypoxic conditions in a HIF dependent manner. Hematopoietic stem cell and endothelial precursor cells (EPC) share a common stem cell origin from hemangioblasts and are present in bone marrow. During angiogenesis circulating and pre-existing EPC cells are attracted to the site of injury and differentiate into endothelial cells.

Although the final objective of endothelial growth is to form new mature vessels for oxygen, substrates and blood cells, other functions could also be carried out before the new vessels are formed. Thus, in the initial phases of the inflammatory response, the new endothelial cells could have antioxidant and anti-immune properties favoring the resolution as well as the progression of the angiogenic vascular phenotype. Angiogenesis is regulated by numerous "classic" factors, including VEGF-A, also known as vascular permeability factor (VPF), FGF-2, TGF, angioproteins, PDGF, thrombospondin-1 and angiostatin. Non-classic endogenous stimulators of angiogenesis include erythropoietin, angiotensin II, endothelins, adrenomedullin, adipokines (leptin, adiponectin), neuropeptide-Y, vasoactive intestinal peptide (VIP) and substance P. VEGF and FGF-2 occupy the center stage in the angiogenesis field. They act in synergy to stimulate endothelial cell function during angiogenesis in tissue repair.

Granulation tissue formation starts about three or four days after injury. The main cell types driving the generation of the new tissue are macrophages, endothelial cells, fibroblasts and kertinocytes. Angiogenesis is associated with granulation tissue formation, as the newly forming cellular complex must be supplied with oxygen and nutrients. As granulation tissue forms in the healing wound, the vascular cells intermingle with the provisional matrix, which is composed mainly of fibrin, fibronectin and vibronectin. Then the new blood vessels associated with fibroblasts and macrophages replace the fibrin matrix with granulation tissue, forming a new substrate for keratinocyte migration.

As the inflammatory response regresses by certain stop signals at appropriate checkpoints, edema production and leukocyte traffic into the traumatized tissue are prevented. The pro-inflammatory mechanisms probably

are counterbalanced by endogenous anti-inflammatory signals that serve to temper the severity and limit the duration of the early phases, which leads to their resolution, an active rather than a passive process. The resolution of the inflammatory response is mainly mediated by families of local-activity mediators that are biosynthesized from essential fatty acids eicosapentaenoic acid and docosahexaenoic acid. These resolution mediators were termed resolvins and resolution is protectins. Inflammation also mediated by lipoxins, trihydroxystearin-containing eicosanoids that are generated within the vascular lumen through platelet-leukocyte interactions. Lipoxins were the first mediators identified to have both direct anti-inflammatory and proresolving properties. The spectrum of lipoxin's anti-inflammatory activity, particularly lipoxin A4, include inhibiting the production of ROS, attenuating the microvascular fluid leak, attenuating the expression of adhesion molecules on leukocytes and endothelial cells, diminishing the production of chemokines and preventing neutrophil adhesion and transmigration through the endothelium and stimulating the uptake of apoptotic polymorphonuclear leukocytes by macrophages.

It has been also proposed that regulatory T cells (Treg cells) have evolved to provide a complementary immunological arm to a physiological tissue-protecting mechanism driven by low oxygen tension (i.e. hypoxia) in inflamed tissues. The hypoxia-adenosinergic pathways might govern the production of immunosuppressive molecules that have already been implicated in the activities of Treg cells. In this way, by virtue of acting in hypoxia and extracellular adenosine-rich tissue, Treg cells could exert their suppressive function with local downregulation of immune response, inducina "immunodormancy" and protecting tissues from continuing collateral tissue damage, thus improving healing. Also B-1 lymphocytes, which represent the main B-lymphocyte population in the peritoneal and pleural cavities, secrete IL-10 and exert a downregulatory effect on inflammation and wound healing in mice.

The progressive resolution of the inflammation favors wound reepithelization. This begins one to two days after injury by the migration of keratinocytes from the epidermis at the wound edge and from injured appendages. Keratinocytes at these epidermal fronts move forward between the injured dermis and the fibrin clot. Epithelial cell migration requires the disassembly of desmosomes and hemidosmosomes, which provide anchorage of the basal keratinocytes with neighboring epithelial cells and the underlying basement membrane respectively. This disassembly and keratinocyte migration requires a cross-talk between growth factors, metalloproteinases, integrins and structural proteins. In addition to lamellipodia extension, basal keratinocytes leapfrog over the basal cell near the wound. The keratinocytes that are behind the leading edge in larger wounds proliferate and mature and finally restore the barrier function of the epithelium. This could involve the proliferation of epidermal stem cells.

The migration and proliferation of epithelial cells occurs under the control of EGF, TGF- α and PDGF. Cell migration may also require the upregulation of tissue plasminogen activator, urokinase plasminogen activator and metalloproteinases (MMP-1, MMP-9, MMP-10). All these factors act as molecular scissors and help regulate the provisional matrix, collagen degradation and cellular movements. Lastly, integrins are also important for wound re-epithelialization, particularly β_1 -integrins. It has been proposed that the phenotypic impairments developed by the keratinocytes during reepithelialization constitute a partial epithelial-mesenchymal transition. Following the completion of wound-repair, keratinocytes revert their mesenchymal-like phenotype to the epithelial phenotype.

The interstitium is considered the battle field where inflammation develops and the stroma is its equivalent in tissues and organs. At the same time, the most abundant cell type of tissue stroma is the fibroblast, an active heterogeneous population of cells.

Fibroblasts can also contribute to the resolution of inflammation by withdrawing survival signals and normalizing chemokine gradients, thereby allowing infiltrating leukocytes to undergo apoptosis or leave the tissues through the draining lymphatics. Lastly, fibroblasts may also provide important positional signals for wound healing and tissue regeneration. In addition to their role in producing an extracellular matrix, they may facilitate angiogenesis by production and release growth factors.

Fibroblasts, which deposit large amounts of extracellular matrix, repopulate the wounded area in parallel to angiogenesis. Several days after injury, a subset of wound fibroblasts differentiate into myofibroblasts which are responsible for wound contraction and for the deposition of additional matrix proteins. Transforming growth factor-beta (TGF- β) and platelet-derived growth factor (PDGF) initiate phenotype changes, converting fibroblasts into myofibroblasts. Matrix formation requires the removal of granulation tissue with revascularization. A framework of collagen and elastin fibers replaces the granulation tissue and leads to progressive tissue sclerosis. This framework is then saturated with proteoglycans and glycoproteins. This is followed by tissue remodeling involving the synthesis of new collagen, mediated by TGF- β , and the breakdown of old collagen by PDGF. During the tissue remodeling phase, the initial collagen type III present in the granulation tissue is gradually substituted and dominated by collagen type I and the resultant larger collagen fibrils are abnormally arranged in parallel bundles. Remodeling begins two to three weeks after injury and lasts for a year or more. Most of the endothelial cells, macrophages and myofibroblasts, undergo apoptosis, leaving a mass that contains few cells and consists mostly of collagen and other extracellular-matrix proteins. However, the prognosis of extensive and deep wounds is not entirely satisfactory because of scar formation and the loss of normal function and skin appendages. Therefore, reducing the formation of scars and reestablishing the normal anatomy and function of the skin and its appendages have become the aim of regenerative medical research.

During the evolution of the inflammatory response, the traumatized tissue losses its more specialized functions and structures. In this progressive deconstruction a redistribution of immediate constituents is produced. In this context, the redistribution of metabolic resources responds to the different trophic requirements of the traumatized tissue as the inflammation progresses. It has been proposed that progressive tissue deconstruction is developed, among other mechanisms, by autophagy. This process is unique since it converts topologically intracellular material into topologically extracellular material. Most of the autophagy genes are present in higher eukaryotes, indicating that this process has been evolutionarily conserved. However, consumption of the substrate deposits and the dysfunction or failure of the specialized cells in the traumatized tissues could also represent an accelerated process of dedifferentiation. The hypothetical ability of the tissues to involute or dedifferentiate could represent a return to early stages of development. Therefore it could develop an effective defense mechanism against injury since it could make retracing a well-known route possible i.e. the prenatal specialization phase during the evolution of the inflammatory response. This specialization would require a return of the prominence of oxidative metabolism with a subsequent angiogenesis in the affected tissue to create the capillary bed that would make regeneration of the specialized tissue possible.

The ability to use oxygen in the oxidative metabolism is recovered when the tissues recover their capillary function and, therefore, nutrition is mediated by them. This type of metabolism is characterized by a large production of ATP (coupled reaction), which is used to drive multiple specialized cellular processes with limited heat generation. It would determine the onset of healing.

The blood cells that occupy the interstitial space in this latter phase of the inflammatory response are red blood cells. To carry out this interstitial occupation, the red blood cells are transported by the newly formed blood capillaries and, therefore, angiogenesis is considered to play the main role in this inflammatory period.

References

- Aller MA, Arias JL, Nava MP, Arias J. Post-traumatic inflammation is a complex response based on the pathological expression of the nervous, immune and endocrine functional systems. *Exp Biol Med (Maywood)* 229: 170-181, 2004.
- Aller MA, Arias JL, Sanchez-Patan F, Arias J. The inflammatory response: An efficient way of life. *Med Sci Monit* 12: RA225-RA234, 2006.
- Aller MA, Arias JI, Arias J. Pathological axes of wound repair: gastrulation revisited. *Theor Biol Med Model* 7: 37, 2010.

- Aller MA, Arias JI, Giner M, Losada M, Cruz A, Alonso-Poza A, Arias J. Oxygen-related inflammatory wound phenotypes. In: Middleton JE. Ed. Wound Healing Process, Phases and Promoting. Chapter 2. Huntington NY USA: Nova Sciences Publishers 1-26, 2011.
- Aller MA, Blanco-Rivero J, Arias JI, Balfagon G, Arias J. The wound-healing response and upregulated embryonic mechanisms: brothers-in-arms forever. *Exp Dermatol* 21: 497-503, 2012.
- Aller MA, Arias JI, Prieto I, Gilsanz C, Arias A, Yang H, Arias J. Surgical inflammatory stress: the embryo takes hold of the reins again. *Theor Biol Med Model* 10: 6, 2013.
- Arias JI, Aller MA, Arias J. Surgical inflammation: a pathophysiological rainbow. *J Transl Med* 7: 19, 2009.
- Kwan P, Hori K, Ding J,Tredget EE. Scar and contracture: biological principles. *Hand Clin* 25: 511-528, 2009.
- Kawasumi A, Sagawa N, Hayashi S, Yokoyama H, Tamura K. Wound healing in mammals and amphibians: Towards limb regeneration in mammals. *Curr Top Microbial Immunol* 2013;367:33-49.
- Leung A, Crombleholme TM, Keswani SG. Fetal wound healing: implications for minimal scar formation. *Curr Opin Pediatr* 24: 371-378, 2012.
- Profyris C, Tziotzios C, Do Vale I. Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics. Part I. The molecular basis of scar formation. J Am Acad Dermatol 66: 1-10, 2012.
- Reinke JM, Sorg H. Wound repair and regeneration. Eur Surg Res 49: 35-43, 2012.
- Rolfe KJ, Grobbelaar AD. A review of fetal scarless healing. *ISRN Dermatol* 2012: 2012: 698034.
- Yokoyama H. Initiation of limb regeneration: The critical steps for regenerative capacity. *Develop Growth Differ* 50: 13-22, 2008.