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Aging and Bone

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Introduction

This chapter will provide a general overview of the aging process followed by the potential effect that aging may have in bone biology. Three important aspects will be considered: decreased number of osteoblasts, increasing adipogenesis, and significant osteoblast/osteocytes apoptosis during the aging process in bone.

Aging—A Definition

In clinical medicine, aging may be best defined by the words Supreme Court Justice Potter Stewart used to describe pornography: “I know it when I see it” (JACOBELLIS v. OHIO, 378 U.S. 184 (1964)). Every physician has witnessed the effects of aging, both in individual patients followed over a period of time and in their collective patient population base. Nevertheless, a number of definitions of aging have been offered, and these have been elegantly summarized in Carrington’s review entitled: “Aging bone and cartilage: cross-cutting issues” (1). In the clinical care setting, aging is generally associated with the loss of a wide range of physiological processes (1) (Table 2.1). These include decreased fertility (2), decreased resilience in response to environmental stressors such as infections, surgery, or physical attack (3), and decreased physical strength. Inevitably, aging is also associated with end of life and death (2,4).

At the cellular level, several fundamental and interconnected processes accompany aging *in vitro* and *in vivo* (1) (Table 2.1). Hayflick first

defined the process of cellular senescence, demonstrating that “normal” diploid cells can undergo a limited number of cell doublings *in vitro* (5). These pioneering observations set the framework within which much of our understanding of aging is now predicated. Consequently, the Hayflick model for replicative senescence has been employed in biogerontology research to unravel mechanisms of age-related cellular defects (6). Using this model, several investigators have reported an inverse relationship between the donor age and maximal proliferative potential of the cells *in vitro* (7). The Kassem laboratory has characterized a Hayflick model for replicative senescence of human osteoblasts (8,9). During continuous culture *in vitro*, human osteoblasts exhibited typical senescence-related phenotype including senescent-associated decrease in osteoblast marker production (alkaline phosphatase [AP], osteocalcin, collagen type I), decrease in mean telomere fragment length, and increase in the number of senescence-associated β -galactosidase (SA β -gal) positive cells (10,11). With each progressive mitotic cycle, each telomere, located at the ends of individual chromosomes, decreases in length; and this has been associated with senescence (12). It has been postulated that the telomere length acts as a “mitotic clock,” and it is known that the overexpression of telomerase, the enzyme responsible for maintaining telomere length, leads to cell immortalization (13). The Kassem laboratory has further examined the effect of donor age on the maximal proliferative potential of bone marrow stem cells (BMSC). An age-related decline in the maximal life span from

TABLE 2.1. Macro- and Micro-Manifestations of Aging (1)

Clinical	Cellular
Decreased fertility	Increased senescence
Decreased physical strength and/or mental acuity	Increased oxidative damage
Decreased resilience and stress response	Altered apoptosis or programmed cell death
Increased mortality	Increased AGEs

AGEs, advanced glycation end products.

41 ± 10 population doublings (PD) in young donors to 24 ± 11 PD in elderly donors was observed. These results thus suggest that human aging is associated with reduced maximal proliferation potential of BMSC. However, the proliferation potential of aged BMSC is still very high; thus, the contribution of the observed *in vitro* age-related decreased maximal proliferative potential of osteoblasts to age-related decreased bone formation *in vivo* is not clear.

It seems that the Hayflick model of replicative senescence is useful for studying some aspects of the aging process, and it has been employed extensively in biogerontology research to investigate changes at both the genetic and epigenetic levels. Somatic mutations increase (1,4) and DNA methylation and histone acetylation patterns alter (14), leading to altered gene expression profiles and differentiation function. Other DNA changes accompany senescence in somatic cells. Reduced oxidative phosphorylation in the mitochondria of senescent cells leads to reduced energy availability and metabolic function (15). In parallel, mitochondrial dysfunction results in elevated levels of free radicals in the form of reactive oxygen species (ROS), and this has long been postulated as a causative factor in cellular senescence and aging (16,17). The generation of ROS has been associated with altered signal transduction responses to growth factors (18). In addition, elevated levels of ROS cause increased expression of pro-apoptotic or programmed cell death regulators within differentiated cell types (19). These changes may cause the cells to be more sensitive to exogenous stress to the endoplasmic reticulum and other subcellular organelles that lead to subsequent apoptosis (20). An additional biochemical event associated with aging is the formation of advanced

glycation end products (AGEs), formed through the non-enzymatic interaction of glucose with amino groups, known as the Maillard reaction (21). Glycated forms of collagen and other proteins accumulate in tissues with low levels of cellular turnover, such as bone (21). Whereas AGEs have been well established as the target for diagnostic and prognostic clinical testing in diabetes, they may have an equivalent potential as biomarkers for aging. Likewise, receptors for AGEs, also known as RAGEs, may be responsible for alterations associated with aging and chronic disease (22,23). The gene for one of these receptors lies within the major histocompatibility locus and has been associated with the inflammatory response (22); its activation induces the NF κ B transcription factor responsible for regulating the expression of pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)- α (24). The accumulated impact of each of these biochemical events results in the cellular changes characterized as “aging.”

Aging and Bone Physiology

Bone development is a dynamic process that begins in the embryo and extends throughout the lifetime of the individual (Figure 2.1). The osteogenic process in the embryo provides a paradigm for our understanding of the physiology of bone in the adult and the consequences of aging. The condensation of mesenchymal stem cells (MSCs) give rise to intramembranous and endochondral bone formation in the embryo (25). In the former case, the progenitor/stem cells differentiate directly into osteoblasts, whereas in the latter, the cells form chondrocytes first, which subsequently mineralize their extracellular matrix and become osteoblasts (25). These events are closely linked with angiogenesis and the secretion of angiogenic factors such as vascular endothelial growth factor (VEGF) in a coordinated and time dependent manner (25). Bone accumulation reflects a life-long balance or homeostasis between bone formation by osteoblasts and bone resorption by osteoclasts. As will be discussed further, multiple hormonal, cytokine, biomechanical, nutritional, and environmental factors influence these events. Shortly after birth, adipogenesis, or the formation

Events in the Progression of Bone Development

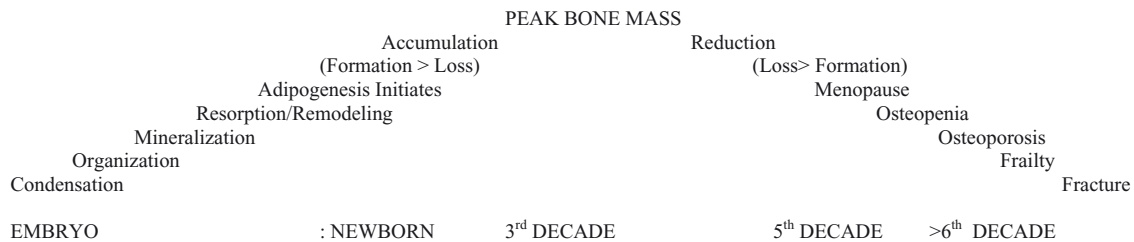


FIGURE 2.1. Events in the progression of bone development.

of fat cells, occurs within the marrow cavity of the distal phalanges and tarsal bones, and this advances proximally towards the skeleton of the thorax throughout life. These events are regulated, in part, by the body's hematopoietic demands. The human body reaches its peak bone mass during the third decade of life. After this point, bone mass decreases by as much as 1–2% per year. In women, the rate of loss is briefly accelerated during the years surrounding menopause. This puts women at greater risk of osteopenia and osteoporosis at a younger age than men. Extreme cases of osteoporosis are associated with frailty in the elderly and contribute to the high incidence of fracture in the aged population.

MSCs

The mesenchymal cells in the developing embryo give rise to the “anlagen,” or condensation that ultimately forms bone. Alexander Friedenstein and his colleagues performed pioneering studies in the 1960s and 1970s identifying a subpopulation of bone marrow fibroblasts with the ability to differentiate along multiple lineage pathways, including adipocyte, chondrocyte, and osteoblast (26). Over the years, these cells have been identified by many different names, including fibroblast stem cells (27), mechanocytes (26), nurse cells (28), reticuloendothelial cells (28,29), stromal cells (30,31), stromal stem cells (32,33), and Westin-Bainton cells (29). Now recognized as mesenchymal stem cells or stromal cells (34), it has been determined that MSCs continue to

reside in the bone marrow microenvironment throughout life. Studies have documented that cloned MSCs retain their multipotent differentiation characteristics, consistent with the identification of a true “stem cell” (35–37). These studies have led to a new appreciation of the existence of “adult” or “somatic” stem cells in multiple tissues of the body, terms that were formerly restricted to the progenitors of the hematopoietic lineages, (i.e., hematopoietic stem cells [HSCs]). A simple assay used to quantify the number of MSCs is based on their ability to form colonies when cultured *in vitro*, known as colony forming unit-fibroblast (CFU-F). Nucleated bone marrow cells are plated at limiting dilutions on a plastic surface and the number of cell “colonies” (defined as groups of more than 50 cells) with fibroblast morphology are determined after a 1- to 3-week expansion period. Based on this approach, studies have found that the number of murine bone marrow MSCs decreases with advancing age (38). Likewise, in humans, the number of CFU-F decreases during the first decade of life (39). In the later decades of life, between the ages of 20 to 70, the number of CFU-F remains relatively constant (40,41). In conclusion, human studies show that with aging there is maintenance of CFU-F cell population size in the bone marrow, and that the observed decline in the number of CFU-F in early adulthood may represent changes in the skeletal dynamics from a modeling mode characteristic of skeletal growth and consolidation to a remodeling dynamic characteristic of the adult skeleton. This may also explain why experiments employing rodents showed a decline in the

CFU-F number as they continue to grow throughout their lifespan.

The Inverse Relationship Between Adipocytes and Osteoblasts

Clinical epidemiological observations have established that a relationship exists between adipocytes and osteoblast functions in the bone marrow microenvironment. Autopsy studies of large patient population bases of varying ages demonstrated that the percentage of the marrow cavity occupied by fat increased with advancing age (42–46). Adipose accumulation was observed in the femur, iliac crest, and vertebral bodies. More recent, non-invasive studies using magnetic resonance imaging (MRI) have further documented the age-dependent increase in marrow fat (47).

Work by Meunier et al. (48) in the early 1970s extended the initial pathological studies. Using bone marrow biopsies, they were able to draw a correlation between osteoporosis and the degree of adipogenesis in the iliac crest bone marrow cavity in a cohort of 84 subjects (48). In the early 1990s, Beresford and colleagues performed pivotal studies regarding the differentiation of MSCs that provide a mechanistic understanding of these clinical observations (49). They observed that cultures of bone marrow stromal cells could select the adipogenic or osteoblastic lineage pathways equally under controlled culture conditions. If, however, they delayed the addition of glucocorticoid or vitamin D3, they were able to promote osteoblast or adipocyte differentiation, respectively (49). They concluded that the MSC response to nuclear hormone receptor ligands could regulate an inverse relationship between the number of adipocytes and osteoblasts in bone marrow (49). Other laboratories later confirmed these important findings (50). It is now recognized that a wide range of exogenous and endogenous factors can regulate MSC adipogenesis and osteogenesis in an inverse or reciprocal manner (Table 2.2). The levels of such factors may change with aging. Recent work by the Kassem laboratory has demonstrated that sera from elderly females are less able to support osteoblastic function in human

TABLE 2.2. Pathways Regulating Bone Marrow MSC Adipogenic and/or Osteogenic Differentiation (52)

Nuclear hormone receptors	Transmembrane signal transduction pathways	Adipocyte-derived adipokines and factors
Vitamin D3	BMP	Adiponectin
Estrogen/Androgen	Insulin	Angiotensin
Glucocorticoids	Parathyroid hormone	Free fatty acids
LXR	TGF- β	Leptin
PPAR	Wnt Signaling	Oxidized LDLs

MSC, mesenchymal stem cell; BMP, bone morphogenetic protein; LXR, liver X receptor; TGF, transforming growth factor; PPAR, peroxisome proliferator-activated receptor; LDL, low-density lipoproteins.

MSCs as compared to that from younger females (51). In contrast, both sera were equally effective in supporting adipocyte differentiation (51). The specific serum components responsible for this remain to be determined.

Biochemical Signaling Pathways

Nuclear hormone receptors are a large family of transcription factors that control a broad range of physiological and metabolic responses. These proteins respond to small lipophilic ligands, which move easily across cell membranes as well as between cells and organs. These lipophilic activators range from fatty acids to steroids, making the nuclear hormone receptors important targets for therapeutic intervention in metabolic disorders (53,54).

The peroxisome proliferator-activated receptor gamma (PPAR γ) is activated by fatty acids derived from dietary and metabolic sources, and is the target of the anti-diabetic thiazolidinedione class of insulin-sensitizing drugs such as rosiglitazone and pioglitazone. PPAR γ is essential for the development of adipose cells, including the adipose depots of the bone marrow (55). *In vitro* studies using bone marrow-derived MSCs find that PPAR γ -mediated induction of adipogenesis inhibits osteoblastic bone formation (49,50). The reciprocal relationship between PPAR γ activity and osteogenesis is particularly evident with increased age (56,57). Recent evidence indicates that the use of thiazolidinediones in older diabetic adults may

be associated with bone loss in women (58). These studies indicate that therapeutic approaches to the treatment of diabetes that target PPAR γ may lead to enhanced bone loss in women at risk for osteoporosis.

The glucocorticoid receptor is another nuclear hormone receptor whose activation has important therapeutic implications. Glucocorticoids are widely used because of their anti-inflammatory effects (reviewed in 59). The side effects associated with long-term use of glucocorticoids includes increased fat accumulation and osteoporosis. *In vitro* studies show that dexamethasone treatment of bone marrow-derived mesenchymal cells leads to increased expression of genes required for adipogenesis (60). These changes are associated with decreased expression of genes that regulate osteoblast formation, suggesting glucocorticoids stimulate production of bone marrow-derived adipocytes at the expense of bone formation. The effects of glucocorticoid receptor activation are particularly problematic in the aging population, which is associated with decreased osteoblast formation (61).

Nuclear hormone receptors are closely linked with transmembrane signaling via the Wnt/ β -catenin signaling pathway. Wnt pathways are important regulators of developmental and endocrine functions. Interaction between nuclear receptors and the Wnt pathway plays a prominent role in bone and adipocyte development. Activation of Wnt signaling blocks the formation of adipocytes by inhibiting the expression of PPAR γ and C/EBP α (62,63). Human studies of mutant forms of the Wnt co-receptor, the low-density lipoprotein (LDL)-related protein 5 (LRP5), demonstrate the importance of Wnt signaling in bone formation. Loss of LRP5 function is associated with decreased bone mass (64) whereas gain-of-function mutations in LRP5 lead to increased bone mass (65). *In vitro* studies of MSCs attribute Wnt-dependent stimulation of osteogenesis to Wnt10b (66), a Wnt signaling protein found in stromal vascular cells, but not adipocytes (63).

The bone morphogenetic proteins (BMP) belong to the transforming growth factor beta (TGF- β) family and are important determinants of bone and fat formation. Recent studies of human bone

marrow mesenchymal cells indicate BMP and Wnt signaling cooperate in regulating inhibition of adipocyte development (67). In particular, BMP signaling regulates expression of Wnt10b and LRP5, both components of the Wnt pathway involved in inhibiting adipocyte formation.

Adipocytes secrete a number of proteins (“adipokines”) that function as hormones through an endocrine pathway. Leptin is a 16-kDa peptide hormone that binds to the leptin receptor, a member of the cytokine receptor signaling pathway (68). Originally identified as a satiety factor, leptin’s role has expanded to include a range of effects, including the regulation of bone formation. Murine studies indicate that age-related loss of bone strength is accompanied by decreased serum leptin levels (69). Studies of elderly men show that leptin exerts a modest effect on bone strength independent of fat mass (70). Further studies demonstrate that MSCs exhibit high-affinity leptin binding when undergoing either adipogenesis or osteogenesis (71). Leptin binding was decreased in mesenchymal cells derived from post-menopausal osteoporotic donors, supporting a role for leptin in determining bone strength in an elderly population.

Adiponectin is another adipocyte-secreted protein that links body weight with regulation of bone mass. Adiponectin is well-described as being secreted by white adipose tissue and having a positive effect on insulin sensitivity. Recent studies show that bone marrow-derived mesenchymal cells contain adiponectin receptors and also produce adiponectin (72,73). The *in vitro* evidence suggests a complex role for adiponectin in regulating bone density. Adiponectin may act directly on bone via endocrine or autocrine pathways and indirectly via improvement of insulin sensitivity.

Resistin, a newly discovered adipokine associated with insulin resistance (74), is also expressed in bone marrow-derived mesenchymal cells (75). Resistin levels are inversely related to bone density (76) in aging men, suggesting a role for resistin in determining bone formation. Although the mechanism of action of these adipokines is not well understood, the relationship between bone and fat formation makes these proteins an important target for therapeutic intervention.

Why Fat?

The role of adipocytes in the bone marrow cavity remains an area of active investigation and speculation (77). A number of teleological hypotheses have been posed:

- That adipocytes fill up space in the marrow cavity that is not required for hematopoiesis. The marrow cavity occupies a greater volume of the adult organism relative to that of a newborn or child. Consequently, less than 100% of the volume may be required at any given time for blood cell production (passive role).
- That adipocytes in the marrow contribute to the overall synthesis, processing, and storage of lipids and triglycerides (active role).
- That adipocytes in the marrow serve as an energy reserve for local or systemic events requiring a rapid metabolic response (active role).
- That adipocytes retain functions associated with other MSC lineages, such as HSC support, through the release of regulatory cytokines and the surface expression of HSC adhesion factors, and/or osteogenesis and mineralization (active role).
- That adipocytes provide bone with mechanical advantages to withstand stresses associated with physical activity (active role).

Which Fat?

The bone marrow is just one of many adipose depots in the body (Table 2.3). Each serves a different function and has greatest importance at specific human developmental stages. Brown

adipose tissue (BAT) acts as a non-shivering heat source, and is located around vital organs such as the heart, carotid arteries, kidneys, and gonads. During the critical period following birth, BAT provides neonatal humans with a survival advantage, allowing them to maintain their core body temperature with a minimum expenditure of energy. Later in life, human BAT stores disappear; however, this is not the case in small rodents or hibernating mammals. Changes in ambient temperature and daylight cycles signal the BAT stores in these animals to increase in size and activity. The BAT provides the necessary energy and heat to allow these animals to survive the winter without significant loss of body mass or function. Bone marrow adipose tissue displays some features in common with BAT. The Nobel Laureate, Charles Huggins, correlated the degree of bone marrow adiposity with the core temperature of the marrow cavity. He found that the femur and ulna (lower core temperatures) contained more marrow fat than the vertebra and ribs (higher core temperatures) (78–80). Further independent studies have confirmed these initial findings (81,82). In the armadillo, which has bony plates exposed close to the skin's surface, the marrow cavity transitions between a red (hematopoietic) and yellow (fatty) phenotype in accordance with the season and ambient temperature (82). Comparable manipulation of the marrow fat can be achieved using hematopoietic stressors or stimuli. Under conditions of anemia, owing to exposure to phenylhydrazine, prolonged hypoxia, or in response to sickle cell disease, adiposity within the marrow cavity is reduced (83–88). Under conditions of artificial polycythemia (hypertransfusion), in contrast, marrow adiposity is increased (89).

Bone marrow adipose tissue displays features in common with white adipose tissue (WAT) as well. In some species, such as rabbit, bone marrow fat plays an active role in clearing chylomicrons and triglycerides from the circulation (90,91). Under conditions of extreme starvation or anorexia, bone marrow adipose depots are depleted to an extent equivalent to WAT (92). It remains to be determined if bone marrow adipose tissue provides any weight-bearing advantage to bone from a biomechanical/bioengineering perspective.

TABLE 2.3. Adipose Tissue Depots in Man (93)

Type of adipose tissue depot	Function
Brown	Non-shivering thermogenesis
Bone marrow	Multiple—hematopoietic, energy and lipid metabolism, other?
Mammary	Lactation support
Mechanical	Weight-bearing stress protection
White	Energy reservoir

What Makes It Bone Versus Fat?

Genetic factors exert considerable influence over the physiology and pathology of MSC differentiation and bone formation or loss. Specific genes have been identified that are associated with exceptionally strong or weak bone phenotypes. An example is LRP5, which functions as a receptor for the Wnt signal transduction pathway. In families with a dominant negative mutation in LRP5, inheritance of the gene leads to a condition known as osteoporosis-pseudoglioma, associated with defective bone formation (64). Likewise, in families with a constitutively active mutation in LRP5, inheritance gives subjects a bone phenotype that appears to be impervious to fracture (65,94). These clinical findings are consistent with *in vitro* and *in vivo* murine studies. Activation of Wnt signaling transduction inhibits the adipogenic pathway in cell models (62,63). When transgenic mice over-express Wnt10b under the control of an adipocyte-specific promoter, their bone marrow lacks adipocytes and displays increased evidence of osteoblast activity (66). At a broader level, genetic factors associated with ethnicity influence bone physiology. For example, the risk of osteoporosis is greater in Caucasian and Asian women as compared to those of African-American origins; however, the genetic basis for this remains an area of active investigation. Nevertheless, there is little evidence that this phenomenon is caused by bone instead of fat formation.

Epigenetic factors exert a level of influence comparable to genetic factors. Physical activity has a direct relationship to bone mass and bone health. In industrialized societies, even “healthy” individuals spend less time each day in physical activity as they enter the work force. An individual’s level of high impact exercise correlates with increased bone formation and bone strength. Weight-bearing activities, such as gymnastics and high-impact exercise, enhance bone metabolism and remodeling. In contrast, enforced bed rest is associated with a reduction in bone mass and bone strength. Patients with chronic illness who are bed-ridden, a condition more frequently observed in aged populations, are therefore at increased risk of osteoporotic changes. With prolonged space flight, physicians and investigators

have determined that weightlessness is detrimental to osteogenesis. The net bone loss may reflect both osteoblastic bone formation and/or enhanced osteoclastic bone resorption.

The physical environment also determines an individual’s sun exposure and, consequently, the biosynthesis of vitamin D and its active metabolites. These nuclear hormone receptor ligands play a critical role in regulating calcium metabolism in the bone, intestine, and kidney, with subsequent consequences on parathyroid hormone action. Whether an individual works indoors or outdoors will have a direct bearing on vitamin D pathways. In many elderly, the hours spent outdoors decrease as fitness declines, resulting in low or inadequate levels of vitamin D receptor ligands.

Nutrition has been a target to offset the risk of vitamin D deficiency. We now fortify milk products with Vitamin D3 to insure that individuals receive a minimum daily level; however, because many elderly reduce their intake of dairy products for reasons of taste or lactose intolerance, this strategy is not always effective. Nutrition exerts other effects on bone and fat metabolism. Dietary components such as flavinoids and antioxidants have been linked to osteoblast differentiation and longevity (*see apoptosis*). Conjugated linoleic acid (CLA), a component of animal fats, has been found to reduce adipose tissue depots in animal models (95). Independent studies indicate that CLA can increase bone mass (96), and this appears to be mediated through effects inhibiting the formation and activation of osteoclasts via the receptor activator of the NFκB ligand (RANKL) signaling pathway (97).

When dietary nutrition leads to a state where net energy consumption exceeds energy demands, it often results in obesity. Although obesity manifests as an abundance of extramedullary WAT, it correlates with enhanced bone mass (98). Several factors may account for this. First, with increased weight, an individual’s skeleton is forced to bear greater loads. Biomechanical stimuli may enhance bone formation relative to bone resorption. Second, obesity alters circulating hormone levels, directly or indirectly. Adipocytes express aromatase, allowing these cells to generate estrogenic-like compounds (98). Adipocytes secrete insulin-like growth factors, and obesity is

associated with hyperinsulinemia secondary to insulin resistance, both of which can lead to bone protection; clinical analyses support this hypothesis (98). Obesity has also been associated with elevated levels of parathyroid hormone (99). Third, adipokines such as leptin have been associated with positive effects on osteoblast differentiation and mineralization in murine *in vitro* and *in vivo* models while inhibiting adipogenesis (100,101). These leptin effects seem to be mediated through peripheral mechanisms acting locally within the marrow microenvironment. Independent studies suggest that leptin administered by intra-ventricular injection causes bone loss through centrally mediated mechanisms involving the hypothalamus (102,103). The development of leptin resistance and the activity of the blood brain barrier may account for the apparent discrepancy in these data. Another adipokine, adiponectin, has been associated with MSC differentiation and altered bone mineral density. Unlike other adipokines, adiponectin decreases with obesity (104). When added to murine bone marrow stromal cells, adiponectin inhibited adipocyte differentiation through a COX2-mediated pathway (105). Transgenic mice over-expressing adiponectin displayed increased bone mass owing to enhanced osteoblast activity and suppressed osteoclast function (106). Both adiponectin and its receptors have been detected in human MSCs (72), and adiponectin levels have been inversely correlated to bone mineral density in clinical studies (104,107). As with leptin, the mechanism of adiponectin actions will require further investigation.

Menopause is associated with a rapid decline in circulating estrogen, and as a consequence there is trabecular bone loss, which results in a loss of bone strength. Paradoxically, there are increases in bone size (medullary bone and periosteal diameter) after menopause. The increase in size is caused by increased periosteal apposition, which partially preserves strength (108). Loss of bone mass that follows the loss of ovarian function is associated with an increase in the rates of bone resorption and bone formation, with the former exceeding the latter, and an increase in the number of osteoclasts in trabecular bone. Post-menopausal bone loss is associated with excessive osteoclast activity. In addition to

these marrow changes, menopause is associated with a gain in fat mass and a loss of lean body mass, but these changes in body composition are not prevented by hormone replacement therapy (109). It is clear that the loss of ovarian function causes dramatic changes to bone marrow cell activity as well as extramedullary cell activity. In addition, menopause results in quite dramatic changes in susceptibility to certain diseases such as cardiovascular disease. It is complex to tease out what drives these changes because of the complexity of the cell systems involved and the interplay between different cell types. In terms of bone turnover, there appear to be effects on the development and activity of both osteoblasts and osteoclasts.

Data indicate that changes in estrogen status *in vivo* are associated with the secretion of mononuclear cell immune factors *in vitro* and suggest that alterations in the local production of bone-acting cytokines may underlie changes in bone turnover caused by surgically induced menopause and estrogen replacement (110). There is now a large body of evidence suggesting that the decline in ovarian function with menopause is associated with spontaneous increases in pro-inflammatory cytokines. The cytokines that have obtained the most attention are interleukin (IL)-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF- α . The exact mechanisms by which estrogen interferes with cytokine activity are still incompletely known but may potentially include interactions of the estrogen receptor with other transcription factors, modulation of nitric oxide activity, antioxidative effects, plasma membrane actions, and changes in immune cell function. Experimental and clinical studies strongly support a link between the increased state of pro-inflammatory cytokine activity and post-menopausal bone loss (111).

The production of IL-6 by stromal-osteoblastic cells, as well as the responsiveness of bone marrow cells to cytokines such as IL-6 and IL-11, is regulated by sex steroids. When gonadal function is lost, the formation of osteoclasts as well as osteoblasts increases in the marrow, both changes apparently mediated by an increase in the production of IL-6. These changes may also be due to an increase in the responsiveness of bone marrow progenitor cells not only to IL-6 but also to other

cytokines with osteoclastogenic and osteoblastogenic properties. This is supported by both *in vitro* and *ex vivo* experimental data. Osteoclast formation in response to either IL-6 in combination with the soluble IL-6 receptor or IL-11 is significantly greater in cultures of bone marrow from ovariectomized mice than in cultures from mice that have undergone sham operations, even when the cultures have the same number of osteoblastic support cells and an IL-6 signal of the same magnitude. These findings indicate that not only the production of the osteoclast precursors but also their responsiveness to IL-6 (and to IL-11) are enhanced in a state of estrogen deficiency.

Studies of the effect of ovariectomy on the formation of osteoblast progenitors in cultures of bone marrow suggest that loss of ovarian function increased osteoblastic activity. The number of fibroblast CFUs is increased several-fold in ovariectomized mice. At this stage there is no mechanistic explanation for the observation that the formation of osteoclasts and the formation of osteoblast progenitors in the marrow increase simultaneously after the loss of ovarian function. It has been hypothesized that changes in levels of systemic hormones alters the sensitivity of osteoblast and osteoclast precursors to several cytokine signals by modulating glycoprotein 130 (112). It is clear that there is still considerable work to be done before we fully understand the control of marrow cell development and activity under normal physiological condition and after menopause. It will be interesting to understand whether sex steroids themselves positively drive activity and/or development of osteoclast and osteoblast progenitors and menopause results in the removal of this activity or, paradoxically, whether gonadal steroids inhibit/control bone formation and resorption and menopause results in the relief of this repression.

Apoptosis and the Aging Bone

Apoptosis, or programmed cell death, has been postulated to act as a cellular mechanism accounting for the effects of aging on bone (1) (Table 2.4). Apoptosis is initiated by the activation of a proteolytic enzyme cascade, leading to cellular self-destruction. Unlike cell death caused by necrosis,

TABLE 2.4. Cellular Apoptosis in the Marrow Microenvironment

Cell type	Agonists	Antagonists
Osteoblast/Osteocyte	Glucocorticoids and thiazolidinediones	Bisphosphonates, 1,25(OH) ₂ D ₃ , calcitonin
	AGE	α -Linoleic acid
	TNF	CD40 ligand
	Weightlessness	TGF- β , IL-6, PTH
Osteoclasts	Bisphosphonates	
	β 3 integrin	
Adipocytes	CLA	Glucocorticoids
	TNF	
	Retinoic acid	

AGE, advanced glyated end product; TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin; PTH, parathyroid hormone; CLA, conjugated linoleic acid.

apoptotic cell death is characterized by cell shrinkage and disintegration without damage to the neighboring cells. Pioneering studies by Jilka and colleagues demonstrated that cytokines such as TNF induced apoptosis in MSC-like cell lines *in vitro* (113). To further address the mechanism, Weinstein, Jilka, and colleagues used an *in vivo* murine model to examine the potential apoptotic effects of glucocorticoids (114). Chronic treatment with glucocorticoids activated apoptotic pathways in osteoblasts and osteocytes of the intact bone while reducing osteoblastogenesis (114). Additional causes of osteoblast and osteocytes apoptosis have been identified. Thiazolidinedione compounds, known ligands for the peroxisome proliferator-activated receptor γ adipogenic transcription factor, stimulated osteoblast and osteocytes apoptotic events when administered to mice (115). In rodents maintained under conditions simulating weightlessness, there was a rapid increase in the number of apoptotic osteoblasts within the bone; this was followed by increased numbers of osteoclasts and bone resorption (116). The addition of AGEs to cultures of human MSCs led to increased numbers of apoptotic cells, and this correlated with a reduced capacity for differentiation (117).

A number of agents antagonize apoptosis in osteoblasts and osteocytes. Endocrine factors such as parathyroid hormone and calcitonin increased bone formation by protecting osteoblasts from apoptosis in rodent models (118,119). Similar actions are displayed by the active form of

vitamin D ($1,25(\text{OH})_2\text{D}_3$) (120) and cytokines including TGF- β and those acting through the gp130 receptor pathway, such as IL-6 and oncostatin M (113). Pharmaceutical agents such as the bisphosphonates exert anti-apoptotic effects on osteoblasts through mechanisms involving the extracellular signal-regulated kinases (ERKs) and the connexin43 channel (120). Likewise, lipids such as α -linoleic acid blocked apoptosis in human bone marrow-derived MSCs exposed to TNF- α or hydrogen peroxide (121). It appeared that the α -linoleic acid prevented the generation of reactive oxygen species and subsequent activation of the NF κ B and c-jun N-terminal kinase pathways (121). Finally, because osteoblasts express the TNF receptor-related surface protein CD40, interaction with the CD40 ligand serves to protect them from apoptosis initiated by a variety of agents, including glucocorticoids, TNF- α , and proteasomal activators (122).

Despite these findings, without apoptosis, bone formation may be impaired. Studies of mice deficient in the enzyme caspase-3, critical to the apoptotic cascade, found that they displayed reduced bone formation *in vivo* and reduced bone marrow-derived MSC differentiation *in vitro* (123). These findings could be mimicked using a caspase-3 inhibitor in wild-type mice (123). Biochemical studies implicated the TGF- β /Smad signal transduction pathway as the underlying mechanism (123). Independent studies created a transgenic mouse over-expressing the bcl2 anti-apoptotic protein under an osteoblast-selective promoter (124). Although the osteoblasts isolated from the transgenic bone were resistant to glucocorticoid-induced apoptosis, the cells displayed reduced mineralization. The transgenic mice were smaller than their wild-type littermates (124). Thus, osteoblastic apoptosis is a complex phenomenon that may have both positive and negative effects on bone formation.

Apoptotic events influence the activity of other cell types within the bone marrow microenvironment. Osteoclasts undergo apoptosis in response to bisphosphonates or in the absence vitronectin, the natural ligand for $\alpha\beta$ 1 integrin (125,126). Bisphosphonates are the accepted standard of care for the treatment of osteoporosis in the elderly. Whereas few, if any, studies have been performed on bone marrow-derived adipocytes,

evidence from extramedullary adipocytes indicates that they are relatively resistant to apoptotic stimuli caused by induced levels of bcl2 (127). Nevertheless, adipocytes undergo apoptosis in response to TNF- α (128), although this occurs in a depot-specific pattern; adipocytes from omental fat were more susceptible than those from subcutaneous fat (129). The relative apoptotic sensitivity of bone marrow adipocytes has not been reported. Additional agents exert apoptotic actions on adipocytes, including CLA, retinoic acid, botanical extracts, and cytokines acting through the gp130 receptor (95,130,131). Some investigators postulate that pharmaceutical agents and/or functional foods targeting the adipocyte apoptotic pathway will have the combined benefit of reducing obesity while improving bone growth by reducing bone marrow adipogenesis and enhancing osteoblast function (131).

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