Glucocorticoids Are Not Always Deleterious for Bone

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ABSTRACT
A 23-year-old man with the rare sclerosing bone disorder van Buchem disease presented with progressively worsening headaches that eventually became persistent and associated with papilledema. Increased intracranial pressure was diagnosed, and the patient had a ventriculoperitoneal drain inserted as well as simultaneously receiving treatment with prednisone. Before starting treatment, there was biochemical evidence for increased bone turnover and for steady increases in bone mineral density (BMD) at the spine and total hip despite the patient having reached his peak height of 197 cm at the age of 19 years. Treatment with prednisone for 2 years resulted in biochemical and histologic suppression of bone formation as well as of bone resorption and arrest of further bone accumulation. Our data suggest that glucocorticoids (GCs) may represent an attractive alternative to the high-risk surgical approaches used in the management of patients with progressive sclerosing bone disorders. Our findings also suggest that whereas sclerostin may not be required for the action of GCs on bone formation, it may well be important for the action of GCs on bone resorption. The exact mechanism by which sclerostin may be involved in the regulation of bone resorption is as yet to be explored.

KEY WORDS: BONE RESORPTION; BONE FORMATION; VAN BUCHEM DISEASE; PREDNISONE; SCLEROSTIN

Introduction

Van Buchem disease is a rare bone sclerosing disorder described for the first time in 1955.(1) It belongs to the group of craniofacial hyperostoses and is characterized by progressive generalized osteosclerosis, particularly of the mandible and the skull, owing to excessive bone formation.(2) It is caused by a 52-kb deletion 35 kb downstream of the SOST gene, which encodes sclerostin, on chromosome 17q12-21.(3,4) This protein is produced in the skeleton exclusively by the osteocytes and inhibits bone formation by antagonizing the Wnt signaling pathway.(5) Clinical manifestations of the disease are due to entrapment of cranial nerves often associated with facial palsy and loss of hearing and smell.(2) Van Buchem disease is thought to have milder clinical manifestations than sclerostosis, a craniofacial hyperostosis with similar phenotype owing to inactivating mutations of the SOST gene.(6,7) Management of the complications of both these sclerosing dysplasias is surgical, aiming at removal of the excess of bone, a technically difficult and sometimes dangerous procedure.(8–10) No medical treatment is available for either sclerosing disease. Glucocorticoids (GCs) are known inhibitors of bone formation,(11,12) and we hypothesized that administration of these agents to patients with complications due to bone overgrowth may arrest its further growth.

We present here sequential observations of a patient with van Buchem disease with life-threatening increased intracranial pressure who was treated successfully with prednisone.

Case Report

The patient first came under our care at the age of 10 years with an established diagnosis of van Buchem disease. The disease was diagnosed clinically and radiologically in infancy and later confirmed genetically by the finding of a 52-kb homozygous deletion 35 kb downstream the SOST gene on chromosome 17q12-q21 (the patient was briefly described (patient 15) by Staeling-Hampton and colleagues(3)). The parents are consanguineous and were both confirmed to be heterozygotes for the disease. There were 3 phenotypically normal sisters in whom no genetic testing has been so far undertaken.

As described in this disorder, clinical manifestations started early in childhood. The patient had a facial palsy at the age of 3 years and developed progressive deafness requiring the use of a hearing aid by the age of 10 years, followed by bilateral bone-anchored hearing aids. He has otherwise been well with normal growth development along the 95th centile, reaching a final height of 197 cm by the age of 19 years. He completed his
secondary education and is employed as office assistant manager. He married at the age of 20 years, and he is the father of 3 healthy children.

The patient demonstrated the typical clinical and radiologic features of van Buchem disease, with enlarged head and mandible and no syndactylly or other digit malformations. During the 15-year duration of follow-up, there were no other clinical signs or symptoms, and blood pressure was normal. Hematologic and biochemical parameters, including those of mineral metabolism, demonstrated no abnormalities over the years. Skeletal radiographs showed thickening of the calvarium, base of the skull, and long bones and sclerosis of the vertebrae (Fig. 1). Bone mineral density (BMD) values of the spine and hip were markedly increased at presentation ($Z$-score $+6.2$) and continued to increase in parallel with that of his healthy peers without, however, attaining a peak (highest $Z$-score being 7.7). Biochemical markers of bone turnover always were increased compared with normal values for age but followed a normal pattern of change with a further increase during the growth spurt and a progressive decline thereafter, although never reaching the normal range (Fig. 2).

At the age 23 years, the patient complained of progressive headaches that eventually became persistent and were associated with dizziness and signs of increased intracranial pressure in the form of papilledema. The diagnosis was confirmed radiologically, and a ventricular-peritoneal drain was implanted, and the patient was concomitantly started on prednisone 30 mg/day that was reduced to 10 mg/day within 1 month. In the following 2 years he received different doses of prednisone, as depicted in Fig. 3, but no calcium or vitamin D supplements. These interventions were followed by rapid improvement of his symptoms, and the improvement was sustained during the follow-up period. There were no appreciable changes in metabolic parameters with treatment (highest values of serum cholesterol and glucose were 5.6 and 6.1 mmol/L, respectively, and of urinary calcium excretion 7.8 mmol/24 hours).

**Methods**

The biochemical markers of bone turnover procollagen type I N propeptide (P1NP) and C-terminal telopeptide of type I collagen ($\beta$-CTX) were measured in serum at regular intervals using the E-170 system (Roche BV, Woerden, Holland). BMD was measured by dual-energy X-ray absorptiometry (DXA; Hologic QDR 4500, Waltham, MA, USA). An iliac crest biopsy was obtained after in vivo labeling with two courses of tetracycline separated by 12 days. Bone histomorphometry was performed on undecalci-fied histologic bone sections by Dr Pascale Chavassieux (INSERM Unit 831, University of Lyon, Faculty of Medicine R Laennec, Lyon, France). Immunohistochemical staining for sclerostin was performed in our laboratory using a previously described technique.\(^{13}\)

**Results**

**Biochemical markers of bone turnover**

The changes in serum P1NP and $\beta$-CTX before and during prednisone treatment are depicted in Fig. 3. Before treatment, values of both markers of bone turnover were elevated and decreased to within the normal reference adult range within 4 weeks of starting treatment with prednisone. The effect of prednisone on bone turnover depended on the dose administered, and attempts to reduce the dose below 5 mg/day were associated with increases in serum markers of bone turnover. It was notable that during treatment, there was a close relationship between serum $\beta$-CTX and P1NP values, with the two markers demonstrating parallel changes during adjustments of the dose of prednisone, suggesting a tight coupling of bone resorption and bone formation. There was a highly significant correlation between the two markers throughout the 2-year period of follow up ($R^2 = 0.765$).

**BMD**

The changes in BMD measured at the spine and hip for the 6 years preceding the start of prednisone treatment and for 2 years thereafter are shown in Table 1. Despite high baseline values, BMD continued to increase steadily during adulthood by about 4% every 2 years, demonstrating no further increase after 2 years of treatment with prednisone.

**Bone histology**

On an iliac crest biopsy taken 2 years after the start of prednisone treatment, there was sclerosis and no evidence of active bone remodeling. Cancellous bone volume was clearly increased, and bone trabeculae were thick and well connected. The extent of eroded surfaces was very low (0.4%; normal $3.1\% \pm 1.1\%$), and Howship’s lacunae were devoid of osteoclasts. In addition, no osteoid seams were seen, and there was no tetracycline uptake on examination under fluorescent light. As expected and described previously,\(^{14}\) osteocytes did not stain for sclerostin.
Discussion

This case illustrates the beneficial effect of prednisone treatment on bone metabolism in a patient with van Buchem disease and life-threatening increased intracranial pressure. Treatment resulted in a histologically documented dramatic decrease in bone formation. Following therapy, there was also no further increase in BMD at the spine and hip. Although clinical manifestations of increased intracranial pressure improved significantly, this cannot be attributed solely to treatment with prednisone because the patient had a ventriculoperitoneal drain implanted simultaneously at the time of starting prednisone.

Before prednisone treatment, the patient had an increased rate of bone turnover, as assessed biochemically, associated with a continuous increase in BMD of the spine and hip. The biochemical markers of bone formation, P1NP and osteocalcin, have been reported previously to be elevated in 6 patients with van Buchem disease compared with their levels in disease carriers, being above the upper limit of the normal range in 3 of them. Urinary cross-linked N-telopeptide of type I collagen (NTX) levels were higher in 4 patients with the disease compared with carriers. Bone density measured in the phalanges by radiographic absorptiometry was elevated in all these patients. There are, however, no longitudinal data reported to date in patients with van Buchem disease. In our patient, at least up to the age of 23 years, both biochemical markers of resorption and formation were increased. The clinical progression of the disease, which was due to bone overgrowth, as also evidenced by the steady increase in BMD, prompted us to use GCs in an attempt to arrest the process of bone accumulation.

The beneficial use of GCs has been reported previously in a patient with craniotubular hyperostosis owing to an unidentified genetic defect. In this patient, prednisone given for three courses of 10 weeks each reduced serum osteocalcin but had no effect on urinary deoxypyridinoline (DPD) and there were no reported changes in BMD. In a few patients with progressive diaphyseal dysplasia, a craniotubular hyperostotic disorder distinct from van Buchem disease, which is due to mutations of the gene encoding transforming growth factor β (TGF-β), prednisone treatment during childhood and adolescence led to clinical and in one case radiologic improvement.

![Fig. 2. Sequential measurement of serum alkaline phosphate activity (AP) in units/L, urinary hydroxypyroline/creatinine ratio (OHP/Cr) in μmol/mmol, and height (cm) in a patient with van Buchem disease over a 10-year period. Interrupted lines indicate the upper limit of normal range.](image)

![Fig. 3. Biochemical markers of bone formation and resorption before and during treatment with prednisone. P1NP = diamonds and solid line; β-CTX = closed circles and interrupted line.](image)

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<th>Table 1. Bone Mineral Density Measurements and Height of a Patient With van Buchem Disease Before and After 2 Years of Prednisone Treatment (Date: Month/Year)</th>
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Note: Height in cm, LS BMD = lumbar spine BMD in g/cm², TH BMD = total hip BMD in g/cm².
GLUCOCORTICOIDS IN VAN BUCHEM DISEASE

14. Wergedal JE, Veselov K, Hellan M, et al. Patients with Van Buchem disease, an osteosclerotic genetic disease, have elevated bone for-


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
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-3β [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-canicular 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 1α-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 calcium [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 described 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

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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 acidine 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-β. 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.

Acknowledgments

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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
Glucocorticoid-Induced Bone Disease
Robert S. Weinstein, M.D.

This Journal feature begins with a case vignette highlighting a common clinical problem. Evidence supporting various strategies is then presented, followed by a review of formal guidelines, when they exist. The article ends with the author’s clinical recommendations.

A 55-year-old woman with severe, persistent asthma requiring glucocorticoid therapy for the past 3 months presents for care. Her medications include albuterol, inhaled fluticasone with salmeterol, montelukast, and prednisone (at a dose of 10 mg per day). In the past, she received several intermittent courses of prednisone at a dose of 15 mg or more per day. Her weight is 45.5 kg (100 lb), and her height 157.5 cm (62 in.); the body-mass index (the weight in kilograms divided by the square of the height in meters) is 18. Scattered wheezing is heard during expiration. Findings on vertebral percussion and rib-cage compression are unremarkable. How should her case be evaluated and managed to minimize the risk of fractures?

The Clinical Problem
Glucocorticoid therapy is the most common cause of secondary osteoporosis and the leading iatrogenic cause of the disease.1-3 Often, the presenting manifestation is fracture, which occurs in 30 to 50% of patients receiving long-term glucocorticoid therapy.4 Glucocorticoid-induced osteoporosis predominantly affects regions of the skeleton that have abundant cancellous bone, such as the lumbar spine and proximal femur. In patients with glucocorticoid-induced osteoporosis, the loss of bone mineral density is biphasic; it occurs rapidly (6 to 12% loss) within the first year and more slowly (approximately 3% loss yearly) thereafter.5 However, the risk of fracture escalates by as much as 75% within the first 3 months after the initiation of therapy, typically before there is a substantial decline in bone mineral density, suggesting that there are adverse effects of glucocorticoids on bone that are not captured by bone densitometry.6 Several large case–control studies have shown strong associations between exposure to glucocorticoids and the risk of fractures.4,6,7 An increase in the risk of vertebral and hip fractures occurs rapidly after the start of treatment and has been reported to occur with doses as small as 2.5 to 7.5 mg of prednisolone per day (equivalent to 3.1 to 9.3 mg of prednisone per day). In a cohort study involving patients 18 to 64 years of age, continuous treatment with 10 mg of prednisone per day for more than 90 days, for a variety of indications, as compared with no exposure to glucocorticoids, was associated with an increase in hip fractures by a factor of 7 and an increase in vertebral fractures by a factor of 17.7 Furthermore, an increase in the risk of fractures has been reported with the use of inhaled glucocorticoids, as well as with alternate-day and intermittent oral regimens.3

Risk Factors
Risk factors associated with glucocorticoid-induced osteoporosis are listed in Table 1. One factor whose importance has been recognized in the past decade is the activity of the 11β-hydroxysteroid dehydrogenase (11β-HSD) system, a prereceptor modulator of glucocorticoid action.11 Two isoenzymes, 11β-HSD1 and 11β-HSD2, catalyze...
conversion between hormonally active glucocorticoids (e.g., cortisol or prednisolone) and inactive glucocorticoids (e.g., cortisone or prednisone). The 11β-HSD1 enzyme is an activator, and the 11β-HSD2 enzyme is an inactivator. The increased risk of fracture with glucocorticoid administration in the elderly may be explained in part by the increase in 11β-HSD1 that occurs with aging. The risk of glucocorticoid-induced osteoporosis appears to be similar in men and women and among various ethnic groups.

**PATHOGENESIS**

Histomorphometric studies in patients with glucocorticoid-induced osteoporosis consistently show fewer osteoblasts and an increased prevalence of osteocyte apoptosis, as compared with normal controls. The increased osteocyte apoptosis is associated with decreases in vascular endothelial growth factor, skeletal angiogenesis, bone interstitial fluid, and bone strength. Thus, glucocorticoid-induced apoptosis of osteocytes could account for the loss of bone strength that occurs before the loss of bone mineral density and the observed mismatch between bone mineral density and the risk of fracture in patients with glucocorticoid-induced osteoporosis. Glucocorticoid excess also directly reduces osteoclast production, but the lifespan of osteoclasts is prolonged, in contrast to the decrease in the lifespan of osteoblasts. Therefore, with long-term therapy, the number of osteoclasts is usually maintained in the normal range, whereas the number of osteoblasts plummets and bone formation is substantially reduced. These histologic features contrast with the increased bone formation and resorption that are typical of postmenopausal osteoporosis or increased parathyroid hormone secretion and indicate that, contrary to previous assumptions, hypogonadism and secondary hyperparathyroidism are not central to the pathogenesis of glucocorticoid-induced osteoporosis.

**STRA TE GIES AND EVIDENCE**

**EVALUATION**

Physicians who prescribe glucocorticoids should educate their patients about side effects and complications, including not only osteoporosis and osteonecrosis but also cataracts and glaucoma.

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**Table 1. Risk Factors for Glucocorticoid-Induced Osteoporosis.**

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Evidence of a Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced age</td>
<td>Patients 60 to 80 years of age receiving glucocorticoid therapy, as compared with patients 18 to 31 years of age, had a relative risk of vertebral fracture of 26 and a shorter interval between initiation of treatment and the occurrence of fracture</td>
</tr>
<tr>
<td>Low body-mass index (&lt;24)†</td>
<td>Low body-mass index is a risk factor for glucocorticoid-induced osteoporosis and probably fractures as well</td>
</tr>
<tr>
<td>Underlying disease</td>
<td>Rheumatoid arthritis, polymyalgia rheumatica, inflammatory bowel disease, chronic pulmonary disease, and transplantation are independent risk factors</td>
</tr>
<tr>
<td>Prevalent fractures, smoking, excessive alcohol consumption, frequent falls, family history of hip fracture</td>
<td>All are independent risk factors for osteoporosis but have not been extensively studied in patients receiving glucocorticoids</td>
</tr>
<tr>
<td>Glucocorticoid receptor genotype</td>
<td>Individual glucocorticoid sensitivity may be regulated by polymorphisms in the glucocorticoid receptor gene</td>
</tr>
<tr>
<td>Increased 11β-HSD1 expression</td>
<td>11β-HSD1 expression increases with the age of the patient and with glucocorticoid administration</td>
</tr>
<tr>
<td>High glucocorticoid dose (high current or cumulative dose; long duration of therapy)</td>
<td>Risk of fracture escalates with increased doses and duration of therapy; alternate-day or inhaled therapies also confer risks of glucocorticoid-induced osteoporosis</td>
</tr>
<tr>
<td>Low bone mineral density</td>
<td>Glucocorticoid-induced fractures occur independently of a decline in bone mass, but patients with very low bone mineral density may be at higher risk</td>
</tr>
</tbody>
</table>

*11β-HSD1 denotes 11β-hydroxysteroid dehydrogenase 1.
† The body-mass index is the weight in kilograms divided by the square of the height in meters.
hypokalemia, hyperglycemia, hypertension, hyperlipidemia, weight gain, fluid retention, susceptibility to bruising, decreased resistance to infection, impaired healing, myopathy, adrenal insufficiency, and the glucocorticoid withdrawal syndrome. Patients receiving long-term glucocorticoid therapy should wear medication identification jewelry. Malpractice suits precipitated by a failure of physicians to document disclosure of the skeletal complications to patients are not rare, yet these complications are often ignored in clinicians’ discussions with patients about the use of glucocorticoids.

Measurement of the patient’s height is important, since loss of height suggests the possibility of prevalent vertebral fractures, with an associated increase in the risk of future fractures. Laboratory testing that should be performed before treatment is prescribed includes measurements of serum 25-hydroxyvitamin D, creatinine, and calcium levels (in addition to glucose, potassium, and lipid levels). Since bone turnover after long-term glucocorticoid therapy is low, tests of biochemical markers of bone metabolism are usually not helpful. Measurement of bone mineral density and vertebral morphologic assessment or plain films are often recommended to assess the patient for vertebral fractures, but the disparity between bone quantity and bone quality in glucocorticoid-induced osteoporosis makes measurements of bone mineral density insensitive for identifying patients at risk. However, measurements of bone mineral
density may be useful for follow-up assessments after an intervention. The use of the World Health Organization fracture prevention algorithm (FRAX) is not recommended in the case of patients with glucocorticoid-induced osteoporosis, since it does not take into account the current and cumulative dose of glucocorticoids and the duration of therapy and underestimates the risk of glucocorticoid-induced fractures. Furthermore, bone mineral density at the femoral neck is used in the algorithm, but vertebral fractures are more common than hip fractures in patients with glucocorticoid-induced osteoporosis, and the inclusion of the common risk factors for postmenopausal osteoporosis in the algorithm may not be applicable to patients with glucocorticoid-induced osteoporosis.24

In patients treated with glucocorticoids who report persistent hip, knee, or shoulder pain, especially pain that occurs with joint movement or that is associated with tenderness or reduced range of motion, magnetic resonance imaging should be performed to rule out osteonecrosis.23 The incidence of osteonecrosis among patients who take glucocorticoids has been estimated to be between 5 and 40%; higher doses of glucocorticoids and prolonged treatment are associated with greater risk, although osteonecrosis may also occur with short-term exposure to high doses, including those administered intraarticularly (typically 40 to 80 mg of methylprednisolone) and in the absence of osteoporosis. The mechanisms that have been postulated for the development of osteonecrosis include fat embolism, vascular thrombosis, and osteocyte apoptosis.28,29

TREATMENT
All patients should receive adequate calcium supplementation (1200 mg per day in divided doses) and adequate vitamin D supplementation (800 to 2000 U per day), but these precautions alone are not sufficient to prevent fractures.4,24-27 Bisphosphonates are considered to be the first-line options for the treatment of glucocorticoid-induced osteoporosis (Table 2); alendronate, risedronate, and zoledronic acid are approved by the Food and Drug Administration (FDA) for this indication, although there is controversy regarding the doses and duration of glucocorticoid treatment that necessitate intervention to reduce the risk of fractures. In randomized, double-blind, placebo-controlled trials, including patients with a variety of underlying diseases and irrespective of bone mineral density, alendronate and risedronate increased bone mineral density at the lumbar spine and femoral neck and reduced the relative risk of glucocorticoid-induced vertebral fractures by about 40%.6,27,32,33; patients in these trials typically had been taking 10 to 20 mg of prednisone daily or the equivalent for at least 1 year before enrollment, although the dose range and the duration of treatment varied widely. In another randomized trial involving patients treated with glucocorticoids, zoledronic acid was noninferior to risedronate in increasing bone mineral density at the lumbar spine.30

Alendronate decreases glucocorticoid-induced apoptosis of osteocytes,34 which may play a role in the preservation of bone strength.17 However, glucocorticoids antagonize the effects of nitrogen-containing bisphosphonates in inducing apoptosis of osteoclasts and inhibiting bone resorption.35,36 Perhaps as a consequence, bisphosphonates appear to be less effective in the protection of bone mineral density in patients with glucocorticoid-induced osteoporosis than they are in patients with other forms of osteoporosis. The average percentage increase in bone mineral density at the lumbar spine and femoral neck in patients with glucocorticoid-induced osteoporosis after treatment with alendronate at a dose of 10 mg per day for 2 years was 3.9% and 0.6%, respectively — considerably less than that reported in women with postmenopausal osteoporosis (about 7% and 3.6%, respectively) or in men with osteoporosis (7% and 2.5%, respectively), even though the latter two groups were, on average, 10 years older than the patients with glucocorticoid-induced osteoporosis.32,37,38 Moreover, the evidence to support the use of bisphosphonates in the treatment of patients with glucocorticoid-induced osteoporosis is not as strong as the evidence for their use in the treatment of patients with postmenopausal osteoporosis; the primary end point in the trials of glucocorticoid treatment was bone mineral density rather than the occurrence of fractures, and most trials were only 12 to 24 months in duration and were not powered to study hip fractures.

A limitation of oral bisphosphonate therapy is poor adherence to treatment — a well-recognized problem even in the case of agents that are administered weekly or monthly. Administration of zoledronic acid as a once-yearly infusion avoids this problem and provides rapid skeletal protection. For protection from fractures in patients who have received prolonged glucocorticoid ther-
Alendronate, risedronate, zoledronic acid, and teriparatide have been approved by the Food and Drug Administration for the treatment of glucocorticoid-induced osteoporosis in Europe, only once-daily oral bisphosphonate regimens, zoledronic acid, and teriparatide are approved for the treatment of glucocorticoid-induced osteoporosis. Bisphosphonates

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alendronate, 10 mg/day or 70 mg/wk, taken orally</td>
<td>Osteoclast inhibition reduces bone loss and reduces vertebral fractures in patients with glucocorticoid-induced osteoporosis; alendronate also prevents glucocorticoid-induced osteocyte apoptosis; if glucocorticoid therapy is discontinued, these drugs can be stopped</td>
<td>Antiresorptive agents do not directly address the decreased bone formation that is characteristic of glucocorticoid-induced bone disease and have not been shown to reduce hip fractures; gastrointestinal side effects may occur; musculoskeletal discomfort, osteonecrosis of the jaw, uveitis, and atypical femoral fractures have occurred in rare cases; bisphosphonates should be avoided in patients with creatinine clearance of ≤30 ml/min; patients have poor adherence to oral therapy; as compared with intravenous therapy, a longer time is required to obtain skeletal protection</td>
</tr>
<tr>
<td>Risedronate, 5 mg/day or 35 mg/wk, taken orally</td>
<td>Osteoclast inhibition reduces bone loss; as compared with oral treatment, there is increased adherence to intravenous treatment and more rapid onset of skeletal effects; gastrointestinal side effects are unlikely</td>
<td>Acute-phase reaction (influenza-like syndrome) may occur within 2 to 3 days and last 3 days or less, particularly after first dose, but can be effectively managed with acetaminophen or ibuprofen</td>
</tr>
<tr>
<td>Zoledronic acid, 5 mg/yr, given intravenously</td>
<td>Osteoclast inhibition reduces bone loss; as compared with oral treatment, there is increased adherence to intravenous treatment and more rapid onset of skeletal effects; gastrointestinal side effects are unlikely</td>
<td>Costs are greater than with oral or intravenous bisphosphonates; daily injections are required; response is reduced when teriparatide is given with high-dose glucocorticoids; it has not been studied in patients with elevated parathyroid hormone levels; adverse effects include mild hypercalcemia, headache, nausea, leg cramps, and dizziness; caution must be taken in patients with preexisting nephrolithiasis; serum calcium should be checked at least once 16 hours or more after injection and oral calcium intake adjusted as needed</td>
</tr>
<tr>
<td>Teriparatide, 20 µg/day, given subcutaneously, for 2 years, followed by bisphosphonate treatment for as long as glucocorticoids are required</td>
<td>Teriparatide directly addresses the increase in osteoblast and osteocyte apoptosis and the decrease in osteoblast number, bone formation, and bone strength that are characteristic of glucocorticoid-induced osteoporosis and reduces vertebral fractures</td>
<td>Denosumab does not address the reduced bone formation caused by glucocorticoid excess; hypocalcemia and vitamin D deficiency must be treated before the use of denosumab</td>
</tr>
<tr>
<td>Denosumab, 60 mg every 6 mo, subcutaneously</td>
<td>Denosumab is a potent inhibitor of osteoclasts, with ease of administration; it can be stopped if glucocorticoids are discontinued; it can be used in patients with creatinine clearance of ≤30 ml/min</td>
<td>Denosumab does not address the reduced bone formation caused by glucocorticoid excess; hypocalcemia and vitamin D deficiency must be treated before the use of denosumab</td>
</tr>
</tbody>
</table>

*Alendronate, risedronate, zoledronic acid, and teriparatide have been approved by the Food and Drug Administration for the treatment of glucocorticoid-induced osteoporosis. In Europe, only once-daily oral bisphosphonate regimens, zoledronic acid, and teriparatide are approved for the treatment of glucocorticoid-induced osteoporosis.*

apy (e.g., 10 mg per day or more of prednisone for longer than 90 days), intravenous bisphosphonate therapy may be preferable to oral therapy. On the basis of estimates that the maximal absorption of alendronate when it is taken orally on an empty stomach is about 0.7% and that the molar potency of alendronate is lower than that of intravenous zoledronic acid by a factor of 10, it is estimated that a patient would need 90 days of treatment with alendronate at a dose of 70 mg per week to receive a dose equivalent to 5 mg of zoledronic acid delivered in 15 minutes, although these regimens have not been compared with respect to rates of fracture in patients with glucocorticoid-induced osteoporosis. Since substantial loss of bone mineral density has been observed in patients who discontinue bisphosphonate therapy while continuing to take glucocorticoids, it is usually recommended that bisphosphonate therapy be continued for at least as long as the glucocorticoids are prescribed; drug holidays are not considered to be appropriate for patients who are being treated with glucocorticoids.

In a 2-year, randomized, controlled, open-label trial involving patients with osteonecrosis of the femoral head, patients who received alendronate therapy, as compared with those who received no treatment, had decreased pain and delayed expansion of lesions and were less likely to need surgery. A prospective, observational study showed that patients with osteonecrosis had a sustained reduction in pain and improvement in ambulation within months after the initiation of alendronate therapy. In both studies, the most common cause of osteonecrosis was the use of glucocorticoids. Although bisphosphonates are useful in treating osteonecrosis of the hip, these drugs are associated with the development of osteonecro-
osis of the jaw.\textsuperscript{43} Osteonecrosis of the jaw is characterized by exposed maxillofacial bone for at least 8 weeks and typically occurs after a dental extraction or other invasive procedure.\textsuperscript{49} Most reported cases of osteonecrosis of the jaw have occurred in patients with osteolytic breast cancer or multiple myeloma who have received frequent, high doses of intravenous bisphosphonates. In patients with osteoporosis treated with bisphosphonates, the estimated risk of osteonecrosis of the jaw is 1 case per 10,000 to 100,000 patient-years.\textsuperscript{43} Before prescribing bisphosphonates, the clinician should perform an oral examination and encourage the patient to be examined by a dentist. Concurrent use of bisphosphonates and glucocorticoids may slightly increase the risk of osteonecrosis of the jaw. Bisphosphonates may also be associated with atypical subtrochanteric femoral fractures, but if there is an association, the risk is low (about 2 cases per 10,000 patient-years).\textsuperscript{44}

An alternative to bisphosphonates is teriparatide, recombinant human parathyroid hormone 1-34, which is approved by the FDA for the treatment of glucocorticoid-induced osteoporosis. In an 18-month, randomized, double-blind trial comparing teriparatide with alendronate in patients with glucocorticoid-induced osteoporosis, teriparatide increased spinal bone mineral density over a shorter period and to a greater extent than did alendronate and also reduced vertebral fractures by 90\%.\textsuperscript{45} Daily subcutaneous administration of parathyroid hormone prevents the expected glucocorticoid-induced increase in osteoblast and osteocyte apoptosis and decrease in osteoblast number, bone formation, and bone strength.\textsuperscript{46} However, the effect of teriparatide is somewhat compromised by high-dose glucocorticoid therapy;\textsuperscript{47} a lesser increase in bone mineral density at the lumbar spine has been noted in patients taking more than 15 mg of prednisone per day, as compared with those taking less than 5 mg per day. In addition, host factors (e.g., the underlying illness and associated weight loss, medications, reduced renal function, and low levels of insulin-like growth factor 1) may contribute to the diminished efficacy of teriparatide in patients with glucocorticoid-induced osteoporosis, as compared with patients who have other forms of osteoporosis.\textsuperscript{46} Disadvantages of teriparatide include the cost and the risk of mild hypercalcemia (Table 2).\textsuperscript{31}

Another potential treatment option is denosumab, a humanized monoclonal antibody to the receptor activator of nuclear factor-κB ligand (RANKL), which is approved by the FDA for the prevention of vertebral, nonvertebral, and hip fractures in women with postmenopausal osteoporosis but is not currently approved for the treatment of glucocorticoid-induced osteoporosis.\textsuperscript{48} Administered as a subcutaneous injection every 6 months, denosumab rapidly decreases bone resorption. In a subgroup analysis of a 12-month, randomized, placebo-controlled trial of denosumab in patients receiving methotrexate treatment for rheumatoid arthritis, patients receiving denosumab, prednisone (≤15 mg per day), and methotrexate had increases in bone mineral density at the lumbar spine and total hip that were similar to those in patients receiving methotrexate and denosumab alone, and the rate of side effects was similar in these groups.\textsuperscript{49} Denosumab may have a role in treating patients taking glucocorticoids who have stable serum calcium levels and who are not candidates for bisphosphonate or teriparatide therapy because of side effects or a history of renal insufficiency, although, as with other agents, more data are needed regarding the effect of denosumab on the risk of fracture.

Vertebroplasty and kyphoplasty are sometimes performed to treat painful vertebral fractures, but in controlled trials, these procedures have not been found to be superior to sham procedures, and the risks include leakage of the cement and an increase in the risk of additional fractures in patients receiving glucocorticoids.\textsuperscript{50}

\textbf{Areas of Uncertainty}

More data are needed to predict the risk of fractures among patients taking glucocorticoids and to establish clinical thresholds for intervention.\textsuperscript{2} Effective strategies are required to educate physicians about the importance of counseling patients who are receiving long-term glucocorticoid therapy regarding the risk of fractures. Additional studies are needed to determine the minimum dose of glucocorticoids and duration of therapy that warrant interventions to prevent fractures and to better understand the ways in which other risk factors for bone loss should guide decisions regarding therapy. Some clinicians prescribe drugs for the prevention of fractures for virtually every patient who requires glucocorticoid therapy and discontinue these drugs only when the glucocorticoids are discontinued, but the benefits, risks, and cost-effectiveness of this strategy, as compared with a more selective approach to the initiation of...
pharmacotherapy, have not been assessed. There is no evidence that medication to prevent fractures is needed with occasional dose-pack prescriptions, annual short-term (e.g., 7 to 10 days) high-dose intravenous or oral therapy (<1 g of cumulative exposure), or replacement therapy for patients with hypopituitarism, adrenal insufficiency, or congenital adrenal hyperplasia, provided that the replacement doses are not excessive.

**GUIDELINES**

Guidelines from the American College of Rheumatology, the National Osteoporosis Foundation, the Royal College of Physicians, and the Belgium Bone Club vary somewhat in their recommendations (Table 3).24-27 The well-recognized early increase in the risk of fracture associated with the use of glucocorticoids, the lack of certainty with respect to a known minimum dose and duration of glucocorticoid therapy that does not increase the risk of fracture, and available trials showing increased bone density (and in some cases reductions in the rate of vertebral fracture) with antiresorptive therapy in patients taking prolonged courses of glucocorticoids (mostly at doses greater than 10 to 20 mg of prednisone per day) serve as the basis of the recommendations. However, high-quality data are lacking to determine the precise risk of fractures associated with doses of prednisone that are less than 5 to 7.5 mg daily or with intermittent regimens of glucocorticoids, as well as the appropriate care of patients who are taking these regimens.

**CONCLUSIONS AND RECOMMENDATIONS**

The woman described in the vignette, who is slender, has been taking prednisone at a dose of 10 mg daily for 3 months, and previously received higher doses of glucocorticoids, is at considerable risk for glucocorticoid-induced osteoporosis. Other asthma therapies should be used as efficiently as possible in an effort to taper the prednisone. The assessment should include a mea-

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**Table 3. Guidelines for Management of Glucocorticoid-Induced Osteoporosis.**2

<table>
<thead>
<tr>
<th>Variable</th>
<th>American College of Rheumatology24</th>
<th>National Osteoporosis Foundation25</th>
<th>Royal College of Physicians of London26</th>
<th>Belgian Bone Club27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose and duration of glucocorticoid treatment warranting pharmacologic intervention†</td>
<td>≥7.5 mg/day for at least 3 months, but patients at increased risk require treatment with any dose or duration</td>
<td>≥5 mg/day for at least 3 months</td>
<td>Any oral dose for at least 3 months in patients ≥65 years of age and those with a prior fragility fracture</td>
<td>≥9.3 mg/day for at least 3 months</td>
</tr>
<tr>
<td>BMD threshold for treatment if dose and duration qualify</td>
<td>Threshold to be based on the FRAX algorithm in addition to “higher daily and cumulative dose, intravenous usage, and declining BMD”</td>
<td>T score, −2.5, unless patient is at high risk on the basis of a modified FRAX model</td>
<td>T score, −1.5</td>
<td>T score, −1.0 to −1.5</td>
</tr>
<tr>
<td>Yearly BMD testing recommended</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Prevalent vertebral fractures as justification for pharmacologic intervention</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Calcium and vitamin D supplementation</td>
<td>1200–1500 mg of calcium per day and 800–1000 units of vitamin D per day for all patients‡</td>
<td>1200 mg of calcium per day and 2000 units of vitamin D per day for all patients‡</td>
<td>Only for patients with low calcium intake (&lt;1 g/day) or vitamin D deficiency (not defined)‡</td>
<td>For all patients</td>
</tr>
<tr>
<td>Pharmacologic intervention</td>
<td>Bisphosphonates; teriparatide reserved for patients at highest risk</td>
<td>Bisphosphonates; teriparatide only for patients at high risk</td>
<td>Bisphosphonates as first-line options, followed by teriparatide</td>
<td>Bisphosphonates</td>
</tr>
</tbody>
</table>

*BMD denotes bone mineral density, and FRAX fracture prevention algorithm.
†Glucocorticoid doses are given in prednisone equivalents.
‡The recommended calcium intake refers to the total daily intake (diet and supplements).
measurement of bone mineral density, and I would also recommend a vertebral morphologic assessment or plain films to look for vertebral fractures. Adequate intake of calcium and vitamin D should be encouraged. Because of her long-term use of glucocorticoids, her age, and the low body-mass index, if prednisone cannot be discontinued (or if she has low bone mineral density or vertebral fractures), she should be advised about therapies to reduce her risk of fracture; bisphosphonates (alendronate, risedronate, and zoledronic acid) and teriparatide are approved by the FDA for these indications and should be continued for as long as the patient requires prednisone. In the absence of data from trials directly comparing the risk of fracture associated with the various therapies, the choice of medication should take into account the cost, the convenience of administration, and the side effects. The more rapid onset of action with zoledronic acid or teriparatide, as compared with oral bisphosphonates, is a potential advantage of these medications, but that advantage must be weighed against the greater costs of those drugs and, in the case of teriparatide, the need for daily injections.

Dr. Weinstein reports holding a patent for in vitro and in vivo models for screening compounds to prevent glucocorticoid-induced bone destruction. No other potential conflicts of interest relevant to this article were reported.

Disclosure forms provided by the author are available with the full text of this article at NEJM.org.

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CLINICAL PRACTICE


46. Weinstein RS, Jilka RJ, Roberson PK, Manolagas SC. Intermittent parathyroid hormone administration counteracts the adverse effects of glucocorticoids on osteoblast and osteocyte viability, bone formation, and strength in mice. Endocrinology 2010;151:2641-9.


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Glucocorticoid dose determines osteocyte cell fate

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ABSTRACT In response to cellular insult, several pathways can be activated, including necrosis, apoptosis, and autophagy. Because glucocorticoids (GCs) have been shown to induce both osteocyte apoptosis and autophagy, we sought to determine whether osteocyte cell fate in the presence of GCs was dose dependent by performing in vivo and in vitro studies. Male Swiss-Webster mice were treated with slow-release prednisolone pellets at 1.4, 2.8, and 5.6 mg/kg/d for 28 d. An osteocyte cell line, MLO-Y4 cells, was treated with various doses of dexamethasone. We found that GC treatments dose dependently decreased activation of antioxidant-, autophagy-, and antiapoptosis-focused RT-PCR gene pathways in mouse cortical bone. The activation of antioxidant genes was correlated with autophagy gene expression after the GC treatments. The presence of osteocyte autophagy, as detected by immunostaining for LC3, increased ~50% at the distal femur cortical bone region but not at trabecular bone region at the 1.4 and 2.8 mg/kg/d GC dose levels. The number of apoptotic osteocytes was increased at the cortical bone region by ~40% initially observed at the 2.8 mg/kg/d dose level. In addition, the presence of the osteocyte autophagy was associated with an increased protein level of cathepsin K in vitro after the GC treatments. In summary, we found that GC treatment dose-dependently decreased antioxidant gene expression, with lower GC doses activating autophagy, whereas a higher dose increased apoptosis. These data suggest that autophagy may provide a mechanism for osteocytes to survive the stress after GC exposure and provide further insight into how GCs alter bone cell fate.—Jia, J., Yao, W., Guan, M., Dai, W., Shahnazari, M., Kar, R., Bonewald, L., Jiang, J. X., Lane, N. E. Glucocorticoid dose determines osteocyte cell fate. FASEB J. 25, 3366–3376 (2011).

Key Words: osteoporosis · apoptosis · autophagy · antioxidant · MLO-Y4 cell · LC3

Glucocorticoids (GCs) are frequently used in clinical medicine to treat noninfectious inflammatory diseases. Epidemiological studies show that 50% of patients with rheumatoid arthritis treated with chronic GCs will have an osteoporotic fracture; baseline data from randomized clinical trials report the prevalence in vertebral fracture is nearly 30% (1–4). Patients treated with GCs may require the treatment for a long period of time, thereby increasing their risk of fractures. Clinical studies of GC-treated subjects observed that the initiation of GC treatment is associated with a change in bone metabolism. In turn, this leads to a rapid reduction in bone mass at sites rich in trabecular bone, e.g., the vertebrae and femur, with incident vertebral fracture risk elevated within 1 yr of initiation of GC treatment (5–7). However, the loss of trabecular mass and architecture does not explain the increase in fracture risk in individuals treated with GCs, because these GC-treated subjects frequently experience fractures with higher bone mineral density values than do women with postmenopausal osteoporosis (6). Therefore, a more comprehensive understanding of the biology of GC-induced bone loss could empower clinicians to effectively prevent and treat this disease.

Osteocytes, the most abundant type of cells in bone, are buried in the bone matrix and are now known to contribute bone mineral homeostasis (8). Osteocytes are connected to one another and to the bone surface. Osteocyte lacunae have been reported to change size in clinical situations when there is a calcium deficiency, including with lactation, GC treatment, hypophosphatemic rickets, and prolonged estrogen deficiency (9–12). The increase in osteocyte lacunae size in these metabolic states may occur because of an insufficient trabecular and endocortical bone surface area for osteoclasts to resorb bone and maintain serum calcium balance when calcium is in high demand (13). For example, in the case of lactation, osteocyte lacunae are enlarged during lactation and return to the normal size postlactation, presumably after the calcium demand from lactation is diminished (10). Osteocytes synthesize and secrete a number of “osteocytic” specific pro-
teins, such as dentin matrix acidic phosphoprotein, matrix extracellular phosphoglycoprotein, and fibroblast growth factor 23, that contribute to the regulation of both calcium and phosphorus metabolism (14–18), and the synthesis of these proteins by the osteocyte has been associated with changes in the perilacunar mineral around the osteocyte. However, it is unclear whether GC would affect osteocyte cell fates and would alter localized perilacunar mineralization changes around the osteocyte and whole bone strength.

Aging and glucocorticoid treatments are associated with accumulations of destroyed proteins, damaged nucleic acids, and accumulated oxygen radicals (19–21). Cells rely on autophagy, the only known intracellular degradative mechanism, to remove the dysfunctional organelles and/or oxidized proteins (22–24). The autophagic process is also activated when the cell is under stress, as an attempt to survive (25, 26). Once the autophagic process is initiated, parts of the cytoplasm and intracellular organelles are sequestered within autophagic vacuoles, which are eventually delivered to lysosomes for bulk degradation (27). Although the process of autophagy can preserve cell viability as a survival strategy, it can also lead to a self-destructive process, resulting in programmed cell death with excessive activation of this self-degrading system (28, 29). Although autophagy may prolong cell survival under stressful conditions, it is an inefficient process, and over time cells accumulate metabolic debris, which results in a decline in both cell and organ functions (30). Endogenous GCs, secreted by the adrenal glands, are essential in the body’s ability to respond to stress. GCs are known to impair the enzymatic antioxidant defenses or directly induce oxidative stress in various tissues (31–36) and are associated with cell fate in a number of disease states. In lymphoid malignancies, Laane et al. (37) reported that dexamethasone (Dex) induced lymphoid cell death through the induction of autophagy before apoptosis. The induction of osteocyte apoptosis was thought to be the primary mechanism for GC-induced osteoporosis and changes in bone quality (38–42). We recently reported that prolonged GC treatment in mice resulted in osteocyte autophagy. Inhibition of autophagy with 3-methyladenine, an inhibitor of endogenous GCs, secreted by the adrenal glands, are essential in the body’s ability to respond to stress. GCs are known to impair the enzymatic antioxidant defenses or directly induce oxidative stress in various tissues (31–36) and are associated with cell fate in a number of disease states. In lymphoid malignancies, Laane et al. (37) reported that dexamethasone (Dex) induced lymphoid cell death through the induction of autophagy before apoptosis. The induction of osteocyte apoptosis was thought to be the primary mechanism for GC-induced osteoporosis and changes in bone quality (38–42). We recently reported that prolonged GC treatment in mice resulted in osteocyte autophagy. Inhibition of autophagy with 3-methyladenine, an inhibitor of endogenous autophagy, led to osteocyte apoptosis (19, 43). Based on these data, we hypothesized that autophagy is one of the pathways in which osteocytes respond to GC exposure. The purpose of this investigation was to further characterize the dose-dependent effects of GC-induced osteocyte cell fates in vivo and in vitro.

MATERIALS AND METHODS

Animals and experimental procedures

Three-month-old male Swiss-Webster mice were obtained from Charles River, Inc. (San Jose, CA, USA). The mice were maintained on commercial rodent chow (22/5 Rodent Diet; Teklad, Madison, WI, USA) with 0.95% calcium and 0.67% phosphate, available ad libitum. Mice were housed in a room that was maintained at 20°C with a 12 h light-dark cycle. The mice were randomly assigned to 4 experimental groups of 6–16 animals/group. Slow-release pellets (Innovative Research of America, Sarasota, FL, USA) of prednisolone (GC) were implanted as follows: group 1, the control group, was implanted with a placebo pellet (PL); group 2 was implanted with a 2.5 mg/60 d slow-release GC pellet, which is equivalent to 1.4 mg/kg/d for 28 d; group 3 was implanted with a 5 mg/60 d slow-release GC pellet, which is equivalent to 2.8 mg/kg/d for 28 d; and group 4 was implanted with a 10 mg/60 d slow-release GC pellet, which is equivalent to 5.6 mg/kg/d for 28 d. At 48 h before the mice were sacrificed, a fluorescent-conjugated monoclonal antibody for the autophagy marker, LC3 (LC3-5F10; 30 µg/mouse; NanoTools, San Diego, CA, USA) was injected into 3 mice in each of the 4 experimental groups to label the autophagic osteocytes. All animals were treated according to the U.S. Department of Agriculture animal care guidelines with the approval of the University of California at Davis Committee on Animal Research.

Biochemical methods

Serum levels for cortisol (R&D Systems, Minneapolis, MN, USA), osteocalcin (Biomedical Technology, Stoughton, MA, USA), and cathepsin K (Alpco, Salem, NH, USA) were measured in duplicate by ELISA, following the manufacturers’ instructions. The within-run variations in our laboratory are between 4 and 6%, and between-run variations are ~5%, which allow us to determine true changes between treatment groups (43–50).

Osteocyte culture and experiments

MLO-Y4 cells were cultured on collagen-coated (rat tail collagen type I, 0.15 mg/ml) surfaces (BD, Franklin Lakes, NJ, USA) and were grown in phenol red-free α-modified essential medium (α-minimal essential medium) supplemented with 2.5% FBS and 2.5% bovine calf serum (Invitrogen, Carlsbad, CA, USA) and incubated in a 5% CO₂ incubator at 37°C as described previously. The cells were treated with Dex (Sigma-Aldrich Corp., St. Louis, MO, USA) at 10−8 to 10−5 M for 24 h (19, 51). For the cell GFP-LC3 transfection experiments, the cells were transfected with the GFP-LC3 vector for 48 h. The cells were then treated with 10−8 to 10−6 M doses of Dex for 24 h, fixed with 4% paraformaldehyde, and examined under an Olympus BX61 motorized reflected fluorescence microscope (Olympus, Tokyo, Japan) with an AMCA filter (excitation, 535 nm; emission, 490 nm) for DAPI and FITC filter (excitation, 480 nm; emission, 535 nm) using SlideBook4.1 software (Intelligent Imaging Innovations, Denver, CO, USA). Autophagic cells were quantified by counting cells exhibiting GFP-LC3 punctate staining.

To evaluate the colocalization of the LC3 and lysosomes, MLY-O4 cells were plated on coverslips in a 12-well plate at 1 × 10⁵ cells/cm². After 24 h of plating, the cells were transfected with 1 µg of GFP-LC3 using 5 µl of lipofectamine in optimum I reduced serum medium (Invitrogen). Both GFP-LC3 and lipofectamine were diluted in optimum I reduced serum medium to two separate tubes to a final volume of 100 µl. After 5 min of incubation, GFP-LC3 and lipofectamine were mixed together in one tube and were incubated for 15 min at room temperature. Then the mixture of DNA-lipofectamine was added to cells dropwise in 1 ml of...
optimum I medium, and the plates were swirled gently and incubated in a 37°C incubator. After 3 h of transfection, medium was replaced with full-growth medium. After 24 h of transfection, cells were treated with 10⁻⁸ to 10⁻⁶ M Dex in 1% serum (FBS + bovine calf serum) phenol red-free medium for 24 h. Serum starvation was used as a positive control for autophagy (52, 53). After 24 h of transfection, cells were treated with 10⁻⁸ to 10⁻⁶ M LysoSensor Blue (Invitrogen) that was added directly to the treatment medium. The medium was removed after 30 min of incubation with LysoSensor Blue. Cells were washed once with fresh medium, and live cell images were taken to visualize the colocalization of GFP-LC3 puncta and the lysosomes.

**Real-time RT-PCR**

Total RNA was obtained from the tibiae or from MLO-Y4 cell cultures. For the tibiae, joint and bone marrow was removed, and total RNA was isolated using a modified two-step purification protocol with homogenization (PRO250 Homogenizer, 10 mm x 105-mm generator; PRO Scientific Inc., Oxford, CT, USA) in TRIzol (Invitrogen) followed by purification over an RNeasy column (Qiagen, Valencia, CA, USA). The antioxidation autophagy and antiapoptosis focus RT-PCR gene pathway arrays were purchased from SABiosciences (Frederick, MD, USA). Each pathway gene array has a preselected panel of 96 genes, which are related to antioxidant, autophagy, or apoptosis pathways, housekeeping genes, and no primer or cDNA controls. Detailed gene information can be found online (http://www.sabiosciences.com/RTPCR.php). We excluded genes that had values of Ct ≥ 35 because low expression levels can result in large fold changes, but the differences were not significant. After the exclusions, we reported 80 test genes in the autophagy RT-PCR gene array (see Fig. 2) and genes that were significantly different from PL after GC treatments for antioxidant and antiapoptosis (Tables 1 and 2).

**Immunohistochemistry**

The right distal femurs were decalcified in 10% EDTA for 2 wk and embedded in paraffin. Sections (4 μm) were prepared for immunohistochemistry using primary antibodies against the autophagy protein LC3-phosphatidylethanolamine conjugate antibody (LC3B antibody; Cell Signaling Technology, Danvers, MA, USA). LC3 detection was performed using a Vectastain ABC system (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with methyl green. Apoptosis was determined using an In Situ Fluorescein Cell Death Detection Kit (Roche, Indianapolis, IN, USA) following the manufacturer’s instructions. Results are presented as the percentage of the positive stained osteocytes/trabecular or cortical bone volume 0.5 to 3 mm distal to the growth plate at the distal femurs.

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All genes expressed significantly from PL (P<0.05).
A Bioquant imaging analyzing system (Bioquant, Nashville, TN, USA) was used for the measurements.

**Western blot**

The cells were lysed in RIPA buffer with homogenization. The bone lysates or cell lysates were resolved on SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies that included β-actin (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LC3 (1:1000; Cell Signaling Technology) or anti-cathepsin K (Cell Signaling Technology) followed by species-specific horseradish peroxidase secondary antibody. Anti-LC3 antibody recognizes both LC3-I, which is cytoplasmic and LC3-II that binds to the autophagic membranes. Immunoreactive materials were detected by chemiluminescence (Pierce Laboratories, Rockford, IL, USA), imaged with a Kodak Gel Logic 100 Digital Imaging System, and quantitated by Kodak 1D 3.6 image analysis software (Eastman Kodak, Rochester, NY, USA).

**Statistical analysis**

Group means and sds were calculated for all outcome variables. The nonparametric Kruskal-Wallis test was used to determine the differences between the groups. The 2-tailed Spearman correlation test was used to determine the association between the activation (fold changes from PL) for all genes in the antioxidation, autophagy, or antiapoptosis gene arrays (version 12; SPSS Inc., Chicago, IL, USA).

**RESULTS**

**Dose-dependent effects of GC on serum chemistry values, bone turnover, and strength**

The serum cortisol level was not changed at the 1.4 mg/kg/d GC dose level but increased by >200% at the 2.8 mg/kg/d dose level and 600% at the 5.6 mg/kg/d dose level after 28 d of treatment. Bone formation, measured by the serum osteocalcin level or surface-based mineralizing surface at the distal femurs, decreased nonsignificantly by ∼10–40% at a 1.4 mg/kg/d GC dose level and 40–60% at the two higher GC dose levels (P<0.05). The maximum vertebral compressive stress was significantly lowered at the 5.6 mg/kg/d GC dose level compared with that for PL (Fig. 1).

**Excess GC induced an osteocyte autophagy in vivo**

To evaluate the dynamic and integrative nature of GCs on osteocyte stress response and cell fate, we obtained RNA from the long bones (tibiae, n=6/group) of mice treated with PL or 3 doses of GCs for 28 d and performed real time RT-PCR gene arrays for antioxidants, autophagy, and antiapoptosis. We found that GC dose-dependently decreased the activation of oxidative stress responsive gene expressions (Table 1). For exam-
ple, the expression of superoxide dismutase (Sod) 1, soluble increased ~12-fold at the 1.4 mg/kg/d dose level and increased 6- and 9-fold, respectively, at the 2.8 and 5.6 mg/kg/d GC dose levels, Sod2 (mitochondrial) increased 15 fold at the 1.4 mg/kg/d dose level and increased 6-fold at the 2.8 and 5.6 mg/kg/d GC dose levels; and Sod3 (extracellular) increased 30-fold at the 1.4 mg/kg/d GC dose level and increased 23- and 11-fold, respectively, at the 2.8 and 5.6 mg/kg/d GC dose levels. GC at the 1.4 mg/kg/d dose level activated a number of genes that are associated with autophagy by an average increase of 20- to 30-fold from the PL treatment and by ~10-fold at the 2.8 mg/kg/d dose level and was similar to PL (1-fold) at the 5.6 mg/kg/d dose level (Fig. 2A). Activation of the antioxidant gene pathway was positively correlated with activation of the autophagic gene array, especially at the 1.4 mg/kg/d GC dose level (Fig. 2B). For example, the expressions of autophagy-related proteins (Atg) 12 and 7, both of which are essential for the formation of double-membrane vesicles and autophagosomes, increased 23- and 42-fold, respectively, at the 1.4 mg/kg/d GC dose level and increased 14- and 11-fold, respectively, at the 2.8 mg/kg/d GC dose level but did not differ from PL at the 5.6 mg/kg/d GC dose level. In contrast, the expression of genes associated with apoptosis increased by ~5-fold from PL at the 1.4 mg/kg/d GC group but were significantly decreased by an average of ~10-fold in the 2.8 mg/kg/d GC dose group and ~2 to ~30-fold in the 5.6 mg/kg/d GC dose group (Table 2). For example, the expression of B-cell leukemia/lymphoma 2 (Bcl2) was not changed at the 1.4 mg/kg/d GC dose level but decreased 1.6-fold at the 2.8 mg/kg/d GC dose and decreased 9-fold at the 5.6 mg/kg/d GC dose level; the expression of IL-10 increased 3-fold at the 1.4 mg/kg/d GC dose level but decreased 6-fold at the 2.8 mg/kg/d GC dose and decreased 35-fold at the 5.6 mg/kg/d GC dose level. There was no significant correlation between the activation of antioxidant and antiapoptosis genes. In addition, there was no significant correlation between activation of autophagy and antiapoptosis genes (data not shown).

GC treatment at all three doses did not increase the percentage of LC3\textsuperscript{\textdagger} osteocytes in the trabecular bone region (Fig. 3A). However, GC treatment increased autophagic osteocytes in the cortical bone region of the distal femurs at the 1.4 and 2.8 mg/kg/d (Fig. 3B) measured by immunohistochemical staining against LC3 (Fig. 3C) or by injecting the fluorescent-conjugated LC3 antibody (LC3-5F10) into the mice (Fig. 3D). GC exposure did not significantly change osteocyte apoptosis in the trabecular bone region of the distal femur at either dose level but increased apoptotic osteocytes significantly in the cortical bone region at the 2.8 and 5.6 mg/kg/d GC dose levels (Fig. 4).

**GC increased osteocyte autophagy and cathepsin K secretion in vitro**

To determine the dose-dependent effect of GCs on autophagy in vitro, osteocytic MLO-Y4 cells were transected by GFP-LC3 for 48 h and then were treated with various concentrations of Dex (10\textsuperscript{−8} to 10\textsuperscript{−6} M) for 24 h. GFP-LC3 was diffusely distributed in the cytoplasm in the absence of Dex (control). In contrast, treatment with Dex increased the number of GFP-LC3 puncta, indicating that LC3 was recruited and aggregated in the cytoplasm (Fig. 5A). Increased colocalization of GFP-LC3 was seen, and lysosomes were observed accumulating in the cytoplasm in Dex-treated cells, especially in 10\textsuperscript{−7} to 10\textsuperscript{−6} M Dex (Fig. 5B). Western blot confirmed the increase in GFP-LC3-II levels after Dex treatment at all doses starting from the lowest dose (10\textsuperscript{−8} M), and the maximal response was seen at 10\textsuperscript{−7} M Dex (Fig. 5C, D). The enzyme for matrix metabolism, cathepsin K, was increased in the MLO-Y4 cells (Fig. 5C, D).
D) treated with Dex, as well as in the culture medium, which was most significant at the 10^{-2} M dose level (Fig. 5E). Interestingly, when the MLO-Y4 cells were treated with Dex (10^{-8} to 10^{-5} M) and evaluated for the antioxidant gene expressions, we did not find that Dex treatment in vitro activated the antioxidant pathway significantly (data not shown).

DISCUSSION

After 28 d of low-dose GC treatment in mice, there was significant activation of the autophagy pathway and increased osteocyte autophagy in the cortical bone region of the distal femur that were significantly different from placebo treatment. A decreased antiapoptosis response and osteocyte apoptosis were observed at the cortical bone region after the higher-dose GC treatment.

Weinstein et al. (38, 42) reported that reduced bone formation in GC-treated mice was associated with increased apoptosis of osteoblasts and osteocytes. Plotkin et al. (54) also reported in vitro evidence of apoptosis of osteocytes exposed to GCs using 3 separate assays (trypan blue exclusion, nuclear morphology, and annexin V/propidium iodine ratios by FACS analysis) to accurately confirm that there was osteocyte apoptosis present (54). However, these investigations were performed before reagents were available to assess the
presence of autophagy. Our study evaluated the dose response of osteocytes to GCs and found that osteocyte apoptosis increased after a higher dose of GC at the cortical bone region. Our results (43) are similar to those reported by Weinstein et al. (38, 42), who also reported that in male mice treated with higher doses of GCs nearly 20% of the osteocytes at the cortical bone region of the tibiae had undergone apoptosis. These investigators also demonstrated that the increased osteocyte apoptosis was significantly associated with a reduction in whole bone strength.

In addition to apoptosis, we found that autophagic osteocytes were observed in the cortical bone region of the lumbar vertebral bodies in mice that had received 56 d of GC treatment (19). The autophagy pathway is one of the most important biological processes that

**Figure 3.** Distal femurs from PL- or GC-treated mice were embedded in paraffin at d 28. A, B) Numbers of LC3$^+$ osteocytes present in a defined trabecular bone area (A) or cortical bone area (B) were calculated. C) Immunohistochemical staining was performed using an anti-LC3 antibody (LC3$^+$ cells were stained in red; red arrows). D) Autophagic osteocytes were also quantitated by injecting fluorescent-conjugated monoclonal antibody for LC3 (LC3–5F10; green arrows) into mice at 48 h before sacrifice. n = 8/group, 3 sections/animal were analyzed. *P < 0.05 vs. PL.

**Figure 4.** Presence of apoptosis in the trabecular and cortical bone of the right distal femurs in mice treated with PL or GC. Apoptosis was determined by *in situ* fluorescence TUNEL staining. Apoptotic osteocytes (A, yellow arrows) in both the trabecular bone (B) and cortical bone (C) regions were measured. n = 8/group. *P < 0.05 vs. PL.
enables the cells to survive stress and starvation and helps to maintain cellular homeostasis by degrading damaged organelles (22, 25, 28, 55). The hallmark of autophagy is the formation of autophagosomes, also known as autophagic vacuoles that are lined by two membranes with the recruitment of LC3-II to the autophagosomal membranes, a characteristic for autophagosomes, whereas LC3-I remains in the cytoplasm (56). Aging is associated with an increase in the intracellular overabundance of oxidative products, including reactive oxygen species (e.g., oxygen ions and free radicals) and increased defense mechanisms for oxidative stress such as glutathione (GSH), thioredoxin, and GSH peroxidase, which convert the peroxides to harmless materials, and the expressions of superoxide dismutase, the antioxidant enzymes in bone tissues (57–62). Likewise, GCs also activate the oxidative pathway and accelerate the aging process in bone tissue (34, 36, 63–65). GC treatment in vitro did not activate the antioxidative pathway but significantly increased the overall stress level in vivo in mice by increasing the systemic circulating cortisol levels. On the basis of our data, it appears that antioxidative responses were provoked by GC-induced stress, and the osteocytes may have responded with autophagy. An increased LC3-II protein level and autophagic osteocytes were observed primarily in the cortical bone regions. This autophagic attempt to “survive” the insult from the GC exposure may be successful if the dose is low. However, with the increasing doses of GCs, the cells’ antioxidative defenses were overwhelmed, and the ability of the osteocyte to survive was reduced, which could then direct the cells to apoptosis. Other investigators have suggested that osteocyte cell fate may be related to the dose of GC (66, 67). Our data clearly demonstrate the dynamics of the osteocyte cell fate with different GC doses within the
cortical bone. The lack of osteocyte response in the trabecular bone regions may due to the fact that trabecular and cortical bone have different remodeling rates, and these two bone compartments have different responses to various stimuli, such as immobilization and exercise (68–72). The life spans of trabecular bone osteocytes and cortical bone osteocytes are also different: cortical bone osteocytes live significantly longer, and this may help to explain the differing response to GC treatment (73). The activation of the autophagic gene pathway and osteocyte autophagy was significantly increased when the cells or mice were treated with a low dose of GC. The higher doses of GC activated the gene pathway for apoptosis and osteocyte apoptosis was then significantly increased.

During the initial autophagic process, cells may be able to remain viable during periods of metabolic stress (26, 74, 75). However, in the later stage of autophagy, the digestion of autophagic materials involves the fusion of autophagosomes with lysosomes to form autolysosomes or the degradative autophagic vacuole. This catabolic process releases cathepsins and other hydrolases from the lysosomal lumen into the cytosol or, if the autolysosome fuses with the plasma membrane, into the surrounding tissue (76, 77). This extrusion of the contents of the phagolysosomes is in some degree similar to the virus shedding from a lymphocyte (78, 79). We observed increased colocalization of GFP-LC3 and lysosomes in osteocytes treated with Dex as well as increased cathepsin K levels both in the osteocytes and in the circulation. Our findings suggest that GC increased the release of the cathepsins and other hydrolases secreted into the perilacunar bone matrix. Over time, the localized release of cathepsin K with other hydrolases from the lysosome may change the perilacunar matrix composition, and over time it may also change the localized material properties of the bone. Therefore, osteocyte cell fate through autophagy may have a different effect on the localized perilacunar composition and bone quality than death by apoptosis. Histological sections of osteocyte death by autophagy generally have only empty lacunae. However, the apoptotic cell may send signals to the bone cell surface to activate osteoclast-mediated bone remodeling.

In summary, GC treatment dose dependently decreased antioxidant, autophagy, and antiapoptosis responses. A low GC dose induced osteocyte autophagy, and higher GC doses induced osteocyte apoptosis, principally in the cortical bone. Although GC-induced autophagy has been reported, the dose response of autophagy and apoptosis within the cortical bone is novel, and additional studies are now warranted to elucidate these findings and determine whether interventions directed to alter autophagy or the inhibition of cathepsin K may be a useful approach to reduce GC-induced bone fragility.

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