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Glucocorticoids and Osteocyte Autophagy

Wei Yao¹, Weiwei Dai¹, Jean X. Jiang², and Nancy E. Lane¹

¹Department of Medicine, University of California at Davis Medical Center Sacramento, CA 95818

²Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229

Abstract

Glucocorticoids are used for the treatment of inflammatory and autoimmune diseases. While they are effective therapy, bone loss and incident fracture risk is high. While previous studies have found GC effects on both osteoclasts and osteoblasts, our work has focused on the effects of GCs on osteocytes. Osteocytes exposed to low dose GCs undergo autophagy while osteocytes exposed to high doses of GCs or for a prolonged period of time undergo apoptosis. This paper will review the data to support the role of GCs in osteocyte autophagy.

Keywords

Glucocorticoids; autophagy; bone fragility

Introduction

Glucocorticoids (GCs) are used in clinical medicine as effective therapy for inflammatory/autoimmune diseases. However, GC use creates rapid bone loss that results in a high incident fracture risk. Epidemiologic studies find 50% of rheumatoid arthritis (RA) patients in the United States today are still treated with chronic GCs and; baseline data from clinical trials in RA patients report a prevalence in vertebral fracture of 30-50% [1-6]. Other studies find that both old and young, men and women and all ethnic groups studied have bone loss with GC treatment, making this an important public health problem [7]. Because patients treated with GCs may require the treatment for a long period of time, there is a high medical need to understand the biology of GC induced bone loss so that clinicians can effectively prevent and treat this disease. Interestingly, the loss of trabecular mass, trabecular architecture, and integral bone mass does not explain the increase in fracture risk from GCs, as individuals treated with GCs frequently experience fractures at higher Bone Mineral Densities (BMDs) than women with postmenopausal osteoporosis [8]. In addition, after withdrawal of GC treatment, there can be some recovery of BMD suggesting maintenance of bone architecture despite a change in bone fragility [8-10]. Recently, atypical fractures have been documented to occur more often in the shaft or subtrochanteric regions of the femur in patients treated with long-term bisphosphonates, especially for those who were treated for 6 months or longer with GCs (Girgis C. et al., ASBMR 2010). Although more epidemiologic and pathophysiologic research is needed to better define the risk, the adverse

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Corresponding Author Information: Nancy E. Lane, MD, Center for Musculoskeletal Health, 4625 2nd Avenue, Suite 1002, Sacramento, California 95817, Telephone: 916-734-0758, FAX: 916-734-4773, nelane@ucdavis.edu.

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effects of GCs on the cortical bone quality that may be independent of BMD loss warrant further investigation [11].

Biology of GC-Induced Bone Loss

GC treatment results in changes in bone remodeling [12, 13]. Observations of surface and biochemically-based turnover in clinical studies of GC-induced osteoporosis show a reduction in trabecular bone volume, thickness and bone formation [12, 14-16]. The influence of glucocorticoids (GCs) on bone resorption was thought to be indirect and related in part to reduced calcium absorption and increased renal calcium excretion [17]. However, recent studies have found that GCs act directly on osteoclasts to decrease the apoptosis of mature osteoclasts [18]. Kim *et al.* found that GCs *in vitro* inhibited the proliferation of osteoclasts from bone marrow macrophages in a dose-dependent manner. In addition, higher GC doses had no effect on osteoclast maturation but inhibited osteoclasts from reorganizing their cytoskeleton [19]. Therefore, excess GC results in an increase in osteoclast number, but in an apparent inhibition of function with impaired spreading and degradation of mineralized matrix [19].

GCs also alter osteoblast and osteocyte function, which contributes to GC-induced osteoporosis [17]. GCs directly inhibit cellular proliferation and differentiation of osteoblast lineage cells [20], reduce osteoblast maturation and activity [13], and also induce osteoblast and osteocyte apoptosis *in vivo* [21]. The suppression of osteoblast function by GCs is reported to be associated with alteration of the Wnt signaling pathway [22], a critical pathway for osteoblastogenesis [23, 24]. GCs enhance Dickkopf 1 expression [25], one of the Wnt antagonists that prevents soluble Wnt proteins from binding to their receptor complex [26]. GCs maintain levels of glycogen-synthase kinase-3 [27], a key kinase that phosphorylates β -catenin, thereby preventing the translocation of β -catenin into the nucleus and the initiation of transcription in favor of osteoblastogenesis. GCs may also enhance bone marrow stromal cell development towards the adipocyte lineage rather than towards the osteoblast lineage [24, 28]. Moreover, the loss of osteocytes by GC-induced apoptosis [29] may disrupt the osteocyte-canalicular network, resulting in a failure to direct bone remodeling at the trabecular surface. GC-induced changes in osteocyte function also result in a weakening of the localized material properties around osteocytes as well as in decreased whole bone strength [30].

Mineral Metabolism and Osteocytes

GC treatment is known to alter calcium metabolism. Treatment with GCs reduces the gastrointestinal absorption of calcium and increases urinary excretion of calcium, which leads to a calcium deficit [17, 31, 32]. Over time this calcium deficit and low serum ionized calcium levels can stimulate PTH release; PTH then catalyzes 1- α -hydroxylase enzyme production in the kidney, which in turn increases 1,25(OH)₂ vitamin D₃ levels, and this is followed by gastrointestinal absorption of both calcium and phosphorus. If the calcium deficit continues, gastrointestinal absorption of these minerals continues, resulting in elevation of serum phosphorus that then stimulates the production of fibroblast growth factor 23 (FGF23) by osteocytes in an attempt to lower serum phosphorus. FGF23 is a hormonal factor that is produced primarily by osteocytes and reduces serum phosphorus and 1,25(OH)₂ vitamin D₃ levels by acting on the kidney through FGF receptors and Klotho [33-35]. The production and circulating levels of FGF23 appear to be tightly regulated but the mechanisms responsible are still under investigation.

The association between FGF23, osteocytes and mineralization has recently been explored [36]. FGF23 serves as a phosphaturic factor synthesized by osteocytes and inhibits 1,25(OH)₂ vitamin D₃ production by the kidney to maintain the balance between phosphate

homeostasis and skeletal mineralization [37]. A recent *in vitro* study demonstrated that overexpression of FGF23 suppressed osteoblast differentiation and matrix mineralization [38]. Another study evaluated the proteins associated with osteocytes and bone mineralization and found that FGF23 co-localized to the secondary spongiosa of trabecular bone and areas of cortical bone where the osteocyte lacunar system was mature, suggesting that FGF23 produced by osteocytes would then be part of the bone-renal axis that is central to proper mineral metabolism [39, 40]. Elevated levels of serum FGF23 have been found in individuals with autosomal hypophosphatemic rickets with mutations in *DMP-1* (dentin matrix protein-1) and other forms of rickets and chronic kidney disease exhibit elevated levels of FGF23 despite normal calciuria [41, 42]. In contrast, mice with deletion of *Klotho* developed elevated DMP-1, hyperphosphatemia and low FGF23 levels [43]. Overexpression of FGF23 in primary rat calvaria cell cultures suppressed matrix mineralization [38]. In one pilot study, increased FGF23 expression in ovine callus was associated with delayed fracture healing [44]. It appears that changes in the production and local concentration of this phosphaturic factor by the osteocyte may result in a reduction in osteocyte-driven mineral metabolism, thereby compromising local bone strength [45-47]. In GC-treated mice, we have observed a dose-dependent increase in serum FGF23, with a decrease in serum phosphorus and 1,25(OH) vitamin D₃, suggesting that GC use may influence mineral metabolism through FGF23 [48]. The altered perilacunar mineralization around GC-treated osteocytes may be secondary to increased FGF23 production. If this was the case, adequate calcium supplementation or restricted phosphate dietary intake may prevent some of the changes in the bone renal axis that occur with GC treatment.

GC induced bone loss clearly does affect the osteocyte

Osteocytes are terminally differentiated osteoblasts that lie below the bone surface and are connected both to other osteocytes and the bone surface via dendritic processes that travel through canaliculi [46, 49-53]. Our *in vivo* mouse studies showed that with GC treatment, a number of the osteocyte lacunae were enlarged as measured by a modified atomic force microscopy/scanning probe microscopy (AFM/SPM). Raman microscopy of the perilacunar area of GC treated osteocytes revealed an enlarged area of demineralization, and AFM/SPM revealed reduced elastic modulus around the enlarged osteocyte lacunae (nearly 40% below the other bone matrix) in a number of the osteocytes [30]. A review of the literature described that we had rediscovered “osteocytic osteolysis” a term initially used to describe enlarged lacunae in patients with hyperparathyroidism [54], immobilized rats [55], X-linked hypophosphatemic rickets, and lactation [56, 57]. Osteocyte lacunar architecture can also be modified by poor mineralization when the bone is being formed, such as with renal osteodystrophy which is distinctly different from “osteocytic osteolysis”. Our observation of the removal of mineral by osteocytes (over weeks or months) would certainly be slower than the bone removal by osteoclasts and may involve a different process. As we also found reduced mineral and elastic modulus surrounding the GC treated osteocyte, we postulated that the osteocyte in the presence of GCs modified the pre-existing mineral of its surrounding matrix creating “osteocyte halos” as initially used by Heuck for the pericanicular demineralization in X-linked hypophosphatemic rickets [58].

To try to elucidate how the osteocyte could be changing its perilacunar matrix we performed microarray analysis, RT-PCR and immunohistochemistry on selected genes and found with GC (1.4 mg/kg/d, low dose) exposure for either 28 or 56 days, the expression of genes associated with inhibition of bone formation (*Dkk-1*, *SOST*, *Wif1*), inhibition of mineralization (*FGF23*) and lysosomes/matrix degradation (*MMPs*, *cathepsin*, *proteinases*) were significantly higher compared to the placebo-control at day 0 (preliminary data). In summary, we determined that GC induced changes in the osteocyte metabolism resulted in a number of the osteocytes developing an increase in osteocyte lacunar size with perilacunar

demineralization, localized reduction in elastic modulus and production of proteins that inhibit osteoblast formation and bone mineralization. However, we did not find much evidence for either pro-apoptotic gene expression, or the presence of apoptotic osteocytes at the low GC dose (1.4 mg/kg/d). In contrast, mice treated for 28 days with a higher GC dose (2.4 mg/kg/d) had apoptotic osteocytes present in the cortical bone. Their changes to the osteocyte in its localized microenvironment with exposure to low dose GC for 28 days suggested to our research group that non-apoptotic programmed cell death, such as autophagy, may also play a role in osteocyte's response to the GC induced stress.

Does autophagy explain the osteocyte response to GCs?

The autophagy pathway is one of the most important biologic processes that enable cells to survive stress and helps to maintain cellular homeostasis by degrading damaged organelles [59-62]. Autophagy is defined by the formation of autophagosomes, also known as autophagic vacuoles that are lined by two membranes with the recruitment of microtubule-associated protein light-chain 3 (LC3)-phosphatidylethanolamine conjugate (LC3-II) to the autophagosomal membrane, a characteristic for autophagosome [63]. When the autophagosomes fuse with the lysosomes and form autolysosomes, degradation occurs and the amino acids or other small molecules are delivered to the cytoplasm for energy production or recycling. If the cells are subjected to long periods of time under GC stress, this may result in extensive recycling of damaged organelles that may lead to cell death or apoptosis [60, 64, 65]. Autophagy can be inhibited by chloroquine (CQ) as it accumulates within autophagosomes, and inhibits the fusion with lysosomes thereby preventing the formation of autolysosomes. This reduction by chloroquine in the final phase of autophagy that provides a pathway for the breakdown of proteins and removal of metabolic debris from the cell, may augment apoptosis [66-68] or rescue osteocyte from cell death [69]. Recently Xia et al reported that dexamethasone treatment of an osteocytic cell line, MLO-Y4 cells, increased autophagy markers and the accumulation of autophagosome vacuoles as detected by several standard approaches based on recently published guidelines including fluorescent GFP-LC3 punctate dots, MDC fluorescence, LC3 lipidation and electron microscopy imaging in addition to conventional acridine orange staining [70]. The enhancement of autophagy was also validated in isolated primary osteocytes isolated from embryonic chicks treated with dexamethasone and in vivo from osteocytes in bone from mice chronically treated with prednisolone. In addition, gene microarray analysis of the cortical bone from mice after 28 days of prednisolone treatment showed increased messenger RNA for several autophagy markers including autophagy-related 16 like 2, autophagy-related 7, LC-3 and LC-3 . Conversely, gene markers for pro-apoptosis were not significantly increased until after a longer prednisolone treatment (56 days of chronic GC exposure) [24, 30]. We also observed gene and protein expression for matrix proteolysis, including matrix metalloproteinases, caspases and cathepsins increased in the cortical bones following GC treatment [24]. Because the interior of a lysosome is strongly acidic, as it releases the contents of its vacuole through autophagic flux into the microenvironment of the osteocyte, it may induce matrix proteolysis, and demineralization of bone around the osteocyte that over time may weaken both the localized bone tissue and whole bone strength [30].

We also found that dexamethasone reduced the number of metabolically normal osteocytes and this effect was augmented when autophagy was inhibited [70]. This study implies that autophagy could be an attempt by osteocytes to attenuate the effect of GC on osteocyte. Autophagy is reported to act as a “double-edge sword” involved in both cell protection and cell death [62, 71]. The cell protective function of autophagy is likely to occur under short or moderate stress conditions. Our cell viability study showed that cells under the autophagic state are very much alive and are likely under metabolic stress. Autophagy is a probable mechanism by which osteocytes can repair damaged organelles or cell membranes.

However, higher, or more prolonged stress may result in an accumulation of autophagosomes and cell death. Interestingly, after 56 days of a treatment with a relatively high dose of prednisolone (5.6mg/kg/d) in mice, we studied the trabecular bone from the vertebral bodies and observed increased apoptotic tunnel-positive labeling [21, 72]. Therefore, these studies demonstrate that low dose GC (less than 2.8 mg/kg/d in mice) treatment resulted in osteocyte autophagy both *in vitro* and *in vivo*. During the initial period of GC treatment, gene array studies revealed that the oxidative pathway [73-77] was activated and simultaneously autophagy was activated suggesting that the osteocytes responded with autophagy in an attempt to “save themselves”. However, with the prolonged GC exposure or higher doses of GCs (5.6mg/kg/d), the cell may undergo apoptosis and or necrosis. The outcome may be related to either the duration of GC treatment or the dose of GC or both [78, 79]. It is possible that suppression or the prevention of autophagy may be a promising new target in the prevention of GC induced bone fragility. If we find that low dose GCs induce osteocyte autophagy that does not affect bone formation and whole bone strength, as opposed to higher doses of GCs that induce osteocyte apoptotic induced bone remodeling and increased fragility, this represent a major paradigm shift for the mechanism responsible for GC-induced bone fragility. Treatments for GC-induced osteoporosis would focus on the inhibition or augmentation of autophagy.

Why do GCs induce osteocyte autophagy

Chronic GC treatment decreases bone formation and increases bone fragility that resembles an accelerated aging process [12, 13]. We found that there was a dose-dependent decrease in the activation of autophagy and anti-oxidative defense gene expression in the cortical bone of mice. GCs at a lower dose increased anti-oxidative responsive as well as autophagic pathways by an average of 30-fold (Figure 1A). In addition, the DNA damage and anti-oxidant pathways were significantly increased both at the lower GC dose and within the first days of the GC exposure, suggesting that cells were being “over-activated” in response to the initial GC treatment. Prolonged exposure or higher doses of GCs reduced both the expression of genes encoding proteins that are anti-oxidants and the number of autophagic osteocytes [80], supporting a relationship between the cells anti-oxidant ability and autophagy following GC exposure [81, 82] (Figure 1B). Bone formation, measured by serum osteocalcin and surface based histomorphometry was greatly reduced by chronic or high dose GC treatments. MicroCT evaluation of trabecular structure showed reduced trabecular bone volume and thickness, as compared to control mice [30, 83]. Similar observations of surface and biochemical based turnover in clinical studies of GIOP have been made including the reduction in trabecular bone volume, thickness and reduced bone formation [12, 14-16]. In summary, GC treatment effects on bone formation were very similar to that observed with aging in that GCs reduced the activation of anti-oxidant gene expression, decreased bone marrow osteogenic potential, reduced autophagy and bone formation. Based on these studies, we propose that modulation of the oxidative and autophagic pathways may provide promising new targets for maintaining bone formation in the presence of GCs or aging, which over time may preserve bone mass.

Therefore, these studies demonstrate that low dose GC treatment (1.4mg/kg/d for 28 days) resulted in autophagy in osteocytes both *in vitro* and *in vivo*. However, with the continued stress of prolonged GC exposure or higher doses of GCs (5.6mg/kg/d for 28 days), the cell may undergo apoptosis and or necrosis. The outcome may be related to either the duration of GC treatment or the dose of GC or both [78, 79]. Autophagy may provide a promising new target in the prevention of GC induced bone fragility (Figure 2). If we find that low dose GCs induce osteocyte autophagy that does not affect bone formation and whole bone strength, as opposed to higher doses of GCs that induce osteocyte apoptotic induced bone remodeling and increased fragility, this represents a major paradigm shift for the mechanism

responsible for GC-induced bone fragility. Treatments for GC-induced osteoporosis would focus on the inhibition or augmentation of autophagy.

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Figure 1.

RNA was extracted from the tibial cortical bone in mice that were treated with PL or various doses of GC. RT-PCR gene arrays were performed for antioxidant defense (A). Correlations between gene expressions associated with antioxidant and autophagy following GC treatments (B).

Figure 2. Proposed mechanisms for osteocyte autophagy and glucocorticoid-induced bone fragility