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Interplay of RANKL in Oxidative Stress Induced Bone Loss

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ABSTRACT

Oxidative stress, the source of Reactive Oxygen Species is a very often and potent factor contributing to bone loss. The expression of both (ROS) and RANKL are considered to be interdependent. RANKL mediated ROS generation is carried out especially by TRAF-6, Rac1 and NOX1. TNF-α also augments RANKL expression and enhance ROS levels. Nrf2 guards the ROS production and is inhibited by Keap1. RANKL, produced from osteoblast cells by ROS aid in formation of matured and activated osteoclasts from its precursors which is inhibited by osteoprotegerin, a natural decoy receptor of RANKL. Natural sex steroid estrogen has a potential role in preventing bone loss and its deficiency leads to ROS accumulation accomplished by T-cell activation stimulating RANKL and promoting bone loss. The generated ROS acts on both osteoblast and osteoclast cells upregulating multiple signaling cascades like kinases, Foxo transcription and NF-κB culminates in osteoclastogenesis resulting in bone loss.

INTRODUCTION

Oxidative stress (OS) results from the excess production of reactive oxygen species (ROS), which plays a vital role in inducing bone loss in two ways:

ABBREVIATIONS:

ALP, alkaline phosphatase; ATF, activating transcription factor; CREB, cAMP response element-binding protein; ERK, extracellular signal regulated kinase; FoxO, Fork head box O; HSF, heat shock factor; IFN, interferon; IL, Interleukin; Keap1, kelch-like ECH associated protein 1; M-CSF, macrophage colony stimulating factor; NOX, Nicotine-amide adenine dinucleotide phosphate oxidase; Nrf2, nuclear factor E2-related factor 2; Ob, osteoblasts; OC, osteoclasts; OPG, Osteoprotegerin; OS, Oxidative stress; PTH, parathyroid hormone; RANKL, receptor activator of NF-κB ligand; ROS, Reactive oxygen species; STAT, Signal Transducer and Activator of Transcription; TGF-β, transforming growth factor β; TNF, tumor necrosis factor; TRAF, Tumor necrosis factor receptor associated factor.

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suppression of osteoblastic differentiation and enhancing the maturation of osteoclast cells. ROS are small molecules recognized as defense against invading organisms but can also functions by involving in various signaling pathways via acting as a secondary messenger or as a modulator, especially as an intracellular secondary mediator in the pathogenesis of bone loss related diseases, as evident from decrease in plasma antioxidants found in aged osteoporotic women [1]. The status of OS depends on the balance between oxidant and antioxidant enzymatic activities. The magnitude and duration of the stress is an important factor in determining the respective activated signaling cascade. ROS are generated in cells at multiple sites including the plasma membrane, mitochondria, endoplasmic reticulum and cytoplasm which involve several enzymatic complexes such as Nicotine amide adenine dinucleotide phosphate oxidase (NOX), cytochrome P450, monoamine oxidase, cyclooxygenase and lipoxygenase. OS attenuates the bone formation process that has been proved based on the study wherein osteoblastic differentiation of primary rabbit bone marrow stromal cells (BMSC) was suppressed, when osteoblasts (Ob) were treated with H_2O_2 resulted in waning of differentiation markers such as alkaline phosphatase (ALP, a major enzyme involved in mineralisation), type I collagen, colony forming unit formation, and nuclear phosphorylation of the transcription

factor Runx2[2]. Runx2 (a core binding factor 1, binds to the Ob specific cis acting element) regulates the expression of Ob related genes (osteocalcin, type I collagen, and ALP) in the promoter region[3]. So with the repression of these markers, lifespan of Ob is declined.

ROS Generation

Generation of ROS by RANKL stimulation occurs through TNF (tumor necrosis factor) receptor associated factor (TRAF), small guanosine triphosphatase (GTPase) Rac1, and NOX as explained in figure 1. TRAF is an adaptor signaling protein, directs the expression of OC specific genes recommended for differentiation and activation of OC. TRAF's are of different types TRAF 2, 5 and 6 but among these TRAF-6 has a significant role related to osteoclastogenesis even though all these three types possess affinity towards RANKL. Multiple isoforms of Nox were also reported categorized as Nox 1 to 5 having differential expression in various cells of which Nox 4 is predominantly expressed by pre-osteoblasts and is essential for causing demineralization of bone.

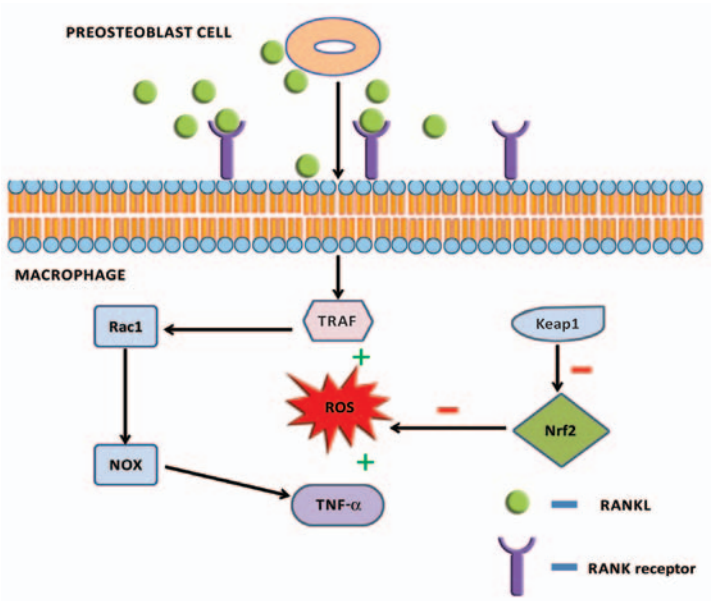

FIGURE 1: Generation of Ros in Osteocells.

Stimulation of RANKL leads to differentiation of bone marrow monocyte macrophage lineage (BMM) cells into osteoclasts (OC). This proceeds through a signaling cascade involving TRAF 6, Rac1, and Nox1, resulting in ROS generation[4]. RANKL stimulation is followed by interaction of TRAF6 to the cytoplasmic domain of RANK, which brings about activation of distinct signaling pathways induced by ROS. The Rac1, predominant in macrophages and monocytes is a cytosolic component of NOX, further activates the NOX and exhilarates its activity. Thus TRAF6 and its down streaming cascade Rac1 and Nox1, are required for RANKL mediated ROS production and osteoclastogenesis. Apart from RANKL , TNF-α also has shown to instigate ROS production in OC lineage cells by inflating RANKL expression[5]. There are some cellular protective mechanisms resistant to oxidative stress. One of these include the transcription factor, nuclear factor E2-related factor 2 (Nrf2) which can induce the expression of antioxidant enzymes, there by subsiding the ROS levels contributing to reduction in the number of tartrate resistant acid phosphatase (TRAP) positive multi-nucleated cells thus attenuating the bone loss. Activation of kelch-like ECH associated protein 1 (Keap1), a negative regulator of Nrf2, demotes the Nrf2/Keap1 ratio under RANKL stimulation, abrogates cytoprotective enzymes and leading to supplementation of bone loss[6].

ROS induced RANKL stimulation and role of RANKL

Generation of ROS and stimulation of RANKL are interdependent. So, understanding the role of RANKL and how it generates ROS provides better information regarding OS induced bone loss. RANKL is a member of the TNF family, exists either as a membrane bound protein or as a secreted form[7]. RANKL is ubiquitously expressed and is originated from various cells including bone marrow stromal cells, osteoblastic lineage, synovial cells, activated T cells, B cells, fibroblasts, mammary epithelial cells, chondrocytes and endothelial cells. Osteoprotegerin (OPG) is a soluble decoy receptor that negatively controls RANKL expression. Any imbalance in the RANKL:OPG ratio implicates in devastating condition like bone loss. It associates with RANK receptor, a type I trans-membrane protein obtained by cloning from dendritic cells. The influence of RANKL in osteoclastogenesis from bone marrow macrophage lineage cells and how OPG prevents the process is quoted briefly in figure 2. Factors like PTH, prostaglandin E2 (PGE2), dexamethasone, inflammatory cytokines such as interleukin-1 (IL-1), TNF-α, vitamin D3 can trigger RANKL expression, where as in contrast TGF-β negotiates its function. Sex steroids also have a significant impact in bone physiology of which estrogen gains a relevant role in postmenopausal osteoporosis. ROS levels are highly exacerbated in estrogen deficiency promoting bone loss and their levels were mitigated after estrogen supplementation. This drastic effect of decreased levels of estrogen, its contribution to bone loss in relation with ROS mediated through TNF activation and the mechanism involving in it is described in figure 3.The activation of TNF-α, promotes TGF-β down regulation, accomplished by production of IFN-y that is mediated by interaction of estrogen to estrogen responsive element (ERE) in the promoter region of TGF-β[13].Thus enriching RANKL expression relaying to impaired osteoclastogenesis.

Effect of oxidative stress on osteoblasts

P38 and ERK are the kinase mediated pathways resulting in augmented levels of RANKL expression in Ob by ROS generation. Besides these activating transcription factor (ATF), cAMP response element-binding protein (CREB) and heat shock factor (HSF) also has a crucial role in bone loss by enhancing RANKL concentration, their by activating several osteotropic factors like TGFβ, PTH, basic fibroblast growth factor, IL-1, and PGE_2, inducing osteoclast differentiation[7]. It was found that ATF-3

contributed to increased gene expression of RANKL in mouse osteoblast following the stimulation of NOX activity and ROS production by ethanol, also activates CREB and HSF2 ultimately results in RANKL expression as mentioned in figure 4. ROS also activates phospholipase c-γ1 and ERK-dependent NF-κB activation, resulting in impaired osteoblastic differentiation. ERK stimulates Ob related gene expression by binding with extracellular matrix integrin receptor or under the regulation of growth factors or by phosphorylating Runx2. WNT signaling prevents proteosomal degradation of β-catenin and aid in its accumulation in the cytoplasm, then translocates in to nucleus and provokes osteoblastic markers resulting in osteoblastic differentiation. Runx2 is the upstream cascade of β-catenin. In addition to this β -catenin exhibits an additive effect with T cell factor proteins and regulates the expression of OPG, a major inhibitor of osteoclast differentiation. As β-catenin exerts a positive role in bone homeostasis, stabilization of this in differentiated Ob results in high bone mass.

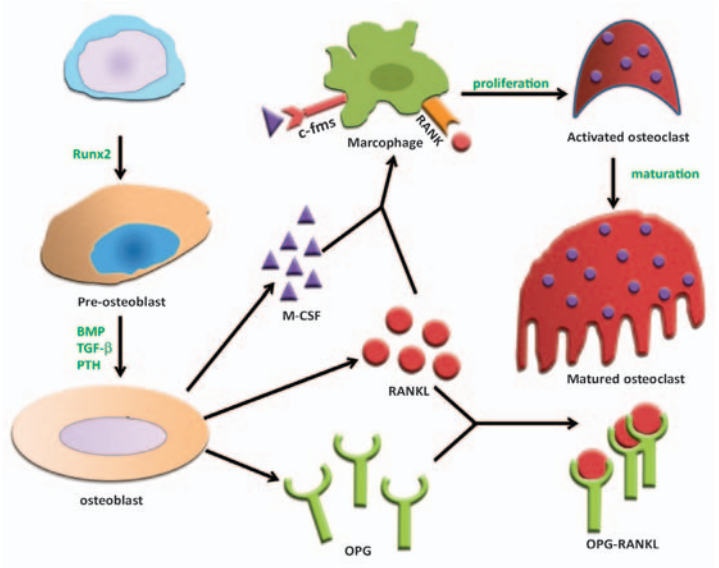

Figure 2. RANKL formation and its role in bone loss.

Mesenchymal progenitors emerged into Ob through a process that involves the consecutive action of the transcription factors Runx2 and Osterix, a downstream of Runx2 [8]. Bone morphogenetic proteins (BMPs), transforming growth factor β (TGF-β) and parathyroid hormone (PTH) signals specifically to pre-osteoblast cells to convert into Ob , which regulates RANKL, M-CSF and OPG production under the influence of factors like pro-receptive hormones, cytokines and growth regulators [9]. RANKL, its receptor RANK and inhibitor OPG (an endogenous antagonistic decoy receptor) all together are considered as molecular triad, which plays a significant role in proliferation and activation of OC contributing to bone turnover. RANKL belongs to TNF family having a promising function that stimulates the fusion of pre-osteoclasts into multinucleated cells, its attachment to bone followed by sequential actions such as activation and survival of osteoclast. OPG binds with RANKL and prevents the interaction of RANKL to its receptor RANK their by down stairing osteoclastogenesis, in a reversible manner but it does not have any profound effect on Ob cells. M-CSF binds to its receptor, colony-stimulating factor receptor 1 (c-Fms) augmenting the proliferation of OC precursors and enhances survival of matured OC. Via these various osteoclastogenic mediators, the proliferation of pre-osteoclast is progressed into matured OC, upgrading the bone loss.

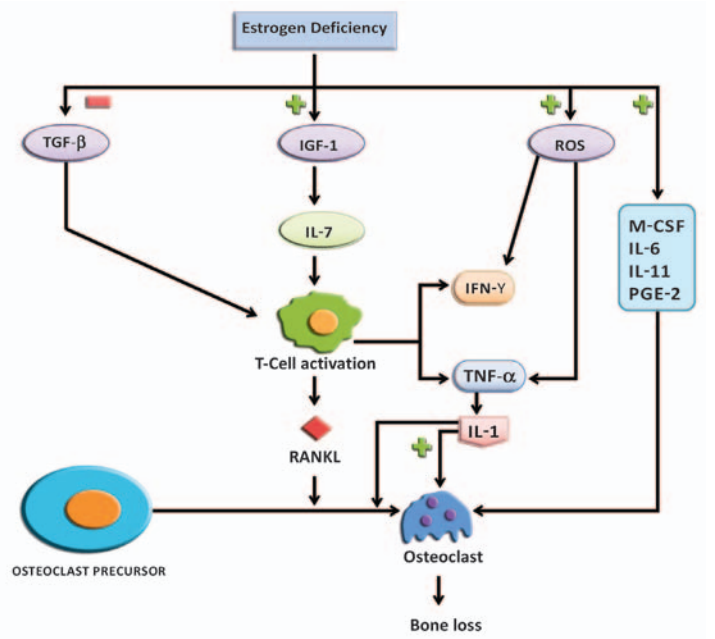

Figure 3: Role of estrogen in bone loss via OS and other mechanisms.

Estrogen modulates bone homeostasis by various means. Consequences of estrogen deficiency may include 1) inhibition of TGF-β, which inturn promotes T cell activation 2)elevating the levels of IL-7, a down streaming cascade of insulin like growth factor-1 (IGF-1) accomplishing in T cell activation 3) generation of ROS which activates the functions of IFN-γ and TNF-α. ROS amplifies T cell activation in turn exacerbates the egression of osteoclastogenic factors RANKL and TNF. 4) esculates the levels of M-CSF, IL-6, IL-11 and PGE-2, regulating OC activity[10]. Once T cell is activated it intensifies the RANKL expression, converting the OC precursors into matured OC, contributing to bone loss and burgeons the production of inflammatory cytokines like IFN-γ, TNF-α and IL-1 which diminish the activity of mature Ob and stimulates OC formation[11].IFN- further expedites TNF-α followed by up regulation of IL-1. IL-7 exhibits power anti apoptotic effects by promoting RANKL expression in T- cells and enhances osteoclsatogenesis. IL-1 may directly stimulate osteoclast precursors and also can enhance TNF activity [12]. Thus estrogen regulates the expression of genes present in osteoclastogenic factors like IL-6, TNF-α, RANKL and M-CSF responsible for bone loss.

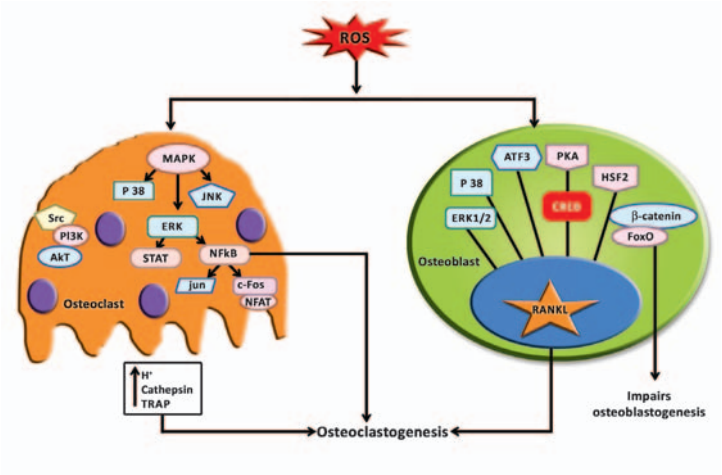

Figure 4: Signaling cascade of ROS in both osteoblast and osteoclast cells

In osteoblasts, ROS induced phosphorylation of p53 and p66sh allows sequestration of FoxO transcription factor with β-catenin through JNK, their by diverting â-catenin, restricting the proliferation of Ob progenitors leading to impaired osteoblastogenesis resulting in decreased bone formation culminating bone loss [14]. ROS also promotes phosphorylation of ATF-3, promotes CREB binding to the CRE domain in the RANKL promoter region, also assist the binding of HSF2 to the heat shock element in the RANKL promoter region, increases RANKL gene expression[15]. The enhanced level of RANKL hastens bone resorption by promoting the proliferation of OC precursors to multinucleated matured OC cells. Strikingly, in these cells ROS avails osteoclastogenesis by activating kinase signaling pathways like p38 and ERK1/2, ERK being an upstream stimulator of CREB or HSF. In OC ROS provokes various signaling cascades mediated by protein kinases including JNK, p38 and ERK. ERK function is associated either by NF-κB directly or by having down streaming effect on STAT during estrogen deficiency. Expression of c-Jun and c-Fos transcription factors is possible down streaming effect of NFkB, the expressed c-Fos inturn binds with nuclear factor of activated T cells (NFATc1), accelerating osteoclastogenesis. ROS also up regulates the signaling cascade Src (non receptor tyrosine kinase factor), fosters OC activation by lipid kinase phosphatidylinositol 3-OH kinase (PI3K) and serine/threonine protein kinase AKT enhancing RANKL expression[16]. The OC secretes H^+, TRAP and cathepsin K which boosts up bone degradation.

Impact of oxidative stress on osteoclasts

Expression of RANKL takes place in Ob where as it's signaling and upstream cascade is observed when the RANK ligand binds to its receptor RANK present on precursors of OC. This interaction is accomplished by recruitment of some adaptor proteins such as TRAF and growth factor receptor bound protein-2 (Gab-2) promoting OC activation by different signaling pathways. The key pathways in bone cell differentiation by redox signalling in correlation with RANKL in OC cells are mitogen-activated protein kinases (MAPKs) and NFkB. ROS modulates phosphorylation of tyrosine kinase either by stimulating tyrosine kinase receptors or by inhibiting tyrosine phosphatases[17]. ERK signaling activates NF-κB through phosphorylation of IκB proteins, promoting the protease-mediated degradation of IκB, contributing to the nuclear translocation of active NF-κB and mediates OC differentiation[18]. Activated NF-κB by RANKL up regulates c-Fos and c-jun transcription and RANKL activate Src implicating in secretion of H^+, formation of cathepsin and TRAP leading to bone demineralization. All the above discussed pathways stimulates RANKL mediates osteoclastogenesis. Thus OS and osteoclastogenic factors like RANKL are interrelated via various signaling cascades, together resulting in bone loss by altering bone microenvironment. So targeting all these cascades can be a beneficial way to prevent bone loss specifically supplementation of estrogen is a promising approach in postmenopausal osteoporosis. All the possible ways catalyzing ROS as depicted above may potentially be targeted and inhibited effectively in near future research.

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