

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**FERRITIN: A POTENT INHIBITOR OF VASCULAR CALCIFICATION
AND OSTEOBLAST ACTIVITY**

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The Examination takes place at the Library of Division of Nephrology, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 19th September 2014, at 11:00 AM

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INTRODUCTION

Vascular Calcification

Cardiovascular disease is a major cause of morbidity and mortality among patients with chronic kidney disease (CKD). Vascular calcification plays a role in the pathogenesis of atherosclerosis, diabetes and CKD and is an independent risk factor associated with cardiovascular disease and mortality. Furthermore, vascular calcification has been associated with cardiovascular complications such as atherosclerotic plaque burden, myocardial infarction, coronary artery disease, postangioplasty dissection, and increased ischemic episodes in peripheral vascular disease. The first description of vascular calcification was provided by Rudolf Ludwig Karl Virchow, the father of cellular pathology, who noted the presence of stiff, “bone-like” consistency in atheroma in 1863. It is now evident that vascular calcification occurs in 2 layers of arterial wall: tunica intima and tunica media. Tunica intima is a layer of endothelial cells supported by internal elastic lamina, while tunica media comprises a smooth muscle layer and elastic tissue. In atherosclerosis, endothelial injury is followed by adhesion of leukocytes as well as maturation of monocytes into macrophages with lipid uptake. Smooth muscle cells (SMC) migrate from the media to intima and proliferate. Fatty streaks and fibrous plaques enlarge and bulge into the arterial wall in which calcification causes narrowing of the lumen. Intimal or atherosclerotic calcification is more prevalent in large arteries such as aorta, and occurs more frequently in elderly, hypertensive, dyslipidemic and diabetic patients. Medial calcification was first described in 1903, as a sheet-like calcification in the SMC layer of arterial wall without lipid or cholesterol deposit. The increase in arterial stiffness caused by such medial calcification can result in poor arterial compliance, systolic hypertension, widening of pulse pressure, left ventricular hypertrophy, impaired coronary perfusion and myocardial ischemia. Furthermore, arterial stiffness has been found to be a predictor of mortality in hemodialysis and peritoneal dialysis patients.

Medial calcification is particularly common in patients with end stage renal disease (ESRD) and frequently found in peripheral arteries, such as epigastric, femoral, and radial arteries. Earlier evidence revealed substantially higher prevalence of vascular calcification in young adults on chronic hemodialysis compared to the general population in the same age range, indicating the influence of CKD-related risk factors on the development of vascular calcification. In 1979, Ibels et al. studied the pathology of arteries obtained from dialysis patients and discovered an increase in arterial calcification compared with a normal population of the same age. In CKD, studies reported prevalence of vascular calcification ranges from 47%-92%. Vascular calcification has been shown to predict cardiovascular events and mortality in the entire spectrum of patients with CKD as well as in kidney allograft recipients. Such overall vascular stiffness due to vascular calcification has a significant negative effect upon survival for patients requiring renal replacement therapy. For instance, in a well-designed study, quantitative computed tomography (CT) scanning was used to assess the relationships among progressive calcification of the superficial femoral artery, concomitant femoral arterial stiffness, and mortality. At two years, progressive calcification was seen in 57% of patients with increases in calcification of the vascular tree correlating with increases in arterial stiffness. Additionally such vascular calcification was present in almost all patients who died (95%). In contrast, most patients with an initial calcification score of zero did not develop calcification over this period.

In addition to traditional cardiovascular risk factors including aging, smoking, diabetes, dyslipidemia, inflammation, hypoalbuminemia and elevated c-reactive protein, CKD related risks such as phosphate (Pi) retention, excessive calcium intake, past dialysis experience, decreased calcification inhibitors, vitamin D deficiency and increased FGF-23 are also associated with the severity and progression of vascular calcification.

Vascular calcification was in the past considered to be an imminent, passive, degenerative process involving advanced atherosclerotic lesions; however, recent research has revealed numerous similarities with actively controlled processes occurring in the bone tissue. Such active and regulated

process has created a great deal of interest and research to better understand the pathophysiology of vascular calcification. These studies have revealed that the mechanism of vascular calcification is complex and multifactorial. In this regard, increased level of Pi that is a common finding in later stages of CKD has been shown to be a major culprit of transdifferentiation of vascular smooth muscle cells into osteoblast like cells. Advanced CKD patients develop hyperphosphatemia due to impaired renal Pi excretion. High serum Pi levels have been shown to have detrimental consequences on cardiovascular homeostasis and might be considered as a ‘vascular toxin’.

Elevated levels of Pi may directly induce vascular injury and indirectly stimulate osteoblastic differentiation through a type III sodium-dependent phosphate co-transporter (Pit-1). Studies suggest that elevated intracellular Pi may directly stimulate SMC to transform into calcifying cells by activating genes associated with osteoblastic functions. This process includes stimulation of a key osteogenic transcription factor, namely core binding factor alpha-1 (Cbfa-1) that is also known as RUNX2. Activation of Cbfa-1 is accompanied by a cascade of signaling pathways that promote differentiation, bone matrix gene expression, and consequently, bone mineralization. This entails expression of specific osteoblastic genes such as alkaline phosphatase (ALP) which is an important enzyme in early osteogenesis and osteocalcin, a major noncollagenous protein found in bone matrix that is believed to regulate mineralization and is also known as a pathognomonic feature of osteoblastic terminal differentiation. Therefore, based on mounting evidence hyperphosphatemia has been recognized as a major risk factor in patients with severe impairment of renal function.

Heme Oxygenase/Ferritin System

The heme oxygenase was first discovered in the late 1960s to catalyze degradation of heme and generation of bilirubin. HO-1 is the stress inducible isoform of the enzyme that catalyzes the rate-limiting step in heme breakdown resulting in the production of equimolar amounts of biliverdin, iron, and carbon monoxide (CO). Iron subsequently induces the expression of ferritin. Although initially recognized for its

role in heme catabolism and erythrocyte turnover, it has become increasingly evident that HO-1, plays an important protective role in numerous injury settings and clinical conditions.

The mechanism underlying this cytoprotective effect involves the ability of HO-1 to catabolize free heme, which is a potent pro-oxidant and prevent it from sensitizing cells to undergo programmed cell death. Furthermore, such protective biological activities are conferred via antioxidant, anti-inflammatory, anti-apoptotic and pro-angiogenic properties that have been attributed to CO, biliverdin, bilirubin and ferritin. These findings have been confirmed not only by animal models but also the discovery of two cases of human HO-1 deficiency. Heme degradation results in release of iron that in turn induces the expression of ferritin. Iron is essential for almost all living organisms. It participates in a wide variety of fundamental metabolic processes, including oxygen transport, DNA synthesis, and electron transport. However, when present in excess, iron poses a threat to cells and tissues. The toxicity of iron is largely based on its ability to catalyze the generation of free radicals, which attack and damage cellular membranes, protein and DNA. In fact iron overload has been associated with pathological conditions including neurodegenerative disorders, liver dysfunction, diabetes and even carcinogenesis among others. Therefore, it is apparent that meticulously orchestrated mechanisms to move iron across biological membranes and to ensure that its distribution in multicellular organisms when needed is essential. One such crucial mechanism is the induction of ferritin. Ferritin is the most ancient molecule of iron homeostasis with highly conserved three-dimensional structure that carries fundamental functions and new roles arose during evolution.

As an iron storage protein, ferritin plays a key role in iron metabolism. Its ability to sequester the iron element gives ferritin the dual functions of iron detoxification and iron reserve. The importance of these functions is emphasized by ferritin's ubiquitous distribution among living species. Ferritin molecules isolated from vertebrates are composed of two types (heavy [H] and light [L] chain) whose proportion depends on the iron status of the cell, the tissue, and the organ.

The H-chain has ferroxidase activity that is essential to prevent cellular damage provoked by reactive oxygen species. This site catalyzes the oxidation of Fe(II) with the production of H₂O₂, which is used as an oxidant for further oxidation of Fe(II). Thus, the ferroxidase activity can consume both reagents of the Fenton reaction: H₂O₂ and Fe(II), thereby minimizing potential injury induced by the activity of the Fenton reaction. The L chain has no enzymatic activity but its presence even in small proportions (2-4 chains per shell) accelerates the transfer of iron to the iron core and improves the overall iron sequestering process.

Consequently the presence of the two subunit-types enhances the functionality of the molecule. In mammals the H and L chains assemble in the proportion determined by the tissue and the cellular development. The H-rich ferritins are found in heart and brain, have higher ferroxidase activity and have a more pronounced anti-oxidant activity, while the L-rich ferritins of spleen and liver are physically more stable, may contain larger amount of iron and a more pronounced iron storage function. The first study that identified ferritin heavy subunit as a potent anti-oxidant was reported by Balla and colleagues. Prior to this study, ferritin was regarded as a housekeeper iron storage protein. Numerous studies have since confirmed the findings reported in Balla et al.'s report. Through these studies a distinct portrait has evolved that identifies ferritin as major protective mechanism to prevent oxidative damage. The indispensable nature of FtH has been confirmed in a transgenic mouse model where disruption of FtH by homologous recombination led to early embryonic lethality. The protective nature of the HO-1/ferritin system in a diverse range of clinical conditions led us to hypothesize that its induction may play a beneficial role in vascular calcification.

Iron and osteoporosis

Bone is a distinct form of connective tissue that is composed of specialized cells and extracellular matrix. The main feature of bone that distinguishes it from many other forms of connective tissue is the mineralization of its matrix, which produces a rigid tissue capable of not only providing support and

posture but also protection of vital organs. Osteogenesis is dependent on a specialized cell type, osteoblast that is a secretory cell with the ability of dividing and proliferation. Hence, the degree of bone formation and overall homeostasis is highly dependent on well-functioning osteoblasts. While accumulation of excessive amounts of iron has been linked with various organ dysfunctions, its association with bone loss has only recently been emerging. Traditionally, decreased levels of sex steroids had been suggested to be major mediator of decreased osteoblastic activity in patients with hemochromatosis.

Emerging evidence however, indicates that iron and its overload have direct deleterious effects on bone, concluding that the effect of iron is to decrease bone deposition rather than to increase bone resorption. Despite strong *in vitro* and *in vivo* evidence though, the precise mechanism by which iron can cause a decrease in bone mass and its mineralization was yet to be thoroughly studied. The latter part of this thesis investigated the mechanism by which iron overload causes decreased bone mass.

Aims of the study

Part 1

Vascular calcification is a major cause of morbidity and mortality. More recent advances have demonstrated that the process of vascular calcification is not a mere passive process as previously thought. In fact it involves transdifferentiation of SMC into osteoblasts that is accompanied by expression of bone specific genes followed by deposition of hydroxyapatite minerals. Patients with ESRD are at increased risk of developing vascular calcification irrespective of age and other comorbidities. Despite improved knowledge of pathomechanisms leading to vascular calcification, therapeutic and/or preventive measures have remained elusive.

HO-1/ferritin system has been shown to be protective in many models of injury and clinical conditions including cardiovascular diseases. However, little was known as to whether activation of HO-1/ferritin system plays any beneficial role in mitigating vascular calcification.

Aim 1:

- Study the effect of HO-1/ferritin induction in high Pi induced SMC calcification
- Identify mechanism of inhibition of vascular calcification by ferritin

Part 2

Iron overload has been associated with osteoporosis and osteopenia. Despite such association the exact role of iron in decreased bone density was unknown. Based on the results of the first part of study we were prompted to investigate the mechanism by which iron may lead to decreased bone mass.

Aim 2:

- Investigate the pathological role of iron overload in decreased osteoblast activity
- Examine the role of ferritin in iron induced inhibition of osteoblast activity and gene expression

MATERIALS AND METHODS

Cell culture and reagents

SMC were purchased from Cambrex, FBS from Gibco, biliverdin was obtained from MP Biomedicals, tin-Protoporphyrin (SnPP) from Frontier Scientific, 1% CO gas from Linde gas and the gas chamber from Billups-Rothenburg Inc. Unless otherwise mentioned all other reagents were obtained from Sigma. Cell cultures were maintained in growth medium (GM) DMEM (high glucose) containing 15% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin and neomycin, and 1 mM of sodium pyruvate. Cells were grown to confluence and used from passages 4 to 8. Iron was introduced as ammonium ferric citrate or ferric sulfate as well as ferrous form. To keep the ferrous state the medium were supplemented with 200 µmol/L of Ascorbic acid. Iron was dissolved in deionized water. Small interfering RNA (siRNA) specific to HO-1 and negative control siRNA were obtained from Ambion and were transfected with Oligofectamine Reagent (Invitrogen) 24 hours prior to the experiment. Heme, biliverdin and bilirubin were dissolved in NaOH. Final concentration of NaOH was kept below 2 mmol/L in all experiments. This amount of NaOH caused a little change in the pH of the medium (7.40 vs. 7.46) which did not influence calcification, and underlying gene expression of SMC.

Induction of calcification in SMC

At confluence, cells were switched to calcification medium, which was prepared by adding 4 mmol/L of inorganic phosphate to the growth medium. Both growth medium and calcification medium were changed every 2 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0.

Quantification of calcium deposition

Cells grown on 48-well plates were washed twice with PBS and decalcified with 0.6 mol/L HCl for 24 hours at 37°C. Calcium content of the supernatants was determined by the QuantiChrome Calcium Assay Kit (Gentaur). After decalcification, cells were solubilized with a solution of NaOH 0.1 mol/L and SDS 0.1% and protein content of samples were measured with BCA protein assay kit (Pierce). Calcium content of the cells was normalized to protein content and expressed as $\mu\text{g}/\text{mg}$ protein. Mineralization was determined by von Kossa staining.

Alkaline phosphatase activity assay

Cells grown on 6-well plates were washed with PBS twice, solubilized with 1% Triton X-100 in 0.9% NaCl and assayed for ALP activity. Briefly, 130 μl of Alkaline Phosphatase Yellow Liquid Substrate (Sigma) was combined with 50 μg of protein samples, incubated at 37°C for 30 min, and then the kinetics of p-nitrophenol formation was followed for 30 min at 405 nm. Maximum slope of the kinetic curves was used for calculation.

Heme oxygenase enzyme activity assay

Cells grown on P100 dishes were washed twice with HBSS, scraped and centrifuged at 2000 x g for 15 min at 4°C. Cells were resuspended in 300 μl potassium-phosphate (100 mmol/L, pH 7.4) buffer containing 2 mmol/L MgCl_2 , frozen and thawed three times, sonicated and centrifuged at 18,000 x g for 10 minutes at 4°C. The supernatant containing cell microsomes was used to measure HO activity as described previously. HO activity is expressed as pmol bilirubin formed/mg cell protein/60 min.

Western blot to detect HO-1, ferritin H-, and L-chain

To evaluate HO-1 protein expression cell lysate was electrophoresed in 12.5% SDS-PAGE. For ferritin H- and L-chain detection cell lysate was subjected to 8% non-denaturing PAGE. Western Blotting was

performed with a polyclonal anti-HO-1 antibody at 1:2500 dilution (Calbiochem) or with mouse anti-human ferritin H- or L-chain antibodies (from P. Arosio) at 1:1000 dilution followed by HRP-labelled anti mouse IgG antibody. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (Amersham Biosciences). After detection membranes were stripped and reprobed for GAPDH.

Quantification of ferritin and osteocalcin

Ferritin content of cell lysate was measured with the IMx ferritin enzyme immunoassay (Abbott Laboratories). For osteocalcin detection extracellular matrix of cells grown on 6-well plates was dissolved in 300 μ L of EDTA (0.5 mol/L, pH 6.9). Osteocalcin content of the EDTA-solubilized extracellular matrix samples was quantified by an enzyme-linked immunoabsorbent assay (Bender MedSystems).

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated, reverse transcribed and HO-1 mRNA was determined as described previously. To measure mRNA levels the 25 μ L reaction mixture contained 5 μ L of reverse transcribed sample, 0.3 nmol/L of primers and 12.5 μ L of iQ SYBR Green Supermix (Bio-Rad) were used. PCRs were carried out using the iCycler iQ Real Time PCR System (Bio-Rad). Results were normalized by GAPDH mRNA levels.

Ferritins and ceruloplasmin

Apoferritin and ceruloplasmin were from Sigma. Human recombinant wild type H chain and L chain ferritins and the H-chain mutant 222 deleted ferroxidase activity were expressed in *E. coli* and purified as described previously. Final concentrations of ferritins were 2 mg/mL, which correspond to 4.5 μ mol/L for Apoferritin, 3.95 μ mol/L for H-ferritin, 4.19 μ mol/L for L-ferritin. Final concentration of ceruloplasmin was 4 mg/mL which corresponds to 32.7 μ mol/L.

Phosphate measurement

Pi content of the cell lysate was determined by the QuantiChrome phosphate Assay Kit (Gentaur). After 24 hours incubation, cells were washed twice with PBS and solubilized with 1 % Triton and the cell lysates were assayed for Pi. Phosphate content of the cells was normalized to protein content and expressed as $\mu\text{m/L/mg}$ cell protein.

Carbon monoxide exposure

CO at a concentration of 1% (10,000 parts per million; ppm) in compressed air was mixed with compressed air containing 5% CO₂ before being delivered into the culture incubator, yielding a final concentration of 400 ppm CO. The incubator was humidified and maintained at 37 °C. A CO analyzer was used to determine CO levels in the chamber. After the chamber had stabilized, no oscillations were measured in the CO concentration.

Osteoblast cell culture and reagents

Human osteoblasts were purchased from Cambrex and FBS from Invitrogen. Unless otherwise mentioned all other reagents were obtained from Sigma. Cell cultures were maintained in growth medium (GM) DMEM (high glucose) containing 15% FBS, 100 U/ml penicillin, 100 $\mu\text{g/mL}$ streptomycin and neomycin, and 1 mM of sodium pyruvate. Cells were grown to confluence and used from passages 3 to 7. Iron was introduced as ammonium ferric citrate dissolved in deionized water. Apoferritin and ceruloplasmin were from Sigma. Human recombinant wild type H chain ferritins and the H-chain mutant 222 deleted ferroxidase activity were expressed in E. coli and purified as described previously . Final concentrations of ferritins were 2 mg/mL and ceruloplasmin was 3 mg/mL.

Induction of calcification in OB

At confluence, cells were switched to calcification medium, which was prepared by adding 2.5 mmol/L of inorganic phosphate to the growth medium. Both growth medium and calcification medium were changed every 2 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0.

Quantification of calcium deposition

Cells grown on 48-well plates were washed twice with PBS and decalcified with 0.6 mol/L HCl for 24 hours. Calcium content of the supernatants was determined by the QuantiChrome Calcium Assay Kit (Gentaur). After decalcification, cells were solubilized with a solution of NaOH 0.1 mol/L and SDS 0.1% and protein content of samples were measured with BCA protein assay kit (Pierce). Calcium content of the cells was normalized to protein content and expressed as $\mu\text{g}/\text{mg}$ protein. Mineralization was also determined by von Kossa staining.

Statistical analysis

Data are shown as mean \pm SD. Statistical analysis was performed by ANOVA test followed by post hoc, Newmann-Keuls test for multiple comparisons. A value of $P < 0.05$ was considered significant and marked with one asterisk, and $P < 0.01$ was considered highly significant and marked with two asterisks.

RESULTS

Heme decreases SMC calcification in a dose responsive manner

To develop an *in vitro* model we cultured SMC in calcification medium. Granular deposits developed in SMC grown in calcification medium for 9 days, but not in the control culture grown in normal growth medium. Intriguingly, we found that addition of heme (50 $\mu\text{mol/L}$, 9 days) to the calcification medium inhibited calcium deposition as shown by von Kossa staining. Extracellular calcium measurements showed that elevated phosphate-induced calcification is time dependent and that the inhibitory effect of heme on extracellular calcification is dose dependent, with a highly significant suppression at a dose of 25 $\mu\text{mol/L}$. Heme is a strong inducer of HO-1, and as expected we found that HO-1 mRNA, protein and HO activity were elevated in the cells cultured in heme containing medium. Pi level of the medium did not affect this heme-mediated induction of HO-1. In addition we found that heme did not significantly alter HO-2 expression and it induced expression of ferritin regardless of Pi level of the medium.

Ferritin and ferroxidase activity attenuate SMC calcification

Heme induces HO-1 and ferritin, thus it was of interest to analyze which of the two had a major effect on calcification. We also analyzed the role of the end products of HO catalyzed heme degradation by adding them exogenously to the calcification medium. We found that iron, regardless of its ferric or ferrous state completely inhibits calcification. Biliverdin at the concentration of 50 $\mu\text{mol/L}$ provided a little but significant decrease in calcification. Addition of CO (1%) or bilirubin did not influence calcification. On the other hand, addition of apoferritin or recombinant H-chain ferritin to the calcification medium abolished calcification. These two ferritin types have ferroxidase activity, thus we tested another protein with ferroxidase activity, ceruloplasmin. Ceruloplasmin was found to mimic the effect of ferritins at a concentration of 4 mg/ml. The protective effect of L ferritin was minor compared with that of H-

ferritin and ceruplasmin. This may have the following explanation. The L- ferritin chains taken up by the cells may coassemble with the endogenous ferritin and thus expand the pool of active ferritins. The H- mutant 222 ferritin which lacks both ferroxidase activity and iron storing capability was not protective at all against mineralization of SMC.

In order to confirm the protective role of ferritin, we inhibited HO using SnPP, a well-known inhibitor of HO activity, and also transfected the cells with small interfering RNA (siRNA) specific for HO-1. We confirmed the efficiency of siRNA and observed around 70% decrease of HO-1 protein expression for up to 4 days post transfection. In fact, cells treated with heme in the presence of SnPP or siRNA showed very low HO enzyme activity. Treatment with SnPP or siRNA did not affect the heme-mediated ferritin induction and, more importantly, did not influence heme mediated inhibition of calcification indicating the paramount role of ferritin in this protection.

To further confirm the function of ferritin in the heme or iron induced inhibition of calcification, we selectively downregulated heme-, or iron-induced ferritin synthesis by the iron chelator, deferoxamine (DFO). Treatment of the cells with DFO together with equimolar amount of heme or Fe resulted in a complete block of heme-, or Fe-induced ferritin synthesis of both H- and L-chains as shown by western blot. Downregulation of ferritin synthesis by DFO lead to complete loss of inhibition of calcification by iron. Moreover, co-treatment with heme and DFO resulted in downregulation of both chains of ferritin, but not of HO-1, that was accompanied by substantial decrease in inhibition of calcification. Mild, but significant inhibition of calcification was noted which may be attributed to biliverdin derived from HO mediated heme degradation.

Ferritin inhibits osteoblastic differentiation of SMC

It has been shown that vascular calcification in vivo shares similarities with bone mineralization, therefore we asked whether ferritin and its ferroxidase activity solely inhibits mineralization or does it suppress the phenotype transition of SMC into osteoblast-like cells. We examined the activity of alkaline

phosphatase an important enzyme in early mineralization. SMC maintained in calcification medium for 9 days showed around a 7-fold increase in ALP activity compared to control. Supplementation with heme provided a decrease in ALP activity. Similarly, exposures of cells to iron abolished high Pi induced ALP activity. Biliverdin (caused some inhibition while other end-products of HO mediated heme degradation – bilirubin and CO – failed to decrease ALP activity. Co-treatment of the cells with heme and SnPP demonstrated similar changes in ALP activity as heme alone; on the other hand co-treatment with heme and DFO did not affect the increased ALP activity. Importantly, apoferritin, H ferritin and ceruloplasmin also decreased the activity of ALP to the level seen in controls but the H mutant 222 ferritin was totally ineffective.

Next we investigated the presence of another bone specific protein, osteocalcin in the extracellular matrix. Maintaining of SMCs in calcification medium for 9 days resulted in more than 10-fold increase in osteocalcin content compared to control. Heme decreased upregulation of osteocalcin, and SnPP did not alter this effect. In contrast, co-treatment of the cells with heme and DFO lead to the loss of osteocalcin downregulation by heme. Iron inhibited upregulation of osteocalcin similarly to heme. In addition, biliverdin had a mild but significant effect, while other products of HO reaction – bilirubin and CO – failed to downregulate high Pi induced osteocalcin expression. Apoferritin, H ferritin and ceruloplasmin abolished expression of osteocalcin while H mutant 222 had no effect at all.

Finally, to explore the mechanism underlying the inhibition of mineralization, we examined the level of Cbfa-1 the “master gene” of osteoblast differentiation in our in vitro model. Culturing SMC in calcification medium for 48 hours resulted in a 1.8 fold increase in Cbfa-1 mRNA level compared to cells maintained in normal growth medium. Heme inhibited induction of Cbfa-1 mRNA. Accordingly, also apoferritin significantly suppressed this Cbfa-1 induction.

We also tested the intracellular levels of Pi and as our results indicate neither apoferritin, nor ceruloplasmin alter intracellular Pi levels after 24 hours. Iron causes slight but significant decrease in the level of intracellular Pi that may be attributed to its phosphate binding

capacity. Notice, such decrease in intracellular Pi levels does not result in significant inhibition of calcification. Furthermore, we have also examined the role of aluminum which is both a trivalent cation and is a strong phosphate binder. Although there was some inhibition of calcification, the extent was one third of that observed with heme or iron.

Iron inhibits calcification of osteoblasts in a dose responsive manner

To develop an *in vitro* model of osteoblastic activity and mineralization we cultured human OB in calcification medium which was prepared by addition of 2.5 mmol of Pi to the growth medium. Granular deposits developed in OB grown in calcification medium for 14 days, but not in the control culture grown in normal GM as confirmed by von Kossa staining. We found that addition of iron to the calcification medium suppresses granular deposit development and extracellular calcium deposition in a dose responsive manner, causing highly significant inhibition at a concentration of 25 $\mu\text{mol/L}$, and a complete inhibition while using 50 $\mu\text{mol/L}$ of iron. As iron is a very potent inducer of ferritin, next we tested whether the observed inhibitory effect of iron on calcium deposition is mimicked by apoferritin. We found that iron-free apoferritin abolishes granule-formation and dose dependently inhibits calcium deposition causing complete inhibition when applied at a concentration of 2 mg/ml. Then we asked if decreasing the level of available iron with the iron chelator deferoxamine– which in turn leads to the posttranscriptional downregulation of ferritin synthesis–could alter the level of calcification. Indeed addition of DFO to the calcification medium increased the levels of calcium deposition by approximately 20%. In fact, both endogenous up-, and downregulation of ferritin H- and L-chains with Fe or DFO respectively and exogenous administration of apoferritin caused alterations of calcium deposition of OB. We found a strong negative correlation between the ferritin levels of the cells and the observed calcium deposition.

Ferroxidase activity is responsible for inhibition of calcification in osteoblasts

To investigate whether the inhibitory effect of ferritin is due to its iron sequestration capacity or its ferroxidase activity or both we tested the effect of ceruloplasmin, a protein, which possesses ferroxidase activity but not iron sequestration capacity. In fact as indicated in exogenous ceruloplasmin dose dependently inhibited mineralization causing abolishment of calcium deposition at a dose of 3 mg/ml. Experiments using recombinant H-ferritin and the H-mutant 222 ferritin which lacks both ferroxidase activity and iron storing capability provided further evidence of the role of ferroxidase activity in the observed inhibitory effect, while H-ferritin attenuated calcium deposition at a dose of 2 mg/ml, the H-mutant 222 ferritin did not alter calcification at all when applied at the same dose.

Ferritin-ferroxidase activity downregulates expression and subsequent activity of ALP

To investigate whether the observed effects are only restricted to reduced calcification or they influence OB gene expression as well, we examined the role that ferritin may play regarding OB specific genes. First we examined the ALP gene since its activity is a good marker of osteoblastic activity. ALP activity is crucial in early osteogenesis by increasing local levels of Pi and therefore facilitating the formation of hydroxyapatite crystals.

Basal ALP activity of OB was quite high when culturing them in a normal GM and addition of Pi to the GM caused a mild but significant increase in ALP activity. However addition of iron to the high Pi containing calcification medium led to a significant decrease of ALP activity, which became much lower than the basal ALP activity of OB cultured in GM. Accordingly, this inhibitory effect was attenuated when using equimolar concentrations of iron and DFO together. Addition of 10 $\mu\text{mol/L}$ of DFO alone caused mild but significant increase in the level of ALP activity. Supplementation of the calcification media with apoferritin caused a dose responsive inhibition of ALP expression and activity. The role of ferroxidase activity behind the observed inhibitory effect of iron and ferritin was also assessed by using wild type and mutant H-ferritin and ceruloplasmin. While addition of both H-ferritin and ceruloplasmin to

the calcification medium strongly downregulated ALP activity, H- mutant 222 ferritin did not influence ALP activity at all confirming ferroxidase activity as the central element of this inhibition.

Ferritin-ferroxidase activity suppresses the exclusive OB product: Osteocalcin

While maintaining OB in calcification medium for 14 days results in ~ 13 fold increase in the amount of osteocalcin deposition in the newly synthesized extracellular matrix, addition of iron decreased the level of osteocalcin dose responsively and causing very significant inhibition at doses of 25 $\mu\text{mol/L}$ and almost a complete inhibition.

Accordingly, supplementation with apoferritin also caused a dose dependent inhibition in the amount of osteocalcin providing very significant inhibition at 0.5 mg/ml and a complete inhibition was resulted at a dose of 2 mg/ml. To further confirm that such inhibition is mainly provided by ferroxidase activity, ceruloplasmin and H-ferritin (wild type and mutant 222) were added to the calcification medium. While ceruloplasmin and H-ferritin abolished osteocalcin production, H-mutant 222 ferritin did not cause any significant inhibition regarding the amount of osteocalcin deposition.

Intracellular Pi concentrations are not affected by apoferritin or ceruloplasmin

Because ferric iron is known to bind phosphate and to examine the mechanism by which both mineralization and OB gene expression are downregulated, we measured Pi uptake of OBs after 24 hours of incubation in calcification medium. Our results indicate that iron caused a mild but significant decrease in the level of intracellular Pi. This is probably due to the phosphate-binding capacity of ferric iron. On the contrary, neither apoferritin nor ceruloplasmin altered intracellular Pi concentrations 24 hours after incubation. Hence iron is capable of binding to phosphate and hence causing a minor decrease in the level of extracellular Pi, but this seems to contribute only in minor proportion to the mechanism of action of iron on bone deposition. This notion is firmly confirmed by the inhibition caused by apoferritin, H-ferritin, and ceruloplasmin, which are all free of iron.

Ferritin inhibits OB specific transcription factor Cbfa-1

It is well recognized that OB maturation and differentiation is powerfully influenced (if not dictated) by transcription factor Cbfa-1. Hence we investigated if ferritin could downregulate expression of Cbfa-1 and therefore put forward a possible explanation for the observed inhibitory effects of ferritin on OB activity. Indeed, supplementation of the calcification medium with apoferritin caused a dose dependent downregulation of Cbfa-1 protein expression.

Ferritin prevents calcification of human 143-B cells

In order to confirm the inhibitory effect of ferritin and ferroxidase activity on mineralization, we employed another human osteoblastic cell line: 143-B osteosarcoma cells. Granular deposits developed in 143-B cells grown in calcification medium for 7 days but not in the control culture grown in normal GM, as demonstrated by alizarin red staining. We found that addition of iron to the calcification medium prevented granular deposit development and extracellular calcium deposition. Furthermore, exposure of cells to apoferritin also abolishes granule formation and completely inhibits calcium deposition. Importantly, cells treated with ceruloplasmin fail to exhibit granule formation and extracellular calcium accumulation at a concentration of 3 mg/mL. These results confirm that ferroxidase activity acts as an inhibitor in OB mineralization.

Summary

The studies that are presented in this thesis have focused on two different, yet overlapping clinical conditions. First, our findings have focused to provide molecular understanding into the mechanism of inhibition of high Pi induced vascular calcification. Our results suggest that induction of ferritin is a potent inhibitor of SMC calcification and transdifferentiation into osteoblast like cells. Second, we sought to investigate the mechanism of iron overload induced osteoporosis. We provide evidence that iron inhibits osteoblast activity via upregulation of intracellular ferritin. In both studies we found that ferroxidase activity plays a central role in inhibition of vascular calcification and osteoblast activity. These studies, for the first time, provide mechanistic insight into two very detrimental clinical conditions and hence offer novel avenues of understanding and future research that may lead to innovative therapeutic modalities.

A relationship between calcification and iron metabolism has never been explored, although it should be noted that most patients who have CKD and require renal replacement therapy have vascular calcification and deranged iron homeostasis. Moreover, in patients with CKD, there is an accumulation of iron in reticuloendothelial cells that is accompanied by higher levels of plasma ferritin; however, this increase largely results because most of such iron is sequestered by reticuloendothelial cells and its availability to other cells is significantly reduced. This translates to depletion of intracellular ferritin and subsequent anemia of chronic disease. In inflammatory diseases such as CKD, cytokines released by activated leukocytes and other cells exert multiple effects. These contribute to the reduction in hemoglobin levels and increased hepatic synthesis of hepcidin that in turn binds to ferroportin, the transporter that allows egress of iron from reticuloendothelial macrophages and from intestinal epithelial cells. Binding of hepcidin leads to internalization and degradation of ferroportin. The corresponding sequestration of iron within the macrophages limits iron availability to all cells. On the basis of our observations, we suggest that such derangements in iron metabolism may facilitate Pi-induced vascular calcification; therefore, parenteral iron administration may be considered not only to replete iron and

correct anemia but also to prevent vascular calcification via increasing intracellular ferritin expression and decreasing extracellular Pi level, especially when the inflammation is well controlled.

The latter part of this thesis revealed seminal findings that identify ferritin induction by iron overload to be the mechanism that decreases osteoblast activity subsequently leading to osteopenia and osteoporosis. Therefore, These findings will expand the knowledge of both osteogenesis and pathogenesis of iron caused bone defects, and by exposing ferritin as the cause of decreased osteoblast maturation may offer new understanding into better planning strategies to prevent or reverse iron-induced osteoporosis and osteopenia.

Novel Findings

Heme prevents calcification of vascular smooth muscle cells in a dose responsive manner

The observed inhibition is dependent on upregulation of ferritin

Ferroxidase activity is central to mitigation of calcification

The inhibitory mechanism is via suppression of Cbfa-1 (osteoblast specific transcription factor) expression

Osteoblast activity is suppressed by iron in a dose dependent manner which is dependent on induction of ferritin

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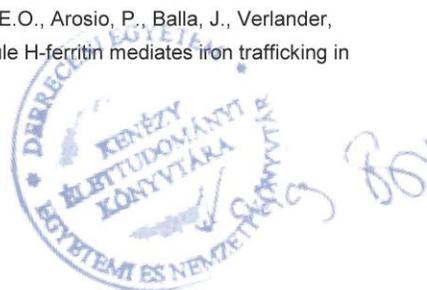
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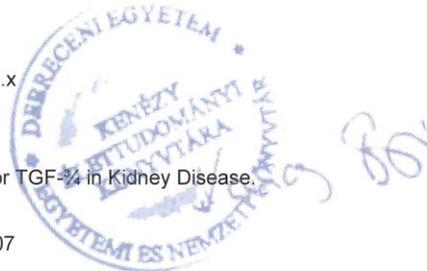




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