FACTA UNIVERSITATIS
Series: **Medicine and Biology** Vol. 18, No 1, 2016, pp. 6–11 UDC 612.017:616.71-001.5

Review Article

INTERACTIONS BETWEEN SKELETAL SYSTEM AND MACROPHAGES IN HOMEOSTASIS AND BONE INJURY

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Abstract. *New insights about close connection between skeletal and immune systems have expanded vistas of modern medicine and tissue engineering. Intensive progress of osteoimmunology enabled the understanding of processes related to bone tissue from a completely new angle, both in physiological and pathological conditions. In this respect, macrophages stand out as cells which affect bone through the ability to secrete a range of different cytokines. Macrophages' activation is directed by environmental conditions which determine the phenotype and function of these cells. Acquired phenotypic and functional characteristics of macrophages are changed according to changes in their environment. Thanks to these abilities, macrophages have great impact on bone development, bone homeostasis and osteoreparatory process. During bone development, macrophages can affect osteoblast differentiation and matrix mineralization. Coordinated action of osteoclasts and osteoblasts is important in bone tissue remodeling process. Also, during osteoreparation macrophages are among the first cells that will come to the site of bone injury. Their impact on bone is particularly visible during inflammatory phase of fracture healing. Better understanding of mechanisms by which macrophages exert their influence on bone would be an important step in approach to more specific therapies that would modulate activity of these cells and might accelerate healing of bone defects.*

Key words: *macrophages, bone, bone homeostasis, osteogenesis, fracture, osteoreparation*.

Introduction

The belief that bones represent inert structures has been disproved long ago by abundant evidence that bone tissue is very dynamic and that it is in constant process of resorption and formation [1, 2]. There are numerous data on direct correlation between skeletal and immune systems. Among various cells of immune system, macrophages are those that stand out by their secretory products which directly affect osteogenesis and osteoreparation [3–5]. In addition, macrophages are very plastic cells since they adjust their activity and change their phenotype according to general state of the environment. They are involved in several stages of osteoreparation, and are especially important actors during initiation of bone tissue healing [4]. Therefore, the possibility of modulating macrophages' activity would be a useful tool in an attempt to control osteogenesis and osteoreparation, especially after bone tissue injury or in pathological conditions.

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Received January 21st, 2016, accepted for publication July 3rd, 2016

Macrophages' Differentiation and Activation

Macrophages belong to a group of professional phagocytes which perform their functions thanks to numerous surface receptors and secretory products [6]. Almost all organs of the body contain tissue-resident macrophages, which play an important role in homeostatic processes [7, 8]. Macrophages have a wide range of morphological characteristics that correspond to their functional state and environmental conditions. Different subpopulations of tissue-resident macrophages exist in various tissues [8–10]. Depending on the tissue they are placed, tissue-resident macrophages include osteoclasts (bone), alveolar macrophages (lung), microglial cells (CNS), histiocytes (connective tissue), Kupffer cells (liver), and Langerhans cells (skin) [11].

The process of macrophages' differentiation should be distinguished from activation process, which means that differentiated macrophages through further stimulation increase their capability to exert certain functions. Tissue-resident macrophages are quiescent and characterized by low oxygen consumption, low expression level of major histocompatibility complex class II gene (MHC II), a little cytokine production and by preserved proliferative capacity. It is believed that there are two levels of macrophages' activation. Initial activation (priming) leads to increased expression of the MHC II gene, increased production of cytokines and reduced proliferative capacity.

Priming is usually achieved by low concentrations of interferons (IFNs) and it is a process of preparing for a quick response and reaction to other cytokines, although macrophages are not still fully activated [12]. Macrophages then react to secondary signals (e.g. tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS)) and become fully activated, which means that they lose their ability to proliferate, but gain ability to kill parasite and tumor cells, and all this is accompanied by increase in oxygen consumption, cytokines, reactive oxygen species (ROS) and nitric oxide (NO) secretion [13, 14].

Macrophages' Classification according to their Functional Characteristics

Macrophages show a remarkable plasticity through the ability to adapt their phenotype and function to environmental changes. Tissue injury, infections or tissue reaction to a foreign body excite quick response of these cells. Macrophage classification arises from their functional characteristics, surface markers and type of produced cytokines. According to their functional characteristics macrophages are usually classified as M1 or M2, i.e. classically and alternatively activated macrophages. This nomenclature is based on the type of T cells (Th1 or Th2) which influence macrophages' activation by distinct cytokines [15].

M1, i.e. classically activated macrophages, are referred as inflammatory and can be activated by IFN-γ, TNF-α and LPS. They are involved in defending the host against various pathogens and tumors. Macrophages of M1 type produce ROS and NO, high level of interleukin-12 (IL-12) and low level of IL-10 and also produce numerous pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 [6].

M2, i.e. alternatively activated macrophages, are referred as anti-inflammatory according to their anti-inflammatory function, but they also regulate wound healing [6]. Within this type of functional macrophages there are three subtypes of cells with different physiological roles. M2a macrophages are involved in later events of tissue repair, and they are activated by cytokines IL-4 and IL-13. M2c macrophage subtype is induced by IL-10 or glucocorticoids, and this subtype has anti-inflammatory function. M2b macrophages also achieve anti-inflammatory activity via IL-10, but also synthesize pro-inflammatory cytokines (IL-1 and TNF-α), like M1 type macrophages [16].

The relationship between Skeletal and Immune Systems from Macrophages' Perspective

At the beginning of the new millennium osteoimmunology was defined as new branch of science that deals with interactions between cells of immune system and bone tissue cells [17]. Immune cells produce cytokines which can have a part in normal bone tissue healing [4], but also can affect appearance and flow of different pathological conditions [18].

The connection between bone and immune system exists on at least three levels. Firstly, bone marrow is anatomically located in bones, so the mutual interaction of immune and bone cells is unavoidable. Secondly, cells of immune system originate from hematopoietic stem cells of bone marrow, similar to osteoclasts which structurally and functionally belong to bone tissue. Thirdly, the two systems share various cytokines, growth factors, signaling molecules and transcription factors [19].

Connection and conditionality between cells of bone and immune system is clearly represented trough osteoclastogenesis, since many factors that affect precursors of osteoclasts can be synthesized by inflammatory cells too. Furthermore, osteoclasts and immune cells share the same progenitors through differentiation process [20]. Osteoclasts originate from bone marrow pluripotent hematopoietic stem cell and are by themselves specialized bone tissue macrophages [11, 21]. Likewise, individual macrophages can fuse together to form osteoclasts [22]. The two most important cytokines that are necessary for unobstructed osteoclastogenesis are receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which can be secreted among others by inflammatory cells. RANKL is a cytokine expressed by osteoblasts, stromal cells, and activated T lymphocytes [23] and belongs to TNF superfamily. RANKL binds to RANK-receptor which exists on the surfaces of osteoclast precursors. Osteoprotegerin (OPG), secretory product of osteoblasts and numerous hematopoietic cells, is RANKL-competitor and has anti-osteoclastogenic function [24]. M-CSF is produced by bone marrow stromal cells, osteoblasts and T lymphocytes and it is responsible for proliferation and survival of osteoclast progenitors, as well as mature osteoclasts [25]. The fact that these two factors can be synthesized by cells of immune system indicates that in this way immune system can affect bone tissue. This correlation is particularly visible in some bone diseases [26].

Macrophages/monocytes can regulate bone development and homeostasis through secretion of numerous cytokines and other molecules, although their role in abovementioned processes is still not fully understood. Many of these secretory products are pro-angiogenic and pro-osteogenic [27]. It has been experimentally proved that macrophages are involved in osteoblast differentiation [3] and mineralization process [3, 28]. In addition, macrophages may activate other cells from their environment to secrete certain cytokines important for the osteogenic process [27]. Chang and coworkers point to macrophage population termed OsteoMacs in murine and human osteal tissue, significant in bone homeostasis. OsteoMacs are defined as stellate-shaped resident bone tissue macrophages located on endosteal and periosteal surfaces. Difference between OsteoMacs and osteoclasts is, among others, based on $F4/80^{+}TRAP^{-}$ phenotype of OsteoMacs and $F4/80^{-}$ $TRAP^{+}$ phenotype of osteoclasts. Also, OsteoMacs in

physiological conditions are not osteoclast precursors, while they may be in pathological conditions. These cells interact with osteoblasts, regulate their function and mineralization process through induction of gene for osteocalcin *in vitro*. [29]. OsteoMacs at bone modeling and remodeling sites form canopy structures over mature osteoblasts. Depletion of these cells leads to disappearance of mature osteoblasts from bone modeling surfaces. During bone remodeling OsteoMacs, like osteoclasts, provide coupling signals, most probably transforming growth factor beta (TGF-β) and ephrin B2 to osteoblasts, affecting bone formation [30].

Another unique ability of macrophages is to quickly respond to chemoattractants from the site of tissue injury. During fracture healing, macrophages come to the site of injury and release various cytokines that promote angiogenesis and recruitment of mesenchymal stem cells [27]. Presence of blood vessels and mesenchymal stem cells at defect site is crucial for proper osteoreparatory process [31, 32]. All of these macrophages' capabilities are in favor of their potential use in bone tissue engineering.

Inflammatory process plays an important role in initiating bone regeneration after injury. On the other hand, some inflammatory diseases or reactions to implanted material can lead to chronic inflammation, which has a destructive effect on bone tissue [33]. One such example of bone destruction associated with inflammation is reumatoid arthritis [34]. Therefore, studies concerning control of inflammatory signal are of the great significance.

The role of macrophages in the process of fracture healing is discussed in the following sections.

Repair of Bone Defects

Bone healing process usually goes through three dynamic phases that overlap each other and are named inflammatory, reparative and remodeling phase. Therefore, repair of bone defects (fractures) is characterized by an initial inflammatory reaction accompanied by cell proliferation and remodeling, which ultimately leads to bone reconstruction. The main actors of inflammatory process are macrophages, which migrate to the site of injury [4]. These cells also release factors involved in the formation and resorption of bone tissue.

Inflammatory phase

Together with bone damage, as consequence of fracture, damage of surrounding tissues and blood vessels also develops. Blood coagulation results in formation of hematoma. Due to blood vessels injury in the zone of bone fracture, lack of oxygen and nutrients occurs, leading to premature cell apoptosis and to the formation of necrotic tissue. Necrotic tissue, platelet-derived growth factor (PDGF) from blood clot and growth factors from extracellular matrix (TGF-β for example) act as chemoattractants for inflammatory cells (macrophages, monocytes, lymphocytes and neutrophils) and fibroblasts, and provoke acute inflammatory response. This initial phase of bone tissue healing reaches its maximum 24-48 h after injury and completes in about 1 to 2 weeks [35, 36]. Actually, it is believed that these first 2 weeks are the milestone in bone healing process [37].

Inflammatory phase is characterized by dynamic processes such as formation of granulomatous tissue, ingrowth of blood vessels and migration of mesenchymal stem cells to the fracture site [38, 39]. Likewise, levels of several pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, IL-11 and IL-18 are significantly increased [36, 38]. Although it is known that extended or chronic expression of pro-inflammatory cytokines might have negative effect on bone, short-term and highly specific secretion of these molecules is extremely important for tissue regeneration [40, 41]. These signals recruit inflammatory cells and promote angiogenesis [38]. It is believed that TNF-α as a product of inflammatory cells, especially macrophages, mediates the induction of secondary pro-inflammatory signals, which are chemoattractants for different cells and also can induce osteogenic differentiation of osteoblast-like cells [42, 43, 44]. Along with them, TGF-β1 and PDGF from blood clot also serve as guides to differentiation and proliferation of mesenchymal stem cells [45]. Over time, the acute inflammatory response is being replaced by the next phase.

Reparative phase

Reparatory phase starts with reorganization of hematoma. Numerous cells which came to the fracture site during inflammatory phase produce callus. Callus consists of cartilage and immature bone tissue and has function to increase stability of the fracture. Formed cartilage through ossification process becomes bone, under the influence of TGF-β2, TGF-β3, bone morphogenetic proteins (BMPs) and other signaling molecules [35, 38, 42, 46]. During reparative phase inflammatory cells and pro-inflammatory cytokines are absent [39].

Remodeling phase

During remodeling phase through the activity of osteoblasts and osteoclasts initial immature woven bone is replaced by mature lamellar bone. This phase, which begins 8 to 12 weeks after injury, is strongly osteoclast-dependent and it is regulated by a number of pro-inflammatory signals like IL-1, IL-6, IL-11, IL-12 and TNF-α [36, 38, 39]. Remodeling phase is the longest phase during bone healing process and can last up to several years.

The role of Macrophages during Fracture Healing Process

Macrophages play a significant role in bone healing process, in initial as well as the final stage. Immediately after fracture, macrophages along with neutrophils and lymphocytes penetrate into hematoma. Monocytes/

macrophages produce BMP-2, one of the key factors involved in the early osteogenic process. In fact, BMP-2 directs stem cells toward osteoblast differentiation *in vitro*, as well as *in vivo*. Pirraco and coworkers used experiments with co-cultures of human peripheral blood monocytes/macrophages and human bone marrow stromal cells (hBMSCs) which have shown that hBMSCs from co-cultures have higher proliferative capacity and higher alkaline phosphatase activity in regard to hBMSCs monocultures [47]. Schlundt and colleagues have worked with murine experimental model which included macrophage reduction using clodronate liposomes during bone healing process. In their experiments macrophages' reduction had no effect on early stages of fracture healing, while they had altered endochondral ossification through delayed hard callus formation [48].

Bone tissue is well vascularized so angiogenesis and vascularization are essential for unobstructed repair of bone tissue after injury [31, 49, 50]. According to literature data it is known that macrophages are able to affect all stages of angiogenesis thanks to their secretory products [51]. Stimulated macrophages release pro-angiogenic cytokines and growth factors, as well as enzymes that degrade extracellular matrix and enable releasing of "trapped" growth factors (bFGF, TGF-beta, GM-CSF) which also have proangiogenic activity [52]. Inclusion of macrophages (induced from THP-1 monocytic cell line treated with PMA (phorbol-12-myristate-13- acetate)) in co-culture made of human outgrowth endothelial cells (OECs) and primary osteoblasts leads to multiplying of microvessel-like structures formed by OECs and higher production of vascular endothelial growth factor (VEGF) compared to co-culture. Likewise, in triple-culture expression of IL-6, IL-8 and TNF-α was upregulated, indicating beneficial effects of pro-inflammatory cytokines in osteoreparation [53].

M1 type macrophages are the first that could be found at the site of tissue injury, with role to engulf necrotic material and to synthesize pro-inflammatory cytokines, ROS and NO. Guihard and coworkers found that M1 type macrophages stimulate osteogenic process through production of Oncostatin M (OSM), member of IL-6 cytokine family, which induce osteoblast differentiation and mineralization. [3]. Other experiments based on juxtacrine interaction in co-cultures composed of primary mouse macrophages and bone marrow stromal cells (BMSCs) resulted in enhanced proliferation and migration of stem cells which was mediated with increased macrophage IL-6 production in these co-cultures [54].

M1 macrophages are later replaced by M2 type that produces IL-10, TGF-β, as well as other anti-inflammatory cytokines, which are essential for proper wound healing. Actually, due to their plasticity macrophages can switch from M1 to M2 phenotype [55, 56]. It has been experimentally proved in mouse osteotomy model that induction of M2 macrophages during fracture healing process enhances bone formation [48]. Loi and colleagues had investigated the effect of M1 and M2 type macrophages on osteogenesis *in vitro* in co-cultures of polarized primary murine macrophages and preosteoblastic MC3T3-E1 cells [57]. In each co-culture type osteogenic differentiation of MC3T3-E1 cells was increased and switching of macrophage phenotype from M1 to M2 through IL-4 application had enhanced osteogenic ability of MC3T3-E1 cells in co-cultures. It has been confirmed by these experiments that inflammatory phase is necessary before healing process is initiated.

Above all, action of these two types of macrophages had to be balanced. If M1 type macrophage activity overcomes macrophages of M2 type, that can lead to further tissue damaging, while the opposite case can lead to fibrosis [58].

During inflammatory phase macrophages remove necrotic tissue and secrete a number of pro-inflammatory cytokines such as TNF-α and IL-1. The aforementioned pro-inflammatory cytokines reach maximal concentration 24 h after tissue injury [59]. At fracture site TNF-α can have a dual function that depends on which of the two cell receptor (TNFR1 and TNFR2) TNF-α binds [36]. IL-1 can exists in two forms: IL-1α and IL-1β. While IL-1α upregulates inflammation [60], IL-1β is thought to have a positive effect on mesenchymal stem cells differentiation into osteoblasts [61] and proliferation of osteoblast-like cells [43].

During remodeling phase TNF-α concentration rises again [59]. This cytokine binds to TNFR1 which exists on preosteoclasts' surfaces [62] and in this way has impact on osteoclastogenesis [36]. At the same time, along with TNF-α, concentration of IL-1 increases and affects degradation of cartilage matrix during its maturation into bone matrix [36].

Macrophages as *in vitro* Model in Bone Tissue Engineering

In some cases, when large bone defects occurred, bone tissue is not able to compensate the loss so it is necessary to use different bone substitutes. Bone substitutes most often include biomaterials based on tricalcium phosphates, hydroxyapatites, collagen and composites made from both inorganic and organic compounds. Also, 3D scaffolds are very useful because of their characteristic 3D structure that mimics the structure of living tissue. All of these materials can produce inflammatory reactions of macrophages *in vivo* when implanted into the defect area. Intensity of inflammation can greatly affect the course of the healing. Bearing in mind that injury itself creates local inflammatory reaction, if materials further stimulate this process, that could lead to the creation of fibrous tissue and inadequate healing process. Biomaterials that are nowadays increasingly used in bone tissue engineering are designed to have a stimulating effect on osteogenic process without having potential to induce or prolong inflammatory response of macrophages at injury site. Therefore, it is very important to show that biomaterials

are immunocompatible. For assessing the response of macrophages to different biomaterials, which can be potentially applied in bone tissue engineering and regenerative medicine, *in vitro* models of macrophages are used very often. The examination can be carried out on peritoneal macrophages, peripheral blood monocytes and different cell lines. Most commonly used cell line for these purposes is RAW 264.7 cell line. As previously stated in this paper, for the normal flow of healing process it is essential that there is a balance between M1 and M2 macrophages. It is therefore important to examine how biomaterial of interest affects the polarization of macrophages [63–65]. Another very important characteristic that biomaterials should have is to induce controlled and moderate phagocytosis by macrophages. Different *in vitro* approaches of materials testing on macrophages are used, such as direct or indirect contact assays with both direct application of materials' particles or application of materials' extracts. In both assays phagocytosis can be measured quantitatively by using standard phagocytosis tests such as NBT test [5] or Neutral red uptake test, or analyzed through materials' particles uptake assay by transmission electron microscope (TEM) [65, 66]. For assessing the production of pro-inflammatory and anti-inflammatory cytokine release from macrophages stimulated with biomaterial particles or extracts, the most frequently performed method is determination of cytokine level by ELISA assay [67–70]. For this purpose, biological assay such as L929 assay can also be used [5]. Macrophages can also be used to simulate an inflammatory state *in vitro* in order to examine how different factors released from activated macrophages can influence the osteogenic differentiation of cells [71].

Conclusion

Science progress and better understanding of pleiotropic role of macrophages in a variety of biological and pathological processes put them at the top of "cell pyramid" because of their great influence on all aspects of tissue homeostasis and tissue reparation. It is believed that these phagocytes, as well as molecules they are secreting (especially during inflammatory phase), are the key factors for the successful bone tissue repair. Future research should be directed toward modulation of macrophage's activity which might have positive influence on the final result of osteogenesis and osteoreparatory process.

Acknowledgments: This paper is supported by Project Grant III41017 funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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