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Chapter 4

How Homocysteine Modulates the Function of Osteoblasts and Osteocytes

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http://dx.doi.org/10.5772/intechopen.76398

Abstract

Over the years, numerous mechanisms have been identified through which homocysteine affects osteoblast functioning. These include alterations in collagen structure, epigenetic modifications and changes in RANKL-OPG production by osteoblasts. These mechanisms are reviewed in this chapter. We have also herein discussed how homocysteine affects osteocyte behavior. With onset of hyperhomocysteinemia induction of osteocyte specific genes particularly the mineralization genes like Dmp1 and Sost is facilitated producing untoward mineralization, osteocyte apoptosis, deviations from regular bone remodeling process and onset of targeted remodeling in bone. These modifications have immense effect on the overall mechanical stability of bone. Homocysteine thus represents an independent risk factor for bone fragility.

Keywords: homocysteine, osteoblast, osteocyte, sclerostin, dentine matrix protein1

1. Introduction

Bone remodelling is a process that occurs throughout life. This process occurs to replace old mineralized bone with new bone, preserve bone mass, mineral homeostasis, pH balance, repair microdamage, maintain glucose homeostasis and preserve male fertility. Bone remodeling is a highly co-ordinated process that requires the controlled activities of many systemic and local factors like calcitriol, parathyroid hormone, growth hormone, thyroid hormones, glucocorticoids, bone morphogenetic proteins, prostaglandins, sex hormones, various cytokines and the molecular triad comprising of OPG (osteoprotegerin), receptor activator of nuclear factor-κB ligand (RANKL) and receptor activator of nuclear factor-κB (RANK). The cells involved in the process are osteoblasts, osteoclasts, osteocytes, immune cells, megakaryocytes and osteomacs.

With senescence, decreased production of sex hormones like estrogen and testosterone, susceptibility to genetic and environmental factors and life style modifications; the process of bone remodeling process gets hampered increasing the rate of bone resorption as compared to that of bone formation. Such deviations from normal bone remodeling process deplete the bone of its minerals like calcium and proteins like collagen affecting the overall mass and mechanical property of the bone, escalating the risk of bone fractures [1, 2].

Many metabolic substances also affect bone. One such factor is homocysteine. Since its discovery in 1932, homocysteine has remained an important aspect for research. Homocysteine is basically a metabolite of methionine metabolism that exists at a critical biochemical point in the methionine cycle from where it is used to synthesize cysteine and glutathione. When remethylation and transsulfuration cycles in methionine cycle collapse owing to an enzyme or co-factor deficiency, homocysteine accumulate in blood resulting in a clinical condition called hyperhomocysteinemia (>15 micromol/L). The prevalence of hyperhomocysteinemia varies with geography, age, sex and ethnicity. Till date high level of methionine, deficiency of enzymes in methionine metabolism like cystathionine synthase (a pyridoxal phosphate dependent enzyme), methionine synthase (a folate and vitamin B12 dependent enzyme) and methylenetetrahydrofolate reducatase, deficiency of vitamins like folate and vitamin B_{12} in diet (caused by losses of these sensitive vitamins by methods of food processing such as milling of grains, canning, extraction of sugars and oils, radiation and chemical additives), environmental elements, life-style habits, hormonal changes, drugs and diseases like cardiovascular, cancer and type 2 diabetes have been found to be causes for hyperhomocysteinemia.

The negative effect of homocysteine on the bone is well supported by the demonstrations of loss of bone physiology in experimental animals of hyperhomocysteinemia generated by administering a methionine-enriched diet (with low folate) as well in genetic models of enzyme deficiency [3]. This chapter will present the current findings on how hyperhomocysteinemia alters the functions of two types of bone cells—the osteoblast and the osteocyte.

1.1. How homocysteine affects osteoblast function?

Studies on the effect of homocysteine on bone forming osteoblasts have shown that homocysteine is different from a conventional oxidant and exerts its effects on cells via multiple modes. Over the years diverse mechanisms were identified by which homocysteine affects osteoblast functioning. These include alterations in collagen structure, epigenetic modifications and changes in RANKL-OPG production by osteoblasts. The first example to cite how homocysteine alters osteoblast machinery was a clinical study by Hermann et al. in 2005 [4]. The results of this study showed that a positive correlation occurs between hyperhomocysteinemia and circulating concentrations of osteocalcin, an osteoblast activity marker. In contrast to this finding was a report by Sakamoto et al. in the same year 2005 [5], which demonstrated that homocysteine, stimulates only osteopontin and has an attenuating effect on osteocalcin. The paper also revealed that homocysteine represents an independent risk factor for osteoporosis. In the subsequent years 2007–2008, clinical studies by Hermann et al. [6, 7] published the following: Accumulation of homocysteine caused by "reduction in co-factors of methionine

metabolism like folate, vitamin B_{12} and vitamin B_6" do not cause any change in the activities of alkaline phosphatase, osteocalcin and pro-collagen type I N-terminal peptide (PINP) in serum. However such low concentrations of folate, vitamin B_{12} and vitamin B_6 are enough to produce a stimulatory effect on osteoclast activity. This study also rationalized the mechanistic role of low B-vitamin concentrations for bone degradation. The same research group also demonstrated how direct exposure of primary human osteoblasts to increasing concentrations of homocysteine stimulates cellular activity. This report brought about the awareness that homocysteine "not inhibits but alters" osteoblast function, one of the reasons why some of results of clinical and experimental studies were in disagreement with each other. In the subsequent years Thaler et al. [8–10] reported noteworthy mechanisms regarding how homocysteine affected the bone matrix. The authors showed that homocysteine altered collagen cross linking by inhibiting the expression of lysyl hydroxylase and lysyl oxidase, enzymes required for formation of stable bone matrix. Collagen cross linking in the bone is a post translational modification of collagen molecules that play integral role in tissue differentiation and render mechanical support to the bone. The authors revealed that homocysteine uncovers RGD motif (a tripeptide of Arg-Gly-Asp) in collagen by RelA protein activation. Collagens are important structural proteins that form the extracellular matrix and play important role in shaping and organizing a tissue and the major collagen found in the bone is type 1 collagen. When denatured, type 1 collagen unwinds its triple helical structure causing the exposure of RGD motifs in it. Such exposure is basically a mechanism by which signals are presented to cells for regulating cell behavior, promoting tissue repair and regeneration. But when exposed to homocysteine such RGD exposure elicits serum amyloid A3 expression and overexpression of matrix degrading enzymes and cytokines like MMP-13 (metalloproteinase-13), Ccl_5, Ccl_2, Cxcl10 and interleukin-6, substances otherwise known to hamper the proper collagen cross linking of bone matrix [10]. This group also reported that homocysteine increased the expression of genes for epigenetic DNA methylation like cytosine-5-methyl transferases1 (Dnmt1) and lymphoid specific helicase. The mechanism was found to be by increasing the expression of Fli1 (Friend leukemia virus integration 1), a transcription factor important for Dnmt1 stimulation. The authors also discovered that homocysteine caused hypermethylation of Lox (lysyl oxidase) proximal promoter that caused Lox repression. Lox is an extracellular copper dependent enzyme that catalyzes the formation of aldehydes from lysine residues in collagen precursors. In 2011, Lv et al. [11] reported a similar hypermethylation effect of homocysteine on promoter A region of estrogen receptor-alpha that cause repression of estrogen receptor alpha expression. The authors concluded that such inhibitory mechanisms can elicit postmenopausal osteoporosis in women, a bone disorder encountered by most females upon menopause. It was in the same year that Kriebitzsc et al. [12] established a link between homocysteine and vitamin D3. A microarray experiment by these authors on MC3T3-E1 murine pre-osteoblasts treated with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) revealed the induction of a cluster of genes including the *cbs* (cystathionine β-synthase gene). Since CBS is an enzyme that converts homocysteine to cystathionine, thereby committing transsulfuration pathway to cysteine synthesis, the authors were intrigued to find out how vitamin D3 regulated the level of homocysteine in the osteoblast. They then discovered that *Cbs* mRNA levels were very much higher when osteoblasts obtained sufficient exposure to vitamin D3. Importantly, the

chromatin immunoprecipitation on chip and transfection studies demonstrated a functional vitamin D response element in the second intron of *cbs*. The possible clinical relevance of these findings were investigated by these authors, and the human data from the Longitudinal Aging Study Amsterdam suggested a correlation between vitamin D status ($25(OH)D_3$ levels) and homocysteine levels. The authors drew conclusions that *cbs* is a primary $1,25(OH)_2D_3$ target gene which renders homocysteine metabolism responsive to $1,25(OH)_2D_3$.

In 2012, the role of homocysteine mediated endoplasmic reticulum (ER) stress in inducing apoptosis was reported revealing that osteoblast death can also occur during hyperhomocysteinemia. Homocysteine increases the expression of glucose-regulated protein 78, inositol-requiring transmembrane kinase and endonuclease 1α (IRE-1α), spliced X-box binding protein, activating transcription factor 4, and C/EBP homologous protein to carry out cell death [13].

Our group in 2013 reported how homocysteine altered the production of proteins in osteoblasts [14]. We were mainly interested in evaluating how homocysteine affected the synthesis of ligands like RANKL and OPG by osteoblasts. Proteins like RANKL, OPG and RANK (receptor on osteoclast) form a molecular trio which is one of the important regulators of bone remodeling. RANKL is required for RANK activation, development of multi-nucleated osteoclasts and induction of bone resorption. To regulate the osteoclast activity, the osteoblast also synthesizes OPG that serves as a decoy receptor for RANKL that binds it and prevents it from activating RANK. Our studies on how homocysteine affected the synthesis of OPG also threw light that this ligand production in osteoblast is coupled to the insulin-MAPK (mitogen activated protein kinase) signaling cascade and antioxidant defense machinery. The dephosphorylations of insulin receptor and associated downstream targets caused by homocysteine induce phosphorylation of PP2A (protein phosphatase 2A), a negative modulator of the insulin signaling. This increase in phosphatase activity also inhibited phosphorylation of p38 mitogen activated protein kinase, a pathway important for OPG synthesis by osteoblast cells. We were intrigued to find that dephosphorylations of insulin receptor signaling also produced increased nuclear translocation of ForkheadO1 transcription factor and activation of MnSod (manganese superoxide dismutase), an antioxidant. The RANKL synthesis however occurred independently and involved activation of c-Jun/JNK MAP kinase (JNK) signaling pathway. Thus the oxidative stress imparted by homocysteine altered the osteoblast behavior shifting the balance between bone formation and bone resorption.

1.2. Does homocysteine affect osteocytogenesis?

Osteocyte represents the third major cell type in the bone, which regulates the functions of osteoblasts and osteoclasts. These cells originate from mesenchymal stem cells via osteoblast lineage differentiation, and only 10–20% of such osteoblasts develop into osteocytes. Osteocytes also have an extraordinary long-life of 10–20 years and consequently constitute 95% of the cellular component of adult living bone. Mature osteocytes inhabit in cavities called "osteocyte lacunae" measuring some hundreds of μm^3 in volume that shape into an interconnected network *via* tiny canals or canaliculi to form the lacunar-canalicular pore

system (LCS). LCS buried within the mineralized matrix positions osteocytes to derive nutrients from the blood supply, feel external mechanical signals, connect among themselves and with other cells on bone surfaces and control structural reorganization following bone remodeling [15, 16]. It was formerly thought that osteocytes are inert cells, however these are now contemplated to be superior cell type with endocrine functions. Upon stimulation, osteocytes secrete substances like RANK ligand, OPG, fibroblast growth factor23 (FGF23), prostanoids, nitric oxide, nucleotides, cytokines and growth factors that regulate bone remodeling. FGF23 produced by osteocytes regulate serum phosphate level by increasing renal phosphate excretion. Sclerostin and DKK1 specifically inhibit Wnt-B-catenin pathway that regulate bone formation [17, 18].

The process of "osteocytogenesis" is the evolution of a bone forming osteoblast to an osteocyte when it gets deeply buried in the bone matrix. The process involves three different phases: (*i*) type I osteoblastic-osteocyte, (*ii*) type II osteoid-osteocyte and (*iii*) type III preosteocyte (*surrounded by matrix*) [19]. Some of the noteworthy proteins involved in the process are: (a) Pdpn (E11), a trans-membrane glycoprotein that is required for the formation of dendrites, (b) osteocalcin, a non-collagenous protein for proper mineralization of marix, (c) dentine matrix protein or Dmp1 for regulation of crystal mineral size and osteocyte maturation [17, 20], (d) Phex, a metalloproteinase that binds to the inhibitor of Dmp1 *viz.* MEPE and regulates Dmp1 activity, (e) AHSG or FetuinA which regulates mineralization around developing osteocytes [21, 22] and (f) Sclerostin, a regulator of bone remodeling which can inhibit bone formation *via* downregulation of Wnt Lrp5/6 signaling, the major anabolic pathway in bone, which can activate osteoclasts and regulate mineralization [18, 23].

Till date there are only few reports that substantiate that osteocytes affected during hyperhomocysteinemia. One of the reasons for this is the difficulty in isolating osteocytes from mineralized tissues for obtaining these in sufficient numbers and purity. Over the years a cell line MLO-Y4 was developed by Lynda F Bonewald and many methods to isolate osteocytes from bone tissue were developed but none gave a complete picture as to how osteocytes responded to external stimuli. This is mainly because both the osteocytes and its extensive connections are not possible to be replicated in concert in vitro. Yet, investigations have been done on using the MLO-Y4 cell line to evaluate how osteocyte responds homocysteine. The results showed that homocysteine induced apoptosis in osteocyte culture via Nox and AMPK activation [24]. Nox or NADPH oxidase family of superoxide and hydrogen peroxide producing proteins represent an important source of reactive oxygen species whilst AMPK or adenosine monophosphate activated protein kinase is an energy sensor that regulates oxidative stress. The study by Takeno et al. did not render a complete picture as osteocytes are embedded deep inside the bone where it is not exposed to high concentrations of homocysteine. Thus we investigated how homocysteine affected osteocytes in vivo by administering mice with homocysteine i.p. and then evaluating how homocysteine in circulation modulated osteocytes employing microCT50, immunohistochemistry and Real Time PCR [25]. These techniques enabled us to identify time dependent changes in osteocyte lacunar numbers and osteocyte markers with onset of hyperhomocysteinemia. It was interesting to find that with induction of hyperhomocysteinemia, there was initially an increase in osteocyte lacunar numbers coupled to an increase in transcription and protein expression of many osteocyte markers.

But with time we observed that homocysteine mainly increased the protein expressions of Dmp1 and sclerostin that were otherwise involved in mineralization of osteocyte lacunae. This is in fact a pathogenic mechanism since increased mineralization is also a cause for bone instability. Another trait seen in these bone was osteocyte apoptosis. Erstwhile studies have previously demonstrated that apoptotic osteocytes are not "debris" but necessary regulators of a process called "targeted remodeling" wherein apoptotic osteocytes signal nearby cells to release factors like RANKL, VEGF, ATP, sphingosine-1-phosphate and chemokines for endothelial cell activation and recruitment of bone cell precursors, including osteoclasts and osteoblasts, to the site of injury to enable repair *via* BMU-mediated remodeling process [26, 27]. It has already been shown that homocysteine induces RANKL synthesis. Thus, homocysteine mediated induction of RANKL production by both osteoblasts and osteocytes is therefore an important determinant that drives bone to rapid bone resorption and thereby increased bone remodeling during hyperhomocysteinemia. Our findings thus provide an interesting avenue for future research into the role of osteocytes in disease-mediated changes in bone mineralization.

2. Conclusions

The effect homocysteine has on bone remodeling is dynamic. Unlike any other oxidant that generates free radicals, homocysteine exerts effects at multiple ways to induce cellular damage. We have seen that in some cases homocysteine induces gene methylations to render certain genes like Lox inactive so that collagen architecture is altered whereas in other cases homocysteine over expresses genes like Dmp and Sost to promote mineralization, a process that can produce adverse effects on long run. Understanding the complexities involved in hyperhomocysteinemia is therefore vital for designing therapeutics for treatment of bone disorders.

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