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RAGE and its ligands in bone metabolism

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1. ABSTRACT

The receptor for advanced glycation end products (RAGE), a member of the immunoglobulin super-family transmembrane proteins, has multiple ligands, thus, is implicated in the pathogenesis of various diseases, including diabetic complications, neurodegenerative disorders, and inflammatory responses. Its function in normal physiology is beginning to be defined, and recent studies have pointed to an important role for RAGE and its ligands [e.g., HMGB1 (high mobility group box 1)] in innate immune response. In addition, RAGE and its ligands are also implicated in osteoclast activation and bone remodeling. Understanding how RAGE and its ligands regulate bone remodeling may provide insight into the pathogenesis of diabetes and chronic inflammation associated bone loss. Recent progress relevant to the functions of RAGE and its ligands in bone remodeling is discussed in this review.

2. INTRODUCTION

Skeletal system, including bone and cartilage, consists of bone cells and extracellular matrix. Bone, a specialized connective tissue, performs mechanical, protective, and homeostatic functions for body. Bone cells, including osteoblasts, osteoclasts, and osteocytes, maintain bone architecture, homeostasis, and mechanical properties by balancing the production and degradation of bone matrix. Bone matrix is largely mineralized and constantly regenerated throughout life by bone cells. Cartilage consists of chondrocytes and extracellular matrix released from chondrocytes. Thus, bone remodeling is a life-long, multi-cell orchestration under both patho- and physiological conditions. Any dysfunction of bone cells and/or abnormity of bone matrix could lead to dysfunction of skeletal system, further influencing the life quality.

Osteoclasts are our body's principal bone resorbing cells that not only play a crucial role in skeletal

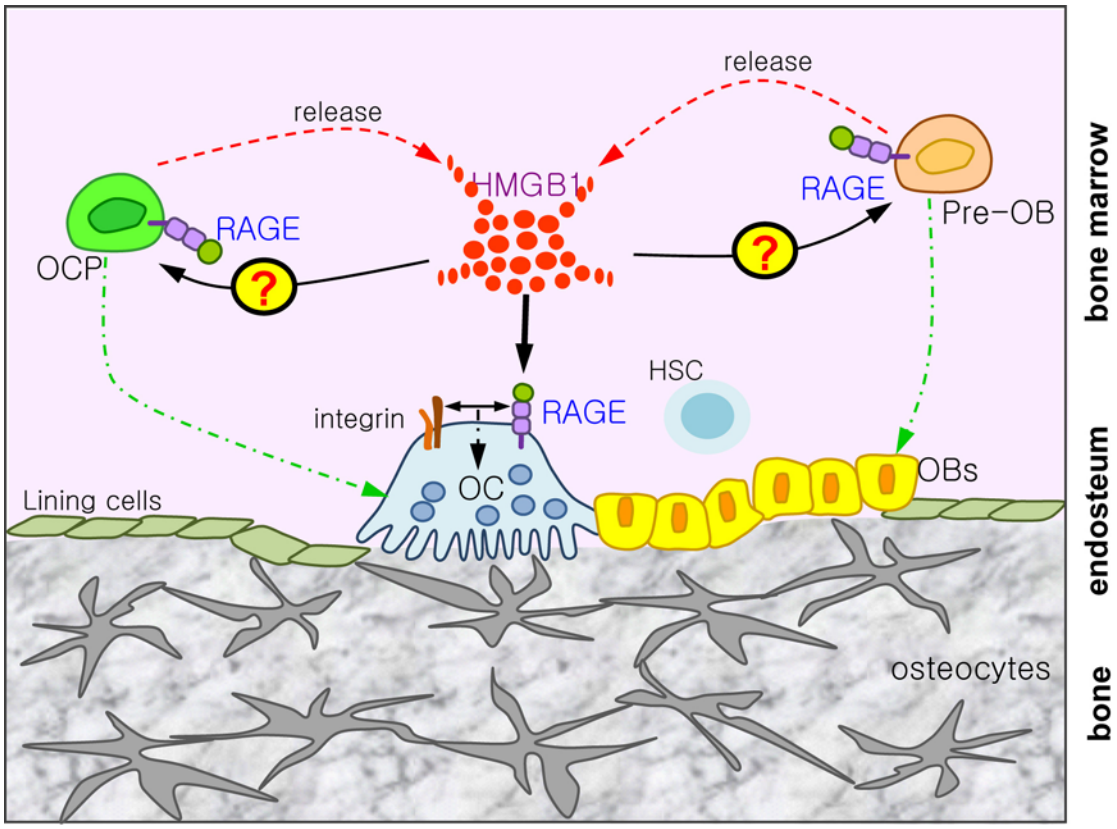

Figure 1. A model to illustrate potential functions of RAGE and HMGB1 in bone remodeling**.** The bone cells [osteoblasts (OBs), osteoclast (OC), pre-osteoblast (Pre-OB), osteolcast precursor (OCP), and osteocytes) are indicated. RAGE (marked by 3 Ig motifs) is expressed in nearly all of these cells. The extracelluar HMGB1, released from both OCP and Pre-OB, as well as bone marrow macrophages and chondrocytes, promotes OC differentiation in a RAGE dependent manner. HMGB1's function in OB is unclear. It is suggested that HMGB1 may play a role in recruitment of pre-OBs and OCPs to the site of bone remodeling.

development and maintenance but also are implicated in the pathogenesis of various bone diseases including postmenopausal osteoporosis (1-2). Osteoclasts are multinucleated giant cells that differentiate from cells of hematopoietic origin (1-3)(Figure 1). Hematopoietic stem cells give rise to circulating mononuclear cells which are attached to bone-resorbing sites to differentiate into osteoclasts in response to two key osteoclastogenic factors M-CSF (macrophage colony stimulating factor) and RANKL (receptor activator for nuclear factor κ B ligand)(4-6). The adhesion of mature osteoclast to bone matrix is also essential for its resorptive function (Figure 1). In addition to bone resorption, osteoclasts also regulate osteoblast differentiation, promote hematopoietic stem cell mobilization from the bone marrow to the blood stream, and participate in immune response (7). *Osteoblasts* are major bone formation cells (8). The origin of osteoblasts is from local mesenchymal stem cells. The major function of osteoblasts is to synthesize and secrete type I collagen and other bone matrix proteins. Osteoblasts also regulate osteoclast differentiation by secreting soluble factors [e.g., RANKL and osteoprotegerin (OPG)] and by interacting with osteoclast precursor cells. *Osteocytes* are the most abundant cells in adult skeleton, account for over 90-95% of all bone cells (7)(Figure 1). They originate from terminal differentiation of osteoblasts and are embedded in the bone matrix. Morphologically, osteocytes extend their dendritic processes within calcified bone matrix to connect with other osteocytes or bone cells (e.g., osteoblasts and osteoclasts) on the bone surface (Figure 1). The functions of osteocytes are thought to mediate mechanical stimulation via its cellular processes, orchestrate bone remodeling by regulating the activity of osteoblast and osteoclast, and to be involved in phosphate homeostasis. In addition to these bone cells, *chondrocytes* are the cartilage formation cells. Like osteoblasts, the progenitor of chondrocytes is also mesenchymal stem cells, and the major function of chondrocytes is to produce and maintain the cartilaginous matrix, which consists mainly of collagen and proteoglycans.

RAGE, a member of the immunoglobin (Ig) superfamily of cell surface receptors, contains three N-terminal Ig domains, a single transmembrane domain, and a short, acidic C-terminal cytosolic tail (9)(Figure 1). Among the three Ig domains, the extreme IgV domain is believed to serve as the ligand-binding site, and the C-terminal cytosolic domain appears to be required for the signal transduction (9). Rage gene encodes multiple isoforms, including membrane and soluble forms. The soluble RAGE

(sRAGE), a form of the RAGE lacking the cytosolic and transmembrane domains, appears to have a 'decoy' function, as it has the ligand binding capability and competes with RAGE for its ligand binding, without relaying signaling transduction (10). RAGE is a multi-ligand receptor, and its ligands include AGEs, beta amyloid peptide (Abeta), HMGB1, S100 family, and Mac-3 1/beta2 integrin (9). RAGE is thus implicated in the pathogenesis of multiple disorders, including AGE-associated diabetes, beta amyloid peptide associated Alzheimer's disease, HMGB1, Mac-3 1/ beta2 integrin, and S100 family associated inflammation and arthritis (9-11). RAGE regulation of innate immune response may be an essential mechanism underlying its involvement in multiple disorders (11). Whereas more is known about RAGE in the immune system than that in skeletal biology, considerable evidence is mounting that RAGE and its ligands actively participate in bone metabolism and are implicated in the pathogenesis of bone diseases. Here we summarize recent studies to provide an overview of RAGE and its ligands in bone remodeling, which may be useful for our understanding of additional therapeutic targets in the treatment of bone diseases. As other chapters in this review series provide comprehensive reviews on general aspects and immune functions of RAGE and its ligands, we would like to refer our readers to these Chapters.

3. DISCUSSION

3.1. RAGE in bone remodeling

3.1.1. RAGE & sRAGE in osteoclast activation

RAGE is expressed in both primary cultured bone marrow macrophages (BMMs) and macrophage cell lines (13). Its expression, both at transcript and protein levels, is up-regulated during *in vitro* osteoclast differentiation (13). RANKL appears to induce RAGE expression in a time and dose dependent manner (13) (unpublished observation). These results implicate RAGE in osteoclast differentiation and activation. Indeed, BMMs derived from RAGE mutant mice exhibit defective osteoclast differentiation and activation in response to RANKL *in vitro* (13). The mice lacking RAGE show increased bone mass and bone mineral density and decreased bone matrix resorptive activity (13, 14). These results support a role for RAGE in RANKL induced osteoclast maturation and activation *in vitro* and *in vivo*. Further supporting this view is that blockade of cellular RAGE using sRAGE diminished alveolar bone loss in a murine diabetic periodontal disease model (15), and that elevated level of auto-antibodies for RAGE is correlated with less erosive course of rheumatoid arthritis (RA)(16). It is notable that there are controversial reports regarding the function of sRAGE in the progression of RA. It appears that local increase verses systematic raise of sRAGE may have different effects on the course of RA.

Signaling mechanisms underlying RAGE regulation of osteoclast maturation and activation remain to be delineated, though *in vitro* studies have pointed to RAGE regulation of integrin signaling in this event (Figure 1)(12). Osteoclasts derived from RAGE mutant mice show disrupted actin ring structures and defective integrin signaling (12), a pathway that is necessary for osteoclast activation. Multiple mechanisms may underlie RAGE regulation of integrin signaling. RAGE may regulate integrin signaling at the transcriptional level, at the cell surface (by possible direct association with integrins), and intracellularly. Endothelial RAGE has been shown to associate with beta2 integrin in leucocytes (17). It would be of interest to examine whether RAGE acts as a co-receptor of beta2 integrin in BMMs or osteoclasts, thereby enhancing integrin activation and promoting osteoclast activation. Moreover, RAGE may regulate integrin signaling intracellularly. Hudson *et al* have reported that RAGE intracellular tail binds to the FH1 domain of mammalian Diaphanous-1 (mDia-1), an important protein for Rac-1 and CDC42 activation, actin cytoskeleton remodeling, and integrin signaling (18). It remains to be examined if mDia-1 mediates RAGE regulation of integrin activation in osteoclasts.

3.1.2. RAGE in osteoblasts and chondrocytes

In addition to osteoclasts, RAGE is also expressed in osteoblasts, chondrocytes (19), and articular chondrocytes (20) (Figure 1). RAGE expression in MSCs (mesenchymal stem cells) and osteoblasts appears to be up-related by its ligand stimulation. It is reported that AGE accumulation can induce RAGE expression in MSCs and osteoblast, paralleling with the bone loss (21). However, exact functions of RAGE in both osteoblasts and chondrocytes remain to be investigated. A recent study shows that RAGE deficiency leads to attenuated gene expression associated with osteoblast differentiation, including ALP, Collagen I, Runx2 and osterix, and therefore, reducing bone accrual rate and cortical bone volume (22). Other studies, however, show an increase of bone mass in young RAGE mutant mice (13-14), implicating a negative role of RAGE in OB function. The different results may be due to the age or species difference of RAGE mutant mice characterized by different laboratories, an issue remains to be clarified.

3.2. HMGB1 in bone remodeling

HMGB1 (high mobility group box 1), also called amphoterin or HMG1, is a 30 kDa, non-histone nuclear protein that is abundantly expressed in all eukaryotic cells. It belongs to HMGB family proteins, which include HMGB1, HMGB2, and HMGB3 with ~80% amino acid identity and are composed of two basic HMG-box domains (A and B) and a long acidic C-terminal tail (24). HMGB1 can translocate from the nuclear compartment to the cytoplasm via HMGB1 acetylation and can be released into extracellular environment from activated macrophages/monocytes actively or injured/dead cells passively (24, 25). Its function varies under different locations. In the nuclei, HMGB1 regulates chromosome architecture and influences transcription by binding to DNA to modify its conformation and structure. In the extracellular space, HMGB1 displays pro-inflammatory cytokine-like properties and serve as an alarmin to activate the innate immunity and mediate a wide range of physiological and pathological responses (24).

3.2.1. HMGB1 expression and secretion in bone cells

HMGB1 is found to be expressed in and released by bone cells, and involved in ossification, bone remodeling and arthritis pathogenesis (26-33). Bidwell's laboratory reported that HMGB1 is not only expressed in bone cells (osteoblasts and osteoclasts), but also detected in the culture medium of primary osteoblast and pre-osteoclast cultures, demonstrating that HMGB1 can be secreted from both cells (26-28)(Figure 1). HMGB1 secretion appears to be regulated. In cultures of primary osteoblasts and MC3T3-E1 osteoblast-like cell line, HMGB1 release is attenuated by PTH (parathyroid hormone), a regulator of bone remodeling (26-28). However, HMGB1 release is increased by PTH in UMR106-01 cells, a rat osteosarcoma cell line (26). Moreover, HMGB1 release is increased in primary BMMs (bone marrow macrophages) and RAW276.4. macrophage cell line by multiple cytokines, including RANKL, TNF□lpha, and LPS (34). The mechanisms underlying HMGB1 release remain to be further investigated. HMGB1 acetylation may contribute to its translocation from the nuclear compartment to the cytoplasm (35). However, this needs to be studied in osteoblasts stimulated by PTH or macrophages in response to cytokines.

3.2.2. HMGB1 functions in bone remodeling

HMGB1 appears to behave similarly to other osteoclastogenic cytokines (e.g., TNFalpha), exerting its effects through receptors on stromal cells, osteoblasts, and/or osteoclasts (Figure 1). The source of extracelluar HMGB1 may come from both pre-osteoblasts and osteoclast precursors (Figure 1). It may function to stimulate osteoclastogenesis, as HMGB1 function blocking antibody inhibits RANKL-induced *in vitro* and *in vivo* osteoclastogenesis (34). RAGE and TLR2/4/9 have been implicated as receptors for HMGB1. In BMMs (and cells of the innate immune response in general), TLR4 appears to be critical for HMGB1 early signaling events (e.g., activation of Erk and NF-κB), whereas RAGE may have a modulatory role in later events that sustain/propagate the host response. The latter could occur independently of TLR2/4, but coordinately with integrin activation and actin cytoskeleton organization. In pre-osteoclasts, RAGE appears to be necessary for integrin signaling and HMGB1 cross-talk to integrin signaling (34). Such an effect (regulating integrin engagement-induced signaling and actin ring formation) may underlie HMGB1 induction of osteoclastogenesis from pre-OCs *in vitro* and *in vivo* (34). HMGB1 has been found to induce polarized co-distribution of RAGE with beta2-integrin in neutrophils (36). RAGE associates with beta2 integrins not only *in trans* (endothelial RAGE interacts with neutrophil beta2 integrins) (17), but also *in cis* (neutrophil RAGE binds to neutrophil beta2 integrins) (36). Such interactions seem to be needed for inflammation-associated neutrophil recruitment (17, 36). It would be of interest to examine if such an interaction also present in BMMs and/or OCs and is involved in RAGE regulation of integrin signaling.

3.2.3. HMGB1 functions in chondrocytes

In addition to osteoclasts and osteoblasts, HMGB1 is also expressed in and secreted from developing cartilages and differentiated chondrocytes (e.g. hypotrophic chondrocytes) (37). Using HMGB1 mutant embryos, Noboru Taniguchi *et al* have demonstrated a requirement of HMGB1 in endochondral ossification (37). Embryos deficient in HMGB1 show a delay of cartilage invasion by osteoclasts, osteoblasts, and blood vessels (37). They also show by immunohistochemical analysis that HMGB1 protein is accumulated in the cytosol of hypertrophic chondrocytes at growth plates, and HMGB1 is released from the chondrocytes in organ culture (37). Thus, they have proposed a model in which HMGB1 is released from hypotrophic chondrocytes and functions as a chemoattractant for osteoclasts and osteoblasts, as well as for endothelial cells to promote cartilage invasion (37). These studies also open the door to many questions. Does HMGB1 act as an osteoclastogenic cytokine in this stage? Does PTH regulate HMGB1 release in chondrocytes *in vitro* and *in vivo*? Is this HMGB1 function in chondrocytes dependent on RAGE? What is the contribution of nuclear HMGB1 in cartilage invasion? Of note is that HMGB2, a HMGB1 related chromatin protein, is reported recently to be involved in articular cartilage surface maintenance by promoting Lef-1 mediated Wnt/ beta-catenin pathway (38). It remains to be studied if nuclear HMGB1 behaves in a similar manner as nuclear HMGB2.

3.3. AGE, S100, and Abeta in bone remodeling

3.3.1. AGE in bone remodeling

Advanced glycation endproducts (AGEs) are non-enzymatic chemical modifications of proteins by aldose sugars (39). During the Maillard reaction, the carboxyl group of sugars reacting with amino group of protein to form Schiff bases, Amadori products and eventually oxidized into AGEs. AGE accumulation is an inevitable process with age. This process may enhance RAGE expression. It is believed that AGE interaction with its receptor RAGE activates and sustains MAP kinase pathway and NF-kB signaling, altering the expression of inflammatory cytokines, growth factors and adhesion molecules (9-10). This further influences cellular activity and function. The formation of AGEs is implicated in the development of complications of diabetes mellitus, as well as the pathogenesis of cardiovascular, renal, and neurodegenerative diseases (40-41).

The characteristic lifelong remodeling and long-lasting mineralized bone matrix protein make them particularly susceptible to AGE generation, accumulation and modification (42). The potential mechanisms of AGE regulation of bone turnover include influencing the bone cellular activity and functions, and impairing bone quality by non-enzymatic collagen crosslink (42).

In osteoblasts, multiple functions appear to be induced by AGEs, including inhibiting MSC proliferation and differentiation (43), inducing apoptosis in osteoblast cell lines (44-45), and disturbing osteoblast function by impairing matrix mineralization. In addition, it is reported that AGEs decrease expression of bone formation associated genes (e.g., alkaline phosphatase and osteocalcin) (46) and reduce synthesis and secretion of bone matrix proteins (e.g., collagen). Those altered gene

expression and protein level may account for the impaired matrix mineralization and mature bone nodule formation (43, 47). Furthermore, AGEs may diminish bone healing physiologically and pathologically (48).

Those AGE effects in osteoblasts may be mediated by RAGE pathway. This is supported by observations that AGE treatment induces RAGE expression at both mRNA and protein levels in bone cells (46, 49-53), and that RAGE functional blocking antibodies partially prevent AGE-induced cellular events. (43). However, it remains to be examined if other AGE binding receptors (e.g., AGE-R2, AGR-R3, and ScR-II) are involved in AGEs' effects in osteoblasts. In osteoclasts, although AGE effect appears to be controversial, several lines of observations support a role for AGEs in promoting osteoclastic activity. A much greater resorption than control is observed by implanting AGE-containing bone particles subcutaneously (53). Using un-fractioned bone cells cultured on AGEs-modified dentin slices, Miyata *et al* **(**54**)** also found an increase of pit formation, without any effect on newly formed OCs. These observations suggest an increased osteoclastic activity, but not OC formation, in response to AGEs.

Another AGE functional site is extracellular bone matrix. Collagens, the most abundant extracellular matrix in bone, appear to be a major target of AGEs. AGEs may reduce bone strength and increase bone fragility via modification of collagens (such as type I collagen) (39, 42, 55).

Taken together, AGEs may affect bone cell functions by activation of RAGE downstream signaling pathways, and by change of bone matrix biomechanical properties via non-enzymatic cross-linking. Mechanisms underlying AGE modification of bone matrix and/or bone cells need to be further investigated.

3.3.2. S100 in bone remodeling

S100/calgranulin proteins are a family of small proteins carrying the Ca^{2+}-binding EF-hand motif. They contain at least 21 different types of S100 proteins (56-58). Intracellularly, like other EF-hand proteins, S100 proteins serve as Ca^{2+} sensor proteins and transmit signal by Ca^{2+}-dependent binding to target protein. Some S100 proteins, such as S100B, S100A4, S100A8, S100A9, S100A12, and S100A13, are secreted to the extracellular space. These extracellular S100 proteins act as cytokines, which may function through binding to RAGE.

S100 protein is widely expressed in bone tissues, including osteoblastic cells in osteogenic tumors, bone callus, bone lesions and Paget's disease. (59-60). It is expressed in bone cells, such as osteoblast and chondrocyte, and may be involved in the process of cartilage calcification (60-61). It is also expressed in cells of bone tumors, including osteosarcoma, chondrosarcoma, osteoid osteoma, ossifying fibromyoloid tumor, and bone cells in teratoma (62-66). In terms of their function in bone remodeling, S100A4 is found to inhibit osteoblast differentiation and mineralization. Its expression is decreased during osteoblast maturation and mineralization; Reduced S100A4 synthesis leads to increased *in vitro* bone nodule formation (67-68). It is also reported that S100A4 directly inhibit osteoclast differentiation, possibly through its induction of osteoblast production of soluble RAGE (69). In addition to S100A4, S100A6 is found to be increased in osteosarcoma (70). Over-expression of S100A6 enhanced osteoblast proliferation and expression of ALP, an osteoblast differentiation and mineralization marker. Silencing S100A6 inhibited cell adhesion and promoted cell migration and invasion in osteosarcoma cell lines (70). Whereas S100 is implicated in bone remodeling, the underlying mechanisms remain largely unclear.

3.3.3. APP/Abeta in bone remodeling

Beta-amyloid (Abeta) peptide, the central player in the pathogenesis of Alzheimer's disease (AD), is another ligand of RAGE. Abeta peptide is generated by the proteolytic cleavage of amyloid precursor protein (APP), a large transmembrane protein (71-72). AD, one of the most dreaded neurodegenerative disorders, is characterized by cortical and cerebrovascular Abeta deposits, neurofibrillary tangles, chronic inflammation, and neuronal loss. Increased bone fracture rates and reduced bone mass are commonly observed in patients with AD, suggesting a common denominator(s) between both AD and bone loss. However, very few studies are available that have addressed this issue. APP is expressed ubiquitously, and Abeta is a normal catabolic product of APP metabolism in most cells, including bone cells. The proteases, beta- and gamma-secretases, that cleave APP for the generation of Abeta are also expressed in bone cells (73). Transgenic mice expressing mutant APP exhibits reduced bone volume (73), implicating a function of mutant APP in bone remodeling. Exactly how APP/Abeta regulates bone remodeling and whether it is through RAGE signaling remain to be addressed in future.

4. SUMMARY AND PERSPECTIVE

Recent studies of RAGE and its ligands in bone remodeling have demonstrated the importance of RAGE signaling not only in physiological condition, but also in pathological bone disorders. Several lines of evidence support a role for RAGE and its ligands in stimulating osteoclastic activity and osteoclast maturation. In addition, HMGB1-RAGE signaling is implicated in recruitment of osteoclasts, osteoblasts, and blood vessels during endochondral bone formation. AGE-RAGE signaling may play a negative role in osteoblast differentiation and function. The function of Abeta-RAGE signaling in bone remodeling remains largely unclear. Understanding RAGE signaling in bone remodeling may provide important insights into multiple disorder associated bone loss, including chronic inflammation, diabetes, and Alzheimer's disease associated bone loss.

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